DEVELOPMENT OF ASSAYS FOR COENZYME Q_{10} AND VITAMIN K, AND THEIR APPLICATION IN CLINICAL TRIALS

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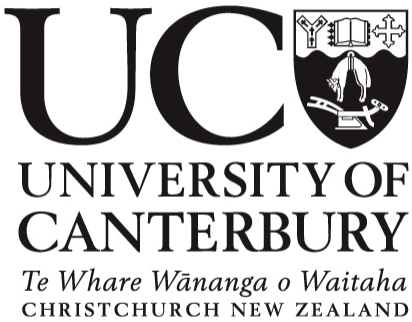

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Abstract

This thesis describes the development of separate assays to measure coenzyme Q_{10} (CoQ_{10}) and vitamin K. Coenzyme Q is essential for the mitochondrial electron transport chain, and vitamin K for the blood coagulation cascade. Vitamin K deficiency is associated with haemorrhagic disease of the new-born, and CoQ_{10} deficiency with HMG-CoA-reductase inhibitor (statin) therapy and heart failure. Coenzyme Q and vitamin K are usually measured by HPLC, using electrochemical and ultraviolet, and electrochemical and fluorescence detection, respectively.

For vitamin K_1, the limit of detection achieved using fluorescence and electrochemical detection was 0.28 and 0.12 nmol/L, respectively. Sensitivity of fluorescence detection is improved by using protic solvents in the mobile phase, and platinum-black catalysed alcohol reduction. The lipophilicity and low endogenous concentrations of vitamin K_1 hinder its measurement, and further work is required to produce a rapid, reliable and robust assay for its measurement in human plasma.

The limits of detection achieved using fluorescence, ultraviolet and electrochemical detection to measure CoQ_{10} were 29, 4.8, and 0.34 nmol/L, respectively. Plasma CoQ_{10} is not stable during long term storage at –13 ºC, but at –80 ºC it is stable for at least 18 months.

The reference interval for plasma total CoQ_{10} in the New Zealand population is 0.47 – 1.80 µmol/L. There is no clinical requirement for stratification of the reference interval according to gender. Coenzyme Q_{10} in human plasma is homeostatically controlled, varying little over a two month interval in healthy young males.

Coenzyme Q_{10} supplements have significantly different bioavailability, with the median increase in plasma CoQ_{10} ranging from 0.14 to 0.59 µmol/L for seven different supplement brands. There is a large inter-individual variation in CoQ_{10} absorption, and hence plasma concentrations should be monitored during supplementation. A plateau in CoQ_{10} absorption, from a single dose, at approximately 200 mg suggests that the maximum dose ingested at one time should be 200 mg or less. Q-Gel capsules containing 30 mg of CoQ_{10} are twice as effective at raising blood CoQ_{10} as 100 mg capsules.

Plasma CoQ_{10} in patients with chronic heart failure are significantly lowered by approximately 33% when these patients receive Atorvastatin for six weeks. The

absolute decrease in CoQ_{10} showed a significant correlation with worsening endothelial function ($r = + 0.548$, $p = 0.011$).

Coenzyme Q_9 was shown to be present in human plasma with a reference interval of 8.8 – 47.0 nmol/L.

Publications and Presentations

Publications

Molyneux, S.L.; Florkowski, C.M.; Lever, M.; George, P.M. (2004). The Bioavailability of coenzyme Q_{10} supplements available in New Zealand differs markedly. *The New Zealand Medical Journal.* **117**(1203): U1108.

Strey, C.H.; Young, J.M.; Molyneux, S.L.; George, P.M.; Florkowski, C.M.; Scott, R.S.; Frampton, C.M. (2005). Endothelium-ameliorating effects of statin therapy and coenzyme Q_{10} reductions in chronic heart failure. *Atherosclerosis*. **179**:201-206

Molyneux, S.L.; Florkowski, C.M.; Lever, M.; George, P.M. (2005). Biological variation of coenzyme Q_{10}. *Clinical Chemistry.* **51**(2): 455-457

Molyneux, S.L.; Lever, M. (2005). Fluorescence is less sensitive than ultraviolet or electrochemical detection for measurement of coenzyme Q_{10} in plasma. *Clinica Chimica Acta*. **358**:198-200.

Molyneux S.L.; Florkowski, C.M.; George, P.M.; Lever, M. Dose Response to the Coenzyme Q_{10} Supplement Q-Gel®. *In preparation*.

Molyneux, S.L.; Lever, M.; Florkowski, C.M.; George, P.M.. Reference interval and biological variation of, CoQ_9 in the New Zealand population. *In preparation*.

Presentations

Poster presented at the Third International Coenzyme Q_{10} Association Conference, London, 21st-24th November, 2002 "An alternative detection system for coenzyme Q_{10} assay by HPLC" Sarah Molyneux, Michael Lever, Peter George, Alison Daines, Murray Munro.

Poster presented at COMBIO Conference, Melbourne, September 2003 "Quantifying biologically important quinones". Molyneux, S.L.; Lever, M.; George, P.M.; Florkowski, C.M.

Presented at the South Island Seminar of the New Zealand Institute for Medical Laboratory Science, Shantytown, Greymouth, 6th May 2004 "Coenzyme Q_{10}. Importance, measurement, and some clinical examples"

Presented at the University of Canterbury Postgraduate Conference, University of Canterbury, 1st September 2004 "Coenzyme Q_{10}: Importance and Bioavailability"

Presented at the Canterbury Health Science Research Conference, Christchurch School of Medicine and Health Sciences, September 5th – 8th 2004. "Coenzyme Q_{10}: Importance and Bioavailability"

Presented at the New Zealand Society for Biochemistry and Molecular Biology, Canterbury Branch Regional Meeting, September 16th, 2004. "Coenzyme Q_{10}: Is that supplement supplementing you, and why do you need it?"

Poster presentation at the Medical Sciences Congress 2004, Queenstown 30th November to 3rd December, 2004. "Biological Variation of Coenzyme Q_{10}" Molyneux, S.L.; Florkowski, C.M.; Lever, M.; George, P.M.

Poster presented at the Fourth International Coenzyme Q_{10} Association Conference, Los Angeles, 14th-17th April 2005. "Bioavailability, Biological Variation, and Reference Interval for Coenzyme Q_{10}". Sarah Molyneux, Chris Florkowski, Michael Lever, Peter George.

Presented at the NZ Institute of Medical Laboratory Science Annual Scientific Meeting NZ Society of Cytology 36th Annual Conference, Christchurch, 14th-18th August 2005. "Coenzyme Q_{10}".

Poster presented at the Australian Atherosclerosis Society Annual Scientific Meeting, Darwin, 19th-22nd October 2005. "Coenzyme Q_{10} in heart failure and healthy subjects." Molyneux, S.L.; Florkowski, C.M.; George, P.M.; Lever, M.

Invited speaker at the RCPA –AACB Chemical Pathology Course, Christchurch, 13th February 2006. "Coenzyme Q_{10}".

Poster presented at the 31st FEBS Congress, Molecules in Health and Disease, Istanbul, 24th – 29th June 2006. "Coenzyme Q_{10} in heart failure and healthy subjects". S.L. Molyneux. Abstract published in FEBS Journal. (2006). **273**(Supplement 1):216

Invited speaker at the HSANZ, ANZSBT and ASTH Combined Conference, Hobart, 15th – 18th October 2006. "An overview of the assessment of plasma vitamin K status". S.L. Molyneux; M. Lever; P.M. George.

Abbreviations

A_{max}	Wavelength at which maximum absorbance occurs
ATP	Adenosine triphosphate
AUC	Area under curve
b.l.	Below limit of detection
BMI	Body mass index
CoQ	Coenzyme Q
CoQ_9	Coenzyme Q_9
CoQ_{10}	Coenzyme Q_{10}
CoQ_9H_2	Coenzyme Q_9 quinol
$CoQ_{10}H_2$	Coenzyme Q_{10} quinol
C_{max}	The maximum concentration
CMC	Critical micelle concentration
%CV	Percent coefficient of variation
CV_a	Analytical variation
CV_g	Between-subject variation
CV_i	Within-subject variation
CV_p	Pre-analytical variation
CV_t	Total variation
DNA	Deoxyribonucleic acid
e^-	Electron
EDTA	Ethylenediaminetetraacetic acid
Em_{max}	Wavelength at which maximum emission occurs
ESR	Electron spin resonance
Gla	γ-carboxyglutamate
HDL	High-density lipoprotein
HDN	Haemorrhagic disease of the new-born
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	High-performance liquid chromatography
II	Index of individuality
l-NMMA	N^G-monomethyl l-arginine
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantitation
min	Minute
MK-n	Menaquinone-n
NADH	Reduced nicotinamide adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide
NCCLS	National Committee on Clinical Laboratory Standards
n.d.	Not determined
n.s.	Not stated
ox	Oxidised
Φ	Fluorescence quantum efficiency
PIVKA-II	Proteins in vitamin K absence
PRP	Polymer reverse phase
RCV	Reference change value
REML	Residual maximum likelihood ratio
ROS	Reactive oxygen species
S_1	First singlet state
S_2	Second singlet state
SD	Standard deviation
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
t_{max}	Time at which maximum occurs
UV	Ultraviolet
VLDL	Very low density lipoprotein
vs	Versus
v/v	Volume per volume

Chapter 1

Introduction

1.1. Introduction

Coenzyme Q (ubiquinone, CoQ) and vitamin K (phylloquinone, vitamin K_1, and menaquinone) are quinones. The electron and proton transfer functions of these compounds are of fundamental importance to all life forms; ubiquinone in the mitochondria of eukaryotes, plastoquinone and vitamin K_1 (phylloquinone) in the chloroplast of plants, vitamin K_1 in the blood coagulation cascade of animals, and menaquinone in bacteria. The work described in this thesis started from a previous project to develop an assay to measure vitamin K_1 in the plasma of new born babies, and in human and cow milk (1). This new test was intended for monitoring and investigating the prevalence of haemorrhagic disease of the new-born, a condition associated with low circulating vitamin K. As found in this study it was not practicable to meet the clinical expectations (measuring low concentrations with small sample sizes) with the analytical resources available. While this work was in progress there was escalating interest in CoQ. Although their physiological roles are different, vitamin K and CoQ are chemically similar, and it was expected that the experience with developing the vitamin K assay could be adapted to measuring CoQ (1). Thus the work described here emphasises CoQ. With the increasing popularity of statin therapy, a medication of choice for preventing cardiovascular disease, a need to monitor the effect of statin therapy on CoQ has arisen. Coenzyme Q has also become a popular health supplement. There is little reliable data on the clinical biochemistry of CoQ and the work described here is a contribution to improving this.

1.2. Quinones

The name “quinone” was given to a compound first described in 1838 (2), which was obtained by oxidising the natural product quinic acid. This compound is now usually called “benzoquinone”, and the term quinone is used generically for a whole class of compounds that can be regarded as derivatives of benzoquinone. Quinones are unsaturated diketones, and are related to aromatic compounds. There are two isomeric benzoquinones, 1,4-benzoquinone (Figure 1.1), also called a paraquinone, and a 1,2-benzoquinone (called an orthoquinone). However, almost all of the biochemically important quinones, and all of those of clinical importance, are 1,4-quinones. Quinones with condensed ring systems are common in nature, so as well as benzoquinones, there are naphthoquinones and

anthroquinones. The basic structures and numbering systems of these are shown in Figure 1.1.

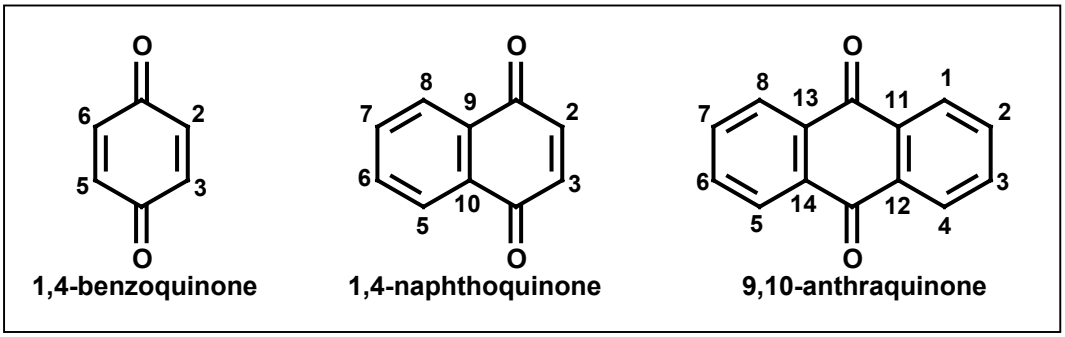


Figure 1.1 The basic structures and numbering systems of common biological quinones.

Quinones show a characteristic reduction and oxidation (redox) chemistry, and the biological importance of quinones centres on the resulting electron and proton transfer functions of these compounds. Oxidation (or reduction) of quinols (or quinones) occurs in two steps, with one hydrogen ion being abstracted from the quinol (or donated to the quinone) to form a relatively stable radical, called a semiquinone (Figure 1.2), which is resonance-stabilised. A second hydrogen ion can be removed (or added) to complete the oxidation (or reduction) to a quinone (or quinol).

The transfer of electrons during oxidation and reduction of quinones can be used to quantify them, using electrochemical detection. Semi-quinones have unpaired electrons, and can therefore be measured with electron spin resonance (ESR).

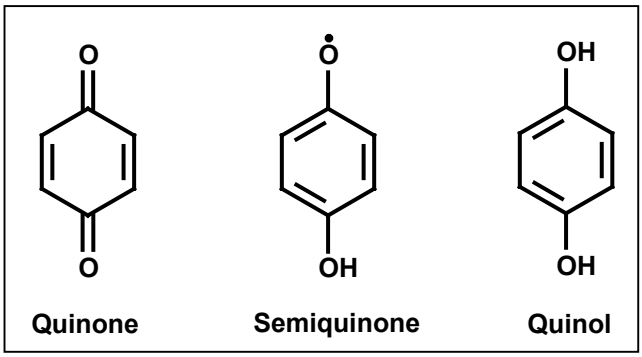


Figure 1.2 The redox states of quinones.

Because the reduction of quinones (or oxidation of quinols) involves hydrogen ions, the potential of the reaction is strongly pH dependent, and can be used to measure pH. The redox potential of different quinones also depends on their structure and environment. Naphthoquinones are more stabilised by the condensed ring than benzoquinones and are weaker oxidising agents. Anthroquinones are weaker still. These differences are significant for their biological roles and affect the approach to measuring them.

The quinols are aromatic diphenols. The phenolic hydroxyl groups of quinols are weakly acidic. At high pH phenoxide ions are formed, which are very easily oxidised, and are sensitive to oxygen.

Methods for measuring quinones in the laboratory are mostly based on either their spectroscopic properties or on their electrochemical properties. Quinones are typically coloured compounds with absorbances in the visible region and intense near-UV absorbance. Quinols, being aromatic compounds, typically absorb at shorter wavelengths than the quinones but they are usually fluorescent.

The biologically important quinones contain long hydrophobic side chains, giving them excellent lipid solubility. The chemical structures of some biologically important quinones, CoQ, vitamin K, and α-tocopherol, are shown in Figure 1.3.

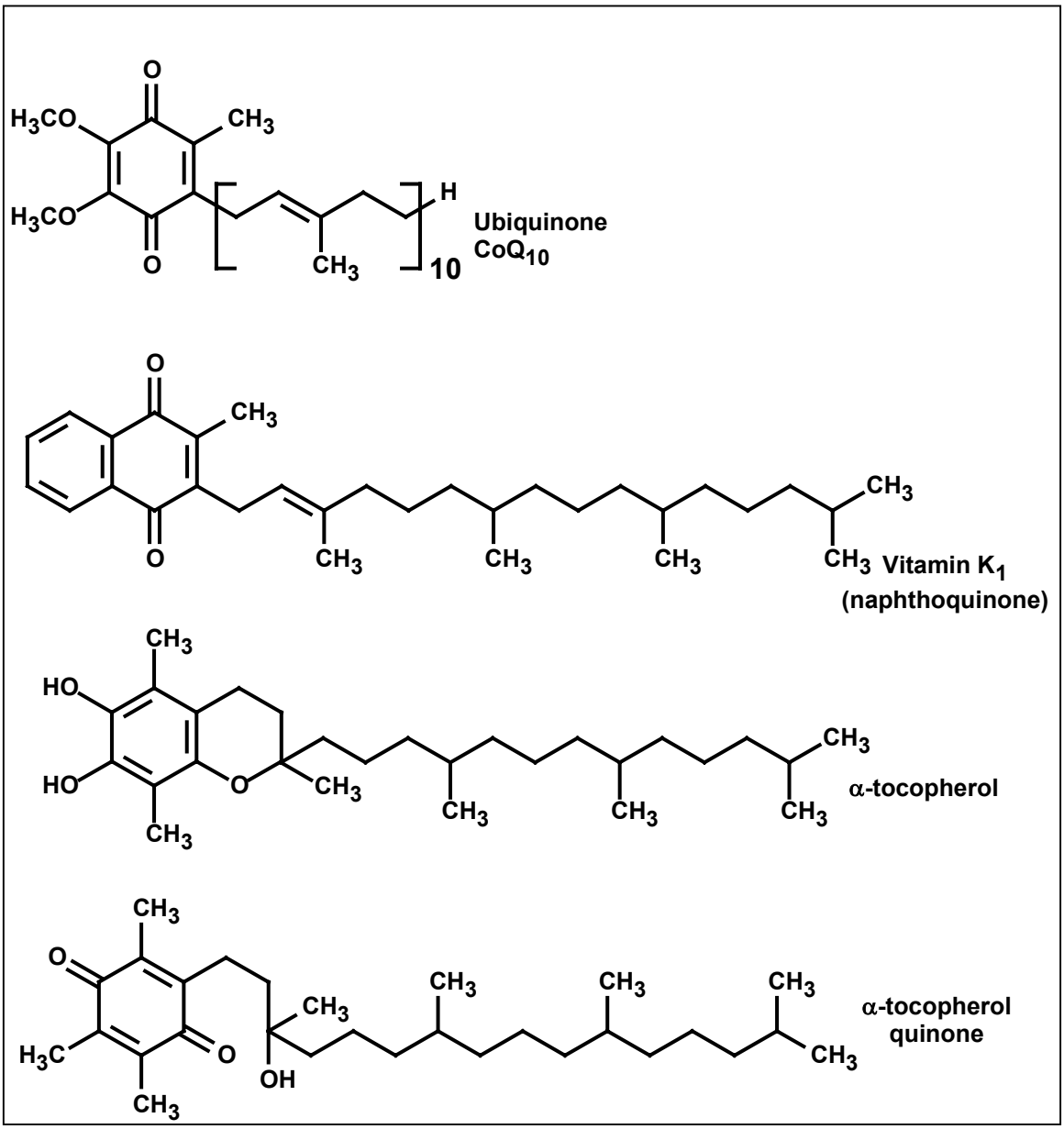


Figure 1.3 The structures of some biologically important quinones, CoQ_{10}, vitamin K_1, α-tocopherol, and α-tocopherol quinone.

1.2.1. Coenzyme Q

Coenzyme Q_{10} was first isolated from beef heart mitochondria by Frederick Crane of Wisconsin, USA, in 1957 (3). Coenzyme Q_{10} is a 1,4-benzoquinone with a long (50-carbon) isoprenoid side chain (Figure 1.3). Different CoQ analogues contain various numbers of isoprenoid units in the sidechain, and the length of this side chain varies between species, but is predominantly ten units in humans. The dominant CoQ homologue

in rats is CoQ_9 (4), with CoQ_{10}, CoQ_8 and CoQ_7 also being present in rat plasma although CoQ_7 appears to be of dietary origin (4). Both the reduced (ubiquinol) and oxidised (ubiquinone) forms of coenzyme Q_{10} (Figure 1.4) are present in the body, with the intermediate (semiquinone) form being transient in nature, and unable to be isolated. Oxidised CoQ_{10} is reduced to $CoQ_{10}H_2$ in the mitochondria by flavoenzymes such as mitochondrial succinate dehydrogenase and NADH (5).

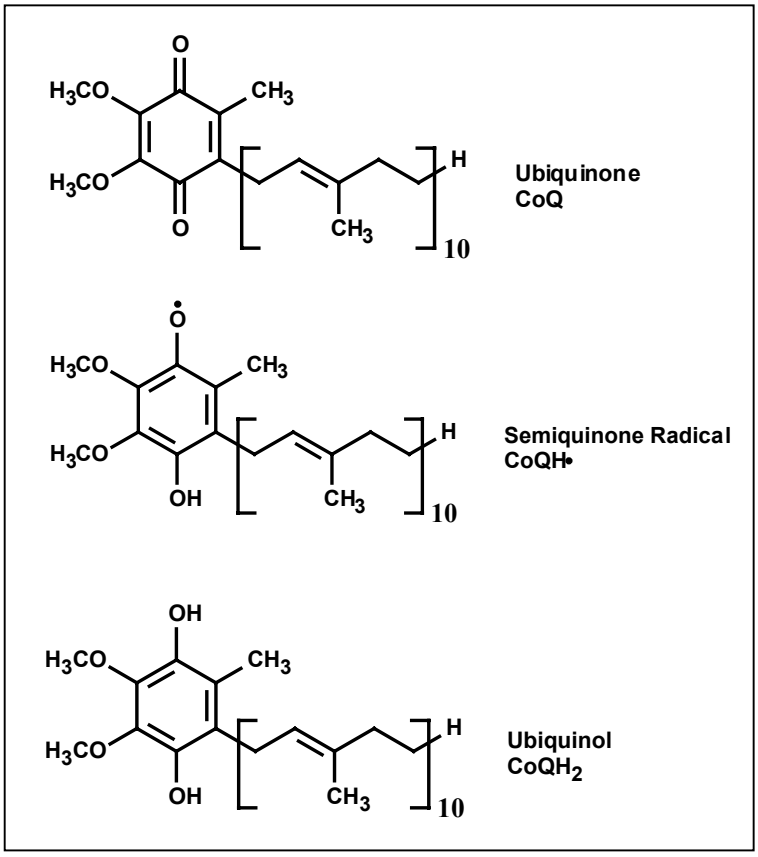


Figure 1.4 Coenzyme Q_{10} and its redox states.

The isoprene side chain gives the molecule excellent lipid solubility and allows its localisation inside cellular membrane bilayers (Figure 1.5).

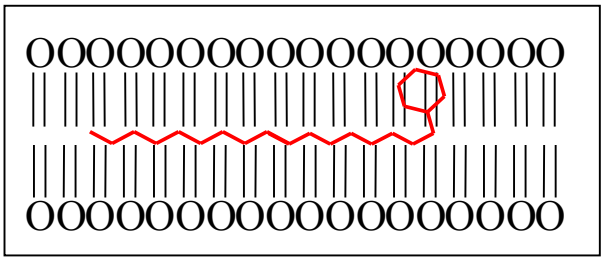


Figure 1.5 The location of CoQ_{10} in the lipid bilayer

The percentage $CoQ_{10}H_2$ of total CoQ_{10} in plasma in a healthy individual is about 96% (6). In human liver, pancreas and intestine, all CoQ_{10} is $CoQ_{10}H_2$, but in brain and lung most CoQ_{10} (80%) is in the oxidised state (7). Coenzyme $Q_{10}H_2$ is easily oxidised in the presence of oxygen. Therefore, it has been proposed that the ratio of $CoQ_{10}H_2$ to CoQ_{10} may indicate oxidative stress in the body (8, 9). It is generally assumed that systemic antioxidant deficiency precedes and predisposes to the deleterious effects of oxidative stress (10, 11). Kontush *et al.,* (1997) (12) reported that the proportion of $CoQ_{10}H_2$ of total CoQ_{10} decreases in the order young adults>aged controls>hyperlipidaemic patients without complications>hyperlipidaemic patients with complications. However, Lenaz *et al.,* (2002) (13) proposed that antioxidant defences should actually increase in response to oxidative stress, as an adaptation to the increased stress. Evidence of an increase in antioxidant defences is provided in several animal studies where CoQ_9 is significantly increased in response to the pro-oxidant effects of diabetes mellitus (14-16). However, none of these studies evaluated changes in the CoQ redox state. It is possible that the body adapts to an oxidative stress by producing more total CoQ, so that while the percentage $CoQH_2$ of total CoQ decreases, the absolute amount of $CoQH_2$ is similar to that in a healthy individual.

Additionally, the ratio of $CoQ_{10}H_2$ to CoQ_{10} changes in a biological sample due to oxidation of $CoQ_{10}H_2$ by atmospheric oxygen. The percentage $CoQ_{10}H_2$ of total CoQ_{10} in plasma has been reported to be between 51 and 96% (4, 6, 17-25). The simple, robust and well validated method of Tang *et al.,* (2001) (6) suggests that in healthy individuals (n = 25) the percentage of $CoQ_{10}H_2$ is tightly regulated, at 96.3 ± 2.0 %. It is possible that the reports of lower percentages of $CoQ_{10}H_2$ in healthy individuals are actually a result of sample handling, especially when some methods recommend analysis of one sample at a time (23, 24). Therefore, strict sample handling is required to ensure that the measured ratio is the actual ratio and not an artefact of sample handling.

1.2.2. Roles of CoQ_{10}

1.2.2.1. Electron transport chain

The best-known role of coenzyme Q_{10} is to transfer electrons between complexes I and II, and complexes III and II in the mitochondrial electron transport pathway, which ultimately produces adenosine triphosphate (ATP) (Figure 1.6). Peter Mitchell received the Nobel

Prize in 1978 for his contribution to the understanding of biological energy transfer through the formation of the chemiosmotic theory, which includes the vital protonmotive role of CoQ_{10} in energy transfer systems (26).

The overall reaction of the electron transport pathway is the reaction $2H^+ + ½O_2 \rightarrow H_2O$, which is made to occur in small steps so that most of its energy can be converted into storage instead of being released to the environment as heat. The electrons are passed through the respiratory chain while the protons escape into the aqueous surroundings. These protons are returned only after the electrons reach the end of the respiratory chain, when they neutralise the negative charges created by the final addition of the electrons to the oxygen molecule.

The electron transport begins when a hydride ion ($H:^-$) is removed from NADH to regenerate NAD^+, and the hydride ion is converted into a proton and two electrons ($H:^- \rightarrow H^+ + 2e^-$). These two electrons are passed into the first of many electron carriers embedded in the inner mitochondrial membrane. At this stage the electrons are at very high energy, and their energy is gradually reduced as they pass along the sequence of more than fifteen different electron carrier molecules in the respiratory chain. The electrons pass through metal atoms and cytochromes via 1-electron transfers. Different metal atoms are tightly bound in a different way to a protein, which alters the electron affinity of the bound atom so that electron transfer occurs. The simplest of the electron carriers is CoQ, which, via the semiquinone intermediate, can pick up or donate either one or two electrons. Coenzyme Q temporarily picks up a proton from the medium along with each electron that it carries, and functions as a mobile electron carrier.

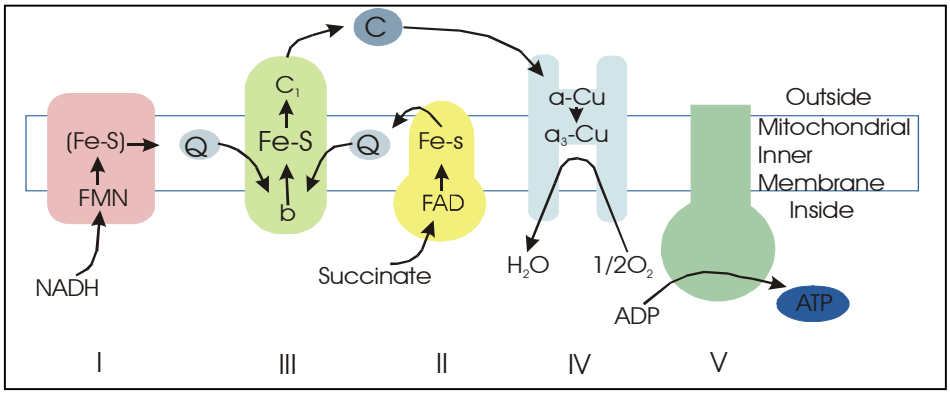


Figure 1.6 The mitochondrial electron transport chain. Coenzyme Q_{10} is indicated as Q. Arrows indicate direction of electron flow through this pathway. Fe-S = the iron-sulfur protein, FMN = flavin mononucleotide, NADH = nicotinamide adenine dinucleotide, b = cytochrome b, C_1 = cytochrome C_1, C = cytochrome C, FAD = flavin adenine dinucleotide, a-Cu = copper associated with cytochrome a, a_3-Cu = copper associated with cytochrome a_3, ADP = adenosine diphosphate, ATP = adenosine triphosphate.

Coenzyme Q is also present in protein-bound form in complex III, where it is necessary for the function of the "Q-Cycle" of electron transfer, linked to proton translocation inside the complex. This cycle (Figure 1.7) involves the Rieske iron-sulfur centre, cytochromes b_{566} and b_{560}, cytochrome c_1 and ubiquinones (27).

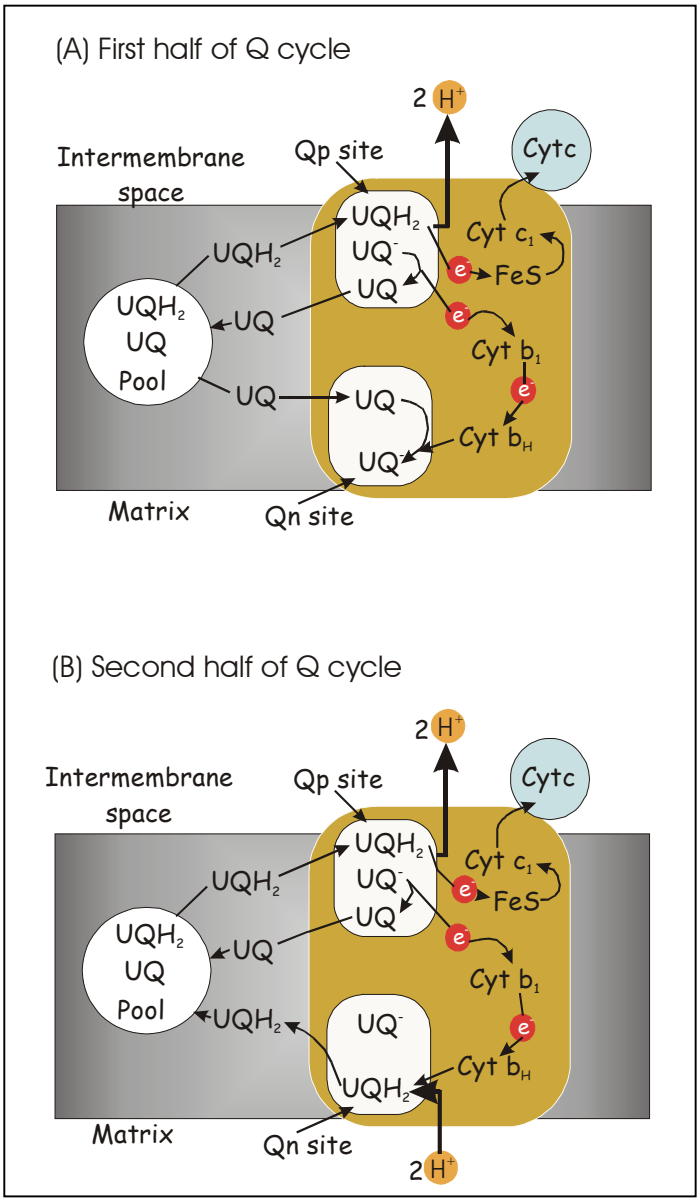


Figure 1.7 The Q-cycle, showing the transfer of electrons in complex III of the mitochondrial electron transport chain. The Qp site is the first quinone-binding site, and the Qn site is the second quinone-binding site. Cytc = cytochrome C, Cyt b = cytochrome b. UQH_2 = reduced CoQ, UQ- = semiquinone, UQ = oxidised CoQ. FeS is the Rieske protein. (Figure adapted from (28)).

Coenzyme Q has also been shown to be a native constituent of a lysosomal electron transport chain, which promotes proton translocation across the lysosomal membrane (29). Lysosomes are membrane-bound cytoplasmic organelles involved in intracellular protein degradation, and the lumina of the lysosome is acidic. The role of CoQ_{10} in this organelle may therefore be to pump protons to regulate the luminal pH (30).

1.2.3. Concentration of CoQ in plasma and tissue

The range of CoQ_{10} plasma concentrations reported in healthy people falls within 0.22 – 2.31 μmol/L (6, 25, 31-35). The presence of CoQ_9 in plasma is controversial, but has been reported to be around 46 nmol/L (20). Coenzyme Q_9 is used as an internal standard in some assays for plasma CoQ_{10}, and hence it is desirable to confirm the concentration of endogenous CoQ_9, to ensure that endogenous CoQ_9 does not affect estimation of plasma CoQ_{10}.

The presence of both CoQ_{10} and CoQ_9 in human and rat tissues has been reported, with CoQ_{10} in humans being present in the highest concentrations in heart, kidney and liver. Coenzyme Q_9 contributes only 2-5% of total CoQ in humans (Table 1.1) (7).

Table 1.1 Distribution of CoQ_9 and CoQ_{10} in human and rat tissues (from (7)).

Tissue	Rat		Human	
	CoQ_9 (μmol/kg)	CoQ_{10} (μmol/kg)	CoQ_9 (μmol/kg)	CoQ_{10} (μmol/kg)
Heart	254.0 ± 23.3	19.6 ± 2.0	3.1 ± 0.4	132.0 ± 10.7
Kidney	155.8 ± 14.2	25.5 ± 2.2	4.1 ± 0.3	77.0 ± 7.6
Liver	164.6 ± 18.6	24.7 ± 2.3	2.3 ± 0.3	63.6 ± 4.7
Muscle	53.6 ± 7.0	3.6 ± 0.3	1.3 ± 0.1	46.0 ± 5.0
Brain	47.0 ± 4.1	21.9 ± 1.7	1.3 ± 0.1	15.5 ± 1.2
Pancreas	46.8 ± 4.0	3.2 ± 0.3	2.0 ± 0.3	37.9 ± 3.2
Spleen	28.8 ± 2.5	10.7 ± 0.9	0.9 ± 0.1	28.5 ± 4.2
Lung	21.3 ± 1.8	2.8 ± 0.3	0.8 ± 0.1	9.1 ± 0.9
Thyroidea	54.7 ± 4.7	7.6 ± 0.9	1.5 ± 0.3	28.6 ± 2.3
Thymus	31.4 ± 2.8	8.3 ± 0.8	n.d.	n.d.
Testis	40.7 ± 3.6	5.4 ± 0.6	0.5 ± 0.1	12.2 ± 1.3
Intestine	64.0 ± 9.1	22.0 ± 1.9	0.6 ± 0.1	13.3 ± 1.9
Colon	59.7 ± 4.9	9.7 ± 0.8	0.5 ± 0.1	12.4 ± 2.2
Ventricle	69.9 ± 6.2	6.1 ± 0.6	n.d.	13.7 ± 0.9

The values are means ± SD (n = 6). n.d. = not determined.

In rats, although CoQ_9 constitutes 92% of total CoQ in the liver, heart, and skeletal muscle, only 67% of total CoQ in the brain is CoQ_9 (7, 36, 37).

The proportion of $CoQH_2$ in tissues has been shown to be negatively correlated with CoQ content (38). In rats under mitochondrial impairment, CoQ redox control is tissue specific (38). In rats, the highest concentration of CoQ_9H_2 is found in the liver (85%) and the lowest amount in the brain (28%) (37). In mice, the highest amounts of CoQ_9H_2 are present in the heart (89%), liver (61%), and brain (58%), with only 35% of CoQ_9 in muscle being CoQ_9H_2 (39). In mice, the percentage of $CoQ_{10}H_2$ is very similar to that of CoQ_9H_2 (39). In contrast to humans where 95% of plasma CoQ_{10} is $CoQ_{10}H_2$ (6), in rats 50% of plasma CoQ is $CoQH_2$ (37).

1.2.4. CoQ and antioxidant activity

CoQ was initially thought to be a mitochondrial reactive oxygen species (ROS) generator (40), only to be later gradually accepted as an antioxidant. While evidence for the antioxidant function of CoQ is now overwhelming, a definitive role for CoQ in mitochondrial oxygen radical generation has not been directly demonstrated.

The earliest observations of an antioxidant function of $CoQ_{10}H_2$ date back to the sixties, and show that this substance could, like α-tocopherol, protect against the light-catalysed peroxidation of mitochondrial lipids (41). These observations have been extensively confirmed during the last two decades. Coenzyme QH_2 is the only lipid-soluble antioxidant synthesised by animal tissues *in vivo*. Coenzyme $Q_{10}H_2$ can act as an antioxidant directly, by preventing initiation and propagation of mitochondrial lipid peroxidation, or indirectly, by recycling α-tocopherol. Coenzyme $Q_{10}H_2$ can also protect against oxidative damage to other important cellular macromolecules such as proteins and DNA.

The reactivity of $CoQ_{10}H_2$ with peroxyl radicals is much slower than that of α-tocopherol, and because $CoQ_{10}H_2$ is a highly hydrophobic molecule located in the middle of the phospholipid bilayer, it has considerably less intramembrane mobility than α-tocopherol, which would tend to decrease its radical scavenging potential. Therefore, this suggests that α-tocopherol, rather than $CoQ_{10}H_2$, acts as the direct scavenger of peroxyl radicals within the mitochondrial inner membrane.

Supporting the hypothesis that $CoQ_{10}H_2$ recycles α-tocopherol, several in-vivo observations have shown that levels of CoQ_{10} correlate with the concentration of α-tocopherol (18, 42, 43). Electron spin resonance studies have shown that in homogeneous solutions (44), as well as cellular membranes (45), the presence of CoQ_{10} can prevent oxidation of α-tocopherol. The ability of CoQ_{10} to regenerate α-tocopherol may also explain why an increase of vitamin E has been reported after CoQ_{10} supplementation in mice (36, 46) and rats (47), although this effect of CoQ_{10} supplementation on vitamin E was not seen in humans (48). Simultaneous supplementation with vitamin E and CoQ_{10} has been shown to result in lower increases in plasma CoQ_{10} levels (31) of up to 50%. The decreased absorption is dependent on the dose of vitamin E given being less when lower concentrations of vitamin E are administered (49). This effect of co-supplementation with vitamin E on CoQ_{10} levels may be due to CoQ_{10} regenerating α-tocopherol, since a decrease in $CoQ_{10}H_2$ has been observed when vitamin E was supplemented . Additionally, it is possible that there is competitive absorption, transport, and/or uptake between CoQ_{10} and vitamin E in the gut (49), or that the plasma concentrations are regulated at the lipoprotein level, since plasma low-density-lipoprotein-cholesterol (LDL-cholesterol) and plasma total CoQ_{10} concentrations correlate (12, 18, 22, 33, 43, 50, 51).

1.2.5. Biosynthesis of CoQ_{10}

In mammals, CoQ is synthesised by the body via stepwise addition of isoprenoid units to generate a decaprenyl unit, which is then added to a quinoid moiety that has been derived from tyrosine or phenylalanine (Figure 1.8). The biosynthetic pathway by which the isoprenoid units are added to build the sidechain is the mevalonate pathway. The mechanism of intracellular transport of CoQ_{10} from the site of biosynthesis to the site of action has not yet been established.

Vitamin B_6, as pyridoxal 5′-phosphate, is required for the initial transamination step, which produces 4-hydroxyphenylpyruvic acid from tyrosine. Thus an adequacy of vitamin B_6 is essential for the synthesis of CoQ_{10} (52).

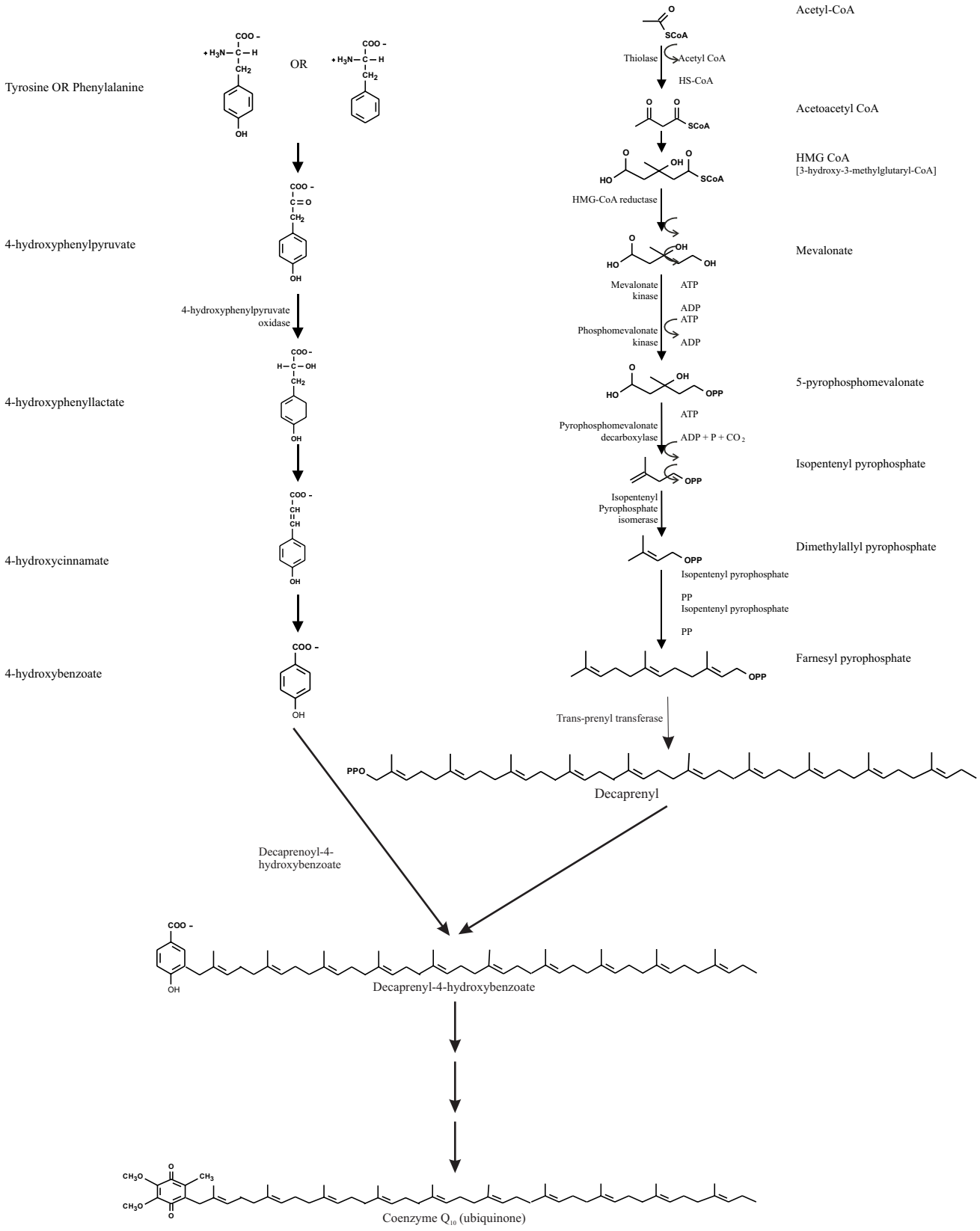


Figure 1.8 The biosynthesis of CoQ.

1.2.6. Dietary CoQ

Coenzyme Q_{10} is also obtained from the diet. A proposed pathway of dietary CoQ_{10} absorption and elimination is shown in Figure 1.9. Dietary-derived CoQ_{10} accumulates first in liver before it is detectable in other peripheral tissues (46, 53). CoQ_{10} is absorbed at a constant (zero-order) rate in the gastrointestinal tract with an absorption time of 6.2 hours per 100 mg, and then distributed sequentially to (1) chylomicrons and tissues, which rapidly absorb the chylomicron-packaged CoQ_{10}, (2) the liver, and (3) a compartment representing very low-density lipoproteins (VLDL) and tissues, which rapidly absorb the VLDL-packaged CoQ_{10} (54). The half-life of CoQ_{10} is approximately 33 hours (55). Laaksonen *et al.*, (1995) (56) reported that the LDL-cholesterol fraction was the major vehicle for endogenous/supplemented ubiquinone during all three supplementation phases, containing 46 to 62% of the serum CoQ_{10}. Coenzyme Q_{10} was not detected in lipoprotein-deficient serum either before or during the supplementation periods, indicating that all endogenous and exogenous CoQ_{10} is carried in circulating lipoproteins (57). Supplemental CoQ_{10} is rapidly reduced to maintain the CoQ_{10} to $CoQ_{10}H_2$ ratio to that already present (58, 59).

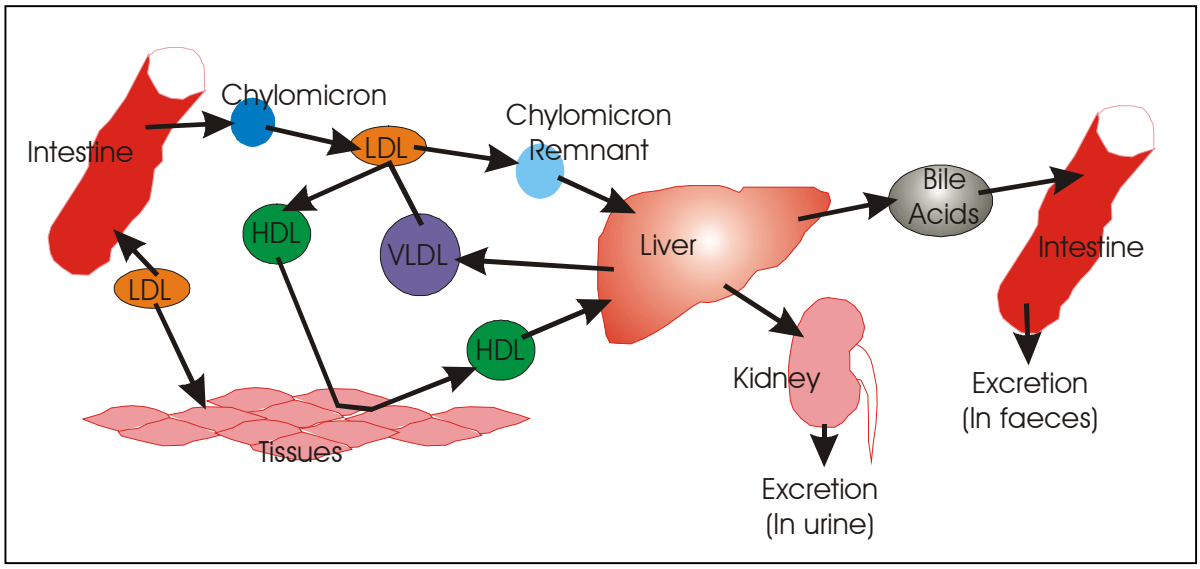


Figure 1.9 Absorption of dietary CoQ_{10} from the intestines. After absorption CoQ_{10} is transported by chylomicrons to the liver. VLDL then initiates movement from liver to peripheral tissues. VLDL = very low-density lipoproteins, LDL – low density lipoproteins, HDL = high-density lipoprotein.

Vitamin E has a similar absorption pattern and is transported from liver to blood via transport proteins. It has not yet been established whether transport proteins are required for CoQ_{10} to be moved from liver to the bloodstream.

In terms of elimination of CoQ_{10} from the body, faecal elimination has been proposed to involve 62.5% of it, with the main elimination occurring via bile . Bile contains bile acids (derivatives of cholesterol synthesised in the hepatocyte that are conjugated to either glycine or taurine), which are facially amphipathic, enabling them to emulsify lipid aggregates, and solubilise and transport lipids in an aqueous environment.

Small amounts of CoQ_{10} are excreted in the urine, with Okamoto *et al.,* (1985) (4) reporting CoQ_{10} levels in urine to be in the range 23 – 98 μmol/kg of creatinine. Daily urinary excretion levels of CoQ_{10} were therefore estimated to be 20 – 128 μg (23 – 148 nmol), based on the daily urinary excretion levels of creatinine in normal subjects (4).

Supplemental CoQ_{10} is generally well tolerated. Forty percent of treated patients with Huntington's disease receiving 1200 mg/day reported minor side effects (including headache, heartburn, fatigue, and increased involuntary movements) that may or may not have been directly related to the supplemental CoQ_{10} (60).

1.2.7. Dietary sources of CoQ_{10}

Coenzyme Q_{10} is found in the mitochondria of every eukaryotic cell, based on which it was also named 'ubiquinone'. Hence, dietary CoQ_{10} is found in many sources, but is most concentrated in sources containing high levels of mitochondria such as meat. Meat has been reported to provide 64% of total dietary CoQ_{10} in the Danish diet (61). Weak positive associations between cholesterol-adjusted serum CoQ_{10} and the average daily consumption of meat and meat products (43) have been shown. Intake of eggs, vegetables and roots are also positively associated with plasma CoQ_{10} (33). Conversely, intake of fish and dairy products has been shown to have a negative association with plasma CoQ_{10} (33). In all, dietary intake of CoQ_{10} has only a marginal effect on plasma CoQ_{10} concentrations (33), and hence it is very difficult to

accurately estimate the effect of the diet on CoQ_{10} levels. It is unlikely that vegetarians would be deficient in CoQ_{10}, since it is synthesised by the body.

1.2.7.1. Absorption of dietary and supplemental CoQ

It can be expected that absorption of CoQ_{10} from the gastrointestinal tract will be slow due to its high molecular weight and lipophilicity. Maximum plasma concentrations occur approximately six hours after ingestion of a CoQ_{10} supplement (48, 62-64). CoQ_{10} supplements have been shown to have varying bioavailability (48, 62-66). This can be due to the form of CoQ_{10} (reduced or oxidised), or due to excipients in the supplement.

The absorption of nutrients often diminishes with increasing dose due to saturation of the transport system in the intestine. Upon supplementation, a dose-dependent increase in plasma CoQ_{10} level has been observed up to a daily dose of 200 mg, which resulted in a 6.1-fold increase in plasma CoQ_{10} levels (31). Laaksonen *et al.,* (1995) (56) supplemented eight healthy male participants (22-49 years old) for one week at 60, 120, and 240 mg, and reported an increase in mean serum CoQ_{10} concentrations of 85, 180, and 362% respectively. Zita *et al.,* (2003) (67) reported that expressing the change in serum CoQ_{10} concentration as a function of dose shows that each mg of a 30 mg and a 100 mg dose increases serum CoQ_{10} concentration by about 0.03 µmol/L and 0.02 µmol/L respectively. Kurowska *et al.,* (2003) (63) reported that the absorption (defined as percent of initial dose present in the plasma at t_{max} assuming a 2.5 litre total plasma volume) of both a reduced CoQ_{10} formulation and a commercial grade oxidised formulation was 1.2 and 0.7 % respectively.

The oxidised and reduced forms of CoQ_{10} may be differentially absorbed due to their different lipophilicities. While the oxidised form of CoQ_{10} has a substantial hydrophobicity and tendency to aggregate, the reduced form is more hydrophilic. Kurowska *et al.,* (2003) (63) compared a commercial grade CoQ_{10} powder with a 'bio-transformed' $CoQ_{10}H_2$ supplement, obtained by fermentation of a soy-based, CoQ_{10} rich media with bakers yeast. They reported enhanced bioavailability of the $CoQ_{10}H_2$ supplement (63). Miles *et al*., (2002) (64), also reported a 25% higher bioavailability of $CoQ_{10}H_2$ as compared to a supplement containing solubilised oxidised CoQ_{10}, although this increase was not significant.

Turunen *et al.,* (1999) (68) administered CoQ_{10}, acetylated CoQ_{10}, and succinylated CoQ_{10} (Figure 1.10) to 6-week old male rats for three weeks via the diet. Uptake of CoQ_{10} into the blood was 40% and 70% greater in rats given the succinylated and acetylated forms, respectively (68). All three forms of CoQ_{10} increased total CoQ_{10} in the liver (about 100%) and the spleen (about 130%) to the same extent (68). Neither CoQ_{10} nor its esterified forms were taken up into the kidney, heart, muscle or brain (68). Therefore, esterification of CoQ_{10} increases the uptake of dietary CoQ_{10} into blood, but derivatisation does not contribute to its elevation in the various organs (68). In the blood, about 5% of the total uptake of CoQ_{10} could be identified as the derivative (using HPLC), but in liver and spleen, no succinylated or acetylated forms were detected (68). Therefore, esterases present in blood and tissues efficiently hydrolyse the derivatised forms of CoQ_{10} (68).

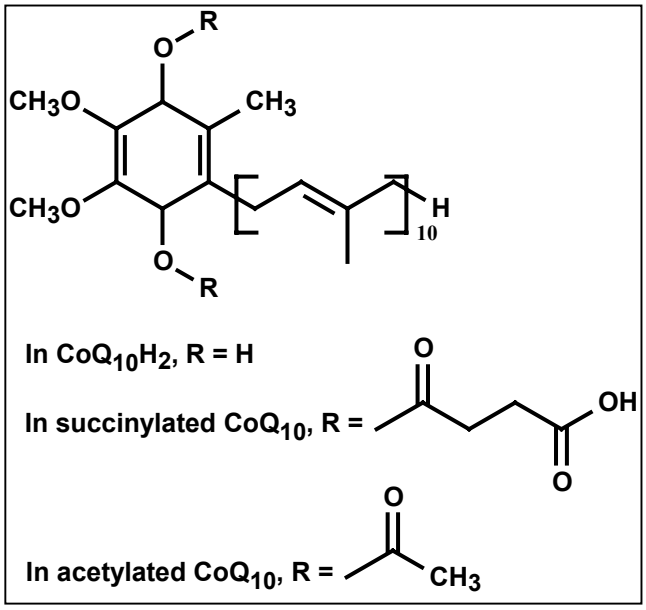


Figure 1.10 The structures of $CoQ_{10}H_2$, succinylated CoQ_{10} and acetylated CoQ_{10}, the bioavailability of which was compared by Turunen *et al.*, (1999) (68).

Excipients used in CoQ_{10} supplements include soybean oil which has been reported to increase the bioavailability of CoQ_{10} above that seen in CoQ_{10} supplements formulated with other inert substances (69, 70). Additionally, emulsifying agents have been added to supplements to increase the dispersion of CoQ_{10}. The concentration of emulsifying agent is crucial. Surfactants, as emulsifying agents, at concentrations

below their critical micelle concentration (CMC), can increase the solubility and dissolution rate of drugs from dosage forms and thus the drug becomes available for absorption (71, 72). In addition, surfactants can penetrate and disrupt the normal structure of biological membranes resulting in increased membrane permeability. However, surfactants above their CMC solubilise and retain the drug thereby causing an overall reduction in the amount of drug released and absorbed (71). Below the CMC individual lipid molecules predominate. Nearly all the lipid added above the CMC spontaneously forms micelles.

Emulsifying agents that have been added to CoQ_{10} supplements include polysorbate-80 (polyoxyethylene (20) sorbitan monooleate), a non-ionic surfactant and emulsifier capable of forming micelles, and lecithin, a liposome forming phospholipid. Animal experiments have shown that lecithin increases the bioavailability of CoQ_{10} over that of a powder formulation (73). However, Weis *et al.,* (1994) (62) found no significant difference between a reference formulation and two formulations with micelle/liposome forming additives (polysorbate 80/lecithin) in humans (n = 10). Only the soybean oil formulation significantly increased bioavailability (62). In the study of Weis *et al.,* (1994) (62), the exact ingredients in the reference formulation were not given which makes it difficult to compare this study with animal studies. It is possible that the concentrations of lecithin and polysorbate-80 were not ideal for aiding bioavailability in the study of Weis *et al.,* (1994) (62), especially since the emulsifying agents significantly lowered the bioavailability of the CoQ_{10} as compared to an identical formulation without any emulsifying agents. Further studies examining the exact effect of excipients are required.

Topical application of CoQ_{10} (as a 0.05% CoQ_{10} containing cream) significantly increases CoQ_{10} in the sebum without significantly affecting the stratum corneum or plasma concentration of total CoQ_{10} or vitamin E (74).

There is large inter-individual variability in absorption of CoQ_{10} from supplements, and this variability has been reported for many different formulations of CoQ_{10} supplement (48, 62, 63, 67, 75). Zita *et al.,* (2003) (67) found that in the group supplemented with 30 mg CoQ_{10} daily for 2 months, 25 of 28 men showed an increase in plasma CoQ_{10} concentration (increase ranged from –0.56 to 1.95 μmol/L). In the

group supplemented with 100 mg daily for 2 months, 32 of 36 men showed an increase (increase ranged from –0.66 to 5.34 μmol/L) (67). In the studies of Kaikonnen *et al.,* (1997) (48) and Weis *et al.,* (1994) (62), the bioavailabiilty of CoQ_{10} varied more between subjects than between preparations.

Correlating demographic variables with changes in plasma CoQ_{10} concentration after supplementation may help explain the large inter-individual variation in absorption. However, there is little information available on such correlations, and that which is available is contradictory. For example, Zita *et al.,* (2003) (67) reported that the change in CoQ_{10} concentration was not dependent on baseline CoQ_{10} concentration, age or body weight. However, Wolters *et al.,* (2003) (43) reported that, after supplementation, changes in cholesterol-adjusted CoQ_{10} concentrations were inversely correlated to the baseline values. This aspect of supplementation deserves more attention as knowledge of what affects absorption may aid determination of the optimal dosage to supplement and also aid in the comparison of results from different study populations. Krone *et al.,* (2001) (76) proposed that one possible explanation for the variable absorption of CoQ_{10} between human subjects is *Candida* colonisation of the gastrointestinal tract. Uptake and utilisation of supplemental CoQ_{10} by this yeast could diminish availability for the human subject. The normal endogenous CoQ in this yeast is CoQ_9, but Krone *et al.,* (2001) (76) showed that growth of the yeast is enhanced in the presence of CoQ_{10}, suggesting CoQ_{10} is biologically functional in this yeast. Many common medical treatments including antibiotics and anti-hyperchlorhydric agents (antacids and proton pump inhibitors) increase the risk of gastrointestinal tract *Candida* colonisation and hence may affect absorption of supplemental CoQ_{10}.

While the concentration of CoQ_{10} in erythrocytes (which do not contain mitochondria) is not dependent on the concentration of CoQ_{10} in plasma, the platelet concentration follows the increase in plasma concentration after CoQ_{10} supplementation, and the exogenous CoQ_{10} enters the mitochondria in platelets (59). This suggests that CoQ may enter inner cell membrane compartments, but not outer cell membrane compartments (59). A CoQ derivative that contains a CoQ analogue covalently coupled to an alkyltriphenylphosphonium cation has been developed (77)

which targets the CoQ analogue to the mitochondria. Oral administration of this compound to mice resulted in its accumulation in heart, brain, liver, and muscle (78).

In a case of severe CoQ_{10} deficiency, described as a rare encephalomyopathy (due to defects in either mitochondrial or nuclear DNA), where three siblings had severe neurohormonal and muscular symptoms, CoQ_{10} supplementation led to a dramatic improvement in the childrens' health (79). This improvement suggests that CoQ_{10} was distributed to its proper localisation in subcellular organelles and membranes, and that the uptake of dietary CoQ_{10} does occur when there is a deficiency in the target organs.

The effect of CoQ_{10} supplementation on tissue levels of CoQ is most easily studied in animal models. CoQ_{10} supplementation increases CoQ_{10} in mice and rats in both plasma (36, 37, 46, 47, 49, 80) and tissues including spleen (49), liver (37, 46, 49, 80) and kidney (46, 80). Heart muscle mitochondria (36), skeletal muscle mitochondria (36, 80), liver mitochondria (36, 46, 49, 80), mitochondria of spleen (49) and mitochondria of kidney (80) have also shown an increase in CoQ_{10} with CoQ_{10} supplementation. Using older mice (24 months old), Lass *et al.,* (1999) (46) reported no increase in CoQ_{10} in heart or skeletal muscle. The increase in CoQ_{10} in the brain may be dose-dependent since both no increase (36, 81, 82) and an increase (47, 80) have been reported. Kamzalov *et al.,* (2003) (36) reported an increase in CoQ_{10} in brain mitochondria only in rats fed high dose CoQ_{10} (654 mg/kg/day). The uptake of CoQ into tissues may therefore be dependent on the age of the animal and the dose administered.

Many of the reported animal studies are difficult to extrapolate to humans since impractically high doses of CoQ_{10} are often supplemented. It has been suggested that uptake of CoQ_{10} by tissues such as heart, skeletal muscle and brain is low or negligible unless the endogenous levels fall below a critical physiological threshold, which is known to occur during aging (37, 81, 83). The most accurate way to measure uptake of supplemental CoQ by tissues is also arguable since the CoQ concentration is 4-250 times higher in mitochondria than in the tissue homogenate (36). Therefore, while the concentration of CoQ in the mitochondria may increase substantially, homogenisation of tissue may suggest the increase in CoQ concentration in the whole tissue is minor. Thus, measurement of CoQ in tissue mitochondria may give a more

sensitive and accurate assessment of tissue uptake than measuring total tissue homogenates (80).

Finally, although rats and mice contain predominantly CoQ_9, supplementation with CoQ_{10} may also increase CoQ_9. An increase in CoQ_9 after CoQ_{10} supplementation has been reported in plasma (80), spleen (49) and liver (49). An increase in CoQ_9 in mitochondria has been reported in mitochondria of heart muscle (36), skeletal muscle (36, 80), kidney (80), and liver only in rats fed a high dose of CoQ_{10} (654 mg/kg/day) (36). Ibrahim *et al.,* (2000) (49) reported a decrease in CoQ_9 in muscles and no change in CoQ_9 in serum, kidney or heart after CoQ_{10} supplementation. Similarly, Kwong *et al.,* (2002) (80) reported no change in CoQ_9 in liver and kidney. The effect of CoQ_{10} supplementation on CoQ_9 concentrations in the brain are controversial with reports of an increase in cerebral CoQ_9 (47), no change in brain CoQ_9 (36), and an increase in CoQ_9 in brain mitochondria only in rats fed a high dose of CoQ_{10} (654 mg/kg/day) (36). The increase in CoQ_9 during CoQ_{10} supplementation may be due to the *in vivo* modification of the isoprene moiety, and/or the antioxidative protection of mitochondrial CoQ_9 by exogenous CoQ (84).

1.2.8. Effect of supplementation on oxidation resistance

There is conflicting evidence on the effect of supplemental CoQ_{10} on oxidation resistance mainly because there is no easy way to measure oxidation resistance. Because the reduced form of CoQ_{10} has antioxidant capacity, and since supplementation with the oxidised form of CoQ_{10} does increase levels of $CoQ_{10}H_2$ (58, 59), it is reasonable to assume that supplemental CoQ_{10} increases oxidative resistance of plasma. However, despite there being many methods available for determining the oxidative resistance of plasma, none provide clear answers. For example, Kaikonnen *et al*., (1997) (48) reported that after 2 months of supplementation, none of the CoQ_{10} supplements they tested increased the oxidative resistance of the VLDL + LDL-cholesterol fractions suggesting the measurement techniques used to determine oxidative resistance of the VLDL + LDL-cholesterol fraction may have lacked sensitivity. Because CoQ_{10} is an endogenous substance, it is difficult to achieve multiple fold elevations in its content in lipoproteins, which could lead to a lack of measurable increase of oxidative resistance or a decrease in plasma

malonaldehyde (a degradation product of lipid peroxidation) concentration. Also, the oxidative resistance may not increase with an increase in CoQ_{10} concentration because the exogenous CoQ_{10} may play a pro-oxidative role. If the antioxidant role of CoQ_{10} is mainly that of regenerating α-tocopherol, then a low plasma α-tocopherol concentration may limit the increase in oxidative resistance due to increased CoQ_{10}.

Autooxidation of the CoQ semiquinone intermediate in the electron transport chain is the major source of mitochondrial oxidants. However, CoQ_{10} supplementation in rats does not appear to have an effect on the rate of mitochondrial H_2O_2 generation (80). Additionally, CoQ_{10} supplementation does not result in a decrease of antioxidative enzymes in skeletal muscle and liver homogenates in rats (80). Plasma homocysteine content, and the ratio of reduced to oxidised glutathione, may be appropriate indices of *in-vivo* oxidative stress (80). After 13 weeks of CoQ_{10} supplementation in rats, no change in the level of plasma reduced glutathione and methionine was observed. However, plasma oxidised glutathione content was decreased by about 60% resulting in an increase of about 2-fold in the ratio of reduced to oxidised glutathione (80). There was also a 25% decrease in cysteine–glycine, and a 45% decrease in homocysteine. CoQ_{10} supplementation may therefore attenuate oxidative stress by altering the plasma aminothiol redox balance towards a more reductive, and less pro-oxidative, environment (80).

1.2.9. Factors affecting levels of CoQ_{10}

1.2.9.1. Genetic/ethnic and gender influences

There are relatively few reports of genetic and ethnic influences on CoQ_{10}. A very rare primary CoQ_{10} deficiency due to an autosomal recessive disorder has been reported on several occasions worldwide, and is detected in early childhood/adolescence. This disorder has a clinical spectrum that encompasses three major phenotypes:

1) A myopathic form, characterised by exercise intolerance, mitochondrial myopathy, myoglobinuria, epilepsy and ataxia, which was first reported by Ogasahara *et al*., (1989) (85).
2) A generalised infantile variant with severe encephalopathy and renal disease (Rötig *et al*., 2000) (79).

3) An ataxic form, dominated by ataxia, seizures, and cerebellar atrophy (Musumeci *et al*., 2001) (86).

Significant differences in CoQ_{10} with respect to gender have been documented for human adults (33, 87), with males having approximately 22.9% higher total CoQ_{10} concentration, or 13.3% higher cholesterol-adjusted CoQ_{10} concentration, than females (33). Conversely, a lack of difference between genders in respect to plasma CoQ_{10} concentration has been reported (4, 32, 75). The association of gender and CoQ_{10} observed in adults does not occur in children (51, 59). It is possible that the gender difference seen in adults may not be clinically relevant, and perhaps is a result of higher concentration of lipids in older males (88). The gender effect may be compounded by genetic differences since Miles *et al.,* (2003) (87) found no significant difference between healthy black males and females for CoQ_{10}, $CoQ_{10}H_2$, or the percentage of $CoQ_{10}H_2$ of total CoQ_{10}. However, healthy white males had a significantly higher concentration of total CoQ_{10} and $CoQ_{10}H_2$, and a higher total CoQ_{10} to total cholesterol index than healthy white females (87). Additionally, American blacks had significantly higher concentrations of plasma CoQ_{10} than whites in all cases except for the $CoQ_{10}H_2$ to total CoQ_{10} fraction (87). The total CoQ_{10} to LDL-cholesterol index and the total CoQ_{10} to total cholesterol index were 21 and 25% higher, respectively, in blacks than in whites (87).

The gender difference in CoQ_{10} has been extended to supplementation with CoQ_{10}. Wahlqvist *et al.,* (1998) (75) reported that, 4 hours after administration of supplemental CoQ_{10}, plasma CoQ_{10} was significantly higher in men compared with women. Similarly, Weis *et al.*, (1994) (62) reported a significant difference in absorption between males and females with the increase in serum CoQ_{10} levels being 27.7% and 40.6% for females and males respectively. However, because more females than males in this cohort smoked, it is possible that this significant finding was due to a type-1 error. It is therefore possible that men have better absorption, and/or a lower clearance rate for CoQ_{10} than women but this finding needs to be confirmed.

1.2.9.2. Body mass index and plasma lipids

Total CoQ_{10} levels have been positively correlated with body mass index (BMI) (33, 67) but this correlation has been shown to disappear when levels are adjusted for cholesterol (43). This is probably because BMI and plasma total cholesterol positively correlate (89). Plasma CoQ_{10} is positively associated with plasma total cholesterol (12, 18, 22, 33, 43, 50, 51) and plasma triglycerides (33, 43, 90). Adjusting CoQ_{10} concentration for total cholesterol eliminates the correlation of plasma CoQ_{10} and triglycerides (43). The correlation of fasting plasma CoQ_{10} and lipids is expected due to the hydrophobicity of the CoQ_{10} molecule.

1.2.9.3. Age

Reports on the correlation of plasma CoQ_{10} with age are conflicting, possibly because the age-related CoQ_{10} changes are dependent on circulating lipids and hence disappear when CoQ_{10} concentrations are adjusted for lipids (43). There are reports of a positive association with age (33, 91, 92) but also of no association, either adjusted for lipids or not (11, 87). A negative correlation exists between the year of life of infant and the CoQ_{10} concentration adjusted for lipids (59).

Young children have increased lipid-adjusted total CoQ_{10} concentrations compared with adults (11) and older children (0.1 – 7 years vs 9 – 19 years) (59, 93). Miles *et al.,* (2004) (11) reported a significant age-related decrease in the CoQ_{10} redox ratio after 18 years of age which may be related to the early effects of oxidative stress. There is a marked increase in plasma CoQ_{10} during the first few weeks of life (11) and young children tend to have low CoQ_{10} redox values (11, 94) suggesting infants may require weeks or even months to attain a $CoQ_{10}H_2/CoQ_{10}$ ratio within the reference range for children (11).

A decrease of CoQ_{10} levels with increasing age has been reported in both human and rat tissues (95). The exception to this trend is the brain, where no decrease has been observed in rats, while in humans a decrease is apparent only at 90 years of age (95, 96). This decrease of tissue CoQ_{10} with ageing could be due to reduced synthesis, or age-dependent increases in lipid peroxidation, that can reduce CoQ_{10} levels (84). It is not clear whether the decrease in tissue CoQ_{10} is a result of CoQ_{10} leaving mitochondria, or extra-mitochondrial regions. Therefore, whether the observed

decrease in CoQ_{10} with age results in a real deficiency syndrome is not clear, since it may just be a general adaptation to a decreased metabolic rate with age (42).

1.2.9.4. 'Recreational substances'

The effect of alcohol on CoQ_{10} is unclear and probably depends on the volume and duration of alcohol consumption. A weak positive association between cholesterol-adjusted serum CoQ_{10} and alcohol has been shown (33, 43), and serum γ-glutamyltransferase (a marker of liver damage) was associated with increased plasma CoQ_{10} levels (33). In cirrhotic patients and in chronic alcoholics, plasma CoQ_{10} levels have been shown to be decreased (97).

The effect of smoking on CoQ_{10} is also controversial. As in the case of alcohol, the effect probably depends on the number of cigarettes smoked and the number of years of smoking. Smoking has been shown to positively associate with CoQ_{10} (33, 67) whereas smoking did not have a significant effect on CoQ_{10} in the studies of Lagendijk *et al.,* (1996) (22) and Miles *et al*., (2003) (87), plasma CoQ_{10} levels being adjusted for lipids in the study of Lagendijk *et al.,*(1996) (22). Zita *et al*., (2003) (67) suggested that the higher CoQ_{10} concentration in smokers may be due to increased oxidative stress and hence an increased demand for CoQ_{10} in smokers. However, Lagendijk *et al*., (1996) (22) reported no significant difference in plasma concentrations of $CoQ_{10}H_2$, CoQ_{10}, or the $CoQ_{10}H_2/CoQ_{10}$ ratio between male smokers and non-smokers. In contrast to these studies, plasma CoQ_{10} was found to be reduced amongst smokers by Kontush *et al.,* (1997) (12).

1.2.9.5. Exercise

The effect of intense exercise on CoQ_{10} is difficult to determine since it is not clear whether plasma levels reflect tissue levels of CoQ_{10}. A negative association between intense exercise and plasma CoQ_{10} has been reported (33). In rats, exercise training resulted in tissue-specific increases in the mitochondrial CoQ content (98), with the concentration being significantly increased in red quadriceps and soleus muscles as well as in white and brown adipose tissue (98). The ratio of CoQ_9 to CoQ_{10} in rats is not significantly influenced by exercise training (98).

1.2.9.6. Medications

The effect of medications on CoQ_{10} has mainly focused on HMG-CoA reductase inhibitors (statins). Statins are currently indicated for nearly all dyslipidaemias associated with coronary artery disease (99). Statins partially inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which catalyses the rate limiting step in cholesterol biosynthesis (Figure 1.11) resulting in lower intracellular cholesterol levels and a secondary up-regulation of LDL-cholesterol receptor expression, leading to increased LDL-cholesterol clearance (100, 101). Statin-induced reductions in cardiovascular disease risk are greater than can be explained by lipid lowering effects alone, and clinical benefits of statins that are LDL-cholesterol-independent (globally termed pleiotropic effects) include anti-thrombotic, anti-oxidant, anti-proliferative, anti-inflammatory, vasodilatory and plaque stabilising effects (102).

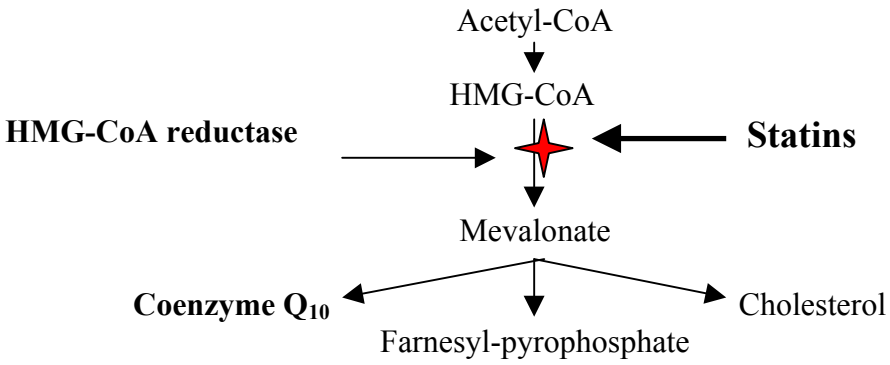


Figure 1.11 The site of action of statins in the mevalonate pathway.

Statin therapy is associated with a reduction in plasma CoQ_{10} concentrations of up to 54% (103-108). This reduction is thought to be due to competitive inhibition of HMG-CoA reductase or to a decrease in circulating LDL-cholesterol (109).

In view of the controversy as to whether decreased plasma CoQ_{10} concentrations are indicative of decreased tissue CoQ_{10}, and because statins are relatively new drugs, the long-term consequence of CoQ_{10} lowering by statins is not currently known. Two studies have shown a CoQ_{10} decrease in blood, myocardium and skeletal muscle in rat and hamster (110, 111) during statin therapy whereas in another study, patients with hypercholesterolaemia given simvastatin for four weeks had a reduced serum level of coenzyme Q_{10} but increased tissue CoQ_{10} concentration (57). Statin therapy has been associated with muscle pain and fatigue in approximately 13.6% of New Zealanders on simvastatin (112). This side effect of statin therapy may be due to a statin-induced CoQ_{10} deficiency (106). Further investigation of the role of CoQ_{10} in muscle pain experienced in some patients on statin therapy is necessary.

1.2.9.7. Disease

The literature on endogenous CoQ_{10} levels and the incidence of disease or ill-health, and on the effect of supplemental CoQ_{10} on disease and ill-health, is limited. There are many reports of associations of CoQ_{10} and disease, but most need confirming with large, well planned clinical trials.

Coenzyme Q_{10} deficiency has been reported in patients with cancer (113), patients with Steinert's myotonic dystrophy (114), children with Prader-Willi syndrome (115), patients on haemodialysis (116), patients with phenylketonuria consuming natural protein-restricted diets (117), and patients with hyperthyroidism (35). An altered redox state has been shown for platelet CoQ_{10} in Parkinson's patients (118). Miles *et al*., (2004) (90) reported that a comparison of groups with minimal-risk and high-risk of developing the metabolic syndrome revealed an increased ratio of $CoQ_{10}H_2$ to CoQ_{10} in the high-risk group. Total CoQ_{10} to total cholesterol index, total CoQ_{10} to LDL-cholesterol index, and total CoQ_{10} to triglyceride index are significantly higher, higher and lower respectively in subjects with the metabolic syndrome as opposed to those without (119).

Folkers (1996) (120) suggests that a cause of cancer may be a deficiency of one or more of the DNA bases (thymidine, guanine, adenine, and cytosine) that require vitamin B_6 for their biosynthesis. Vitamin B_6 is also necessary for CoQ_{10} synthesis such that a deficiency of CoQ_{10} may correlate with, but not be causative for, cancer.

Supplemental CoQ_{10} has reportedly been beneficial in patients with breast cancer (121, 122), diabetes (123), patients on haemodialysis (123), patients maintained on continuous ambulatory peritoneal dialysis (123), and patients with chronic stable angina pectoris (124). Supplemental CoQ_{10} reportedly improves *in-vivo* cardiac and skeletal muscle bioenergetics in patient's with Friedreich's ataxia (125), aids patients with Kearns-Sayre syndrome (126), provides mild symptomatic benefit in patients with Parkinson's disease (127) and increases sperm cell motility in patients with idiopathic asthenozoospermia (128). In hypertensive patients, CoQ_{10} supplementation decreases the need for antihypertensive therapy and significantly reduces both systolic and diastolic blood pressure (129-131).

