CAPILLARY ELECTROPHORESIS

IMPROVING CLINICAL MEASUREMENT OF CLOZAPINE

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BGE	background electrolyte
CE	capillary electrophoresis
CE-MS	capillary electrophoresis – mass spectrometry
CGE	capillary gel electrophoresis
CZE	capillary zone electrophoresis
CITP	capillary isotachophoresis
CIEF	capillary isoelectric focusing
CV	coefficient of variation
CD	cyclodextrins
DTNBP1	dysbinding dystrobrevin binding-protein 1
EOF	electro-osmotic flow
μ_e	electrophoretic mobility
μ_{EOF}	electro-osmotic flow mobility
EQC	external quality control
EPS	extrapyramidal side effects
HPFA-CE	high performance frontal analysis-capillary electrophoresis
HPLC	high performance liquid chromatography
HS-γ-CD	highly sulphated γ-CD
i.d.	internal diameter
MEKC	micellar electrical chromatography
MCGE	microchip capillary gel electrophoresis
MEEKC	microemulsion electrokinetic chromatography
t	migration time
MARTA	multi-acting receptor-targeted antipsychotics
NRG1	neuregulin 1
$H_3PO_4^-$	phosphoric Acid
$KH_2PO_4 \cdot H_2O$	potassium dihydrogen phosphate buffer
Na_2CO_3	sodium carbonate solution
SD	standard deviation
SDA	serotonin-dopamine antagonists
$NaH_2PO_4 \cdot H_2O$	sodium dihydrogen phosphate solution
SDS	odium dodecyl sulphate
NaOH	sodium hydroxide

TD	tardive dyskinesia
N	theoretical plates
TCA	trichloroacetic acid
μ_{tot}	total mobility
ζ	zeta potential

ABSTRACT

Schizophrenia is a mental disorder affecting approximately one percent of the population worldwide. The introduction of the second generation antipsychotic drug, atypical antipsychotic, clozapine, has demonstrated 80% reduction in suicide incident. This drug showed effectiveness in the treatment of resistant schizophrenia, however, high concentrations of clozapine and N-desmethylclozapine in plasma exhibit the development of agranulocytosis, a possible lethal blood disorder. Therefore, constant therapeutic drug monitoring is important for patients who receive clozapine.

High performance liquid chromatography (HPLC) is the current assay for clinical clozapine measurement. A different assay, the capillary electrophoresis (CE) was explored in this study. It was found the use of a background electrolyte (BGE) concentration of 60 mM, pH at 2.5, temperature at 22 ^{0}C, voltage applied at 10 kV and sample injection at 23 kV for 1.5 seconds is the optimal condition for clozapine separation using a fused-silica capillary 75 μm in internal diameter (i.d.). The use of 75 μm (i.d.) fused-silica capillary not only permits a larger sample size, but also provided longer detection pathlength which increased the limit of detection for CE.

One hundred and eight patients' samples were analysed by CE and compared with HPLC results obtained from the Canterbury Health Laboratory. A linear regression line of 1.100 was obtained. Seven External Quality Control (EQC) samples were also analysed and compared to the HPLC results gained from the EQC program world wide. A linear regression line of 1.008 and 1.043 were obtained from clozapine and N-desmethylclozapine separation respectively. The developed CE method has shown to be a valid assay for clozapine and N-desmethylclozapine separation and a more cost effective method compared to HPLC.

INTRODUCTION

1.1 Overview

Schizophrenia is a mental disorder affecting approximately one percent of the population worldwide. This mental illness is often described as a “split mind”, because schizophrenic patients may experience hallucinations and delusions. Unfortunately, suicide claims 10% of lives afflicted by this debilitating mental disorder (Murray et al,. 1996; Meltzer et al., 2003). Antipsychotic drug treatment includes both typical and atypical antipsychotic. Clozapine is an atypical antipsychotic, known as a second generation drug for schizophrenia treatment. Clozapine has been shown to give improvement in positive and negative psychotic symptoms, and it shows high effectiveness in treatment-resistant schizophrenia patients (Breier et al., 1994, Iqbal et al., 2003). However, it was found approximately 1-2% of patients who received clozapine, without monitoring, developed agranulocytosis, a potentially fatal blood disorder (Amsler et al., 1977). The major metabolite of clozapine, N-desmethylclozapine, also exhibits toxici effects at high concentrations (Gerson et al., 1994). Therefore, it is relatively important to perform regular therapeutic drug monitoring for schizophrenic patients who receive clozapine.

High performance liquid chromatography (HPLC) is the current method for clozapine measurement at Canterbury Health Laboratories. This study will explore a different technique, capillary electrophoresis (CE), for clozapine quantification from plasma samples. A protocol for CE will be developed in this study, and the results will be compared with HPLC.

1.2 Schizophrenia

Schizophrenia is one of the top ten causes of disability in developed countries (Murray et al,. 1996), and found in one percent of the population worldwide. People with

schizophrenia behave differently, they may hear ‘voices’ or talk to things that do not exist, they may also become ‘flat’ and, eventually, socially withdrawn. Therefore, the effects of schizophrenia have been a great concern for many years. It is estimated 50% of the patients with schizophrenia have attempted suicide, and 10-13% of them have succeeded (Meltzer et al., 2003).

Eugene Bleuler is the first person to characterise the symptoms of schizophrenia into two categories, “positive” and “negative” symptoms. “Positive” symptoms are additional sensations