Coenzyme Q_{10} has often been associated with heart disease since the heart is a site of major energy expenditure and is critical to life. A deficiency of plasma $CoQ_{10}H_2$ and total CoQ_{10} in patients with ischaemic heart disease has been recorded (132, 133). CoQ_{10} supplementation has been used for the treatment of mild congestive heart failure in Japan since 1974. Whether CoQ_{10} supplementation does improve health of patients with heart failure or cardiomyopathy is controversial, particularly because clinical studies exploring the effect of CoQ_{10} supplementation on heart failure differ in the dose of CoQ_{10} given (low doses were given up until the 1990s when it was discovered that high doses of supplemental CoQ_{10} are well tolerated), the duration of supplementation, the severity of heart failure when supplementation was initiated, the number of subjects enrolled in trials, and the measured endpoints. A positive effect of CoQ_{10} supplementation in patients with heart failure (134-143) has been reported more often than a neutral effect (144-146). Large, multicentre, and well designed studies on the efficacy of CoQ_{10} supplementation in heart failure and cardiomyopathy are required, but these are difficult to conduct because funding is not easily forthcoming for trials on non-patentable, over-the-counter therapies. One study, called 'Q-SYMBIO', a randomised double-blind multicentre trial with CoQ_{10} as an adjunctive therapy in chronic heart failure, is currently under way (147).

It is clear that further work is necessary to confirm the effect of disease on CoQ_{10}, and the efficacy of CoQ_{10} supplementation on disease.

1.2.10. Determination of CoQ_{10}

In an early report of measurement of CoQ_{10}, Kröger *et al*., (1978) (148) used a dual wavelength spectrophotometer (in the ultraviolet region) to determine simultaneously menaquinone and CoQ_{10} in both the reduced and oxidised forms. The method was used to determine CoQ in mitochondria and sub-mitochondrial particles of bacteria, cell-free bacterial homogenates and purified preparations of bacterial membranes. Extraction of quinones from aqueous suspensions of membrane preparations was carried out with light petroleum in the presence of methanol. The quinone forms of CoQ and menaquinone were determined using the absorbance difference caused by reduction to the quinol by potassium borohydride. The quinols were measured by recording the oxidation reaction with oxygen under alkaline conditions. Besides using dual-wavelength spectrophotometry, the difference in the spectra of extracts (reduced by potassium borohydride vs untreated or oxidised) was evaluated using the same wavelength-pairs as proposed for dual-wavelength recording.

Recently, Hagerman *et al.,* (2003) (149) reported an assay for the measurement of plasma CoQ_{10} based on the binding of CoQ to a CoQ binding peptide (149). The 14-amino acid binding peptide was chemically synthesised, and conditions for immobilising the peptide on microfuge tubes were established (149). CoQ_{10} was selectively bound to the immobilised peptide, eluted, and determined spectrophotometrically (149). The limit of detection was 0.25 – 5 nmol CoQ, and recoveries ranged from 99-102% of the values obtained with HPLC (149). This assay allows high sample throughput using Elisa plates, but relatively long sample preparation times are required. The assay has been applied to measure CoQ_6 in yeast, but was not adapted to measure CoQ in human plasma or tissues.

High-performance liquid chromatography (HPLC) has been the most commonly used method for determination of tissue and plasma CoQ in human and rodent samples. Ultraviolet detection lacks sensitivity to measure the ratio of CoQ to $CoQH_2$, but has been used to measure total CoQ (4, 32, 35, 150-152). Electrochemical detection after HPLC has been the preferred detection method to determine the ratio of CoQ to $CoQH_2$ (6, 18, 20-25, 39, 59, 153, 154) although simultaneous detection using ultraviolet and electrochemical detection has also been used to measure this ratio

(155-158). The results presented by Daines (2001) (1) suggest that fluorescence detection may be sufficiently sensitive to measure the ratio of CoQ_{10} to $CoQ_{10}H_2$ in plasma samples. Reduction of endogenous CoQ_{10} to the fluorescent $CoQ_{10}H_2$ after separation on an analytical column is necessary, and the most efficient method of achieving this reduction (electrochemical, chemical, or platinum-black catalysed alcohol reduction) is discussed in Chapter 2.

Some assays have required a concentration step after liquid-liquid extraction (using evaporation to dryness and reconstitution of the residue), to measure total CoQ by ultraviolet or diode-array detection (4, 32, 150, 152), or to measure the ratio of CoQ to $CoQH_2$ using electrochemical detection (20, 21, 24, 59, 153, 154, 158), or when using a combination of electrochemical and ultraviolet detection (155, 157). Less complex assays that do not require concentration of the extract have been reported for measuring total CoQ with ultraviolet detection (35, 151), and the ratio of CoQ to $CoQH_2$ using electrochemical detection (6, 18, 22, 23, 25, 39).

1.2.11. Stability of CoQ

Reports on the stability of CoQ_{10} in plasma, standards, and extracts are difficult to interpret because they do not always give full experimental details, eg what anticoagulant was used, whether the sample was protected from light, and, in the case of plasma, what the total volume of plasma in each aliquot was. Light protection is critical (24). The aliquot volume may affect the stability of CoQ_{10} in plasma because the surface area (exposed to light and air) is often proportionally higher for smaller sample volumes.

1.2.11.1. Photostability of CoQ

Coenzyme Q_{10} is photochemically degraded so its stability under full laboratory lighting is unclear. Tang *et al.,* (2001) (6) suggest that CoQ_{10} is stable (variation < 2%) under normal laboratory lighting for at least 7 hours at room temperature when a pool of plasma is exposed. However, Kaikkonen *et al.,* (1999) (24) suggest that 10% of CoQ_{10} degrades during exposure to light during extraction, evaporation and reconstitution. These results suggest that laboratories setting up an assay to measure

CoQ_{10} should confirm whether or not samples need to be protected from light during the selected extraction procedure.

1.2.11.2. Heat stability of CoQ

CoQ_{10} is not stable at temperatures above 40 ºC during evaporation of solvents under a stream of nitrogen, as shown by peaks of decomposed products after samples had been exposed to a 40 ºC water bath (34).

1.2.11.3. Stability of CoQ in plasma and plasma extracts

There is no clear data on the stability of $CoQ_{10}H_2$ in extracts of plasma or tissues, with reports of it being stable for 2 hours (155) and 4 hours (39). Reports of stability for times intermediate between these two are also available (18, 156, 157). Discrepancies may be due to extraction procedure, light exposure, volume stored and assay sensitivity.

Tang *et al.,* (2004) (39) provide evidence that $CoQ_{10}H_2$ is more stable in 1-propanol extracts than in the tissue itself since, in a 1-propanol extract stored on ice for 4 hours, $CoQH_2$ remained stable, while >50% of the tissue $CoQH_2$ oxidised in the same time frame. The use of cold 1-propanol therefore seems to prevent tissue ubiquinols from oxidation (39). This finding is supported by Leary *et al*., (1998) (153) who reported that methanol-water extracts of tissue $CoQH_2$ remain unchanged when kept on ice for 3 hours.

Anticoagulants markedly affect the stability of $CoQ_{10}H_2$, with the ratio of $CoQ_{10}H_2$ to CoQ_{10} decreasing 30% in whole blood specimens containing EDTA (stored in open Vacutainers at 4 ºC for 7 hours) in contrast to remaining stable in lithium heparinised blood samples stored in the same way for the same length of time (159). The ratio of $CoQ_{10}H_2$ to CoQ_{10}, is reasonably stable in lithium heparinised plasma for up to 24 hours at 4 ºC when oxygen contact is limited, (i.e. whole blood stored in closed Vacutainers) (159). The $CoQ_{10}H_2$ to CoQ_{10} ratio in lithium heparinised plasma stored in closed containers at 4 ºC decreases by approximately 6 and 28% after 48 and 72 hours, respectively (159). However the volume stored and protection from light are not discussed. In contrast, in heparinised plasma stored at room temperature, the $CoQ_{10}H_2$ to CoQ_{10} ratio is reportedly stable for only 30 minutes, with a 39% increase

in CoQ_{10} after 24 hours at room temperature (154). Whether or not this sample was exposed to the light is not mentioned and oxygen contact is not discussed.

Lagendijk *et al.,* (1996) (22) and Kaikonnen *et al.,* (1999) (33) reported approximately 20% loss of $CoQ_{10}H_2$ in EDTA plasma on ice for 3 hours, but this was not compared with the stability of $CoQ_{10}H_2$ in lithium heparin plasma stored under the same conditions.

Mosca *et al.,* (2002) (151) showed that total CoQ_{10} in lithium heparinised plasma stored at or below 22 ºC for up to 4 days was stable. However the volume stored and whether the samples were protected from light was not reported.

1.2.11.4. Stability of CoQ during freeze-thaw cycles

Knowledge of the stability of CoQ_{10} during freezing is important, to validate sample storage. Standard $CoQ_{10}H_2$ is stable at –20 ºC for several weeks (155). $CoQ_{10}H_2$ in EDTA plasma is not stable at –20 ºC, with a 60% loss occurring within one month (158). Addition of butylated hydroxytoluene accelerates this loss, and addition of reduced glutathione delays but does not inhibit it (158).

Plasma total CoQ_{10} has been shown to be stable during repeated freeze-thaw cycles (7 cycles) to –80 ºC ($n = 5$), although a non-significant ($p = 0.080$) decrease was apparent (mean concentration 0.80 and 0.72 μmol/L before and after freeze-thawing, respectively) (24). Yamashita and Yamamoto (1997) (23) reported that one freeze-thaw cycle does not significantly alter $CoQ_{10}H_2$ or CoQ_{10} in plasma samples, but that freeze-thawing should be minimised when measuring this ratio (24).

Coenzyme Q_{10} in both heparinised and EDTA plasma is stable at –80 ºC for 3 years (24). Tissue CoQ probably has similar stability to that in plasma. Tang *et al.,* (2004) (39) reported that CoQ_9H_2, CoQ_9, $CoQ_{10}H_2$, and CoQ_{10} in mouse tissue were stable at –80 ºC over 3 months.

1.3. Vitamin K

Vitamin K was discovered by Henrik Dam in the late 1920s (160) when he was investigating the role of cholesterol by feeding chickens a cholesterol-depleted diet. After several weeks on a cholesterol depleted diet, the chickens developed lengthened blood clotting times, anaemia and haemorrhage, defects that were not reversed when pure cholesterol was added to the chickens diet. Hence, Dam was led to conclude that a second compound, termed the 'coagulation vitamin' had been extracted from the food along with cholesterol. The new vitamin received the letter K because these initial discoveries were reported in a German journal in which it was designated as Koagulations vitamin. Edward Adelbert Doisy, of St Louis University carried out much of the research that lead to the discovery of the structure and chemical nature of vitamin K. Dam and Doisy shared the 1943 Nobel Prize for medicine for their work on vitamin K.

Vitamin K is the family name for a series of fat-soluble compounds. These all have a common 2-methyl-1,4-naphthoquinone nucleus but differ in the structure of a side chain at the 3-position (Figure 1.12). Vitamin K_1 is synthesised by plants and has a phytyl side chain with only one double bond. Vitamin K_1 is present in biological samples as oxidised K_1 (K_1 naphthoquinone), reduced K_1 (K_1 naphthoquinol), and the epoxide metabolite (161). Only the *trans*-isomer of vitamin K_1 is biologically active. However, the *cis*-isomer is present at about 10-20% in synthetic preparations (162). Menaquinone comprises a group of structures that are synthesised by bacteria. Each menaquinone structure has a side-chain with a varied number of isoprene units. These structures are designated menaquinone-n (MK-n) according to the number (n) of prenyl units. Some bacteria also synthesise menaquinones in which one or more of the double bonds is saturated.

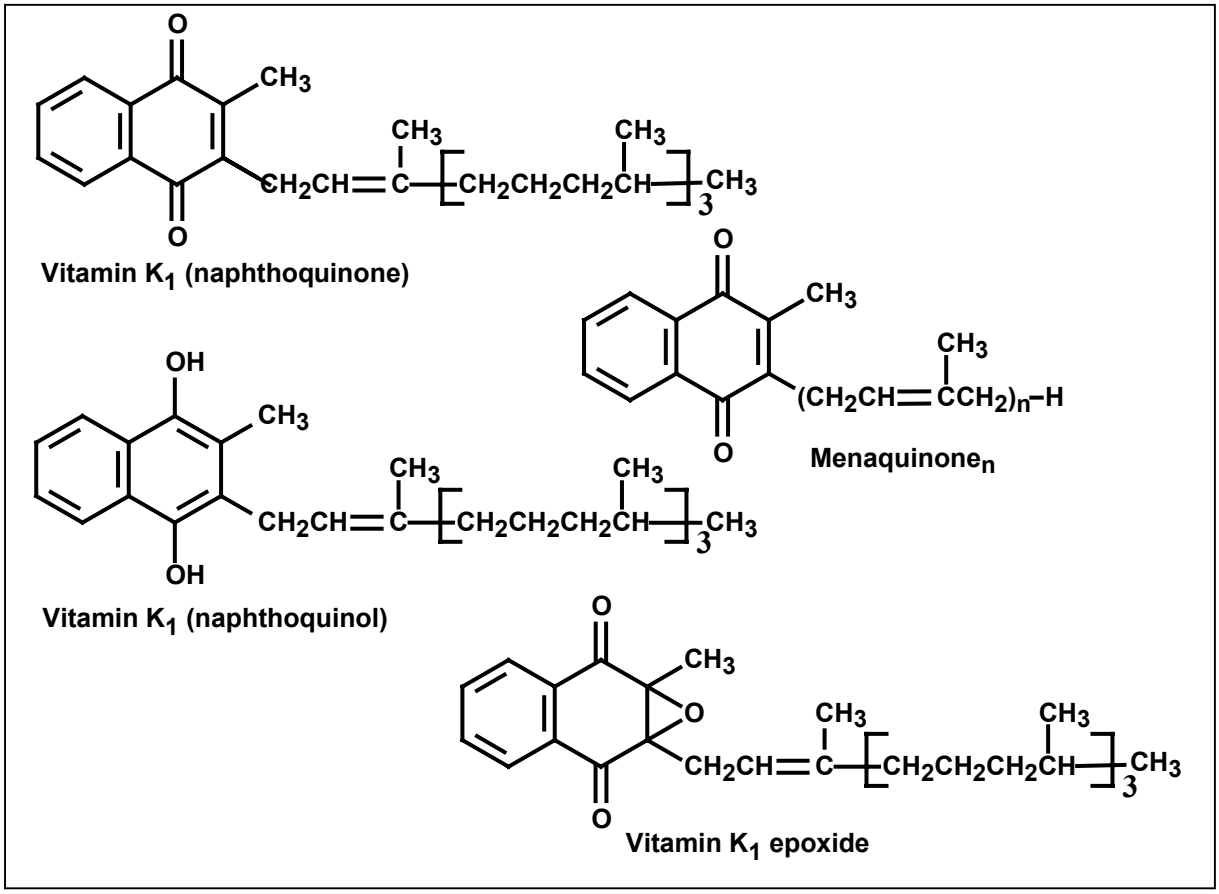


Figure 1.12 The structures of vitamin K_1 naphthoquinone, vitamin K_1 naphthoquinol, vitamin K_1 epoxide, and menaquinone.

The 2-methyl-1,4-naphthoquinone nucleus (common name menadione) does not occur in nature but does possess biological activity in vertebrates because of their ability to add a geranylgeranyl side chain at the 3 position (thus turning it into menaquinone-4). In this way, menadione can be thought of as a provitamin. Menadione, as water soluble salts, is used as a feed supplement in animal husbandry and therefore may indirectly enter the human food chain presumably as preformed menaquinone-4 present in meats.

The body obtains vitamin K_1 from the diet with the majority being obtained from leafy green vegetables and four vegetable oils (soybean, cottonseed, canola and olive) that contain high amounts (163). Menaquinone is present in meats but is also synthesised by bacteria in the colon and absorbed into the body. There is some evidence in rats of tissue-specific formation of menaquinone-4 from vitamin K_1 as a metabolic transformation that does not require bacterial transformation to menadione as an intermediate (164).

1.3.1. Absorption of dietary vitamin K

Dietary vitamin K is absorbed in the proximal intestine by the bile salt-mediated pathway that operates for dietary lipids (165). Upon oral consumption, radiolabelled vitamin K is absorbed from the small intestine, becomes associated with chylomicrons in blood, and disappears from circulation at the same rate as chylomicrons (166). Vitamin K is transported with lipoproteins (167). It has been estimated that healthy adults absorb approximately 80% of an oral dose of vitamin K_1 given in its free form (165).

The liver plays an exclusive role in the metabolic transformations that lead to the excretion of vitamin K from the body. Tracer experiments with labelled vitamin K_1 have shown that a sizeable fraction of a single dose is rapidly catabolised and excreted in either the urine (20% within three days) or bile (40-50%) (165).

1.3.2. Role of vitamin K

The precise function of vitamin K was not discovered until 1974 when Stenflo *et al.,* (1974) (168) isolated the vitamin K-dependent coagulation factor, prothrombin, from cows that had received a high dose of the vitamin K antagonist, warfarin. It was shown that normal prothrombin contained 10 unusual amino acid residues which were identified as γ-carboxyglutamate (Gla). Prothrombin isolated from warfarin-treated cows has normal glutamate (Glu) at the Gla-positions and is designated as descarboxyprothrombin (168). The extra carboxyl group in Gla made clear that vitamin K plays a role in the carboxylation reaction during which Glu is converted into Gla (168).

The essential biochemical role of vitamin K is in the post-translational chemical modification of a group of proteins that have calcium-binding properties collectively called the vitamin K-dependent proteins, or Gla-proteins. These proteins are synthesised in the liver and comprise protease clotting factors II, VII, IX, and X. These factors are procoagulants which arrest and prevent bleeding. Vitamin K acts as a cofactor for the specific carboxylation reaction that transforms selective Glu residues in zymogens to Gla residues, revealing active protease clotting factors. The Gla residues are efficient chelators of calcium ions. In the presence of Gla and calcium ions, these proteins bind to the surface of membrane phospholipids of

platelets and endothelial cells where, together with other cofactors, they form membrane-bound enzyme complexes.

Some vitamin K-dependent proteins are synthesised in other tissues including the bone protein, osteocalcin, and matrix Gla protein, suggesting a link between vitamin K deficiency and bone health. The importance of vitamin K in bone maintenance is revealed by the osteoporosis and fractures resulting from long-term use of warfarin (the mechanism of which is discussed below) which inhibits the bone-building effect of vitamin K (169).

Within the body, vitamin K is cycled between the naphthoquinone, the epoxide and the naphthoquinol forms (Figure 1.13). It is during the conversion between the naphthoquinol and epoxide forms (catalysed by vitamin K γ-glutamyl carboxylase) that Glu residues on zymogens are carboxylated to become Gla residues. Warfarin is a coumarin drug that inhibits the recycling of vitamin K in the body (Figure 1.13). This is achieved by inhibition of the enzymes that catalyse the conversion of vitamin K epoxide to the naphthoquinone (vitamin K epoxide reductase) and which catalyse the reduction of the vitamin K naphthoquinone to the naphthoquinol (vitamin K reductase). Plasma concentrations of phylloquinone epoxide are increased when patients are on warfarin treatment (170), and phylloquinone epoxide is converted to vitamin K_1 via reduction (171). Dietary sources of vitamin K can be converted into the naphthoquinol form via an NADH-dependent pathway, a step that is not inhibited by warfarin.

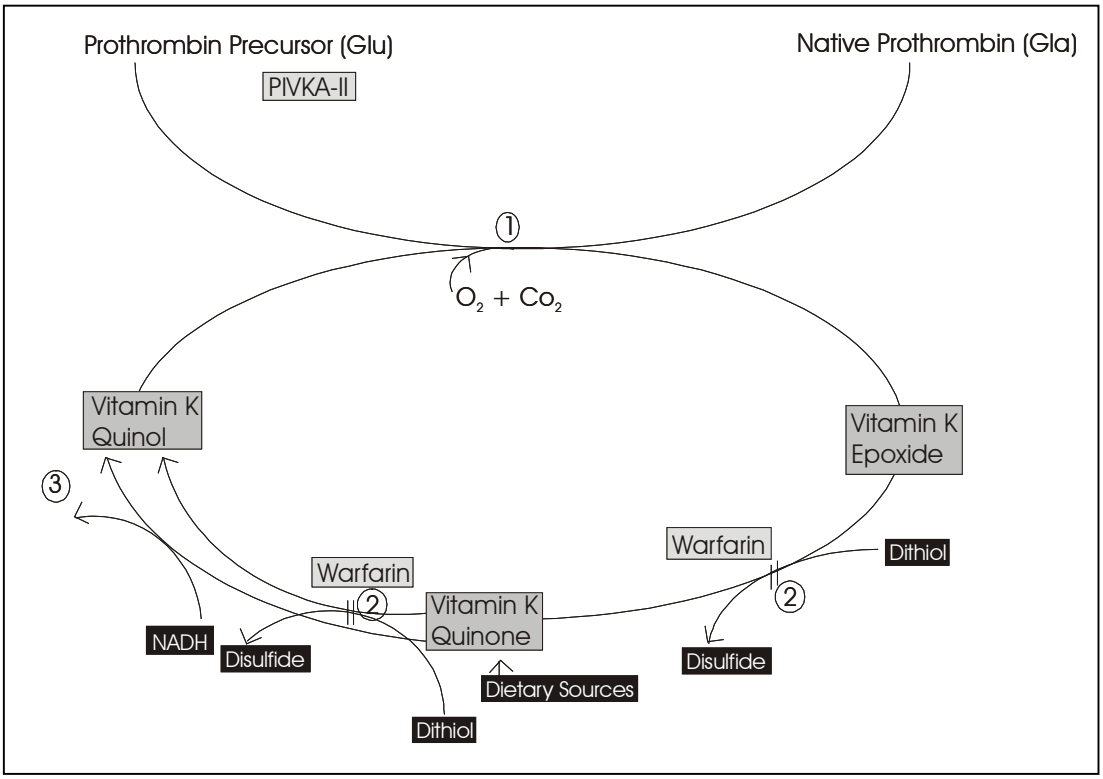


Figure 1.13 The vitamin K epoxide cycle. Enzyme (1) is vitamin K γ-glutamyl carboxylase, (2) is vitamin K epoxide reductase, and (3) is vitamin K reductase. PIVKA II = proteins in vitamin K absence, as described later.

1.3.3. Reference range and factors affecting vitamin K levels

Vitamin K values in a population are distributed log-normally (161, 172) with no significant difference between genders (172). A moderately strong correlation between age and serum or plasma vitamin K has been reported (172). Reference ranges reported for plasma vitamin K vary significantly (Table 1.2). This variation may be due to differences in assays to measure plasma vitamin K or due to genetic or environmental differences. As discussed below, the plasma vitamin K concentration is probably dependent on the diet. Different assays give different estimates of plasma vitamin K because of differences in the extraction and reduction (where fluorescence detection is used) techniques as is discussed in section 1.2.4. There are no clear trends to explain the large range of values for vitamin K shown in Table 1.2, except that the three reports of higher concentrations of plasma vitamin K (161, 173, 174) use photochemical, platinum oxide catalysed, and platinum black catalysed reduction of vitamin K, and not zinc reduction. Whether this is an actual result or an artefact is not clear.

Table 1.2 Reported reference ranges for vitamin K

Publication	Measuring	n	Male/Female (Age (years))	Vitamin K (nmol/L)	Comments
(161)	n.s.	40		5.8 mean 2.0 – 17.3 range	
(175)	K_1	15	10 F 5 M (51-81)	0.83 mean	
(175)	K_1	16	15F 1M (69-86)	0.22 mean	Osteoporotic patients
(171)	K_1	22	6M 16F	1.24 mean 0.20 – 4.70 range	
(175)	K_1	6	M	0.95 mean 0.51 – 2.09 range	
(175)	K_1	16	F	1.55 mean 0.20 – 4.70 range	
(172)	n.s.	50	29M 21F (24-87)	0.55 median 0.14 – 2.17 range	
(172)	n.s.	29	M	0.57 median 0.15 – 2.19 range	
(172)	n.s.	21	F	0.52 median 0.12 – 2.21 range	
(173)	K_1	5	M	6.52 mean 1.55 – 18.4 range	The mean (range) for K_1 in 5 people was 3.55 (1.55 – 6.43) nmol/L, except one person had a value of 18.4 nmol/L
(176)	K_1	298	(7-19)	0.6 mean 0.09-2.22 range	
(177)	K_1	263	M and F (68.5 ± 6.3)	0.7 mean 0.17-2.34 interfractile interval (2.5 – 97.5%)	
(174)	K_1			4.0 ± 2.4 (mean ± SD)	
(174)	K_1			1.38 ± 0.25 (mean ± SD)	Osteoporotic patients treated with menaquinone-4

n.s.= not stated

As stated previously, the actual concentration and form of vitamin K is probably dependent on the diet (173). Vitamin K_1 and menaquinones-4,5,6,7, and 8 are present in plasma, but the common hepatic forms (menaquinones-9-13) are not (173, 174, 178). Longer chain menaquinones, especially menaquinones-10 and menaquinone-11 are found in large amounts

in the healthy human liver (179) and are probably derived from the intestinal flora via the portal vein (179). The absence of these menaquinones in the plasma might be a consequence of a different route of absorption (for example, the possibility of a portal route for long-chain menaquinones versus a lymphatic route for vitamin K_1), but might suggest that, once in a target tissue, the long-chain menaquinones, which are extremely lipophilic, are not easily mobilised (178).

Vitamin K deficiency is not common in healthy adults. However, a deficiency may arise if broad-spectrum antibiotics are consumed. Several mechanisms have been proposed to account for antibiotic-associated hypoprothrombinemia, including eradication of gastrointestinal bacteria, direct inhibition of vitamin K-dependent coagulation, and indirect inhibition of coagulation (180). Deficiency may also occur if absorption of vitamin K_1 from any source is incomplete.

The concentrations of menaquinones-10, 11, and 12 are significantly lower in livers of patients with chronic hepatitis and cirrhosis which may be one cause of haemorrhagic tendency in liver disease (179).

1.3.3.1. Vitamin K in bone health

Vitamin K deficiency has been associated with bone defects. A high prevalence of low vitamin K status (as determined by a high serum undercarboxylated osteocalcin concentration) has been reported in elderly women in Japan (181), but no correlation between low vitamin K status and low bone mineral density was observed. Reduced plasma concentrations of vitamin K_1 and menaquinones-7 and 8 have been reported in patients with recent femoral fractures or prior vertebral compression fractures (182, 183). Serum concentrations of vitamin K_1 and menaquinones-7 and 8 in elderly women after hip fracture were found to be significantly lower than those in age-matched healthy controls (184).

In osteoporotic patients treated with menaquinone-4, plasma menaquinone-4 is significantly elevated and levels of vitamin K_1 and menaquinone-7 are significantly lowered (174).

1.3.3.2. Vitamin K in haemorrhagic disease of the new born

The major concern with vitamin K deficiency is focused on new born babies, where a deficiency of vitamin K can lead to uncontrolled bleeding and haemorrhage. This is commonly called haemorrhagic disease of the new born (HDN). This disease is life-threatening and is the reason infants are often given supplemental vitamin K at birth.

1.3.4. Measurement of vitamin K

For several decades after its discovery in the late 1920s, the vitamin K deficient chick model was the only method for quantifying vitamin K in various foods (185). The chicks were made vitamin K deficient and subsequently fed with known amounts of vitamin K-containing food. The extent to which blood coagulation was restored by the diet was taken as a measure of its vitamin K content.

The concentration of vitamin K in plasma is difficult to assess, partly because vitamin K is present in blood at very low concentrations. Additionally, there are many different forms of vitamin K present in plasma, vitamin K_1, vitamin K_1 epoxide (present at around 32 pmol/L (171)), and menaquinones (present at 0 – 11.4 nmol/L). Most published methods to determine vitamin K in plasma (or serum) use HPLC, with a preliminary extraction of vitamin K from plasma and an extract purification step before analysis (Appendix 3). Extraction procedures commonly use liquid-liquid extraction, followed by solid-phase extraction, and concentration of the sample (172, 186). Detection of vitamin K after HPLC is most commonly achieved using fluorescence detection (161, 170-174, 176, 177, 179, 186-196). Early reports of HPLC measurement of vitamin K utilised ultraviolet detection (161, 197-199), although sensitivity was not high. Fluorescence detection has markedly better selectivity and sensitivity for vitamin K_1 than ultraviolet detection (161). Electrochemical detection has also been utilised for determination of vitamin K after HPLC separation (175, 200, 201). There is one report of the use of gas chromatography to measure vitamin K in plant material after HPLC separation (202), and another reporting the use of GC/MS for measurement of plasma vitamin K after HPLC (203).

Other methods of quantifying vitamin K include conventional coagulation assays (time for blood to clot) to detect overt vitamin K-deficient states (and hence associated risk of uncontrolled bleeding) (204). A more sensitive assay, known as PIVKA II, or proteins in

vitamin K absence, involves immunoassay of under-carboxylated prothrombin (factor II), where an increase is related to the degree of functional vitamin K deficiency (205). Another test for vitamin K status measures urinary Gla, whose excretion is decreased in vitamin K deficiency (206).

1.3.4.1. Reduction

There is a need to reduce vitamin K before fluorescence detection since the naphthoquinone does not fluoresce. The most common reduction method for vitamin K is to use zinc metal in the presence of zinc ions (195) (Equation 1.1).

$$Zn \rightarrow Zn^{2+} + 2e^-$$
$$2H^+ + 2e^- + K_1 \rightarrow K_1H_2 \qquad \textbf{1.1}$$
$$Zn + 2H^+ + K_1 \rightarrow Zn^{2+} + K_1H_2$$

One disadvantage of zinc reduction in a column is that zinc particles in the reducing column are consumed by oxygen and analyte reduction, such that the resulting smaller zinc particles are able to pass through the filter frits on the column, and enter the HPLC system. This limits the useful life of the reducing column and can damage the HPLC system (186).

Vitamin K can be reduced by electrochemical (coulometric) reduction, but the coulometric reduction has low efficiency (approximately 60%) (171), because of residual oxygen. The oxygen can be scrubbed using a zinc metal-packed reactor (171) (Equation 1.2). Removal of oxygen is also essential for elimination of fluorescence quenching.

$$Zn + O_2 + 2H^+ \rightarrow Zn^{2+} + H_2O_2 \qquad \textbf{1.2}$$
$$Zn + H_2O_2 + 2H^+ \rightarrow Zn^{2+} + 2H_2O$$

Vitamin K can also be reduced via platinum catalysed alcohol reduction (1, 173, 174, 179, 186, 193) (Figure 1.14).

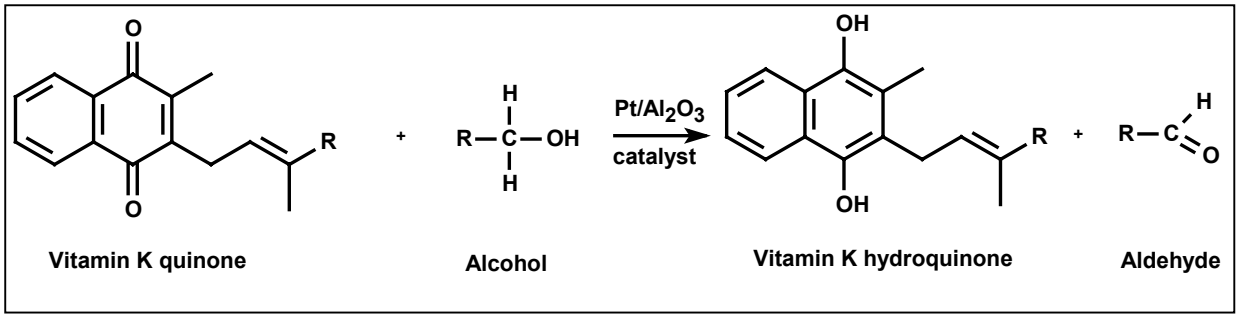


Figure 1.14 The platinum-catalysed reduction of vitamin K by alcohol.

If the reduction of vitamin K by platinum-black catalysed alcohol reduction is shown to be equally or more efficient than that by coulometric or zinc reduction, it should be utilised, since there is no requirement to replace the platinum often, or to include additives in the mobile phase. The assay is therefore simplified.

1.3.4.2. Internal standard

Internal standards are very important in the quantification of plasma vitamin K with HPLC since the recoveries achieved are commonly low and variable (Appendix 3). The internal standard is required to show the same analytical behaviour as the analyte and to not be endogenously present in the specimen being analysed. The internal standards that have been used in vitamin K assays are shown in Table 1.3.

Table 1.3 The internal standards that have been used in vitamin K assays.

Publication	Measuring in	Internal standard
(197)	Human and cow milk	Vitamin K_1 epoxide
(171)	Plasma	Dihydro vitamin K_1
(172)	Plasma	Vitamin $K_{1(25)}$
(173)	Plasma	Menaquinone-3
(186)	Plasma	Menaquinone-4
(176)	Plasma	2′,3′-dihydrophylloquinone

1.3.5. Stability of vitamin K

Vitamin K_1 is stable in breast milk for at least 5 months at –20 ºC (197) and in infant formula in the dark at room temperature for 12 months (197). Boiling does not affect vitamin K_1 in cows milk (197).

Vitamin K is photochemically degraded and K vitamins (menaquinone-3-10) at low concentration (0.1 ng/mL) in clear Pyrex tubes decompose almost completely (3-9% remaining) when allowed to stand in light for 1 day. In contrast, 95% of the K vitamins remain intact in a brown Pyrex tube (173).

Saponification cannot be used when extracting vitamin K because of the instability of vitamin K_1 during alkaline hydrolysis (191).

1.4. Research described in this thesis

The quinones CoQ and vitamin K are of fundamental importance to all life forms specifically because of their electron and proton transfer functions. The initial focus of the work reported in this thesis was to develop an assay to determine vitamin K_1 in plasma of new born babies in order to clarify the mechanisms behind vitamin K and haemorrhagic disease of the new born. The clinical requirements of the vitamin K assay were that:

1) Both plasma vitamin K_1 and menaquinone-4 be quantified.
2) The limit of detection in plasma be lower than 0.2 nmol/L, which represents a low-normal plasma concentration.
3) The coefficient of variation be less than 10% at a plasma concentration of 0.2 nmol/L.
4) The sample volume required for the assay be as low as possible and not more than 500 µL (for a 2.7-3.6 kg baby the recommended maximum blood withdrawal at any one time is 2.5 mL (207) of which approximately 1 mL will be plasma).
5) The assay be as simple, robust, and inexpensive as possible.
6) The assay have the shortest turn-around time possible making it suitable for use in newborns with HDN.

Development of an assay for vitamin K that meets these requirements was initiated by Daines (1) using HPLC with fluorescence detection, and platinum-black catalysed alcohol reduction

of vitamin K. In this thesis (Chapter 7) it is shown that these clinical requirements for a vitamin K assay cannot be met using existing fluorescence technology.

During work on the vitamin K assay, demand from clinicians for an assay to measure plasma CoQ_{10} increased. With the exploding use of statin drugs for cardiovascular disease, the need to measure CoQ_{10} and to investigate the effect of statins on CoQ_{10} increased. Additionally, public knowledge of CoQ_{10} has increased substantially and an Internet search for coenzyme Q can reveal all manner of claims for the health benefits of CoQ_{10} supplementation. Scientific testing of these claims requires a reliable method for measuring CoQ_{10} in plasma.

The work of Daines (2001) (1) provided evidence that fluorescence detection of $CoQ_{10}H_2$ should provide sufficient sensitivity to measure endogenous CoQ_{10} and $CoQ_{10}H_2$ in a single HPLC run, after separation on the analytical column and platinum-black catalysed alcohol reduction.

The clinical requirements of the assay for plasma CoQ_{10} were that the assay:

1) Be capable of measuring total plasma CoQ_{10} and possibly plasma CoQ_{10} and $CoQ_{10}H_2$.
2) Be rapid, reliable, robust and inexpensive.
3) Have a limit of detection corresponding to a plasma concentration of not greater than 0.2 μmol/L.
4) Have a coefficient of variation between assays of less than 10% at the low-normal concentration (0.2 μmol/L).

After development of a suitably validated assay (presented in Chapter 3), clinical biochemical data for CoQ_{10} was required such as the biological variability in healthy individuals and the reference interval in the healthy population. This is presented in Chapter 4. The relative absorption from different CoQ_{10} supplements and the dose-response to a CoQ_{10} supplement are presented in Chapter 5.

1.5. Research hypotheses

The studies described in this thesis form part of the research and development programme of Canterbury Health Laboratories, and were intended to underpin both clinical research and service development. This section outlines research hypotheses that the research group aspires to address. Although only a few of these hypotheses are directly addressed in this

thesis, much of the work described is aimed at developing the tools and background information, that are necessary to test the wider hypotheses.

1.5.1. Clinical research on CoQ

- CoQ_{10} can be limiting in human tissues, and an insufficiency causes cellular dysfunction.
- Muscle tissue, with its high energy requirement, will be particularly susceptible to CoQ_{10} insufficiency.
- CoQ_{10} insufficiency will exacerbate cardiovascular disease.
- Statin therapy can cause CoQ_{10} insufficiency and this is a factor in the muscular side-effects of statin therapy.

1.5.2. Laboratory assessment of CoQ status

- Circulating plasma CoQ_{10} concentrations are a measure of CoQ_{10} sufficiency.
- The healthy New Zealand population has a range of plasma CoQ_{10} concentrations, and it is possible to define a lower limit below which clinical insufficiency can be presumed.
- The intra-individual variation in plasma CoQ is less than the inter-individual variation, and relatively small changes in plasma CoQ concentrations are clinically significant.

1.5.3. Clinical research on vitamin K

- Some groups in the New Zealand population have a sub-clinical deficiency of vitamin K.
- High risk groups include the newborn, lactating mothers and the elderly.

1.5.4. Laboratory assessment of vitamin K status

- Vitamin K is present as multiple active species and measuring one of these is not a sufficient guide to vitamin K status.
- Vitamin K status may be better assessed by measuring metabolic markers of vitamin K status rather than the vitamin itself.

1.5.5. Analytical development

- Coenzyme Q_{10} and vitamin K concentrations can be determined by measuring the ultraviolet absorbance of the quinone forms, the fluorescence of the quinol forms, or their redox properties.
- The ultraviolet absorbance of the quinones will not provide sufficient sensitivity for measuring plasma concentrations of vitamin K_1, CoQ_{10} and $CoQ_{10}H_2$ in human samples, but fluorescence detection will provide the necessary sensitivity.
- Catalytic reduction using an in-line platinum-black reactor in an HPLC system will convert the quinones to quinols more efficiently and robustly than alternative techniques in common use.
- Electrochemical detection is an alternative sensitive method for measuring CoQ and vitamin K.

1.6. References for Chapter 1

1. Daines AM. New Assays for Biologically Active Quinones. PhD Thesis. Christchurch: University of Canterbury, 2001.
2. Woskresensky A. Uber die zusammensetzung der chinasäure. Justus Liebigs Annalen der Chemie 1838;27:257-70.
3. Crane FL, Hatefi Y, Lester RI, Widmer C. Isolation of a quinone from beef heart mitochondria. Biochimica et Biophysica Acta 1957;25:220-1.
4. Okamoto T, Fukui K, Nakamoto M, Kishi T, Okishio T, Yamagami T, et al. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. Journal of Chromatography 1985;342:35-46.
5. Lass A, Sohal RS. Electron transport-linked ubiquinone-dependent recycling of α-tocopherol inhibits autooxidation of mitochondrial membranes. Archives of Biochemistry and Biophysics 1998;352:229-36.
6. Tang PH, Miles, M.V., DeGrauw, A., Hershey, A., Pesce, A. HPLC analysis of reduced and oxidised coenzyme Q10 in human plasma. Clinical Chemistry 2001;47:256-65.
7. Åberg F, Appelkvist E-L, Dallner G, Ernster L. Distribution and redox state of ubiquinones in rat and human tissues. Archives of Biochemistry and Biophysics 1992;295:230-4.
8. Stocker R, Bowry VW, Frei B. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α-tocopherol. Proceedings of the National Academy of Sciences of the United States of America 1991;88:1646-50.
9. Dudman NPB, Wikken DEL, Stocker R. Circulating lipid hydroperoxide levels in human hyperhomocysteinemia: relevance to development of arteriosclerosis. Arteriosclerosis and Thrombosis 1993;13:512-.
10. Crane FL. Biochemical functions of coenzyme Q_{10}. Journal of the American College of Nutrition 2001;20:591-8.
11. Miles MV, Horn PS, Tang PH, Morrison JA, Miles L, DeGrauw T, Pesce AJ. Age-related changes in plasma coenzyme Q_{10} concentrations and redox state in apparently healthy children and adults. Clinica Chimica Acta 2004;347:139-44.

12. Kontush A, Reich A, Baum K, Spranger T, Finckh B, Kohlschütter A, Beisiegel U. Plasma ubiquinol-10 is decreased in patients with hyperlipidaemia. Atherosclerosis 1997;129:119-26.
13. Lenaz G, Bovina C, D'Aurelio M, al e. Role of mitochondria in oxidative stress and aging. Annals of the New York Academy of Sciences 2002;959:199-213.
14. Kucharska J, Govozdjakova A, Stefek M, Sotnikova R, Sumbalova Z. Adaptive changes of antioxidant status in development of experimental diabetes. Bratislavske Lekarske Listy 2001;102:515-9.
15. Palmeira CM, Santos DL, Seica R, Moreno AJ, Santos MS. Enhanced mitochondrial testicular antioxidant capacity in Goto-Kakizaki rats: role of coenzyme Q. American Journal of Physiology Cell Physiology 2001;281:c1023-c8.
16. Ferreira FM, Seica R, Oliveira PJ, al e. Diabetes induces metabolic adaptations in rat liver mitochondria: role of coenzyme Q and cardiolipin contents. Biochimica et Biophysica Acta 2003;1639:113-20.
17. Takada M, Ikenoya S, Yuzuriha T, Katayama K. Simultaneous determination of reduced and oxidised ubiquinones. Methods in Enzymology 1984;105:147-55.
18. Edlund PO. Determination of Coenzyme Q_{10}, α-tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. Journal of Chromatography 1988;425:87-97.
19. Grossi G, Bargossi AM, Fiorella PL, Piazzi S, Battino M, Bianchi GP. Improved high performance liquid chromatographic method for the determination of coenzyme Q_{10} in plasma. Journal of Chromatography 1992;593:217.
20. Wakabayashi H, Yamato S, Nakajima M, Shimada K. Simultaneous determination of oxidized and reduced Coenzyme Q and α-Tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. Biological and Pharmaceutical Bulletin 1994;17:997-1002.
21. Finckh B, Kontush A, Commentz J, Hübner C, Burdelski M, Kohlschütter A. Monitoring of Ubiquinol-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Analytical Biochemistry 1995;232:210-6.
22. Lagendijk J, Ubbink JB, Delport R, Hayward WJ, Human JA. Measurement of the ratio between the reduced and oxidized forms of coenzyme Q10 in human plasma as a possible marker of oxidative stress. Journal of Lipid Research 1996;37:67-75.

23. Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Analytical Biochemistry 1997;250:66-73.
24. Kaikkonen J, Nyyssönen K, Salonen JT. Measurement and stability of plasma reduced, oxidised and total coenzyme Q10 in humans. Scandinavian Journal of Clinical and Laboratory Investigation 1999;59:457-66.
25. Wang Q, Lee BL, Ong CN. Automated high-performance liquid chromatographic method with precolumn reduction for the determination of ubiquinol and ubiquinone in human plasma. Journal of Chromatography B 1999;726:297-302.
26. Mitchell P. Possible molecular mechanisms of the protonmotive function of cytochrome systems. Journal of Theoretical Biology 1976;62:327-67.
27. Mitchell P. The vital protonmotive role of coenzyme Q. In: Folkers K, Littarru GP, Yamagami T, eds. Biomedical and Clinical Aspects of Coenzyme Q, Vol. 6: Elsevier, Amsterdam, 1991:3-10.
28. Garrett RH, Grisham CM. Biochemistry. Second Edition ed: Saunders College Publishing, 1995.
29. Gille L, Nohl H. The existence of a lysosomal redox chain and the role of ubiquinone. Archives of Biochemistry and Biophysics 2000;375:347-54.
30. Nohl H, Gille L. Lysosomal ROS formation. Redox Report : Communications in Free Radical Research 2005;10:199-205.
31. Kaikkonen J, Tuomainen TP, Nyyssönen K, Salonen JT. Coenzyme Q10: Absorption, antioxidative properties, determinants, and plasma levels. Free Radical Research 2002;36:389-97.
32. Kaplan P, Sebestianová N, Turiaková J, Kucera I. Determination of Coenzyme Q in human plasma. Physiological Research 1995;45:39-45.
33. Kaikkonen J, Nyyssönen K, Tuomainen TP, Ristonmaa U, Salonen JT. Determinants of plasma coenzyme Q_{10} in humans. FEBS Letters 1999;443:163-6.
34. Lu W-L, Zhang Q, Lee H-S, Zhou T-Y, Sun H-D, Zhang D-W, et al. Total Coenzyme Q10 concentrations in Asian men following multiple oral 50-mg doses administered as coenzyme Q10 sustained release tablets or regular tablets. Biological and Pharmaceutical Bulletin 2003;26:52-5.
35. Jiang P, Wu M, Zeheng Y, Wang C, Li Y, Xin J, Xu G. Analysis of coenzyme Q10 in human plasma by column-switching liquid chromatography. Journal of Chromatography B 2004;805:297-301.

36. Kamzalov S, Sumien N, Forster MJ, Sohal RS. Coenzyme Q intake elevates the mitochondrial and tissue levels of coenzyme Q and α-tocopherol in young mice. Journal of Nutrition 2003;133:3175-80.
37. Zhang Y, Turunen M, Appelkvist E-L. Restricted uptake of dietary coenzyme Q is in contrast to the unrestricted uptake of alpha-tocopherol into rat organs and cells. The Journal of Nutrition 1996;126:2089-97.
38. Galinier A, Carrière A, Fernandez Y, Bessac AM, Caspar-Bauguil S, Periquet B, et al. Biological validation of coenzyme Q redox state by HPLC-EC measurement: relationship between coenzyme Q redox state and coenzyme Q content in rat tissues. FEBS Letters 2004;578:53-7.
39. Tang PH, Miles MV, Miles L, Quinlan J, Wong B, Wenisch A, Bove K. Measurement of reduced and oxidized coenzyme Q_9 and coenzyme Q_{10} levels in mouse tissues by HPLC with coulometric detection. Clinica Chimica Acta 2004;341:173-84.
40. Boveris A, Cadenas E, Stoppani AOM. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. The Biochemical Journal 1976;156:435-44.
41. Mellors A, Tappel AL. Quinones and quinols as inhibitors of lipid peroxidation. Lipids 1966;1:282-4.
42. Dallner G, Sindelar PJ. Regulation of ubiquinone metabolism. Free Radical Biology and Medicine 2000;29:285-94.
43. Wolters M, Hahn A. Plasma ubiquinone status and response to six-month supplementation combined with multivitamins in healthy elderly women - results of a randomized, double blind, placebo-controlled study. International Journal of Vitamin and Nutrition Research 2003;73:207-14.
44. Stoyanovsky DA, Osipov AN, Quinn PJ, Kagan VE. Ubiquinone-dependent recycling of vitamin E radicals by superoxide. Archives of Biochemistry and Biophysics 1995;323:343-51.
45. Kagan V, Serbinova E, Packer L. Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. Biochemical and Biophysical Research Communications 1990;169:851-7.
46. Lass A, Forster MJ, Sohal RS. Effects of coenzyme Q_{10} and α-tocopherol administration on their tissue levels in the mouse: Elevation of mitochondrial α-tocopherol by coenzyme Q_{10}. Free Radical Biology and Medicine 1999;26:1375-82.

47. Matthews RT, Yang L, Browne S, Baik M, Beal MF. Coenzyme Q_{10} administration increases brain mitochondrial concentrations and exerts neuroprotective effects. Proceedings of the National Academy of Sciences of the United States of America 1998;95:8892-7.
48. Kaikkonen J, Nyyssönen K, Porkkala-Sarataho E, Poulsen HE, Metsä-Ketelä T, Hayn M, et al. Effect of oral coenzyme Q10 supplementation on the oxidation resistance of human VLDL + LDL fraction: Absorption and antioxidative properties of oil and granule-based preparations. Free Radical Biology and Medicine 1997;22:1195-202.
49. Ibrahim WH, Bhagavan HN, Chopra RK, Chow CK. Dietary coenzyme Q10 and vitamin E alter the status of these compounds in rat tissues and mitochondria. The Journal of Nutrition 2000;130:2343-8.
50. Tomasetti M, Littarru GP, Stocker R, Alleva R. Coenzyme Q10 enrichment decreases oxidative DNA damage in human lymphocytes. Free Radical Biology and Medicine 1999;27:1027-32.
51. Menke T, Niklowitz P, de Sousa G, Reinehr T, Andler W. Comparison of coenzyme Q10 plasma levels in obese and normal weight children. Clinica Chimica Acta 2004;349:121-7.
52. Willis R, Anthony M, Sun L, Honse Y, Qiao G. Clinical implications of the correlation between coenzyme Q_{10} and vitamin B_6 status. BioFactors 1999;9:359-63.
53. Yuzuriha T, Takada M, Katayama K. Transport of [14C]coenzyme Q10 from the liver to other tissues after intravenous administration to guinea pigs. Biochimica et Biophysica Acta 1983;759:286-91.
54. Tomono Y, Hasegawa J, Seki T, Motegi K, Morista N. Pharmacokinetic study of deuterium-labelled CoQ in man. International Journal of Clinical Pharmacology, Therapy and Toxicology 1986;24:536-41.
55. Bhagavan HN, Chopra RK. Coenzyme Q10: absorption, tissue uptake, metabolism and pharmacokinetics. Free Radical Research 2006;40:445-53.
56. Laaksonen R, Riihimäki A, Laitila J, Mårtensson K, Tikkanen MJ, Himberg JJ. Serum and muscle tissue ubiquinone levels in healthy subjects. Journal of Laboratory and Clinical Medicine 1995;125:517-21.
57. Laaksonen R, Jokelainen K, Sahi T, Tikkanen MJ, Himberg J-J. Decreases in serum ubiquinone concentrations do not result in reduced levels in muscle tissue during short-term Simvastatin treatment in humans. Clinical Pharmacology and Therapeutics 1995;57:62-6.

58. Mohr D, Bowry VW, Stocker R. Dietary supplementation with coenzyme Q_{10} results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation. Biochimica et Biophysica Acta 1992;1126:247-54.
59. Niklowitz P, Menke T, Andler W, Okun JG. Simultaneous analysis of coenzyme Q10 in plasma, erythrocytes and platelets: comparison of the antioxidant level in blood cells and their environment in healthy children and after oral supplementation in adults. Clinica Chimica Acta 2004;342:219-26.
60. Feigin A, Kieburtz K, Como P, Kickey K, Claude K, Abwender D, et al. Assessment of coenzyme Q10 tolerability in huntington's disease. Movement Disorders 1996;11:321-3.
61. Weber C, Bysted A, Hølmer G. The Coenzyme Q10 content of the average Danish diet. International Journal of Vitamin Nutrition Research 1997; 67:123-9.
62. Weis M, Mortensen SA, Rassing MR, Møller-Sonnergaard J, Poulsen G, Rasmussen SN. Bioavailability of four oral coenzyme Q_{10} formulations in healthy volunteers. Molecular Aspects of Medicine 1994;15:s273-s80.
63. Kurowska EM, Dresser G, Deutsch L, Bassoo E, Freeman DJ. Relative bioavailability and antioxidant potential of two coenzyme Q_{10} preparations. Annals of Nutrition and Metabolism 2003;47:16-21.
64. Miles MV, Horn P, Miles L, Tang P, Steele P, DeGrauw T. Bioequivalence of coenzyme Q10 from over-the-counter supplements. Nutrition Research 2002;22:919-29.
65. Chopra RK, Goldman R, Sinatra ST, Bhagavan HN. Relative bioavailability of coenzyme Q10 formulations in Human subjects. International Journal of Vitamin and Nutrition Research 1998;68:109-13.
66. Wahlqvist ML, Wattanapenpaiboon N, Savige GS, Kannar D. Bioavailability of two different formulations of coenzyme Q_{10} in healthy subjects. Asia Pacific Journal of Clinical Nutrition 1998;7:37-40.
67. Zita C, Overvad K, Mortensen SA, Sindberg CD, Moesgaard S, Hunter DA. Serum coenzyme Q_{10} concentrations in healthy men supplemented with 30 mg or 100 mg coenzyme Q_{10} for two months in a randomised controlled study. BioFactors 2003;18:185-93.

68. Turunen M, Appelkvist E-L, Sindelar P, Dallner G. Blood concentration of coenzyme Q_{10} increases in rats when esterified forms are administered. The Journal of Nutrition 1999;129:2113-8.
69. Kishi H, Kanamori N, Nishii S, Hiraoka E, Okamoto T, Kishi T. In: Biomedical and Clinical Aspects of Coenzyme Q, K Folkers and Y Yamamura, (eds) Elsevier Science, Amsterdam 1984;Chapter 4:131-42.
70. Ozawa Y, Mizushima Y, Koyama I, Akimato M, Yamagata Y, Hayashi H, Murayama H. Intestinal absorption enhancement of CoQ with a lipid microsphere. Arzneim Forsch Drug Res 1986;36:4689-90.
71. Gibaldi M. Biopharmaceutics and clinical pharmacokinetics 3rd edn Lea & Febiger, Philadelphia, PA, USA, 1984.
72. Bateman NE, Uccelline DA. Effect of formulation on the bioavailability of retinol, D-alpha-tocopherol and riboflavine. The Journal of Pharmacy and Pharmacology 1984;36:461-4.
73. Pozzi F, Longo A, Lazzarini C, Carenzi A. Formulations of ubiquinone with improved bioavailability. European Journal of Pharmaceutics and Biopharmaceutics 1991;37:243-6.
74. Passi S, De Pità O, Grandinetti M, Simotti C, Littarru GP. The combined use of oral and topical lipophilic antioxidants increases their levels both in sebum and stratum corneum. BioFactors 2003;18:289-97.
75. Walhlqvist ML, Wattanapenpaiboon N, Savige GS, Kannar D. Bioavailability of two different formulations of coenzyme Q_{10} in healthy subjects. Asia Pacific Journal of Clinical Nutrition 1998;7:37-40.
76. Krone CA, Elmer GW, Ely JTA, Fundenberg HH, Thoreson J. Does gastrointestinal *Candida albicans* prevent ubiquinone absorption? Medical Hypotheses 2001;57:570-2.
77. Smith RAJ, Kelso GF, James AM, Murphy MP. Targeting coenzyme Q derivatives to mitochondria. Methods in Enzymology 2004;382:45-67.
78. Smith RAJ, Porteous CM, Gane AM, Murphy MP. Delivery of bioactive molecules to mitochondria *in vivo.* Proceedings of the National Academy of Sciences of the United States of America 2003;100:5407-12.
79. Rötig A, Appelkvist E-L, Geromel V, Chretein D, Kadhom N, Edery P, et al. Quinone-responsive multiple respiratory-chain dysfunction due to widespread coenzyme Q10 deficiency. The Lancet 2000;356:391-5.

80. Kwong LK, Kamzalov S, Rebrin I, Bayne A-CV, Jana CK, Morris P, et al. Effects of coenzyme Q_{10} administration on its tissue concentrations, mitochondrial oxidant generation, and oxidative stress in the rat. Free Radical Biology and Medicine 2002;33:627-38.
81. Zhang Y, Åberg F, Appelkvist E-L, Dallner G, Ernster L. Uptake of dietary coenzyme Q supplement is limited in rats. The Journal of Nutrition 1995;125:446-53.
82. Beal MF, Matthews RT. Coenzyme Q_{10} in the central nervous system and its potential usefulness in the treatment of neurodegenerative diseases. Molecular Aspects of Medicine 1997;18:s169-s79.
83. Bentinger M, Dallner G, Chojnacki T, Swiezewska E. Distribution and breakdown of labelled coenzyme Q_{10} in rat. Free Radical Biology and Medicine 2003;34:563-75.
84. Forsmark-Andrée P, Lee C-P, Dallner G, Ernster L. Lipid peroxidation and changes in the ubiquinone content and the respiratory chain enzymes of submitochondrial particles. Free Radical Biology and Medicine 1997;22:391-400.
85. Ogasahara S, Engel AG, Frens D, Mack D. Muscle coenzyme Q deficiency in familial mitochondrial encephalomyopathy. Proceedings of the National Academy of Sciences of the United States of America 1989;86:2379-82.
86. Musumeci O, Naini A, Slonim AE, Skavin N, Hadjigeorgiou GL, Krawiecki N, et al. Familial cerebellar ataxia with muscle coenzyme Q10 deficiency. Neurology 2001;56:849-55.
87. Miles MV, Horn PS, Morrison JA, Tang P, DeGrauw T, Pesce AJ. Plasma coenzyme Q_{10} reference intervals, but not redox status, are affected by gender and race in self-reported healthy adults. Clinica Chimica Acta 2003;332:123-32.
88. Nagashima K, Yamasawa I, Kamohara S, Shiota M, Komori T, Watanabe Y, et al. Changes regarding age and correlations between serum lipids and body mass index in humankind. Methods of Information in Medicine 2002;41:202-8.
89. Wakabayashi I. Relationships of body mass index with blood pressure and serum cholesterol concentrations at different ages. Aging Clinical and Experimental Research 2004;16:461-6.
90. Miles MV, Morrison JA, Horn P, Tang P, Pesce AJ. Coenzyme Q10 changes are associated with metabolic syndrome. Clinica Chimica Acta 2004;344:173-9.
91. Komorowski, Muratsu K, Nara, Willis, Folkers K. Significance of biological parameters of human blood levels of CoQ10. Biofactors 1988;1:67-9.

92. Pedersen, Mortensen, Rohde, Deguchi, Mulved, Bjerregaard, Hansen. High serum coenzyme Q10, positively correlated with age, selenium, and cholesterol, in Inuit of Greenland. A pilot study. Biofactors 1999;9:319-23.
93. Artuch R, Moreno J, Quintana M, Puig RM, Vilaseca MA. Serum ubiquinone-10 in a pediatric population. Clinical Chemistry 1998;44:2378-9.
94. Hara K, Yamashita S, Fujisawa A, Ishiwa S, Ogawa T, Yamamoto Y. Oxidative stress in newborn infants with and without asphyxia as measured by plasma antioxidants and free fatty acids. Biochemical and Biophysical Research Communications 1999;257:244-8.
95. Zhang Y, Appelkvist E-L, Kristensson K, Dallner G. The lipid compositions of different regions of rat brain during development and aging. Neurobiology of Aging 1996;17:869-75.
96. Söderberg M, Edlund C, Kristensson K, Dallner G. Lipid compositions of different regions of the human brain during aging. Journal of Neurochemistry 1990;54:415-23.
97. Bianchi, Fiorella, Bargossi, Grossi, Marchesini. Reduced ubiquinone plasma levels in patients with liver cirrhosis and in chronic alcoholics. Liver 1994;14:138-40.
98. Gohil K, Rothfuss L, Lang J, Packer L. Effect of exercise training on tissue vitamin E content. Journal of Applied Physiology 1987;63:1638-41.
99. Stamler J, Daviglus ML, Garside DB, Dyer AR, Greenland P, Neaton JD. Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. JAMA 2000;284:311-8.
100. Grundy SM, Bilheimer DW. Inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase by mevinolin in familial hypercholesterolemia heterozygotes: Effects on cholesterol balance. Proceedings of the National Academy of Sciences of the United States of America 1984;81:2538-42.
101. Bilheimer DW, Grundy SM, Brown MS, Goldstein JL. Mevinolin and colestipol stimulate receptor-mediated clearance of low density lipoprotein from plasma in familial hypercholesterolemia heterozygotes. Proceedings of the National Academy of Sciences of the United States of America 1983;80:4124-8.
102. Davignon J, Laaksonen R. Low-density lipoprotein-independent effects of statins. Current opinion in Lipidology 1999;10:543-59.

103. Folkers K, Langsjoen P, Willis R, Richardson P, Xia L-J, Ye C-Q, Tamagawa H. Lovastatin decreases coenzyme Q levels in humans. Proceedings of the National Academy of Sciences of the United States of America 1990;87:8931-4.
104. Watts GF, Castelluccio C, Rice-Evans C, Taub NA, Baum H, Quinn PJ. Plasma coenzyme Q (ubiquinone) concentrations in patients treated with Simvastatin. Journal of Clinical Pathology 1993;46:1055-7.
105. Ghirlanda G, Oradei A, Manto A, Lippa S, Ucciolo L, Caputo S, et al. Evidence of plasma CoQ10-lowering effect by HMG-CoA reductase inhibitors: A double-blind, placebo-controlled study. The Journal of CLinical Pharmacology 1993;33:226-9.
106. Laaksonen R, Ojala J-P, Tikkanen MJ, Himberg J-J. Serum ubiquinone concentrations after short- and long-term treatment with HMG-CoA reductase inhibitors. European Journal of Clinical Pharmacology 1994;46:313-7.
107. De Pinieux G, Chariot P, Ammi-saïd M, Louarn F, Lejonc JL, Astier A, et al. Lipid-lowering drugs and mitochondrial function: effects of HMG-CoA reductase inhibitors on serum ubiquinone and blood lactate/pyruvate ratio. British Journal of Clinical Pharmacology 1996;42:333-7.
108. Mortensen SA, Leth A, Agner E, Rohde M. Dose-related decrease of serum coenzyme Q10 during treatment with HMG-CoA reductase inhibitors. Molecular Aspects of Medicine 1997;18(s):s137-s44.
109. Elmberger PG, Kalèn A, Lund E, Reihnér E, Eriksson M, Berglund L, et al. Effects of Pravastatin and cholestyramine on products of the mevalonate pathway in familial hypercholesterolemia. Journal of Lipid Research 1991;32:935-40.
110. Rousseau G, DesRosiers C, Varin F. A comparison of the effects of Lovastatin and Pravastatin on ubiquinone tissue levels in rats. Current Therapeutic Research 1998;59:666-79.
111. Rousseau G, Véronneau M, DesRosiers C, Varin F. Effects of lovastatin and pravastatin on ubiquinone and 4-hydroxynonenal tissue levels in the hypercholesterolemic hamster. Current Therapeutic Research 1999;60:87-104.
112. Scott RS, Lintott CJ, Wilson MJ. Simvastatin and side effects. The New Zealand Medical Journal 1991;104:493-5.
113. Folkers K, Osterborg A, Nylander M, Morita M, Mellstedt H. Activities of vitamin Q10 in animal models and a serious deficiency in patients with cancer. Biochemical and Biophysical Research Communications 1997;234:296-9.

114. Siciliano G, Mancuso M, Tedeschi D, Manca ML, Renna MR, Lombardi V, et al. Coenzyme Q10 , exercise lactate and CTG trinucleotide expansion in myotonic dystrophy. Brain Research Bulletin 2001;56:405-10.
115. Butler MG, Dasouki M, Bittel D, Hunter S, Naini A, DiMauro S. Coenzyme Q10 levels in Prader-Willi syndrome: Comparison with obese and non-obese subjects. American Journal of Medical Genetics Part A 2003;119A:168-71.
116. Triolo L, Lippa S, Oradei A, De Sole P, Mori R. Serum coenzyme Q10 in uremic patients on chronic hemodialysis. Nephron 1994;66:153-6.
117. Artuch R, Vilaseca MA, Moreno AJ, Lambruschini N, Cambra FJ, Campistol J. Decreased serum ubiquinone-10 concentration in phenylketonuria. The American Journal of Clinical Nutrition 1999;70:892-5.
118. Götz ME, Gerstner A, Harth R, Dirr A, Janetzky B, Kuhn W, et al. Altered redox state of platelet coenzyme Q10 in Parkinson's disease. Journal of Neural Transmission 2000;107:41-8.
119. Tomasetti M, Alleva R, Solenghi MD, Littarru GP. Distribution of antioxidants among blood components and lipoproteins: significance of lipids/CoQ10 ratio as a marker of increased risk for atherosclerosis. Biofactors 1999;9:231-40.
120. Folkers K. Relevance of the biosynthesis of coenzyme Q10 and of the four bases of DNA as a rational for the molecular causes of cancer and a therapy. Biochemical and Biophysical Research Communications 1996;224:358-61.
121. Lockwood K, Moesgaard S, Folkers K. Partial and complete regression of breast cancer in patients in relation to dosage of coenzyme Q10. Biochemical and Biophysical Research Communications 1994;199:1504-8.
122. Lockwood K, Moesgaard S, Yamamoto T, Folkers K. Progress on therapy of breast cancer with vitamin Q10 and the regression of metastases. Biochemical and Biophysical Research Communications 1995;212:172-7.
123. McDonnell M, Archbold G. Plasma ubiquinol/cholesterol ratios in patients with hyperlipidaemia, those with diabetes mellitus and in patients requiring dialysis. Clinica Chimica Acta 1996;253:117-26.
124. Kamikawa T, Kobayashi A, Yamashita T, Hayashi H, Yamazaki N. Effects of coenzyme Q10 on exercise tolerance in chronic stable angina pectoris. The American Journal of Cardiology 1985;56:247-51.

125. Lodi R, Hart PE, Rajagopalan B, Taylor DJ, Crilley GJ, Bradley JL, et al. Antioxidant treatment improves in vivo cardiac and skeletal muscle bioenergetics in patients with Friedreich's ataxia. Annals of Neurology 2001;49:590-6.
126. Ogasahara S, Yorifuji S, Nishikawa Y, Takahashi M, Wada K, Hazama T, et al. Improvement of abnormal pyruvate metabolism and cardiac conduction defect with coenzyme Q10 in Kearns-Sayre syndrome. Neurology 1985;35:372-7.
127. Müller T, Büttner T, Gholipour A-F, Kuhn W. Coenzyme Q10 supplementation provides mild symptomatic benefit in patients with Parkinson's disease. Neuroscience Letters 2003;341:201-4.
128. Balercia G, Mosca F, Mantero F, Boscaro M, Mancini A, Ricciardo-Lamonica G, Littarru GP. Coenzyme Q10 supplementation in infertile men with idiopathic asthenozoospermia: an open, uncontrolled pilot study. Fertility and Sterility 2004;81:93-8.
129. Yamagami T, Shibata N, Folkers K. Bioenergetics in clinical medicine. VIII. Adminstration of coenzyme Q10 to patients with essential hypertension. Research Communications in Chemical Pathology and Pharmacology 1976;14:721-7.
130. Singh R, Niaz MA, Rastogi SS, Shukla PK, Thakur AS. Effect of hydrosoluble coenzyme Q10 on blood pressures and insulin resistance in hypertensive patients with coronary artery disease. Journal of Human Hypertension 1999;13:203-8.
131. Langsjoen P, Willis R, Folkers K. Treatment of essential hypertension with coenzyme Q_{10}. Molecular Aspects of Medicine 1994;15:s265-s72.
132. Hanaki Y, Sugiyama S, Ozawa T, Ohno M. Ratio of low-density-lipoprotein cholesterol to ubiquinone as a coronary risk factor. The New England Journal of Medicine 1991;325:814.
133. Yalcin A, Kilinc E, Sagcan A, Kultursay H. Coenzyme Q10 concentrations in coronary artery disease. Clinical Biochemistry 2004;37:706-9.
134. Hofman-Bang C, Rehnqvist N, Swedberg K, Wiklund I, Åström H. Coenzyme Q_{10} as an adjunctive in the treatment of chronic congestive heart failure. Journal of Cardiac Failure 1995;1:101-6.
135. Morisco C, Trimarco B, Condorelli M. Effect of coenzyme Q10 therapy in patients with congestive heart failure: a long-term multicenter randomised study. The Clinical Investigator 1993;71:S134-S6.
136. Langsjoen P, Vadhanavikit S, Folkers K. Response of patients in classes III and IV of cardiomyopathy to therapy in a blind and crossover trial with coenzyme Q_{10}.

Proceedings of the National Academy of Sciences of the United States of America 1985;82:4240-4.

137. Judy WV, Hall JH, Toth PD, Folkers K. Double blind-double crossover study of coenzyme Q10 in heart failure. Biomedical and Clinical Aspects of Coenzyme Q 1986;5:315-23.

138. Mortensen SA, Vadhanavikit S, Muratsu K, Folkers K. Coenzyme Q10: Clinical benefits with biochemical correlates suggesting a scientific breakthrough in the management of chronic heart failure. International Journal of Tissue Reactions 1990;XII:155-62.

139. Rossi E, Lombardo A, Testa M, Lippa S, Oradei A, Littarru GP, et al. Coenzyme Q10 in ischaemic cardiopathy. In: Folkers K, Yamamura Y, Littarru GP, eds. Biomedical and Clinical Aspects of Coenzyme Q, Vol. 6: Elsevier, 1991:321-6.

140. Poggesi L, Galanti G, Comeglio M, Toncelli L, Vinci M. Effect of coenzyme Q10 on left ventricular function in patients with dilative cardiomyopathy. A medium-term randomised double-blind study versus placebo. Current Therapeutic Research 1991;49:878-86.

141. Schneeberger W, Müller-Steinwachs J, Anda LP, Fuchs W, Zilliken F, Lyson K, et al. A clinical double blind and crossover trial with coenzyme Q10 on patients with cardiac disease. Biomedical and Clinical Aspects of Coenzyme Q 1986;5:325-33.

142. Vanfraechem JHP, Picalausa C, Folkers K. Effects of CoQ10 on physical performance and recovery in myocardial failure. Biomedical and Clinical Aspects of Coenzyme Q 1986;5:371-7.

143. Baggio E, Gandini R, Plancher AC, Passeri M, Carmosino G, Investigators. OtbotCds. Italian multicenter study on the safety and efficacy of coenzyme Q10 as an adjunctive therapy in heart failure. Molecular Aspects of Medicine 1994;15:s287-s94.

144. Watson PS, Scalia GM, Galbraith A, Burstow DJ, Bett N, Aroney CN. Lack of effect of coenzyme Q on left ventricular function in patients with congestive heart failure. Journal of the American College of Cardiology 1999;33:1549-52.

145. Khatta M, Alexander BS, Krichten CM, Fisher ML, Freudenberger R, Robinson SW, Gottlieb SS. The effect of coenzyme Q_{10} in patients with congestive heart failure. Annals of Internal Medicine 2000;132:636-40.

146. Permanetter B, Rössy W, Klein G, Weingartner F, Seidl KF, Blömer H. Ubiquinone (coenzyme Q10) in the long-term treatment of idiopathic dilated cardiomyopathy. European Heart Journal 1992;13:1528-33.

147. Mortensen SA. Symptomatic effects of coenzyme Q_{10} in heart failure: Q-SYMBIO study status. Fourth Conference of the International Coenzyme Q10 association 2005;Abstract.
148. Kröger A. Determination of contents and redox states of ubiquinone and menaquinone. Methods in Enzymology 1978;53:579-91.
149. Hagerman RA, Willis RA, Hagerman AE. Ubiquinone binding protein used for determination of coenzyme Q. Analytical Biochemistry 2003;320:125-8.
150. Greenspan MD, Lee Lo C-Y, Hanf DP, Yudkovitz JB. Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector. Journal of Lipid Research 1988;29:971-6.
151. Mosca F, Fattorini D, Bompadre S, Littarru G. Assay of coenzyme Q10 in plasma by a single dilution step. Analytical Biochemistry 2002;305:49-54.
152. Sharma SK, Ebadi M. An improved method for analyzing coenzyme Q homologues and multiple detection of rare biological samples. Journal of Neuroscience Methods 2004;137:1-8.
153. Leray C, Andriamampandry MD, Freund M, Gachet C, Cazenave JP. Simultaneous determination of homologues of vitamin E and coenzyme Q and products of alpha-tocopherol oxidation. Journal of Lipid Research 1998;39:2099-105.
154. Menke T, Niklowitz P, Adam S, Weber M, Schlüter B, Andler W. Simultaneous detection of ubiquinol-10, ubiquinone-10, and tocopherols in human plasma microsamples and macrosamples as a marker of oxidative damage in neonates and infants. Analytical Biochemistry 2000;282: 209-17.
155. Lang JK, Gohil K, Packer L. Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Analytical Biochemistry 1986;157:106-16.
156. Podda M, Weber C, Traber MG, Milbradt R, Packer L. Sensitive high-performance liquid chromatography techniques for simultaneous determination of tocopherols, tocotrienols, ubiquinols, and ubiquinones in biological samples. Methods in Enzymology, Vol. 299, 1999:330-41.
157. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. Journal of Lipid Research 1996;37:893-901.

158. Hectors MPC, van Tits LJH, de Rijke YB, Demacker PNM. Stability studies of ubiquinol in plasma. Annals of Clinical Biochemistry 2003;40:100-1.
159. Tang PH, Miles MV, Steele P, DeGrauw A, Chuck G, Schroer L, Pesce A. Anticoagulant effects on plasma coenzyme Q10 estimated by HPLC with coulometric detection. Clinica Chimica Acta 2002;318:127-31.
160. Dam H. Biochim Zeitschr 1929;215:475.
161. Lefevere MF, De Leenheer AP, Claeys AE, Claeys IV, Steyaert H. Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. Journal of Lipid Research 1982;23:1068-72.
162. Indyk HE, Woollard DC. Determination of free myo-inositol in milk and infant formula by high-performance liquid chromatography. Analyst 1994;119:397-402.
163. Booth SL, Suttie JW. Dietary intake and adequacy of vitamin K. The Journal of Nutrition 1998;128:785-8.
164. Davidson RT, Foley AL, Engelke JA, Suttie JW. Conversion of dietary phylloquinone to tissue menaquinone-4 in rats is not dependent on gut bacteria. The Journal of Nutrition 1998;128:220-3.
165. Shearer MJ, McBurney A, Barkhan P. Studies on the absorption and metabolism of phylloquinone (Vitamin K_1) in man. Vitamins and Hormones 1974;32:513-42.
166. Ichihashi T, Takagishi Y, Uchida K, Yamada H. Colonic absorption of menaquinone-4 and menaquinone-9 in rats. The Journal of Nutrition 1992;122:506-12.
167. Kohlmeier M, Salomon A, Saupe J, Shearer MJ. Transport of vitamin K to bone in Humans. Journal of Nutrition 1996;126:1192s-6s.
168. Stenflo J, Fernlund P, Egan W, Roepstorff P. Vitamin K dependent modifications of glutamic acid residues in prothrombin. Proceedings of the National Academy of Sciences of the United States of America 1974;71:2730-3.
169. Simon RR, Beaudin SM, Johnston M, Walton KJ, Shaughnessy SG. Long-term treatment with sodium warfarin results in decreased femoral bone strength and cancellous bone volume in rats. Thrombosis Research 2002;105:353-8.
170. Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high-performance liquid chromatography using postcolumn chemical reduction. Methods in Enzymology 1997;282:408-21.
171. Haroon Y, Bacon DS, Sadowski JA. Liquid-chromatographic determination of vitamin K_1 in plasma, with fluorometric detection. Clinical Chemistry 1986;32:1925-9.

172. Lambert WE, De Leenheer AP, Lefevere MF. Determination of vitamin K in serum using HPLC with post-column reaction and fluorescence detection. Journal of Chromatographic Science 1986;24:76-9.
173. Shino M. Determination of endogenous vitamin K (phylloquinone and menaquinone-n) in plasma by high-performance liquid chromatography using platinum oxide catalyst reduction and fluorescence detection. Analyst 1988;113:393-7.
174. Kamao M, Suhara Y, Tsugawa N, Okano T. Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. Journal of Chromatography B 2005;816:41-8.
175. Hart JP, Shearer MJ, McCarthy PT. Enhanced sensitivity for the determination of endogenous phylloquinone (vitamin K_1) in plasma using high-performance liquid chromatography with dual-electrode electrochemical detection. Analyst 1985;110:1181-3.
176. Jakob E, Elmadfa I. Rapid and simple HPLC analysis of vitamin K in food, tissues and blood. Food Chemistry 2000;68:219-21.
177. Wang LY, Bates CJ, Yan L, Harrington DJ, Shearer MJ, Prentice A. Determination of phylloquinone (vitamin K_1) in plasma and serum by HPLC with fluorescence detection. Clinica Chimica Acta 2004;347:199-207.
178. Shearer MJ, Bach AU, Kohlmeier M. Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. The Journal of Nutrition 1996;126:1181S-6S.
179. Usui Y, Nishimura N, Kobayashi N, Okanoue T, Kimoto M, Ozawa K. Measurement of vitamin K in human liver by gradient elution high-performance liquid chromatography using platinum-black catalyst reduction and fluorimetric detection. Journal of Chromatography 1989;489:291-301.
180. Schentag JJ, Welage LS, Grasela TH, Adelman MH. Determinants of antibiotic-associated hypoprothrombinemia. Pharmacotherapy 1987;7:80-6.
181. Okano T. Vitamins D and K, and bone mineral density,. Clinical Calcium 2005;15 (abstract, article in Japanese):1489-94.
182. Hart JP, Shearer MJ, Klenerman L, Catteral A, Reeve L, Sambrook PN, et al. Electrochemical detection of depressed circulating levels of vitamin K_1 in osteoporosis. The Journal of Clinical Endocrinology and Metabolism 1985;60:1268-9.

183. Hodges SJ, Pilkington MJ, Stamp CB, Catterall A, Shearer MJ, Bitensky L, Chayen J. Depressed levels of circulating menaquinones in patients with osteoporatic fractures of the spine and femoral neck. Bone 1991;12:387-9.

184. Hodges SJ, Akesson K, Vergnaud P, Obrant K, Delmas PD. Circulating levels of vitamins K_1 and K_2 decreased in elderly women with hip fracture. Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research 1993;8:1241-5.

185. Dam H, Kruse I, Sondergaard E. Determination of vitamin K by the curative technique in chicks. Acta Physiologica Scandinavica 1951;22:238-45.

186. MacCrehan WA, Schönberger E. Determination of vitamin K_1 in serum using catalytic-reduction liquid chromatography with fluorescence detection. Journal of Chromatography B 1995;670:209-17.

187. Indyk H, Woollard DC. Vitamin K in milk and infant formulas: Determination and distribution of phylloquinone and menaquinone-4. Analyst 1997;122:1-5.

188. Indyk H, Woollard DC. Determination of vitamin K in milk and infant formulas by liquid chromatography: Collaborative study. Journal of AOAC International 2000;83:121-30.

189. Speek AJ, Schrijver J, Schruers WHP. Fluorimetric determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes by high-performance liquid chromatography with post-column derivatization. Journal of Chromatography 1984;301:441-7.

190. Booth SL, Davidson KW, Sadowski JA. Evaluation of an HPLC method for the determination of phylloquinone (vitamin K_1) in various food matrices. Journal of Agricultural and Food Chemistry 1994;42:295-300.

191. Ware GM, Chase GW, Eitenmiller RR, Long AR. Determination of vitamin K_1 in medical foods by liquid chromatography with postcolumn reduction and fluorometric detection. Journal of AOAC International 2000;83:957-62.

192. Hirauchi K, Sakano T, Notsumoto S, Nagaoka T, Morimoto A, Fujimoto K, et al. Measurement of K vitamins in animal tissues by high-performance liquid chromatography with fluorimetric detection. Journal of Chromatography 1989;497:131-7.

193. Usui Y. Assay of phylloquinone and menaquinones in human liver. Methods in Enzymology 1997;282:438-47.

194. Lambert WE, De Leenheer AP. Simplified post-column reduction and fluorescence detection for the high-performance liquid chromatographic determination of vitamin $K_{1(20)}$. Analytica Chimica Acta 1987;196:247-50.
195. Haroon Y, Bacon DS, Sadowski JA. Chemical reduction system for the detection of phylloquinone (Vitamin K_1) and menaquinones (Vitamin K_2). Journal of Chromatography 1987;384:383-9.
196. Cham BE, Roeser HP, Kamst TW. Simultaneous liquid-chromatographic determination of vitamin K_1 and vitamin E in serum. Clinical Chemistry 1989;35:2285-9.
197. Haroon Y, Shearer MJ, Rahim S, Gunn WG, McEnery G, Barkhan P. The content of phylloquinone (vitamin K_1) in human milk, cows milk and infant formula foods determined by high-performance liquid chromatography. The Journal of Nutrition 1982;112:1105-17.
198. Bueno MP, Villalobos MC. Vitamins and other nutrients. Journal of AOAC International 1983;66:1063-7.
199. Indyk H, Littlejohn VC, Lawrence RJ. Liquid chromatographic determination of vitamin K_1 in infant formulas and milk. Journal of AOAC International 1995;78:719-23.
200. Isshiki H, Suzuki Y, Yonekubo A, Hasegawa H, Yamamoto Y. Determination of phylloquinone and menaquinone in human milk using high performance liquid chromatography. Journal of Dairy Science 1988;71:627-32.
201. Piironen V, Koivu T, Tammisalo O, Mattila P. Determination of phylloquinone in oils, margarines and butter by high-performance liquid chromatography with electrochemical detection. Food Chemistry 1997;59:473-80.
202. Seifert RM. Analysis of vitamin K_1 in some green leafy vegetables by gas chromatography. Journal of Agricultural and Food Chemistry 1979;27:1301-4.
203. Fauler G, Leis HJ, Schalamon J, Muntean W, Gleispach H. Method for the determination of vitamin $K_{1(20)}$ in human plasma by stable isotope dilution/gas chromatography/mass spectrometry. Journal of Mass Spectrometry 1996;31:655-60.
204. Suttie JW. Vitamin K and human nutrition. Journal of the American Dietetic Association 1992;92:585-90.
205. Motohara K, Kuroki Y, Kan H, Endo F, Matsuda I. Detection of vitamin K deficiency by use of an enzyme-linked immunosorbent assay for circulating abnormal prothrombin. Pediatric Research 1985;19:354-7.

206. Levy RJ, Lian JB. Gamma-Carboxyglutamate excretion and warfarin therapy. Clinical Pharmacology and Therapeutics 1979;25:562-70.

207. www.drgreene.com/21_1616.html.

Chapter 2

Redox Properties and Effect of Solvents on the Fluorescence of Coenzyme Q and Vitamin K, and Stability of Coenzyme Q_{10} in Plasma

2.1. Introduction

The chemical properties of CoQ and vitamin K, particularly their photosensitivity and easy oxidation, combined with their low concentrations in plasma, make them difficult to measure. Both CoQ and vitamin K are most commonly measured with HPLC, using fluorescence and electrochemical detection for vitamin K, and ultraviolet and electrochemical detection for CoQ. Both CoQ_{10} and vitamin K_1 are present in plasma in the quinol and quinone forms. Because these compounds are present at such low concentrations, maximum assay sensitivity is achieved when their total concentrations are measured, by reducing or oxidising samples before analysis. The quinone form is most stable with the quinols being readily oxidised by atmospheric oxygen. Therefore, it is more practical to measure the quinone when measuring total concentrations.

As outlined in Chapter 1, Section 1.4, it was hypothesised that use of fluorescence detection after HPLC would provide sufficient sensitivity to measure plasma vitamin K_1 and menaquinone-4, as well as plasma total CoQ and possibly the ratio of CoQ_{10} to $CoQ_{10}H_2$. Only the quinols of vitamin K and CoQ are fluorescent and hence their reduction before fluorescence detection is essential.

Fluorescence response is determined by the solvent that fluorescence is occurring in, due to interactions of the solvent with the excited fluorophore. To optimise assay sensitivity, knowledge of the solvent in which the highest fluorescence occurs is important. The compound 4-methoxy-1-naphthol is a vitamin K_1 analogue that does not contain the hydrophobic side-chain, and CoQ_0H_2 is identical to $CoQ_{10}H_2$ but without the hydrophobic side-chain. Investigation of the effect of solvent on fluorescence of these two compounds as well as the parent compounds allowed insight as to the role of the hydrophobic sidechain on fluorescence response in different solvents.

Knowledge of the stability of an analyte, especially during storage, is essential since this determines how often a routine assay must be run as well as the practicalities of sample handling during clinical trials.

Experiments designed to address these issues are presented in this Chapter.

2.2. Redox properties of CoQ and vitamin K

The reduction/oxidation (redox) potential ($E_{1/2}$) for the reversible CoQ_{10}-$CoQ_{10}H_2$ redox couple is 0.1 V at pH 7.4. The redox equilibrium involves 2 protons (Equation 2.1), and is pH-dependent ($\Delta E_{1/2}/\Delta pH = -0.059$).

$$CoQ + 2H^+ + 2e^- \rightarrow CoQH_2 \qquad \textbf{2.1}$$

Coenzyme Q_{10} and $CoQ_{10}H_2$ have different retention times on reversed-phase C18 columns, since the reduced form contains a more polar head group. An oxidation or reduction step before or during HPLC is therefore necessary when measuring total CoQ_{10}.

The reduction potential (E_0) for vitamin K_1 is 0.363 volts. The redox equilibrium involves 2 protons (Equation 2.2).

$$\text{Vitamin K} + 2H_2 + 2e^- \rightarrow \text{Vitamin K}H_2 \qquad \textbf{2.2}$$

Vitamin K_1 is present in biological samples as oxidised K_1, reduced K_1 and the epoxide metabolite (1) (Figure 1.12). The vitamin K_1 epoxide metabolite is reduced to vitamin K_1.

In HPLC the detection method determines the form (reduced or oxidised) that the analyte needs to be in. The extinction coefficients for CoQ_{10} and $CoQ_{10}H_2$ are 14020 and 3940 at absorbance maxima of 275 and 290 nm, respectively. For vitamin K_1 the extinction coefficient is 18600 at an absorbance maximum of 243 nm in ether. Hence, CoQ_{10} absorbs light more strongly than $CoQ_{10}H_2$ and the ultraviolet detection of CoQ_{10} is more sensitive when oxidised CoQ_{10} is measured.

It is difficult to completely eliminate oxygen during sample preparation and therefore it usually results in oxidation of $CoQ_{10}H_2$, and probably vitamin K. Thus, measurement of the ratio of reduced to oxidised CoQ_{10} requires strict sample handling, to ensure the ratio is not an artefact of sample handling.

In the case of vitamin K, extraction procedures are complex (as discussed in Chapter 7) and it can be assumed that reduced vitamin K is oxidised during extraction. Indeed, Kroger

et al., (1978) (2) report that menaquinol is autooxidised to menaquinone during a methanol/light petroleum liquid-liquid extraction, followed by evaporation and reconstitution in n-heptane and ethanol.

Early methods to detect CoQ_{10} involved long and tedious extraction techniques before HPLC separation and detection, such that the majority of CoQ_{10} was in the oxidised form by the time it was quantified (3, 4). Additionally, the commonly used hexane and ethanol extraction reportedly results in conversion of $CoQ_{10}H_2$ to CoQ_{10} (5) whereas 2-propanol, which along with 1-propanol is now commonly used in single-step extractions of CoQ_{10} from plasma, protects $CoQ_{10}H_2$ from oxidation (5). Therefore, with the advent and popularity of a single step dilution for extraction of CoQ_{10}, it became a necessity to include a specific step to ensure that all CoQ_{10} is in the oxidised form when ultraviolet detection was to be used to measure total CoQ_{10}. $CoQ_{10}H_2$ oxidation has been achieved using autooxidation under alkaline conditions (6), ferric chloride (7), gold (II) chloride (8), cupric sulfate (9), potassium hexacyanoferrate (III) (4), and cupric chloride (10). The oxidation by silver oxide (11) is unreliable because the complete separation of the oxidant (which is necessary before measurement) is difficult to achieve (2). However, 1,4-benzoquinone oxidises $CoQ_{10}H_2$ efficiently and rapidly and can be left in the sample during HPLC analysis (12) without interferance.

The work of Daines (2001) (13) suggested that fluorescence detection should allow measurement of plasma total CoQ_{10}. Because of the sensitivity of vitamin K_1 naphthoquinol and $CoQ_{10}H_2$ to oxygen, it is desirable that the reduction step be integrated into the HPLC set-up, eliminating avenues for oxidation.

Zinc reduction is most commonly used before fluorometric detection of vitamin K during HPLC (14-23). The zinc powder filled columns require frequent repacking due to the zinc particles being consumed by oxygen and the analyte reduction (24), and the presence of zinc ions in the mobile phase. Platinum-black catalysed reduction of vitamin K by alcohols has also been reported (13, 24-28) and has the advantage that platinum-black-filled columns do not require frequent repacking (13), and, aside from requiring an alcohol, there is no requirement for additives in the mobile phase.

Vitamin K_1 has also been reduced using sodium borohydride. The reaction is temperature dependent, occurring more completely as the temperature increases, up to 50 ºC (29).

Photoreduction of quinones has also been reported (30) in a post-column reactor of an HPLC system during which the analyte is reduced to the corresponding quinol while methanol (the hydrogen atom donor) is oxidised to formaldehyde.

Reduction of CoQ_{10} before HPLC with electrochemical detection has been reported. This on-line reduction of CoQ_{10} has been achieved using electrochemical cells (9, 31-35) and chemical reduction (36-39). The method of Leary *et al.,* (1998) (38) uses zinc catalysed reduction in a post-column reactor consisting of a column (20 × 4.6 mm stainless steel) dry packed (under vibration) with zinc particles. The method of Yamashita *et al.,* (1997) (37) utilises a reduction column (IRICA RC-10) immediately prior to the analytical cell. The method of Wang *et al.,* (1999) (36) uses a complex set-up for automated pre-column reduction of CoQ_{10} with sodium borohydride which is made fresh by the automated system every three samples, and injected into the extract. Wakabayashi *et al.,* (1994) (39) report the use of a post-column platinum catalyst for reduction of CoQ. This consisted of a stainless steel column (10 × 4.6 mm) packed with catalyst (5% on alumina, 10 µm), and placed between the analytical column and the detector (39). The reduction ability of the platinum catalyst was unchanged by successive injections of biological samples (39).

Reduction of CoQ_{10} to obtain a $CoQ_{10}H_2$ standard is necessary when measuring the ratio of $CoQ_{10}H_2$ to CoQ_{10} (4, 9, 37, 39, 40). The most common reducing agent used to obtain a pool of standard reduced CoQ_{10} is sodium borohydride (4, 9, 37, 39, 40). Tang *et al.,* (2004) (9) mixed sodium borohydride and CoQ (at a ratio of 23 nmoles of CoQ to 250 nmoles of sodium borohydride) for 30 minutes in the dark at room temperature, whereas Yamashita *et al.,* (1997) (37) mixed sodium borohydride and CoQ for 5 minutes in the dark at room temperature. Excess sodium borohydride can be removed from the sample by washing with water (37). The disadvantages of using sodium borohydride as a reducing agent are that (a) it reduces methanol and to a lesser degree ethanol resulting in hydrogen bubbles, and (b) it has limited solubility in non-polar solvents. Tetrabutylammonium borohydride has increased solubility in organic solvents, offering more flexibility.

Sodium dithionite has also been used as a reducing agent for obtaining $CoQ_{10}H_2$ (38, 41-43). Lang *et al.,* (1986) (41) mixed sodium dithionite and CoQ_{10} for 30 minutes in the dark at room temperature, before extracting $CoQ_{10}H_2$ with hexane, evaporating the hexane and reconstituting the $CoQ_{10}H_2$ in ethanol.

Using an electrochemical cell, Gohil *et al.,* (1987) (44) obtained $CoQ_{10}H_2$ by setting up a loop consisting of a pump and a guard cell, with the potential on the guard cell set to –1000 mV. A CoQ standard was pumped through the loop system in the dark for approximately 3 hours. The conversion rate of CoQ_{10} to $CoQ_{10}H_2$ was approximately 99% (44).

2.2.1. Oxidising plasma $CoQ_{10}H_2$ for measurement of total CoQ_{10}

In an attempt to simplify the assay of Mosca *et al.,* (2002) (12) where an aqueous solution of 1,4-benzoquinone is added to plasma before liquid-liquid extraction with 1-propanol, the 1,4-benzoquinone was added to 1-propanol as a stock solution. This was diluted with 1-propanol and used as the extraction solvent to oxidise and extract CoQ_{10} from plasma in a single step. This simplification was found to work effectively in terms of efficient oxidation of plasma $CoQ_{10}H_2$ and efficient extraction of CoQ_{10} from plasma.

An investigation of the practicalities of this simplification involved determining the stability of the standard solution of 1,4-benzoquinone in 1-propanol (5 mmol/L) over time.

2.2.1.1. Experimental

A 5 mmol/L solution of 1,4-benzoquinone in 1-propanol was stored protected from light at –13 ºC. At various time points between 0 and 60 days, an aliquot was diluted to 33.3 µmol/L and the absorbance at 243 nm (the absorption maxima) was determined.

2.2.1.2. Results

The 1,4-benzoquinone standard solution decomposed by approximately 10% and 20% after 10 and 20 days storage, respectively (Figure 2.1). Therefore, this standard solution needs to be made fresh at least every week when stored at –13 ºC.

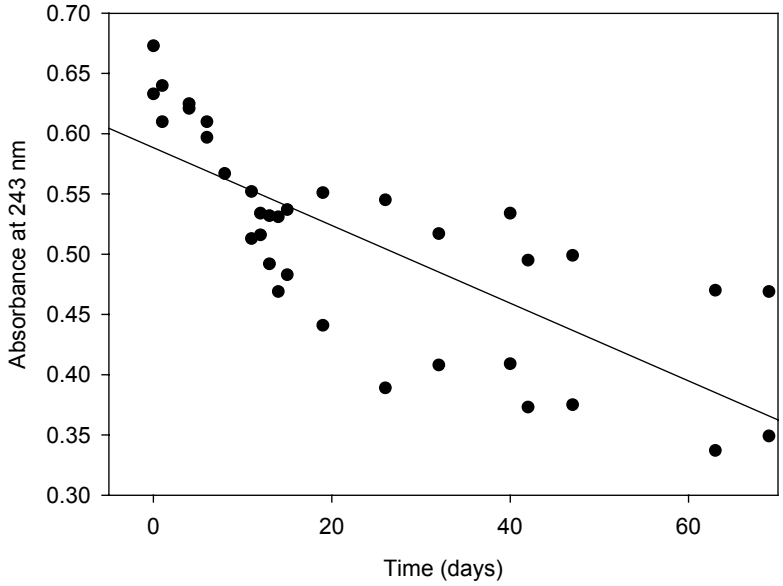


Figure 2.1 The change in absorbance over time of a benzoquinone standard solution (5 mmol/L) stored at –13 ºC.

2.2.2. Reducing CoQ_{10} and vitamin K_1 for fluorescence detection - comparison of reduction efficiency of an electrochemical cell, zinc, and an alcohol in the presence of platinum-black.

The efficiency of reduction of CoQ_{10} and vitamin K achieved with electrochemical reduction, zinc and alcohol (in the presence of platinum-black) was compared. Sodium borohydride reduction was not used due to reduction of the alcohol producing gas in the HPLC system. Additionally, de Barros *et al.,* (1994) (45) examined the extent of decomposition of sodium borohydride in absolute ethanol after time. After a decrease of 35.5%, the content of sodium borohydride almost stabilised in the interval of 2.5 – 6 hours. The reducing power of sodium borohydride in absolute ethanol therefore decreases with time and fresh solutions need to be made regularly.

Coenzyme Q_0 (CoQ_0) and 4-methoxy-1-naphthol were also included in this experiment, as inclusion of CoQ_0 would allow insight into the effect of the hydrophobic side chain on reduction and fluorescence, and 4-methoxy-1-naphthol is an intrinsically fluorescent vitamin K analogue, without the side chain.

2.2.2.1. Experimental

The mobile phases used are outlined in Table 2.1. Fluorescence detection was used to estimate the reduction achieved for both CoQ_{10} (excitation 290 nm, emission 370 nm) and vitamin K_1 (excitation 249 nm, and emission 408 nm). For electrochemical reduction of both CoQ_{10} and vitamin K_1 the electrochemical cell was set to a potential of –800 mV.

Table 2.1 The mobile phases used for comparison of reduction efficiency of CoQ_{10} and vitamin K_1.

Reduction method	CoQ_{10}	Vitamin K_1
Platinum-black	30/70 methanol/ethanol (v/v)	100% methanol
Electrochemical	30/70 methanol/ethanol (v/v), 15 mL/L glacial acetic acid and 50 mmol/L sodium acetate trihydrate	100% methanol, 15 mL/L glacial acetic acid and 50 mmol/L sodium acetate trihydrate
Zinc	30/70/0.55 methanol/ethanol/aqueous solution of 2 mol/L zinc chloride; 1 mol/L glacial acetic acid, and 1 mol/L sodium acetate (v/v/v)	99.45/0.55 methanol/aqueous solution of 2 mol/L zinc chloride; 1 mol/L glacial acetic acid, and 1 mol/L sodium acetate (v/v)

An ethanolic standard of vitamin K_1 (5 nmol/L, 200 µL) was injected. For CoQ_{10}, a 10 µmol/L standard in 1-propanol was injected (200 µL). The analytical column used for both CoQ_{10} and vitamin K_1 was a Phenomenex Luna C18(2) column, (250 × 4.6 mm, 5 µm).

2.2.2.2. Results

The reduction of CoQ_{10} by an electrochemical cell was not as efficient as that by a zinc or alcohol with platinum-black as a catalyst. However electrochemical reduction of CoQ_{10} was more consistent than zinc reduction (Table 2.2). This supports the findings of Haroon *et al.,* (1987) (14) who reported that 95% of injected quinones (K-vitamins) could be reduced to their corresponding hydroquinones by zinc metal compared to 60% reduction with electrochemical cells. In situations where an electrochemical detector is connected to a dual-cell analytical cell, a lower limit of detection and more accurate assay could be obtained using a platinum-black catalysed alcohol reduction system.

Electrochemical reduction of vitamin K was poor and zinc reduction was not as efficient for vitamin K_1 as it was for CoQ_{10} (Table 2.2).

Table 2.2 The percentage reduction (± SD) of CoQ_{10} and vitamin K achieved using three different methods of reduction (n = 3).

Reduction method	CoQ_{10}	Vitamin K_1
Alcohol with platinum-black catalysis	100 ± 1.0	100 ± 2.45
Electrochemical	84 ± 3.8	40 ± 1.2
Zinc	100 ± 11.1	92 ± 6.7

The electrochemical reduction of Vitamin K is inefficient because of residual oxygen in the system. In fact complete removal of oxygen is essential for both efficient electrochemical reduction and elimination of fluorescence quenching (46, 47). It can be hypothesised that insertion of a platinum-black filled reactor prior to the injector in the HPLC system would remove oxygen from the mobile phase and increase the efficiency of electrochemical reduction of vitamin K_1.

2.2.3. Reduction of quinones to determine the effect of solvent on fluorescence

Before the effect of solvent on fluorescence could be quantified, a reliable and efficient method to reduce CoQ_{10}, CoQ_0, and vitamin K_1 was required. As already discussed, sodium borohydride does reduce CoQ_{10} and vitamin K_1, but it has limited solubility in non-polar solvents and degrades in methanol and (to a lesser degree) ethanol. Thus solutions need to be made fresh regularly.

Electrochemical reduction of CoQ_{10}, as described by Galinier *et al.,* (2004) (5) did not produce $CoQ_{10}H_2$. Briefly, 100 mL of 100 µmol/L CoQ_{10} in 1-propanol was recycled through an electrochemical cell set to a potential of –1000 mV. All parts of the system were protected from light using aluminium foil. There was no evidence of formation of $CoQ_{10}H_2$ as determined by measuring the change in absorbance of the solution at 275 nm (A_{max} for CoQ_{10}).

Tetrabutylammonium borohydride in 1-propanol (approximately 19 mmol/L) was used to reduce CoQ_{10}, CoQ_0, and vitamin K_1. This concentration was determined to be the minimum concentration at which all CoQ_{10}, CoQ_0 and vitamin K_1 was reduced. A small volume of the tetrabutylammonium borohydride solution was freshly made each time it was required and each sample was reduced just before measurement to ensure minimal oxidation of $CoQ_{10}H_2$ occurred before analysis.

Reduction of the quinones by tetrabutylammonium borohydride was most probably complete since (a) the results for the replicates were similar, (b) the results showed similar trends to that obtained using HPLC, where a different reduction system was employed, and (c) similar results were obtained for vitamin K_1 and its analogue 4-methoxy-1-naphthol, which did not require reduction to fluoresce. These results are presented in section 2.3.4.3.

When using HPLC to determine the effect of solvent on fluorescence, vitamin K_1 and CoQ_{10} were reduced by alcohol using platinum-black as a catalyst. Heptane was used in the mobile phase to reduce peak tailing. Standards contained 20% methanol. Most peaks had secondary peaks at the end (Figure 2.2) – this may have been due to analyte absorbing onto the surface of the platinum-black, so that when the mobile phase (with a higher eluting power) moved through the reactor the absorbed analyte was washed off (Figure 2.2). This suggests that the reactor is contributing to the chromatography and confirms the findings of Usui, (1989) (25).

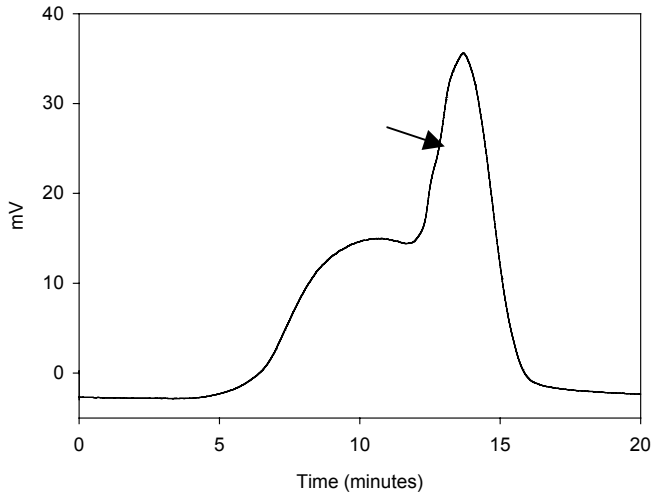


Figure 2.2 The peak tailing observed with the platinum-black reactor, the solvent is 1-propanol. Arrow points to the secondary peak.

Some solvents (for example, acetonitrile) affected the performance of the reactor which consequently affected the results depending on the sequence of solvents run. This is a disadvantage of using this method to investigate the effect of solvent on fluorescence.

2.2.4. Hydrodynamic voltammograms

For analysis of a compound by electrochemical detection, it is first necessary to confirm the potentials required to reduce or oxidise the compound under the conditions (solvent, column, electrochemical cells) to be used in the method. Therefore, hydrodynamic voltammograms of both CoQ_{10} and vitamin K_1 were obtained. For CoQ_{10}, the standard solution was 0.2 μmol/L in 1-propanol/water 9/1 v/v and for vitamin K_1 it was 5 nmol/L in ethanol. Injection volume was 50 μL. The potential at the final electrode was altered by 50 mV increments until the graph of applied potential vs peak height reached a plateau (Figures 2.3 and 2.4).

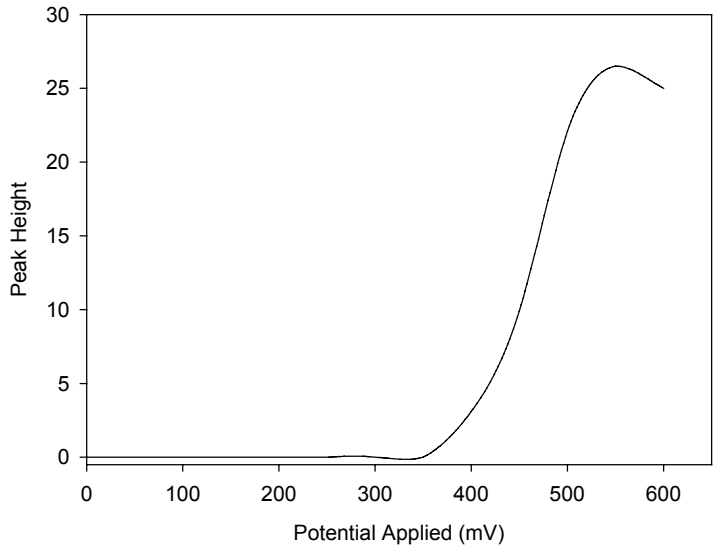


Figure 2.3 Hydrodynamic voltammogram for CoQ_{10}.

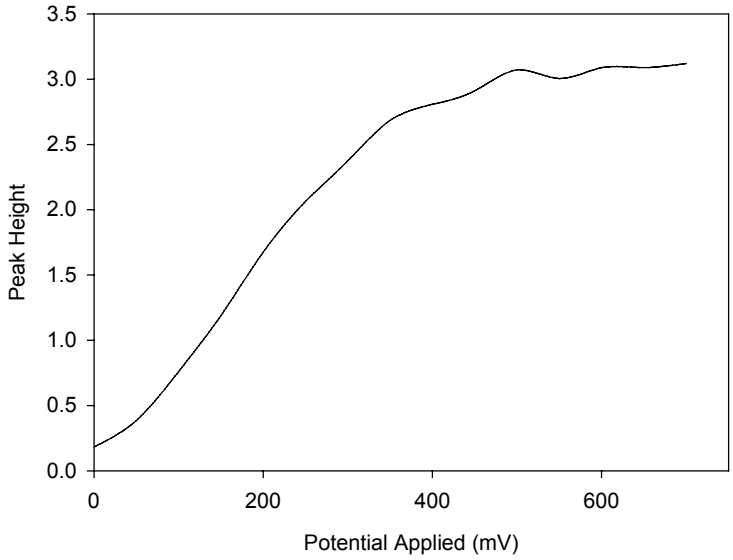


Figure 2.4 Hydrodynamic voltammogram for vitamin K_1.

For electrochemical detection of CoQ_{10}, it is therefore possible to set the first of the analytical cells on the electrochemical detector to a potential below 300 mV, at which no CoQ_{10} will be oxidised but any impurities that are oxidised at or below 300 mV will be. The impurities oxidised by the first cell will therefore not be detected by the second cell which is set to 550 mV. This 'screening' process results in cleaner chromatograms.

2.3. Absorbance and fluorescence of CoQ_{10}, and vitamin K_1

2.3.1. Absorbance of Vitamin K_1 and CoQ_{10}

Ultraviolet detection in HPLC has been utilised to measure total CoQ_{10} (3, 4, 12, 48-50). A preliminary absorbance scan serves to determine (a) the wavelength at which maximal absorption occur, and (b) alternative wavelengths that may be used if there are interfering compounds detected at the wavelength of maximal absorption.

2.3.1.1. Absorbance scans of CoQ_{10} and vitamin K_1

The absorbance scans of CoQ_{10} and vitamin K_1 in 1-propanol and ethanol, respectively, show the absorbance maxima for CoQ_{10} to be at 275 nm (Figure 2.5) and for vitamin K_1 to be at 330 nm (Figure 2.6).

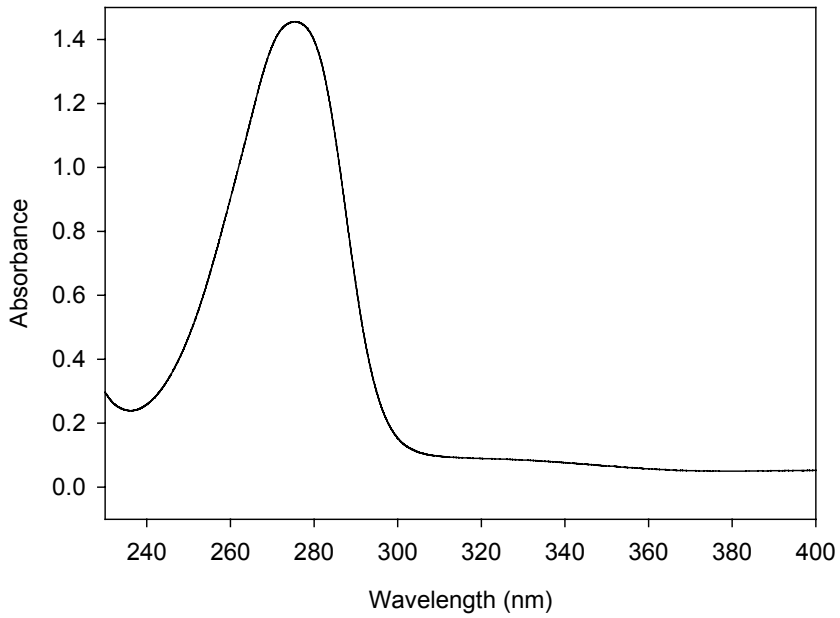


Figure 2.5 Absorbance scan of CoQ_{10} in 1-propanol. Concentration is 100 µmol/L. The absorbance maximum of 1.42 occurred at 275 nm.

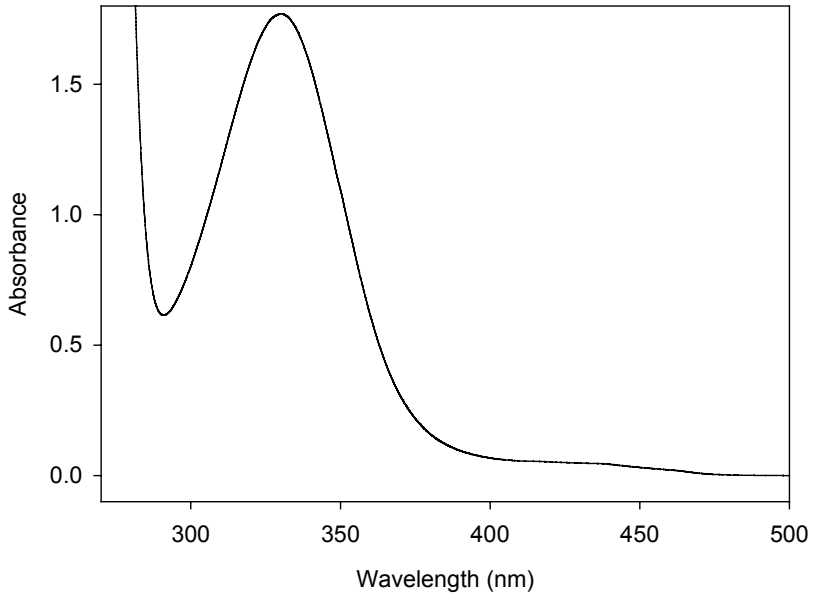


Figure 2.6 Absorbance scan of vitamin K in ethanol. Concentration is 500 µmol/L. The absorbance maximum of 1.7 occurred at 330 nm.

2.3.2. Fluorescence of $CoQ_{10}H_2$, CoQ_0H_2, vitamin K_1 naphthoquinol, and 4-methoxy-1-naphthol.

Fluorescence of $CoQ_{10}H_2$ has been reported (51), but fluorescence detection of $CoQ_{10}H_2$ after HPLC has not. In contrast, fluorescence detection of the vitamin K naphthoquinol after HPLC is the most commonly used detection method (1, 14, 15, 17-29, 52-55).

2.3.3. Effect of solvent on fluorescence

In fluorescence, spectral shifts occur due to the general effect of solvent polarity, specific fluorophore-solvent interactions, and charge separation in the excited state.

Polar solvent molecules decrease the energy of the excited state by stabilising it (the fluorophore) thereby shifting the emission to lower wavelengths (bathochromic shift). In general, only fluorophores which are themselves polar display a large sensitivity to solvent polarity. Non-polar fluorophores, such as unsubstituted hydrocarbons, are much less sensitive to solvent polarity.

The dipole moment determines the asymmetry of a charge distribution and is defined as the product of the total amount of positive or negative charge and the distance between their centroids. Upon absorption of an exciting photon, a dipole moment is created in the fluorophore (usually of different magnitude and direction from the ground state dipole). The orientation of this dipole moment relative to the nuclear framework, and its magnitude, will be determined by the nature of the substituents on the molecule. The dielectric constants and dipole moments for some solvents that are compatible with HPLC are shown in Table 2.3.

Table 2.3 The dielectric constants and dipole moments for some solvents that are compatible with HPLC.

Solvent	Structure	Polarity index (according to Snyder)	Dielectric constant (20 or 25 °C)	Dipole moment (according to Debye)
Heptane	C_7H_{16}	-	1.9	0
Hexane	C_6H_{14}	0.0	1.9	0
1-Butanol	$CH_3(CH_2)_3OH$	3.9	17.8	1.66
Acetonitrile	CH_3CN	6.2	37.5	3.44
1-Propanol	$CH_3(CH_2)_2OH$	4.3	20.1	1.68
2-Propanol	$CH_3CH(OH)CH_3$	4.3	18.3	1.66
Ethyl acetate	$CH_3COOC_2H_5$	4.3	6.0	1.78
Ethanol	C_2H_5OH	5.2	24.3	1.70
1.4-Dioxane	$C_4H_8O_2$	4.8	2.2	0.40
Tetrahydrofuran	C_4H_8O	4.2	7.4	1.63
Methanol	CH_3OH	6.6	32.6	1.70

Intermolecular interactions can cause important changes in the excited state decay mechanism. Additionally, the external solvent parameters (for example, viscosity of the solvent) can influence the rate of intermolecular processes especially in the case where efficient charge separation or internal nuclear motions are involved.

Absorption occurs with the solvent in the arrangement characteristic of the ground state of the molecule. However, before fluorescence occurs, the solvent molecules relax into a new arrangement, which is preserved during the subsequent radiative transition (56). Therefore excitation spectra change very little with different solvents.

Investigation of the effects of solvent on CoQ_{10} and vitamin K_1 fluorescence is important since an increase in the fluorescence yield gives an increase in sensitivity. However, the solvent that gives the best fluorescence yield may not be the most favourable for the chromatographic separation. Therefore a compromise between the best solvent for chromatography and the best solvent for sensitivity may be necessary.

2.3.4. Determination of the effect of solvent on fluorescence of $CoQ_{10}H_2$, CoQ_0H_2, vitamin K_1 naphthoquinol and 4-methoxy-1-naphthoquinol.

2.3.4.1. Aim

The aim of this work was to determine the effect of different solvents on the fluorescence of $CoQ_{10}H_2$, vitamin K_1 naphthoquinol, CoQ_0H_2 and 4-methoxy-1-naphthol (Figure 2.7). Another aim was to determine whether the side-chains of $CoQ_{10}H_2$ and the vitamin K_1 naphthoquinol are implicated in the fluorescence response to solvents.

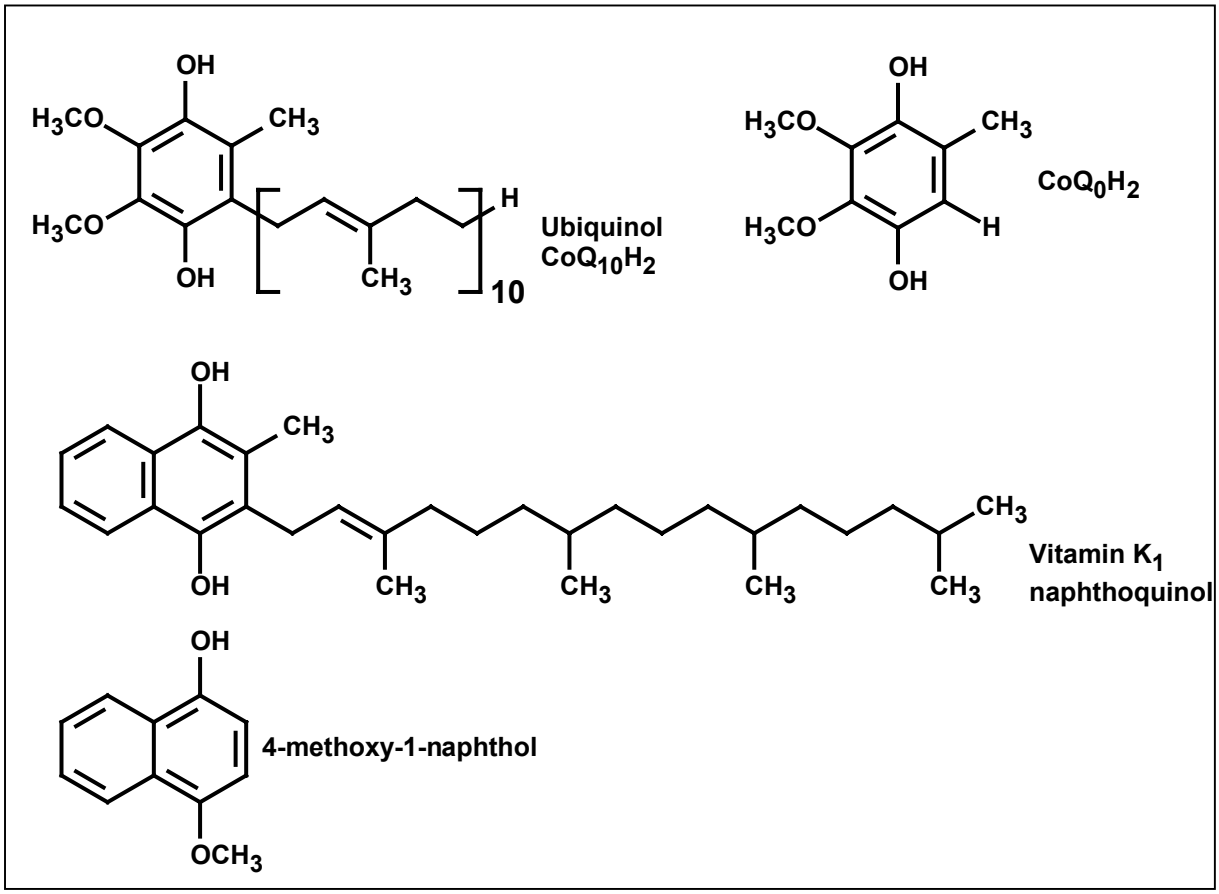


Figure 2.7 The structures of $CoQ_{10}H_2$, CoQ_0H_2, vitamin K_1 naphthoquinol, and 4-methoxy-1-naphthol.

2.3.4.2. Experimental

Fluorescence was measured using a Cary Varian Eclipse fluorescence spectrophotometer.

Vitamin K_1, CoQ_{10}, and CoQ_0 were reduced by tetrabutylammonium borohydride, as described in Section 2.2.3. Fresh solutions of tetrabutylammonium borohydride (approximately 19 mmol/L) in 1-propanol were made by addition of 1-propanol (300 µL) to tetrabutylammonium borohydride (approximately 0.0015g). The mixture was left to stand for 5 minutes to ensure complete dissolution of the tetrabutylammonium borohydride.

For CoQ_{10} and CoQ_0, the standard solutions were 100 µmol/L in 1-propanol. The borohydride solution (50 µL) was added to a quartz curvette followed by 750 µL of standard. After shaking for approximately four seconds, 2200 µL of the solvent under study was added to the curvette. After inversion of the curvette three to five times, it was positioned in the fluorometer with the cell holder set at 25 ºC. After five minutes an excitation scan (260 – 320 nm, emission set to 370 nm) and an emission scan (320 – 450 nm, excitation set to 290 nm) were obtained. Duplicate samples were scanned for each solvent tested. A blank consisting of 750 µL 1-propanol, 50 µL of tetrabutylammonium borohydride solution and 2200 µL of the solvent being tested was scanned for excitation and emission as per the samples. The resulting spectrum was subtracted from the sample spectra.

For vitamin K_1, the standard solution was 500 µmol/L in 1-propanol. The standard (5 µL) was added to 50 µL of borohydride solution in a curvette. After shaking for approximately 4 seconds, 2945 µL of the solvent to be tested was added to the curvette and the curvette mixed by inversion three to five times. The curvette was placed in the cell holder (held at 25 ºC) for 5 minutes before readings were taken. Excitation (230 – 270 nm, emission at 408 nm) and emission (360 – 490 nm, excitation set to 249 nm) spectra were scanned on duplicate samples and on a blank (5 µL 1-propanol, 50 µL borohydride solution, and 2945 µL appropriate solvent). The blank spectra were subtracted from the sample spectra.

For 4-methoxy-1-naphthol the standard solution was 100 µmol/L in 1-propanol. To 55 µL of standard, 2945 µL of the solvent being tested was added. After inversion three to five times, the curvette was placed in the cell holder (at 25 ºC) and excitation (230 – 270 nm, emission at 408 nm) and emission (360 – 490 nm, excitation set to 249 nm) spectra were scanned. Duplicate samples were analysed, and a blank (55 µL 1-propanol and 2945 µL of the appropriate solvent) was run. The blank spectra were subtracted from sample spectra.

The mean of the duplicate spectra was calculated using the fluorometer software. The resulting data were fitted to a Gaussian curve of wavenumber versus peak height. The maximum peak height and corresponding wavenumber were then determined from this curve using the non-linear regression function in SigmaStat (SPSS, Chicago, Inc).

The effect of solvent on fluorescence of CoQ_{10} and vitamin K_1 was also investigated on-line, with HPLC and fluorescence detection (Shimadzu RF-551, Spectrofluorometric detector). Standards (50 µmol/L) were injected in 20/80 (v/v) methanol/solvent of interest, with a mobile phase of 20/80 (v/v) heptane/methanol. The flow rate was 0.1 mL/minute, with no analytical column. Injection of 400 µL of standard gave a 4-minute plateau of fluorescence, the height of which related to the fluorescence intensity of the standard in each solvent.

2.3.4.3. Results

For $CoQ_{10}H_2$, the highest fluorescence intensity was observed in the aprotic solvents ethyl acetate, dioxane, acetonitrile and tetrahydrofuran, when measured with a fluorometer (Table 2.4) and HPLC (Table 2.7). Acetonitrile could not be tested in the HPLC system since it 'poisons' the platinum-black reactor (13). A slight bathochromic shift was observed in the emission maxima for $CoQ_{10}H_2$ with the aprotic solvent 1-butanol (Table 2.4, Figure 2.8). Kruk and Strzalka (1993) (51) also reported that the emission band of $CoQ_{10}H_2$ (at 371 nm) was not sensitive to solvent polarity (Table 2.5) and hypothesised that the methoxy groups of $CoQ_{10}H_2$ may form additional hydrogen bonds with protons in the protic solvents. Since this quenches the fluorescence, maximum fluorescence occurs in aprotic solvents.

Supporting this hypothesis further is the fact that the fluorescence response of $CoQ_{10}H_2$ and CoQ_0H_2 in the various solvents was very similar suggesting that the isoprenoid side-chain of $CoQ_{10}H_2$ does not contribute to the fluorescence response. Additionally, there was no correlation between the dielectric constant ($r = -0.22$, $p = 0.524$) and $CoQ_{10}H_2$ fluorescence, or the dielectric constant and the $CoQ_{10}H_2$ Stokes shift ($r = -0.19$, $p = 0.573$). This hypothesis also provides an explanation as to why much higher concentrations of CoQ_0H_2 and $CoQ_{10}H_2$ were required for investigation of their fluorescence as compared to vitamin K_1 naphthoquinol.

Table 2.4 Fluorescence response of $CoQ_{10}H_2$ and CoQ_0H_2 in various solvents as determined using a fluorometer (data are mean ± SE)

	Coenzyme $Q_{10}H_2$			Coenzyme Q_0H_2		
Solvent	Emission Maxima (wavenumber, cm^{-1})	Emission Response (± SE)	R^2 for Fit	Emission Maxima (wavenumber, cm^{-1})	Emission Response (± SE)	R^2 for Fit
Methanol	27072 ± 10	9.57 ± 0.04	0.988	27410 ± 12	14.91 ± 0.08	0.985
Ethanol	26997 ± 12	11.50 ± 0.06	0.985	27323 ± 14	17.04 ± 0.11	0.979
1-butanol	25853 ± 24	14.35 ± 0.13	0.939	24473 ± 57	33.57 ± 0.56	0.885
2-butanol	26830 ± 11	6.58 ± 0.03	0.985	27106 ± 13	7.54 ± 0.04	0.981
1-propanol	27029 ± 10	11.65 ± 0.05	0.988	27663 ± 16	16.88 ± 0.13	0.978
2-propanol	26924 ± 12	12.19 ± 0.06	0.983	27627 ± 14	18.06 ± 0.12	0.981
Acetonitrile	26885 ± 8	19.29 ± 0.07	0.993	27199 ± 11	23.63 ± 0.12	0.987
Hexane	27130 ± 10	12.15 ± 0.05	0.990	27670 ± 15	15.61 ± 0.11	0.978
Heptane	26979 ± 13	8.94 ± 0.05	0.980	27518 ± 14	15.85 ± 0.10	0.980
Dioxane	26683 ± 9	20.76 ± 0.07	0.991	26926 ± 12	35.78 ± 0.17	0.985
Ethyl Acetate	26768 ± 8	21.55 ± 0.07	0.992	27014 ± 14	21.78 ± 0.12	0.980
Tetrahydrofuran	26693 ± 8	24.48 ± 0.09	0.992	27174 ± 17	19.85 ± 0.14	0.969

Table 2.5 The absorption maxima (A_{max}), emission maxima (Em_{max}), and fluorescence quantum efficiency (Φ), with excitation set to 290 nm of $CoQ_{10}H_2$ in various solvents, from Kruk and Strzalka (1993) (51).

Solvent	A_{max}	Em_{max}	Φ
Methanol	290.1	371.0	0.003
Ethanol	290.3	371.0	0.004
Ethylacetate	291.4	371.0	0.032
Hexane	291.0	369.5	0.033
50 % Methanol in H_2O	288.0	370.5	0.012
25 % Methanol in H_2O	289.0	371.0	0.01

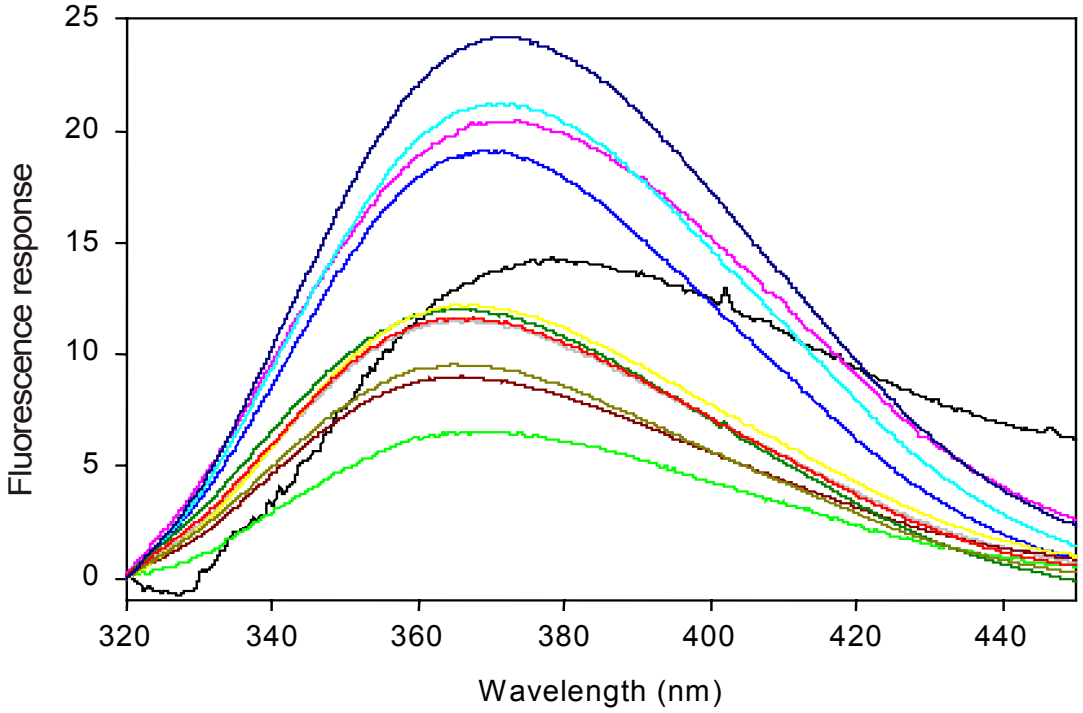
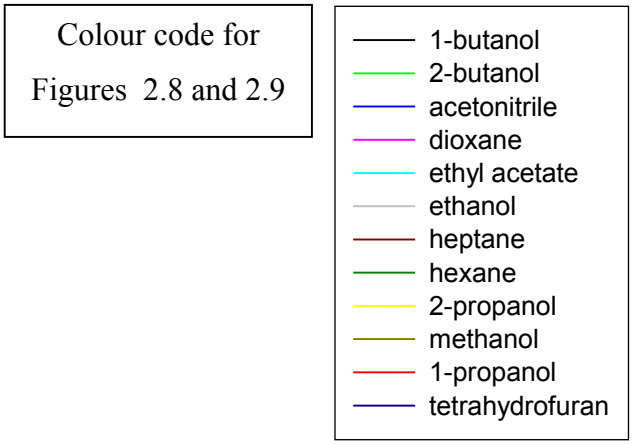


Figure 2.8 Emission scan of CoQ_{10} in various solvents using excitation at 290 nm. See colour code below for corresponding solvents.



As expected, the fluorescence responses of vitamin K_1 and 4-methoxy-1-naphthol in various solvents were similar, and were highest in protic solvents such as ethanol and 1-propanol (Figure 2.9 and Table 2.6). The exception was with tetrahydrofuran and dioxane in which 4-methoxy-1-naphthol showed high fluorescence but vitamin K_1 naphthoquinol did not. The emission maxima shifted slightly toward shorter wavelengths with aprotic solvents. These results support the hypothesis of Kruk and Strzalka (1993) (51), who investigated the effect of solvent on the fluorescence intensity of the chemically similar plastoquinol-9 and α-tocopherol quinone. They reported that the fluorescence quantum efficiency changed significantly in solvents of different polarities, being highest in ethanol and lowest in hexane (51). Kruk and Strzalka (1993) (51) hypothesised that, in a solution of an apolar solvent, the absorption is to S_1 which is non-fluorescent or from which fluorescence has a low efficiency. However, in solvents of high polarity, if the energy distance between S_1 and S_2 is low, the latter state is more strongly stabilised and its energy is lower than that of S_1 (51). The molecule, after absorption to S_1 followed by thermal deactivation to S_2, will emit energy in the form of fluorescence (emission from S_2 is highly efficient) (51). In such a case, the absorption remains unaffected by a change in the solvent polarity from non-polar to polar, whereas the quantum efficiency of the fluorescence strongly increases and a long-wavelength shift in the emission maxima occurs (51). This hypothesis is further supported by the significant correlation between vitamin K_1 fluorescence and dielectric constant ($r = + 0.67$, $p = 0.047$), and between the vitamin K_1 Stokes shift and dielectric constant ($r = + 0.65$, $p = 0.031$). The fluorometer results for vitamin K_1 agree with those obtained using the HPLC method. A quenching of vitamin K_1 fluorescence by dichloromethane as an HPLC solvent has been reported previously (47).

The fluorescence of 4-methoxy-1-naphthol was higher in aprotic solvents than that of vitamin K_1 (Table 2.6), which may be either due to interactions of the phytyl side-chain on vitamin K_1, or due to the methoxy group on 4-methoxy-1-naphthol reacting with the solvents. There was a shift to shorter wavelengths for the emission maxima of 4-methoxy-1-naphthol in the aprotic solvents hexane, heptane, dioxane, ethyl acetate and tetrahydrofuran (Table 2.6).

Table 2.6 Fluorescence response of reduced vitamin K_1, and 4-methoxy-1-naphthol in various solvents, as determined using a fluorometer (data is mean ± SE).

	Vitamin K_1 naphthoquinol			4-methoxy-1-naphthol		
Solvent	Emission Maxima (wavenumber, cm^{-1})	Emission Response (± SE)	R^2 for Fit	Emission Maxima (wavenumber, cm^{-1})	Emission Response (± SE)	R^2 for Fit
Methanol	22889 ± 8	79.56 ± 0.32	0.989	24617 ± 7	176.41 ± 1.20	0.993
Ethanol	23044 ± 9	191.98 ± 0.82	0.987	24800 ± 6	221.28 ± 1.18	0.995
1-butanol	23269 ± 9	76.22 ± 0.35	0.986	24798 ± 7	175.43 ± 1.02	0.994
2-butanol	23058 ± 9	5.99 ± 0.03	0.985	24798 ± 8	4.65 ± 0.03	0.992
1-propanol	22933 ± 8	183.43 ± 0.74	0.989	24862 ± 1	335.83 ± 2.77	0.996
2-propanol	23004 ± 9	137.56 ± 0.61	0.987	24840 ± 6	236.24 ± 1.22	0.995
Acetonitrile	23521 ± 10	119.53 ± 0.64	0.980	25027 ± 6	204.68 ± 0.88	0.996
Hexane	23359 ± 8	2.80 ± 0.01	0.989	25658 ± 4	125.27 ± 0.25	0.999
Heptane	23507 ± 8	6.05 ± 0.02	0.986	25608 ± 5	146.48 ± 0.34	0.999
Dioxane	23177 ± 38	0.29 ± 0.01	0.813	25780 ± 4	236.87 ± 0.48	0.999
Ethyl Acetate	24152 ± 10	1.90 ± 0.01	0.981	25874 ± 5	4.71 ± 0.01	0.999
Tetrahydrofuran	23275 ± 30	4.28 ± 0.05	0.861	25779 ± 5	230.31 ± 0.5	0.999

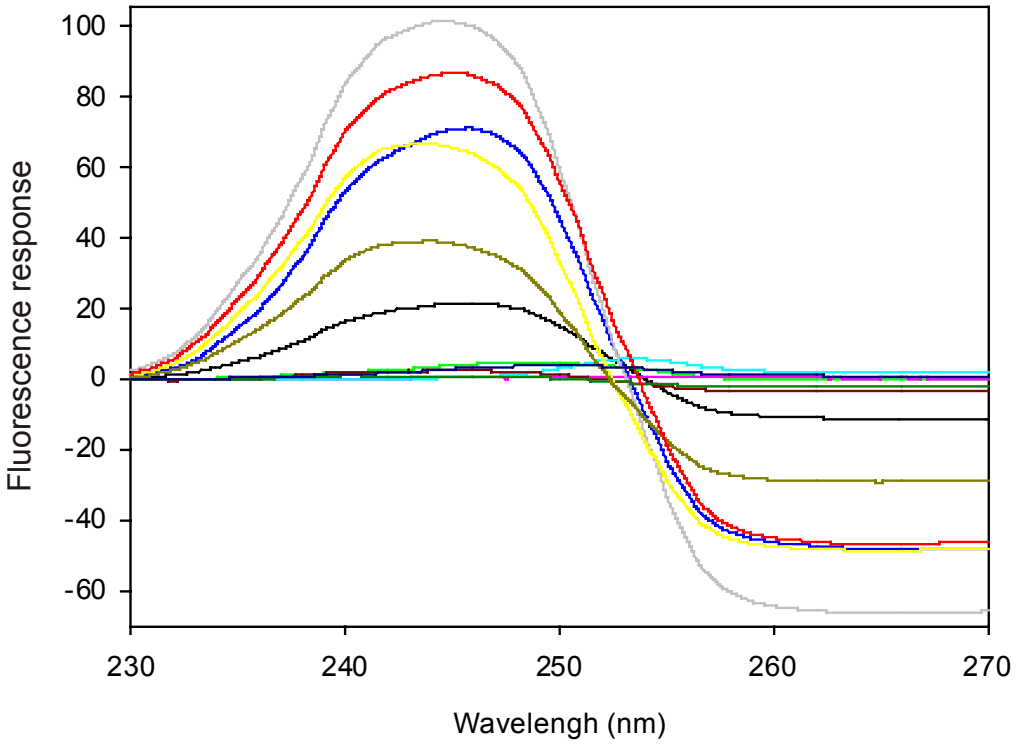


Figure 2.9 Emission scan for vitamin K_1 in various solvents. See colour code (below Figure 2.8) for corresponding solvents.

Table 2.7 Fluorescence peak height for $CoQ_{10}H_2$ and vitamin K_1 naphthoquinol as determined using HPLC (mean ± SD).

Solvent	Coenzyme Q_{10}	Vitamin K_1
Methanol	24.0 ± 1.5	436
Ethanol	25.9 ± 0.4	620
1-Butanol	16.9 ± 1.8	459
1-Propanol	17.0 ± 2.2	443
2-Propanol	12.8 ± 5.0	501
Dioxane	35.4 ± 0.1	195
Ethyl Acetate	26.8 ± 0.6	162
Tetrahydrofuran	11.2 ± 4.7	268

In the HPLC study, repeat measurements were not obtained for vitamin K_1 due to the solvents affecting the performance of the reactor, which consequently affected the results depending on the sequence of solvents run.

The results presented here apply directly to the fluorescence detection of CoQ_{10} and vitamin K_1 after HPLC. Therefore, the presence of 2 and 27% 1-propanol in the vitamin K_1 and CoQ_{10} solutions, respectively (or 20% methanol in the case of the HPLC experiment) for all solvents tested is not expected to be a problem. It was anticipated that biological samples injected into the HPLC would be, for example, 1-propanol extracts of CoQ_{10}. A more accurate estimate of the effect of solvents on fluorescence would have been obtained if pure solvents were used. However, because the primary aim of this project was to develop or improve HPLC assays for CoQ_{10} and vitamin K, this investigation of solvent effect on fluorescence was tailored to give results relevant to the aim of the project as opposed to an exact chemical demonstration of the effect of solvent on fluorescence.

It is possible that the tetrabutylammonium borohydride used to reduce the quinones increases the pH of the final solution, favouring formation of the semi-quinone form which would have different fluorescence properties to the quinol form. Additionally, a basic solution may cause a bathochromic shift in the emission maxima and affect the fluorescence yield. de Barros *et al.,* (1994) (45) reported that fluorescence of primin (Figure 2.10), after reduction with sodium borohydride, was just below 80% of the relative fluorescence intensity when no acid was added, and the relative fluorescence intensity increased on addition of 0 – 40 µmoles of acid in 10 µmol/L, but plateaued after 40 µmol/L (45). For lapachol (Figure 2.10) (87 µmol/L) reduced with borohydride, the relative fluorescence intensity was 75% with no acid and increased with up to 60 µmol/L acid added, then plateaued with addition of extra acid (45).

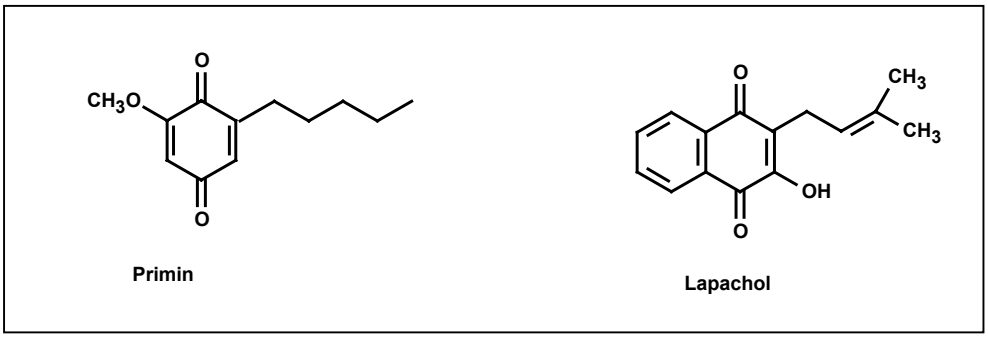


Figure 2.10 The stuctures of primin and lapachol which were reduced by borohydride by de Barros *et al.*, (1994) (45).

However, in the present investigation, the pH of the solution after addition of tetrabutylammonium borohydride was close to seven (as tested using Litmus paper), suggesting that the concentration of tetrabutylammonium borohydride added did not alter the pH significantly.

2.4. Stability of Coenzyme Q_{10} during extraction and frozen storage

An important factor to consider when setting up a clinical assay is the stability of the analyte. This determines the practicalities of sample collection (how soon after collection of the sample must the analyte be measured?), extraction (will the analyte degrade before it is purified?), and logistics (can the sample be stored until a time suitable for analysis?). In the case of routine laboratory analysis, stability of the analyte during long term frozen storage is generally not an issue. However, where clinical studies are being conducted, long-term storage may be necessary.

There is a lack of good analytical data recording the stability of CoQ_{10}. Most reports that do discuss stability of CoQ_{10} in whole blood, EDTA and lithium heparinised plasma, and in solvent extracts of plasma neglect to mention whether the sample was stored in the light or the dark. This is important given that CoQ_{10} is photochemically decomposed (57). It is also possible that the volume of sample stored affects stability, as other antioxidants present in plasma may protect CoQ_{10} from degradation. It is evident that $CoQ_{10}H_2$, and probably CoQ_{10}, is more stable in heparinised than EDTA plasma (32, 58). The exact mechanism for this is not known. Many of the reports on the stability of CoQ_{10} were carried out using EDTA plasma.

It has been known since the 1960s that ultraviolet light and sunlight destroy CoQ_{10} (57). Some methods for measurement of plasma CoQ_{10} recommend that samples be protected from light whenever possible to avoid photochemical decomposition of CoQ_9 and CoQ_{10} (4, 31, 33, 36, 41, 59). Kaikkonen *et al.,* (1999) (10) investigated the effect of light on the stability of CoQ_{10} in plasma. Samples (n = 8) were either covered with aluminium foil or exposed to light during sample pre-treatment. Plasma total CoQ_{10} was 10.1% lower in the samples kept in the light during the long extraction procedure (consisting of evaporation and reconstitution) and this effect was independent of plasma CoQ_{10} concentration and proportionally similar for all samples (10).

The stability of CoQ_{10} during long term storage has not been well documented. Edlund (1988) (33) reported that $CoQ_{10}H_2$ (and presumably total CoQ_{10}) was stable in plasma stored at –70 ºC for at least 5 months, but storage at -20 ºC resulted in oxidation of $CoQ_{10}H_2$. It has also been reported that $CoQ_{10}H_2$ (and presumably total CoQ_{10}) is stable for at least 12 months (32, 60) in plasma stored at –75 ºC and –80 ºC respectively. On a limited number of samples (n = 8), Kaikkonen *et al.,* (1999) (10) reported that total CoQ_{10} is stable in lithium heparinised plasma stored for 3 years at –80 ºC.

2.4.1. General methods

2.4.1.1. HPLC assay for total CoQ_{10}

In the following experiments, the concentration of total CoQ_{10} was measured using HPLC with electrochemical detection as described in Chapter 3, Section 3.6.2.

2.4.2. Short-term total CoQ_{10} stability

2.4.2.1. Aim

To investigate the photostability of total CoQ_{10} in standard solution and plasma.

2.4.2.2. Experimental

Aliquots (25 µL) of plasma (containing 0.38 µmol/L CoQ_{10}) and standard CoQ_{10} in 1-propanol (3.0 µmol/L) were stored in clear plastic Eppendorf tubes on the bench (approximately 21 ºC) either in the light (under fluorescent lighting) or protected from light (wrapped in aluminium foil). After 0, 2, 4, 6, 34, and 48 hours, duplicate aliquots of standard or plasma were extracted by addition of 225 µL 1-propanol, then subjected to HPLC analysis.

2.4.2.3. Results

Coenzyme Q_{10} is light sensitive being almost completely decomposed after 24 hours exposure to light (Figures 2.11 and 2.12). This degradation did not occur when samples were kept protected from light (Figures 2.11 and 2.12). Therefore keeping samples protected from light during long extraction procedures (> 2 hours) is desirable. These results support the statement by Kaikkonen *et al.,* (1999) (10) that samples for plasma total CoQ_{10} measurement can be pre-treated in normal laboratory lighting conditions. It is

probable that the lag time in CoQ_{10} degradation in the presence of light is due to a free radical reaction. During sample analysis an autosampler that keeps samples in the dark is important.

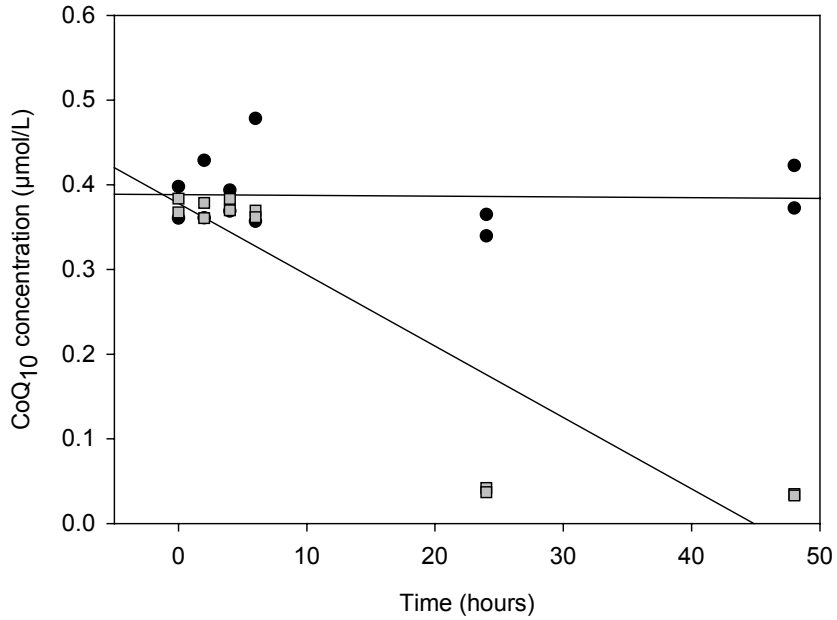


Figure 2.11 The CoQ_{10} concentration of a plasma sample (initial CoQ_{10} concentration 0.38 µmol/L) over time during exposure to (squares) and protection from (circles) light.

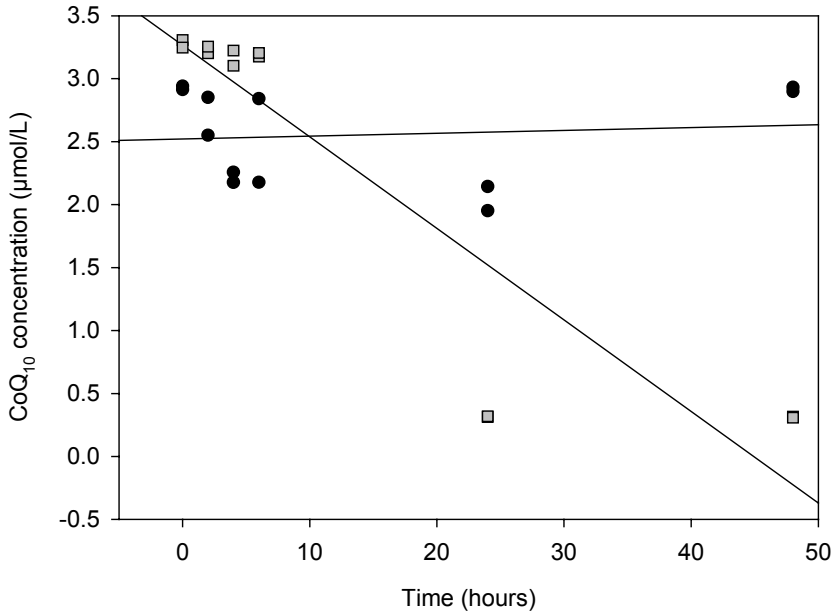


Figure 2.12 The CoQ_{10} concentration of a standard solution of CoQ_{10} in 1-propanol (3 µmol/L) over time, exposed to (squares) and protected from (circles) light.

2.4.3. Long-term stability of total CoQ_{10} at –13 ºC

2.4.3.1. Aim

To determine the stability of total CoQ_{10} at –13 ºC in serum, and EDTA and lithium heparinised plasma samples (stored aliquots > 1 mL).

2.4.3.2. Experimental

Lithium heparinised plasma, EDTA plasma, and serum samples were obtained from 10 staff at Canterbury Health Laboratories (written informed consent was obtained from all volunteers). Blood specimens were centrifuged within 1 hour of collection, and the plasma or serum stored at 4 ºC until HPLC analysis (within 4 hours of collection).

After measuring baseline total CoQ_{10} samples were stored in the dark at –13 ºC for 12 months. After this time samples were thawed, and total CoQ_{10} was measured again using the same methodology.

2.4.3.3. Results

There was a significant decrease in the percentage of total CoQ_{10} in the plasma samples after storage at –13 ºC for 12 months, with the mean (± SD) percentage of CoQ_{10} left in each sample type being 40 ± 13, 63 ± 25, and 54 ± 19 for EDTA, serum, and lithium-heparinised plasma, respectively (Figure 2.13).

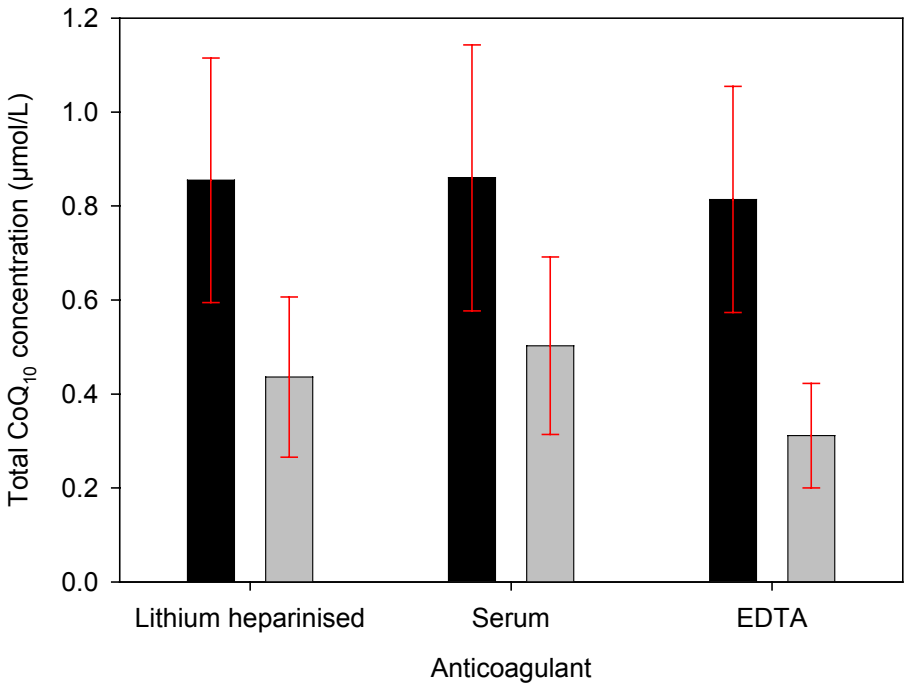


Figure 2.13 The concentration of total CoQ_{10} in lithium heparinised plasma, EDTA plasma and serum samples before (black) and after (grey) storage at –13 ºC for 12 months. Error bars are standard deviations. (n = 10).

2.4.4. Long-term stability of total CoQ_{10} at –80 ºC

2.4.4.1. Aim

To investigate the stability of total CoQ_{10} in lithium heparinised plasma samples stored at –80 ºC for 18 months.

2.4.4.2. Experimental

Baseline (fasted) samples (n = 70) were obtained from healthy males for a study investigating the bioavailability of CoQ_{10} supplements (Chapter 5, section 5.4). Blood specimens were centrifuged and plasma removed within 1 hour of collection. Plasma (lithium heparinised) was immediately stored at –80 ºC, and total CoQ_{10} was analysed within 4 months of collection.

Immediately after analysis, samples were returned to –80 ºC. Eighteen months after the initial analysis of total CoQ_{10}, samples were thawed, and total CoQ_{10} measured again using identical methodology.

2.4.4.3. Results

There was no change in the total CoQ_{10} concentration of the samples after 18 months of storage (mean ± SD 1.25 ± 0.63 vs 1.33 ± 0.78 μmol/L before and after storage, respectively) at –80 ºC. This result is consistent with the findings of Kaikkonen *et al.,* (1999) (10).

2.4.5. Stability of total CoQ_{10} in glass and plastic containers

2.4.5.1. Aim

To test whether CoQ_{10} may be absorbed by plastic or plasticisers when stored in plastic containers.

2.4.5.2. Experimental

Aliquots of fresh plasma (300 μL) were added to glass and plastic (polycarbonate) tubes. Aliquots were left at room temperature or immediately frozen to –13 ºC. Duplicate samples stored in glass and plastic were assayed for total CoQ_{10} after standing protected from light at room temperature for 2 hours. After 24 hours under the same conditions, a further set of aliquots in glass and plastic (in duplicate) were assayed for total CoQ_{10}. Total CoQ_{10} was assayed after 18 and 90 days.

2.4.5.3. Results:

There was no difference in total CoQ_{10} concentration of plasma stored in either glass or plastic tubes (Table 2.8) for up to 90 days.

Table 2.8 The concentration of CoQ_{10} in plasma stored in glass and plastic tubes for varying lengths of time.

Storage Time (days)	Storage Temperature (ºC)	Total CoQ_{10} in Glass Tubes (μmol/L)	Total CoQ_{10} in Plastic Tubes (μmol/L)
0.08	21	0.59	0.61
1	21	0.54	0.57
18	-13	0.69	0.67
90	-13	0.50	0.49

2.5. Discussion

Both CoQ_{10} and vitamin K are lipophilic. Thus when extracting them from plasma, lipids are co-extracted. Sample clean-up steps are often costly in terms of time and resources and may lead to degradation of the analyte. Hence, any advance in measurement techniques that allows measurement of the analyte in a less pure, or less concentrated, extract is welcomed. If co-extracted compounds co-elute with the analyte during HPLC and are detected by the detector (hence interfering with chromatography), altering detector settings may make it possible to 'screen out' these interfering compounds. This requires knowledge of the properties of the analyte under the detection method being used. For example, when measuring CoQ_{10} by electrochemical detection, because the potential required to oxidise CoQ_{10} is high (550 mV) and very little, if any, CoQ_{10} is oxidised at potentials lower than 350 mV, an electrochemical cell prior to the analytical electrochemical cell in the HPLC system can be set to a potential of 350 mV resulting in oxidation of any interfering compounds that are oxidised at or below this potential. Once oxidised, these compounds will then not be detected by the analytical electrochemical cell and hence will not appear on the chromatogram.

Endogenous vitamin K is present in plasma at very low concentrations. Therefore, any factor that can increase sensitivity of an assay for vitamin K is important. Use of a mobile phase containing a protic solvent increases the fluorescence yield and hence the sensitivity of the fluorescence assay. Furthermore, use of a platinum-black filled reactor to catalyse reduction of vitamin K by an alcohol present in the mobile phase may increase the sensitivity, and will certainly increase the reproducibility of an assay for vitamin K as opposed to using zinc reduction. The platinum-black reactor does not require frequent repacking, or additives in the mobile phase as is required for zinc reduction.

Fluorescence detection of coenzyme Q_{10} after HPLC has not been reported. Results presented in Section 2.4.1 show that the fluorescence yield of coenzyme Q_{10} is low, which may be due to quenching of fluorescence in protic solvents. Because of this fact, higher fluorescence yields were found in aprotic solvents for CoQ_{10} and CoQ_0. For platinum-black-catalysed reduction of CoQ_{10} by an alcohol, a protic solvent is required in the mobile phase. Hence fluorescence detection of CoQ_{10} in biological samples is not as effective as it is for vitamin K.

Knowledge of the temperature and light stability of an analyte is essential when carrying out routine measurement in biological samples. Protection of samples from light during analysis can waste time if this precaution is not required. However, inaccuracies in determination of the actual concentration due to sample handling may give erroneous results. Stability results for vitamin K were not able to be obtained due to the inability to develop an acceptable assay for measuring vitamin K in biological samples. Stability results for CoQ_{10} show that there is no need to protect samples from light during extraction, as long as the time that samples are exposed to light is kept to a minimum, and is no longer than 2 hours. Additionally, CoQ_{10} is not stable during storage at –13 ºC for 12 months, but it is stable during storage at –80 ºC for at least 18 months. Preliminary evidence shows that CoQ_{10} has similar stability when stored in either glass or plastic tubes, and is not absorbed onto plastic.

2.6. References for Chapter 2

1. Lefevere MF, De Leenheer AP, Claeys AE, Claeys IV, Steyaert H. Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. Journal of Lipid Research 1982;23:1068-72.
2. Kröger A. Determination of contents and redox states of ubiquinone and menaquinone. Methods in Enzymology 1978;53:579-91.
3. Kaplan P, Sebestianová N, Turiaková J, Kucera I. Determination of Coenzyme Q in human plasma. Physiological Research 1995;45:39-45.
4. Okamoto T, Fukui K, Nakamoto M, Kishi T, Okishio T, Yamagami T, et al. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. Journal of Chromatography 1985;342:35-46.
5. Galinier A, Carrière A, Fernandez Y, Bessac AM, Caspar-Bauguil S, Periquet B, et al. Biological validation of coenzyme Q redox state by HPLC-EC measurement: relationship between coenzyme Q redox state and coenzyme Q content in rat tissues. FEBS Letters 2004;578:53-7.
6. Crane FL, Barr R. Determination of ubiquinones. Methods in Enzymology 1971;18:137-65.
7. Kröger A, Klingenberg M. On the role of ubiquinone in mitochondria. II. Redox reactions of ubiquinone under the control of oxidative phosphorylation. Biochemische Zeitschrift 1966;344:317-36.
8. Redfern ER. Isolation and determination of ubiquinone. Methods in Enzymology 1967;10:381-4.
9. Tang PH, Miles MV, Miles L, Quinlan J, Wong B, Wenisch A, Bove K. Measurement of reduced and oxidized coenzyme Q_9 and coenzyme Q_{10} levels in mouse tissues by HPLC with coulometric detection. Clinica Chimica Acta 2004;341:173-84.
10. Kaikkonen J, Nyyssönen K, Salonen JT. Measurement and stability of plasma reduced, oxidised and total coenzyme Q10 in humans. Scandinavian Journal of Clinical and Laboratory Investigation 1999;59:457-66.
11. Szarkowska L, Klingenberg M. On the role of ubiquinone in mitochondria. Spectrophotometric and chemical measurements of its redox reactions. Biochemische Zeitschrift 1963;338:674-97.
12. Mosca F, Fattorini D, Bompadre S, Littarru G. Assay of coenzyme Q10 in plasma by a single dilution step. Analytical Biochemistry 2002;305:49-54.

13. Daines AM. New Assays for Biologically Active Quinones. Christchurch: University of Canterbury, 2001.
14. Haroon Y, Bacon DS, Sadowski JA. Chemical reduction system for the detection of phylloquinone (Vitamin K_1) and menaquinones (Vitamin K_2). Journal of Chromatography 1987;384:383-9.
15. Booth SL, Davidson KW, Sadowski JA. Evaluation of an HPLC method for the determination of phylloquinone (vitamin K_1) in various food matrices. Journal of Agricultural and Food Chemistry 1994;42:295-300.
16. Jakob E, Elmadfa I. Application of a simplified HPLC assay for the determination of phylloquinone (vitamin K_1) in animal and plant food items. Food Chemistry 1996;56:87-91.
17. Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high-performance liquid chromatography using postcolumn chemical reduction. Methods in Enzymology 1997;282:408-21.
18. Indyk H, Woollard DC. Vitamin K in milk and infant formulas: Determination and distribution of phylloquinone and menaquinone-4. Analyst 1997;122:1-5.
19. Jakob E, Elmadfa I. Rapid and simple HPLC analysis of vitamin K in food, tissues and blood. Food Chemistry 2000;68:219-21.
20. Indyk H, Woollard DC. Determination of vitamin K in milk and infant formulas by liquid chromatography: Collaborative study. Journal of AOAC International 2000;83:121-30.
21. Ware GM, Chase GW, Eitenmiller RR, Long AR. Determination of vitamin K_1 in medical foods by liquid chromatography with postcolumn reduction and fluorometric detection. Journal of AOAC International 2000;83:957-62.
22. Wang LY, Bates CJ, Yan L, Harrington DJ, Shearer MJ, Prentice A. Determination of phylloquinone (vitamin K_1) in plasma and serum by HPLC with fluorescence detection. Clinica Chimica Acta 2004;347:199-207.
23. Haroon Y, Bacon DS, Sadowski JA. Liquid-chromatographic determination of vitamin K_1 in plasma, with fluorometric detection. Clinical Chemistry 1986;32:1925-9.
24. MacCrehan WA, Schönberger E. Determination of vitamin K_1 in serum using catalytic-reduction liquid chromatography with fluorescence detection. Journal of Chromatography B 1995;670:209-17.

25. Usui Y, Nishimura N, Kobayashi N, Okanoue T, Kimoto M, Ozawa K. Measurement of vitamin K in human liver by gradient elution high-performance liquid chromatography using platinum-black catalyst reduction and fluorimetric detection. Journal of Chromatography 1989;489:291-301.
26. Usui Y. Assay of phylloquinone and menaquinones in human liver. Methods in Enzymology 1997;282:438-47.
27. Kamao M, Suhara Y, Tsugawa N, Okano T. Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. Journal of Chromatography B 2005;816:41-8.
28. Shino M. Determination of endogenous vitamin K (phylloquinone and menaquinone-n) in plasma by high-performance liquid chromatography using platinum oxide catalyst reduction and fluorescence detection. Analyst 1988;113:393-7.
29. Cham BE, Roeser HP, Kamst TW. Simultaneous liquid-chromatographic determination of vitamin K_1 and vitamin E in serum. Clinical Chemistry 1989;35:2285-9.
30. Poulsen JR, Birks JW. Photoreduction fluorescence detection of quinones in high-performance liquid chromatography. Analytical Chemistry 1989;61:2267-76.
31. Tang PH, Miles, M.V., DeGrauw, A., Hershey, A., Pesce, A. HPLC analysis of reduced and oxidised coenzyme Q10 in human plasma. Clinical Chemistry 2001;47:256-65.
32. Lagendijk J, Ubbink JB, Delport R, Hayward WJ, Human JA. Measurement of the ratio between the reduced and oxidized forms of coenzyme Q10 in human plasma as a possible marker of oxidative stress. Journal of Lipid Research 1996;37:67-75.
33. Edlund PO. Determination of Coenzyme Q_{10}, α-tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. Journal of Chromatography 1988;425:87-97.
34. Menke T, Niklowitz P, Adam S, Weber M, Schlüter B, Andler W. Simultaneous detection of ubiquinol-10, ubiquinone-10, and tocopherols in human plasma microsamples and macrosamples as a marker of oxidative damage in neonates and infants. Analytical Biochemistry 2000;282: 209-17.
35. Niklowitz P, Menke T, Andler W, Okun JG. Simultaneous analysis of coenzyme Q10 in plasma, erythrocytes and platelets: comparison of the antioxidant level in blood

cells and their environment in healthy children and after oral supplementation in adults. Clinica Chimica Acta 2004;342:219-26.

36. Wang Q, Lee BL, Ong CN. Automated high-performance liquid chromatographic method with precolumn reduction for the determination of ubiquinol and ubiquinone in human plasma. Journal of Chromatography B 1999;726:297-302.
37. Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Analytical Biochemistry 1997;250:66-73.
38. Leray C, Andriamampandry MD, Freund M, Gachet C, Cazenave JP. Simultaneous determination of homologues of vitamin E and coenzyme Q and products of alpha-tocopherol oxidation. Journal of Lipid Research 1998;39:2099-105.
39. Wakabayashi H, Yamato S, Nakajima M, Shimada K. Simultaneous determination of oxidized and reduced Coenzyme Q and α-Tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. Biological and Pharmaceutical Bulletin 1994;17:997-1002.
40. Pastore A, Di Giovamberardino G, Bertini E, Tozzi G, Gaeta LM, Federici G, Piemonte F. Simultaneous determination of ubiquinol and ubiquinone in skeletal muscle of pediatric patients. Analytical Biochemistry 2005;342:352-5.
41. Lang JK, Gohil K, Packer L. Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Analytical Biochemistry 1986;157:106-16.
42. Finckh B, Kontush A, Commentz J, Hübner C, Burdelski M, Kohlschütter A. Monitoring of Ubiquinol-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Analytical Biochemistry 1995;232:210-6.
43. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. Journal of Lipid Research 1996;37:893-901.
44. Gohil K, Rothfuss L, Lang J, Packer L. Effect of exercise training on tissue vitamin E content. Journal of Applied Physiology 1987;63:1638-41.
45. De Barros Alcanfôr SK, Cardoso SV, de Lima CG. Fluorimetric studies of some quinones and quinonoid compounds after reduction reaction. Analytica Chimica Acta 1994;289:273-90.

46. Haroon Y, Bacon DS, Sadowski JA. Reduction of quinones with zinc metal in the presence of zinc ions: Application of post-column reactor for the fluorometric detection of vitamin K compounds. Biomedical chromatography : BMC 1987;2:4-8.
47. MacCrehan WA, May WE. Oxygen removal in liquid chromatography with a zinc oxygen-scrubber column. Analytical Chemistry 1984;56:625-8.
48. Greenspan MD, Lee Lo C-Y, Hanf DP, Yudkovitz JB. Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector. Journal of Lipid Research 1988;29:971-6.
49. Jiang P, Wu M, Zeheng Y, Wang C, Li Y, Xin J, Xu G. Analysis of coenzyme Q10 in human plasma by column-switching liquid chromatography. Journal of Chromatography B 2004;805:297-301.
50. Sharma S, Kheradpezhou M, Shavali S, El Refaey H, Eken J, Hagen C, Ebadi M. Neuroprotective actions of coenzyme Q_{10} in Parkinson's disease. Methods in Enzymology 2004;382:488-509.
51. Kruk J, Strzalka K. Fluorescence properties of plastoquinol, ubiquinol and a-tocopherol quinol in solution and liposome membranes. Journal of Photochemicals and Photobiology B: Biology 1993;19:33-8.
52. Speek AJ, Schrijver J, Schruers WHP. Fluorimetric determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes by high-performance liquid chromatography with post-column derivatization. Journal of Chromatography 1984;301:441-7.
53. Hirauchi K, Sakano T, Notsumoto S, Nagaoka T, Morimoto A, Fujimoto K, et al. Measurement of K vitamins in animal tissues by high-performance liquid chromatography with fluorimetric detection. Journal of chromatography 1989;497:131-7.
54. Lambert WE, De Leenheer AP, Lefevere MF. Determination of vitamin K in serum using HPLC with post-column reaction and fluorescence detection. Journal of Chromatographic Science 1986;24:76-9.
55. Lambert WE, De Leenheer AP. Simplified post-column reduction and fluorescence detection for the high-performance liquid chromatographic determination of vitamin $K_{1(20)}$. Analytica Chimica Acta 1987;196:247-50.

56. Atkins. Physical Chemistry. 6th ed: Oxford university press, Oxford., 1998:page 503, Chapter 17pp.
57. Hatefi Y. Coenzyme Q (Ubiquinone). Advances in Enzymology and Related Subjects of Biochemistry 1963;25:275-328.
58. Tang PH, Miles MV, Steele P, DeGrauw A, Chuck G, Schroer L, Pesce A. Anticoagulant effects on plasma coenzyme Q10 estimated by HPLC with coulometric detection. Clinica Chimica Acta 2002;318:127-31.
59. Andersson S. Determination of coenzyme Q by non-aqueous reversed-phase liquid chromatography. Journal of Chromatography 1992;606:272-6.
60. Hectors MPC, van Tits LJH, de Rijke YB, Demacker PNM. Stability studies of ubiquinol in plasma. Annals of Clinical Biochemistry 2003;40:100-1.

Chapter 3

Determination of Coenzyme Q_{10}

3.1. Introduction

A rapid and reliable assay to quantify plasma CoQ_{10} was required in order for testing of research hypotheses (Chapter 1, Section 1.5). These hypotheses included that CoQ_{10} can be limiting in human tissues, and an insufficiency causes cellular dysfunction, that muscle tissue, with its high energy requirement, will be particularly susceptible to CoQ_{10} insufficiency, that CoQ_{10} insufficiency will exacerbate cardiovascular disease, and that statin therapy can cause CoQ_{10} insufficiency and this is a factor in the muscular side-effects of statin therapy.

Plasma coenzyme Q_{10} (CoQ_{10}) is usually determined using high-performance liquid chromatography (HPLC), with ultraviolet (UV) or electrochemical detection. An assay for CoQ_{10} was required at Canterbury Health Laboratories to enable monitoring of plasma CoQ_{10}, in patients receiving statin therapy. It was not a requirement that the assay be capable of quantifying both reduced and oxidised forms of CoQ_{10}, since this ratio changes readily in the presence of atmospheric oxygen. Sample handling is critical when measuring the ratio of CoQ_{10} to $CoQ_{10}H_2$ in plasma, to avoid a change in the ratio from the *in-vivo* situation. The requirement for strict sample handling makes measuring the ratio inappropriate in a routine diagnostic test.

Measurement of total CoQ_{10} is less subject to artefactual interference from sample handling and is less demanding on detector sensitivity since only 4% of CoQ_{10} is in the reduced form requiring a detection limit of 22.5 nmol/L in plasma. While measurement of the $CoQ_{10}H_2$ to CoQ_{10} ratio was not a priority, the possibility of measuring it was not totally neglected.

Desired attributes of a routine assay are that sample turnaround time is short and assay costs are low. Therefore, extraction of the analyte from the biological matrix should be simple and rapid, the equipment used should be inexpensive and robust, and the solvents used should be easily obtainable and inexpensive.

There are four important steps to consider when developing a routine test to quantify a blood analyte by HPLC. The first step is to make sure that the desired detection method is

sensitive enough to measure the analyte at the expected concentrations. The second step is the extraction of the compound from the biological matrix, the third is the development of the chromatography system and the final step is to confirm that the method gives accurate and reproducible values for the analyte and to build in quality assurance factors to ensure consistency of the method over time.

When setting up an assay for an analyte, it is important to critically review other published assays for that analyte. From this review, either a novel assay can be developed or a reported assay can be set up and improved if necessary. As discussed in Chapter 1, the large majority of assays for CoQ_{10} in biological samples utilise HPLC. A summary of (a) extraction procedures that have been used to extract CoQ_{10} from biological samples (Appendix 1), and (b) HPLC systems that have been used to measure CoQ_{10} in extracts (Appendix 2) is attached.

Two fast and reliable HPLC assays for the determination of plasma CoQ_{10}, one using electrochemical detection (1) and the other ultraviolet detection (2), were used as the basis for work presented in this Chapter. The initial expense and general lack of availability of electrochemical detectors as opposed to ultraviolet detectors makes them less desirable. Fluorescence detection has not been reported for detection of CoQ_{10} despite being the most commonly used detector for measurement of the chemically similar analyte, vitamin K. As has been discussed (Chapter 1, Section 1.4), it was deduced from work by Daines (2001) (3) that fluorescence detection should offer sufficient sensitivity to measure the endogenous CoQ_{10} to $CoQ_{10}H_2$ ratio. It is important to also consider the analytical qualities (such as the sensitivity and specificity) of detection methods before deciding which detector to use.

Three methods of detection (electrochemical, fluorescence, and ultraviolet) of CoQ_{10} (after HPLC assay) were compared to clarify the advantages and disadvantages of each method. Using the results from this investigation, as well as the equipment initially available, steps were taken to set up an assay to measure plasma total CoQ_{10} using ultraviolet detection. After setting up the assay using ultraviolet detection, an electrochemical detector became routinely available, and hence the transfer and modification of the method to use electrochemical detection is discussed. Finally, the reproducibility and accuracy of the two methods are compared with each other and that of similar reported assays.

3.2. Detector

It was anticipated that, by analogy with the sensitivity gained when using fluorescence detection to measure vitamin K (4-23), fluorescence detection could be useful for measuring both plasma total CoQ_{10} and the plasma CoQ_{10} to $CoQ_{10}H_2$ ratio. Only $CoQ_{10}H_2$ fluoresces (24). Because $CoQ_{10}H_2$ is easily oxidised in the presence of air, reduction on-line in the HPLC is desired. As discussed in Chapter 2, CoQ_{10} can be quantitatively reduced by zinc, or alcohol in the presence of platinum-black as a catalyst, and both these reduction systems can be incorporated into an HPLC system with relative ease. It was proposed that a plasma extract could be injected into the HPLC system, CoQ_{10} and $CoQ_{10}H_2$ separated on the analytical column, and the reduction device placed between the column and the detector, to reduce the endogenous CoQ_{10} to $CoQ_{10}H_2$ for detection. For measurement of total CoQ_{10} using fluorescence detection, the reduction device could be placed before the analytical column, and total CoQ_{10} measured, as $CoQ_{10}H_2$. Alternatively, an oxidising agent could be added to the plasma extract before HPLC and the reduction device placed after the analytical column but before the detector, to reduce CoQ_{10} for detection.

As discussed in Chapter 2, oxidised CoQ_{10} absorbs radiation more intensely than $CoQ_{10}H_2$, with the molar absorptivities being 14,020 and 3,940 respectively. Because CoQ_{10} is present in plasma in both the reduced and oxidised state, plasma CoQ_{10} must be oxidised during the sample preparation process before ultraviolet detection, and total CoQ_{10} is subsequently measured.

Electrochemical detection measures the change in potential as electrons are lost or gained during oxidation or reduction. Most commonly, the loss of electrons during oxidation of CoQ_{10} is measured at the analytical electrochemical cell. While requiring more equipment, the most simple HPLC set-ups using electrochemical detection utilise three electrochemical cells, in the oxidation - reduction - oxidation, or reduction – reduction - oxidation mode (1, 25-29). Total CoQ_{10} can be measured by placing the first electrochemical cell before the analytical column. Both $CoQ_{10}H_2$ and CoQ_{10} can be measured simultaneously by placing the first electrode after the analytical column, as the column separates these two forms of CoQ_{10}. If a fourth electrochemical cell is available, the assay may be improved by placing the fourth electrode between the pump and the

injector, to oxidise any electroactive components present in the mobile phase. Some reported methods for electrochemical detection of $CoQ_{10}H_2$ and CoQ_{10} employ just one electrochemical cell for detection, with a non-electrochemical reduction step being added to the HPLC procedure (30-32). For example, the method of Leary *et al.*, (1998) (32) uses zinc catalysed reduction in a post-column reactor. The zinc reactor reportedly performs adequately for 2 weeks of successive runs before requiring replacement of the zinc filling (32). The method of Yamashita *et al.,* (1997) (31) utilises a reduction column (IRICA RC-10) immediately prior to the analytical cell. The method of Wang *et al.,* (1999) (30) uses a complex instrumentation for automated precolumn reduction of CoQ_{10} with sodium borohydride, which is made fresh by the automated system every three samples and injected into the extract. This sodium borohydride reduction requires duplicate injection of each sample when measurement of the ratio of $CoQ_{10}H_2$ to CoQ_{10} is required, and therefore is not efficient in terms of time. Edlund, (1988) (26) also reports the use of sodium borohydride to reduce all CoQ_{10} pre-column for measurement of total CoQ_{10} with electrochemical detection. As discussed in Chapter 2, the reduction of CoQ_{10} by an electrochemical cell is not as efficient as that by zinc or by alcohol in the presence of a platinum-black filled reactor. Therefore, use of a reduction system such as alcohol and platinum-black, or zinc, prior to electrochemical detection may result in a lower limit of detection due to the more efficient reduction.

3.2.1. Extraction

When using liquid-liquid extraction, the degree to which sample dilution can be tolerated depends on the sensitivity and specificity of the detector for the analyte.

For measurement of total CoQ_{10}, the limit of detection does not need to be as low as when measuring both CoQ_{10} and $CoQ_{10}H_2$ simultaneously, so a more dilute extract can be made. Additionally, a more sensitive detector means that a more dilute sample can be made and the limit of detection will still be acceptable. With electrochemical detection, it is possible to set up multiple electrochemical cells to work in a ‘screening’ fashion, so that only compounds able to undergo reversible redox reactions at the potentials applied are detected at the final analytical cell. This results in cleaner chromatograms.

All assays for measurement of CoQ_{10} use liquid-liquid extraction from plasma (or tissues). In some cases the liquid extract is injected directly, in others it is purified before injection, and in others it is purified on-line in the HPLC system (Appendix 1).

Direct extraction of plasma CoQ_{10} into hexane is not efficient (30). Protein denaturation with methanol before hexane extraction has been reported to be inefficient by some (1), but has apparently given 100% recovery for others (31). Similarly, extraction with hexane after precipitation with ethanol has been reported to be an efficient extraction procedure in some instances (1, 33) but has apparently given only 76 ± 36% recovery for others (34). However, the lower efficiency reported by Finckh *et al.,* (1995) (34) may be due to the evaporation procedure used during extraction. The efficiency depends on the ratio of plasma to solvent and also the ratio of methanol or ethanol (protein precipitation solvent) to hexane. The disadvantage of a direct hexane extraction is that when the extract is injected into the HPLC, the hexane reduces the retention of CoQ on a reversed-phase analytical column. To overcome this problem, either small injection volumes are used (31) or the hexane is evaporated and the residue reconstituted in a more appropriate solvent for reversed-phase chromatography (28, 29, 32-41). Reconstitution of the dry hexane extract requires a solvent that is compatible with the HPLC system and efficiently extracts CoQ_{10} from the dry lipid smear on the side of the vessel.

When measuring the ratio of CoQ_{10} to $CoQ_{10}H_2$, the use of hexane extraction, evaporation under nitrogen and reconstitution has been reported (28). However some reports suggest that this concentration process alters the ratio in favour of CoQ_{10} (25, 31) even when heparinised plasma (in which the ratio is more stable) is used (31).

Comparison of the recovery obtained when different solvents are used for liquid-liquid extraction of CoQ_{10} from plasma indicate that 1-propanol (being the most lipophilic alcohol that is miscible with water) is an efficient solvent for extraction (Table 3.1) (1, 26, 30, 42, 43). Lower alcohols do not efficiently extract CoQ_{10} (Table 3.1).

Table 3.1 Reported recoveries of CoQ_{10} in liquid-liquid extraction with various solvents.

Publication	Solvent	Ratio of plasma to solvent	Recovery (mean ± SD) %
(1)	1-propanol	0.11/1	99 ± 3
(42)	1-propanol	0.2/1	96 ± 9.2
(42)	1-propanol	0.3/1	98 ± 3.5
(42)	1-propanol	0.4/1	95.3 ± 4.0
(42)	1-propanol	0.5/1	90.7 ± 10.7
(26)	1-propanol	0.5/1	88
(26)	1-propanol	0.4/1	100.9
(26)	1-propanol	0.3/1	101.37
(1)	2-propanol	0.11/1	89.5
(42)	2-propanol	0.3/1	108.3 ± 14.4
(26)	2-propanol	0.5/1	46
(1)	Ethanol	0.11/1	88 ± 4
(42)	Ethanol	0.3/1	31
(26)	Ethanol	0.3/1	24
(1)	1-butanol	0.11/1	85 ± 5
(1)	Acetone	0.11/1	88
(42)	Acetone	0.3/1	88
(26)	Acetone	0.5/1	36
(26)	Acetone	0.3/1	40
(1)	Methanol/hexane (0.2/2.5 v/v)	0.11/1	64 ± 10
(1)	Hexane	0.11/1	52 ± 9
(1)	Acetonitrile	0.11/1	19 ± 11
(1)	Methanol	0.11/1	19 ± 10
(1)	Ethanol/hexane	0.11/1	100

To improve the efficiency of extraction of CoQ_{10} from plasma, the use of the surface-active agent dodecylsulfate has been reported (38, 43). However, Edlund, (1988) (26) reported that dodecylsulfate had no effect on extraction.

The reason why liquid-liquid extraction of CoQ_{10} from plasma is difficult is that CoQ_{10} is lipophilic and associates with lipids in the plasma. Since the extraction solvent needs to be hydrophobic to extract CoQ_{10} from plasma, it also extracts some lipids. Saponification can be used to breakdown triglyceride into glycerol and three molecules of a fatty acid salt. The fatty acid salt, usually a sodium or a potassium salt, has a hydrophobic tail and a hydrophilic head. Alkaline saponification of lipids has been used when measuring CoQ_{10} in foodstuffs (44) but has been reported to cause partial isomerization of CoQ_{10} to ubichromenol. In addition, if ethanolic potassium hydroxide is used, an exchange of methoxy groups of CoQ_{10} for ethoxy groups occurs even under mild conditions (45, 46). Greenspan *et al.,* (1988) (47) reported that saponification with potassium hydroxide resulted in complete loss of CoQ_{10} although it is possible that the heating step used in their method contributed to the breakdown and, if the procedure was carried out under full light, photochemical degradation may have also occurred. The use of enzymatic hydrolysis with lipase to remove serum lipids has also been reported (48).

Alternatively, purification of liquid-liquid extracts before HPLC analysis has been reported (41, 49, 50). This purification can eliminate the large solvent front and increase column lifetime. Thin layer chromatography (39, 51) has been used to purify the extract of plasma but poor recovery and accuracy were obtained (51). Grossi *et al.,* (1992) (52) and Kaplan *et al.,* (1995) (41) report solid phase extraction (SPE) for pre-purification, but 2 SPE steps were required which leads to a long procedure and more variation. Kommuru *et al.,* (1998) (49) simplified the procedure to only one step of SPE, but the solvent front was very large (42).

Kaikkonen *et al.,* (1999) (37) compared cartridges and powder (C18 and silica) for SPE of plasma extracts. Estimates of the total CoQ_{10} concentration were significantly higher when samples were purified with cartridges ($p = 0.013$) but a better CV and shorter sampling time supported the use of powders in routine analysis. Kaikkonen *et al.,* (1999) (37) also compared simple hexane extraction followed by evaporation and reconstitution in mobile phase with hexane extraction followed by SPE. The direct liquid-liquid extraction gave higher concentrations than the SPE method but this was probably because oxidation with copper chloride was used before extraction in the direct liquid-liquid extraction method. It appears that silica and C18 solid phase pre-treatments do decrease the number of unknown

peaks in the chromatogram compared with a single hexane extraction. However, these extra steps are not necessary because the extra peaks in the chromatogram do not interfere with either CoQ_{10} or the internal standard (37).

A further alternative method of purification of the liquid-liquid extract of plasma is to use semi-preparative HPLC. For example, Edlund, (1988) (26) reported the use of two reversed-phase columns with pre-fractionation on the first column. All of the compounds that were retained strongly on the first column (packed with Spherisorb ODS-2, 100 × 4.6 mm, 3 μm) were washed into a waste container (with a step-gradient of dichloromethane/methanol (50/50 v/v)) after analysis of each sample, and therefore did not pass through the second column (packed with Chromspher C_{18}, 100 × 3 mm, 5 μm). This system therefore incorporated complex column switching and changing of mobile phases. In the method of Jiang *et al.,* (2004) (42), plasma CoQ_{10} was extracted into 1-propanol and the supernatant was purified on-line on a C18 column, before transfer to a reversed-phase analytical column by a column-switching valve. Determination of CoQ_{10} was by ultraviolet detection at 275 nm. The chromatography achieved by Jiang *et al.,* (2004) (42) is visibly better than that by Mosca *et al.,* (2002) (2) (who injects a 1-propanol extract with no purification step) due to the presence of fewer impurities and a higher concentration of CoQ_{10}. The HPLC run time in the method of Jiang *et al.,* (2004) (42) takes 30 minutes per sample which is twice as long as that in the method of Mosca *et al.,* (2002) (2). The precision and recovery obtained by Jiang *et al.,* (2004) (42) is similar to that of Mosca *et al.,* (2002) (2), making it a viable option for measurement of plasma total CoQ_{10} if the equipment required for the on-line sample clean up and column switching is available. Lagendijk *et al.,* (1996) (25) report injection of a 1-propanol extract of plasma into the HPLC system, which includes a pneumatically activated 2-way valve between the column and the conditioning cell. The valve allows passage of the mobile phase either through the coulometric cells or past the coulometric cells directly to the waste container. The 1-propanol extraction used by Lagendijk *et al.,* (1996) (25) uses a plasma to 1-propanol ratio of 100 to 333 μL (v/v) as opposed to the method of Tang *et al.,* (2001) (1) in which the ratio is 100 to 900 μL. There is no requirement for a similar switching device or a secondary sample clean-up in the method of Tang *et al.,* (2001) (1) because the concentration of interfering co-extracted material is much lower and the electrochemical detector is used in a 'screening' mode of oxidation-reduction-oxidation.

3.2.2. Internal standards

Internal standards that have been used in published methods for measurement of CoQ_{10} are shown in Table 3.2.

Table 3.2 The internal standards used in various CoQ_{10} assays.

Publication	Measuring	Sample type	Internal standard
(39)	CoQ_{10}	Human plasma	CoQ_9
(26)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	A diethoxy CoQ_{10} analogue
(40)	CoQ_{10} $CoQ_{10}H_2$	Biological samples	Menaquinone-8
(34)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	CoQ_7, CoQ_9, CoQ_9H_2
(37)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	CoQ_9
(1)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	CoQ_9
(53)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	Ubiquinol-dicaprilate
(29)	CoQ_{10}	Erythrocytes	Ubihydrochinone-9

The use of CoQ_9 as an internal standard when measuring CoQ_{10} and $CoQ_{10}H_2$ in human plasma (1, 37, 39) is controversial as some reports suggest it is endogenously present in human plasma (54). Tang *et al*., (2001) (1) reported that analysis of 50 plasma samples (25 from healthy subjects and 25 from patients with various illnesses) identified only one sample with approximately 25 µg/L CoQ_9 from a patient with a rare glycogen storage disease (Type 1). Measurable CoQ_9 was not detected in the plasma samples from the 25 healthy individuals, or the remaining 24 patients (1). This issue is discussed further in Chapter 6.

Because mouse and rat plasma and tissues contain predominantly CoQ_9, this cannot be used as an internal standard for quantification of mouse and rat CoQ_9, CoQ_9H_2, CoQ_{10}, and $CoQ_{10}H_2$. Therefore, Tang *et al.,* (2004) (27) report the use of CoQ_6 as the internal

standard when measuring rodent CoQ, as CoQ_6 is not present endogenously in mouse tissue. Similarly, Okamoto *et al.,* (1985) (39) used CoQ_{11} as the internal standard when measuring CoQ_{10} in rat plasma.

3.3. Choice of detector

Things to consider when choosing the detection method include what equipment is already on hand (costs are reduced if new equipment is not necessary), budget, sensitivity required and what needs to be measured (for example, total CoQ_{10} or the $CoQ_{10}H_2$ to CoQ_{10} ratio).

A comparison of fluorescence, electrochemical and ultraviolet detectors was conducted to determine the limits of detection for CoQ_{10} achieved using these three detection methods, and thereby to establish whether the limits of detection were sufficient for an assay for total CoQ_{10} in human plasma. The methods of Tang *et al.,* (2001) (1) and Mosca *et al.,* (2002) (2) using electrochemical and ultraviolet detection, respectively, were used as the basis for the HPLC set-up for this investigation. For fluorescence detection, an electrochemical cell (placed post-column and pre-detector) was used to reduce CoQ_{10}.

3.3.1.1. Experimental

Standards were CoQ_{10} dissolved in water/1-propanol mixtures, 1/5 v/v for ultraviolet and fluorescence detection and 1/9 v/v for electrochemical detection. A saturated aqueous solution of 1,4-benzoquinone was added before HPLC analysis where ultraviolet detection was used (50 µL of saturated aqueous benzoquinone solution to 1.20 mL of standard solution). For ultraviolet detection in plasma, 50 µL was mixed with 12.5 µL of saturated aqueous 1,4-benzoquinone and 250 µL of 1-propanol; for electrochemical detection, 25 µL of plasma was mixed with 225 µL of 1-propanol. The mixtures were vortexed for 10 minutes, centrifuged at 8000g for 5 minutes, and the supernatants injected directly into the HPLC system. Injection (200 µL for ultraviolet and fluorescence detection, 20 µL for electrochemical detection) was via a Shimadzu SIL-10AXL autoinjector. The basic HPLC system used for each detection type is described in Table 3.3.

Table 3.3 The mobile phase, flow rate, column and column temperature used with ultraviolet, fluorescence and electrochemical detection when comparing the three different modes of detection for CoQ_{10} analysis.

Detection Method	Mobile Phase	Flow Rate (mL/min)	Column	Column Temperature (ºC)
Ultraviolet	30/70 methanol/ethanol (v/v)	1	Phenomenex Luna C18(2) (250 × 4.6 mm, 5 µm)	45
Fluorescence and Electrochemical	30/70 methanol/ethanol (v/v) 15 mL/L glacial acetic acid, and 50 mmol/L sodium acetate trihydrate	0.5	Phenomenex Luna C18(2) (250 × 3 mm, 5 µm)	45

For ultraviolet detection, the detector (Linear U*V*IS 200) was set at 275 nm. For fluorescence detection, an ESA Guardstat and post-column guard cell (ESA model 5020) at –800 mV reduced CoQ_{10}. Fluorescence detection (Shimadzu RF-551) was at an emission wavelength of 370 nm and an excitation wavelength of 290 nm. For electrochemical detection, an ESA Guardstat and pre-column guard cell (ESA model 5020) set to +700 mV oxidised $CoQ_{10}H_2$. The electrochemical detector was an ESA Coulochem *III* fitted with an analytical cell (ESA model 5011) with the first electrode set to –650 mV and the second to 550 mV with the range 200 nV.

The limit of detection (LOD) was defined as the concentration at which the signal-to-noise ratio is three, with baseline noise estimated as the standard deviation of the data points in sections of clean baseline in chromatograms of standards. LOD was determined using SigmaStat (SPSS Inc. 1997).

3.3.2. Results

The LOD was calculated for each detection method by taking the average LOD calculated using standards at 4, 2, 1, 0.5, and 0.1 µmol/L (Table 3.4). Peak and noise height, peak width and signal-to-noise ratio were determined for each standard and each detection method (Table 3.4).

Table 3.4 The detection limits, conditions used to determine these limits, and chromatogram characteristics for the 1 µmol/L CoQ_{10} standard using fluorescence, ultraviolet and electrochemical detection.

	Fluorescence	Ultraviolet	Electrochemical
Water/1-propanol Ratio (v/v)	1/5	1/5	1/9
Injection Volume (µL)	200	200	20
Signal Height	20.9	5.4	611
Noise Height	1.03	0.03	0.58
Signal-to-Noise	20.3	187	1059
Peak Width	43.7	16.5	20.8
Limit of Detection (nmol/L) (in injected solution)	29	4.8	0.34
Limit of Detection (fmol injected)	5800	1000	6.8
Limit of Detection (nmol/L) (in plasma sample)	174	30	3

Using electrochemical detection to detect CoQ_{10} in a normal plasma sample (CoQ_{10} content 1.76 µmol/L) there were fewer peaks than with the other detection systems making it easier to detect and quantify the CoQ_{10} peak (Figure 3.1). The signal-to-noise ratio was highest with electrochemical detection (Table 3.4) and equated to a LOD of 0.34, 4.8, and 29 nmol/L for electrochemical, ultraviolet and fluorescence detection, respectively. Thus the injection volume can be reduced ten-fold to 20 µL when electrochemical detection is used.

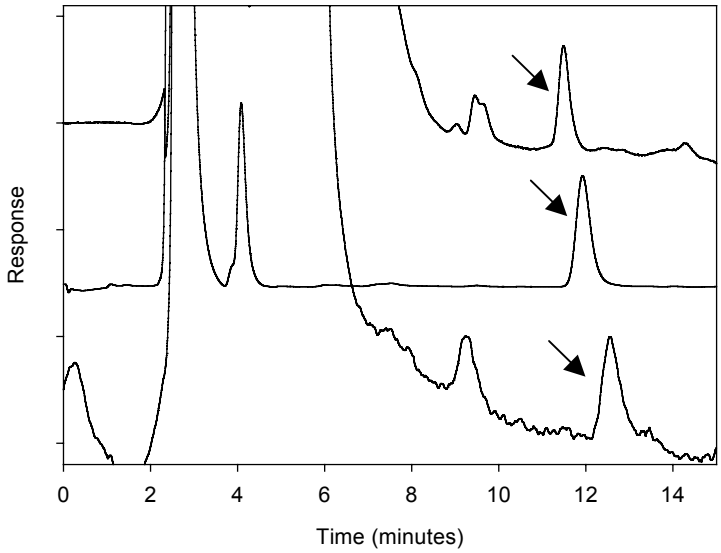


Figure 3.1 A comparison of a plasma extract using ultraviolet (top trace), electrochemical (middle trace) and fluorescence (bottom trace) detection. Arrows point to the CoQ_{10} peaks.

The LOD for ultraviolet detection agrees well with that calculated from the results of Mosca *et al.*, (2002) (2) which was 1.23 pmol/L (on column). The limit of quantitation reported by Mosca *et al*., (2002) (2) of 1.23 nmol/L appears to actually be an LOD, and the units a mis-print for 'pmol/L'.

The LOD reported by Tang *et al.,* (2001) (1) for CoQ_{10} using electrochemical detection was 5.79 nmol/L (in plasma) which agrees well with the results presented here.

It was shown in Chapter 2 that electrochemical reduction of CoQ_{10} is only 84% as efficient as platinum-black catalysed alcohol reduction. Hence it is probable that the LOD for fluorescence detection would be lower if platinum-black catalysed alcohol reduction were used in place of electrochemical reduction. This proposed improvement would not, however, enable fluorescence detection to be used in a clinical assay. As discussed in Chapter 2, fluorescence yields are higher in aprotic solvents, but we were unable to develop practical chromatographic conditions with these solvents and the expected lowering of the LOD would still not make fluorescence detection a more desirable choice than ultraviolet or electrochemical detection.

The cleaner chromatograms obtained with electrochemical detection (Figure 3.1) are due to the screening process whereby only compounds that are able to undergo reversible redox reactions at the potentials applied are detected at the final electrode. The cleaner chromatograms contribute to the lower LOD achieved for electrochemical detection.

3.4. HPLC assay for CoQ_{10} using ultraviolet detection

Results presented in Section 3.3.2 demonstrate that electrochemical detection is unquestionably the most effective detection method for CoQ_{10}, in that it allows measurement of the $CoQ_{10}H_2$ to CoQ_{10} ratio, requires less sample and provides cleaner chromatography. However, results obtained with ultraviolet detection were acceptable and in the absence of a 'spare' electrochemical detector, an assay using ultraviolet detection to measure total CoQ_{10} was set up.

Mosca *et al.,* (2002) (2) reported a simple, rapid and robust method to measure plasma total CoQ_{10} using ultraviolet detection. This assay used addition of 1,4-benzoquinone as an oxidising agent to ensure total CoQ_{10} is measured. After addition of 1,4-benzoquinone to

plasma (50 µL of (approximately) 18.5 mmol/L 1,4-benzoquinone per 200 µL plasma), vortexing and standing for 10 minutes, CoQ_{10} is extracted from plasma by liquid-liquid extraction with 1-propanol (1/5 plasma/1-propanol v/v). After vortexing and centrifugation, 200 µL of the supernatant is injected into the HPLC system. A mobile phase of 35/65 methanol/ethanol (v/v) is pumped at 1 mL/minute and the extract is separated on a C18 column (Supelcosil LC 18 (Supelco), 250 × 4.6 cm, 5 µm). The ultraviolet detector is set to 275 nm. Mosca *et al*., (2002) (2) suggested that this method does not require an internal standard because the efficiency of extraction is satisfactory over a range of concentrations.

As presented in Chapter 2, an attempt to simplify this assay by making an 'extraction solvent' containing 1,4-benzoquinone in 1-propanol was not successful due to the instability of 1,4-benzoquinone in 1-propanol over time. Various other aspects of this assay were investigated, in an attempt to increase sensitivity, improve the chromatography, and generally improve the assay in terms of time and cost required.

3.4.1. Extraction

Because CoQ_{10} is lipophilic, many lipids are extracted with CoQ_{10} and it is generally the lipids in the extract that interfere with the chromatography. The extraction system of Mosca *et al*., (2002) (2) uses 1-propanol liquid-liquid extraction of plasma CoQ_{10}, and direct injection of the extract. This extraction procedure was also used in the method of Jiang *et al.,* (2004) (42) where the ratio of plasma to 1-propanol of 1 to 3.33 (v/v) resulted in a more concentrated extract. However further extract purification on-line was necessary.

In terms of chromatography, injecting CoQ_{10} into the HPLC (equipped with a reversed-phase column) in a relatively polar solvent (one with less eluting power than the mobile phase) results in concentration of CoQ_{10} at the beginning of the analytical column (stacking). This in turn can improve resolution and peak shape. However, because CoQ_{10} is lipophilic, an efficient extraction requires a lipophilic extraction solvent, which leads to a compromise between extraction solvent, extraction efficiency, injection solvent, and chromatography. An alternative is to extract with a lipophilic solvent, evaporate, and reconstitute with a relatively polar solvent, a procedure that is commonly used for related analytes such as vitamins A, E, and K, but which leads to erratic recoveries. It was

postulated that this could be because blood lipids have poor solubility in polar organic solvents such as ethanol and that CoQ_{10}, and presumably related lipophilic solutes, partition into the extracted blood lipids such that the recovery depends on the lipid content of the sample. Alternative extraction procedures were investigated to evaluate which procedure gave both good chromatography and had the minimum concentration of impurities that interfere with chromatography.

3.4.1.1. Experimental

Unless otherwise stated, the mobile phase was 30/70 methanol/ethanol (v/v). Detection was with an ultraviolet detector (Linear Uvis200) set to 275 nm.

Olive oil was used as a model triglyceride to test the hypothesis that blood lipids are responsible for poor recoveries in those procedures based on extraction with hexane, heptane, or other non-polar solvents, followed by evaporation. A CoQ_{10} standard in ethanol (10 mL, 20 μmol/L) was mixed with 1 mL olive oil for 80 minutes in the dark. The two phases were separated by centrifugation. After 100-fold dilution (with 1/9 (v/v) water/1-propanol) the concentration of CoQ_{10} in the olive oil was measured using HPLC with electrochemical detection, as described in Chapter 3, section 3.6.2. After 10-fold dilution (with 1/9 (v/v) water/1-propanol) the concentration of CoQ_{10} in the ethanol layer was determined using the same methodology. The CoQ_{10} concentration in the olive oil was 26 times that in the ethanol phase. This suggests that this extraction procedure will not give efficient recovery of CoQ_{10}, as the CoQ_{10} will be occluded with the lipids on evaporation. Thus the recoveries of CoQ_{10} and related compounds are likely to be erratic in this extraction procedure depending on the blood lipid concentration. 1-Propanol is the most polar alcohol that reliably dissolves triglycerides and presumably other blood lipids, and up to about 15 % water can be added to these solutions before two phases separate. This constrains the choice of extraction solvent.

Diluting a 100% 1-propanol extract 50/50 (v/v) with acetonitrile, and injecting twice the injection volume used for the 100% propanol extract resulted in good peak shape and good resolution but added an extra step to the assay. Extraction of CoQ_{10} into 40/60 1-propanol/acetonitrile (v/v) resulted in sharp peaks with good resolution, but the recovery of the extraction was poor as compared to a 100% 1-propanol extraction. Additionally, a

minor peak that was found to interfere with the CoQ_{10} peak when 1-propanol plasma extracts were injected was still present.

Saponification of lipids has been used when measuring the CoQ_{10} content of foodstuffs (44). The saponification used by Mattila *et al.,* (2001) (44) involved adding potassium hydroxide (KOH) to a homogenate of the foodstuff, ascorbic acid and methanol. This mixture was heated in a boiling water bath for 10 minutes. After cooling, sodium chloride was added to avoid emulsion formation, and the CoQ was extracted using n-hexane (44).

To determine whether saponification of plasma lipids affects CoQ_{10} chromatography, a CoQ_{10} standard was subjected to the following conditions. To 200 µL of water was added 575 µL of ethanol and 25 µL of 100 mmol/L CoQ_{10} stock in 1-propanol. A solution of KOH (0.2 mol/L in methanol) (200 µL) and 200 µL of phosphoric acid (0.1 mol/L in ethanol) were added. A control sample containing 200 µL water, 575 µL ethanol, 25 µL 100 mmol/L CoQ_{10} stock in 1-propanol, 200 µL methanol and 200 µL ethanol was also made. These solutions were assayed with a 40/60 methanol/ethanol (v/v) mobile phase.

The potassium hydroxide saponification of plasma lipids with subsequent addition of phosphoric acid in ethanol to precipitate potassium ions lead to a splitting of the CoQ_{10} peak into two peaks (Figure 3.2), suggesting CoQ_{10} was degraded by the KOH.

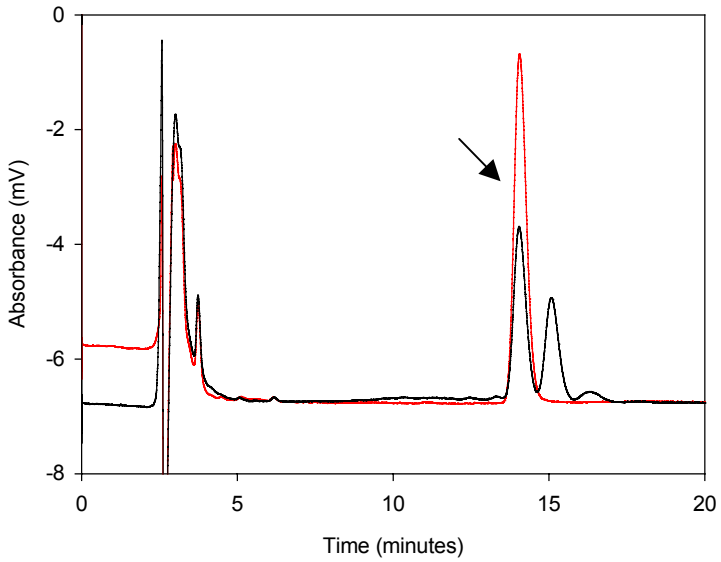


Figure 3.2 A CoQ_{10} standard without (red) and with (black) potassium hydroxide saponification. Arrow points to the CoQ_{10} peak.

Since potassium hydroxide saponification was not viable, calcium hydroxide saponification was investigated. Using a sample of pooled plasma, the following two samples were run: (a) 100 µL plasma, 10 µL of 5 mmol/L 1,4-benzoquinone in 1-propanol and 490 µL of 1-propanol (normal plasma extraction); (b) 100 µL plasma, 10 µL 5 nmol/L 1,4-benzoquinone in 1-propanol, 490 µL 1-propanol and 15 mg of calcium hydroxide. After vortexing for 15 minutes, approximately 20 mg of crushed dry ice was added to neutralise the acid and remove calcium as insoluble calcium carbonate. Finally, 100 µL was injected into the HPLC system.

This procedure resulted in a single CoQ_{10} peak with similar recovery to the control. However, the saponification had a very minor effect on the chromatography, suggesting that either the interfering compounds are not lipid, or that there was limited saponification of the lipids. Water present in the plasma may stop saponification of the plasma lipids, requiring the sample to be heated so that the lipids are hydrolysed. Alternatively, the water needs to be removed from the samples so that saponification can occur.

Prior precipitation of plasma proteins and removal of water (CoQ_{10} would remain with the proteins) could have several advantages for extraction of CoQ_{10} from plasma. Firstly, water present in the plasma mixes with the 1-propanol reducing its lipophilicity, and potentially reducing the extraction efficiency. Secondly, the dilution factor of the extraction would be lower leading to a more concentrated extract. Thirdly, if ethanol is used to precipitate the plasma proteins, any plasma constituents that are soluble in ethanol will be discarded with the ethanol, which would lead to a cleaner CoQ_{10}-containing extract of the protein precipitate. Finally, if the 1,4-benzoquinone is added to the plasma (to oxidise $CoQ_{10}H_2$) before protein precipitation with ethanol, residual 1,4-benzoquinone will dissolve in the ethanol and be removed when the ethanol is discarded.

The use of (a) 5% trichloroacetic acid (TCA) in ethanol, (b) ethanol and (c) 10% TCA in methanol to precipitate plasma proteins was therefore investigated. In some cases, CoQ_{10} was extracted from the protein pellet with the mobile phase solvent (30/70 methanol/ethanol v/v) instead of 1-propanol because the eluting power of 1-propanol is too high to use with the mobile phase when the water is removed from plasma.

Proteins in plasma were precipitated with 5% TCA in ethanol by adding 20 µL 5 nmol/L 1,4-benzoquinone in 1-propanol to 200 µL plasma, followed by 800 µL 5% TCA in ethanol. After mixing, centrifugation and removing the supernatant, the protein pellet was extracted with 250 µL 1-propanol, and 200 µL of the supernatant was injected into the HPLC. The original supernatant (200 µL) was also injected.

In this procedure, some CoQ_{10} partitioned into the supernatant during protein precipitation (as determined by the presence of a CoQ_{10} peak upon chromatography of the supernatant obtained during protein precipitation). CoQ_{10} was therefore lost when the supernatant was discarded, making this extraction procedure non-viable.

The same procedure with 100% ethanol also resulted in extraction of some CoQ_{10} into the ethanol supernatant during protein precipitation making this method also non-viable.

Finally, 500 µL 10% TCA in methanol was added to 250 µL plasma. After vortexing and centrifugation (5 minutes at 8000g), the supernatant was discarded. The pellet was resuspended in 250 µL mobile phase (30/70 methanol/ethanol v/v), vortexed and centrifuged again. Separately, 200 µL of the methanol supernatant and the methanol/ethanol supernatant were injected into the HPLC system.

This precipitation allowed the water to be discarded and, due to the low solubility of CoQ_{10} in methanol, the majority of the CoQ_{10} remained with the proteins (determined from the lack of a CoQ_{10} peak in the methanol supernatant) (Figure 3.3). The extraction of CoQ_{10} from the protein pellet with 30/70 methanol/ethanol (v/v) was, however, incomplete, as determined by the smaller peak obtained for CoQ_{10} in this solvent as compared to that achieved when 1-propanol was used to extract CoQ_{10} from the precipitate (Figure 3.3). The incomplete extraction of CoQ_{10} with 30/70 methanol/ethanol (v/v) was confirmed by a secondary extraction of the protein pellet (after 30/70 methanol/ethanol (v/v) extraction) with 1-propanol and the observation of a CoQ_{10} peak in this secondary 1-propanol extract.

Extraction of CoQ_{10} from the protein pellet with 1-propanol lead to a more concentrated extract. However, interfering peaks were still present in the chromatography, at a higher concentration. Therefore, there was no advantage to this procedure over the more simple direct 1-propanol extraction, and it was not investigated further.

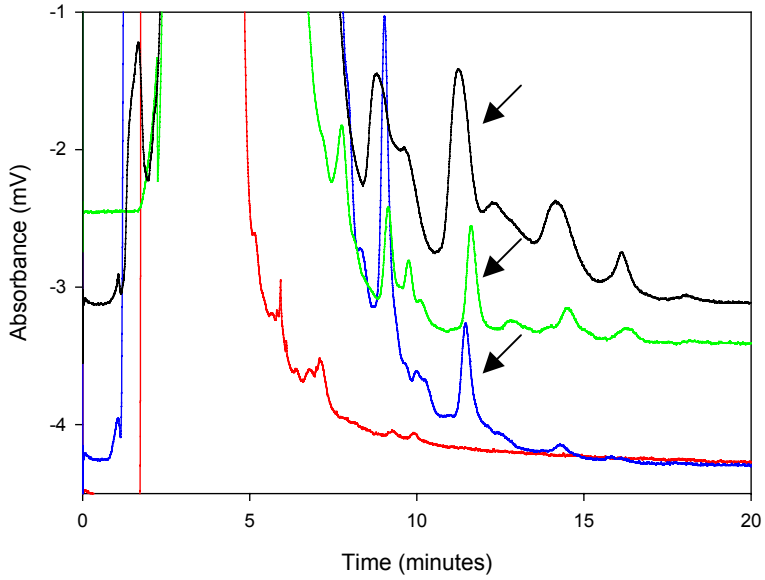


Figure 3.3 Chromatogram of the methanol supernatant after precipitation of proteins (red), the 30/70 methanol/ethanol extract of the plasma proteins (blue), the 1-propanol extract of the plasma proteins (black), and a normal 1-propanol extract of plasma (green). Arrows point to the CoQ_{10} peak in each trace.

The enzymatic hydrolysis of serum lipids with lipase has been used when measuring CoQ_{10} in plasma (48). An attempt was therefore made to reduce the number and size of interfering peaks in the chromatogram by using lipase (Sigma, L-1754) to digest the lipids present in plasma. One unit of lipase hydrolyses 1 mole (1 microequivalent) of fatty acid in 1 hour at pH 7.2 and at 37 ˚C. One triglyceride contains 3 fatty acids, so that assuming a plasma concentration of 10 mmol/L triglycerides (equivalent to a very high plasma triglyceride concentration) means that 200 µL of plasma would contain 6 µmoles of fatty acid and would therefore need 6 µunits of lipase to break down all the fatty acids in 1 hour. The lipase used contains 819 units/mg solid, so that adding 200 µL of a 1 mg/mL solution of lipase to 200 µL of plasma should result in complete breakdown of triglycerides to fatty acids.

To 200 µL of plasma was added 20 µL of 1,4-benzoquinone (5 µmol/L in 1-propanol). Ethanol (800 µL) was added to precipitate proteins, the mixture vortexed for 10 seconds, centrifuged at 8000g for 5 minutes, and the supernatant discarded. The protein pellet was resuspended in 200 µL of tris buffer (pH 7.6) containing (a) 1 mg/mL lipase and (b) no lipase. After heating in a waterbath at 37 ˚C for 15 minutes, 1 mL of propanol was added to

each mixture. The mixtures were vortexed for 10 seconds, centrifuged at 8000g for 5 minutes, and 200 µL of the supernatant injected into the HPLC.

There was minimal change in the chromatogram of lipase-added and lipase-free extracts (Figure 3.4) suggesting that either lipase was not digesting the lipids (possibly due to the methodology used) or that lipids are not the cause of interference in the chromatography.

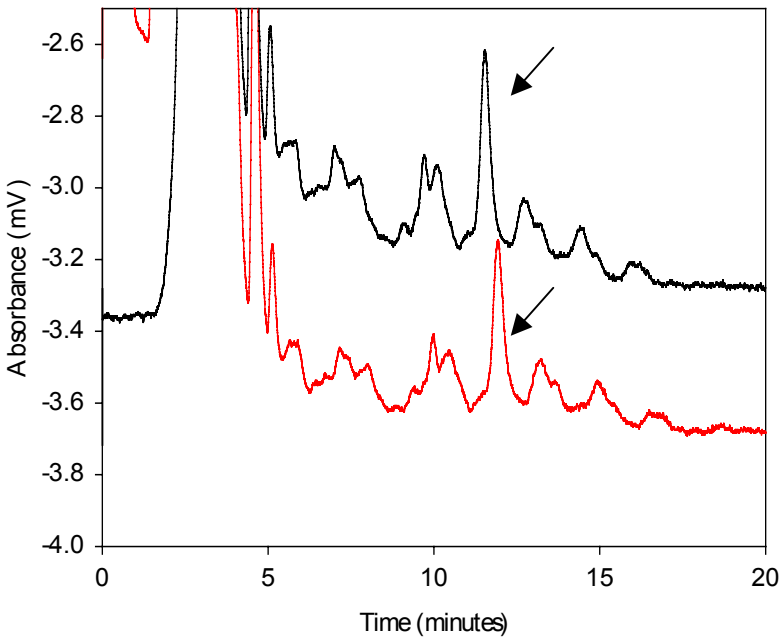


Figure 3.4 Chromatogram obtained with (red) and without (black) lipase added. Arrows indicate the CoQ_{10} peak.

3.4.2. Column

The mobile phase and the analytical column packing material separate the compounds in the injected extract. Together with column length, these factors affect resolution and the limit of detection by affecting the sharpness and separation of peaks. The column packing material used most often for measurement of CoQ_{10} in plasma is C18 (Appendix 2). Reversed-phase C18 columns contain silica, with octadecyl (C18) groups bound to it, and retain non-polar compounds, with analytes being eluted faster with non-polar mobile phases. Normal-phase silica columns contain just silica as the stationary phase, and retain polar compounds, with more polar mobile phases eluting the analytes. Normal-phase diol columns contain two vivinal OH groups, and give similar selectivity to silica columns,

with the added advantage of not being deactivated by small amounts of water. Normal-phase cyano columns contain a CN group (usually cyanopropyl), which gives unique selectivity.

When a C18 column was used with a 30/70 methanol/ethanol (v/v) mobile phase and ultraviolet detection, a small peak co-eluted on the side of the CoQ_{10} peak. The type of C18 column is also important in chromatography and an attempt was made to resolve the interfering peak from the CoQ_{10} peak by changing the type of column.

3.4.2.1. Experimental

Columns (mobile phases) evaluated included silica, diol and cyano (100% heptane and 1/99 1-propanol/heptane), a reversed-phase high carbon-load column (35/65 1-propanol/acetonitrile, 20/80 heptane/methanol, 10/90 heptane/methanol, 15/85 heptane/methanol, and 70/30 ethanol/methanol) and four C18 columns, Phenyl-hexyl, Phenomenex Luna C18(2), Merck LiChroCART Supersphere C18 and Supelco Supelcosil LC18 (70/30 ethanol/methanol). Where not otherwise stated, the plasma extracts used to test the performance of the proposed systems were obtained by extracting 100 µL plasma with 490 µL 1-propanol and 10 µL 5 mmol/L 1,4-benzoquinone in 1-propanol. The mixture was vortexed for 10 minutes, then centrifuged for 5 minutes at 8000g. The supernatant (200 µL) was injected directly into the HPLC equipped with an ultraviolet detector set to 275 nm. Standard solutions of CoQ_{10} were run on all systems tested to identify the CoQ_{10} peak in plasma.

3.4.2.2. Results

Normal-phase columns have been used in the HPLC assay of total CoQ_{10} (47). To assess the practicability of using normal-phase columns to measure CoQ_{10}, CoQ_{10} standards were made up in heptane, rather than 1-propanol which would elute straight from the columns. The anticipated advantage of using silica columns and injecting in hexane, is that hexane has been reported to efficiently extract CoQ_{10} from plasma when combined with protein precipitation with methanol or ethanol (31, 33). A silica column retained CoQ_{10} for at least an hour when a 100% heptane mobile phase (flow rate 1 mL/minute) was used. However, a 1/99 1-propanol/heptane (v/v) mobile phase (flow rate 1 mL/minute) washed the CoQ_{10} straight off the column with no retention.

A diol column retained CoQ_{10} for around 23 minutes when 100% heptane was used as the mobile phase (flow rate 1 mL/minute), but the CoQ_{10} was washed straight off the column when a 1/99 1-propanol/heptane (v/v) mobile phase (flow rate 1 mL/minute) was used. Similarly, a cyano column retained CoQ_{10} for 28 minutes when the mobile phase was 100% heptane (flow rate 1 mL/minute) (although peak shape was poor), and the CoQ_{10} was washed straight through the column when 1/99 1-propanol/heptane (v/v) was used as the mobile phase (flow rate 1 mL/minute).

In the method of Greenspan *et al.,* (1988) (47), the percentage of 2-propanol in the heptane mobile phase was 0.1%. The practicality of using normal-phase columns as part of a routine plasma CoQ_{10} assay is limited since methanol or ethanol is required to precipitate the proteins in the plasma sample and it would not be practical to remove all traces of these solvents before extraction of the CoQ_{10} with heptane. The method of Greenspan *et al.,* (1988) (47) used methanol for precipitation of proteins but the hexane extract is evaporated prior to solid phase extraction which is followed by a further evaporation to remove traces of methanol before the sample is reconstituted in the mobile phase.

It was hypothesised that a reversed-phase high carbon-load C18 column (Phenomenex Ultracarb ODS(30) column, 150 × 3.2 mm, 5µm), would retain CoQ_{10} stronger than a C18 column with a lower carbon load, making it possible to add 1-propanol to the mobile phase while maintaining an acceptable retention time. The anticipated advantage of 1-propanol in the mobile phase was that resolution and chromatography would be improved. On this column, a 35/65 1-propanol/acetonitrile mobile phase (flow rate 0.5 mL/minute) gave longer retention of CoQ_{10} (16 minutes as opposed to 14 minutes with the longer Phenomenex Luna C18(2) column (250 × 3 mm, 5µm), and improved resolution of the CoQ_{10} peak.

It was further hypothesised that using a heptane and methanol mobile phase with the high-carbon load reversed-phase column would result in heptane coating the column packing rather than passing through the column with the methanol. Because CoQ_{10} is more soluble in heptane, this should slow elution of CoQ_{10} through the column. Presuming the interfering compounds wash through the column with the methanol while the CoQ_{10} binds with heptane, the chromatograms would be cleaner. With 20% heptane in methanol (flow rate 0.5 mL/minute), the CoQ_{10} peak co-eluted with another compound in plasma, and the

peak could not be quantified (Figure 3.5). A 10% heptane in methanol mobile phase (flow rate 0.5 mL/minute) resulted in good separation of the CoQ_{10} peak with no interference, but the retention time for CoQ_{10} was too long at 26 minutes (Figure 3.5). A 15% heptane in methanol mobile phase (flow rate 0.5 mL/minute) resulted in a retention time of around 15 minutes but there were interfering compounds in plasma (Figure 3.5). Using a 70% ethanol and 30% methanol mobile phase (flow rate 0.5 mL/minute) with the high-carbon load column resulted in a retention time of 14 minutes but again the CoQ_{10} peak was not resolved from other peaks in the chromatogram (Figure 3.5).

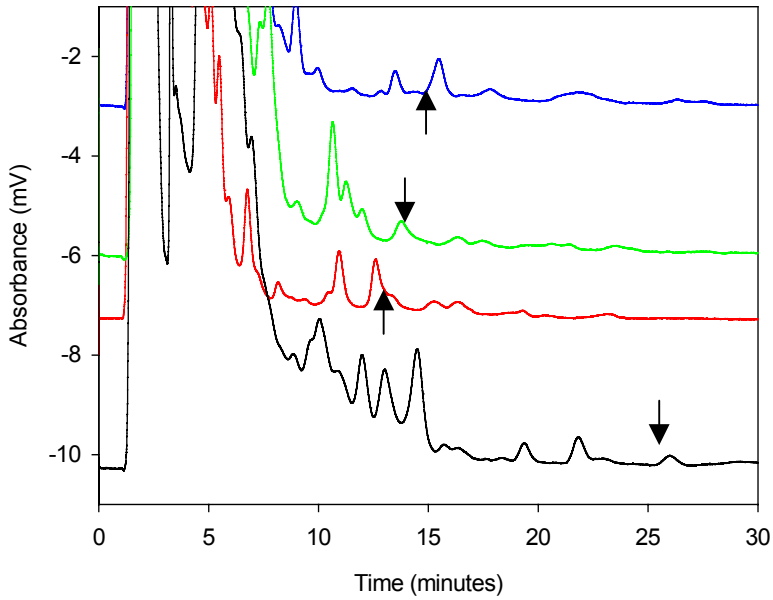


Figure 3.5 CoQ_{10} peaks on the ultracarbon C18 column, with a mobile phase of 20% (red), 15% (blue), and 10% (black) heptane in methanol (v/v), and 30/70 methanol/ethanol (v/v) (green). Arrows point to the peak corresponding to CoQ_{10}.

Since other columns were of little use for quantitation of plasma CoQ_{10}, the decision was made to use a C18 column and investigate chromatography offered by different types of C18 columns, as well as by altering the column temperature and mobile phase. Four different types of C18 columns (Supelco Supelcosil LC18, Phenomenex Luna C18(2), Merck LiChroCART Supersphere C18, and Phenomenex Phenyl-hexyl), of the same length and diameter (250 × 4.6 mm) were compared. Each was run with the same mobile phase (30/70 methanol/ethanol v/v) at the same flow rate (1 mL/min) with the column temperature set at 40 °C. Under these conditions, back pressure varied between columns, being 9.9, 8.7, 8.0, and 17.4 MPa for the Luna, Phenyl-hexyl, Supelcosil and Merck

columns respectively. The particle size in the Supelcosil, Luna, and Phenyl-hexyl columns was 5 μm and in the Merck column 4 μm.

To compare the columns, the same CoQ_{10} standard (corresponding to a plasma concentration of 0.17 μmol/L) and extract of a plasma sample (corresponding to a plasma concentration of 0.24 μmol/L) were injected into each column. The ratio of water (for standards) or plasma to 1-propanol was 1 to 5 (v/v). CoQ_{10} was detected with ultraviolet detection at 275 nm.

The Phenyl-hexyl column gave the best signal-to-noise ratio when standards were run (Table 3.5) which is expected given it has the highest bond density (4 μmol/m^2, as compared to a bonding density of 3 μmol/m^2 for the Luna and Supelco columns). The bonding density is the amount of C18 chains per gram of absorbent and is related to the surface area. It therefore gives a measure of how well the silica surface is shielded.

The Luna column gave better resolution of CoQ_{10} from interfering compounds when the plasma sample was injected (Figure 3.6), and hence gave the better signal-to-noise ratio for plasma samples. Therefore, a Phenomenex Luna C18(2) column was chosen for further assay development.

Table 3.5 The signal-to-noise ratio for a CoQ_{10} standard and plasma extract in four C18 columns.

Column	Standard	Plasma
Luna	9.0	18.3
Merck	10.6	10.9
Supelcosil	10.1	13.2
Phenyl-Hexyl	13.3	14.2

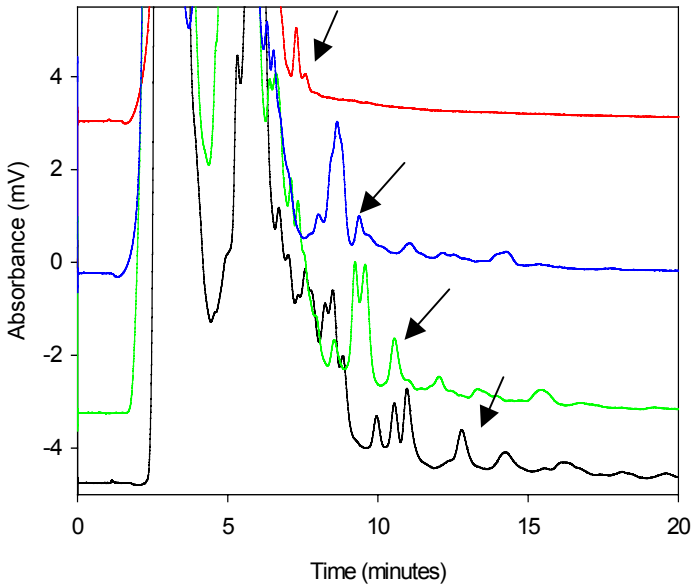


Figure 3.6 Chromatograms obtained with different C18 columns, with arrows indicating the CoQ_{10} peak. Red trace is the Phenyl-hexyl column, blue trace is the Merck column, green trace is the Supelco column and black trace is the Luna column.

A column with a smaller diameter allows the flow rate to be reduced without the chromatography being altered. The advantage is that solvent usage is reduced and better resolution is obtained. Therefore, a smaller diameter column (Phenomenex Luna C18(2) column, 250 × 3 mm, 5 µm) was used in the final assay.

3.4.3. Column temperature

The temperature of the column affects resolution (and hence sensitivity) because pressure in the column and retention are reduced at higher temperatures. Use of very high temperatures causes safety concerns due to boiling points of solvents and can also cause evaporation. The mobile phase used was 70% ethanol and 30% methanol with the ratio of water (for standards) or plasma to 1-propanol being 1 to 5 (v/v).

Comparison of the signal-to-noise ratio when the column (Phenomenex Luna C18(2), 250 × 4.6 mm, 5 µm) was held at 25 °C, 40 °C and 45 °C showed that it increased with increasing column temperature (Table 3.6). A column temperature of 45 ºC achieves optimal sensitivity (Figure 3.7) while remaining safe.

Table 3.6 The signal-to-noise ratio for a CoQ_{10} standard (corresponding to a plasma concentration of 1.34 µmol/L) and a plasma sample (corresponding to a plasma concentration of 0.53 µmol/L) when the HPLC column is held at different temperatures.

Temperature	Standard	Plasma
25 ºC	48	25
40 ºC	71	48
45 ºC	74	54

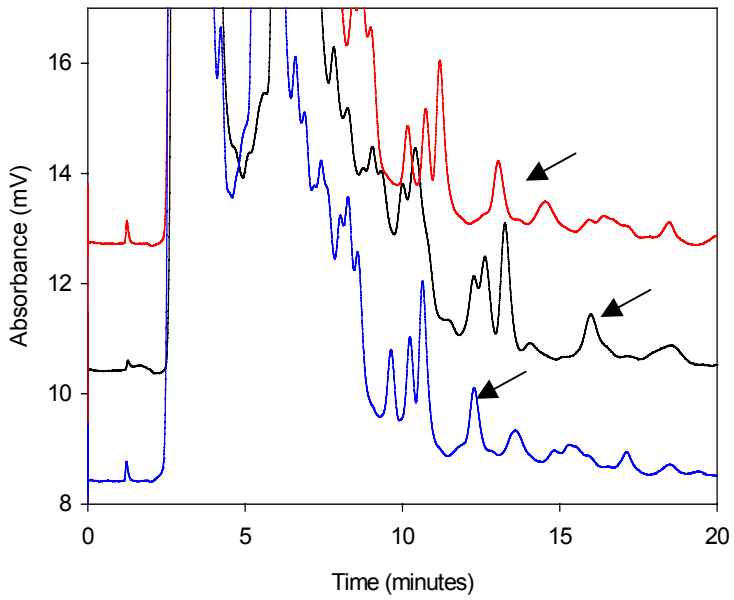


Figure 3.7 Chromatography achieved when the column is held at 25 ˚C (black), 40 ˚C (red), and 45 ˚C (blue). Arrows indicate the peak corresponding to CoQ_{10}.

3.4.4. Mobile phase

When a 30/70 methanol/ethanol (v/v) mobile phase was used with a Phenomenex Luna C18(2) column (250 × 3 mm, 5 µm) to measure CoQ_{10} in plasma, a co-eluting peak on the tail of the CoQ_{10} peak caused interference. Attempts to change the mobile phase to separate this co-eluting peak from the CoQ_{10} peak are described below.

Reported isocratic CoQ_{10} assays have used a mobile phase consisting of 10/90 2-propanol/methanol (v/v) (42); 20/80 1-propanol/methanol (v/v) (25); 5/85 *tert*-butyl alcohol/methanol (v/v) (31); 6/17/77 2-propanol/ethanol/methanol (v/v/v) (28); and 4/21/75 ethanol/methanol/2-propanol (v/v/v) (35). The method of Wang *et al.,* (1999) (30)

used a gradient elution with (a) 100% methanol and (b) 20/80 *tert*-butanol/ethanol. Initial conditions were 90% a and 10% b for the first 4 minutes, followed by a linear change over 6 minutes to 50% a and 50% b, and finally an immediate reversion to the initial conditions for 3 minutes (30).

3.4.4.1. Experimental

The Phenomenex Luna C18(2) column (250 × 3 mm, 5 µm) was held at 45 ˚C for all mobile phases investigated. Detection was by ultraviolet detection at 275 nm. Plasma samples were extracted by adding 490 µL 1-propanol and 10 µL of 5 mmol/L 1,4-benzoquinone in 1-propanol to 100 µL plasma. This mixture was vortexed for 10 minutes then centrifuged at 8000g for 10 minutes. The supernatant (100 µL) was directly injected into the HPLC system. The ratio of water (for standards) or plasma to 1-propanol was therefore 1/5 (v/v) for all extracts.

Changing the mobile phase from 30/70 methanol/ethanol (v/v) to 40/60 ethanol/methanol (v/v) gave no additional separation (Figure 3.8). A 50/50 acetonitrile/ethanol (v/v) mobile phase gave sharper peaks than when methanol was used in the mobile phase and a decreased retention time for CoQ_{10}. However the co-eluting peak was still not resolved from the CoQ_{10} peak (Figure 3.8).

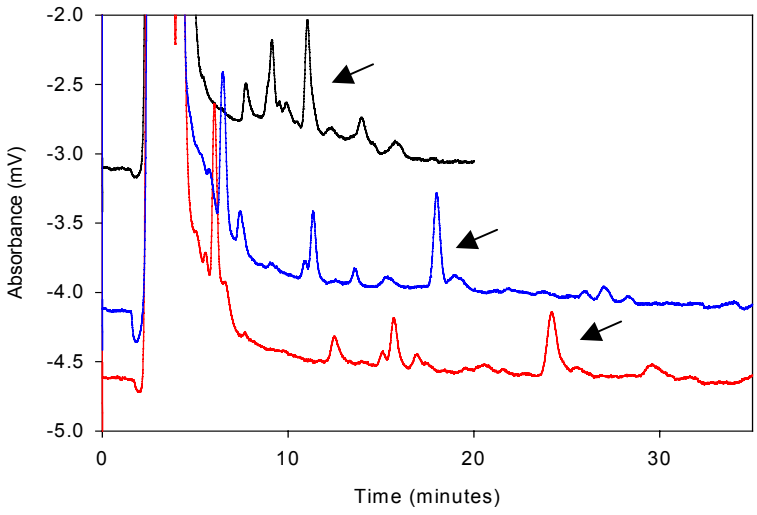


Figure 3.8 Chromatography achieved using a 30/70 methanol/ethanol (v/v) mobile phase (black), a 40/60 ethanol/methanol (v/v) mobile phase (red), and a 50/50 acetonitrile/ethanol (v/v) mobile phase (blue). Arrows indicate positioning of the CoQ_{10} peak.

Replacing the ethanol in the mobile phase with the less polar 1-propanol or 2-propanol (i.e. using 50/50 1- or 2-propanol/ acetonitrile v/v) resulted in a clean CoQ_{10} peak in the chromatogram (Figure 3.9) with retention times of 7 and 9 minutes for 1- and 2-propanol respectively. A mobile phase of 25% 1-propanol and 75% acetonitrile resulted in a retention time of around 27 minutes (Figure 3.9) which is too long for a routine assay. When the 1-propanol content of the mobile phase was 40% (with 60% acetonitrile (v/v)) the retention time was around 10 minutes, but there were co-eluting peaks with the coenzyme Q_{10} peak (Figure 3.9).

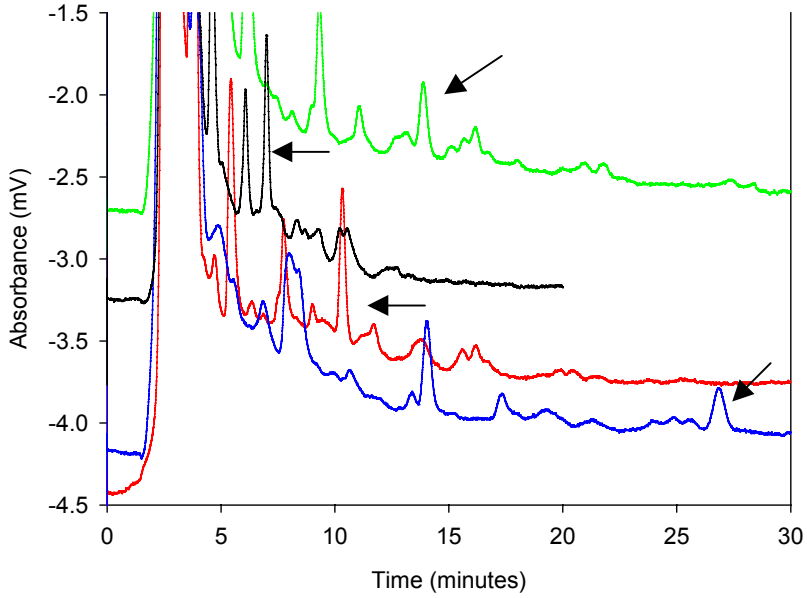


Figure 3.9 Chromatogram of a plasma extract run with a 50/50 1-propanol/acetonitrile (v/v) mobile phase (black), a 25/75 1-propanol/acetonitrile (v/v) mobile phase (blue), a 40/60 1-propanol/acetonitrile (v/v) mobile phase (red), and a 35/65 1-propanol/acetonitrile (v/v) mobile phase (green). CoQ_{10} peaks are identified by arrows.

A mobile phase of 35% 1-propanol and 65% acetonitrile (v/v) resulted in a CoQ_{10} retention time of around 14 minutes. However, when plasma samples were extracted, it became evident that a large peak elutes with the solvent front and tails for 15 minutes (Figure 3.9). This peak masks the CoQ_{10} peak, and hence does not allow quantification of CoQ_{10}.

It became evident that the more polar methanol/ethanol mobile phase washes interfering peaks off the column much faster than the less polar 1-propanol/acetonitrile mobile phase with less peak tailing. It is possible that the interfering compounds contain hydroxyl groups, which are solvated by methanol in the mobile phase, and hence washed from the column. Acetonitrile cannot solvate compounds containing hydroxyl groups which could be why the interference took so long (15 minutes) to wash off the column when the 1-propanol/acetonitrile mobile phase was used.

3.5. Assay for CoQ_{10} using ultraviolet detection

3.5.1. Final assay

A summary of the final assay for CoQ_{10} using ultraviolet detection is outlined below and an example chromatogram is shown (Figure 3.10).

3.5.1.1. Extraction

1,4-Benzoquinone (5 mmol/L) in 1-propanol is stored in the –13 ºC freezer for up to one week. For each assay run, it is diluted into 1-propanol to a concentration of 33 µmol/L, and used as the extraction solvent. The ratio of plasma to extraction solvent is 1/5 (v/v). After addition of extraction solvent, samples are mixed on a vortex mixer for 10 minutes, centrifuged at 8000g for 5 minutes, and the supernatant transferred into an HPLC vial.

3.5.1.2. HPLC set-up

Column: Phenomenex Luna C18(2) (250 × 4.6 mm, 5 µm) at 45 ºC
Mobile phase: 30/70 methanol/ethanol (v/v), flow rate 1 mL/minute
Injection volume: 200 µL
Detector settings: wavelength 275 nm

3.5.1.3. Standards

Standard CoQ_{10} is made to appropriate concentrations ensuring that the water to 1-propanol ratio is always 1/5 (v/v).

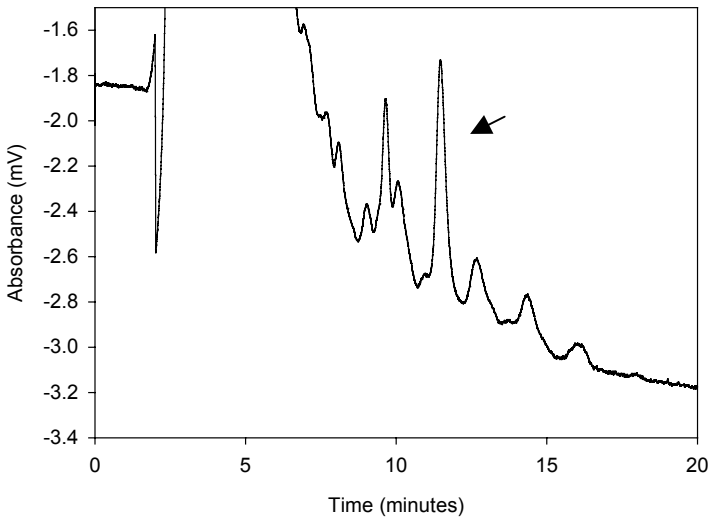


Figure 3.10 An example chromatogram obtained from a plasma extract with ultraviolet detection. Arrow indicates the CoQ_{10} peak.

3.5.2. Calibration curve and linearity

A calibration curve was constructed using standards containing a ratio of 1/5 (v/v) water to 1-propanol (Figure 3.11). The calibration curve is linear to at least 5 µmol/L which supports other reports of linearity for CoQ_{10} with ultraviolet detection (2, 33).

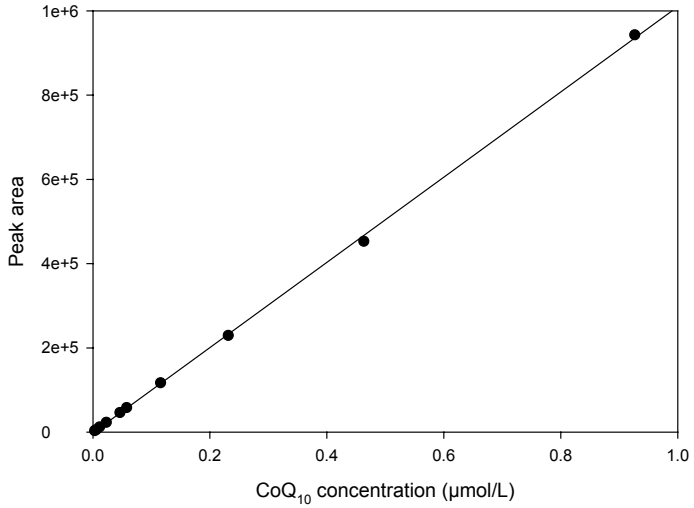


Figure 3.11 A calibration curve for CoQ_{10} using the assay as outlined in Section 3.5.1, with ultraviolet detection (R^2 = 0.9997).

3.5.3. Comparison of anticoagulants in relation to ultraviolet detection

It is possible that the anticoagulants added to blood during phlebotomy may interfere with chromatography or oxidation of plasma $CoQ_{10}H_2$ by 1,4-benzoquinone. It is therefore

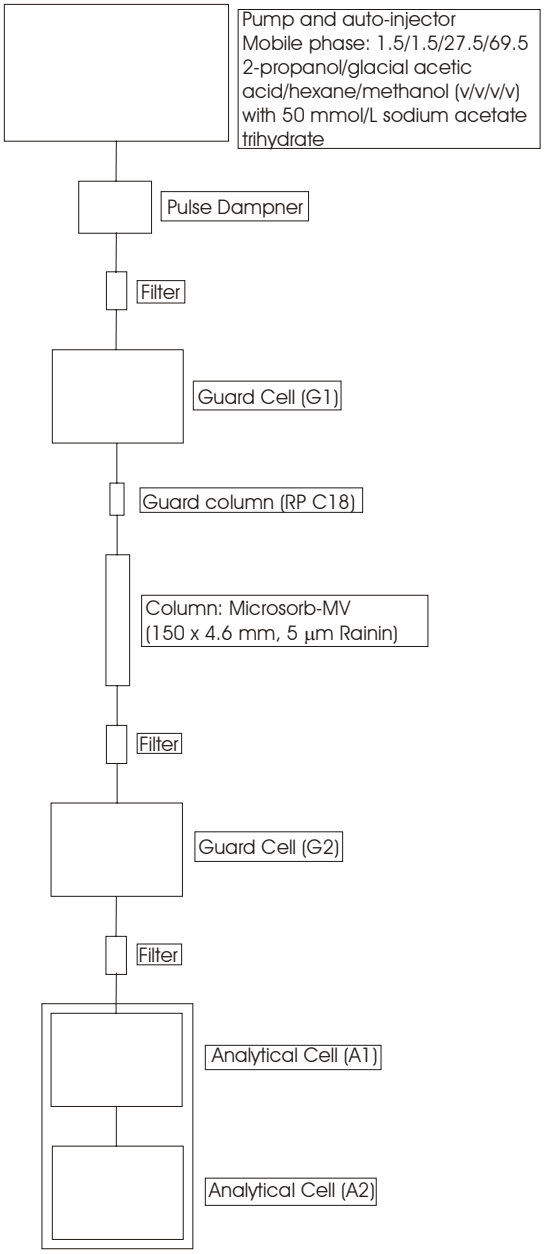
important to confirm the effect of anticoagulants on the CoQ_{10} assay. Serum, lithium heparinised, and EDTA blood samples were taken from ten volunteers (written informed consent was obtained from all participants). Samples were analysed as described in section 3.5.1.

The mean ± SD CoQ_{10} concentration in the lithium heparinised, serum, and EDTA samples for the 10 participants was 1.3 ± 0.5, 1.4 ± 0.7, and 1.4 ± 0.5 μmol/L, respectively, suggesting that there was no effect of the different anticoagulants on the measurement of CoQ_{10}. Therefore, any anticoagulant is acceptable for analysis of total CoQ_{10} using ultraviolet detection.

3.6. Assay for CoQ_{10} using electrochemical detection

After setting up the assay for CoQ_{10} using ultraviolet detection, an electrochemical detector became available. The CoQ_{10} assay was subsequently set up with electrochemical detection, due to the advantages of this detection method (increased sensitivity, ability to measure the ratio of reduced to oxidised CoQ_{10}, and cleaner chromatograms). Tang *et al.,* (2001) (1) recently reported a simple, robust and rapid method for measurement of either total CoQ_{10} or the ratio of $CoQ_{10}H_2$ to CoQ_{10} using electrochemical detection.

3.6.1. Further description of the HPLC set-up used by Tang *et al.*, (2001)(1)



The HPLC set-up described by Tang *et al.,* (2001)(1) involves use of electrochemical detection, and can measure either total CoQ_{10}, or reduced and oxidised CoQ_{10}. There is no pre-extraction oxidation step. Coenzyme Q_{10} is extracted from plasma using 1-propanol at a ratio of 1/9 plasma/1-propanol. For measurement of total coenzyme Q_{10}, an electrochemical cell is situated before the analytical column and set to a potential to oxidise all CoQ_{10}. For measurement of oxidised and reduced CoQ_{10}, the two forms are separated on the analytical column and then a series of oxidation, reduction, and finally oxidation at the analytical cell, is employed. Potentials are set such that only CoQ_{10} and compounds requiring potentials less than CoQ_{10} for oxidation and reduction undergo these reactions. The electrolyte used in the 1.5/1.5/27.5/69.5 2-propanol/glacial acetic acid/hexane/methanol (v/v/v/v) mobile phase is 50 mmol/L sodium acetate trihydrate.

Figure 3.12 The HPLC set-up used by Tang *et al.*, (2001).

A drawback of the assay of Tang *et al.,* (2001) (1) is the use of hexane in the mobile phase. Because of the low boiling point of hexane, evaporation is a concern so that a mobile phase that contains hexane needs to be made fresh regularly to eliminate changes in the composition of the mobile phase due to evaporation. The experience gained from setting up the assay for total CoQ_{10} using ultraviolet detection was therefore integrated into the methodology of Tang *et al.,* (2001) (1), and the final assay is described below.

3.6.2. Final assay for CoQ_{10} using electrochemical detection

A summary of the final assay for coenzyme Q_{10} using electrochemical detection is outlined below. Example chromatograms showing (a) total CoQ_{10}, and (b) the CoQ_{10} to $CoQ_{10}H_2$ ratio as measured using electrochemical detection is shown (Figure 3.13).

3.6.2.1. Extraction

The ratio of plasma to 1-propanol is 1/9 (v/v). After addition of 1-propanol, samples are mixed on a vortex mixer for 10 minutes, centrifuged at 8000g for 5 minutes, and the supernatant is transferred into an HPLC vial.

3.6.2.2. HPLC setup

A pulse dampener is situated between the pump and the injector. A guard cell (set to a potential of + 700 mV when measuring total CoQ_{10}) is situated immediately prior to the analytical column. The first cell of the analytical cell is set to a potential of –700 mV, and the final analytical cell (from which the chromatogram is obtained) is held at a potential of + 550 mV (as determined from the hydrodynamic voltammogram, Chapter 2). When the ratio of $CoQ_{10}H_2$ to CoQ_{10} is to be measured, the guard cell is placed immediately after the analytical column and set to a potential of +700 mV.

Column: Phenomenex Luna C18(2) (250 × 3 mm, 5 μm) at 45 ºC
Mobile phase: 30/70 methanol/ethanol (v/v), 50 mmol/L sodium acetate trihydrate, and 15 mL/L glacial acetic acid, flow rate 0.5 mL/minute
Injection volume: 20 μL
Detector settings: Final analytical cell set to a potential of 550 mV, range setting of 200 nA.

3.6.2.3. Standards

Standard CoQ_{10} is made to appropriate concentrations ensuring that the water to 1-propanol ratio is 1/9 (v/v).

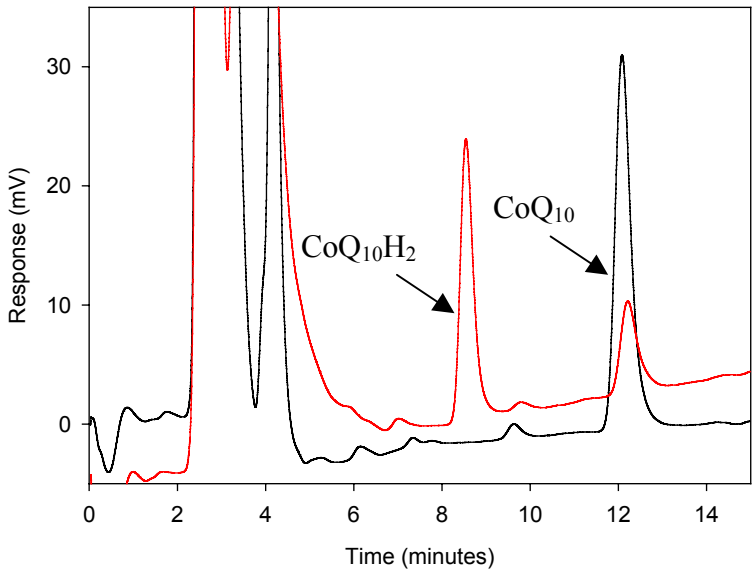


Figure 3.13 Example chromatograms of a 1-propanol plasma extract with CoQ_{10} detected using electrochemical detection, showing total CoQ_{10} (black), and the CoQ_{10} to $CoQ_{10}H_2$ ratio (red).

3.6.3. Calibration curve and linearity

A calibration curve was constructed using standards containing a ratio of 1/9 (v/v) water to 1-propanol (Figure 3.14). The calibration curve is linear to at least 10 µmol/L as has been previously reported (55).

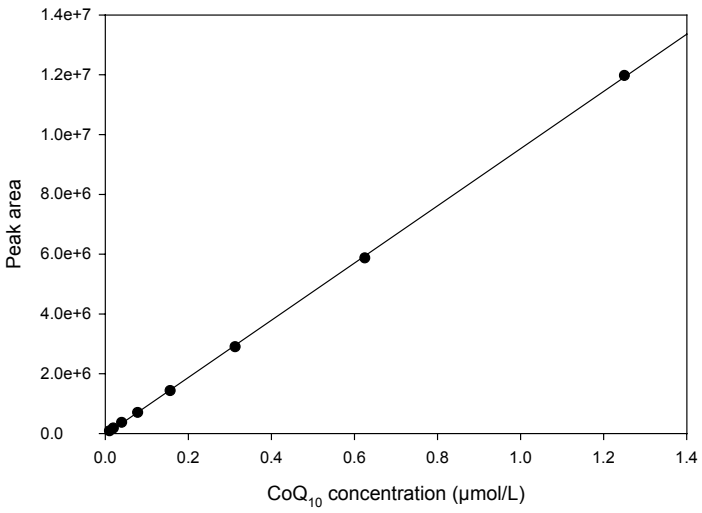


Figure 3.14 A calibration curve of CoQ_{10} with electrochemical detection (R^2 = 0.9998)

3.6.4. Comparison of anticoagulants in relation to electrochemical detection

It has been reported that EDTA interferes with the chromatography when using electrochemical detection to measure CoQ_{10} (56). Therefore, serum, lithium heparinised, and EDTA blood samples were taken from ten volunteers (written informed consent was obtained from all participants). Total CoQ_{10} was measured as described in section 3.6.2.

The CoQ_{10} concentration of EDTA samples was on average 4.4 ± 2.9% lower than that in serum and lithium heparinised samples, which is similar to the 8.8% lower values reported in EDTA plasma as compared to lithium heparinised plasma by Tang *et al.,* (2001) (1). The difference between the three anticoagulants was almost significant ($p = 0.075$). The lower values in the EDTA samples are due to dilution of the plasma by EDTA forcing water out of the red blood cells. The cholesterol concentration of EDTA samples was significantly (6.3 ± 1.8 percent) lower than that in the lithium heparinised samples ($p < 0.001$), which supports this theory. Therefore, EDTA samples are acceptable for the described assay using electrochemical detection, as long as the results obtained from EDTA samples are not compared with results obtained from lithium heparin samples.

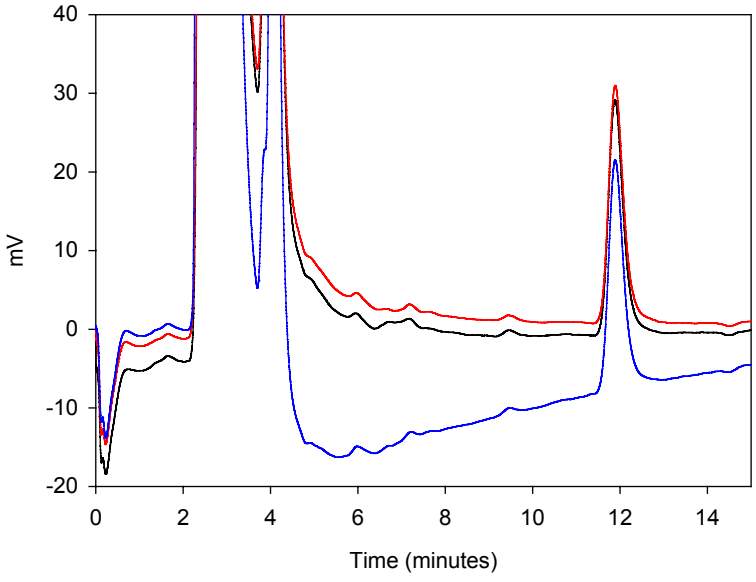


Figure 3.15 Electrochemical detection of an extract of lithium heparin plasma (black), EDTA plasma (blue), and serum (red).

3.7. Precision and recovery of ultraviolet and electrochemical methods for CoQ_{10} detection

Precision and recovery for both the ultraviolet and electrochemical detector based assays, as described above, were calculated.

3.7.1.1. Experimental

Two pools of human plasma with abnormally low CoQ_{10} concentrations (total CoQ_{10} 0.15 (for ultraviolet detection) and 0.24 (for electrochemical detection) μmol/L), prepared by diluting plasma with human serum albumin in saline, were used. Additionally, pooled plasma was spiked by adding CoQ_{10} concentrations of 2.75 (for ultraviolet detection) and 0.97 (for electrochemical detection) μmol/L. The mixtures were allowed to equilibrate for at least 24 hours before use. Precision was calculated by replication at each level, with four aliquots from each pool being analysed each day (within-run) for six days (between-run). Recoveries were determined by calculating the difference between a plasma sample and the same plasma with added CoQ_{10} (four added concentrations from 0.03 to 0.13 μmol/L for electrochemical detection, and 0.08 and 0.19 μmol/L for ultraviolet detection).

3.7.1.2. Results

The precision and recovery of both assays is acceptable by clinical standards, and compares well with previously published methods (Table 3.7).

Table 3.7 The between- and within-run precision, recovery, and the concentration range of samples used to determine these parameters, for CoQ_{10} assays using electrochemical and ultraviolet detection.

	Between-Run %CV	Within-Run %CV	Recovery (%)	Concentration Range (μmol/L)
Present UV assay	3.2	2.4	93-103	0.24-0.98
Present EC assay	3.3	3.2	98-102	0.15-2.76
Tang *et al*., (2001) (1)	1.2-4.9	1.2-4.9	96-101	0.01-4.63
Mosca *et al*., (2002) (2)	2	1.6	96-99	1.02

UV = ultraviolet detection, EC = electrochemical detection.

The between- and within-run precision of total CoQ_{10} assays using either ultraviolet or electrochemical detection is sufficient for clinical use (CV < 3%), though the precision may be overestimated when using ultraviolet detection at low concentrations since the test samples had been diluted with albumin, and the effect of minor peaks has therefore been reduced.

3.8. Discussion

Ultraviolet detectors are more common in an analytical laboratory than electrochemical detectors which generally leads to them being the preferred detection method when setting up an HPLC assay to measure total CoQ_{10} in blood plasma for limited samples. However, a laboratory aspiring to offer measurement of blood plasma CoQ_{10} as a routine hospital test, or to measure the reduced to oxidised CoQ_{10} ratio, would require an electrochemical detector. The within- and between-run precision data and the recovery results for both the electrochemical and ultraviolet detection assays described in this Chapter confirms that both are simple, accurate and reliable.

Ultraviolet detection allows quantification of normal blood plasma CoQ_{10} concentrations, with a 1/5 (v/v) plasma to 1-propanol ratio. The extraction procedure outlined by Mosca *et al.,* (2002) (2) is rapid and simple, and the high recovery values suggest the extraction is complete. The addition of 1,4-benzoquinone to oxidise CoQ_{10} is effective, and does not greatly complicate the extraction procedure. Simplification of this extraction procedure by making a large volume of 1,4-benzoquinone in 1-propanol (5 mmol/L) and storing it at –13 ºC is not feasible, since the 1,4-benzoquinone solution is unstable over time at this temperature (Chapter 2). Taking into account the 1/5 (v/v) sample to 1-propanol extraction procedure, the ultraviolet assay as described has a limit of detection in plasma of 62 nmol/L. A plasma CoQ_{10} concentration of 200 nmol/L would be considered a low result, so therefore the limit of detection using ultraviolet detection allows distinction of low-normal from deficient levels of total CoQ_{10} in human plasma samples. There are many peaks in the chromatogram obtained using ultraviolet detection, and the baseline tends to be noisy, which adversely affects the limit of detection. The method using ultraviolet detection requires a large injection volume (200 µL), however the chromatography is not adversely affected.

Electrochemical detection offers the most sensitive assay to determine total CoQ_{10} in blood plasma. Extraction of CoQ_{10} from blood plasma, as outlined by Tang *et al.,* (2001) (1), is fast and simple. The limit of detection is 20 nmol/L in plasma. The chromatogram obtained using electrochemical detection allows accurate quantification of the CoQ_{10} peak due to the clean baseline, which in turn is due to the 'screening' process applied during HPLC analysis. The oxidation – reduction – oxidation process allows compounds in the extract to be screened out if the electrochemical potentials at these reduction and oxidation steps are appropriate. Advantages of electrochemical detection are the requirement for a smaller sample size (due to the more dilute extracts and smaller injection volumes), and the increased sensitivity (due to a lower limit of detection, and the cleaner baseline).

Electrochemical detection allows analysis of the ratio of reduced to oxidised CoQ_{10} in plasma, with just one injection of the extract. Reduced CoQ_{10} is unstable in the presence of air, being easily oxidised. Hence, when measuring this ratio it is crucial that sample handling is strictly controlled, with samples being frozen to –80 °C as soon as possible if they are to be stored, or else analysed in the shortest possible timeframe if not to be frozen.

Fluorescence detection is not sensitive enough to measure endogenous blood plasma CoQ_{10} unless the extract is concentrated. It is possible that a more sensitive fluorescence detector (such as a laser-induced fluorescence detector) could provide adequate sensitivity but these detectors are expensive. The fluorescence detector used in the present study allows detection of a CoQ_{10} plasma concentration of 760 nmol/L, where a low sample may be 200 nmol/L. The chromatogram obtained using fluorescence detection contains many other peaks, resulting in a noisy baseline, which makes detection of the CoQ_{10} peak difficult. Also, the low sensitivity means that it is necessary to inject a large sample volume, which results in a broad peak.

3.9. References for Chapter 3

1. Tang PH, Miles, M.V., DeGrauw, A., Hershey, A., Pesce, A. HPLC analysis of reduced and oxidised coenzyme Q10 in human plasma. Clinical Chemistry 2001;47:256-65.
2. Mosca F, Fattorini D, Bompadre S, Littarru G. Assay of coenzyme Q10 in plasma by a single dilution step. Analytical Biochemistry 2002;305:49-54.
3. Daines AM. New Assays for Biologically Active Quinones. PhD Thesis. Christchurch: University of Canterbury, 2001.
4. Hirauchi K, Sakano T, Notsumoto S, Nagaoka T, Morimoto A, Fujimoto K, et al. Measurement of K vitamins in animal tissues by high-performance liquid chromatography with fluorimetric detection. Journal of Chromatography 1989;497:131-7.
5. Indyk H, Woollard DC. Vitamin K in milk and infant formulas: Determination and distribution of phylloquinone and menaquinone-4. Analyst 1997;122:1-5.
6. Indyk H, Woollard DC. Determination of vitamin K in milk and infant formulas by liquid chromatography: Collaborative study. Journal of AOAC International 2000;83:121-30.
7. Speek AJ, Schrijver J, Schruers WHP. Fluorimetric determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes by high-performance liquid chromatography with post-column derivatization. Journal of Chromatography 1984;301:441-7.
8. Booth SL, Davidson KW, Sadowski JA. Evaluation of an HPLC method for the determination of phylloquinone (vitamin K_1) in various food matrices. Journal of Agricultural and Food Chemistry 1994;42:295-300.
9. Ware GM, Chase GW, Eitenmiller RR, Long AR. Determination of vitamin K_1 in medical foods by liquid chromatography with postcolumn reduction and fluorometric detection. Journal of AOAC International 2000;83:957-62.
10. Usui Y, Nishimura N, Kobayashi N, Okanoue T, Kimoto M, Ozawa K. Measurement of vitamin K in human liver by gradient elution high-performance liquid chromatography using platinum-black catalyst reduction and fluorimetric detection. Journal of Chromatography 1989;489:291-301.
11. Usui Y. Assay of phylloquinone and menaquinones in human liver. Methods in Enzymology 1997;282:438-47.

12. Haroon Y, Bacon DS, Sadowski JA. Liquid-chromatographic determination of vitamin K_1 in plasma, with fluorometric detection. Clinical Chemistry 1986;32:1925-9.
13. Lambert WE, De Leenheer AP, Lefevere MF. Determination of vitamin K in serum using HPLC with post-column reaction and fluorescence detection. Journal of Chromatographic Science 1986;24:76-9.
14. Lambert WE, De Leenheer AP. Simplified post-column reduction and fluorescence detection for the high-performance liquid chromatographic determination of vitamin $K_{1(20)}$. Analytica Chimica Acta 1987;196:247-50.
15. Lefevere MF, De Leenheer AP, Claeys AE, Claeys IV, Steyaert H. Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. Journal of Lipid Research 1982;23:1068-72.
16. Haroon Y, Bacon DS, Sadowski JA. Chemical reduction system for the detection of phylloquinone (Vitamin K_1) and menaquinones (Vitamin K_2). Journal of Chromatography 1987;384:383-9.
17. Shino M. Determination of endogenous vitamin K (phylloquinone and menaquinone-n) in plasma by high-performance liquid chromatography using platinum oxide catalyst reduction and fluorescence detection. Analyst 1988;113:393-7.
18. Cham BE, Roeser HP, Kamst TW. Simultaneous liquid-chromatographic determination of vitamin K_1 and vitamin E in serum. Clinical Chemistry 1989;35:2285-9.
19. MacCrehan WA, Schönberger E. Determination of vitamin K_1 in serum using catalytic-reduction liquid chromatography with fluorescence detection. Journal of Chromatography B 1995;670:209-17.
20. Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high-performance liquid chromatography using postcolumn chemical reduction. Methods in Enzymology 1997;282:408-21.
21. Jakob E, Elmadfa I. Rapid and simple HPLC analysis of vitamin K in food, tissues and blood. Food Chemistry 2000;68:219-21.
22. Kamao M, Suhara Y, Tsugawa N, Okano T. Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. Journal of Chromatography B 2005;816:41-8.

23. Wang LY, Bates CJ, Yan L, Harrington DJ, Shearer MJ, Prentice A. Determination of phylloquinone (vitamin K_1) in plasma and serum by HPLC with fluorescence detection. Clinica Chimica Acta 2004;347:199-207.
24. Kruk J, Strzalka K. Fluorescence properties of plastoquinol, ubiquinol and a-tocopherol quinol in solution and liposome membranes. Journal of Photochemicals and Photobiology B: Biology 1993;19:33-8.
25. Lagendijk J, Ubbink JB, Delport R, Hayward WJ, Human JA. Measurement of the ratio between the reduced and oxidized forms of coenzyme Q10 in human plasma as a possible marker of oxidative stress. Journal of Lipid Research 1996;37:67-75.
26. Edlund PO. Determination of Coenzyme Q_{10}, α-tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. Journal of Chromatography 1988;425:87-97.
27. Tang PH, Miles MV, Miles L, Quinlan J, Wong B, Wenisch A, Bove K. Measurement of reduced and oxidized coenzyme Q_9 and coenzyme Q_{10} levels in mouse tissues by HPLC with coulometric detection. Clinica Chimica Acta 2004;341:173-84.
28. Menke T, Niklowitz P, Adam S, Weber M, Schlüter B, Andler W. Simultaneous detection of ubiquinol-10, ubiquinone-10, and tocopherols in human plasma microsamples and macrosamples as a marker of oxidative damage in neonates and infants. Analytical Biochemistry 2000;282: 209-17.
29. Niklowitz P, Menke T, Andler W, Okun JG. Simultaneous analysis of coenzyme Q10 in plasma, erythrocytes and platelets: comparison of the antioxidant level in blood cells and their environment in healthy children and after oral supplementation in adults. Clinica Chimica Acta 2004;342:219-26.
30. Wang Q, Lee BL, Ong CN. Automated high-performance liquid chromatographic method with precolumn reduction for the determination of ubiquinol and ubiquinone in human plasma. Journal of Chromatography B 1999;726:297-302.
31. Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Analytical Biochemistry 1997;250:66-73.
32. Leray C, Andriamampandry MD, Freund M, Gachet C, Cazenave JP. Simultaneous determination of homologues of vitamin E and coenzyme Q and products of alpha-tocopherol oxidation. Journal of Lipid Research 1998;39:2099-105.

33. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. Journal of Lipid Research 1996;37:893-901.
34. Finckh B, Kontush A, Commentz J, Hübner C, Burdelski M, Kohlschütter A. Monitoring of Ubiquinol-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Analytical Biochemistry 1995;232:210-6.
35. Hectors MPC, van Tits LJH, de Rijke YB, Demacker PNM. Stability studies of ubiquinol in plasma. Annals of Clinical Biochemistry 2003;40:100-1.
36. Sharma SK, Ebadi M. An improved method for analyzing coenzyme Q homologues and multiple detection of rare biological samples. Journal of Neuroscience Methods 2004;137:1-8.
37. Kaikkonen J, Nyyssönen K, Salonen JT. Measurement and stability of plasma reduced, oxidised and total coenzyme Q10 in humans. Scandinavian Journal of Clinical and Laboratory Investigation 1999;59:457-66.
38. Lang JK, Gohil K, Packer L. Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Analytical Biochemistry 1986;157:106-16.
39. Okamoto T, Fukui K, Nakamoto M, Kishi T, Okishio T, Yamagami T, et al. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. Journal of Chromatography 1985;342:35-46.
40. Wakabayashi H, Yamato S, Nakajima M, Shimada K. Simultaneous determination of oxidized and reduced Coenzyme Q and α-Tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. Biological and Pharmaceutical Bulletin 1994;17:997-1002.
41. Kaplan P, Sebestianová N, Turiaková J, Kucera I. Determination of Coenzyme Q in human plasma. Physiological Research 1995;45:39-45.
42. Jiang P, Wu M, Zeheng Y, Wang C, Li Y, Xin J, Xu G. Analysis of coenzyme Q10 in human plasma by column-switching liquid chromatography. Journal of Chromatography B 2004;805:297-301.
43. Hirota K, Kawase M, Kishie T. Effect of sodium dodecyl sulphate on the extraction of ubiquinone-10 in the determination of plasma samples. Journal of Chromatography 1984;310:204-7.

44. Mattila P, Kumpulainen J. Coenzymes Q_9 and Q_{10}: contents in foods and dietary intake. Journal of Food Composition and Analysis 2001;14:409-17.
45. Crane FL, Barr R. Determination of ubiquinones. Methods in Enzymology 1971;18:137-65.
46. Linn BO, Trenner NR, Arison BH, Weston RGS, C.H., Folkers K. Coenzyme Q. XII. Ethoxy homologs of coenzyme Q10. Artifact of isolation. Journal of the American Chemical Society 1960;82:1647-51.
47. Greenspan MD, Lee Lo C-Y, Hanf DP, Yudkovitz JB. Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector. Journal of Lipid Research 1988;29:971-6.
48. Imabayashi S, Nakamura Y, Sawa Y, Hasegawa J, Sakaguchi T, Fujita T, et al. Determination of individual ubiquinone homologs by mass spectrometry and high performance liquid chromatography. Analytical Chemistry 1979;51:534-6.
49. Kommuru TR, Khan MA, Ashraf M, Kattenacker R, Reddy IK. A simplified chromatographic method for quantitative determination of coenzyme Q10 in dog plasma. Journal of Pharmaceutical and Biomedical Analysis 1998;16:1037-40.
50. Takada M, Ikenoya S, Yuzuriha T, Katayama K. Studies on reduced and oxidized coenzyme Q (ubiquinones). II. The determination of oxidation-reduction levels of coenzyme Q in mitochondria, microsomes and plasma by high-performance liquid chromatography. Biochimica et Biophysica Acta 1982;679:308-14.
51. Vadhanavikit S, Sakamoto N, Ashida N, Kishi T, Folkers K. Quantitative determination of coenzyme Q10 in human blood for clinical studies. Analytical Biochemistry 1984;142:155-8.
52. Grossi G, Bargossi AM, Fiorella PL, Piazzi S, Battino M, Bianchi GP. Improved high performance liquid chromatographic method for the determination of coenzyme Q_{10} in plasma. Journal of Chromatography 1992;593:217.
53. Passi S, De Pità O, Grandinetti M, Simotti C, Littarru GP. The combined use of oral and topical lipophilic antioxidants increases their levels both in sebum and stratum corneum. BioFactors 2003;18:289-97.
54. Artuch R, Moreno J, Quintana M, Puig RM, Vilaseca MA. Serum ubiquinone-10 in a pediatric population. Clinical Chemistry 1998;44:2378-9.

55. Galinier A, Carrière A, Fernandez Y, Bessac AM, Caspar-Bauguil S, Periquet B, et al. Biological validation of coenzyme Q redox state by HPLC-EC measurement: relationship between coenzyme Q redox state and coenzyme Q content in rat tissues. FEBS Letters 2004;578:53-7.

56. Tang PH, Miles MV, Steele P, DeGrauw A, Chuck G, Schroer L, Pesce A. Anticoagulant effects on plasma coenzyme Q10 estimated by HPLC with coulometric detection. Clinica Chimica Acta 2002;318:127-31.

Chapter 4

Coenzyme Q_{10} Reference Interval and Biological Variation

4.1. Introduction

Having developed an assay to measure CoQ_{10} (Chapter 3), the assay was applied to determine the characteristics of CoQ_{10} in the New Zealand population.

As outlined in Chapter 1, decreased CoQ_{10} levels may occur during disease, ill health, or as a side-effect of medication. Because CoQ_{10} is essential for every cell in the body, this decreased CoQ_{10} may have important medical consequences. It is therefore important to be able to estimate CoQ_{10} concentration in the body, either to ensure sufficient CoQ_{10} is present, or to advise supplementation and monitor bioavailability of supplementation if it is not.

An understanding of the biochemistry of a compound, such as the reference interval in healthy people and the biological variation, is essential for interpreting concentrations found in patients.

4.1.1. Reference interval

A reference interval is the range of values that occur in 95% of a given population. Reference intervals may be influenced by genetic and ethnic factors, although the effect of these factors is difficult to isolate from endogenous factors which are inherent in individuals and cannot be modified such as age and sex. Exogenous factors, such as exercise, pregnancy, habitual use of alcohol or other 'recreational substances' can also affect reference intervals.

Determination of the reference interval provides an understanding of the variability of the analyte concentration in the population, and often (if sample size permits) some factors affecting the interval distribution. These include gender, smoking, alcohol and diet (1). One can also determine whether data have a normal (Gaussian) distribution.

To calculate a reference interval, a reference sample group (a number of individuals taken to represent the reference population) is selected from the population of possible individuals. Reference values are obtained on these individuals to give a statistical

dispersion, termed the reference distribution (1). The upper and lower reference limits (2.5 and 97.5% interfractile intervals) of the reference dispersion are usually used to define the reference interval.

Ideally, individuals used to determine a reference interval should: be ostensibly healthy, not be taking oral contraceptives, prescribed or over-the-counter drugs, excessive amounts of alcohol, or tobacco products and other such recreational substances (1). However, often one has to select the most appropriate individuals as it is almost impossible to get the ideal population (1).

When determining a reference interval, one must consider how many people need to be sampled before a statistically valid reference interval is obtained. The following need to be taken into account (1) (1) the size of error that the laboratory and clinicians are willing to accept (2) the magnitude of change that is clinically important and (3) the variability in the measurements.

The Harris and Boyd criteria (2) may be used to determine whether reference intervals should be stratified according to an influencing factor such as gender. This method uses a Gaussian-based z-statistic, with large values indicating that separation is warranted. The criterion for the standard deviations indicates that separate reference intervals are necessary if the ratio of the standard deviations is too large. These criteria are recommended for samples with approximately 60 subjects per group under consideration.

4.1.1.1. Reporting CoQ_{10} adjusted for lipids

The close association of plasma CoQ_{10} and lipids is widely accepted, and is expected since CoQ_{10} is lipophilic. This association implies that lipids should be considered when measuring plasma CoQ_{10} (3, 4) since plasma CoQ_{10} concentrations may be directly influenced by the concentration of plasma lipids.

4.1.1.2. The measurement of plasma CoQ_{10} as opposed to tissue CoQ_{10}

When measuring CoQ_{10}, do plasma and tissue CoQ_{10} concentrations correlate? If tissue and plasma concentrations do not correlate, is measurement of plasma CoQ_{10} relevant? It is possible that tissue levels may be more than adequate when plasma levels are deficient, especially when plasma lipid concentrations are low.

Measurement of tissue CoQ_{10} is difficult due to the invasive sample collection procedure required. Measurement of plasma CoQ_{10} is much more straightforward and routine.

In the study of Laaksonen *et al.,* (1995) (5), median plasma CoQ_{10} concentrations were slightly lower in young endurance trained athletes than in healthy controls but muscle CoQ_{10} concentrations were almost twice that of healthy controls. Interestingly, there was a much larger range in plasma CoQ_{10} concentrations in these athletes compared to that in controls (5). Statistically significant associations between serum and muscle CoQ_{10} concentrations were not observed in all subjects or in different subgroups (5).

The decrease in plasma CoQ_{10} after exercise reported by Kaikkonen *et al*., (1999) (6) suggests that plasma concentrations, at least to some degree, reflect the need for CoQ_{10} by muscles and other organs.

Niklowitz *et al.,* (2004) (7) report that excessive CoQ_{10} supplementation did not influence erythrocyte (a non-mitochondrial containing blood compartment) concentrations, but a positive correlation existed between plasma content and concentrations in platelets (a mitochondria containing blood compartment). Therefore, under physiologically normal conditions, blood cells or organs may regulate their CoQ_{10} content independently from environmental supply (7). Because erythrocyte CoQ_{10} concentration is independent of supplementation, incorporation into outer cell membranes may be limited, but inner compartments, like mitochondrial membranes, may be influenced (7).

4.1.2. Biological variation

Biological variation quantifies fluctuation around a homeostatic set point, and variation when there is no homeostasis. Total variation in measurement of an analyte is a composite of pre-analytical factors (those related to preparation of the individual for sampling, such as posture, and those influenced by sample collection itself, such as tourniquet application time); analytical random error (precision), and inherent biological variation around the homeostatic set point. If the correct procedures are used, the variation around the set point itself can be separated from the other sources of variation contributing to total variation.

The difference between homeostatic set points themselves is the between-subject biological variation (1).

When within-subject variation (CV_i) is much smaller than between-subject variation (CV_g), the analyte is said to have marked individuality (1). The index of individuality (II) for an analyte is calculated as (Equation 4.1)

$$II = [CV_a^2 + CV_i^2]^{1/2}/CV_g \quad \textbf{4.1}$$

Where CV_a is analytical variation. This is most often simplified to CV_i/CV_g, which is satisfactory if analytical variation (CV_a) is less than the within-subject variation (1).

The individuality of analytes significantly influences reference values (1). Where an analyte has high individuality (a low index of individuality, $II < 0.6$), the range of values from each individual span only a small part of the reference interval. Therefore, an individual could have values that were very unusual for them (therefore clinically important) but these results would still lie within the reference interval. In this situation, the population-based reference interval is of limited utility for detecting unusual results in most individuals (1). In contrast, when an analyte has little individuality (II is high, $II > 1.4$) the distribution of values from a single individual cover much of the reference interval, and conventional reference intervals are of significant value (1). It is possible to increase II by stratification (stratify the data by, for example, age or gender), thereby making reference intervals more useful (1).

Sequential analysis of an analyte is required to obtain a reasonable estimate of an individual's homeostatic set point as the result does have intrinsic analytical and within-subject biological variation. To calculate how many samples (N) are needed to ensure that the estimate of the homeostatic set point is within a certain percentage of the true value with a stated probability, equation 4.2 can be applied (1).

$$N = [Z*(CV_a^2 + CV_i^2)^{1/2}/D]^2 \quad \textbf{4.2}$$

Where Z is the Z-score appropriate for the probability, usually 1.96 for $p < 0.05$, and D is the desired percentage of closeness to the homeostatic set point.

Lowering the probability, or widening the allowance for the window of acceptability (increasing D), decreases the number of samples required.

To generate data on components of random biological variation, sequential samples are required from a small group of subjects, usually healthy people, and preferably of both genders (1). The number of subjects is a compromise between the large number that is the ideal for a better estimate, and the smaller number that can be handled in any practical experimental design (1). To have good precision, and negligible bias, the analyses ought to be done with one instrument, by one operator, with only one set of calibrators, and a single lot of reagents and consumables. The best experimental design assays each sample twice, in random order, in a single analytical run (1). This has the advantage that between-run analytical variation is eliminated, and the analytical component of variation can be derived from replicate analyses of subject samples, ensuring that analytical variation is estimated at the same level as subject samples, and that the matrix is identical throughout (1). However, the intensive analytical effort required to analyse samples in this manner limits the number of subjects and samples that can be studied.

Analysing all samples only once in one analytical run allows for a larger number of subjects and samples to be studied, although analytical variation must be assessed using quality control samples. This has the disadvantage that for some tests, precision achieved with quality control samples differs from that obtained with patient samples (1).

Data on within-subject biological variation and analytical precision can be used to determine the change that must occur in an individual's serial results before the change is significant (the reference change value), and also to determine the statistical probability that a change in an individual's serial results is significant (1).

Total variation (CV_t) is a combination of pre-analytical variation (CV_p) (variation related to preparation of the individual for sampling and those influenced by sample collection itself), analytical variation (CV_a), and within-subject biological variation (CV_i) (Equation 4.3).

$$CV_t = (CV_p^2 + CV_a^2 + CV_i^2)^{1/2} \quad \textbf{4.3}$$

To state with confidence that an individual's serial results have changed, the difference in results must exceed that which can be explained by the inherent variation due to these three factors (1). If pre-analytical variation is minimised, then Equation 4.4 is true.

$$CV_t = (CV_a^2 + CV_i^2)^{1/2} \quad \textbf{4.4}$$

This variation is random, and therefore shows a Gaussian distribution (1). The range in which the values will lie with a certain probability can therefore be derived.

For a Gaussian distribution:
The found value will lie within the range value ± 1 CV with 68.3% probability.
The found value will lie within the range value ± 2 CV with 95.5% probability.
The found value will lie within the range value ± 3 CV with 99.7% probability.

The multipliers 1,2, and 3 are the z-scores. Thus, the found value for any analyte lies within $\pm Z (CV_a^2 + CV_i^2)^{1/2}$ with a probability appropriate to the z-score (1). Z-scores are bi-directional, and the 99% and 95% bi-directional Z-scores have probability of 2.58 and 1.96 respectively.

When a sample is analysed in duplicate, total variation is as described in Equation 4.5.

$$\text{Total variation} = 2^{1/2} * Z * (CV_a^2 + CV_i^2)^{1/2}. \quad \textbf{4.5}$$

For serial results to be significantly different, the difference in numerical results must be greater than the combined variation inherent in the two results (1). This value is the 'critical difference' or the 'reference change value' (RCV), derived using Equation 4.6.

$$RCV = 2^{1/2} * Z * (CV_a^2 + CV_i^2)^{1/2} \quad \textbf{4.6}$$

Ninety-five percent probability is conventionally regarded as significant, and 99% probability is conventionally regarded as highly significant.

Alternatively, the probability that a change is significant can be calculated using a rearranged formula (Equation 4.7),

$$Z = \text{change}/[2^{1/2} * (CV_a^2 + CV_i^2)^{1/2}] \quad \textbf{4.7}$$

Comparison of the Z score with the probability table allows determination of whether the change is significant (1).

Existing databases on biological variation can be used in all laboratories, as there is evidence that biological variation is constant with ageing, and disease (especially when the analyte is unaffected by the disease). Thus although the homeostatic set point may change, the variation around it does not (1). The biological variation of CoQ_{10} data described below are the first data describing biological variation of CoQ_{10}.

A reference interval for coenzyme Q_{10} concentrations in the healthy New Zealand population, and quantification of the natural biological variation of coenzyme Q_{10} that occurs in a healthy individual over time are presented in this Chapter.

4.2. General methods

4.2.1. Ethics

These studies were approved by the Canterbury Ethics Committee, Christchurch, New Zealand and written informed consent was obtained from all participants.

4.2.2. Biochemistry

For all studies heparinized plasma specimens were analysed for total CoQ_{10} using HPLC with electrochemical detection, as described in Chapter 3, Section 3.6.2. Total cholesterol, triglycerides and HDL-cholesterol were determined by an enzymatic colorimetric method (Aeroset Analyser Model LN, Abbott Laboratories, Illinois IL, USA). Coefficients of variation for the total cholesterol, triglycerides and HDL-cholesterol assays were 1.6%, 1.1%, and 5.3% respectively. Direct LDL-cholesterol was measured using Roche Diagnostics Reagents, with a coefficient of variation of 1.2%. Between and within-run coefficients of variation for total CoQ_{10} measurement were approximately 3.3%.

4.2.3. Statistics

Statistical analysis was performed using SPSS Base version 10.0 (SPSS, Inc., Chicago, Illinois), and SigmaStat software (SPSS, Inc., Chicago, Illinois). Statistical significance was accepted when $p < 0.05$.

Correlation analysis in the reference interval study was performed with the Pearson correlation coefficient. Outliers were included in determination of the reference interval because the nonparametric analysis allows for these. Comparisons were performed with the Mann-Whitney rank-sum test.

For the study of biological variation of CoQ_{10}, variance estimates for the inter- and intra-individual and analytical variation were determined with a residual maximum likelihood ratio (REML) variance decomposition procedure, and are expressed as CVs.

4.3. Reference interval

4.3.1. Aim

To determine a reference interval for total plasma CoQ_{10}, the plasma CoQ_{10} to LDL-cholesterol ratio, and the plasma CoQ_{10} to total cholesterol ratio in healthy New Zealand adults.

4.3.2. Study design

4.3.2.1. Subjects

Two hundred and five participants, who were self-reportedly healthy and disease free were enrolled from the electoral roll or from responses to advertisements. A screening questionnaire was used to determine entry into the study. Mean age of participants was 46.9 years (range 18.0-83.0), and included both males (n = 90) and females. The group comprised 90% New Zealand Europeans, 5% New Zealand European/Maori, and 5% other ethnicities. There were few vegetarians or vegans (2.4%). Thirty five percent of the participants reported taking herbal supplements at some time. These ranged from multivitamins, minerals and antioxidants to bee pollen, barley juice powder, and deer velvet. None of the participants reported taking CoQ_{10} supplements. Thirty two percent of the group was on medication, with 3% taking hormone replacement therapy, and 11%

currently taking hormonal contraceptive. Eight percent of the participants smoked one or more cigarettes per day.

4.3.2.2. Specimen handling

Blood samples were taken between 0750 and 1015 hours, with 115 participants having fasted overnight. Samples were taken into glass vacuum tubes containing lithium heparin. Blood was centrifuged within 1 hour of collection, and plasma was stored protected from light at –30 °C until analysis. The maximal time samples were stored (at –30 ˚C) before analysis was 112 days.

4.3.2.3. Laboratory assays

Because not all samples in the reference interval study were from fasted subjects, LDL-cholesterol was measured directly and triglycerides and HDL-cholesterol were not measured.

4.3.3. Results

4.3.3.1. Demographic variables

The demographic characteristics and lipid profiles of the population sample used to determine the reference interval are summarised in Table 4.1. There was no significant difference in any variable (total CoQ_{10}, total cholesterol, LDL-cholesterol, CoQ_{10} to total cholesterol ratio, or CoQ_{10} to LDL-cholesterol ratio) between the fasting (n = 115) and non-fasting (n = 90) groups. Therefore, fasting and non-fasting data were pooled for statistical analysis. This lack of difference may be explained by the fact that all samples were taken between 0750 and 1015 hours, at which time non-fasting subjects would have consumed breakfast which (for the greater part of the population) would contain low levels of CoQ_{10} and lipids. Furthermore, dietary CoQ_{10} is not well absorbed so that only a very small amount of the ingested CoQ_{10} would have been absorbed into the blood stream by the time of taking blood. Maximum absorption of CoQ_{10} from supplements is reported to occur six hours after ingestion, as found in Chapter 5.

Table 4.1 Demographic characteristics and lipid profiles of population samples. Data are median (interquartile range). p-Value is for comparison between males and females.

	Females	Males	p-Value	Total Cohort
Age (years)	44.00 (20.80 – 74.60)	45.00 (21.00 – 75.10)	p = 0.918	44.00 (21.00 – 74.80)
BMI (kg/m^2)	24.52 (18.69 – 39.98)	26.05 (18.82 – 37.16)	p = 0.122	25.60 (18.79 – 38.99)
Total Cholesterol (mmol/L)	5.47 (3.78 – 8.51)	5.61 (3.36 - 8.37)	p = 0.541	5.54 (3.54 – 8.50)
LDL – Cholesterol (mmol/L)	2.96 (1.46 – 5.27)	3.24 (1.56 – 4.87)	p = 0.381	3.01 (1.49 – 5.09)

BMI = body mass index.

The lack of significant difference in age, body mass index, total cholesterol, and LDL-cholesterol between males and females confirms that these two groups were well matched.

4.3.3.2. CoQ_{10} in the population – distribution

The distribution of total CoQ_{10} for the complete cohort was not Gaussian, being skewed toward higher concentrations (Figure 4.1) (Skewness (± standard error) = 1.14 ± 0.17). Therefore, non-parametric statistics were used to describe the population.

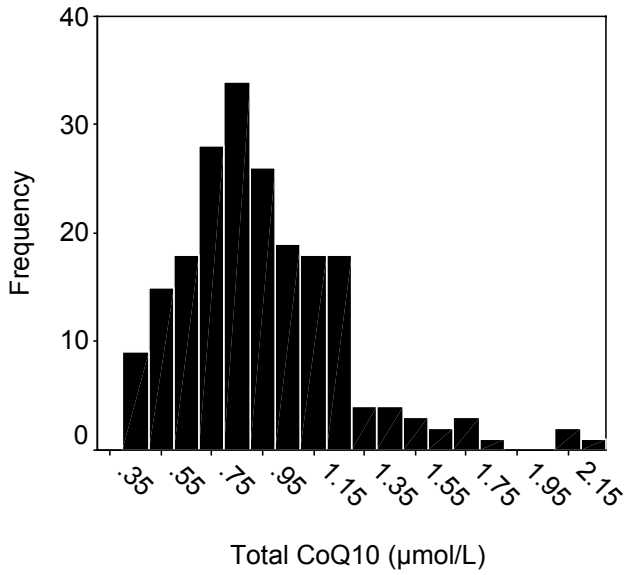


Figure 4.1 Histogram of total CoQ_{10} levels in the complete population sample (n = 205).

The distributions of the total CoQ_{10} to LDL-cholesterol ratio, and the total CoQ_{10} to total cholesterol ratio were closer to a normal distribution than that of CoQ_{10} alone (skewness (± standard error) = 0.93 ± 0.17). However, some skewness to a higher ratio is evident (Figure 4.2).

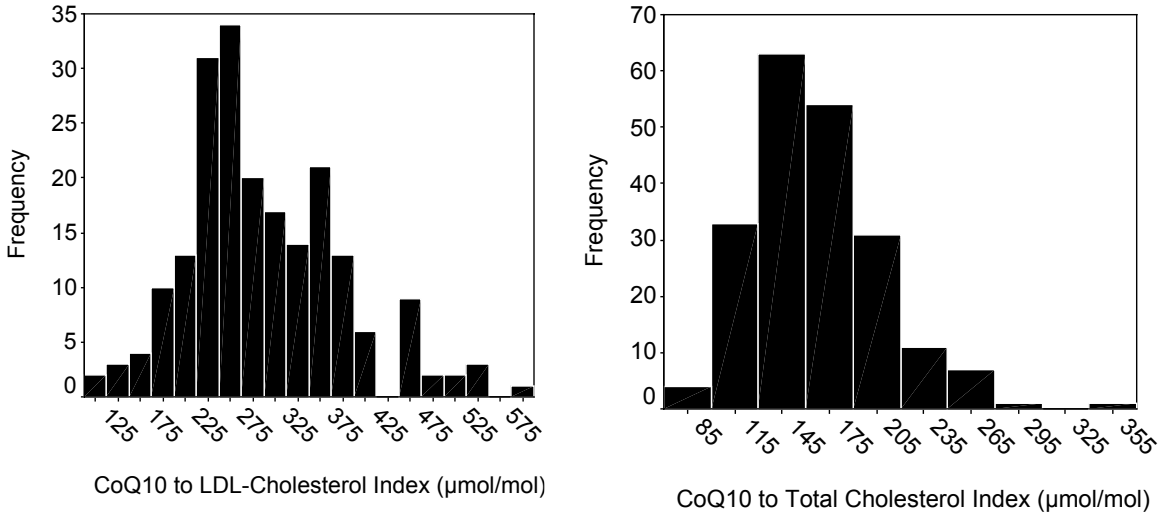


Figure 4.2 Histogram of the total CoQ_{10} to total cholesterol ratio (A) and total CoQ_{10} to LDL-cholesterol ratio (B) in the complete population sample (n = 205).

4.3.3.3. CoQ_{10} and gender

The reference interval for total CoQ_{10}, lipids and the ratios of CoQ_{10} to lipids for males and females separately and combined are shown in Table 4.2.

Table 4.2 The 95% interfractile reference intervals for all analytes, ratios, and subgroups that deserve stratification.

	n	95% Interfractile Reference Interval
Total CoQ_{10}	205	0.46 – 1.78 µmol/L
Total CoQ_{10} – Males	90	0.45 – 2.05 µmol/L [a]
Total CoQ_{10} - Females	115	0.46 – 1.71 µmol/L [a]
Total CoQ_{10} – Age 18 – 44 years	105	0.43 – 1.61 µmol/L
Total CoQ_{10} – Age 45 – 83 years	100	0.57 – 1.95 µmol/L
LDL-Cholesterol	205	1.50 – 4.98 mmol/L
Total CoQ_{10} to LDL-Cholesterol Ratio	205	158 – 522 µmol/mol
Total Cholesterol	205	3.57 – 8.40 mmol/L
Total CoQ_{10} to Total Cholesterol Ratio	205	101 – 265 µmol/mol
Total CoQ_{10} to Total Cholesterol Ratio - Males	90	121 – 284 µmol/mol
Total CoQ_{10} to Total Cholesterol Ratio - Females	115	88 – 244 µmol/mol

[a] These sub-groups are not statistically required to be stratified using Harris and Boyd criteria (2), but the difference is of interest.

There was a significant difference in total CoQ_{10} and the CoQ_{10} to total cholesterol ratio between males and females ($p = 0.008$ and $p < 0.001$, respectively), with males tending to have a higher total CoQ_{10} level than females (Figure 4.3) (2.5 – 97.5% ranges for males and females were 0.45 – 2.05 and 0.46 – 1.71 µmol/L respectively). There was a non-significant trend for a difference in CoQ_{10} to LDL-cholesterol ratio between males and females ($p = 0.075$). A significant difference in total CoQ_{10} between males and females has been previously reported in some studies (6, 8) but not in others (9-11).

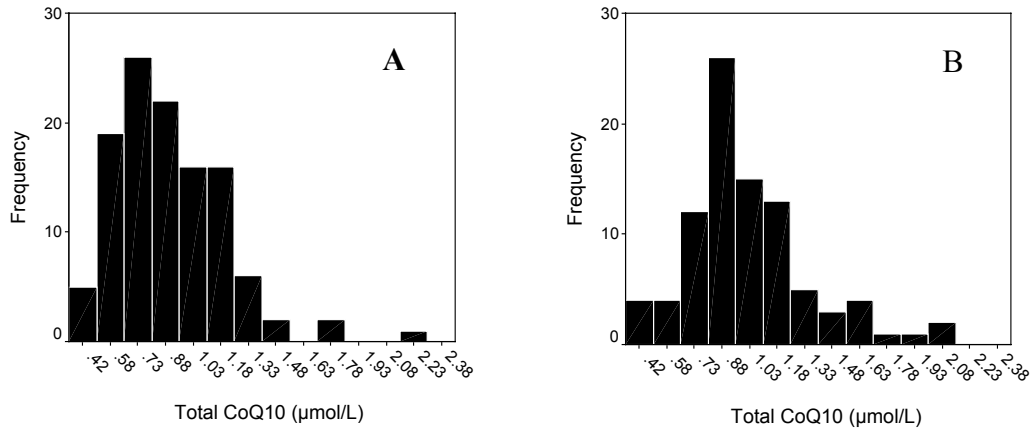


Figure 4.3 Histogram of total CoQ_{10} for females (A) (n = 115) and males (B) (n = 90).

On the basis of the criteria recommended by Harris and Boyd, (1990) (2), and the National Committee for Clinical Laboratory Standards (NCCLS), (1995, 2001) (12), the reference interval for total CoQ_{10} need not be stratified according to gender.

4.3.3.4. Correlation of CoQ_{10} and lipids

The correlations of total CoQ_{10} with both total cholesterol (r = + 0.651) and LDL-cholesterol (r = + 0.600) were significant ($p < 0.001$) (Figure 4.4). This significant correlation is expected due to the lipophilicity of CoQ_{10} and has been previously reported (4, 6, 13-17). A strong correlation between total cholesterol and LDL-cholesterol also existed (Figure 4.5).

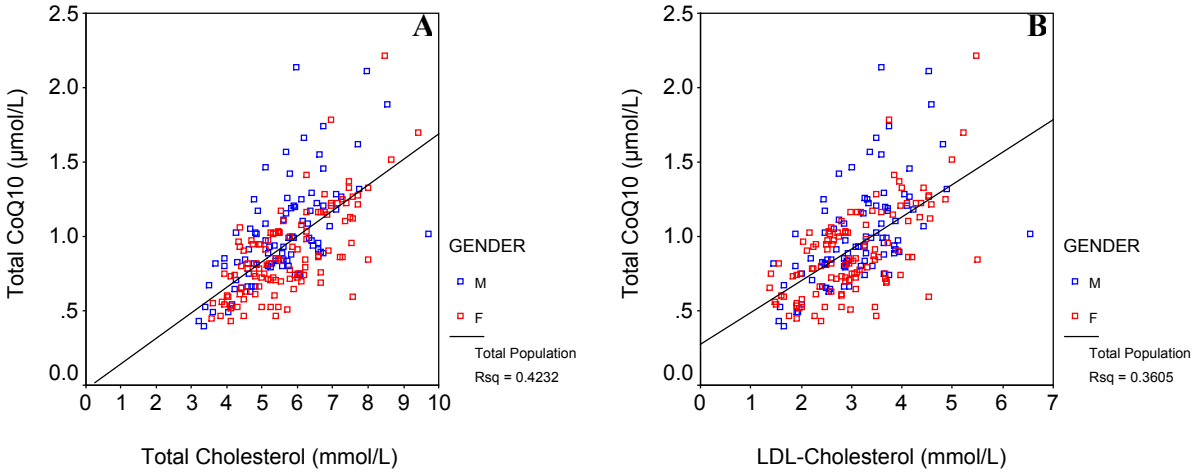


Figure 4.4 The correlation of total CoQ_{10} and total cholesterol (A), and total CoQ_{10} and LDL-cholesterol (B) for the complete population (n = 205), males (n = 90), and females (n = 115).

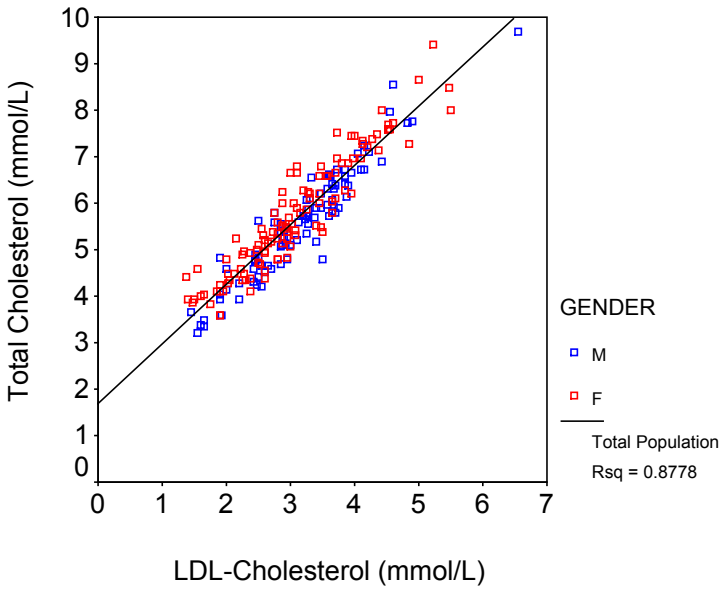


Figure 4.5 The correlation of total cholesterol and LDL-cholesterol in the complete population sample (n = 205), males (n = 90), and females (n = 115).

4.3.3.5. CoQ_{10} and age

Significant positive trends for total CoQ_{10} (Figure 4.6), LDL-cholesterol and total cholesterol (Figure 4.7) to increase with increasing age ($p < 0.001$, $r = + 0.277$, $+ 0.385$, and $+ 0.439$ respectively) were evident and have been previously reported (6, 18, 19). Other reports have described a lack of association between age and CoQ_{10} (8, 20). The correlation of CoQ_{10} and age disappeared when cholesterol was included in a multivariate analysis, which supports the findings of Wolters *et al.*, (2003) (16). Application of the criteria recommended by Harris and Boyd (2) and the NCCLS (21) indicates that separate reference intervals for total CoQ_{10} according to age are justified (Table 4.2). In the ideal situation, more data would be collected for each age group, in order to have more confidence in these results.

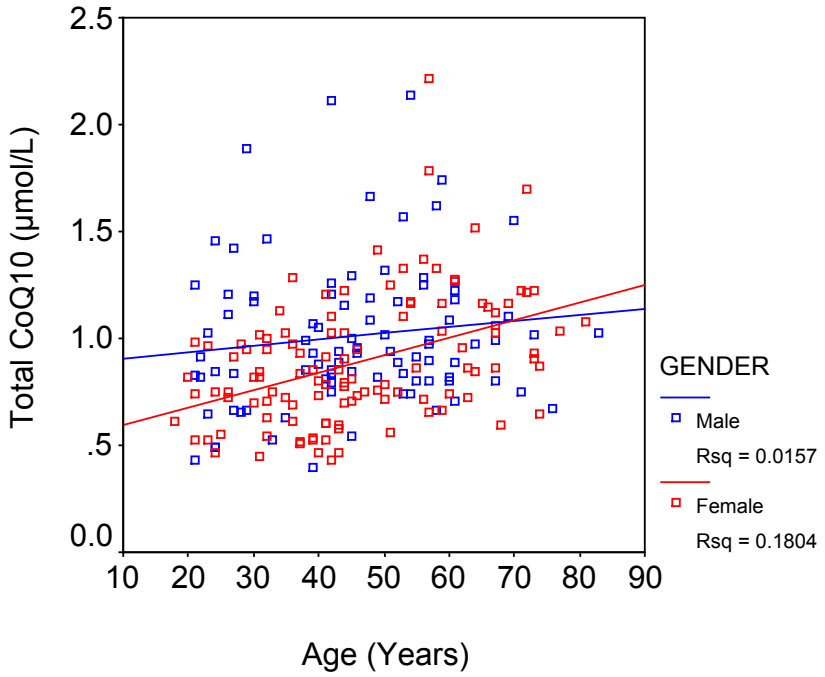


Figure 4.6 The correlation of total CoQ_{10} and age for males (n = 90, p = 0.239), females (n = 115, p < 0.001).

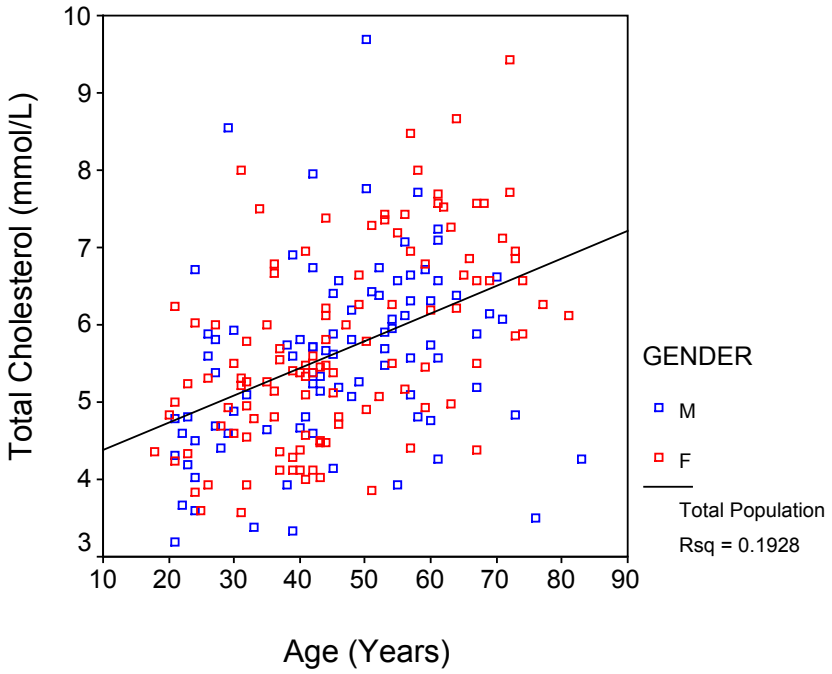


Figure 4.7 The correlation of total cholesterol and age for the complete sample population (n = 205, p < 0.001), males (n = 90), and females (n = 115).

4.3.3.6. CoQ_{10} and BMI

There was a significant correlation ($p < 0.001$) between CoQ_{10} and BMI ($r = + 0.246$) (Figure 4.8), as has been previously reported (6, 8, 16, 22). Related to this was a significant trend for increasing total cholesterol, and LDL-cholesterol ($p = 0.007$ and < 0.001 respectively) with increasing BMI ($r = + 0.188$ and $+ 0.249$ respectively). Wolters *et al.,* (2003) (16) reported that the association of CoQ_{10} and BMI disappeared when CoQ_{10} values were adjusted for lipids, which suggests that the increased circulating cholesterol results in more circulating CoQ_{10}. There is, however, no case for stratifying the reference interval according to BMI (2, 21).

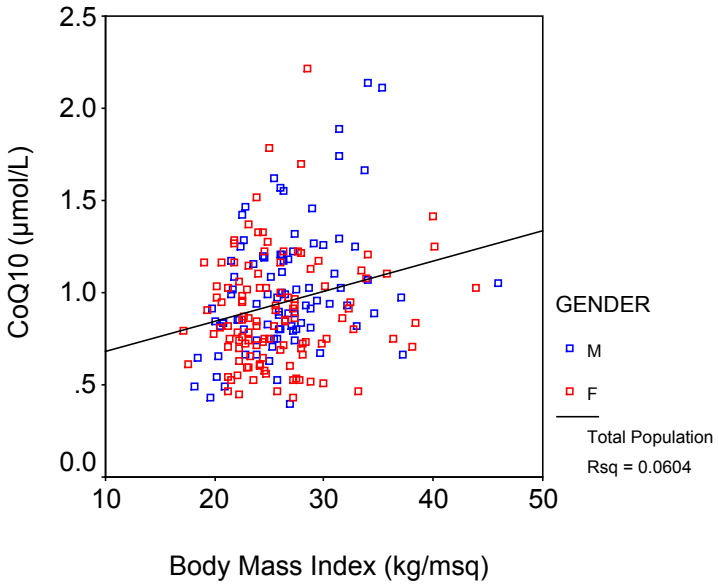


Figure 4.8 The correlation of total CoQ_{10} and body mass index for the complete population sample (n = 205; p < 0.001), males (n = 90), and females (n = 115).

The reference intervals as presented in Table 4.2 can be compared to previously published reference intervals, as shown in Table 4.3.

Table 4.3 Other reported reference intervals for CoQ_{10}.

Publication	Measuring In	Males/ Females	n	Age	Total CoQ_{10} (μmol/L)	Total CoQ_{10}/Total Cholesterol	% Oxidised CoQ_{10} in Total
(10)	Plasma				0.88 ± 0.17		
(10)	Serum				0.89 ± 0.35		
(13)	Serum				0.94 ± 0.28		
(23)	Serum		18		2.41 ± 0.25		44
(24)	Plasma	Both		2 – 6 days	0.56		
(11)	Plasma	Both	31	18 - 56	0.54 ± 0.21 (0.30 – 1.19)		
(5)	Serum				1.58 (0.66 – 3.51)		
(25)			60 smokers		1.01 ± 0.76		
(26)		Males	14	23 – 56			4.4 ± 1.6
(27)	Plasma	Males	256	45-70	1.12 ± 0.29 (0.55 – 2.31)		
(27)	Plasma	Females	264	47 - 70	0.97 ± 0.25 (0.28 – 2.23)		
(27)	Plasma	Males	123	34 - 66	1.22 ± 0.40 (0.54 – 3.44)		
(27)	Plasma	Females	118	42 - 64	1.06 ± 0.27 (0.52 – 1.88)		
(27)	Plasma	Males	11	50 - 67			88.6 ± 1.0 (87.4 – 90.4)
(27)	Plasma	Females	29	51 - 69			87.3 ± 2.0 (80.9 – 90.9)

Table 4.3 Other reported reference intervals for CoQ_{10} (continued).

Author	Measuring In	Males/ Females	n	Age	Total CoQ_{10} (µmol/L)	Total CoQ_{10}/Total Cholesterol	% Oxidised CoQ_{10} in Total
(28)			18		0.38 – 0.93		7
(29)	Plasma				0.32 – 0.97		3.7 ± 2
(30)	Plasma	Males	400	25.1 – 70.1	0.40 – 1.72		
(30)	Plasma	Females	305	22.1 – 70.3	0.43 – 1.47		
(31)		Males	20	19 – 23	1.01 ± 0.56 (0.24 – 2.10)		
(7)	Plasma	Both	50	Children	0.84 ± 0.29	255 ± 91 µmol/mol	
(7)	Plasma	Both	12	Adults	0.96 ± 0.26	199 ± 45 µmol/mol	9.3 ± 2.5
(32)		Both	21 M 37 F	45.2 ± 14.8	0.69 ± 0.29 (0.22 – 1.45)		
(17)	Plasma			Children	0.79 (0.62 – 0.95)		
(16)						0.16 mmol/mol	

4.4. Biological variation

4.4.1. Aim

To determine the intra- and inter-individual variation of plasma total CoQ_{10}, the plasma CoQ_{10} to LDL-cholesterol ratio, and the plasma CoQ_{10} to total cholesterol ratio.

4.4.2. Study design

4.4.2.1. Subjects

Ten healthy adult male volunteers were enrolled via response to advertisements. Participants were excluded from the study if they reported taking any CoQ_{10}, vitamin supplements or other medications within four weeks prior to initiation of the study. All participants were self-reportedly healthy and disease free throughout the study and did not smoke. The median age of participants was 23.5 years (range 21-28 years), the median weight was 69 kg (range 60-100 kg) and the median body mass index was 21.4 (18.5 – 28.6) kg/m^2.

4.4.2.2. Study protocol

Seven baseline fasting blood samples were collected, at least one week apart, over a 2-month period.

4.4.2.3. Sample handling

Blood samples were taken in the morning after a 10 hour overnight fast. Seven samples were taken in total over the two month period. Blood specimens were collected from the median cubital vein into glass vacuum tubes containing lithium heparin. Blood was immediately placed on ice, and tubes were centrifuged within 1 hour of collection at 1800g for 10 minutes at 4 °C. Plasma was immediately transferred to pre-labelled 5 mL serology tubes and stored at -80 °C until analysis, which was within 5 months of collection.

4.4.3. Results

4.4.3.1. Demographic variables

All ten participants had healthy levels of CoQ_{10} and lipids (Table 4.4). Table 4.4 also shows the median ratios for total CoQ_{10} to LDL-cholesterol, and total CoQ_{10} to total cholesterol (n = 70).

Table 4.4 Lipid characteristics for the participants in the biological variation of CoQ_{10} study (n = 70).

	Median (interquartile range)
Total CoQ_{10} (µmol/L)	0.85 (0.66 – 0.99)
Total Cholesterol (mmol/L)	4.75 (4.10 – 5.70)
LDL-Cholesterol (mmol/L)	2.75 (2.24 – 3.28)
HDL-Cholesterol (mmol/L)	1.12 (0.99 – 1.31)
Triglycerides (mmol/L)	1.20 (1.00 – 1.50)
Total CoQ_{10} to LDL-Cholesterol (mmol/mol)	289 (252 – 348)
Total CoQ_{10} to Total Cholesterol (mmol/mol)	169 (147 – 198)

4.4.3.2. Intra- and inter- individual variation

The intra- and inter-individual variation in CoQ_{10} shows that CoQ_{10} in an individual is tightly distributed around a homeostatic set point (Table 4.5). This concept is confirmed by observing graphs of the range of CoQ_{10} values reported for each individual over the seven time points (Figure 4.9). There is greater natural variation in the CoQ_{10} to lipid (total cholesterol or LDL-cholesterol) ratio than there is for total CoQ_{10} alone (Figure 4.10), suggesting that lipids have a larger natural variation than total CoQ_{10}.

Table 4.5 The intra- and inter-individual variation in CoQ_{10} parameters.

	Intra-individual %CV	Inter-individual %CV
Total CoQ_{10}	12	29
Total CoQ_{10} to LDL-Cholesterol Ratio	15	26
Total CoQ_{10} to Total Cholesterol Ratio	14	18

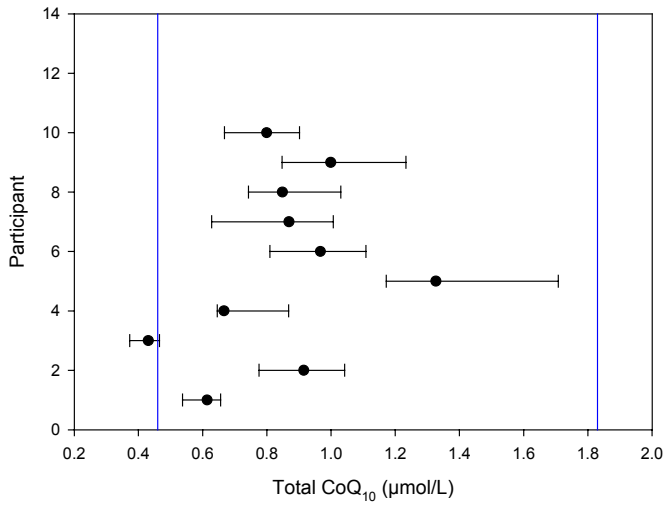


Figure 4.9 Biological variation (median and range) for CoQ_{10} for each participant. Blue vertical lines indicate the reference interval, as determined in the healthy New Zealand population.

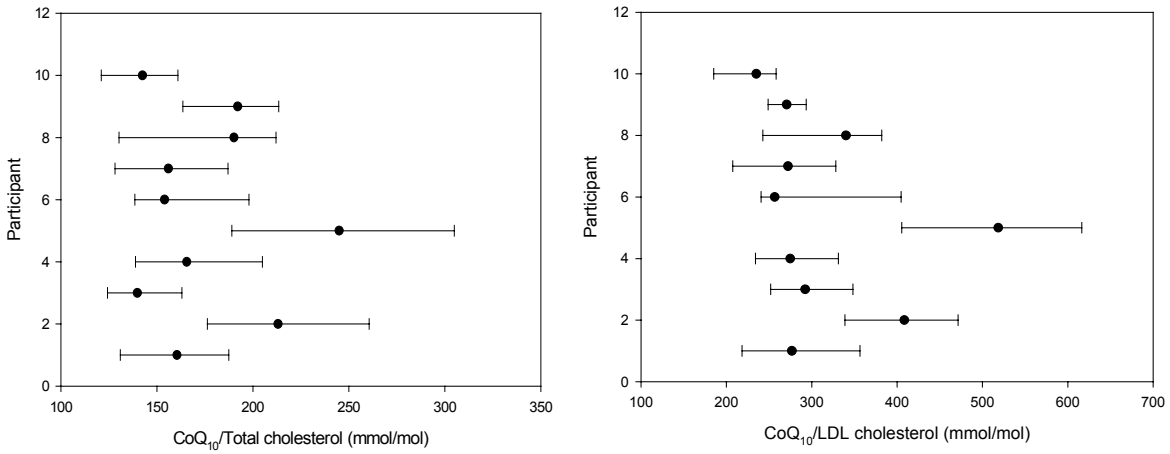


Figure 4.10 Biological variation (the median and range) for the CoQ_{10} to total cholesterol ratio, and the CoQ_{10} to LDL-cholesterol ratio for each participant.

As discussed in the introduction of this Chapter, with knowledge of the biological variation and analytical imprecision of the assay, it is possible to calculate a reference change value (RCV) or 'critical difference' for serial results to be significantly different. Thus, for total CoQ_{10}, with a CV_i of 12% and a CV_a of 3.3%, a 95% significant change is 35.0% and a 99% significant change is 46.1%. Significant changes in CoQ_{10} can therefore occur within the reference interval. For example, with a starting CoQ_{10} concentration of 1.00 µmol/L, the concentration can decrease to 0.65 µmol/L (a 95% significant change) or 0.54 µmol/L

(a 99% significant change) and still be within the reference interval. Biological variation and the reference change value therefore need to be taken into consideration when assessing, for example, whether therapy with statins has resulted in a significant fall in CoQ_{10}, or conversely, whether supplementation has resulted in a significant increase in CoQ_{10}.

Furthermore, for total CoQ_{10}, the index of individuality (CV_i/CV_g) is 12.2 / 29.0 = 0.42 which is low. This further suggests that reference intervals are of little use in deciding whether a significant change in Q_{10} values has occurred (be it an increase due to supplementation or a decrease due to, for example, statin therapy).

Applying Equation 4.2 reveals that seven samples should be evaluated to ensure that the estimate of the homeostatic set point is within 10% of the true value with 95% probability.

4.5. Discussion

For CoQ_{10}, a deficiency state is important since CoQ_{10} is required by every cell in the body. The reference interval gives a basis on which to make medical decisions, since results lying outside the reference interval deserve attention – either in the form of continued monitoring or action such as supplementation. Because the lower (2.5%) limits for males and females in the present study were very similar (0.45 and 0.46 μmol/L respectively), the statistically significant difference in total CoQ_{10} between males and females is not clinically important, and it is therefore valid to use one reference interval for males and females in a clinical setting. CoQ_{10} concentrations do not show a Gaussian distribution. Therefore non-parametric statistics were used since non-parametric (or distribution-free) statistics require no assumption of the distribution. The disadvantage of non-parametric statistics is that they are less powerful than the corresponding parametric tests.

The difference in CoQ_{10} between genders does not appear to be due to differences in total cholesterol levels, since the ratio of CoQ_{10} to total cholesterol was also significantly different in males and females. However, the association of CoQ_{10} and age is probably explained by higher total cholesterol levels found in older people (at least up to 80 years of age, as in the studied population) since the correlation of age and CoQ_{10} disappeared when

cholesterol was included in a multivariate analysis. The association of CoQ_{10} and BMI is also probably due to increased total cholesterol levels in people with higher BMI.

The index of individuality of 0.42 for total CoQ_{10} suggests that individuals have a tightly homeostatically controlled plasma CoQ_{10} concentration, and therefore population based reference intervals are of little use. An individual can have a significant change in their CoQ_{10} concentration, but still have a level that falls within the reference interval. The low index of individuality therefore suggests that monitoring of plasma CoQ_{10} levels over time is more informative than comparing a one off value for an individual to a population based reference interval.

From a clinical perspective, serial changes in CoQ_{10} should be evaluated against the reference change value (RCV) to allow for both biological variation and analytical imprecision. The biological variation study was carried out on young healthy males and it is possible that variation in CoQ_{10} may differ for females, older adults, or children. Males were chosen for this study because males should have more constant biochemistry over a seven week period than females. Documentation on the effect of the menstrual cycle on plasma CoQ_{10} is lacking and may be of interest for interpretation of patient results.

4.6. References for Chapter 4

1. Fraser CG. Biological variation: From principles to practice. USA: AACCPress, American Association for Clinical Chemistry, Inc., 2001.
2. Harris EK, Boyd JC. On dividing reference range data into sub-groups to produce separate reference ranges. Clinical Chemistry 1990;36:265-70.
3. Tomasetti M, Alleva R, Solenghi MD, Littarru GP. Distribution of antioxidants among blood components and lipoproteins: significance of lipids/CoQ10 ratio as a marker of increased risk for atherosclerosis. Biofactors 1999;9:231-40.
4. Kontush A, Reich A, Baum K, Spranger T, Finckh B, Kohlschütter A, Beisiegel U. Plasma ubiquinol-10 is decreased in patients with hyperlipidaemia. Atherosclerosis 1997;129:119-26.
5. Laaksonen R, Riihimäki A, Laitila J, Mårtensson K, Tikkanen MJ, Himberg JJ. Serum and muscle tissue ubiquinone levels in healthy subjects. Journal of Laboratory and Clinical Medicine 1995;125:517-21.
6. Kaikkonen J, Nyyssönen K, Tuomainen TP, Ristonmaa U, Salonen JT. Determinants of plasma coenzyme Q_{10} in humans. FEBS Letters 1999;443:163-6.
7. Niklowitz P, Menke T, Andler W, Okun JG. Simultaneous analysis of coenzyme Q10 in plasma, erythrocytes and platelets: comparison of the antioxidant level in blood cells and their environment in healthy children and after oral supplementation in adults. Clinica Chimica Acta 2004;342:219-26.
8. Miles MV, Horn PS, Morrison JA, Tang P, DeGrauw T, Pesce AJ. Plasma coenzyme Q_{10} reference intervals, but not redox status, are affected by gender and race in self-reported healthy adults. Clinica Chimica Acta 2003;332:123-32.
9. Wahlqvist ML, Wattanapenpaiboon N, Savige GS, Kannar D. Bioavailability of two different formulations of coenzyme Q_{10} in healthy subjects. Asia Pacific Journal of Clinical Nutrition 1998;7:37-40.
10. Okamoto T, Fukui K, Nakamoto M, Kishi T, Okishio T, Yamagami T, et al. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. Journal of Chromatography 1985;342:35-46.

11. Kaplan P, Sebestianová N, Turiaková J, Kucera I. Determination of Coenzyme Q in human plasma. Physiological Research 1995;45:39-45.
12. National Committee for Clinical Laboratory Standards. How to define and determine reference intervals in the clinical laboratory: approved guideline. Villanova, PA.: NCCLS, 1995, 2001.
13. Edlund PO. Determination of Coenzyme Q_{10}, α-tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. Journal of Chromatography 1988;425:87-97.
14. Lagendijk J, Ubbink JB, Delport R, Hayward WJ, Human JA. Measurement of the ratio between the reduced and oxidized forms of coenzyme Q10 in human plasma as a possible marker of oxidative stress. Journal of Lipid Research 1996;37:67-75.
15. Tomasetti M, Littarru GP, Stocker R, Alleva R. Coenzyme Q10 enrichment decreases oxidative DNA damage in human lymphocytes. Free Radical Biology and Medicine 1999;27:1027-32.
16. Wolters M, Hahn A. Plasma ubiquinone status and response to six-month supplementation combined with multivitamins in healthy elderly women - results of a randomized, double blind, placebo-controlled study. International Journal of Vitamin and Nutrition Research 2003;73:207-14.
17. Menke T, Niklowitz P, de Sousa G, Reinehr T, Andler W. Comparison of coenzyme Q10 plasma levels in obese and normal weight children. Clinica Chimica Acta 2004;349:121-7.
18. Komorowski, Muratsu K, Nara, Willis, Folkers K. Significance of biological parameters of human blood levels of CoQ10. Biofactors 1988;1:67-9.
19. Pedersen, Mortensen, Rohde, Deguchi, Mulved, Bjerregaard, Hansen. High serum coenzyme Q10, positively correlated with age, selenium, and cholesterol, in Inuit of Greenland. A pilot study. Biofactors 1999;9:319-23.
20. Miles MV, Horn PS, Tang PH, Morrison JA, Miles L, DeGrauw T, Pesce AJ. Age-related changes in plasma coenzyme Q_{10} concentrations and redox state in apparently healthy children and adults. Clinica Chimica Acta 2004;347:139-44.

21. Standards. NCfCL. How to define and determine reference intervals in the clinical laboratory: approved guideline. Villanova, PA.: NCCLS, 1995, 2001.
22. Zita C, Overvad K, Mortensen SA, Sindberg CD, Moesgaard S, Hunter DA. Serum coenzyme Q_{10} concentrations in healthy men supplemented with 30 mg or 100 mg coenzyme Q_{10} for two months in a randomised controlled study. BioFactors 2003;18:185-93.
23. Wakabayashi H, Yamato S, Nakajima M, Shimada K. Simultaneous determination of oxidized and reduced Coenzyme Q and α-Tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. Biological and Pharmaceutical Bulletin 1994;17:997-1002.
24. Finckh B, Kontush A, Commentz J, Hübner C, Burdelski M, Kohlschütter A. Monitoring of Ubiquinol-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Analytical Biochemistry 1995;232:210-6.
25. Kaikkonen J, Nyyssönen K, Porkkala-Sarataho E, Poulsen HE, Metsä-Ketelä T, Hayn M, et al. Effect of oral coenzyme Q10 supplementation on the oxidation resistance of human VLDL + LDL fraction: Absorption and antioxidative properties of oil and granule-based preparations. Free Radical Biology and Medicine 1997;22:1195-202.
26. Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Analytical Biochemistry 1997;250:66-73.
27. Kaikkonen J, Nyyssönen K, Salonen JT. Measurement and stability of plasma reduced, oxidised and total coenzyme Q10 in humans. Scandinavian Journal of Clinical and Laboratory Investigation 1999;59:457-66.
28. Wang Q, Lee BL, Ong CN. Automated high-performance liquid chromatographic method with precolumn reduction for the determination of ubiquinol and ubiquinone in human plasma. Journal of Chromatography B 1999;726:297-302.

29. Tang PH, Miles, M.V., DeGrauw, A., Hershey, A., Pesce, A. HPLC analysis of reduced and oxidised coenzyme Q10 in human plasma. Clinical Chemistry 2001;47:256-65.
30. Kaikkonen J, Tuomainen TP, Nyyssönen K, Salonen JT. Coenzyme Q10: Absorption, antioxidative properties, determinants, and plasma levels. Free Radical Research 2002;36:389-97.
31. Lu W-L, Zhang Q, Lee H-S, Zhou T-Y, Sun H-D, Zhang D-W, et al. Total Coenzyme Q10 concentrations in Asian men following multiple oral 50-mg doses administered as coenzyme Q10 sustained release tablets or regular tablets. Biological and Pharmaceutical Bulletin 2003;26:52-5.
32. Jiang P, Wu M, Zeheng Y, Wang C, Li Y, Xin J, Xu G. Analysis of coenzyme Q10 in human plasma by column-switching liquid chromatography. Journal of Chromatography B 2004;805:297-301.

Chapter 5

Relative absorption at six hours of Coenzyme Q_{10} Supplements, Dose Range for the Coenzyme Q_{10} Supplement Q-Gel, and the Effect of Statin on Coenzyme Q_{10} in Patients with Chronic Heart Failure

5.1. Introduction

5.1.1. Coenzyme Q_{10} supplementation

Deficiency of CoQ_{10} is important in terms of ill health. Therefore, where medical intervention is required, supplementation will generally be advised. It has been reported that the desired concentration of plasma CoQ_{10} considered to have a therapeutic effect is 2.90 μmol/L (1, 2). CoQ_{10} supplements are available over the counter from health food shops and pharmacies in most countries, and different brands differ in bioavailability because of differing excipients, form of CoQ_{10} (reduced or oxidised), and the type of encapsulation (3-8). Information on the bioavailability of various brands of CoQ_{10} is limited and may be influenced by uninformed sales representatives. As CoQ_{10} supplements are relatively expensive (approximately NZ $1 per day), knowledge of bioavailability is important. While there are reports comparing various formulations of CoQ_{10}, comparison of results between studies is complicated by differences in the CoQ_{10} preparations, subjects, and dosage. Extrapolation of bioavailability results from animal trials to humans is hampered by a common trend to supplement animals with doses that are equivalent to extremely high doses in humans. For example, giving a 75 kg human a 150 mg dose of CoQ_{10} is equivalent to 2 mg/kg, whereas Lass *et al.,* (1999) (9) administered 123 mg/kg/day to mice when determining the effect of CoQ_{10} administration on tissue levels. A survey of the relative absorption at 6 hours of CoQ_{10} supplements available in New Zealand has not been previously reported.

Knowledge of the dose-range for CoQ_{10} is limited but would be very useful when planning and designing studies involving CoQ_{10} supplementation. Therefore, a study investigating the dose-range for the most bioavailable brand of CoQ_{10} supplement, Q-Gel, was completed.

5.2. Coenzyme Q_{10} and statin therapy in patients with chronic heart failure

As discussed in Chapter 1, Section 1.1.9.6, statin (3-hydroxy-3-methylglutaryl-coenzyme-A (HMG CoA) reductase inhibitor) therapy is associated with a decrease in plasma CoQ_{10} concentration (10-15). A multitude of studies have demonstrated that statins can

profoundly improve both coronary and peripheral endothelial function (16-18) even in young, healthy and normocholesterolemic males. Chronic heart failure is associated with endothelial dysfunction (19, 20). The endothelial nitric oxide pathway has been found to be defective in patients with heart failure (19, 20) and the degree of endothelial impairment is related to the severity of heart failure (21). The improvement in tissue perfusion is an important goal in patients with chronic heart failure in terms of both the peripheral and coronary circulation (22). Although not currently indicated for chronic heart failure, statin therapy could result in substantial clinical benefits through its endothelium enhancing properties and other pleiotropic effects. In a retrospective analysis of large statin trials, statin therapy was associated with improved cardiovascular outcome in subgroups with heart failure (23). However, statin therapy may potentially be unfavourable in chronic heart failure patients due to its CoQ_{10} reducing properties (10, 11, 13, 14). CoQ_{10} deficiency has been implicated in chronic heart failure, and the severity of heart failure is correlated with the degree of CoQ_{10} depletion.

Therefore, a study was conducted by the Lipid and Diabetes Research Group of Christchurch Hospital, to investigate the effect of statin therapy on both endothelial function and plasma CoQ_{10} concentrations in people with chronic heart failure.

5.3. General methods

5.3.1. Ethics

These studies were approved by the Canterbury Ethics Committee, Christchurch, New Zealand and written informed consent was obtained from all participants.

5.3.2. Biochemistry

For all studies (unless otherwise specified), heparinized plasma specimens were analysed for total CoQ_{10} using HPLC with electrochemical detection, as described in Chapter 3, section 3.6.2. Total cholesterol, triglycerides, and HDL-cholesterol were determined by an enzymatic colorimetric method (Aeroset analyser Model LN, Abbott Laboratories, Illinois IL, USA). Coefficients of variation for the total cholesterol, triglycerides, and HDL-cholesterol assays were 1.6%, 1.1%, and 5.3% respectively. Direct LDL-cholesterol was measured using Roche Diagnostics reagents, with a coefficient of variation of 1.2%.

Between and within-run coefficients of variation for total CoQ_{10} measurement are approximately 3.3%.

5.3.3. Statistics

Statistical analysis was performed using SPSS Base version 10.0 (SPSS, Inc., Chicago, Illinois), and SigmaStat software (SPSS, Inc., Chicago, Illinois). Statistical significance was accepted when $p < 0.05$.

For the bioavailabilty study of coenzyme Q_{10} supplements, the differences between CoQ_{10} supplements were tested using the non-parametric Friedman test, and Wilcoxon signed-rank test, as appropriate.

5.4. Absorption of coenzyme Q_{10} supplements

5.4.1. Aim

To investigate the absorption of seven different CoQ_{10} supplements marketed in New Zealand.

5.4.2. Study design

5.4.2.1. Subjects

Ten healthy adult male volunteers were enrolled. Participants were excluded if they reported taking any CoQ_{10}, vitamin supplements or other medications within four weeks prior to the study. All participants were self-reportedly healthy and disease free throughout the study, and all were non-smokers. The mean age of participants was 24.2 years (range 21 - 28 years), the mean height was 180 cm (range 173 - 187 cm), and the mean weight was 72 kg (range 60 - 100 kg). The study size (n = 10) was selected based on reported effects of coenzyme Q_{10} supplementation, indicating that CoQ_{10} supplementation would increase C_{max} by 15 – 20% (equates to ± 0.23 - 0.30 μmol/L) at $\alpha = 0.5$ with a power of 80%.

5.4.2.2. Study protocol

Baseline blood samples were obtained after a ten hour overnight fast and CoQ_{10} supplements were administered as a single dose of 150 mg (based on the claimed content on supplement packaging). Supplement brands were given in a randomised order (a

different randomised order for each participant), with a week long washout period between trial days. After administration of the supplement, a standardised vegetarian breakfast and lunch were provided. Lunch was provided as a takeaway package and participants were permitted to leave the study centre after breakfast. Six hours after administration of the supplement, participants returned to the study centre for collection of a second blood sample.

5.4.2.3. Blood collection

Blood specimens were collected from the median cubital vein into glass vacuum tubes containing lithium heparin. Blood was immediately placed on ice and tubes were centrifuged within 1 hour of collection at 1800g for 10 minutes at 4 °C. Plasma was immediately transferred to pre-labelled 5 mL serology tube then stored at -80 °C until analysis, which was within 5 months of sample collection.

5.4.2.4. Assumptions made in this study

This study assumes that maximum absorption from all of the CoQ_{10} supplements occurs 6 hours after ingestion. In several studies looking at the pharmacokinetic properties of CoQ_{10}, t_{max} occurred at approximately six hours (Table 5.1) (3, 4, 7, 8). Whalqvist *et al.,* (1998) (6) reported t_{max} to occur at 4 hours, and Kaikkonen *et al.,* (1997) (4) reported t_{max} for a granule-based supplement to occur at 10 hours, as opposed to 6 hours for an oil-based supplement. Data presented in Section 5.5.3.5 further support the occurrence of t_{max} 6 hours after supplementation. It is possible that time to t_{max} varies more between individuals than between formulations. This is a limitation of this study, as it is very possible that t_{max} occurred at a time other than 6 hours for some individuals and/or some supplement brands.

Table 5.1 t_{max} as determined by various authors.

Publication	Supplement Formulation	t_{max} (hours)
(3)	CoQ and Emcompress	6[a]
	CoQ and soy bean oil	
	CoQ, soy bean oil, polysorbate-80 and phosphatidylcholin	
	CoQ, soy-bean oil, and polysorbate-80	
(24)	Q-Gel	7
	Starch-coated nano beadlets containing CoQ dispersed into a water-soluble gelatin matrix	7
(6)	CoQ in a complex micelle in an emulsion	4[a]
	CoQ as dry powder	
(7)	Solubilised CoQ	6.2 ± 1.6
	Reduced CoQ	8.1 ± 6.3
	Fully solubilised CoQ	5.8 ± 0.7
	CoQ as dry powder	6.7 ± 1.0
(8)	Biotransformed, reduced CoQ	6.0 ± 0.3
	CoQ as dry powder	5.9 ± 3.0

[a]secondary peak observed at 24 hours.

Evidence of a second (lesser) peak at 24 hours after ingestion of the supplement has also been reported (3, 6). It has been suggested that this two-peak pattern occurs because the CoQ_{10} is administered as the oxidised form, and the first peak is due to the blood content of this parent compound. While circulating in the blood, the parent compound (oxidised CoQ_{10}) undergoes reduction somewhere in the peripheral compartment (for example, in the liver). The oxidised CoQ_{10} will easily pass biomembranes into the liver, but the increase in water solubility caused by the reduction of the quinone by the liver enzymes impairs the ability of the reduced CoQ_{10} to pass biomembranes. The result will then be a second peak caused by $CoQ_{10}H_2$ (3, 6). However, one formulation tested by Kurowska *et al.,* (2003) (8) contained $CoQ_{10}H_2$, and the reported t_{max} for this formulation was 6.0 ± 1.3 hours, which is inconsistent with the theory of Whalqvist *et al.,* (1998) and Weis *et al.,* (1994) (3, 6). Redistribution after incorporation into the VLDL fraction in the liver (25) and hepatic recycling (26) have also been suggested as causes for the two-peak pattern.

5.4.2.5. Supplement brands

Supplement brands investigated for relative absorption were Blackmores (Blackmores Ltd), Good Health (Good Health Ltd), Kordel's (distributed by Nutra-Life Health & Fitness (NZ) Ltd), Q-Gel (manufactured by Gel-Tec, Tishcon Corp., USA), Radiance (Health & Herbs International Ltd), Solgar (manufactured by Solgar Vitamin and Herb),

and Thompson's (Thompson Nutrition Ltd). These brands were tested because they are 'popular' brands (as advised by health food shop assistants), or brands that contain differing excipients. The formulation of each supplement brand used in this study is outlined in Table 5.2.

Table 5.2 The excipients and formulation of the seven CoQ_{10} supplement brands investigated for relative absorption.

Supplement	Excipients	Capsule/Tablet Type
Q-Gel	Vitamin E, Annato seed extract, Biosolv® base (lecithin, polysorbate, sorbitin monoleate, and medium chain triglycerides)	Softules containing liquid dispersion
Radiance	Rice bran oil, lecithin, selenium and vitamin E	Softgels containing liquid dispersion
Blackmores	Soy lecithin	Capsules containing liquid dispersion
Solgar	Vegetable cellulose, vegetable magnesium stearate and silica	Vegetable capsules containing dry powder
Kordel's	Evening primrose oil and salmon oil	Capsules containing liquid dispersion
Thompson's	Vegetable oil	Vegetarian capsules containing liquid dispersion
Good Health	Glucose, sucrose, magnesium stearate, calcium phosphate, and natural orange flavour	Chewable tablets

5.4.3. Results

Coenzyme Q_{10} supplementation with all brands was well tolerated with no reported side effects.

5.4.3.1. Coenzyme Q_{10} content of the diet

Participants were fed an identical, vegetarian diet on each study day to limit the effect of dietary CoQ_{10}. The diet of participants on study days consisted of: (breakfast) weet-bix, milk and sugar, two slices of toast, and margarine, with two eggs; (Lunch) one sandwich (white and brown bread, lettuce, tomato, and cheese), apple, and orange juice. This diet contributed approximately 315 µg of CoQ_{10} (Table 5.3).

Table 5.3 The CoQ_{10} content of the diet given to participants on each study day.

Item	Approximate Weight (g)	CoQ_{10} Concentration (µg/g)	Total Contribution (µg)
Milk	128	0.1[a]	12.8
Toast	50	0[a]	0
Jam	10	n.k.	n.k.
Weet-bix	31	0	0
Margarine	9	n.k.	n.k.
Orange Juice	102	0.3[a]	30.6
White bread	60	0[a]	0
Brown bread	60	0[a]	0
Lettuce	6	n.k.	n.k.
Tomato	24	0.9[a]	21.6
Cheese	40	1.3[a]	52.0
Apple	152	1.3[a]	197.6
Total			**314.6 µg**

n.k. = not known; [a] from (27).

5.4.3.2. Supplement adherence

All CoQ_{10} supplements tested contained 100% or more of the claimed CoQ_{10} content (Table 5.4). Six of the seven brands contained approximately 20% more CoQ_{10} than claimed. The brand Good Health had a mean of 100% of the CoQ_{10} concentration claimed with some tablets containing 10% less CoQ_{10} than was claimed on the packaging. Some brands supply a more variable dose than others, Q-Gel being the most consistent (Table 5.4).

Table 5.4 The measured and claimed CoQ_{10} content of the different supplement brands (n = 6 capsules or tablets).

	mg of CoQ_{10} per Capsule/Tablet		
Brand	Claimed	Measured (mean ±SD)	Yield Recovery (%)
Q-Gel	30	41 ± 1.3	137
Radiance	50	63 ± 2.1	125
Blackmores	50	60 ± 4.1	121
Solgar	30	39 ± 4.4	130
Kordel's	75	95 ± 5.5	127
Thompson's	30	36 ± 1.9	121
Good Health	30	30 ± 2.0	100

Because absorption of CoQ_{10} is very poor (8, 28), minor differences in the amount of CoQ_{10} ingested due to variability in dose in each capsule or tablet probably become insignificant, and can be considered to have little effect on the results.

5.4.3.3. Demographic variables

Mean baseline lipids (± SD) for all participants were 4.81 ± 1.04, 2.78 ± 0.75, 1.18 ± 0.30, and 1.28 ± 0.44 mmol/L for total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides, respectively. Mean baseline CoQ_{10} (± SD) was 0.85 ± 0.25 μmol/L. There was no significant change in baseline levels of CoQ_{10}, LDL-cholesterol, HDL-cholesterol, triglycerides, or total cholesterol during the seven weeks of the trial. This confirms that the week-long wash-out period was sufficient for a return to baseline levels, and also that there were no significant dietary changes made by participants during the trial period.

5.4.3.4. Effect of CoQ_{10} supplementation on plasma lipids/dietary compliance

There was no significant effect of CoQ_{10} supplementation on total cholesterol ($p = 0.539$), triglycerides ($p = 0.128$) or LDL- and HDL-cholesterol ($p = 0.910$ and 0.587 respectively). This suggests that participants were compliant with dietary restrictions on study days, since the supplements contained little or no lipid and the diet given contained low concentrations of lipid.

5.4.3.5. Difference in absorption between participants

There was a significant difference ($p = 0.003$) in CoQ_{10} absorption between the ten participants (Figure 5.1). Some participants efficiently absorbed CoQ_{10} from most supplements, while others showed inefficient absorption. This difference in absorption between participants has been previously reported (3, 4, 6, 8, 28).

There was no correlation ($p = 0.56$) between baseline CoQ_{10} levels and absorption of CoQ_{10} from the seven supplement brands which supports the findings of Zita *et al.,* (2003) (28). Conversely, Wolters *et al.,* (2003) (29) reported that changes in cholesterol-adjusted CoQ_{10} concentrations were inversely related to baseline values.

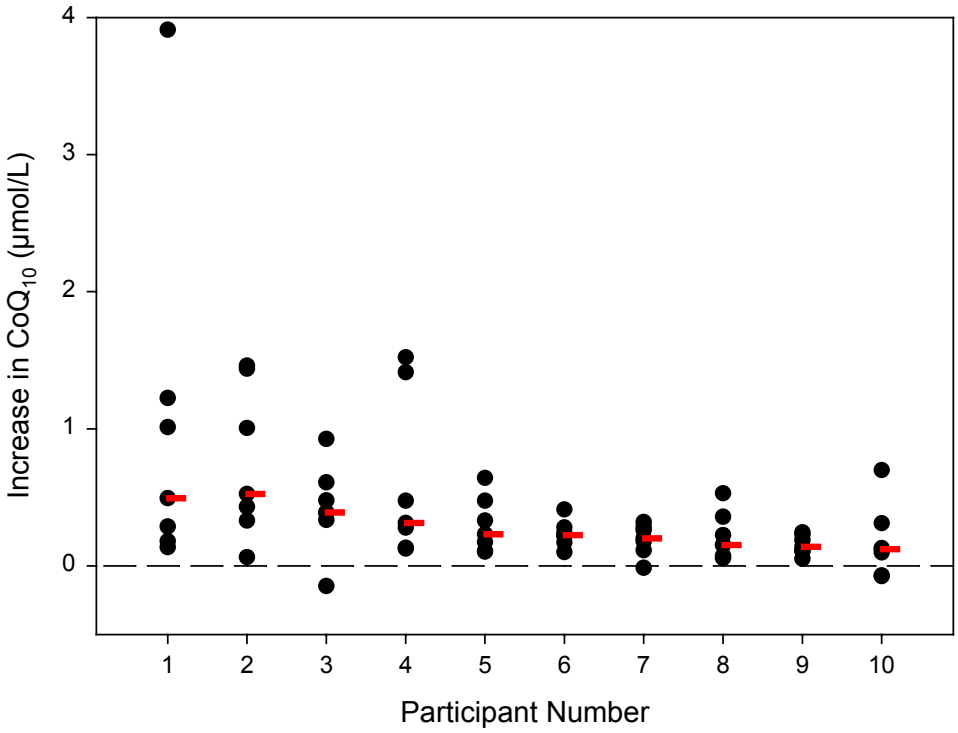


Figure 5.1 The increase in coenzyme Q_{10} concentrations at six hours for individual participants and all supplement brands (n = 7). Horizontal lines show median increase in CoQ_{10} for each participant.

This significant difference in absorption between the participants may be explained on the basis of the significant correlations between baseline LDL-cholesterol concentration and change in CoQ_{10} ($p = 0.004$; $r = + 0.343$), between total cholesterol level and change in CoQ_{10} ($p = 0.004$; $r = + 0.338$), and also between baseline triglycerides and change in CoQ_{10} ($p = 0.035$; $r = + 0.253$). This suggests that higher LDL-cholesterol or triglyceride concentrations may aid absorption of CoQ_{10}.

There was no significant correlation between HDL-cholesterol, weight, or body mass index and CoQ_{10} absorption.

5.4.3.6. Relative absorption of CoQ_{10} brands at six hours

There was a significant difference in relative absorption at six hours between the seven CoQ_{10} brands tested ($p < 0.001$), with Q-Gel having significantly higher relative absorption

at 6 hours than any other supplement brand (p = 0.013). These results are summarised in Table 5.5, which shows the median change in total CoQ_{10} and CoQ_{10} ratios, for the different supplement brands. The significance of these differences is shown in Table 5.6.

There was a significant change in the CoQ_{10} to LDL-cholesterol and CoQ_{10} to total cholesterol ratios between the supplement brands (p = 0.001 for both). The change in these ratios appeared to mirror the change in total CoQ_{10} (Table 5.5).

Table 5.5 The median change in CoQ_{10} at six hours after supplementation with the different brands.

Brand	Change in Total CoQ_{10} concentration (μmol/L)	Change in CoQ_{10} to LDL-Cholesterol Ratio (mmol/mol)	Change in CoQ_{10} to Total Cholesterol Ratio (mmol/mol)
Q-Gel	0.586 (0.349 - 1.424)	0.275 (0.218 - 0.500)	0.125 (0.100 - 0.225)
Radiance	0.321 (0.218 - 1.118)	0.140 (0.120 - 0.373)	0.065 (0.048 - 0.210)
Blackmores	0.229 (0.109 - 0.531)	0.130 (0.072 - 0.253)	0.045 (0.028 - 0.090)
Solgar	0.203 (0.094 - 0.295)	0.075 (0.048 - 0.188)	0.045 (0.020 - 0.075)
Kordel's	0.177 (0.102 - 0.274)	0.075 (0.050 - 0.173)	0.040 (0.018 - 0.078)
Thompson's	0.173 (0.106 - 0.442)	0.080 (0.060 - 0.150)	0.028 (0.060 - 0.073)
Good Health	0.139 (0.105 - 0.297)	0.095 (0.040 - 0.165)	0.040 (0.018 - 0.053)

Values shown are median values, with brackets showing the inter-quartile range.

Table 5.6 Statistical analysis of differences in the change in total CoQ_{10} at 6 hours after a single oral dose[a].

	Q-Gel	Radiance	Blackmores	Solgar	Kordel's	Thompson's
Radiance	0.013					
Blackmores	0.013	0.059				
Solgar	0.013	0.053	0.333			
Kordel's	0.005	0.009	0.203	0.959		
Thompson's	0.005	0.037	0.646	0.386	0.878	
Good Health	0.005	0.022	0.203	0.959	0.386	0.508

[a]Null hypothesis was that there was no significant difference in relative absorption at 6 hours between the seven supplement brands.

As discussed in Chapter 1, different excipients in CoQ_{10} supplements are likely to affect the absorption of the CoQ_{10}. Because CoQ_{10} is lipid soluble, it is likely that administering it as a dispersion, or solubilised in oil will aid absorption into blood, as has been previously reported (30, 31). This was supported by the present study. Conversely, Chopra *et al.,* (1998) (5) found the absorption from powder-filled hardshell capsules and powder-based

tablets to be higher (125% and 128% respectively) than that from a standard softgel capsule containing a CoQ_{10} suspension in oil. A summary of studies on the absorption and bioavailability of CoQ_{10} supplements is shown in Table 5.7.

The high absorption of Q-Gel compared to other coenzyme Q_{10} supplement brands supports the findings of Chopra *et al.,* (1998) (5), who found the absorption of Q-Gel to be 319% better than that from a standard softgel capsule containing a CoQ_{10} suspension in oil, after 3 weeks of a daily 120 mg dose. Additionally, Ullmann *et al.,* (2005) (24), reported Q-Gel to have higher bioavailability than a Q-SorB product (which contains rice bran oil, lecithin, and a beeswax soybean oil mixture as excipients) and slightly higher bioavailability than a new supplement containing starch-coated nano beadlets encasing CoQ dispersed into a water-soluble gelatin matrix

It is possible that the high relative absorption at 6 hours of Q-Gel is due to the presence of both non-ionic surfactants (polysorbate 80) and the natural surfactant lecithin. The brands Radiance and Blackmores showed the second and third highest absorption respectively, and also contain lecithin as an excipient. Lecithin (a liposome forming phospholipid) has been shown to increase the absorption of CoQ_{10} supplements (32), although Weis *et al.,* (1994) (3) reported no increase in bioavailability when micelle and liposome-forming agents were added to the CoQ_{10} supplement. As discussed in Chapter 1, it is not only the presence of liposome and micelle forming agents, but also their concentration that plays a major role in bioavailability, because of critical micelle concentrations (33).

The effect of the capsule on absorption is not clear, since there are no reported studies where the same formulation has been encapsulated in different capsules. Therefore, any perceived differences in absorption due to capsules is actually a combined difference due to formulation and encapsulation.

Table 5.7 A summary of studies investigating the absorption and bioavailability of CoQ_{10} supplements.

Publication	Dose	Duration	Study Design	Number of Subjects (age)	% Male	Baseline Q (μmol/L)	Supplement Type	Final Q
(34)	300 mg (3 x 100 mg dose daily)	11 days	Simple	3 (31 - 35 y/o)	100	$Q_{10}H_2$ 0.8	100 mg Q_{10} powder dissolved in 25 mL milk	$Q_{10}H_2$ 3.2 (during latter half of supplementation)
(3)	100 mg	Single dose	Randomised, crossover	10 (24 – 30 y/o)	50	Not given	1)Hard gelatin capsule with 100mg Q_{10}, 400mg Emcompress 2)Soft gelatin capsule with 100mg Q_{10}, 400 mg soy bean oil 3)Soft gelatin capsule with 100mg Q_{10}, 20mg polysorbate 80, 100mg lecithin, 280mg soy bean oil 4)soft gelatin capsule with 100mg Q_{10}, 20mg polysorbate 80, 380mg soy bean oil	Increase at about six hours 1) 0.9 μmol/L 2) 1.5 μmol/L 3) 0.9 μmol/L 4) 0.9 μmol/L
(4)	30 mg 3 x daily (90 mg total)	2 months	Large block, randomised, single-blind, placebo controlled	(46 ± 7 y/o) 30 30 30	100 (smokers, 23 ± 9 cigarettes/ day)	1.08 ± 0.31 1.07 ± 0.34 0.89 ± 0.33	Granular Oil-based capsule Placebo	Change 1.81 ± 0.82 (168% increase) 1.90 ± 0.97 (178% increase) -0.01 ± 0.26

Table 5.7 A summary of studies investigating the absorption and bioavailability of CoQ_{10} supplements (continued).

Publication	Dose	Duration	Study Design	Number of Subjects (age)	% Male	Baseline Q (μmol/L)	Supplement Type	Final Q
(5)	120 mg	3 weeks	Randomised, double-blind	(20 - 56 y/o)	Not given	0.579 – 0.602		
				6			Oil based capsule	1.586 ± 0.289
				6			Powder filled capsule	1.887 ± 0.243
				6			Tablet	1.853 ± 0.254
				6			Q-Gel	3.833 ± 1.563
(5)	120 mg	4 weeks	Randomised, double-blind	(20 – 56 y/o)	Not given	0.451 ± 0.127		
				12			Oil based capsule	1.459 ± 0.579
				12			Q-Gel	3.242 ± 0.926
(6)	100 mg	Single dose	Randomised, crossover	23 (20 - 43 y/o)	52	0.630 ± 0.165	Dry powder in hard gelatin capsule	@4hr 0.730 ± 0.164 (20 ± 35% increase)
						0.605 ± 0.121	Q in a complex micelle in an emulsion in soft gelatin capsule	@4hr 1.366 ± 0.361 (130 ± 61% increase)
(7)	180 mg	Single dose	Randomised, crossover	9 (23 – 56 y/o)	89	0.602 (mean)	Oxidised in liquid	1.192 ± 0.428 (C_{max})
							Reduced in capsule	1.47 ± 0.764 (C_{max})
							Q-Gel	1.192 ± 0.579 (C_{max})
							powder tablet	0.138 ± 0.057 (C_{max})
(8)	300 mg	1 week	Randomised, two-way crossover, 3-week washout	11 (30.9 ± 10.8 y/o)	55%	2.884 ± 1.089	Crystaline Q, hard gelatin capsule,	C_{max} 0.936 ± 0.645 After 1 week 8.687 ± 3.602 (Change 0.205)
						2.085 ± 1.008	powdered ω-3 fatty acids	
							Biotransformed reduced Q_{10} powder, powdered ω-3 fatty acids, hard gelatin capsule	C_{max} 2.118 ± 1.050 After 1 week 8.096 ± 3.602 (change 0.367)

Table 5.7 A summary of studies investigating the absorption and bioavailability of CoQ_{10} supplements (continued).

Publication	Dose	Duration	Study Design	Number of Subjects (age)	% Male	Baseline Q (μmol/L)	Supplement Type	Final Q
(35)	50 mg (single daily dose)	15 days	Randomised	(19 – 23 y/o) 10 10	100	 0.949 ± 0.37 1.123 ± 0.741	 Regular tablet Sustained release tablet	After 412 hours 1.204 ± 0.359 1.297 ± 0.822
(36)	0.05% Q in cream, once daily 50 mg + cream as above	2 months 2 months	Randomised	25 (35-45 y/o) 25 (35-45 y/o)	0	$Q_{10}H_2$ 0.72 ± 0.14 Q_{10} 0.27 ± 0.12 $Q_{10}H_2$ 0.73 ± 0.15 Q_{10} 0.24 ± 0.10	25 mg Q_{10} + 25 mg of d-RRR-α-tocopherol acetate + 25 μg selenium as selenium aspartate (2 x daily)	(After 60 days) $Q_{10}H_2$ 0.76 ± 0.13 Q_{10} 0.29 ± 0.08 $Q_{10}H_2$ 1.56 ± 0.24 Q_{10} 0.49 ± 0.16 (131% increase Q_{10} in sebum)
(29)	30 mg (plus other antioxidants)	6 months	Double-blind, placebo-controlled	220 (>60 y/o, median 63, range 60-90)	0	Median (interquartile range) Control 0.98 (0.55 – 1.62) Treatment 1.02 (0.54 – 1.81)	Soft gelatin capsules, soy oil and other antioxiants	After supplementation Median (interquartile range) Control 1.30 (0.99 – 2.46) Treatment 1.97 (1.06 – 3.50)
(28)	30 mg and 100 mg	2 months (single daily dose)	Randomised, double-blind, placebo-controlled	99 (20 - 80 y/o)	100	1.459 10 and 90% fractiles (0.949 & 2.119)	Soya oil Soft gelatin capsule	Median increase (μmol/L) 30mg => 0.637 100mg => 1.575 placebo => -0.266

5.5. Dose range for the coenzyme Q_{10} supplement Q-Gel

5.5.1. Aim

To investigate the dose range for the CoQ_{10} supplement Q-Gel, given at 60, 150, and 300 mg doses.

5.5.2. Study design

5.5.2.1. Subjects

Eight healthy adult male volunteers were enrolled. Participants were excluded if they reported taking any CoQ_{10}, vitamin supplements, or other medications within four weeks prior to the study. All participants were self-reportedly healthy, disease free throughout the study and all were non-smokers. The mean age of participants was 24 years (range 20 - 26 years), the mean height was 185 cm (range 178 - 192 cm), and the mean weight was 75 kg (range 63 - 86 kg).

5.5.2.2. Study protocol

Baseline blood samples were obtained after a ten hour overnight fast, and the Q-Gel supplement was administered as a single dose (based on the claimed content on supplement packaging) of 60, 150, and 300 mg, taken as two, five, and ten, 30 mg capsules. A dose of 300 mg taken as three 100 mg Q-Gel capsules was also given. Supplement doses were given in a randomised order (a different randomised order for each participant), with a week long washout period between trial days. A standardised breakfast was given after administration of the supplement, and a standardised lunch and afternoon tea were provided at set time points throughout the day. Blood samples were taken at 2 hourly intervals after administration of the supplement, for 10 hours. Participants were not permitted to leave the study centre at any time during each study day.

5.5.2.3. Specimen handling

Blood specimens were collected from the median cubital vein into glass vacuum tubes containing lithium heparin. Blood was immediately placed on ice, and tubes were centrifuged within 1 hour of collection at 1800g for 10 minutes at 4 °C. Plasma was immediately transferred to pre-labelled 5 mL serology tubes then stored at -80 °C until analysis, which was within 2 months of sample collection.

5.5.3. Results

Coenzyme Q_{10} supplementation with all doses was well tolerated with no reported side effects.

5.5.3.1. Coenzyme Q_{10} content of the diet

Participants were fed an identical, vegetarian diet on each study day to limit the effect of dietary CoQ_{10}. The diet of participants on study days consisted of: (breakfast) four weet-bix, milk and sugar, or four slices of toast, with margarine and jam, with a pottle of preserved fruit and a glass of fruit juice; (Lunch) one filled roll (white bread, lettuce, tomato, and cheese), an apple, and a muesli bar. This diet contributed approximately 533.2 µg of CoQ_{10} (Table 5.8).

Table 5.8 The approximate CoQ_{10} content of the diet given to participants in the dose range study on each study day.

Item	Approximate Weight (g)	CoQ_{10} Concentration (µg/g)	Total Contribution (µg)
Milk	290	0.1[a]	29
Toast	100	0[a]	0
Jam	10	n.k.	n.k.
Weet-bix	100	0	0
Margarine	9	n.k.	n.k.
Orange Juice	170	0.3[a]	51
Pottle fruit	140	1.3	182
White bread	60	0[a]	0
Lettuce	6	n.k.	n.k.
Tomato	24	0.9[a]	21.6
Cheese	40	1.3[a]	52.0
Muesli bar	31	0	0
Apple	152	1.3[a]	197.6
Total			**533.2 µg**

n.k. = not known; [a] from (27).

5.5.3.2. Supplement adherence

Both Q-Gel capsules used contained more than 100% of the claimed CoQ_{10} content. The 30 and 100 mg capsules (n = 6) each contained 44.5 ± 3.4 and 144.3 ± 6.0 mg CoQ_{10} (mean ± SD), respectively. Therefore, at a nominal dose of 300 mg, the actual amount ingested was 445 mg and 432 mg, respectively.

5.5.3.3. Demographic variables

Mean baseline lipids (± SD) for all participants were 4.17 ± 0.59, 2.64 ± 0.41, 1.32 ± 0.29, and 0.89 ± 0.40 mmol/L for total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides respectively. Mean baseline CoQ_{10} (± SD) was 0.66 ± 0.18 μmol/L. There was no significant change in baseline levels of CoQ_{10}, LDL-cholesterol, HDL-cholesterol, triglycerides, or total cholesterol during the four weeks of the trial, further confirming that the week-long wash-out period was sufficient for the CoQ_{10} level to return to baseline, and also that there were no significant dietary changes made by participants during the trial period.

5.5.3.4. Dose range for Q-Gel

The absolute change in CoQ_{10} for each participant and dose is shown in Table 5.9.

Table 5.9 The absolute change in CoQ_{10} at six hours for each participant and dose.

Subject	Change in CoQ at 6 hours (μmol/L)			
	60 mg dose (30 mg capsules)	150 mg dose (30 mg capsules)	300 mg dose (30 mg capsules)	300 mg dose (100 mg capsules)
1	0.39	1.99	1.79	0.65
2	0.18	0.96	0.69	0.98
3	0.28	0.39	0.57	0.46
4	0.65	0.97	2.14	0.54
5	0.64	0.85	1.58	0.78
6	0.26	0.89	0.32	0.19
7	0.34	0.77	1.22	1.17
8	0.17	1.28	1.11	0.37

The median increase in plasma CoQ_{10} concentration with time, after supplementation with four different doseage regimens of CoQ_{10}, is shown in Figure 5.2. The dose vs increase in CoQ_{10} concentration curve for Q-Gel given as 30 mg capsules is shown in Figure 5.3. There was a significantly higher median increase in CoQ_{10} from the 150 mg dose than the 60 mg dose ($p < 0.001$). The median CoQ_{10} increase was not significantly different between the 150 and 300 mg doses made up from 30 mg capsules ($p = 0.114$). The median absorption from the 300 mg doses via 30 or 100 mg capsules was significantly different ($p < 0.001$). The median increase in CoQ_{10} was significantly higher ($p = 0.016$) from the 150 mg dose (via 5 x 30 mg capsules) than from the 300 mg dose via 3 x 100 mg capsules. There appears to be no further increase of CoQ_{10} level from a single dose after approximately 200 mg (Figure 5.3).

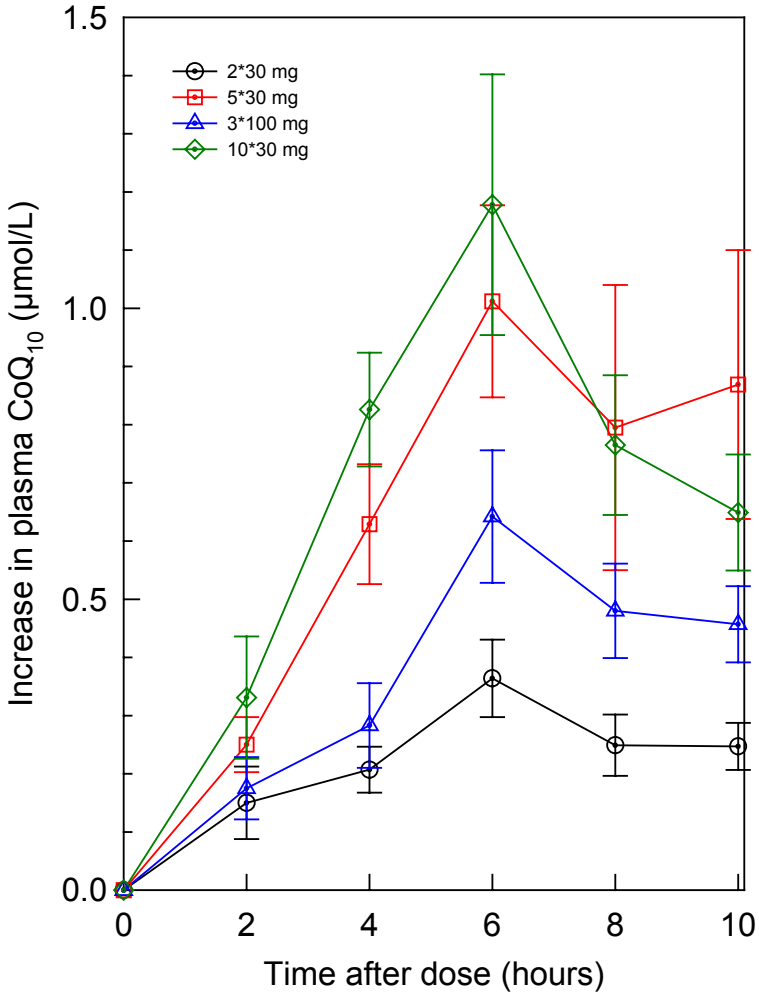


Figure 5.2 The median increase in plasma CoQ_{10} after oral supplementation with 60, 150, and 300 mg CoQ_{10} via 30 mg Q-Gel capsules, and with 300 mg via 100 mg Q-Gel capsules. Error bars indicate the standard error of the mean.

5.5.3.5. Time to t_{max}

The median time to t_{max} for all doses tested was six hours, which confirms the that the six hour time-point used in the bioavailabilty study was accurate, at least for the Q-Gel preparation.

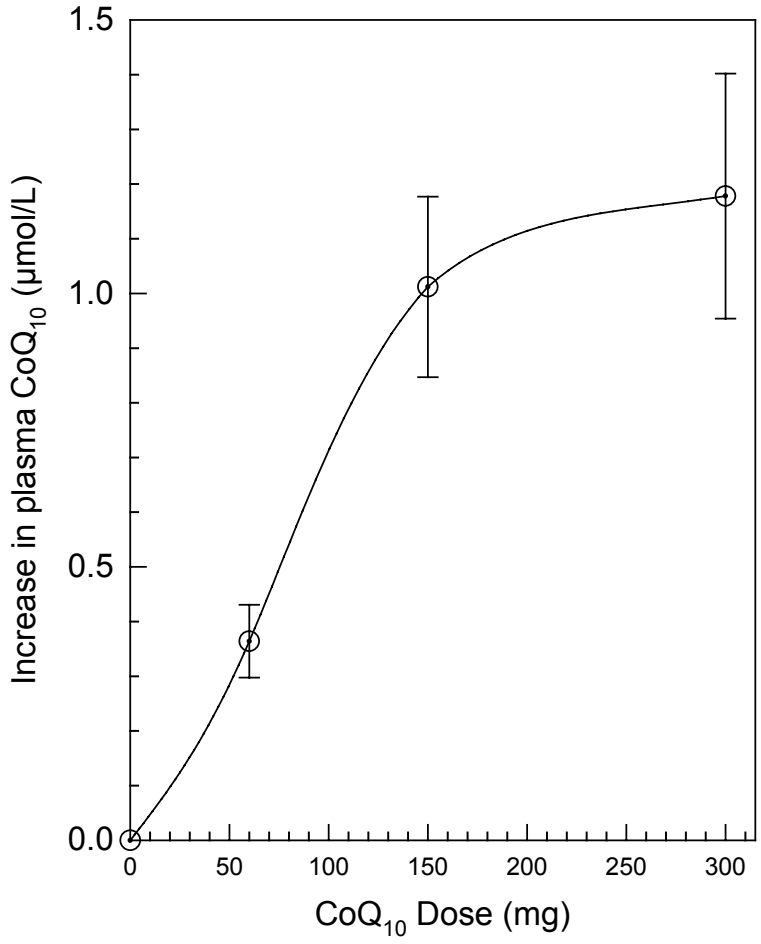


Figure 5.3 The dose range for the coenzyme Q_{10} supplement Q-Gel, administered as 30 mg capsules.

5.6. Coenzyme Q_{10} and statin therapy in patients with chronic heart failure

This study was carried out in collaboration with the Lipid and Diabetes Research Group of Christchurch Hospital.

5.6.1. Aim

To measure the effect of statin therapy on plasma total CoQ_{10} concentrations in patients with chronic heart failure, and to investigate the association of statin therapy, CoQ_{10}, and endothelial function.

5.6.2. Study design

5.6.2.1. Subjects

Twenty four patients were recruited from the Cardiology Department outpatient clinic at Christchurch Hospital. All had symptomatic heart failure (New York Heart Association Functional Class II or III), and reduced left ventricular ejection fraction (<40%) on echocardiography. Four patients had type 2 diabetes mellitus and 8 had hypertension. Patients were receiving standard anti-failure medication of a loop diuretic (n = 18), angiotensin converting enzyme inhibitor (n = 18) or angiotensin II receptor antagonist (n = 4), with or without digoxin (n = 2), a beta-adrenergic blocker (n = 13), and spironolactone (n = 4). It was a requirement that the dosage of anti-failure treatment had not changed for at least three months prior to enrolment, and throughout the study period. Fourteen patients used aspirin throughout the study period.

5.6.2.2. Study protocol

Patients received 40 mg of Atorvastatin or placebo once daily in the evening for 6 weeks in a randomised, placebo-controlled, double blind, crossover design. Placebo was lactose, formulated (by Christchurch Hospital Pharmacy) to visually appear identical to the Atorvastatin. A 2-week wash-out period was used between treatments. Venous blood samples (lithium heparin) were collected at baseline, and after each treatment arm (statin and placebo), for the measurement of plasma lipoproteins and total CoQ_{10}.

5.6.2.3. Measurement of resistance vessel function

Endothelium-dependent and endothelial-independent resistance vessel function was assessed by venous occlusion plethysmography as described by Watts (37) (Hokanson, Bellevue, WA, USA) and discussed in Strey *et al.*, (2005) (38). Area under the curve (AUC) for the forearm blood flow curve was calculated as the first 3 valleys (pulses) after the cuff artefact. An AUC ratio of the infused arm to the non-infused arm was calculated.

5.6.2.4. Measurement of CoQ_{10}

Total plasma CoQ_{10} was measured in lithium heparinised plasma by reversed-phase HPLC with ultraviolet detection, as described in Chapter 3, Section 3.5.1.

5.6.3. Results

Six weeks of Atorvastatin therapy was well tolerated. One patient with longstanding, slowly progressive myotonic dystrophy developed myalgia, but these symptoms were tolerable and the participant chose to complete the study. One participant was withdrawn from the study after the baseline visit due to worsening heart failure. Atorvastatin treatment was associated with reductions in LDL-cholesterol (50%), triglycerides (26%) and plasma CoQ_{10} levels (33%) (Table 5.10). HDL-cholesterol concentrations remained unchanged.

The percentage reduction in CoQ_{10} was associated with improvement in acetylcholine induced, endothelium-dependent vasodilation ($r = + 0.501$, $p = 0.034$) (Figure 5.4). This association did not exist during co-infusion with the nitric oxide antagonist N^G-monomethyl l-arginine (l-NMMA) ($p = 0.598$), or during sodium nitroprusside infusion ($p = 0.123$). Although there was a significant correlation between statin-induced reductions in CoQ_{10} and LDL-cholesterol ($p = 0.017$), multivariate analysis adjusting for reductions in LDL-cholesterol showed that CoQ_{10} remained the significant variable predicting change in endothelial function ($p = 0.041$).

Table 5.10 Effect of 6-week statin therapy on lipoprotein profiles and plasma CoQ_{10} levels.

	Baseline	Placebo	Statin Treatment	% Reduction	p-value
Total Cholesterol (mmol/L)	5.25 ± 0.20 (3.90 – 7.20)	5.50 ± 0.21 (3.90 - 8.00)	3.47 ± 0.15 (2.30 - 5.40)	36.6 ± 1.8 (15.4 – 50.0)	<0.001
LDL-Cholesterol (mmol/L)	3.32 ± 0.16 (2.04 - 4.84)	3.56 ± 0.16 (2.19 - 5.24)	1.77 ± 0.11 (0.73 – 3.02)	50.4 ± 2.1 (26.6 - 66.8)	<0.001
Triglycerides (mmol/L)	1.60 ± 0.13 (0.80 - 3.00)	1.70 ± 0.20 (0.60 - 4.70)	1.15 ± 0.09 (0.60 - 2.50)	26.3 ± 3.6 (0.0 - 57.5)	<0.001
HDL-Cholesterol (mmol/L)	1.22 ± 0.08 (0.67 - 2.16)	1.17 ± 0.07 (0.66 - 2.04)	1.18 ± 0.07 (0.67 - 2.02)	-2.1 ± 2.5 (-24.6 - 30.2)	0.55
Plasma CoQ_{10} (µmol/L)	1.19 ± 0.09 (0.59 - 2.08)	1.13 ± 0.10 (0.56 - 2.27)	0.74 ± 0.06 (0.42 - 1.63)	33.1 ± 2.6 (12.5 – 52.4)	<0.001

Data are mean ± SEM for n = 24 patients with ranges in brackets, p-value = comparison between placebo and treatment.

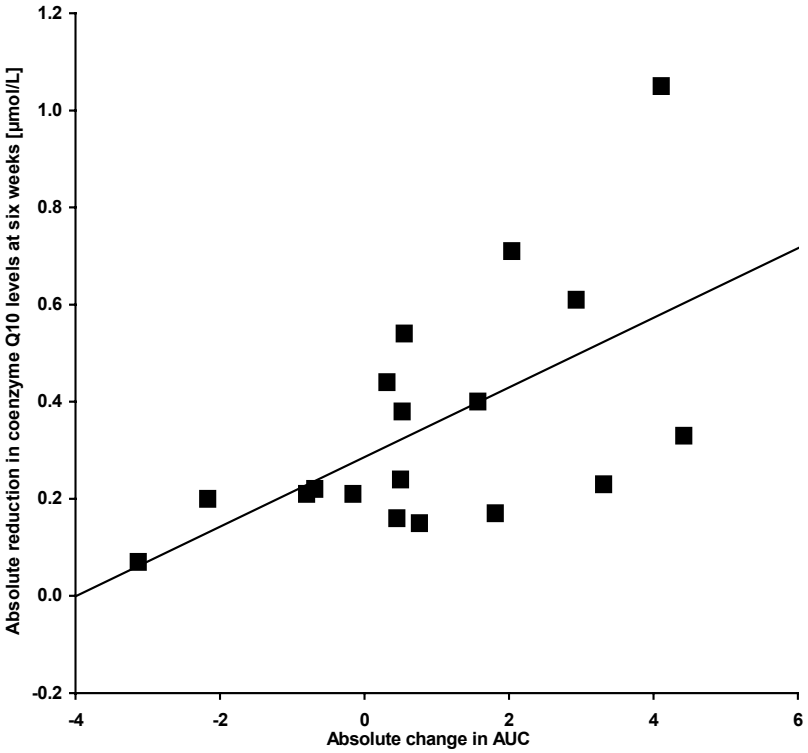


Figure 5.4 Correlation between absolute reduction in plasma CoQ_{10} and improvement in AUC ratio during intra-arterial acetylcholine infusion at 30 µg/min following statin treatment.

5.7. Discussion

Where supplementation with CoQ_{10} is warranted, the significant difference in absorption between participants highlights the need for monitoring CoQ_{10} concentrations. Results presented in this chapter also show that not all supplements have equal absorption at 6 hours. The Q-Gel brand showed better absorption at six hours than other brands tested, so will be the best brand to use in future clinical trials.

It was found that supplements do not all contain the exact nominated dose. The majority of the supplement brands investigated, with the exception of Good Health, contained more CoQ_{10} than stated. This is probably done on purpose by manufacturers to ensure consumers cannot complain for getting less product than they paid for. This additional CoQ_{10} in capsules was not corrected for in our results, because it is minor additional CoQ_{10}, and the absorption of CoQ_{10} is so low that this difference was assumed to make little difference to

the overall results. Additionally, the majority of supplement brands contained 20% more CoQ_{10} than nominated, so were relatively consistent anyway.

Absorption of CoQ_{10} during supplementation correlates significantly with plasma cholesterol, and statins decrease plasma cholesterol. Therefore, patients on statin therapy may show reduced absorption of CoQ_{10} from exogenous sources, due to the low concentration of circulating cholesterol. This needs further investigation.

The dose range results suggest that the maximum single dose of CoQ_{10} (as the supplement brand Q-Gel) that should be administered for effective supplementation is approximately 200 mg. A significantly better absorption is achieved when a 300 mg dose is administered as 10 x 30 mg capsules, as opposed to 3 x 100 mg capsules. The increased absorption from the 30 mg capsules may be due to a lower ratio of CoQ_{10} to oil in these capsules. Additionally, it has been suggested that co-supplementation with vitamin E may decrease absorption of CoQ_{10} (39, 40), and the vitamin E content of the 300 mg dose via 10 x 30 mg capsules was 60 international units, whereas that of the 300 mg dose via 3 x 100 mg capsules was 450 international units.

The results presented in this Chapter show the response to a single dose of CoQ_{10}. Further studies are required to confirm the plasma response to repeated CoQ_{10} dosing over time. It is probable that plasma levels rise over time, possibly until a plateau is reached. If plasma levels plateau with supplementation, it is possible that lower levels of supplementation may maintain levels at the plateau value, once it has been reached. Most people who are supplemented with CoQ_{10} will be supplemented with repeated dosing, which makes this information important.

The 33% decrease in plasma CoQ_{10} due to statin therapy is consistent with that previously reported (10-15). Changes in plasma CoQ_{10} levels correlated with changes in LDL-cholesterol levels, as has been previously reported (13). Other statin trials have been unable to demonstrate any significant association between reductions in CoQ_{10} and LDL-cholesterol concentrations (11). In patients with type 2 diabetes, there is emerging evidence that supplementation with CoQ_{10} may improve endothelial function in conduit vessels (41) and in resistance vessels in combination with fenofibrate (42). In a recently published study, CoQ_{10} or Cerivastatin therapy alone, and the combination of both, improved

endothelial function in conduit vessels of dyslipidemic males with poor endothelial function. Interestingly, the combination did not result in additional benefits for endothelium-dependent vasodilation when compared to each treatment alone (43). Our results show that CoQ_{10} reductions did not eliminate the beneficial effects of statin therapy on endothelium-dependent vasodilation. It remains to be tested whether CoQ_{10} supplementation in statin treated heart failure patients would translate into additional clinical benefits. In our study, participants with the greatest reduction in CoQ_{10} had the most pronounced improvement in endothelium-dependent vasodilation after statin therapy. It is possible that statin-induced reduction of CoQ_{10} levels may limit the maximum favourable effects of Atorvastatin on the microcirculation.

In addition, if our findings that improvements in endothelial function correlate well with CoQ_{10} change can be confirmed, CoQ_{10} measurement could potentially serve as a biochemical marker for statin pleiotropism.

5.8. References for Chapter 5

1. Langsjoen PH, Folkers K, Lyson K, Muratsu K, Lyson T, P.H. L. Effective and safe therapy with coenzyme Q10 for cardiomyopathy. Klinische Wochenschrift 1988;66:583-90.
2. Langsjoen PH, Langsjoen P, Folkers K. Long-term efficacy and safety of coenzyme Q10 therapy for idiopathic dilated cardiomyopathy. American Journal of Cardiology 1989;65:521-3.
3. Weis M, Mortensen SA, Rassing MR, Møller-Sonnergaard J, Poulsen G, Rasmussen SN. Bioavailability of four oral coenzyme Q_{10} formulations in healthy volunteers. Molecular Aspects of Medicine 1994;15:s273-s80.
4. Kaikkonen J, Nyyssönen K, Porkkala-Sarataho E, Poulsen HE, Metsä-Ketelä T, Hayn M, et al. Effect of oral coenzyme Q10 supplementation on the oxidation resistance of human VLDL + LDL fraction: Absorption and antioxidative properties of oil and granule-based preparations. Free Radical Biology and Medicine 1997;22:1195-202.
5. Chopra RK, Goldman R, Sinatra ST, Bhagavan HN. Relative bioavailability of coenzyme Q10 formulations in Human subjects. International Journal of Vitamin and Nutrition Research 1998;68:109-13.
6. Wahlqvist ML, Wattanapenpaiboon N, Savige GS, Kannar D. Bioavailability of two different formulations of coenzyme Q_{10} in healthy subjects. Asia Pacific Journal of Clinical Nutrition 1998;7:37-40.
7. Miles MV, Horn P, Miles L, Tang P, Steele P, DeGrauw T. Bioequivalence of coenzyme Q10 from over-the-counter supplements. Nutrition Research 2002;22:919-29.
8. Kurowska EM, Dresser G, Deutsch L, Bassoo E, Freeman DJ. Relative bioavailability and antioxidant potential of two coenzyme Q_{10} preparations. Annals of Nutrition and Metabolism 2003;47:16-21.
9. Lass A, Forster MJ, Sohal RS. Effects of coenzyme Q_{10} and α-tocopherol administration on their tissue levels in the mouse: Elevation of mitochondrial α-tocopherol by coenzyme Q_{10}. Free Radical Biology and Medicine 1999;26:1375-82.

10. Folkers K, Langsjoen P, Willis R, Richardson P, Xia L-J, Ye C-Q, Tamagawa H. Lovastatin decreases coenzyme Q levels in humans. Proceedings of the National Academy of Sciences of the United States of America 1990;87:8931-4.
11. Watts GF, Castelluccio C, Rice-Evans C, Taub NA, Baum H, Quinn PJ. Plasma coenzyme Q (ubiquinone) concentrations in patients treated with Simvastatin. Journal of Clinical Pathology 1993;46:1055-7.
12. Ghirlanda G, Oradei A, Manto A, Lippa S, Ucciolo L, Caputo S, et al. Evidence of plasma CoQ10-lowering effect by HMG-CoA reductase inhibitors: A double-blind, placebo-controlled study. The Journal of CLinical Pharmacology 1993;33:226-9.
13. Laaksonen R, Ojala J-P, Tikkanen MJ, Himberg J-J. Serum ubiquinone concentrations after short- and long-term treatment with HMG-CoA reductase inhibitors. European Journal of Clinical Pharmacology 1994;46:313-7.
14. De Pinieux G, Chariot P, Ammi-saïd M, Louarn F, Lejonc JL, Astier A, et al. Lipid-lowering drugs and mitochondrial function: effects of HMG-CoA reductase inhibitors on serum ubiquinone and blood lactate/pyruvate ratio. British Journal of Clinical Pharmacology 1996;42:333-7.
15. Mortensen SA, Leth A, Agner E, Rohde M. Dose-related decrease of serum coenzyme Q10 during treatment with HMG-CoA reductase inhibitors. Molecular Aspects of Medicine 1997;18(s):s137-s44.
16. Vita JA, Yeung AC, Winniford M, Hodgson JM, Treasure CB, Klein JL, et al. Effect of cholesterol-lowering therapy on coronary endothelial vasomotor function in patients with coronary artery disease. Circulation 2000;102:846-51.
17. Egashira K, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Inou T, Takeshita A. Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. Circulation 1994;89:2519-24.
18. Vogel RA, Corretti MC, Plotnick GD. Changes in flow-mediated brachial artery vasoactivity with lowering of desirable cholesterol levels in healthy middle-aged men. American Journal of Cardiology 1996;77:37-49.
19. Maguire SM, Nugent AG, McGurk C, Johnston GD, Nicholls DP. Abnormal vascular responses in human chronic cardiac failure are both endothelium dependent and endothelium independent. Heart 1998;80:141-5.

20. Katz S, Schwarz M, Yuen J, LeJemtel T. Impaired acetylcholine-mediated vasodilation in patients with congestive heart failure. Role of endothelium-derived vasodilating and vasoconstricting factors. Circulation 1993;88:55-61.
21. Carville C, Adnot S, Sediame S, Benacerraf S, Castaigne A, Calvo F, et al. Relation between impairment in nitric oxide pathway and clinical status in patients with congestive heart failure. Journal of Cardiovascular Pharmacology 1998;32:562-70.
22. Drexler H. Endothelium as a therapeutic target in heart failure. Circulation 1998;98:2652-5.
23. Kjekshus J, Pedersen TR, Olsson AG, Faergeman O, Pyorala K. The effects of simvastatin on the incidence of heart failure in patients with coronary heart disease. Journal of Cardiac Failure 1997;3:249-54.
24. Ullmann U, Metzner J, Schulz C, Perkins J, Leuenberger B. A new coenzyme Q10 tablet-grade formulation (all-Q®) is bioequivalent to Q-Gel® and both have better bioavailability properties than Q-SorB®. Journal of Medicinal Food 2005;8:397-9.
25. Tomono Y, Hasegawa J, Seki T, Motegi K, Morista N. Pharmacokinetic study of deuterium-labelled CoQ in man. International Journal of Clinical Pharmacology, Therapy and Toxicology 1986;24:536-41.
26. Lücker PW, Wetzelberger N, Hennings G, Rehn D. Biomedical and Clinical aspects of Coenzyme Q10, K Flokers and Y Yamamura (eds) Elsevier Science, Amsterdam 1984;Chapter 3:67-77.
27. Mattila P, Kumpulainen J. Coenzymes Q_9 and Q_{10}: contents in foods and dietary intake. Journal of Food Composition and Analysis 2001;14:409-17.
28. Zita C, Overvad K, Mortensen SA, Sindberg CD, Moesgaard S, Hunter DA. Serum coenzyme Q_{10} concentrations in healthy men supplemented with 30 mg or 100 mg coenzyme Q_{10} for two months in a randomised controlled study. BioFactors 2003;18:185-93.
29. Wolters M, Hahn A. Plasma ubiquinone status and response to six-month supplementation combined with multivitamins in healthy elderly women - results of a randomized, double blind, placebo-controlled study. International Journal of Vitamin and Nutrition Research 2003;73:207-14.
30. Kishi H, Kanamori N, Nishii S, Hiraoka E, Okamoto T, Kishi T. In: Biomedical and Clinical Aspects of Coenzyme Q, K Folkers and Y Yamamura, (eds) Elsevier Science, Amsterdam 1984;Chapter 4:131-42.

31. Ozawa Y, Mizushima Y, Koyama I, Akimato M, Yamagata Y, Hayashi H, Murayama H. Intestinal absorption enhancement of CoQ with a lipid microsphere. Arzneim Forsch Drug Res 1986;36:4689-90.
32. Pozzi F, Longo A, Lazzarini C, Carenzi A. Formulations of ubiquinone with improved bioavailability. European Journal of Pharmaceutics and Biopharmaceutics 1991;37:243-6.
33. Gibaldi M. Biopharmaceutics and clinical pharmacokinetics 3rd edn Lea & Febiger, Philadelphia, PA, USA, 1984.
34. Mohr D, Bowry VW, Stocker R. Dietary supplementation with coenzyme Q_{10} results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation. Biochimica et Biophysica Acta 1992;1126:247-54.
35. Lu W-L, Zhang Q, Lee H-S, Zhou T-Y, Sun H-D, Zhang D-W, et al. Total Coenzyme Q10 concentrations in Asian men following multiple oral 50-mg doses administered as coenzyme Q10 sustained release tablets or regular tablets. Biological and Pharmaceutical Bulletin 2003;26:52-5.
36. Passi S, De Pità O, Grandinetti M, Simotti C, Littarru GP. The combined use of oral and topical lipophilic antioxidants increases their levels both in sebum and stratum corneum. BioFactors 2003;18:289-97.
37. Watts G, O'Brien SF, Silvester W, Millar JA. Impaired endothelium-dependent and independent dilatation of forearm resistance arteries in men with diet-treated non-insulin-dependent diabetes: role of dyslipidaemia. Clinical Science 1996;91:567-73.
38. Strey CH, Young JM, Molyneux SL, George PM, Florkowski CM, Scott RS, Frampton CM. Endothelium-ameliorating effects of statin therapy and coenzyme Q_{10} reductions in chronic heart failure. Atherosclerosis 2005;179:201-6.
39. Kaikkonen J, Tuomainen TP, Nyyssönen K, Salonen JT. Coenzyme Q10: Absorption, antioxidative properties, determinants, and plasma levels. Free Radical Research 2002;36:389-97.
40. Kaikkonen, Nyyssönen, Tomasi, Iannone, Tuomainen, Porkkalla-Sarataho, Salonen. Antioxidant efficacy of parallel and combined supplementation with coenzyme Q10 and D-a-tocopherol in mildly hypercholesterolemic subjects: a randomised placebo-controlled clinical study. Free Radical Research 2000;33.

41. Watts GF, Playford DA, Croft KD, Ward NC, Mori TA, Burke V. Coenzyme Q10 improves endothelial dysfunction of the brachial artery in Type II diabetes mellitus. Diabetologia 2002;45:420-6.

42. Playford DA, Watts GF, Croft KD, Burke V. Combined effect of coenzyme Q10 and fenofibrate on forearm microcirculatory function in type 2 diabetes. Atherosclerosis 2003;168:169-79.

43. Kuettner A, Pieper A, Koch J, Enzmann F, Schroeder S. Influence of coenzyme Q_{10} and cerivastatin on the flow-mediated vasodilation of the brachial artery: results of the ENDOTACT study. International Journal of Cardiology 2005;98:413-9.

Chapter 6

Coenzyme Q_9 – Reference Interval, Biological Variation, and Change with Coenzyme Q_{10} supplementation

6.1. Introduction

Coenzyme Q_9, a homologue of CoQ_{10}, is a benzoquinone unit with nine isoprenoid units. As discussed in Chapter 1, CoQ_9 is the predominant CoQ homologue found in rodents (mice and rats), while most other mammals (including humans) have predominantly CoQ_{10}. There are conflicting reports as to whether CoQ_9 is present in humans. Human serum has been reported to contain (1-4), and not contain (5, 6) CoQ_9. Tang *et al.,* (2001) (7) reported the presence of CoQ_9 in only one individual, a patient with glycogen storage disease, Type I. They found no measurable CoQ_9 in 25 healthy individuals, and 24 other individuals with illness.

If CoQ_9 is present in human plasma in both the reduced and oxidised forms, as CoQ_{10} is, it is possible that the reason why Tang *et al.,* (2001) (7) did not detect CoQ_9 in human plasma is because their method quantifies both reduced and oxidised forms of CoQ_9 and CoQ_{10}, and the limit of detection may not be low enough to measure the low concentrations of endogenous CoQ_9 and CoQ_9H_2.

The presence of CoQ_9 in human plasma has no known medical significance. It is possible that CoQ_9 originates as a metabolite of CoQ_{10} catabolism, as a product of incomplete CoQ_{10} biosynthesis (where the last isoprenoid unit is not added during the biosynthesis of CoQ_{10}), or from the diet.

It has been estimated that the daily dietary CoQ_9 intake is 0 – 1.3 μmol/day (8, 9), but this estimate is unreliable because of the large number of food items that contained CoQ_9 at levels below the detection limit (8). The main dietary sources of CoQ_9 are cereals and edible fats (Table 6.1) (8, 9). If 10% of the dietary intake of CoQ_9 is absorbed, the CoQ_9 plasma level would increase by approximately 50 nmol/L (9).

Table 6.1 The CoQ_9 concentration in various foods.

Food Item	CoQ_9 (nmol/g food) Weber *et al.,* (1997) (8)	CoQ_9 (nmol/g fresh weight) Mattila and Kumpulainen, (2001) (9)
Pork heart	4.9 (2.1 – 7.7)	3.9
Beef	3.3	0.5
Chicken	1.0	0.5
Pork chop	1.3	n.d.
Ham	0.4	1.1
Herring	b.l.	b.l.
Rainbow trout	b.l.	0.4
Salmon	b.l.	n.d.
Bread (rye)	5.9	5.9
Bread (wheat)	1.4	2.6
Rice	b.l.	n.d.
Broccoli	0.8 (0.7 – 0.9)	n.d.
Cauliflower	b.l.	0.1
Potato	b.l.	b.l.
Tomato	b.l.	b.l.
Carrot	b.l.	b.l.
Cucumber	0.1	n.d.
Orange	0.6	b.l.
Apple	b.l.	0.3
Kiwifruit	0.4	n.d.
Yoghurt	0.4	b.l.
Hard cheese	b.l.	b.l.
Cream cheese	b.l.	n.d.
Hens egg	0.5 (0.4 – 0.6)	b.l.
Reindeer	n.d.	10.7
Beef Heart	n.d.	b.l.
Beef liver	n.d.	1.8
Pork liver		1.5
Rapeseed oil	n.d.	b.l.
Tuna (canned)	n.d.	0.4
Crispbread, rye	n.d.	5.9
Pea		0.1
Bean	n.d.	0.1
Blackcurrant	n.d.	1.0
Ligonberry	n.d.	3.6
Strawberry		0.1
Clemintine		b.l.
Orange juice	n.d.	b.l.
Ligonberry juice	n.d.	0.1
Milk (1.5%fat)	n.d.	n.d.

n.d. = not determined, b.l. = below limit of detection.

There are published methods for measurement of CoQ_{10} in human plasma that use CoQ_9 as an internal standard (Table 6.2). The presence of endogenous CoQ_9 in human plasma would rule out this compound as an internal standard.

Table 6.2 Assays for CoQ_{10} in human plasma that use CoQ_9 as an internal standard.

Publication	Measuring	Sample Type	Internal Standard
(7)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	CoQ_9
(10)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	CoQ_7, CoQ_9, CoQ_9H_2
(5)	CoQ_{10}	Human plasma	CoQ_9
(11)	CoQ_{10} $CoQ_{10}H_2$	Human plasma	CoQ_9

This chapter describes work that defines the concentration of endogenous total CoQ_9 in human plasma, establishing a reference interval, biological variation, and the influence of CoQ_{10} supplementation on CoQ_9 concentrations. This is essential information to underpin investigations of the possible clinical significance of a change in the proportion of CoQ_9, and for estimating its quantitative effects on many assay methods for CoQ_{10}.

6.2. Coenzyme Q_9 measurement

The assay for CoQ_9 is as described for CoQ_{10} using electrochemical detection as described in Chapter 3, Section 3.6.2. The assay quantifies both total CoQ_9 and total CoQ_{10} simultaneously. The method gives 100 ± 2% recovery of CoQ_9 (7).

The standard curve for CoQ_9 in 1-propanol is linear from at least 0.5 to 250 nmol/L (Figure 6.1), r = + 0.998.

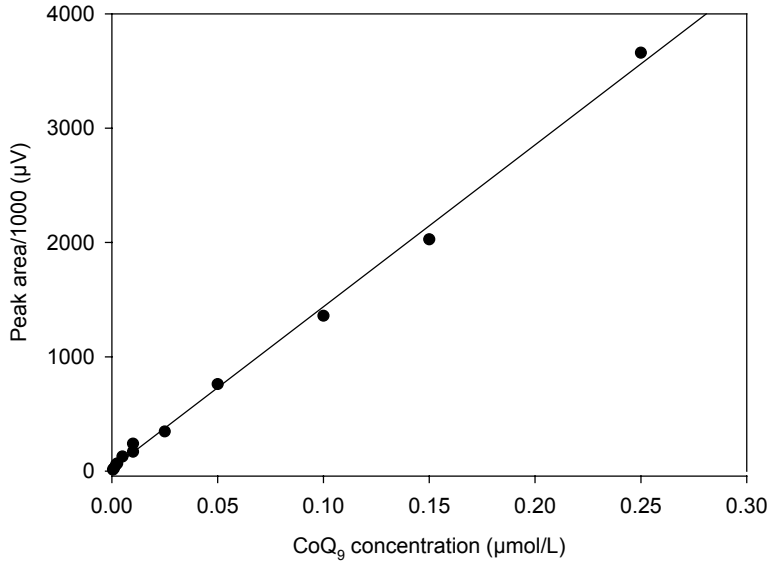


Figure 6.1 Standard curve for coenzyme Q_9 in 1-propanol.

A chromatogram of a CoQ_9 standard, an extract of rat plasma, an extract of human plasma, and an extract of a human plasma that has been spiked with CoQ_9, confirms that a peak for CoQ_9 is evident in human plasma (Figure 6.2).

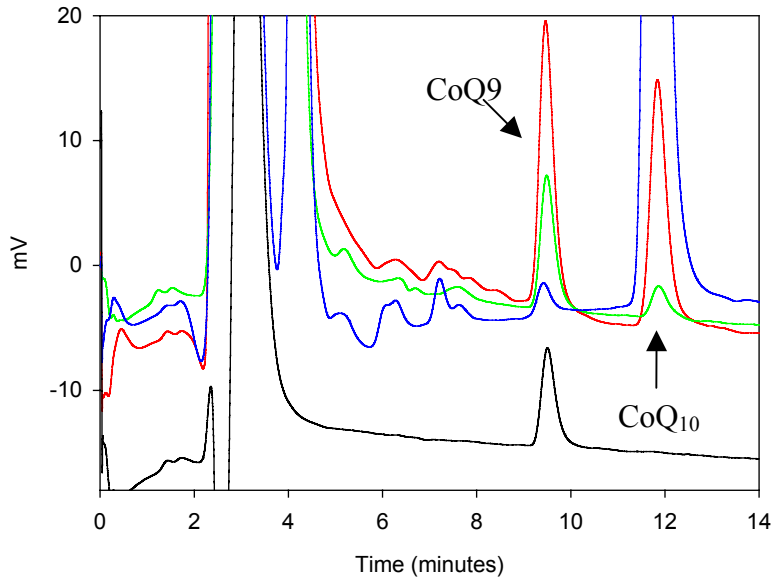


Figure 6.2 Chromatogram of a CoQ_9 standard (0.01 µmol/L) (black), a rat plasma extract in 1-propanol, 163 nmol/L (green), a human plasma extract in 1-propanol, 40.1 nmol/L (blue), and a 1-propanol extract of human plasma spiked with CoQ_9 (red).

Confirmation that the CoQ_9 peak is free of interference was obtained by running the same HPLC system as described, but replacing the analytical column with a Phenomenex Develcosil C30 column (250 × 4.6 mm, 5 µm) and slowing the flow rate to 0.8 mL/minute with the column at 22 ºC. CoQ_9 and CoQ_{10} were retained for longer on the C30 column (retained for 25.2 and 35.3 minutes respectively) than on the C18 column. The plasma and standard CoQ_9 and CoQ_{10} had identical retention times when the C30 column was used (Figure 6.3), confirming the identity of the CoQ_9 peak. Additionally, the CoQ_9 and CoQ_{10} concentrations calculated for 4 plasma samples using both the C18 and C30 columns were very similar (Table 6.3), further confirming that it is CoQ_9 that is being measured.

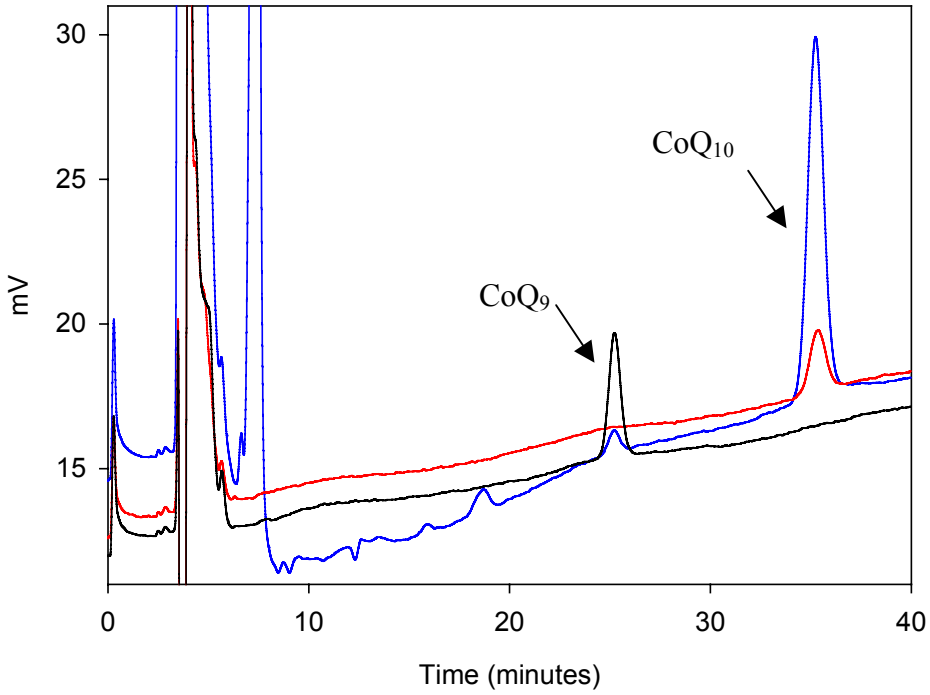


Figure 6.3 Chromatograms of a CoQ_9 standard (0.02 µmol/L) (black), a human plasma extract in 1-propanol, 50 nmol/L (blue), and a CoQ_{10} standard (0.02 µmol/L) (red), using a Phenomenex C30 analytical column

Table 6.3 The concentration of CoQ_9 in four random human plasma samples, as measured using a Phenomenex Luna C18(2) column, and a Phenomenex Develcosil C30 column.

Phenomenex Luna C18(2) Column (CoQ_9 in nmol/L)	Phenomenex Develcosil C30 Column (CoQ_9 in nmol/L)
11.5	7.6
46.7	50.0
14.4	16.4
18.2	18.2

Further confirmation of the identity of the CoQ_9 peak was achieved by spiking a plasma sample with standard CoQ_9, and ensuring that a single peak was obtained from the spiked plasma sample (Figure 6.4) when run on the C30 column.

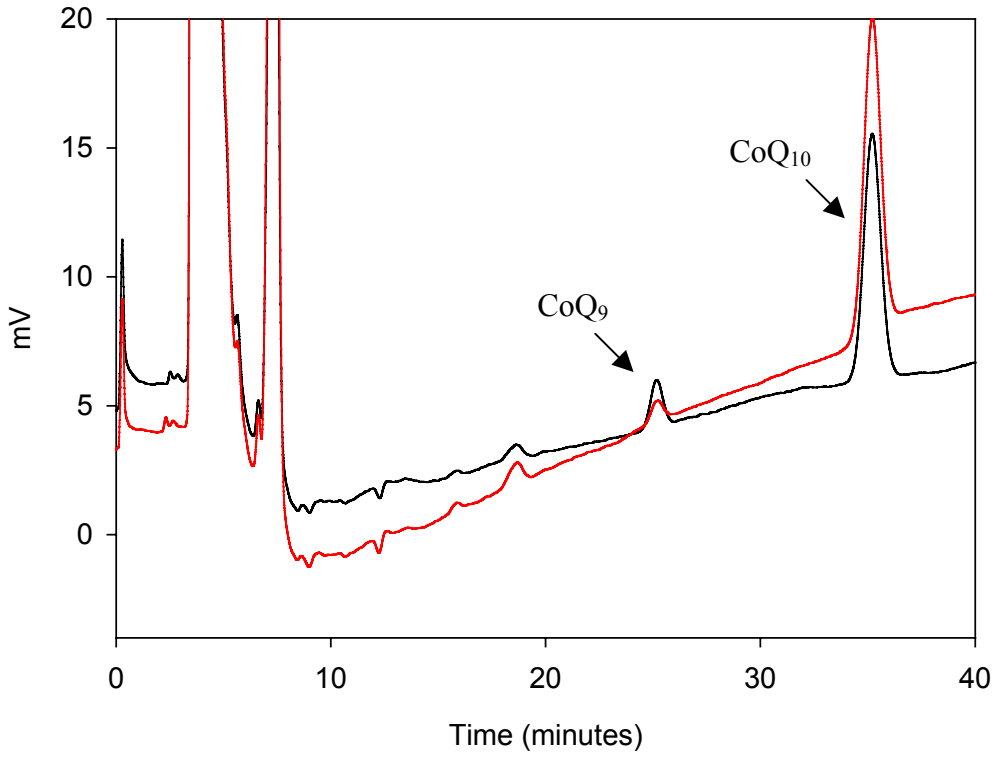


Figure 6.4 A plasma sample spiked with CoQ_9 (black), and the same sample unspiked (red). Endogenous CoQ_9 concentration was 50 nmol/L, and the total CoQ_9 in the spiked sample was 70 nmol/L.

A chromatogram showing reduced and oxidised CoQ_{10} (no oxidation or reduction of the extract before the analytical column) in human plasma, as is used in the method of Tang *et al.,* (2001) (7), reveals a very small peak for CoQ_9 (Figure 6.5). This is approximately the size of the CoQ_9 peak observed in the method of Tang *et al.,* (2001) (7). Of concern, however, is that when using CoQ_9 as an internal standard for measurement of CoQ_{10}, the concentration of CoQ_9 in different individuals will not be identical. This would lead to error in the CoQ_9 concentration, which would transfer to error in the calculated CoQ_{10} concentration when using CoQ_9 as an internal standard. For example, Tang *et al.,* (2001) (7) added 1260 nmol/L CoQ_9 to each plasma sample, which would lead to a 1 to 7% (depending on the endogenous CoQ_9 concentration) underestimation of the actual CoQ_{10} concentration when the peak height or area of the internal standard is used to calculate CoQ_{10} concentration.

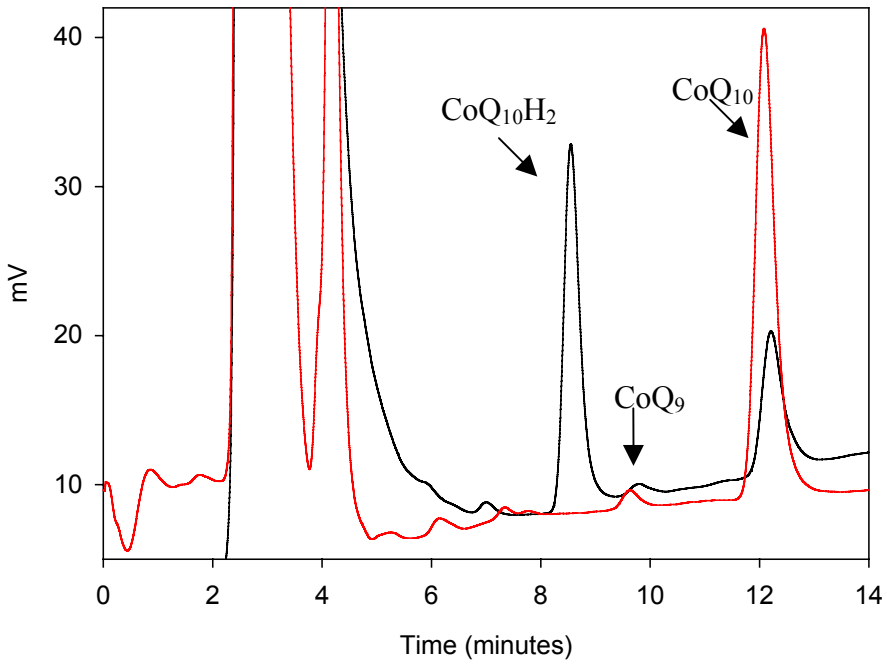


Figure 6.5 Chromatograph showing reduced and oxidised CoQ_{10} in a human plasma sample (black), and the same sample as total CoQ_{10} (red). The concentration of total CoQ_9 and total CoQ_{10} in this sample is 19 nmol/L, and 0.60 µmol/L, respectively.

6.3. Reference interval for Coenzyme Q_9

The endogenous plasma total CoQ_9 concentration was determined in 205 healthy New Zealanders, as described in Chapter 4, Sections 4.3.2 and 4.3.3.

6.3.1. Results

6.3.1.1. Reference interval for CoQ_9 in complete cohort, and in males and females separately.

The distribution of plasma CoQ_9 is skewed to the right (Figure 6.6), as found for CoQ_{10}. Therefore, non-parametric statistics were used to determine the reference intervals. The reference interval (2.5 and 97.5 interfractile intervals) for the complete population is 8.8 – 47.0 nmol/L (n = 193), with the median CoQ_9 concentration being 20.7 nmol/L. There is a non-significant trend (p = 0.350) for males to have a higher CoQ_9 level than females, with the medians (reference interval) for CoQ_9 in males and females being 21.2 (8.3 – 71.8) nmol/L (n = 80) and 19.9 (8.8 – 43.5) nmol/L (n = 113), respectively.

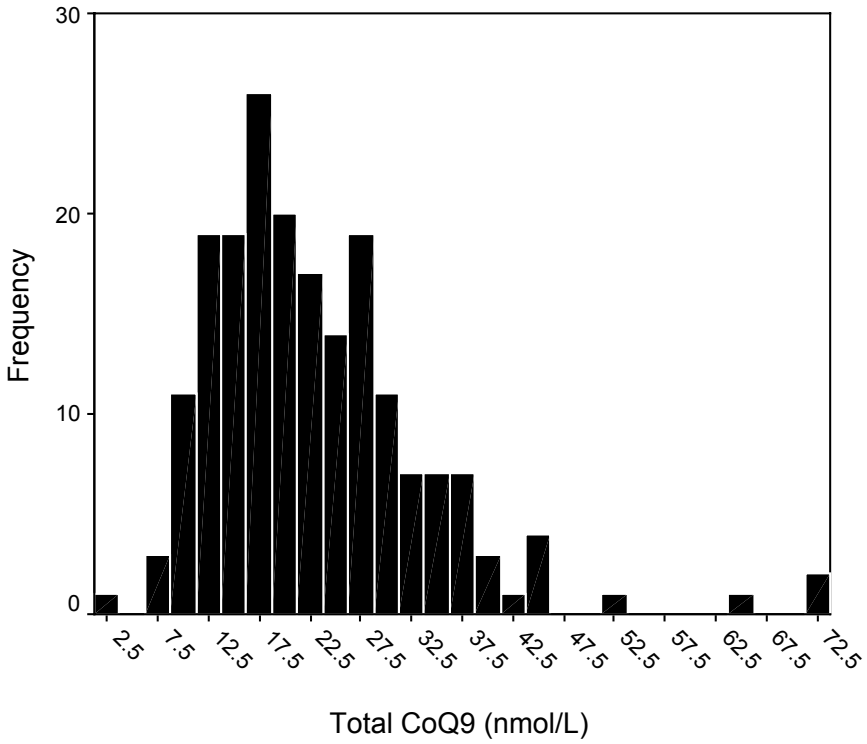


Figure 6.6 A histogram for CoQ_9 in the complete reference cohort (n = 193).

6.3.1.2. Correlation of CoQ_9 with CoQ_{10} and lipids.

Approximately 33% of the variation in CoQ_9 is explained by variation in CoQ_{10}, and the correlation between CoQ_9 and CoQ_{10} in the complete reference cohort (Figure 6.7) was

highly significant (r = + 0.577, p < 0.001). The median ratio of CoQ_9 to CoQ_{10} was 23.9 mmol/mol, with the absolute range of the ratio being 7.6 – 60.1 mmol/mol. The median total CoQ_9 to LDL–cholesterol ratio in the complete reference population was 689 nmol/mol, with the reference interval being 290 – 1880 nmol/mol. The median total CoQ_9 to total cholesterol ratio in the complete reference population was 385 nmol/mol, with the reference interval being 158 – 890 nmol/mol.

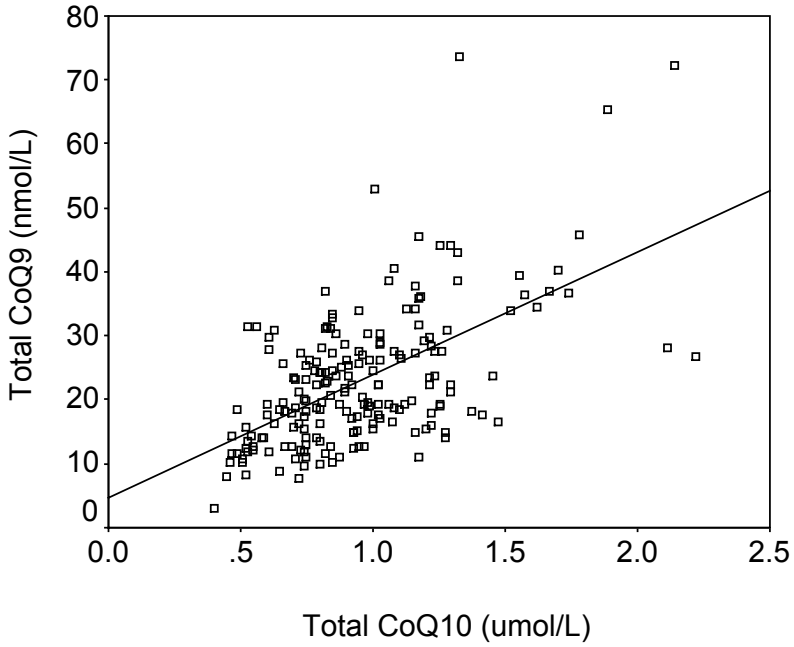


Figure 6.7 Correlation of CoQ_9 and CoQ_{10} in the complete reference cohort (n = 205).

The weak but highly significant correlation of CoQ_9 and CoQ_{10} supports the hypothesis that CoQ_9 is made during CoQ_{10} biosynthesis by an isoprenoid unit being left off. However, this correlation also supports the hypothesis that CoQ_9 is a metabolite of CoQ_{10} catabolism, since it is feasible that a person with high CoQ_{10} has more CoQ_{10} degradation, and hence increased levels of CoQ_9.

Plasma CoQ_9 concentrations correlated weakly, but significantly, with total cholesterol (r = + 0.316, p < 0.001) and direct LDL-cholesterol (r = + 0.245, p = 0.001).

6.4. Biological variation of Coenzyme Q_9

The biological variation of plasma total CoQ_9 was estimated by measuring CoQ_9 in the ten healthy male volunteers used to study the biological variation of CoQ_{10}, as discussed in Chapter 4, Sections 4.4.2, and 4.4.3.

6.4.1. Results

6.4.1.1. Demographic variables

The median ratios for total CoQ_9, the total CoQ_9 to LDL-cholesterol, and the total CoQ_9 to total cholesterol ratio (n = 70) are shown in Table 6.4.

Table 6.4 Lipid characteristics for the participants in the biological variation of CoQ_9 study (n = 70).

	Median (interquartile range)
Total CoQ_9 (nmol/L)	18.03 (7.65 – 31.51)
Total CoQ_9 to LDL-Cholesterol (nmol/mol)	6.76 (3.61 – 1.58)
Total CoQ_9 to Total Cholesterol (nmol/mol)	0.37 (0.23 – 0.74)

Observation of the change in CoQ_9 over the two month period in the 10 healthy males showed that 8 of the 10 participants had CoQ_9 levels that were tightly distributed around a homeostatic set-point, and 2 participants (5 and 6) had less tightly controlled plasma total CoQ_9 concentrations (Figure 6.8). The CoQ_{10} level correlated with the CoQ_9 level in the 10 volunteers (Figure 6.9). The ratios varied widely (Figure 6.10).

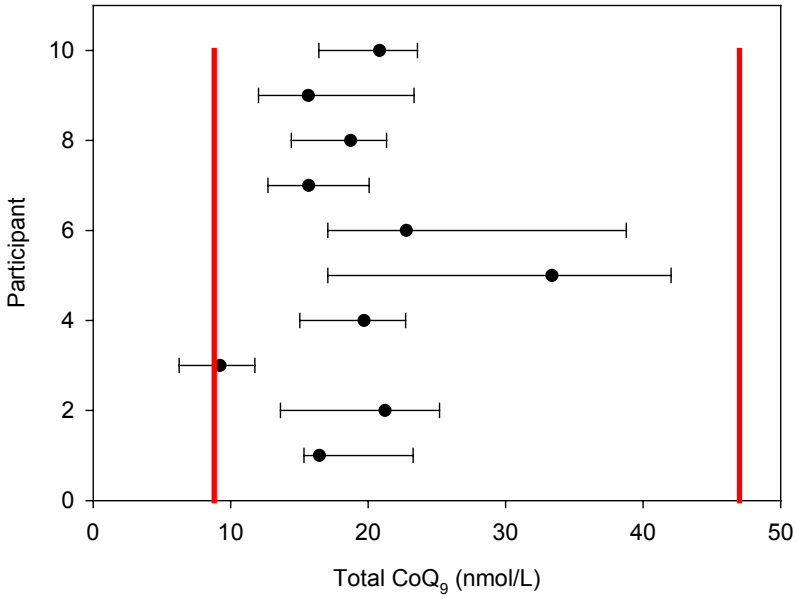


Figure 6.8 Median (·) and range (bars) of CoQ_9 concentrations of seven measurements taken over 2 months from 10 healthy males. Red vertical lines represent the reference interval determined for CoQ_9.

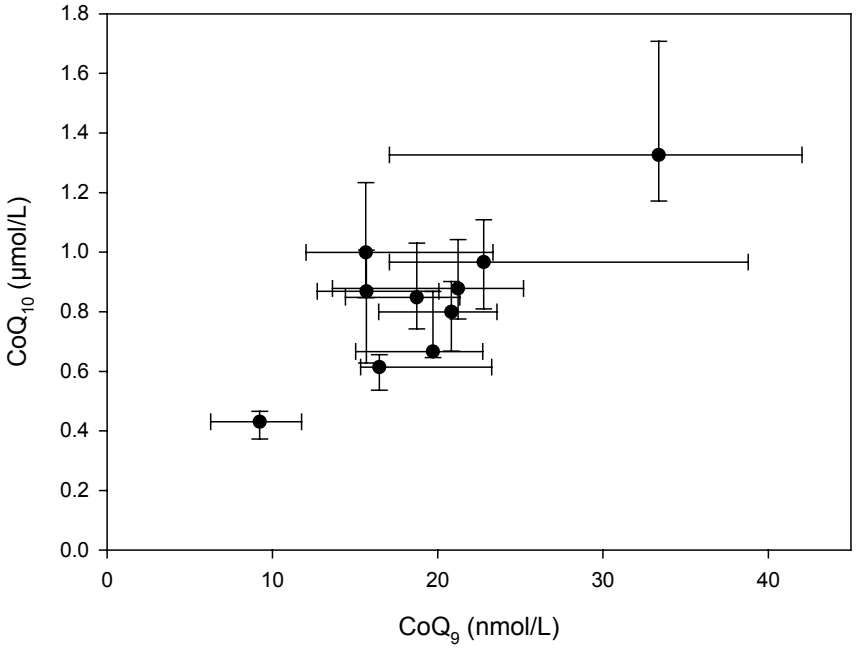


Figure 6.9 Biological variation (median (·) and absolute range (bars)) for CoQ_9 vs CoQ_{10} for each participant.

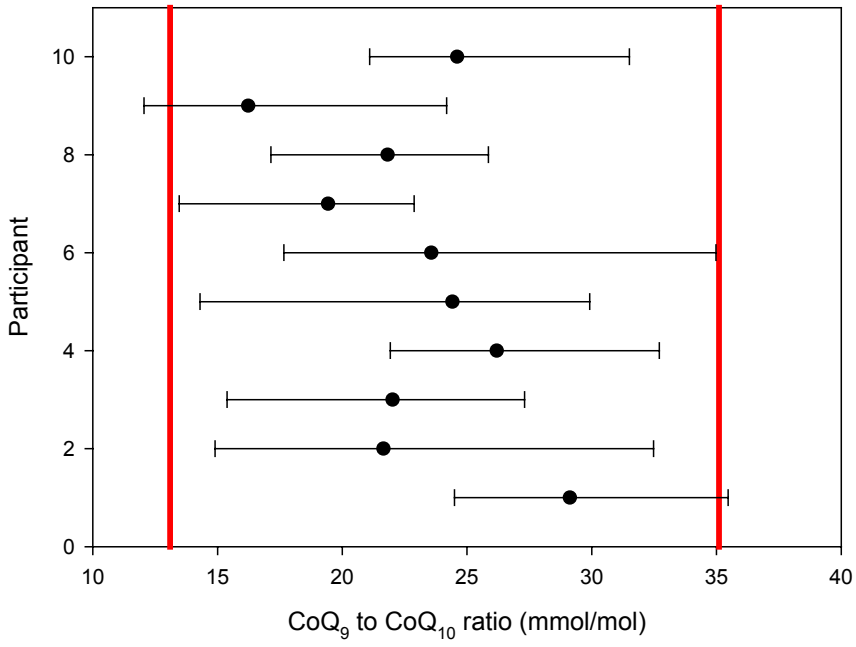


Figure 6.10 Biological variation (the median and range) for the ratio of CoQ_9 to CoQ_{10} for each participant. Red vertical lines indicate the reference interval, as determined in the healthy New Zealand population.

6.5. Effect of CoQ_{10} supplementation on CoQ_9

6.5.1. Study design

Ten healthy male volunteers were supplemented with 150 mg of various CoQ_{10} supplement brands, as outlined in Chapter 5, Section 5.4.

6.5.2. Results

6.5.2.1. Demographic variables

Mean baseline CoQ_9 (± SD) was 19.1 ± 6.8 nmol/L. There was a significant increase in CoQ_9 after supplementation with CoQ_{10} ($p < 0.001$). The median (2.5 – 97.5 percentiles) CoQ_9 concentration increased from 18.0 (7.7 – 39.5) nmol/L to 24.1 (11.7 – 42.1) nmol/L.

There was a weak but significant correlation ($r = + 0.380$, $p = 0.001$) between the change in CoQ_9 and the change in CoQ_{10} after CoQ_{10} supplementation (Figure 6.11).

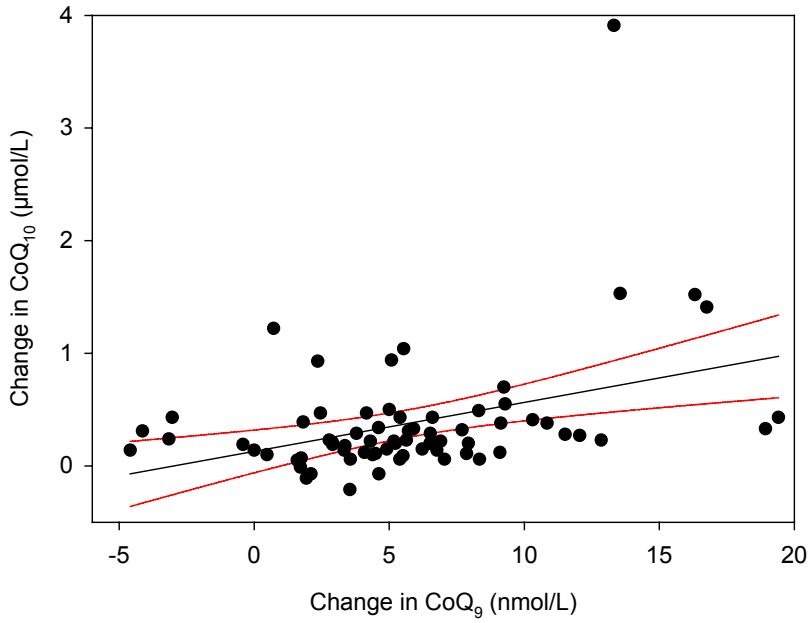


Figure 6.11 Correlation of the change in CoQ_{10} and CoQ_9 after supplementation with 150 mg CoQ_{10} (n = 70). Red lines indicate the 95% confidence intervals.

There was no significant change in the ratio of CoQ_9 to CoQ_{10} after CoQ_{10} supplementation ($p = 0.351$). However, there was much greater variation in the ratio after supplementation than before (Figure 6.12).

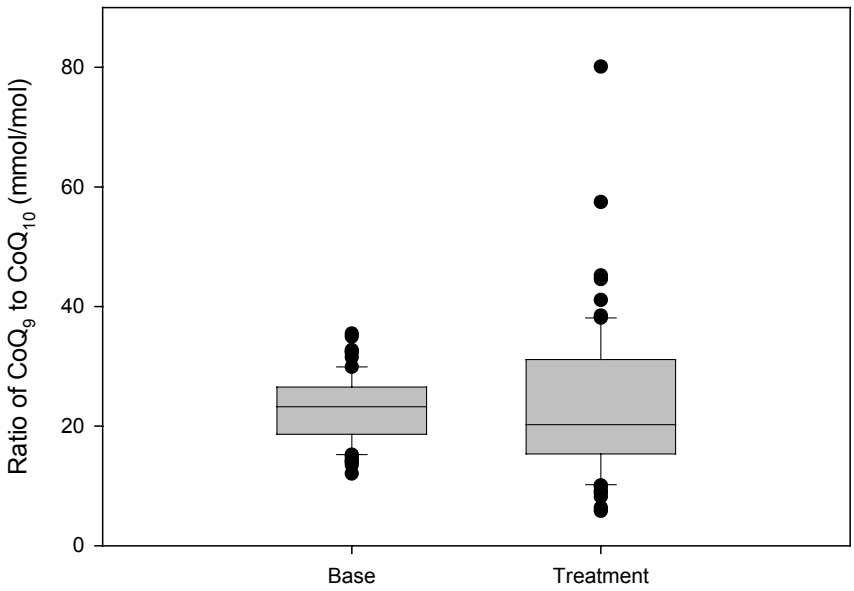


Figure 6.12 The ratio of CoQ_9 to CoQ_{10} at baseline and after supplementation with CoQ_{10} (n = 70).

Analysis of the formulation contents revealed the presence of CoQ_9 in all formulations, at an approximate concentration of 4 – 26 μg (5 - 32 nmol) per unit. It is not expected that this very low concentration of CoQ_9 would affect plasma concentrations substantially.

6.6. Discussion

The question of whether CoQ_9 is present in human plasma has been controversial. These studies have shown that CoQ_9 is present endogenously in humans, with the median CoQ_9 concentration in the reference population being 20.7 nmol/L. This value is lower than the mean CoQ_9 concentration of 46.4 ± 7.8 nmol/L reported in 18 healthy participants by Wakabayashi *et al.,* (1994) (2). It is also lower than the CoQ_9 concentration reportedly found in one patient with a rare glycogen storage disease (Type I) of 31 nmol/L by Tang *et al.,* (2001) (7). However, Tang *et al.,* (2001) (7) report that no detectable CoQ_9 was found in the 25 samples from healthy subjects and 24 samples from other subjects with various illnesses.

The concentration of CoQ_9 in the rat plasma sample was 163 nmol/L which compares with published values of 252 ± 12.6 nmol/L in the blood of 12 month old rats (n = 6) (12). The CoQ_{10} concentration in the rat plasma sample measured here was 46 nmol/L, which compares with the published value of 51.0 ± 4.6 in the blood of 12-month old rats (n = 6) (12).

It can be assumed that CoQ_9 circulates in both the reduced and the oxidised forms since the CoQ_9 peak was larger when all CoQ_9 was oxidised before detection than when the reduced and oxidised forms were separated on the analytical column.

The results presented here provide evidence that CoQ_9 is not an ideal internal standard for measurement of CoQ_{10}, and may result in 1 to 7% underestimation of CoQ_{10} concentrations as compared to methods not using CoQ_9 as an internal standard.

The increase in plasma total CoQ_9 after supplementation with CoQ_{10} agrees with animal data on the effect of CoQ_{10} supplementation on CoQ_9 concentrations in mice and rats. In rats supplemented with CoQ_{10}, an increase in plasma CoQ_9 has been reported (13), but in other studies no change was seen (14). An increase of CoQ_9 in rat tissues after CoQ_{10} supplementation has been reported for spleen (14), liver (14), and cerebral tissues (15). No

change in CoQ_9 concentrations in rat tissues after CoQ_{10} supplementation was reported for brain (14, 16), liver (13), kidney, (13, 14), and heart (14). A decrease in CoQ_9 after CoQ_{10} supplementation in rats has been reported in skeletal muscle (14).

In rat and mouse mitochondria, an increase in CoQ_9 after CoQ_{10} supplementation has been reported in heart muscle (16), skeletal muscle (13, 16), brain (16) and kidney (13). No change in CoQ_9 after CoQ_{10} supplementation was seen in rat liver mitochondria (13).

Whether the effect of CoQ_{10} supplementation on CoQ_9 levels in rats and mice is comparable to the effect in humans is questionable since CoQ_9 is the predominant CoQ homologue in rats and mice. In rats, plasma CoQ_9 is 5-fold higher than CoQ_{10}, tissue CoQ_9 is 3-11-fold higher than CoQ_{10}, and skeletal muscle mitochondrial CoQ_9 is 20-fold higher than CoQ_{10} (13). During CoQ_{10} supplementation of rats, the ratio of CoQ_9 to CoQ_{10} has been reported to remain unaltered in all tissues except skeletal muscle (16).

The increase in CoQ_9 during CoQ_{10} supplementation is not likely to arise from the low concentration of CoQ_9 in the supplements. It may be due to the *in vivo* modification of the isoprene moiety, and/or the antioxidative protection of mitochondrial CoQ_9 by endogenous CoQ (17). It is also possible that the various tissues possess the ability to trim off an isoprenoid unit. The possibility that exogenous CoQ_{10} stimulates the synthesis of endogenous CoQ_9 in rats has been ruled out by Dallner *et al.,* (2000) (18) and Bentinger *et al.,* (2003) (19). Additionally, it is possible that supplemental CoQ_{10} exchanges with tissue CoQ, thus displacing some CoQ_9 into the plasma.

6.7. References for Chapter 6

1. Lang JK, Packer L. Quantitative determination of vitamin E and oxidised and reduced coenzyme Q10 by HPLC with in-line ultraviolet and electrochemical detection. Journal of Chromatography 1987;385:109-17.
2. Wakabayashi H, Yamato S, Nakajima M, Shimada K. Simultaneous determination of oxidized and reduced Coenzyme Q and α-Tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. Biological and Pharmaceutical Bulletin 1994;17:997-1002.
3. Lang JK, Gohil K, Packer L. Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Analytical Biochemistry 1986;157:106-16.
4. Artuch R, Moreno J, Quintana M, Puig RM, Vilaseca MA. Serum ubiquinone-10 in a pediatric population. Clinical Chemistry 1998;44:2378-9.
5. Okamoto T, Fukui K, Nakamoto M, Kishi T, Okishio T, Yamagami T, et al. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. Journal of Chromatography 1985;342:35-46.
6. Zierz S, Jahns G, Jerusalem F. Coenzyme Q in serum and muscle of 5 patients with Kearns-Sayre syndrome and 12 patients with ophthalmoplegia plus. Journal of Neurology 1989;236:97-101.
7. Tang PH, Miles, M.V., DeGrauw, A., Hershey, A., Pesce, A. HPLC analysis of reduced and oxidised coenzyme Q10 in human plasma. Clinical Chemistry 2001;47:256-65.
8. Weber C, Bysted A, Hølmer G. The Coenzyme Q10 content of the average Danish diet. International Journal of Vitamin Nutrition Research 1997; 67:123-9.
9. Mattila P, Kumpulainen J. Coenzymes Q_9 and Q_{10}: contents in foods and dietary intake. Journal of Food Composition and Analysis 2001;14:409-17.
10. Finckh B, Kontush A, Commentz J, Hübner C, Burdelski M, Kohlschütter A. Monitoring of Ubiquinol-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Analytical Biochemistry 1995;232:210-6.
11. Kaikkonen J, Nyyssönen K, Salonen JT. Measurement and stability of plasma reduced, oxidised and total coenzyme Q10 in humans. Scandinavian Journal of Clinical and Laboratory Investigation 1999;59:457-66.

12. Turunen M, Appelkvist E-L, Sindelar P, Dallner G. Blood concentration of coenzyme Q_{10} increases in rats when esterified forms are administered. The Journal of Nutrition 1999;129:2113-8.
13. Kwong LK, Kamzalov S, Rebrin I, Bayne A-CV, Jana CK, Morris P, et al. Effects of coenzyme Q_{10} administration on its tissue concentrations, mitochondrial oxidant generation, and oxidative stress in the rat. Free Radical Biology and Medicine 2002;33:627-38.
14. Ibrahim WH, Bhagavan HN, Chopra RK, Chow CK. Dietary coenzyme Q10 and vitamin E alter the status of these compounds in rat tissues and mitochondria. The Journal of Nutrition 2000;130:2343-8.
15. Matthews RT, Yang L, Browne S, Baik M, Beal MF. Coenzyme Q_{10} administration increases brain mitochondrial concentrations and exerts neuroprotective effects. Proceedings of the National Academy of Sciences of the United States of America 1998;95:8892-7.
16. Kamzalov S, Sumien N, Forster MJ, Sohal RS. Coenzyme Q intake elevates the mitochondrial and tissue levels of coenzyme Q and α-tocopherol in young mice. Journal of Nutrition 2003;133:3175-80.
17. Forsmark-Andrée P, Lee C-P, Dallner G, Ernster L. Lipid peroxidation and changes in the ubiquinone content and the respiratory chain enzymes of submitochondrial particles. Free Radical Biology and Medicine 1997;22:391-400.
18. Dallner G, Sindelar PJ. Regulation of ubiquinone metabolism. Free Radical Biology and Medicine 2000;29:285-94.
19. Bentinger M, Dallner G, Chojnacki T, Swiezewska E. Distribution and breakdown of labelled coenzyme Q_{10} in rat. Free Radical Biology and Medicine 2003;34:563-75.

Chapter 7

Determination of Vitamin K

7.1. Introduction

The plethora of papers describing assays for measurement of vitamin K suggests that it is difficult to develop a simple, accurate, and reliable assay for its measurement. As discussed in Chapter 1, plasma vitamin K is difficult to quantify because (1) it is present at very low levels (approximately 0.2 – 5 nmol/L), and (2) it is associated with the lipid constituents of plasma. Assays to date have used long and tedious sample preparation (Appendix 3), involving liquid-liquid extraction and solid phase purification, with concentration of extracts by evaporation and reconstitution.

The most promising assays for vitamin K to date utilise HPLC (Appendix 3). Because vitamin K deficiency is mostly associated with haemorrhagic disease of the new-born, the sample-size requirements of the assay need to be considered to ensure it can be applied for measurement of vitamin K in this population.

Detection of vitamin K in HPLC is most commonly achieved using fluorescence detection which has been applied for measurement of vitamin K in milk products (1, 2), foods (3-5), animal and human tissues (6-8), and plasma (9-20) (Appendix 4). Early reports of HPLC determination of vitamin K used ultraviolet detection for measurement in plasma (12) and milk products (21-23) although sensitivity was not high. Electrochemical detection has also been used for determination of vitamin K in milk (24), foods (25), and plasma (26). There is one report of the use of gas chromatography to measure vitamin K in plant material after HPLC separation (27), and another reporting the use of GC/MS for measurement of plasma vitamin K (28).

As discussed in Chapter 2, fluorescence detection for vitamin K requires the reduction of naphthoquinone. Because vitamin K naphthoquinol is readily oxidised in the presence of atmospheric oxygen, this reduction is most efficient if carried out online immediately prior to the fluorescence detector. This reduction has been achieved using zinc metal in the presence of zinc ions during extraction (9), using tetramethylammonium octahydridotriborate added to the HPLC column effluent before detection (29) or to the mobile phase (11), using photochemical reduction (12, 30), and using sodium borohydride in ethanol added to the HPLC column effluent before the detector (15). Coulometric

reduction has also been employed (6). Reduction with zinc, utilising a small on-line column packed with zinc powder and zinc ions in the mobile phase, has been the most common reduction method reported when measuring vitamin K with fluorescence detection (1, 2, 4, 5, 13, 17, 18, 20, 31). On-line reduction of vitamin K by alcohol catalysed by platinum (packed in a small on-line column) has also been reported, using 10% platinum-on-alumina (16), platinum-black (7, 8, 19), and platinum oxide (14). In the method of Usui *et al.,* (1989) (7), the platinum-black column and the analytical column were heated to 50 ºC, but this heating was applied solely to improve the chromatography, and was not a necessity for the reduction of vitamin K.

The disadvantage of chemical reduction, for example with sodium borohydride, is that the solution of sodium borohydride degrades and therefore has to be made fresh regularly. Coulometric reduction is sensitive to residual oxygen and in the absence of an oxygen scrubber, the reduction efficiency achieved by a coulometric cell is only approximately 60% (9) as discussed in Chapter 2. Reduction by zinc is efficient, but the zinc powder needs to be replaced on a regular basis and there is a requirement for zinc ions in the mobile phase. Additionally, Lambert *et al.,* (1986) (29) suggested that packed-bed reactors cause serious peak broadening and therefore a loss of assay sensitivity as was confirmed in Chapter 2.

In this Chapter, platinum-black was used as a catalyst for the reduction of vitamin K by alcohol. Platinum catalysed reduction of vitamin K by alcohol is preferred over zinc reduction because it is highly efficient (as shown in Chapter 2) and the reduction efficiency does not decrease over time (7, 14, 16, 32). Therefore, upon exposure to an alcohol mobile phase, the lifetime of the platinum-black catalyst appears indefinite as long as the mobile phase does not contain acetonitrile or tetrahydrofuran, which poison the reactor (7, 16, 32). Additionally, there is no requirement for modifiers in the mobile phase, the only requirement being for alcohol. The results presented in Chapter 2 show that the fluorescence response of the vitamin K hydroquinone is highest in protic solvents so this requirement is more of an advantage than a disadvantage.

Daines (2001) (32) investigated the relative effectiveness of various methanol/metal catalyst systems at reducing vitamin K. Eight different metal catalysts were investigated including 5 different forms of platinum, silver powder, copper (I) oxide, and palladium

black. Each catalyst was packed into a 20 × 2 mm refillable guard column and flushed with methanol at a flow rate of 0.5 mL/min for at least one hour. Platinum compounds had superior reducing efficiency when compared to the other metal catalysts tested (32). The only exception was platinum-on-carbon which, supporting the findings of MacCrehan and Schönberger (1995) (16), showed no reduction activity. It is possible that platinum-on-carbon completely adsorbs the reactants and products yielding no fluorescence or absorbance signal (16, 32). Palladium black gave a much lower fluorescence response than the platinum compounds (32). Silver powder and copper oxide catalysts did not bring about any reduction because these catalysts require high temperatures to reduce (32).

Daines (2001) (32) also investigated the reducing ability of a series of different alcohol mobile phases, and found the order of reducing ability in the presence of platinum-black was methanol>ethanol>2-propanol>butanol>1,2-ethanediol.

The analytical columns used in HPLC assays of vitamin K are almost always C18, with the exception of Cook *et al.,* (1999) (33) who utilised a C30 column. The C30 column allowed the separation of the *cis* and *trans* isomers of vitamin K_1, present in margarines and oils (33). The *trans*-isomer of vitamin K_1 is biologically active, whereas the cis-isomer is relatively inactive (34, 35). It is therefore only necessary to be able to separately quantify these two forms when measuring vitamin K in processed food samples. The assay of Shino (1988) (14) quantified vitamin K_1 and menaquinone-4-8 in plasma as single peaks using a C18 column with a methanol/ethanol/60% perchloric acid (600/400/1.2 v/v/v) mobile phase.

The mobile phases used in HPLC assay of vitamin K usually contain various combinations of methanol, dichloromethane, ethanol, hexane, ethyl acetate, acetonitrile, 2-propanol and water (Appendix 4). Methanol, ethanol and dichloromethane are the three most often used solvents in the mobile phase. As discussed in Chapter 2, the fluorescence of vitamin K naphthoquinol in dichloromethane and ethyl acetate is not as high as in methanol, ethanol or some other protic solvent.

Recovery of vitamin K is often low (Appendix 3). This is in part due to the long and tedious extraction and purification procedures required, and partly because the highly lipophilic material in the crude hexane extract is not readily soluble in a reversed-phase

eluent, and tends to occlude both vitamin K_1 and the menaquinones (12) as discussed in Chapter 3, Section 3.1.1. Even when a less polar alcohol such as 2-propanol is used as the solvent instead of methanol, only 50% of the vitamin K_1 present in a sample is recovered (12).

Many of the purification steps used during extraction use silica/normal-phase chromatography (6-10, 12, 14, 16, 19, 20, 26) (Appendix 3). Alternatively, Cham *et al.,* (1989) (15) and Jakob and Elmadfa (2000) (18) 'washed' the hexane extract with methanol/water (9/1 v/v). Davidson and Sadowski (1997) (17) report the purification of the extract using C18 solid phase extraction. When using GC/MS for measurement of vitamin K, concentration of the hexane extract was necessary but no additional sample purification was required (28).

The concentration and form of vitamin K present in plasma is dependent on diet (14). Shino (1988) (14) showed that in 5 healthy males, the concentration of menaquinone-7 was higher than that of all other forms of menaquinone (Table 7.1).

Table 7.1 The concentration of the various forms of vitamin K in human plasma (from Shino, (1988) (14)).

Form	Mean Concentration (nmol/L)	Concentration Range (nmol/L)
Vitamin K_1	6.5	1.6 – 18.4
Menaquinone-4	0.7	0 – 2.0
Menaquinone-5	1.8	0.4 – 3.9
Menaquinone-6	0.4	0 – 2.1
Menaquinone-7	5.9	1.2 – 11.4
Menaquinone-8	0.4	0 – 1.0

Additionally, Kamao *et al.,* (2005) (19) reported the plasma concentration of menaquinone-4, menaquinone-7 and vitamin K_1 in 20 healthy subjects (mean ± SD) to be 0.76 ± 0.85, 38.63 ± 71.31, and 8.92 ± 5.41 nmol/L respectively. In osteoporotic patients treated with menaquinone-4, plasma menaquinone-4 was significantly elevated and levels of vitamin K_1 and menaquinone-7 were significantly lowered (19). Plasma concentrations of phylloquinone epoxide are increased when patients are on warfarin treatment (17), and

phylloquinone epoxide is converted to vitamin K_1 via reduction (9). The question of what forms of vitamin K should be measured in a routine assay to measure the vitamin K status of individuals therefore remains controversial.

This chapter describes the steps taken to develop a rapid and robust assay to measure vitamin K_1 and menaquinone-4 in the minimum volume of plasma (as requested by clinicians). A range of columns and mobile phases were investigated and a comparison of detection methods after HPLC separation was made.

7.2. Experimental

Unless otherwise stated, all HPLC analysis with fluorescence detection was carried out using a system made up of the following components: a Shimadzu SCL-10A system controller, a Shimadzu SIL-10AXL auto injector, a Shimadzu LC-10AT liquid chromatograph pump, a Shimadzu DGU-14A membrane degasser and a Shimadzu-RF-551 spectrofluorometric detector. Fluorescence detection used an excitation wavelength of 249 nm and an emission wavelength of 408 nm.

HPLC with electrochemical detection was carried out using a system made up of the following components: a Shimadzu-10Advp auto injector, a Shimadzu LC-10AD pump, a Uniflows Degasys DG-2410 degassing system, an ESA model 5020 guard cell, an ESA model 5011 analytical cell and an ESA Coulochem III electrochemical detector. All cell potentials are with respect to an α-hydrogen/palladium electrode.

Platinum-black was used as received from the supplier. Platinum-black was packed into an Alltech refillable guard column (20 × 2 mm) and flushed thoroughly with methanol at a flow rate of 0.1 mL/minute overnight before use. The platinum-black filled column is referred to in this Chapter as a ‘platinum-black reactor’.

The HPLC systems were interfaced through Delta 5.0 Chromatography software for data collection. All solvents used were HPLC grade and used as received from the supplier.

7.3. Comparison of detection methods after HPLC

7.3.1. Limit of detection for vitamin K_1 using fluorescence detection

7.3.1.1. Experimental:

Standards (0.2 – 18 nmol/L) were made up in a solvent mixture consisting of 11% water and 89% 1-propanol. Injection volume was 200 µL. The mobile phase was 30/70 (v/v) methanol/ethanol, pumped at a flow rate of 0.5 mL/minute. The analytical column was a Phenomenex Develosil RPAqueous C30 (250 × 4.6 mm, 5µm), at ambient temperature. The fluorescence detector was an Agilent (1100 series) with the PMT gain set to 12. A platinum-black reactor was placed between the column and the fluorescence detector. Noise was determined by calculating the standard deviation of the data points collected in a clean patch of baseline and the limit of detection taken as the concentration when the peak height was three times the noise.

The calibration curve for determination of vitamin K_1 (in ethanol) using fluorescence detection was linear (Figure 7.1). The average limit of detection from a range of standards (in ethanol) from 0.2 to 18 nmol/L, was 0.28 ± 0.16 nmol/L (mean ± SD).

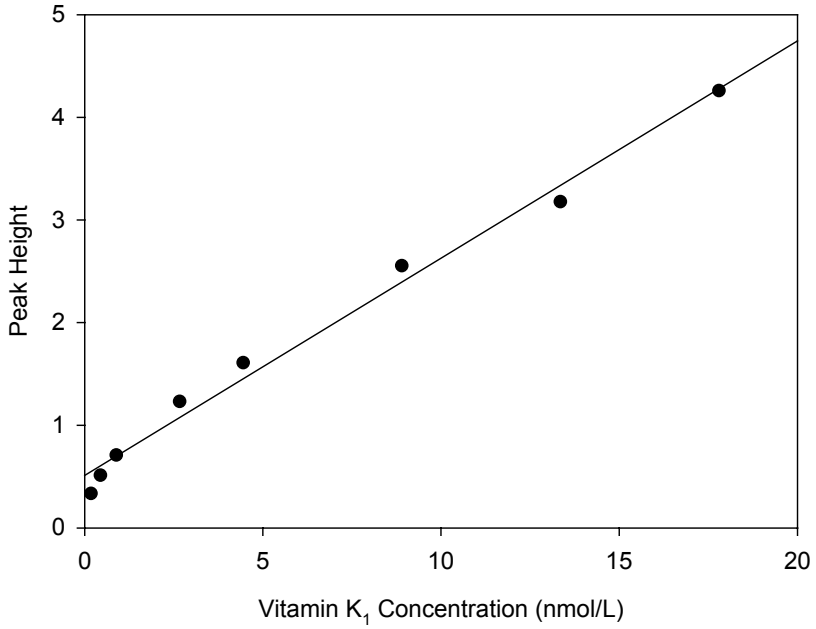


Figure 7.1 The calibration curve for vitamin K_1 using fluorescence detection.

7.3.2. Limit of detection for vitamin K_1 using electrochemical detection

7.3.2.1. Experimental

Standards were as used for fluorescence detection (Section 7.3.1.1) and 50 µL was injected. The analytical column, flow rate and mobile phase were also the same except sodium acetate trihydrate (50 mmol/L) and glacial acetic acid (15 mL/L) were added to the mobile phase as electrolytes. A platinum-black reactor was placed between the pump and the injector, and a second platinum-black reactor was placed between the column and the analytical cell. An electrochemical guard cell (set to a potential of –700 mV) was placed after the first platinum-black reactor, and before the pulse-dampener and injector. The first cell of the analytical cell was set to a potential of 325 mV and the second to a potential of 700 mV. Noise and the limit of detection were determined as for fluorescence detection.

The calibration curve for vitamin K_1 (in ethanol) using electrochemical detection was linear (Figure 7.2). The limit of detection calculated on standards (in ethanol) from 0.2 to 18 nmol/L) was 0.12 ± 0.04 nmol/L (mean ± SD).

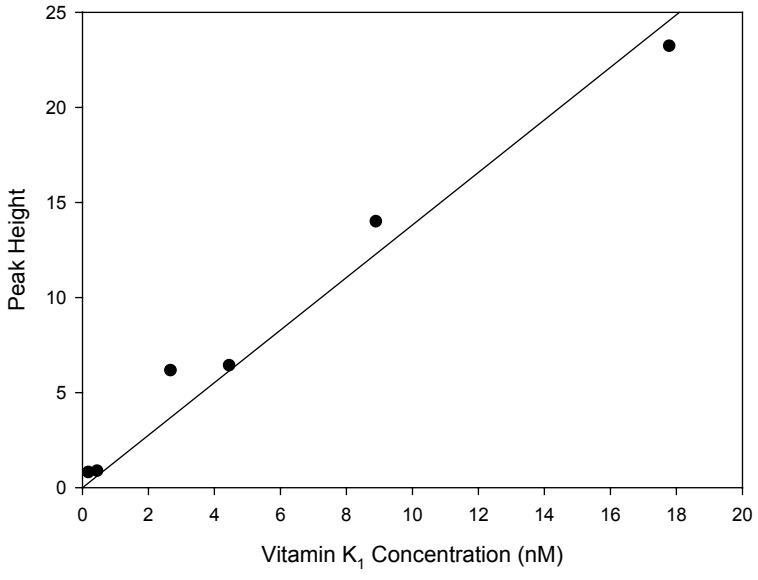


Figure 7.2 The calibration curve for vitamin K_1 with electrochemical detection.

The advantage of using electrochemical detection is that specificity can be increased by using the multiple electrochemical cells in a 'screening' mode, as discussed in Chapter 2, Section 2.2.4. However, there is a requirement for an electrolyte in the mobile phase and, additionally, residual oxygen in the system decreases the efficiency of reduction of vitamin K_1 by electrochemical cells, as discussed in Chapter 2. Removal of residual oxygen has been achieved using a platinum oxide catalyst (36). A platinum-black reactor placed online between the pump and the injector will also scrub oxygen from the system leading to an improved efficiency of reduction.

7.4. Extraction

In a routine clinical assay, the extraction procedure must be relatively quick and straightforward. The extraction must also be efficient as shown by spiking plasma with a vitamin K_1 standard, and measuring recovery.

Liquid-liquid extraction of vitamin K_1 from plasma with hexane, after precipitation of proteins with ethanol, and concentration of the extract by evaporation and reconstitution, was investigated. Plasma (200 μL) was mixed with ethanol (200 μL), before vitamin K was extracted with hexane (400 μL). The hexane layer (300 μL) was evaporated and the residue reconstituted in 1-propanol (200 μL). This method resulted in approximately 87% recovery of the vitamin K_1 from a 50 nmol/L spiked plasma extract (Figure 7.3). However, when the same procedure was applied to an unspiked plasma sample, the vitamin K_1 peak was too small to be quantified. This method has the advantage that the vitamin K can be reconstituted in a range of solvents that are compatible with the HPLC system. However, the disadvantage of this method is that evaporating the supernatant to dryness can result in degradation of vitamin K due to exposure to light and air. Additionally, as discussed in Chapter 3, Section 3.1.1, the recovery of this extraction procedure is dependent on the concentration of lipids in the extract.

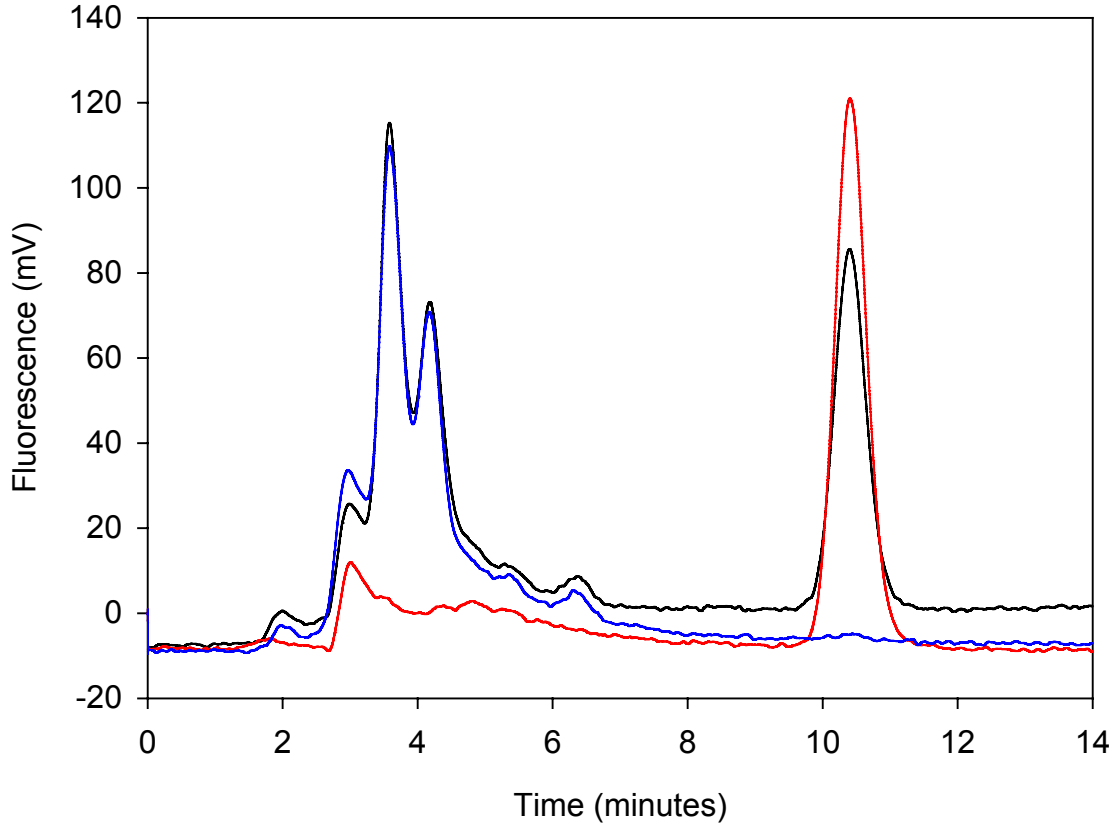


Figure 7.3 Evaporation method for extraction of vitamin K_1 from plasma.

The practicalities of analysing vitamin K in plasma by adding 500 µl of 1-propanol to an equal volume of plasma was investigated. After thorough mixing, the sample was centrifuged, and the supernatant injected directly into the HPLC. Most of the vitamin K remained bound to proteins (determined by the low recovery achieved using this method), making this procedure inefficient. Because vitamin K is lipophilic, large volumes of 1-propanol are required to release plasma vitamin K from the lipids into the 1-propanol. However, because vitamin K is present in plasma at such low concentration, the large volume of 1-propanol required for extraction gives a vitamin K concentration that is too low to measure. There was no phase separation between 1-propanol and water, so the lipophilicity of the 1-propanol is reduced, and the vitamin K_1 is therefore more soluble in the protein pellet than the 1-propanol/water mixture.

A liquid-liquid extraction of vitamin K_1 from plasma using 1- and 2-butanol was investigated by adding 500 µl of 1- or 2-butanol to 500 µl of plasma, vortexing and centrifugation. The top layer (1- or 2-butanol) was removed and injected into the HPLC

system. A problem with this method is that the precipitated plasma proteins form a layer in between the aqueous constituents of plasma and the 1- or 2-butanol organic phase. Therefore, some of the 1- and 2-butanol is associated with the proteins. Three compounds, urea, sucrose and ammonium sulfate, were investigated to determine whether their addition to plasma before the 1- or 2-butanol would decrease the thickness of the protein layer, and reduce the volume of 1- or 2-butanol associated with the protein layer. The addition of a small amount of urea decreased the thickness of the protein layer, possibly because urea denatures plasma proteins. Of the two solvents (1- and 2-butanol), the protein layer was thinner when 2-butanol was used as extraction solvent. Hence this solvent is preferred over 1-propanol.

A possible extraction procedure therefore involves addition of a small amount of urea (approximately 15 mg) to 500 µl plasma, followed by addition of 500 µl 2-butanol. After vortexing and centrifugation, the organic phase can be injected directly into the HPLC. Using this protocol, 72-78% recovery of standards (in an aqueous phase) was achieved. However, when this procedure was applied to plasma, the lipids in the 2-butanol extract complicated quantification of vitamin K in the extract, so additional purification of the extract was still necessary.

7.4.1. PRP column

As described in the column section below (Section 7.5), an HPLC polymer reversed-phase column (PRP) (Phenomenex PolymerX) retained vitamin K when methanol, ethanol, and 2-butanol were used as the mobile phase. It was proposed that PRP could therefore be used as a solid phase extraction medium for purification prior to HPLC analysis.

Plasma (100 µL) was mixed with 900 µL of 1-propanol. The solid phase extraction columns (Phenomenex Strata 100 µm SDB-L syrene Divinyl Benzene 100 mg/mL) were conditioned with 1 mL 1-propanol. The 1-propanol plasma extract was applied to the PRP column and eluted using vacuum. The column was washed with 2 mL 1-propanol, and the vitamin K_1 then eluted with 0.5 mL hexane. The hexane was evaporated at 35 ºC under a stream of nitrogen. The residue was reconstituted in 100 µL 1-propanol, diluted 1/8 (v/v) with water, and injected (50 µL) into the HPLC system (Phenomenex Develosil C30 analytical column (250 × 4.6 mm, 5 µm), 30/70 methanol/ethanol (v/v) with 50 mmol/L

sodium acetate trihydrate and 15 mL/L glacial acetic acid mobile phase, flow rate 0.5 mL/minute) with electrochemical detection. An impurity co-eluted with the vitamin K_1 peak, hence this extraction protocol was not taken any further.

7.5. HPLC columns

The protocol for the vitamin K assay using a platinum-black reactor and fluorescence detection does not enable the use of normal-phase columns, since the quantity of alcohol required to reduce the quinone would wash vitamin K through without retention. Vitamin K is lipophilic and therefore more soluble in non-polar solvents. However, vitamin K cannot be injected onto a reversed-phase column in a non-polar solvent as this would minimise retention and compromise fluorescence yields. The chromatography offered by four different reversed-phase columns was briefly investigated (using fluorescence detection), in order to identify the column with the most promising attributes for the development of a routine vitamin K assay:

(a) A C18 column (Merk Supersphere 100 RP-18 endcapped, 150 × 4.6 mm, 5 μm) retained vitamin K_1 (standard in ethanol) for about 20 minutes when methanol (run at 0.5 mL/min) was used as the mobile phase (Figure 7.4). For practical reasons, vitamin K extracted from biological samples needs to be injected in a polar solvent otherwise retention on the column will be compromised. It is not ideal to inject vitamin K in polar solvents since its solubility is poor.

(b) A Phenyl-Hexyl column (3 μm, 50 × 3 mm, Phenomenex) incorporating a phenyl phase with a hexyl alkyl linker as opposed to the traditional propyl chain, did not retain ethanoic vitamin K_1 when a 60/40 methanol/ethanol (v/v) mobile phase was run at 0.2 mL/min. This column reputedly has an increased retention for polar aromatic compounds, but vitamin K_1 is not polar and its retention was not significant on this column (Figure 7.4). Therefore, no further work was carried out with this column, due to the lack of retention with a polar mobile phase.

(c) A reversed-phase C30 column (Phenomenex Develosil RP aqueous, 250 × 4.6 mm, 5 μm) retained vitamin K_1 for about 18.5 minutes with a 30/70 (v/v) methanol/ethanol mobile phase (Figure 7.4).

(d) A polymer reversed-phase (PRP) column from Phenomenex, PolymerX (150 × 4.6 mm, 5 µm) consists of microporous polystyrene divinylbenzene and is reported to have similar selectivity characteristics to a C18-bonded silica column. This column retained vitamin K_1 when washed with methanol, ethanol, 2-propanol, and 2-butanol, all at a flow rate of 0.5 mL/minute. Vitamin K was eluted from this column by heptane. Figure 7.4 shows a chromatogram of a vitamin K_1 standard (500 nmol/L, low sensitivity on the fluorescence detector) with a 20/40/40 hexane/methanol/2-propanol (v/v/v) mobile phase, retention time approximately 7 minutes. The strong affinity of this column for vitamin K is probably due to a strong interaction between the styrene-divinylbenzene copolymer beads of the stationary phase and the aromatic group of vitamin K. Further work was therefore carried out to determine the most practical mobile phase to use with this column.

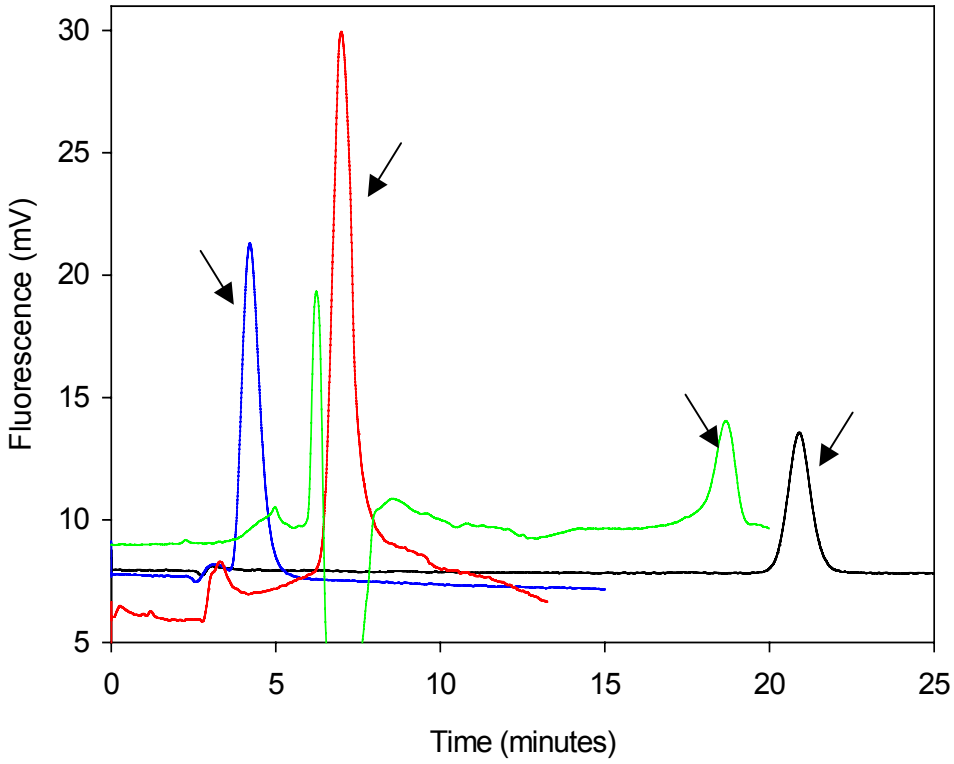


Figure 7.4 Vitamin K_1 standard (50 nmol/L) on a C18 column (black), and a Phenyl Hexyl column (blue), a 500 nmol/L vitamin K_1 standard on a Polymer Reversed-Phase column (red), and a 20 nmol/L vitamin K_1 standard on a C30 column (green). Arrows point to vitamin K peaks.

7.6. Mobile phase

Requirements of the mobile phase are that first a primary or secondary alcohol must be present to act as a reducing agent for the platinum-black catalysed reduction. Secondly, vitamin K must be retained on the column while any co-extracted interfering substances are washed through. Thirdly, run time for the assay needs to be reasonable (such as 30 minutes or less) for it to be practical as a routine clinical test. Fourthly, protic solvents should predominate in the mobile phase since the solvent effect on fluorescence described in Chapter 2 demonstrated that protic solvents result in a greater fluorescence response from vitamin K_1. Finally, it is desirable that the mobile phase separates vitamin K_1 and menaquinone-4.

The Phenomenex PolymerX column (150 × 4.6 mm, 5 μm) described above was used for the study of the mobile phase since of the columns tested, it retained vitamin K most strongly.

(a) Vitamin K_1 eluted at 29 minutes when the mobile phase consisted of 10/90 heptane/methanol (v/v) (flow rate of 0.4 mL/min). Analysis of an extract of a spiked (50 nmol/L) plasma sample, (ethanol (200 μl) was mixed with 500 μl spiked plasma, 450 μl of hexane was then added, and the sample vortexed and centrifuged. The supernatant was removed and evaporated and the sample reconstituted in ethanol) showed that interfering compounds are also extracted from plasma and continue to be eluted from the column at 26 minutes. The interfering compounds masked the vitamin K_1 peak so that it could not be accurately quantified (Figure 7.5).

(b) Vitamin K_1 eluted at 18 minutes when a 60/20/20 hexane/methanol/2-propanol (v/v/v) mobile phase was run. The peak was broad in time and also showed some tailing.

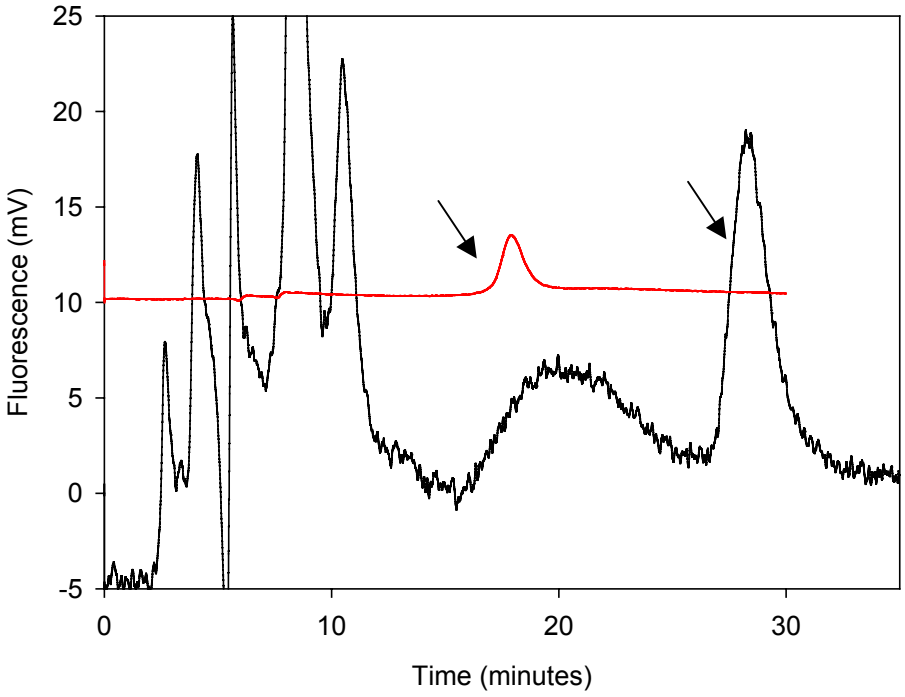


Figure 7.5 Chromatogram of vitamin K_1 spiked plasma with 10/90 heptane/methanol mobile phase on a PRP column (black), and a vitamin K_1 standard (50 nmol/L) with a 60/20/20 hexane/methanol/2-propanol mobile phase (red).

(c) Tetrahydrofuran (THF) was added to the mobile phase to investigate what effect it would have on the chromatography. Vitamin K_1 was not eluted from the column after 30 minutes by a 50/50 THF/methanol (v/v) mobile phase run at 0.5 mL/minute. Vitamin K_1 was eluted without retention when the mobile phase was both 40/40/20 (v/v/v) THF/methanol/heptane, and 20/20/60 (v/v/v) heptane/THF/methanol, run at 0.5 mL/minute. A mobile phase of 5/20/75 THF/heptane/methanol (v/v/v) (run at 0.5 mL/min) resulted in elution of menaquinone-4 at 10 minutes, and vitamin K_1 at 11 minutes. However, the vitamin K_1 and menaquinone-4 peaks overlapped so the two forms could not be quantified separately (Figure 7.6). A 10/10/80 THF/heptane/methanol (v/v/v) mobile phase (at 0.5 mL/minute) eluted vitamin K_1 and menaquinone-4 at 12 minutes with no separation of the vitamin K_1 and menaquinone-4 peaks. A 5/20/75 (v/v/v) heptane/THF/methanol mobile phase (at 0.5 mL/minute) retained vitamin K_1 (retention time 8.2 minutes) for slightly less time than menaquinone-4 (retention time 8.7 minutes). A 25/75 THF/methanol (v/v) mobile phase (at 0.5 mL/minute) retained menaquinone-4 (retention time 9 minutes) slightly

longer than vitamin K_1 (8.5 minutes). Therefore, THF in the mobile phase results in menaquinone-4 being retained for longer than vitamin K_1 on a polymer reversed-phase column. However, heptane in the mobile phase results in vitamin K_1 being retained for longer than menaquinone-4 on a polymer reversed-phase column. The concentration of THF has to be kept low since THF poisons the platinum-black reactor (7, 16, 32).

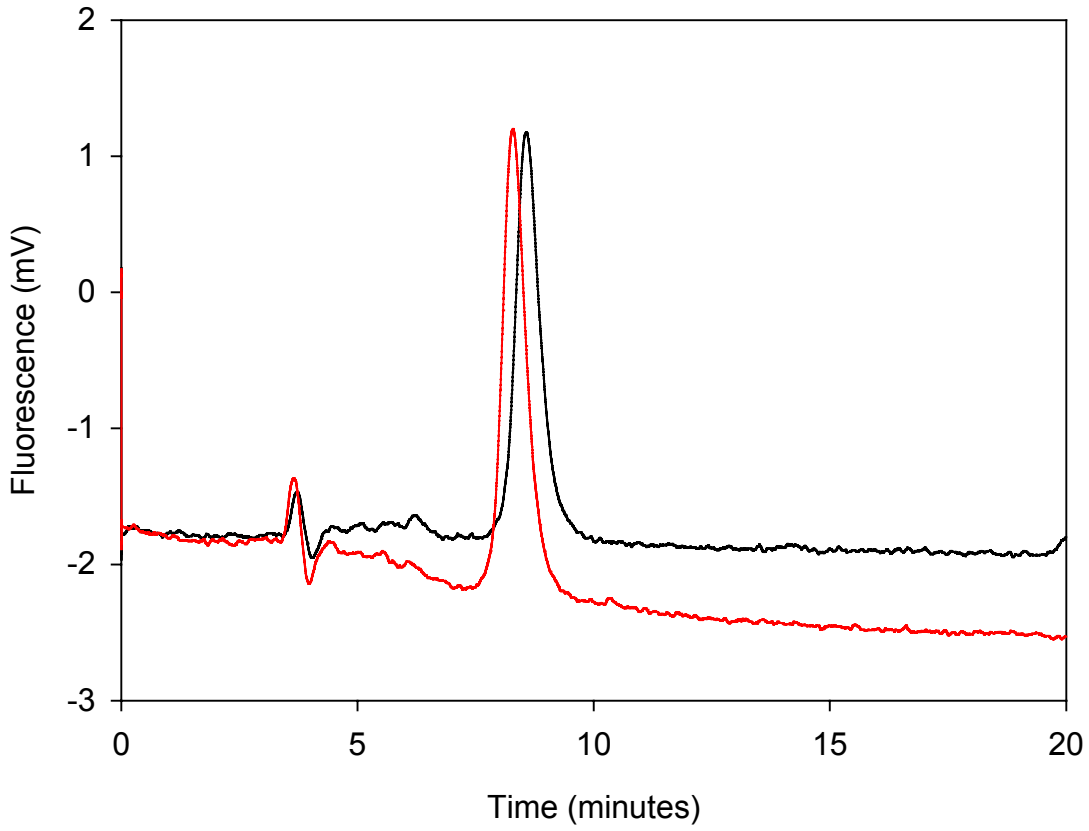


Figure 7.6 Chromatogram of 50 nmol/L vitamin K_1 (black) and 50 nmol/L menaquinone-4 (red), with a 5/20/75 THF/heptane/methanol mobile phase.

(d) When propylene carbonate was added to the mobile phase (50/50 (v/v) propylene carbonate/methanol, at 0.5 mL/minute), no vitamin K_1 peak was seen either with or without a column. It is possible that propylene carbonate may have some intrinsic ultraviolet absorbance, which masked the vitamin K_1 peak.

(e) A 20/80 (v/v) cylcohexane/methanol mobile phase (at 0.5 mL/minute) eluted vitamin K from the column in a similar manner to heptane. As a mobile phase constituent, heptane is preferred over cyclohexane since heptane is less volatile.

These results indicate that a mobile phase consisting of methanol, ethanol, and heptane in various proportions may be most useful for a vitamin K assay, on a Phenomenex polymerX reversed-phase column. The proportions of each solvent in the mobile phase can be altered to lengthen or shorten retention times of vitamin K (for example, to separate vitamin K_1 and menaquinone-4).

(f) Using a 20/80 heptane/methanol (v/v) mobile phase (run at 0.3 mL/min), menaquinone-4 was eluted at 21 minutes and vitamin K_1 at 23 minutes. The vitamin K_1 and menaquinone-4 peaks overlapped slightly so could not be accurately quantified using peak areas (Figure 7.7).

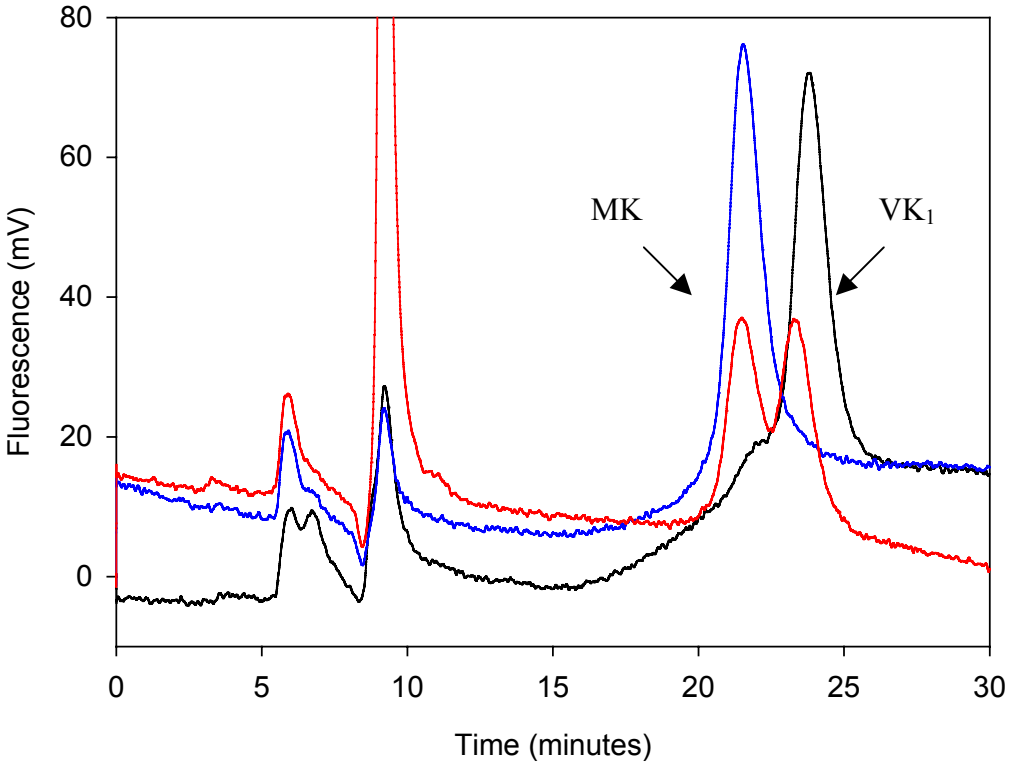


Figure 7.7 Chromatogram of the separation of vitamin K_1 (black, VK_1) and menaquinone-4 (blue, MK), both 50 nmol/L, and of a solution containing 25 nmol/L K_1 and 25 nmol/L menaquinone-4 (red). Mobile phase 20/80 (v/v) heptane/methanol. Detector set on high sensitivity.

(g) A mobile phase of 20/40/40 heptane/methanol/ethanol (v/v/v) (at a flow rate of 0.5 mL/minute) eluted vitamin K_1 (retention time 9.5 minutes) before menaquinone-4 (retention time 10.5 minutes) and the peaks were not baseline separated. A mobile phase of 20/60/20 (v/v/v) heptane/ethanol/methanol (0.4 mL/minute) also did not separate vitamin K_1 (retention time 11 minutes) from menaquinone-4 (retention time 12 minutes).

(h) Replacing ethanol with 1-propanol, (a mobile phase of 20/40/40 heptane/methanol/1-propanol (v/v/v) run at 0.4 mL/minute) separated the vitamin K_1 (retention time 9.5 minutes) and menaquinone-4 (retention time 11 minutes) peaks slightly more but they were still not baseline resolved (Figure 7.8).

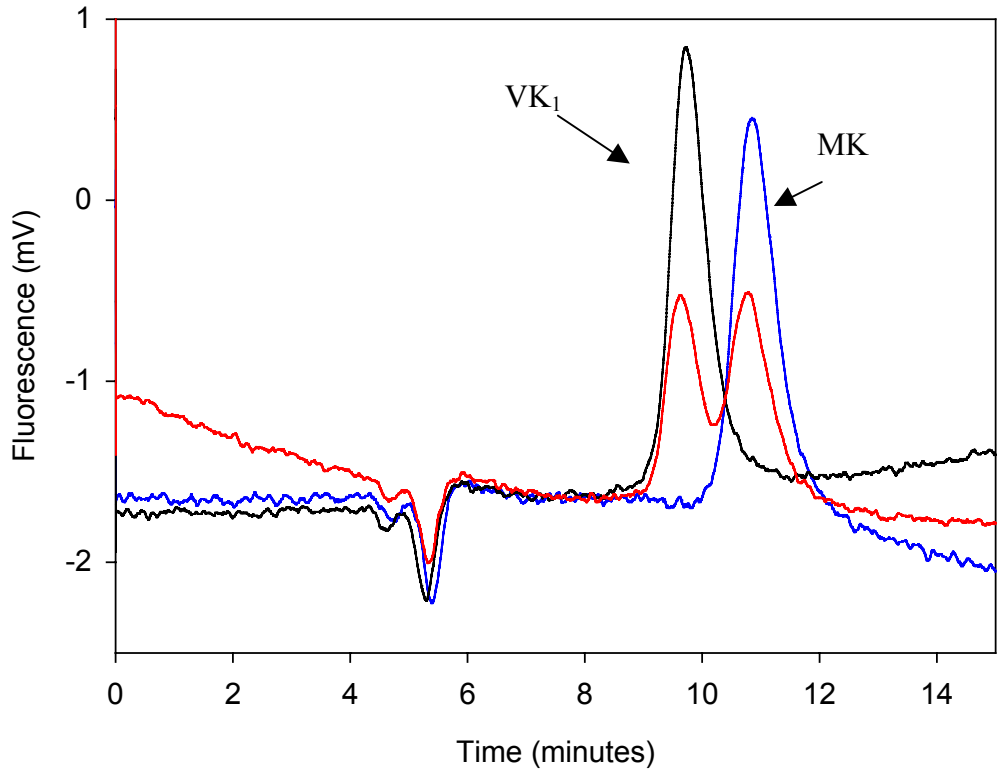


Figure 7.8 Chromatogram of 50 nmol/L vitamin K_1 (black, VK_1), 50 nmol/ menaquinone-4 (blue, MK), and 25 nmol/L vitamin K_1 and 25 nmol/L menaquinone-4, in a 20/40/40 heptane/1-propanol/methanol (v/v/v) mobile phase. Detector set on low sensitivity.

(i) The 1-propanol was replaced with 2-propanol and similar peak separation to that achieved in (h) was evident. Further work used 2-propanol rather than 1-propanol, since 2-propanol is kept in stock at Canterbury Health Laboratories.

(j) Since heptane is the solvent eluting vitamin K from the column, the heptane content of the mobile phase was reduced to 15% to investigate whether the vitamin K_1 and menaquinone-4 peaks were better separated. A 15/45/40 heptane/methanol/2-propanol (v/v/v) mobile phase did increase the resolution but still not sufficiently for a quantitative assay.

(k) The concentration of 2-propanol was increased, so the mobile phase was 15% heptane, 35% methanol and 50% 2-propanol. The two peaks were now baseline resolved allowing quantification of both vitamin K_1 and menaquinone-4. Therefore, a 15/35/50 heptane/methanol/2-propanol (v/v/v) mobile phase (at a flow rate of 0.4 mL/minute) was chosen to quantify vitamin K_1 and menaquinone-4 (Figure 7.9).

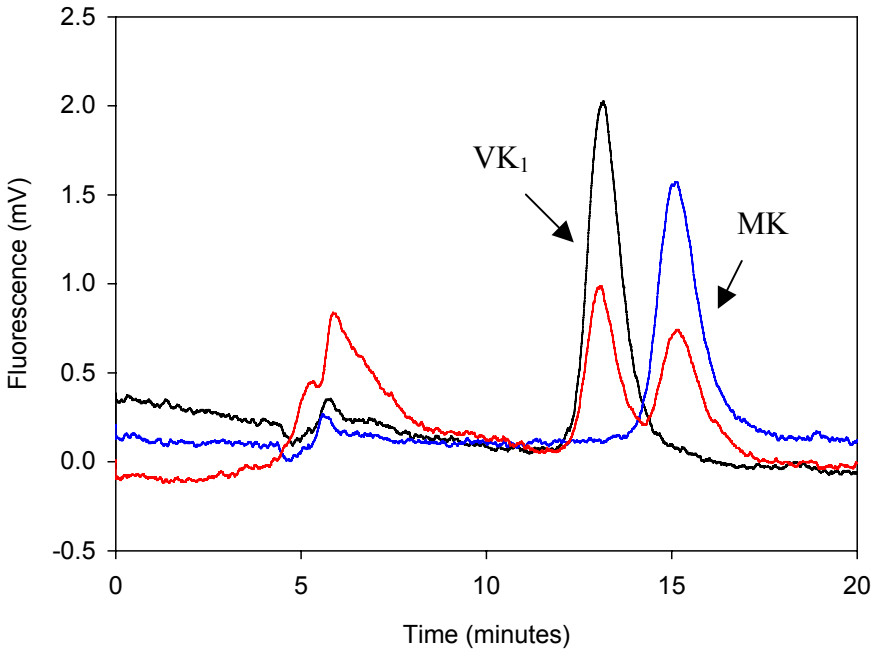


Figure 7.9 Chromatogram of 50 nmol/L vitamin K_1 (black), 50 nmol/L menaquinone-4 (blue), and 25 nmol/L vitamin K_1 and 25 nm menaquinone-4 (red), with mobile phase of 15/50/35 (v/v/v) heptane/2-propanol/methanol.

7.7. Discussion

This Chapter describes attempts to develop a rapid, reliable, and robust assay for quantification of vitamin K_1 and menaquinone-4 in human plasma using minimal sample volume. The extraction procedure and the HPLC system in terms of detection, column and mobile phase were investigated. An extraction procedure consisting of a simple liquid-liquid extraction of vitamin K_1 from plasma appears to be insufficient because impurities that interfere with the chromatography are also extracted and, additionally, the extract needs to be concentrated. Washing a hexane extract with methanol/water, as described by Jakob and Elmadfa (2000) (18) would be a good, simple purification technique which needs to be investigated further. Additionally, the assay of Yamashita *et al.,* (1997) (37), (measuring CoQ_{10}), describes the injection of 5 μL of the hexane extract directly onto a C8 column with a *tert*-butyl alcohol/methanol mobile phase. Because the injection volume is very small, the incompatible solvent does not appear to adversely affect the chromatography.

The Phenomenex PolymerX HPLC column allowed more scope for altering the mobile phase than the traditionally used C18 columns. Of the mobile phases investigated, only 15/35/50 (v/v/v) heptane/methanol/2-propanol gave baseline separation of the vitamin K_1 and menaquinone-4 peaks.

The sensitivity of fluorescence detection is increased by finding the optimal emission and excitation wavelengths for vitamin K_1 in the mobile phase. As discussed in Chapter 2, there is a small Stokes shift with different solvents for vitamin K_1.

MacCrehan and Schönberger (1995) (16) compared deuterium and xenon light sources for the fluorometer. The detection limits (S/N = 3) for a 50 μL injection were 50 and 7 pg for the deuterium and xenon-source fluorometers respectively. The detection limit for oxidative amperometric detection of hydroquinone at a glassy carbon electrode following on-line catalytic reduction was approximately 200 pg injected, and therefore less sensitive than fluorometric detection.

Menaquinone-7 may also be an important vitamin K homologue that requires determination (14, 19). The retention time of phylloquinone epoxide should also be known, to ensure that the assay is measuring vitamin K_1 as opposed to vitamin K_1 plus

phylloquinone epoxide. If the latter is true, then knowledge of whether the patient is currently receiving Warfarin therapy is essential since Warfarin significantly elevates phylloquinone epoxide (17). Additionally, the measurement of vitamin K_1 and menaquinone-4 as separate entities is desirable, since menaquinone-4 is significantly elevated when patients (usually with osteoporosis) are treated with menaquinone-4.

The work described in this Chapter provides a basis on which to further develop a practical vitamin K assay that meets the demands of clinicians. Electrochemical detection using coulometric cells has been shown to provide the highest sensitivity of the detection methods available, especially when combined with platinum-black filled columns for catalysis of alcohol reduction of vitamin K on-line.

7.8. References for Chapter 7

1. Indyk H, Woollard DC. Vitamin K in milk and infant formulas: Determination and distribution of phylloquinone and menaquinone-4. Analyst 1997;122:1-5.
2. Indyk H, Woollard DC. Determination of vitamin K in milk and infant formulas by liquid chromatography: Collaborative study. Journal of AOAC International 2000;83:121-30.
3. Speek AJ, Schrijver J, Schruers WHP. Fluorimetric determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes by high-performance liquid chromatography with post-column derivatization. Journal of Chromatography 1984;301:441-7.
4. Booth SL, Davidson KW, Sadowski JA. Evaluation of an HPLC method for the determination of phylloquinone (vitamin K_1) in various food matrices. Journal of Agricultural and Food Chemistry 1994;42:295-300.
5. Ware GM, Chase GW, Eitenmiller RR, Long AR. Determination of vitamin K_1 in medical foods by liquid chromatography with postcolumn reduction and fluorometric detection. Journal of AOAC International 2000;83:957-62.
6. Hirauchi K, Sakano T, Notsumoto S, Nagaoka T, Morimoto A, Fujimoto K, et al. Measurement of K vitamins in animal tissues by high-performance liquid chromatography with fluorimetric detection. Journal of Chromatography 1989;497:131-7.
7. Usui Y, Nishimura N, Kobayashi N, Okanoue T, Kimoto M, Ozawa K. Measurement of vitamin K in human liver by gradient elution high-performance liquid chromatography using platinum-black catalyst reduction and fluorimetric detection. Journal of Chromatography 1989;489:291-301.
8. Usui Y. Assay of phylloquinone and menaquinones in human liver. Methods in Enzymology 1997;282:438-47.
9. Haroon Y, Bacon DS, Sadowski JA. Liquid-chromatographic determination of vitamin K_1 in plasma, with fluorometric detection. Clinical Chemistry 1986;32:1925-9.
10. Lambert WE, De Leenheer AP, Lefevere MF. Determination of vitamin K in serum using HPLC with post-column reaction and fluorescence detection. Journal of Chromatographic Science 1986;24:76-9.

11. Lambert WE, De Leenheer AP. Simplified post-column reduction and fluorescence detection for the high-performance liquid chromatographic determination of vitamin $K_{1(20)}$. Analytica Chimica Acta 1987;196:247-50.
12. Lefevere MF, De Leenheer AP, Claeys AE, Claeys IV, Steyaert H. Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. Journal of Lipid Research 1982;23:1068-72.
13. Haroon Y, Bacon DS, Sadowski JA. Chemical reduction system for the detection of phylloquinone (Vitamin K_1) and menaquinones (Vitamin K_2). Journal of Chromatography 1987;384:383-9.
14. Shino M. Determination of endogenous vitamin K (phylloquinone and menaquinone-n) in plasma by high-performance liquid chromatography using platinum oxide catalyst reduction and fluorescence detection. Analyst 1988;113:393-7.
15. Cham BE, Roeser HP, Kamst TW. Simultaneous liquid-chromatographic determination of vitamin K_1 and vitamin E in serum. Clinical Chemistry 1989;35:2285-9.
16. MacCrehan WA, Schönberger E. Determination of vitamin K_1 in serum using catalytic-reduction liquid chromatography with fluorescence detection. Journal of Chromatography B 1995;670:209-17.
17. Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high-performance liquid chromatography using postcolumn chemical reduction. Methods in Enzymology 1997;282:408-21.
18. Jakob E, Elmadfa I. Rapid and simple HPLC analysis of vitamin K in food, tissues and blood. Food Chemistry 2000;68:219-21.
19. Kamao M, Suhara Y, Tsugawa N, Okano T. Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. Journal of Chromatography B 2005;816:41-8.
20. Wang LY, Bates CJ, Yan L, Harrington DJ, Shearer MJ, Prentice A. Determination of phylloquinone (vitamin K_1) in plasma and serum by HPLC with fluorescence detection. Clinica Chimica Acta 2004;347:199-207.
21. Bueno MP, Villalobos MC. Vitamins and other nutrients. Journal of AOAC International 1983;66:1063-7.
22. Haroon Y, Shearer MJ, Rahim S, Gunn WG, McEnery G, Barkhan P. The content of phylloquinone (vitamin K_1) in human milk, cows milk and infant formula foods

determined by high-performance liquid chromatography. The Journal of Nutrition 1982;112:1105-17.

23. Indyk H, Littlejohn VC, Lawrence RJ. Liquid chromatographic determination of vitamin K_1 in infant formulas and milk. Journal of AOAC International 1995;78:719-23.
24. Isshiki H, Suzuki Y, Yonekubo A, Hasegawa H, Yamamoto Y. Determination of phylloquinone and menaquinone in human milk using high performance liquid chromatography. Journal of Dairy Science 1988;71:627-32.
25. Piironen V, Koivu T, Tammisalo O, Mattila P. Determination of phylloquinone in oils, margarines and butter by high-performance liquid chromatography with electrochemical detection. Food Chemistry 1997;59:473-80.
26. Hart JP, Shearer MJ, McCarthy PT. Enhanced sensitivity for the determination of endogenous phylloquinone (vitamin K_1) in plasma using high-performance liquid chromatography with dual-electrode electrochemical detection. Analyst 1985;110:1181-3.
27. Seifert RM. Analysis of vitamin K_1 in some green leafy vegetables by gas chromatography. Journal of Agricultural and Food Chemistry 1979;27:1301-4.
28. Fauler G, Leis HJ, Schalamon J, Muntean W, Gleispach H. Method for the determination of vitamin $K_{1(20)}$ in human plasma by stable isotope dilution/gas chromatography/mass spectrometry. Journal of Mass Spectrometry 1996;31:655-60.
29. Lambert WE, De Leenheer AP, Baert EJ. Wet-chemical postcolumn reaction and fluorescence detection analysis of the reference interval of endogenous serum vitamin $K_{1(20)}$. Analytical Biochemistry 1986;158:257-61.
30. Poulsen JR, Birks JW. Photoreduction fluorescence detection of quinones in high-performance liquid chromatography. Analytical Chemistry 1989;61:2267-76.
31. Jakob E, Elmadfa I. Application of a simplified HPLC assay for the determination of phylloquinone (vitamin K_1) in animal and plant food items. Food Chemistry 1996;56:87-91.
32. Daines AM. New Assays for Biologically Active Quinones. Christchurch: University of Canterbury, 2001.
33. Cook KK, Mitchell GV, Grundel E, Rader JI. HPLC analysis for *trans*-vitamin K_1 and dihydro-vitamin K_1 in margarines and margarine-like products using the C_{30} stationary phase. Food Chemistry 1999;67:79-88.
34. Parrish DB. CRC Critical Reviews in Food Science and Nutrition, 1980;377.

35. Suttie JW. Vitamin K. In: Machlin LJ, ed. Handbook of Vitamins, Vol.: Marcel Dekker Inc., 1984.
36. MacCrehan WA, May WE. Oxygen removal in liquid chromatography with a zinc oxygen-scrubber column. Analytical Chemistry 1984;56:625-8.
37. Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Analytical Biochemistry 1997;250:66-73.

Chapter 8

Conclusions and Future Work

Coenzyme Q_{10} (CoQ_{10}) and vitamin K are quinones whose electron and proton transfer functions are essential for all life forms. The work described in this thesis was initiated by a demand from clinicians for a rapid, reliable and robust assay to determine vitamin K in biological samples in order to investigate and monitor the prevalence of haemorrhagic disease of the new born. Initial work on developing this assay was carried out by Daines (2001) (1), and further developed in an attempt to meet the needs of clinicians.

During work on the vitamin K assay, demand for a rapid, reliable and robust assay to determine the chemically similar CoQ_{10} increased. CoQ_{10} deficiency is implicated in the increasingly popular statin therapy, as well as in heart failure and various other pathologies. Additionally, CoQ_{10} has become increasingly popular as a nutritional supplement, with many claims for health benefits of supplementation being made with little scientific backing.

The work described in this thesis was carried out to develop assays for vitamin K and CoQ_{10} that meet the desires of clinicians, and to form a solid basis of knowledge of the biochemistry of CoQ_{10} on which to build future clinical trials.

8.1. Vitamin K

- For measurement of vitamin K, the forms to be quantified need to be defined since the final assay (including the extraction procedure) depends on what is to be measured. For example, if measuring phylloquinone epoxide, the sample cannot be subjected to reducing conditions before separation on the HPLC column, since phylloquinone epoxide is reduced to phylloquinone (vitamin K_1). Additionally, Warfarin therapy significantly elevates the level of phylloquinone epoxide, and patients being treated for osteoporosis are often supplemented with menaquinone-4.

- The low endogenous concentration of vitamin K, combined with its lipophilicity, make it a difficult analyte to quantify in biological samples. With the fluorometer available, fluorescence detection could not meet clinical requirements for determination of low vitamin K levels in small samples (less than 50 µL). Electrochemical detection gave scope to meet clinical requirements but further developmental work is necessary.

- For measurement of vitamin K in plasma, an extraction procedure consisting of simple liquid-liquid extraction of vitamin K_1 appears to be inadequate because impurities, which interfere with the chromatography, are also extracted. Additionally, the extract needs to be concentrated.

- A Phenomenex PolymerX HPLC analytical column allows more scope for altering the mobile phase than the traditionally used C18 columns and will be a good tool to use in further assay development work.

8.2. Coenzyme Q_{10}

- Coenzyme Q_{10} in biological samples can be analysed by HPLC using either ultraviolet or electrochemical detection. Ultraviolet detection allows measurement of total CoQ_{10} (after oxidation of endogenous $CoQ_{10}H_2$ during extraction). Electrochemical detection is required for determination of both reduced and oxidised CoQ_{10}. Fluorescence detection of CoQ_{10} after alcohol reduction with platinum-black as a catalyst (a more efficient reduction technique than electrochemical or zinc reduction) does not allow adequate sensitivity to measure biological levels of CoQ_{10} even when aprotic solvents (which give the highest fluorescence yield for CoQ_{10}) are used in the mobile phase.

- Coenzyme Q_9 (CoQ_9) is present in human plasma with a reference interval of 8.8 – 47.0 nmol/L. These results provide evidence that CoQ_9 is not an ideal internal standard for measurement of CoQ_{10}, and its use leads to erroneous results. The origin of plasma CoQ_9 in humans, be it from catabolism of CoQ_{10} or a minor product of CoQ_{10} synthesis, is not clear. There was a weak but highly significant correlation between plasma total CoQ_9 and CoQ_{10} concentrations and a significant increase in plasma total CoQ_9 concentrations after CoQ_{10} supplementation.

- Coenzyme Q_{10} in biological samples is stable for at least 18 months when stored at –80 ºC. It is not, however, stable at –13 ºC. Coenzyme Q_{10} is photochemically degraded, and exposure to light for periods of longer than 2 hours leads to significant degradation.

- The reference interval for plasma total CoQ_{10} in the healthy New Zealand population was determined to be 0.47 – 1.90 μmol/L. Coenzyme Q_{10} concentrations in healthy young males were shown to be tightly distributed around a homeostatic set point which suggests that interpretation of CoQ_{10} values for a given individual should be based on previously determined values for that individual as opposed to comparison with a reference interval.

- Coenzyme Q_{10} supplements have varied bioavailability and inter-individual differences in absorption of CoQ_{10} are marked. Therefore, plasma CoQ_{10} concentrations should be monitored during supplementation to ensure efficacy.

- There is a plateau in absorption of CoQ_{10} from a single oral dose of the supplement Q-Gel (the most bioavailable brand of the CoQ_{10} supplements tested) which occurs at approximately 200 mg. Therefore, the maximum single oral dose to be taken for maximum absorption efficiency should be 200 mg. The Q-Gel supplements containing 30 mg CoQ_{10} per capsule give approximately twice the plasma levels of those containing 100 mg CoQ_{10} per capsule when equivalent doses are given.

- There is a highly significant 33% reduction in plasma total CoQ_{10} when patients with chronic heart failure are given 40 mg per day Atorvastatin therapy for six weeks. This reduction in plasma CoQ_{10} correlates with an improvement in endothelial function, suggesting that plasma CoQ_{10} concentrations may be a marker for statin-mediated improvements in endothelial function.

8.3. Further work

Further work is needed to investigate whether there is any correlation between tissue and plasma CoQ_{10} levels. This work is essential to ascertain whether measurement of plasma CoQ_{10} levels indicates the CoQ_{10} status in muscle tissue such as myocardium and brain. Collection of muscle tissue for analysis of CoQ_{10} involves an invasive sampling technique, hence is undesirable, however it may be essential if plasma CoQ_{10} levels do not indicate tissue status.

Studies investigating the changes in plasma CoQ_{10} with multiple sequential CoQ_{10} dosing, including bioavailability studies, would be logical next steps from the bioavailability study reported in this thesis.

Further work is also necessary to elucidate the role of CoQ_{10} in the many disease states in which it has been implicated, and hence the benefits of CoQ_{10} supplementation. To complete this further work, it is necessary to have reliable assay methods and reliable data on normal circulating concentrations of CoQ_{10} and how these are affected by disease, drug interactions and supplementation. The work described in this thesis provides an essential basis of background information from which this further work can evolve.

The significant association of reduction in CoQ_{10} and improvement in acetylcholine-induced, endothelium-dependent vasodilation suggests the need for an investigation into whether acetylcholine-induced endothelium-dependent vasodilation is further improved with CoQ_{10} and statin co-therapy, or whether the improvement is negated by CoQ_{10} supplementation.

The well-known hypotheses that CoQ_{10} depletion is the cause of statin-induced myalgia, and that co-therapy of CoQ_{10} and statins will eliminate myalgia symptoms, remain to be thoroughly explored, and a study investigating this would answer a very important question that has remained unanswered for some time now.

Langsjoen and Langsjoen (1999) (2) completed an interesting review of over 40 studies that have been published that relate information on the benefit of CoQ_{10} supplementation in cardiovascular disease. Langsjoen and Langsjoen (1999) (2) concluded that there is some benefit, particularly lowering of New York Heart Association (NYHA) class, improved left ventricular ejection fraction, and 6-minute walk test in heart failure patients who are supplemented with CoQ_{10}. There is currently a large international multicenter study being conducted into the effect of CoQ_{10} supplementation in patients with heart failure, called Q-SYMBIO (3). The principle centre for this study is Denmark, and the results of this study will be very interesting.

Rauchaus *et al*., (2003) (4) recently published an interesting finding that survival in heart failure patients is predicted by their total cholesterol levels, with low levels of total

cholesterol being associated with worse survival. Various potential mechanisms for this association were postulated, including lower metabolic reserve in patients with low total cholesterol, and a protective role for lipoproteins in chronic heart failure. One potential mechanism for this association that was not discussed by Rauchaus *et al.,* (2003) (4) was that CoQ_{10} levels in these patients may be deficient, if their total cholesterol levels are low. This potential explanation for the association would be easily explored in stored samples from a cohort of heart failure patients with a long follow-up period recording survival.

Further work is required to develop a rapid and reliable assay to measure plasma vitamin K. The first step in development of this assay must be to determine exactly what forms of vitamin K are to be quantified. It is possible that laser-induced fluorescence detection will give scope to measure the low plasma concentrations of vitamin K in small sample volumes. Alternatively, electrochemical detection may allow measurement of vitamin K in these small samples after further improvement of the assay extraction procedure.

8.4. References for Chapter 8

1. Daines AM. New Assays for Biologically Active Quinones. Christchurch: University of Canterbury, 2001.
2. Langsjoen PH, Langsjoen AM. Overview of the use of CoQ10 in cardiovascular disease. Biofactors 1999;9:273-84.
3. Mortensen SA. Overview on coenzyme Q_{10} as adjunctive therapy in chronic heart failure. Rationale, design and end-points of "Q-symbio" - A multinational trial. BioFactors 2003;18:79-89.
4. Rauchhaus M, Clark AL, Doehner W, Davos C, Bolger A, Sharma R, et al. The relationship between cholesterol and survival in patients with chronic heart failure. Journal of the American College of Cardiology 2003;42:1933-40.

Appendices

Appendix 1. Summary of extraction procedures used in published assays of CoQ_{10} in biological specimens.

Publication	Sample Type	Measuring	Detection	Anticoagulant	Sample volume	Extraction	Evaporation?	Recovery	Injection volume	Comments
(1)	Plasma	CoQ_{10}	UV	Heparin	500 µL	Liquid –liquid with hexane then TLC	Yes	103.6 ± 3.3%	10	Two evaporation steps. Final reconstitution in ethanol.
(2)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Ox UV	Heparin	200 µL	Liquid-liquid with hexane and 5/95 2-propanol/ethanol	Yes	88.5% for $CoQ_{10}H_2$ and 111.1% for CoQ_{10}	20 µL	Added SDS and BHT to plasma before extraction. Reconstitute in mobile phase
(3)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Ox-red-ox	Heparin	300 µL	Liquid-liquid with 1-propanol (1 mL)	No	101 ± 3.7%	100 µL	Use ferric chloride to oxidise plasma $CoQ_{10}H_2$ to measure total CoQ_{10}
(4)	Tissue	CoQ_{10} Other analytes	Diode array		1g	Liquid-liquid with methanol/chloroform	Yes	92%		Reconstitute in chloroform then SPE with bond-elute column, evaporation and reconstitute in 0.1% 2-propanol in heptane
(5)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD	None (Serum)	100 µL	Liquid-liquid with ethanol and hexane	Yes	97.8 ± 4.4%	10 µL	Reconstitute in ethanol
(6)	Plasma	CoQ_{10} $CoQ_{10}H_2$ Carotenoids tocopherols	ECD Red-partial ox-complete ox	Heparin	5-10 µL	Liquid-liquid with hexane	Yes	>97%	20 µL	Reconstitute in 2.5/47.5/50 2-propanol/ethanol/ methanol

Publication	Sample Type	Measuring	Detection	Anticoagulant	Sample volume	Extraction	Evaporation?	Recovery	Injection volume	Comments
(7)	Plasma	CoQ_{10}	UV	Heparin	1000 µL	Liquid-liquid with methanol and hexane SPE with silica and C18 cartridges	Yes	64 ± 6%		Reconstitute in 2-propanol
(8)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD ox-red-ox	EDTA	300 µL	Liquid-liquid with 1-propanol (1 mL)	No		80 µL	
(9) (10)	Tissues	$CoQ_{10}H_2$ CoQ_{10} CoQ_9H_2 CoQ_9 Vitamin E homologues	ECD UV		50 – 100 mg tissue	50/50 ethanol/hexane (4 mL)	Yes	>90 %		Antioxidants are added before and during extraction procedure
(11)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD ox	Heparin	50 µL	Liquid-liquid with 33/66 (v/v) methanol/hexane	No	99 – 104 %	5 µL	5 µL of the hexane extract injected directly
(12)	Tissue	$CoQ_{10}H_2$ CoQ_{10} CoQ_9H_2 CoQ_9 Vitamin E and α-tocopherol oxidation products	ECD ox		100 mg	Liquid-liquid with hexane (2 × 3 mL)	Yes	>95%		reconstituted extract in 1/3 (v/v) chloroform/methanol
(13)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Red-ox	EDTA or heparin	500 µL	Liquid-liquid with methanol and hexane SPE with either solid phase silica and C18 cartridges, or silica and C18 powders	Yes	69.9% with cartridges 79.5% with powders		
(13)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Red-ox	EDTA or heparin	50 µL	Liquid-liquid with ethanol and hexane	Yes	80.7%		
(14)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD ox	EDTA	50 µL	Liquid-liquid with ethanol (500 µL)	No	90.7 – 107.7 % (mean 99.2 %)	25 µL	

Publication	Sample Type	Measuring	Detection	Anticoagulant	Sample volume	Extraction	Evaporation?	Recovery	Injection volume	Comments
(15)	Plasma	$CoQ_{10}H_2$ CoQ_{10} and tocopherols	ECD Red-red-ox	Heparin	10 µL	Liquid-liquid with hexane (500 µL)	Yes	93 – 97 %		Reconstituted extract in 2.5/47.5/100 (v/v/v) 2-propanol/ethanol/ methanol
(16)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD ox-red-ox	Heparin	100 µL	Liquid-liquid with 1-propanol (900 µL)	No	95.8 - 101.0 %	20 µL	
(17)	Plasma	CoQ_{10}	UV	Heparin	200 µL	Liquid-liquid with 1-propanol (1 mL)	No	96 - 98.5 %	200 µL	1,4-benzoquinone oxidation pre-extraction
(18)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD and UV	EDTA	200 µL	Liquid-liquid with methanol (2 mL) and hexane (4 mL)	Yes		20 µL	Reconstituted extract in 0.3 mL ethanol
(19)	Plasma	CoQ_{10}	UV	Heparin	300 µL	Liquid-liquid with 1-propanol (1 mL)	No	100.9 ± 2.1 %	400 µL	Sample clean-up pre-analytical cell, using column switching
(20)	Erythrocytes and platelets	CoQ_{10}	ECD Red-red-ox	EDTA	2 mL blood	Liquid-liquid with methanol and hexane (800 µL)	Yes	91 – 100 %		Reconstitute in 40 µL ethanol
(21)	Tissue	CoQ_{10} CoQ_9 Other analytes	UV		25 mg	Liquid-liquid with hexane	Yes		10 µL	Reconstituted extract in 50 µL 25/75 (v/v) hexane/methanol
(22)	Mouse tissues	$CoQ_{10}H_2$ CoQ_{10} CoQ_9H_2 CoQ_9	ECD Ox-red-ox		Approx 1.1 g	Liquid-liquid with 1-propanol and water	No	90% for CoQ_9H_2 117% for CoQ_9 86% for $CoQ_{10}H_2$ 256% for CoQ_{10}		

CoQ_{10} = coenzyme Q_{10}; $CoQ_{10}H_2$ = coenzyme $Q_{10}H_2$ quinol; CoQ_9 = coenzyme Q_9; CoQ_9H_2 = coenzyme Q_9 quinol; TLC = thin layer chromatography; UV = ultraviolet detection; ECD = electrochemical detection; Ox = oxidation; Red = reduction; SDS = sodium dodecylsulfate; BHT = butylated hydroxytoluene; SPE = solid phase extraction; EDTA = ethylenediaminetetraacetic acid ; v/v = volume to volume.

Appendix 2. Summary of chromatography systems used in published assays of CoQ_{10} in biological specimens.

Publication	Sample type	Measuring	Detection	Mobile Phase	Column	LOD	% CV	Comments
(1)	Plasma	CoQ_{10}	UV	25/75 n-hexane/methanol	Finepak SIL C_{18-5} (250 × 4.6 mm, 5 µm)	10 ng (S/N = 5)		Used potassium hexacyanoferrate (III) to oxidise tissue $CoQ_{10}H_2$ but not plasma
(2)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Ox UV	4.5/10/85.5 2-propanol/methanol/ethanol + 20 mmol/L lithium perchlorate, 1 mL/min	Altex Ultrasphere ODS (250 × 4.6 mm, 5 m) or Altex Ultrasphere octyl (250 × 4.6 mm, 5 µm)	2 pmol (corresponding to a concentration of 0.1 µmol/L in the extract)	<10%	
(3)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Ox-red-ox	15/85 1-propanol/methanol + 33 mmol/L perchloric acid and 57 mmol/L sodium perchlorate for $CoQ_{10}H_2$ measurement, proportion of 1-propanol increased to 25 for measurement of total CoQ_{10}	Column 1 Spherisorb ODS-2 (100 × 4.6 mm, 3 µm) Column 2 Chromspher C18 (100 × 3 mm, 5 µm)		10%	Dual columns on HPLC – compounds strongly retained on the first column are washed off to waste – CoQ_{10} goes to second column
(4)	Tissue	CoQ_{10} Other analytes	Diode array	0.1/99.9 2-propanol/heptane	Cyanopropyl			
(5)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD	0.8/1.0/8.2 v/v/v methanol/water/ethanol + 0.05 mol/L sodium perchlorate	Capcell Pak C8 (SG 120, 150 × 4.6 mm, 5 µm)	100 pg (S/N = 3)	Intra- 3.1 and Inter- 4.0 % for CoQ_{10}	Post-column platinum catalyst reduction.

Publication	Sample type	Measuring	Detection	Mobile Phase	Column	LOD	% CV	Comments
(6)	Plasma	CoQ_{10} $CoQ_{10}H_2$ Carotenoids tocopherols	ECD Red-partial ox-complete ox	10/24/88 2-propanol/ethanol/methanol (v/v/v) + 13.4 mmol/L lithium perchlorate	SuperPac Pep-S $RP_{C2/C18}$ (250 × 4.0 mm, 5 µm	51 and 60 fmol/20 µL inject for $CoQ_{10}H_2$ and CoQ_{10} respectively (S/N = 3)	Intra- and inter- 12 and 32% for $CoQ_{10}H_2$ and 9 and 55 for oxidised CoQ_{10}	Large inter-run precision Requires minimal sample volume
(7)	Plasma	CoQ_{10}	UV	15/85 hexane/methanol	C18 RP column, Sepharon C18 (150 × 3 mm, 5 µm)	90 µg CoQ_{10} per Litre plasma		
(8)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD ox-red-ox	20/80 1-propanol/methanol + 32.5 mmol/L perchloric acid and 57 mmol/L sodium perchloric	Phase Sep s 5 ODS 2 (50 × 4.6 mm)		Inter- 9.3 – 12.3%, Intra- 3.4 – 7.9%	
(9, 10)	Tissues	$CoQ_{10}H_2$ CoQ_{10} CoQ_9H_2 CoQ_9 Vitamin E homologues	ECD UV	Gradient of (a) 20/80 water/methanol + 0.2% (w/v) lithium perchlorate and (b) ethanol with 0.2 % (w/v) lithium perchlorate	Beckman Ultrashpere ODS C_{18} column (250 × 4.6 mm, 5 µm)	0.3 pmol/L for ubiquinols 0.2 pmol/L for quinones		
(11)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD ox	15/85 (v/v) *tert*-butyl alcohol/methanol + 50 mmol/L sodium perchlorate	Supelcosil LC-8 (250 × 4.6 mm, 5 µm)	4 nmol/L (detection limit of plasma CoQ_{10} and $CoQ_{10}H_2$)		
(12)	Tissue	$CoQ_{10}H_2$ CoQ_{10} CoQ_9H_2 CoQ_9 Vitamin E and α-tocopherol oxidation products	ECD ox	Gradient of (a) 2/98 (v/v) water/methanol + 5 mmol/L zinc chloride, 2.5 mmol/L sodium acetate, and 2.5 mmol/L acetic acid, and (b) 2/49/49 (v/v/v) water/ethanol/methanol + 7.5 mmol/L zinc chloride, 3.75 mmol/L sodium acetate, and 3.75 mmol/L acetic acid	LiChrocart Merck RP column (125 × 4 mm, 5 µm), LiChrosorb 100 RP-18, Merck	0.6 pmol/L for $CoQ_{10}H_2$ 1 pmol/L for CoQ_{10}	Inter- 8 – 13% for $CoQ_{10}H_2$ 3-5 % for CoQ_{10}	Solid-phase post-column reactor dry packed with zinc particles (20 × 4.6 mm stainless steel column) was used for on-line reduction

Publication	Sample type	Measuring	Detection	Mobile Phase	Column	LOD	% CV	Comments
(13)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Red-ox	2.2/660/660/880 (v/v/v/v) 70% perchloric acid/acetonitrile/methanol/ethanol + 0.05 mol/L sodium perchlorate	SuperPac Pep-S (250 × 4 mm, 5µm) or SuperPac Sephasil (250 × 4 mm, 5 µm)			
(13)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD Red-ox					
(14)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD ox	Gradient of (a) 100% methanol + 50 mmol/L sodium perchlorate and 10 mmol/L perchloric acid and (b) 20/80 (v/v) *tert*-butanol/ethanol	Hewlett-Packard Hypersil C_{18} (125 × 4.0 mm, 5 µm)	2.5 nmol/L	Inter- 5.1% Intra- 3.3%	Chromatography is not very good, baseline is not stable
(15)	Plasma	$CoQ_{10}H_2$ CoQ_{10} and tocopherols	ECD Red-red-ox	6/17/77 (v/v/v) 2-propanol/ethanol/methanol + 31.7 mmol/L ammonium formate	Prontosil 120-3-C18-SH PEEK column (150 × 4 mm, 3 µm)	$CoQ_{10}H_2$ 25 fmol/20 µL injection volume CoQ_{10} 28 fmol/20 µL injection volume	Intra- 3-13% for all measured substances	
(16)	Plasma	CoQ_{10} $CoQ_{10}H_2$	ECD ox-red-ox	1.5/1.5/27.5/69.5 Glacial acetic acid/2-propanol/hexane/methanol + 50 mmol/L sodium acetate trihydrate	RP Microsorb MV (150 × 4.6 mm, 5 µm) Rainin.	11.6 nmol/L	Inter- and intra- 1.20 – 4.9 %	
(17)	Plasma	CoQ_{10}	UV	30/70 methanol/ethanol	Supelcosil LC18 (Supelco) (250 × 4.6 mm, 5 µm)	1.23 nmol/inject	Inter- 2%, Intra- 1.6%	
(18)	Plasma	$CoQ_{10}H_2$ CoQ_{10}	ECD and UV	4/21/75 ethanol/methanol/2-propanol + 20 mmol/L lithium perchlorate	Inertsil ODS-2 column (2 × 100 × 3.0 mm, 5 µm)		Inter- 5.9 % for $CoQ_{10}H_2$ and 3.1 % for CoQ_{10}	No limit of detection or recovery data given
(19)	Plasma	CoQ_{10}	UV	10/90 2-propanol/methanol	Hypersil ODS_2 (150 × 4.6 mm, 5 µm)	116 nmol/L	Inter <3%, Intra- <2%	Column switching eliminated polar and strongly retained solutes

Publication	Sample type	Measuring	Detection	Mobile Phase	Column	LOD	% CV	Comments
(20)	Erythrocytes and platelets	CoQ_{10}	ECD Red-red-ox	6/17/77 (v/v/v) 2-propanol/ethanol/methanol + 31.7 mmol/L ammonium formate	Prontosil 120-3-C18-SH PEEK column (150 × 4 mm, 3 µm)		Intra- and inter- 5% for total CoQ_{10} and 12% for oxidised CoQ_{10}	
(21)	Tissue	CoQ_{10} CoQ_9 Other analytes	UV	25/75 (v/v) hexane/methanol		3.6 ± 0.25 ng for CoQ_{10} 3.3 ± 0.15 ng for CoQ_9		Measured lots of things on a single tissue sample, so not optimised for CoQ.
(22)	Mouse Tissues	$CoQ_{10}H_2$ CoQ_{10} CoQ_9H_2 CoQ_9	ECD Ox-red-ox	1.5/1.5/14/83 (v/v/v/v) glacial acetic acid/2-propanol/hexane/methanol + 51.2 mmol/l sodium acetate anhydrous	RP Microsorb-MV (150 × 4.6 mm, 5 µm) Rainin	17 nmol/L	Intra- 2.7 – 5.6% for CoQ_9 and CoQ_{10} Inter 4.6 – 8% for CoQ_9 and CoQ_{10}	

CoQ_{10} = coenzyme Q_{10}; $CoQ_{10}H_2$ = coenzyme $Q_{10}H_2$ quinol; CoQ_9 = coenzyme Q_9; CoQ_9H_2 = coenzyme Q_9 quinol; UV = ultraviolet detection; ECD = electrochemical detection; Ox = oxidation; Red = reduction; EDTA = ethylenediaminetetraacetic acid ; w/v = weight to volume; S/N = signal to noise.

Apendix 3. The extraction procedures used in various methodologies for determining vitamin K.

Publication	Sample	Measuring	Detection	Sample volume	Extraction	Recovery	Injection volume	Comments
(23)	Infant formulas	K_1	Reflectance densiometry	N/A	Liquid-liquid, with diethyl ether and petroleum ether, then preparative column chromatographic separation then TLC	91 ± 3%		
(24)	Plant	K_1	GC	N/A	Liquid-liquid with hexane, purified on alumina column			
(25)	Human and cow milk and infant formula	K_1	UV	N/A	Liquid-liquid with chloroform and methanol (2/1 v/v). Washed extract with water, chloroform layer was evaporated to dryness. Solid phase extraction with silica, washed with light petroleum and eluted with light petroleum/diethyl ether (97/3 v/v). Evaporation and reconstitute residue in 50 – 100 µL mobile phase			Sample clean-up on first HPLC system, followed by second (analytical) HPLC
(26)	Serum	K_1 and menaquinones	Fluorescence and UV in series	2 mL	Precipitate proteins with ethanol, then liquid-liquid with hexane, evaporation, reconstitute in hexane		Complete extract	Sample clean-up on first HPLC system, followed by second (analytical) HPLC
(27)	Infant formulas	K_1	UV	N/A	Enzymatic hydrolysis of lipids, using lipase (1.5 hours incubation)	84 – 103%	10 µL	
(28)	Animal feed	K_3	Fluorescence	N/A	Aqueous extraction, then K3 is converted to menadione.	94.4 ± 6.8% (mean ± SD)	100 µL	
(29)	Plasma	K_1	ECD	3 mL	Liquid-liquid with hexane, and further purified with semi-preparative HPLC		30 µL	

Publication	Sample	Measuring	Detection	Sample volume	Extraction	Recovery	Injection volume	Comments
(30)	Plasma	K_1	Fluorescence	0.5 – 1.0 mL	Liquid-liquid with ethanol and hexane, evaporate hexane and reconstitute residue in hexane. Purify extract with silica 'Sep-Pak' column, which was washed with hexane, and vitamin K_1 eluted with diethyl ether/hexane (3/97 v/v) and evaporate. Redissolve residue in hexane, reduce vitamin K_1 and inject.		100 µL	'Reductive extraction'. Add to exract 70 mmol/L zinc chloride in 3/97 (v/v) acetic acid/acetonitrile (pH 2.1). Add 5-10 mg of zinc metal for 2 minutes, discard hexane layer, and evaporate acetonitrile layer, redissolve in hexane/water (75/25 v/v). Evaporate hexane and reconstitute in mobile phase
(31)	Plasma or serum	$K_{1(20)}$ and $K_{1(21)}$	Fluorescence	2mL	Liquid-liquid with ethanol and hexane. Hexane evaporated and residue redisolved in hexand/diisopropyl ether (98.5/1.5 v/v).	94%		Semi-preparative HPLC prior to analytical HPLC
(32)	Plasma	K_1	Fluorescence					
(33)	Plasma	K_1	Fluorescence	2mL	Liquid-liquid with ethanol and hexane. Hexane evaporated and residue redissolved in hexand/diisopropyl ether (98.5/1.5 v/v).	94%		Semi-preparative HPLC prior to analytical HPLC
(34)	Human and cow milk	K_1 and menaquinones	ECD	N/A	Enzymatic hydrolysis of milk (incubate with lipase for 1.5 hours), then extract hydrolysate with 1-pentane, evaporate and redissolve residue in 2-propanol, subject to semi-preparative then analytical HPLC.	>93%		Semi-preparative HPLC prior to analytical HPLC

Publication	Sample	Measuring	Detection	Sample volume	Extraction	Recovery	Injection volume	Comments
(35)	Plasma	K_1 and menaquinones	Fluorescence	1 mL	Denature proteins with ethanol, liquid-liquid extraction with hexane and diethyl ether. Addition of water then evaporate hexane, reconstitute residue in hexane and apply to a silica gel column, which is washed with hexane/benzene (3/2 v/v). Vitamin K eluted with hexane/benzene (1/2 v/v) and the eluate evaporated. Residue redissolved in hexane, and applied to an alumina column, washed with hexane/benzene (6/1 v/v) then elute with hexane/benzene (3/1 v/v). Eluate evaporated and redissolved in ethanol.		50 µL	Reduction post-column by platinum oxide catalyst column
(36)	Human serum	K_1 and vitamin E	Fluorescence	0.5 – 3 mL	Denature proteins with ethanol, extract lipids into hexane. Wash hexane layer with methanol/water (9/1 v/v). Evaporate hexane layer and reconstitute residue in 120 µL 2-propanol.	80%	100 µL	Reduction post-column with sodium borohydride in ethanol (21 nmol/L)
(37)	Animal tissues	K_1 and menaquinones	Fluorescence	1 g tissue sample	Tissue homogenised with 66% 2-propanol, then mixed with hexane. Hexane layer is evaporated and the residue redisolved in hexane, and applied to a Sep-Pak silica cartridge, which is washed with hexane and K vitamins are eluted with hexane/diethyl ether (96/4 v/v). Eluate is evaporated and reconstituted in hexane, which is then subjected to TLC on a silica gel 60 F_{254} plate. The vitamin K-containing spot is removed and extracted with chloroform, which is then evaporated, the residue redissolved in ethanol for injection	>73.5%	50 µL	Coulometric reduction
(38)	Plant extracts	K_1	Fluorescence	N/A	Dichloromethane extraction of the plant using a soxhlet apparatus. Evaporation (not to completion) and reconstitution of the extract in methanol.			Photochemical reduction

Publication	Sample	Measuring	Detection	Sample volume	Extraction	Recovery	Injection volume	Comments
(39)	Human liver	K_1 and menaquinones	Fluorescence	1g tissue	Tissue homogenised with 66% 2-propanol, then mixed with hexane. Hexane layer is evaporated and the residue redisolved in hexane, and applied to a Sep-Pak silica cartridge, which is washed with hexane and K vitamins are eluted with hexane/diethyl ether (96/4 v/v). Eluate is evaporated and reconstituted in hexane, which is then subjected to TLC on a silica gel 60 F_{254} plate. The vitamin K-containing spot is removed and extracted with chloroform, which is then evaporated, the residue redissolved in ethanol for injection	107.5 ± 3.0% (%CV)	50 µL	Platinum-black reduction column
(40)	Foods	K_1	Fluorescence	N/A	Liquid-liquid extraction into 2-propanol and hexane, then solid-phase extraction on Silica gel column. Sometimes an extra step of reversed-phase solid-phase extraction on C18 was required.			Post-column zinc-reduction
(41)	Infant formulas and milk produces	K_1	UV	N/A	Lipase in phosphate buffer to digest lipids. Semi-preparative normal-phase HPLC prior to reversed-phase HPLC and quantitation.			
(42)	Serum	K_1	Fluorescence	500 µL	Proteins were precipitated with 2-propanol, and sample extracted with hexane. Hexane was evaporated, and the residue redissolved in hexane. The residue was subjected to solid-phase extraction, which was washed with hexane, and vitamin K_1 was eluted with ether/hexane (3/97 v/v), which was evaporated, and the residue reconstituted in ethanol	70 ± 34% (mean ± SD) (n = 30)	50 µL	Reduction catalysed by 10% platinum-on-alumina
(43)	Plasma	K_1	GC/MS	1 mL	Proteins precipitated with methanol (addition of a small amount of water), followed by liquid-liquid extraction with hexane. Evaporated hexane, and redissolve residue in hexane.		10 µL	
(44)			Fluorescence					Zinc reduction

Publication	Sample	Measuring	Detection	Sample volume	Extraction	Recovery	Injection volume	Comments
(45)	Plasma	K_1 and phylloquinone 2,3-epoxide	Fluorescence	1 mL	Proteins precipitated with ethanol (addition of small amount of water), then liquid-liquid extraction with hexane. Hexane evaporated, residue reconstituted in 2-propanol (heated to 50 ºC to help dissolution) and subjected to solid-phase-extraction - C18 column, washed with water/methanol (5/95 v/v), and acetonitrile, and vitamin K eluted with dichloromethane/methanol (20/80 v/v). Eluent evaporated. Redissolve residue in 20 µL of 100% dichloromethane (added first) and 180 µL methanol containing 10 mmol/L zinc chloride, 5 mmol/L acetic acid, and 5 mmol/L sodium acetate (1 L methanol + 5 mL aqueous solution).	75.0 ± 5.0% (mean ± SD)	100 µL	Post-column zinc reduction (50 × 2 mm stainless steel column dry-packed with zinc metal). 100 × 4.6 mm ID column packed with 10% platinum on alumina placed pre-injector
(46)	Infant formulas and milk	K_1 and menaquinones	Fluorescence	N/A	Enzymatic digestion, then extraction	>98%	20 µL	Zinc reduction
(47)	Oils and margarines	K_1	ECD	N/A	Extraction with hexane, followed by straight-phase semi-preparative HPLC for margarine extracts.	98 – 102%	40 µL	ECD reduction
(48)	Human liver	K_1 and menaquinones	Fluorescence	1g tissue	Tissue homogenised with 66% 2-propanol, then mixed with hexane. Hexane layer is evaporated and the residue redissolved in hexane, and applied to a Sep-Pak silica cartridge, which is washed with hexane and K vitamins are eluted with hexane/diethyl ether (96/4 v/v). Eluate is evaporated and reconstituted in hexane, which is then subjected to TLC on a silica gel 60 F_{254} plate. The vitamin K-containing spot is removed and extracted with chloroform, which is then evaporated, the residue redissolved in ethanol for injection	89 – 107%	50 µL	Platinum-black catalysed reduction, held at 50 ºC

Publication	Sample	Measuring	Detection	Sample volume	Extraction	Recovery	Injection volume	Comments
(49)	Milk powders and milk	K_1	Fluorescence	N/A	Samples digested with lipase and extracted into hexane, an alliquot is evaporated, reconstituted into methanol and injected			Post-column reactor, 20 × 4 mm, dry-packed with zinc powder.
(50)	Plasma	K_1	Fluorescence	1 mL	The hexane extract, if necessary after evaporation of non-hexane solvents, was washed with a mixture of methanol and water, as previously described.			Zinc-powder reduction
(51)	Medical foods	K_1	Fluorescence	N/A	Sample enzymatically digested with lipase and α-amylase, and extracted with 1% sodium bicarbonate solution/isoporpanol (1/1 v/v). Then C18 solid phase extraction.	101.6 ± 2.85%		Post-column zinc reduction
(52)	Plasma	K_1	Fluorescence	0.1 – 0.5 mL	Proteins precipitated with ethanol, then hexane extraction. Hexane layer put onto silica cartridges, which are washed with hexane, and vitamin K is eluted with diethylether/hexane 3.5/96.5 v/v). Eluate is evaporated, and the residue reconstituted in 25 µL dichloromethane followed by 75 µL mobile phase component A.	92.3 ± 1.9% (n = 12)	45 µL	Zinc reduction
(53)	Serum or plasma	K_1, menaquinones 4 and 7	Fluorescence	0.5 mL	Plasma diluted with water, internal standards added, and sample extracted with ethanol and hexane. Hexane layer applied to Sep-Pak silica cartridge, which is washed with hexane, and K vitamins eluted with hexane/diethyl ether (97/3). Eluate evaporated and residue redissolved in ethanol.	>92%	40 µL	Platinum-black reduction. One HPLC system for menaquinone 4 and another for K_1 and menaquinone 7.

TLC = Thin-Layer Chromatography

Appendix 4. HPLC assays previously described for the determination of vitamin K.

Publication	Sample type	Measuring	Detection	Mobile phase	Column	LOD	%CV	Comments
(25) [part 1]	Human and cow milk and infant formula	K	UV	Methanol/dichloromethane (8/2 v/v)	Either microparticulate silica or an amino-cyano-bonded phase			Collect eluant containing K, evaporated and reconstitute in 50-100 µL mobile phase
(25) [part 2]	Human and cow milk and infant formula	K	UV (254 nm or 270 nm)	Methanol/dichloromethane (8/2 v/v) (1 mL/min) OR Methanol/dichloromethane (9/1 v/v) OR Acetonitrile/dichloromethane (7/3 v/v) OR Acetonitrile/dichloromethane 17/3 (v/v)	Zorbax-ODS Hypersil-ODS Zorbax-ODS Hypersil-ODS			
(26) [part 1]	Serum	K_1	UV	3% diisopropyl ether in hexane	Silica column (200 × 7 mm, Rsil 5 µm)			Collected elutant containing K_1, evaporated and reconstitute in 40 µL methanol, inject 20 µL
(26) [part 2]	Serum	K_1	UV (248 nm)	100% Methanol, 1 mL/min	RP C18	0.5 ng/mL serum	Within-day 5.3% (mean conc 5.5 ng/mL)	Photochemical induction for reduction, hydroquinone stabilised with ascorbic acid
(27)	Infant formulas	K_1	UV (254 nm)	Acetonitrile/methanol/tetrahydrofuran/water (39/39/16/6 v/v/v/v)				
(28)	Animal feed	K3	Fluorescence (ex 325 nm, em 425 nm)	Water/ethanol 4/6 (v/v) (0.6 mL/minute)	ODS-Hypersil	0.02 µg/g		Reduced K3 using sodium borohydride in ethanol, 21 mmol/L
(29) [part 1]	Plasma	K_1	ECD (red – ox)	1 mL/min	Normal- phase HPLC			Residues dissolved in 70 µL mobile phase
(29) [part 2]	Plasma	K_1	ECD (red – ox)	1 mL/min		50 pg	10% (n=6), mean value was 330 pg/mL.	

Publication	Sample type	Measuring	Detection	Mobile phase	Column	LOD	%CV	Comments
(30)	Plasma (EDTA)	K_1	Fluorescence (ex 248 nm, em 418 nm)	20/80 (v/v) dichloromethane/methanol to each litre of which was added 5 mL of a solution containing 2 mol/L zinc chloride, 1 mol/L sodium acetate, 1 mol/L aceic acid. Flow rate 1 mL/min.	Hypersil-ODS column. (250 × 4.6 mm, 5 µm)	0.05 µg/L	10%	Reduction with dry-packed zinc particles in a 20 × 3.9 mm stainless-steel column, placed post-column.
(31) [part 1]	Plasma or serum	K_1(20) and K_1(21)	Fluorescence	Hexane/diisopropyl ether (98.5/1.5 v/v). 0.85 mL/min	Semipreparative silica column, RoSIL 5 µm 200 × 4.6 mm)	50 pg/mL	Within-run 3.6% (n = 5, mean 311 pg/mL)	
(31) [part 2]	Plasma or serum	K_1(20) and K_1(21)	Fluorescence (ex 325 nm, em 430 nm)	Methanol/ethy acetate (96/4 v/v) at 0.85 mL/min	RSIL C18 HL 5 µm (150 × 3.2 mm)			Reduction after column by adding reducing agent (a methanolic solution of tetramethylammonium octahydridotriborate, 600 mg/100 mL), which is mixed with the column effluent at a flow rate of 0.3 mL/min. This reaction is performed at 80 ºC in a 'knitted coil reactor'.
(32)	Plasma	K_1	Fluorescence	20/80 (v/v) dichloromethane/methanol, with 10 mmol/L zinc chloride.	Hypersil ODS (250 × 4.6 mm)	25 pg		Solid-phase reactor is high-purity 200-mesh zinc particles dry-packed into a 20 × 3 mm stainless steel column.
(33)	Plasma	K_1	Fluorescence	Methanol/ethyl acetate (96/4 v/v) containing 130 mg tetramethylammonium octahydrotribotate per 100 mL) flow rate 0.7 mL/minute	RP C18 (RoSIL C18HL, 5 µm, 150 × 3.2 mm)			Post-column reactor was an open tubular knitted coil, total volume 980 µL) placed in a silicane bath at 80 ºC.
(34) [part 1]	Human and cow milk	K_1 and menaquinones	ECD	Methanol/acetonitrile (1/1 v/v), flow rate 4 mL/minute	Nucleosil C18 (5 µm, 300 × 8 mm), held at 35 ºC		8.3 – 15.2%	Semi-preparative HPLC prior to analytical HPLC.

Publication	Sample type	Measuring	Detection	Mobile phase	Column	LOD	%CV	Comments
(34) [part 2]	Human and cow milk	K_1 and menaquinones	ECD	Methanol/ethanol/60% perchloric acid (600/400/1.2 v/v/v) containing 0.05 mol/L $NaClO_4$ in total solution. Flow rate 1 mL/minute	Partisil ODS-2 (5 µm, 250 × 4.6 mm) for K_1 and menaquinone-4. Partisil ODS-3 (5 µm, 250 × 4.6 mm) for menaquinones 6,7,8, and 9.			
(35)	Plasma	K_1 and menaquinones	Fluorescence Ex 254 nm, em 430 nm	Methanol/ethanol/water (1/2/0.06 v/v/v), hydrogen gas saturated. Flow rate 1.2 mL/min	Nucleosil 5C18 (250 × 4.6 mm)	25 pg injected	4.8% (1.7 ng/mL, n = 4)	
(36)	Human serum	K_1 and vitamin E	Fluorescence (ex 320 nm, em 430 nm)	Ethanol/water (92/8 v/v), flow rate 0.9 mL/minute.	C18 Resolve column (150 × 3.9 mm, 5 µm)	30 pg per injection	Within-run 8.1% Between-run 12.9%	Stainless steel coil (0.8 mm ID × 1m) used as the reactor, held at 55 ºC, ethanolic sodium borohydride added to effluent from analytical column. Flow rate of reducing agent 0.6 mL/min.
(37)	Animal tissues	K_1 and menaquinones	Fluorescence (ex 320 nm and em 430 nm)	92.5 or 97.5 % ethanol containing 0.25% sodium perchlorate. Flow rate 1 mL/minute	Nucleosil C18 (150 × 4.6 mm, 5 µm)	40 pg/g		
(38)	Plant	K_1	Photoreduction fluorescence	Methanol/2-propanol (60/40 v/v)	Du Pont Zorbax ODS column (250 × 4.6 mm, 5 µm)			Solvent resovior equipped with a gas dispersion tube so the mobile phase could be sparged with ‘oxygen-free’ nitrogen. Reactors prepared from 30-gauge PTFE tubing (0.30 mm ID).

Publication	Sample type	Measuring	Detection	Mobile phase	Column	LOD	%CV	Comments
(39)	Human liver	K_1 and menaquinones	Fluorescence (ex 320 nm and em 430 nm)	Gradient – A: 100% methanol and B: 2-propanol/ethanol (4/1 v/v). 100% A for 15 minutes, then B from 0 - 80% from 15 – 90 minutes. Flow rate 1 mL/min	Nucleosil 5C18 (250 × 4.6 mm)	10 pg (S/N =3) for standard, 100 pg in liver		
(40)	Foods	K_1	Fluorescence	Methanol/methylene chloride (90/10 v/v), to each litre of which was added 5 mL of a solution containing 2 mol/L zinc chloride, 1 mol/L acetic acid, and 1 mol/L sodium acetate, flow rate 1 mL/minute.			Inter- and intra-day precision ranged from 6.6 – 13.6%	Reduction with post-column chemical reactor (2 × 50 mm) packed with zinc metal (-200 mesh).
(42)	Serum	K_1	Fluorescence (ex 242 nm, em 430 nm)	Ethanol/methanol 40/60 (v/v), flow rate 1 mL/minute	RP bonded-phase silica column (210TP 54, 250 × 4.6 mm, 5 µm) Vydac Hesperia		Relative standard deviation 32% (n = 30, 420 pg/mL)	Reduction catalysed by 10% platinum-on-alumina (50 × 2 mm column bed), an oxygen reducer column (10% platinum-on-alumina, 100 × 4.6 mm) placed pre-injector
(45)	Plasma	K_1	Fluorescence (ex 244 nm, em 418 nm)	Dichloromethane/methanol (10/90 v/v) to each litre of which is added 5 mL of an aqueous solution of 2 mol/L zinc chloride, 1ol/L glacial acetic acid, and 1mol/L sodium acetate. Flow rate 0.6 mL/minute	BDS-Hypersil (150 × 3 mm, 3 µm)	33 pmol/L	Within-run 5.6% (n=12), between-run 11.8% (n = 14)	
(46)	Infant formulas and milk	K_1 and menaquinones	Fluorescence (ex 243nm, em 430 nm)	Dichloromethane/methanol (100/900 v/v) to which was added a methanolic solution of zinc chloride (10 mmol/L), anhydrous sodium acetate (5 mmol/L), and glacial acetic acid (5 mmol/L). Flow rate 1.5 mL/min	C18 resolve cartridge column (100 × 8 mm ID, 5 µm)	30 pg on column		
(47)	Oils and margarines	K_1	ECD	95% Methanol/sodium acetate buffer (0.05M, pH3). Flow rate 1 mL/minute	Vydac 201 TP54 column (250 × 4.6 mm, 5 µm)	50 pg/injection		

Publication	Sample type	Measuring	Detection	Mobile phase	Column	LOD	%CV	Comments
(48)	Human liver	K_1 and menaquinones	Fluorescence (ex 320 nm and em 430 nm)	Gradient – A: 100% methanol and B: 2-propanol/ethanol (4/1 v/v). 100% A for 15 minutes, then B from 0 - 80% from 15 – 90 minutes. Flow rate 1 mL/min	Nucleosil 5C18 (250 × 4.6 mm). Column held at 50 ºC.	5 pg for MK-4, 10 pg for vitamin K_1, and MK-5 and –6, 20 pg for MK-7-9, and 40 pg for MK-10-13		
(49)	Milk powders, milk formula, and milk	K_1	Fluorescence (ex 243nm, em 430 nm)	Dichloromethane/methanol (100/900 v/v) + 5 mL methanol containing 2 mol/L zinc chloride, 1 mol/L anhydrous sodium acetate, and 1 mol/L glacial acetic acid.	Any C18 column (monomeric or polymeric, containing 5 µm spherical particle silica with >10% carbon loading.			
(50)	Plasma	K_1	Fluorescence	Methanol/dichloromethane, with zinc chloride, sodium acetate, and acetic acid.		0.09 nmol/L		
(51)	Medical foods	K_1	Fluorescence	Methanol/methylene chloride (900/100 v/v) + 10 mL of 2 M zinc chloride, 1 M sodium acetate, and 1 M acetic acid in methanol	Zorbax C18 (250 × 4.6 mm, 5 µm)	8 pg Limit of quantitation 27 pg on column		

Publication	Sample type	Measuring	Detection	Mobile phase	Column	LOD	%CV	Comments
(52)	Plasma	K_1	Fluorescence (ex 244nm, em 430 nm)	Stepwise gradient: Component A: 994.5 mL methanol and 5.5 mL aqueous solution of 2 mol/L ZnCl, 1 mol/L acetic acid, and 1 mol/L sodium acetate. Component B: 100% dichloromethane. 95% component A and 5% B for 10 minutes, then 65% A and 35% B for 13 minutes, and finally 95%A and 5% B for 5 minutes. Flow rate 0.6 mL/minute.	Hypersil BDS C18 column (3.2 × 150 mm, 3 µm) held at 22 ºC.	4 fmol per injection (S/N = 3). Limit of quantitation for 0.25 mL plasma was 0.04 nmol/L	Intra-assay 8.2% (n = 6) and inter-assay 12% (n = 21) for mean K_1 concentration of 1.4 nmol/L	
(53)	Serum or Plasma	K_1, menaquinones 4 and 7	Fluorescence	For menaquinone-4: 95/5 (v/v) methanol/water For menaquinone-7 and vitamin K_1: 95/5 (v/v) methanol/ethanol. Flow rates 1 mL/minute	CAPCELL PAK C18 uG120 (250 × 4.6 mm, 5 µm). Column held at 35 ºC.	LOD for menaquinone-4, vitamin K_1, and menaquinone-7 was 4, 2, and 4 pg respectively	Inter-and intra-assay %CV was 5.7-9.2% for menaquinone-4, 4.9-9.6% for vitamin K_1 and 6.3-19.3% for menaquinone-7	Platinum reduction column: 15 × 4 mm, RC-10, Irica Kyoto, Japan.

UV = ultraviolet detection, HPLC = high-performance liquid chromatography, ECD = electrochemical detection, K_1 = vitamin K_1, red = reduction, ox = oxidation, em = emission wavelength, ex = excitation wavelength, MK = menaquinone.

REFERENCES FOR APPENDICES

1. Okamoto T, Fukui K, Nakamoto M, Kishi T, Okishio T, Yamagami T, et al. High-performance liquid chromatography of coenzyme Q-related compounds and its application to biological materials. Journal of Chromatography 1985;342:35-46.
2. Lang JK, Gohil K, Packer L. Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates, and subcellular fractions. Analytical Biochemistry 1986;157:106-16.
3. Edlund PO. Determination of Coenzyme Q_{10}, α-tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. Journal of Chromatography 1988;425:87-97.
4. Greenspan MD, Lee Lo C-Y, Hanf DP, Yudkovitz JB. Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector. Journal of Lipid Research 1988;29:971-6.
5. Wakabayashi H, Yamato S, Nakajima M, Shimada K. Simultaneous determination of oxidized and reduced Coenzyme Q and α-Tocopherol in biological samples by high performance liquid chromatography with platinum catalyst reduction and electrochemical detection. Biological and Pharmaceutical Bulletin 1994;17:997-1002.
6. Finckh B, Kontush A, Commentz J, Hübner C, Burdelski M, Kohlschütter A. Monitoring of Ubiquinol-10, carotenoids, and tocopherols in neonatal plasma microsamples using high-performance liquid chromatography with coulometric electrochemical detection. Analytical Biochemistry 1995;232:210-6.
7. Kaplan P, Sebestianová N, Turiaková J, Kucera I. Determination of Coenzyme Q in human plasma. Physiological Research 1995;45:39-45.
8. Lagendijk J, Ubbink JB, Delport R, Hayward WJ, Human JA. Measurement of the ratio between the reduced and oxidized forms of coenzyme Q10 in human plasma as a possible marker of oxidative stress. Journal of Lipid Research 1996;37:67-75.
9. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. Journal of Lipid Research 1996;37:893-901.
10. Podda M, Weber C, Traber MG, Milbradt R, Packer L. Sensitive high-performance liquid chromatography techniques for simultaneous determination of tocopherols,

tocotrienols, ubiquinols, and ubiquinones in biological samples. Methods in Enzymology, Vol. 299, 1999:330-41.

11. Yamashita S, Yamamoto Y. Simultaneous detection of ubiquinol and ubiquinone in human plasma as a marker of oxidative stress. Analytical Biochemistry 1997;250:66-73.
12. Leray C, Andriamampandry MD, Freund M, Gachet C, Cazenave JP. Simultaneous determination of homologues of vitamin E and coenzyme Q and products of alpha-tocopherol oxidation. Journal of Lipid Research 1998;39:2099-105.
13. Kaikkonen J, Nyyssönen K, Salonen JT. Measurement and stability of plasma reduced, oxidised and total coenzyme Q10 in humans. Scandinavian Journal of Clinical and Laboratory Investigation 1999;59:457-66.
14. Wang Q, Lee BL, Ong CN. Automated high-performance liquid chromatographic method with precolumn reduction for the determination of ubiquinol and ubiquinone in human plasma. Journal of Chromatography B 1999;726:297-302.
15. Menke T, Niklowitz P, Adam S, Weber M, Schlüter B, Andler W. Simultaneous detection of ubiquinol-10, ubiquinone-10, and tocopherols in human plasma microsamples and macrosamples as a marker of oxidative damage in neonates and infants. Analytical Biochemistry 2000;282: 209-17.
16. Tang PH, Miles, M.V., DeGrauw, A., Hershey, A., Pesce, A. HPLC analysis of reduced and oxidised coenzyme Q10 in human plasma. Clinical Chemistry 2001;47:256-65.
17. Mosca F, Fattorini D, Bompadre S, Littarru G. Assay of coenzyme Q10 in plasma by a single dilution step. Analytical Biochemistry 2002;305:49-54.
18. Hectors MPC, van Tits LJH, de Rijke YB, Demacker PNM. Stability studies of ubiquinol in plasma. Annals of Clinical Biochemistry 2003;40:100-1.
19. Jiang P, Wu M, Zeheng Y, Wang C, Li Y, Xin J, Xu G. Analysis of coenzyme Q10 in human plasma by column-switching liquid chromatography. Journal of Chromatography B 2004;805:297-301.
20. Niklowitz P, Menke T, Andler W, Okun JG. Simultaneous analysis of coenzyme Q10 in plasma, erythrocytes and platelets: comparison of the antioxidant level in blood cells and their environment in healthy children and after oral supplementation in adults. Clinica Chimica Acta 2004;342:219-26.

21. Sharma SK, Ebadi M. An improved method for analyzing coenzyme Q homologues and multiple detection of rare biological samples. Journal of Neuroscience Methods 2004;137:1-8.
22. Tang PH, Miles MV, Miles L, Quinlan J, Wong B, Wenisch A, Bove K. Measurement of reduced and oxidized coenzyme Q_9 and coenzyme Q_{10} levels in mouse tissues by HPLC with coulometric detection. Clinica Chimica Acta 2004;341:173-84.
23. Manes JD, Fluckiger HB, Schneider DL. Chromatographic analysis of vitamin K1; Application to infant formula products. Journal of Agricultural and Food Chemistry 1972;20:1130-2.
24. Seifert RM. Analysis of vitamin K_1 in some green leafy vegetables by gas chromatography. Journal of Agricultural and Food Chemistry 1979;27:1301-4.
25. Haroon Y, Shearer MJ, Rahim S, Gunn WG, McEnery G, Barkhan P. The content of phylloquinone (vitamin K_1) in human milk, cows milk and infant formula foods determined by high-performance liquid chromatography. The Journal of Nutrition 1982;112:1105-17.
26. Lefevere MF, De Leenheer AP, Claeys AE, Claeys IV, Steyaert H. Multidimensional liquid chromatography: a breakthrough in the assessment of physiological vitamin K levels. Journal of Lipid Research 1982;23:1068-72.
27. Bueno MP, Villalobos MC. Vitamins and other nutrients. Journal of AOAC International 1983;66:1063-7.
28. Speek AJ, Schrijver J, Schruers WHP. Fluorimetric determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes by high-performance liquid chromatography with post-column derivatization. Journal of Chromatography 1984;301:441-7.
29. Hart JP, Shearer MJ, McCarthy PT. Enhanced sensitivity for the determination of endogenous phylloquinone (vitamin K_1) in plasma using high-performance liquid chromatography with dual-electrode electrochemical detection. Analyst 1985;110:1181-3.
30. Haroon Y, Bacon DS, Sadowski JA. Liquid-chromatographic determination of vitamin K_1 in plasma, with fluorometric detection. Clinical Chemistry 1986;32:1925-9.

31. Lambert WE, De Leenheer AP, Lefevere MF. Determination of vitamin K in serum using HPLC with post-column reaction and fluorescence detection. Journal of Chromatographic Science 1986;24:76-9.
32. Haroon Y, Bacon DS, Sadowski JA. Chemical reduction system for the detection of phylloquinone (Vitamin K_1) and menaquinones (Vitamin K_2). Journal of Chromatography 1987;384:383-9.
33. Lambert WE, De Leenheer AP. Simplified post-column reduction and fluorescence detection for the high-performance liquid chromatographic determination of vitamin $K_{1(20)}$. Analytica Chimica Acta 1987;196:247-50.
34. Isshiki H, Suzuki Y, Yonekubo A, Hasegawa H, Yamamoto Y. Determination of phylloquinone and menaquinone in human milk using high performance liquid chromatography. Journal of Dairy Science 1988;71:627-32.
35. Shino M. Determination of endogenous vitamin K (phylloquinone and menaquinone-n) in plasma by high-performance liquid chromatography using platinum oxide catalyst reduction and fluorescence detection. Analyst 1988;113:393-7.
36. Cham BE, Roeser HP, Kamst TW. Simultaneous liquid-chromatographic determination of vitamin K_1 and vitamin E in serum. Clinical Chemistry 1989;35:2285-9.
37. Hirauchi K, Sakano T, Notsumoto S, Nagaoka T, Morimoto A, Fujimoto K, et al. Measurement of K vitamins in animal tissues by high-performance liquid chromatography with fluorimetric detection. Journal of Chromatography 1989;497:131-7.
38. Poulsen JR, Birks JW. Photoreduction fluorescence detection of quinones in high-performance liquid chromatography. Analytical Chemistry 1989;61:2267-76.
39. Usui Y, Nishimura N, Kobayashi N, Okanoue T, Kimoto M, Ozawa K. Measurement of vitamin K in human liver by gradient elution high-performance liquid chromatography using platinum-black catalyst reduction and fluorimetric detection. Journal of Chromatography 1989;489:291-301.
40. Booth SL, Davidson KW, Sadowski JA. Evaluation of an HPLC method for the determination of phylloquinone (vitamin K_1) in various food matrices. Journal of Agricultural and Food Chemistry 1994;42:295-300.

41. Indyk H, Littlejohn VC, Lawrence RJ. Liquid chromatographic determination of vitamin K_1 in infant formulas and milk. Journal of AOAC International 1995;78:719-23.
42. MacCrehan WA, Schönberger E. Determination of vitamin K_1 in serum using catalytic-reduction liquid chromatography with fluorescence detection. Journal of Chromatography B 1995;670:209-17.
43. Fauler G, Leis HJ, Schalamon J, Muntean W, Gleispach H. Method for the determination of vitamin $K_{1(20)}$ in human plasma by stable isotope dilution/gas chromatography/mass spectrometry. Journal of Mass Spectrometry 1996;31:655-60.
44. Jakob E, Elmadfa I. Application of a simplified HPLC assay for the determination of phylloquinone (vitamin K_1) in animal and plant food items. Food Chemistry 1996;56:87-91.
45. Davidson KW, Sadowski JA. Determination of vitamin K compounds in plasma or serum by high-performance liquid chromatography using postcolumn chemical reduction. Methods in Enzymology 1997;282:408-21.
46. Indyk H, Woollard DC. Vitamin K in milk and infant formulas: Determination and distribution of phylloquinone and menaquinone-4. Analyst 1997;122:1-5.
47. Piironen V, Koivu T, Tammisalo O, Mattila P. Determination of phylloquinone in oils, margarines and butter by high-performance liquid chromatography with electrochemical detection. Food Chemistry 1997;59:473-80.
48. Usui Y. Assay of phylloquinone and menaquinones in human liver. Methods in Enzymology 1997;282:438-47.
49. Indyk H, Woollard DC. Determination of vitamin K in milk and infant formulas by liquid chromatography: Collaborative study. Journal of AOAC International 2000;83:121-30.
50. Jakob E, Elmadfa I. Rapid and simple HPLC analysis of vitamin K in food, tissues and blood. Food Chemistry 2000;68:219-21.
51. Ware GM, Chase GW, Eitenmiller RR, Long AR. Determination of vitamin K_1 in medical foods by liquid chromatography with postcolumn reduction and fluorometric detection. Journal of AOAC International 2000;83:957-62.
52. Wang LY, Bates CJ, Yan L, Harrington DJ, Shearer MJ, Prentice A. Determination of phylloquinone (vitamin K_1) in plasma and serum by HPLC with fluorescence detection. Clinica Chimica Acta 2004;347:199-207.

53. Kamao M, Suhara Y, Tsugawa N, Okano T. Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. Journal of Chromatography B 2005;816:41-8.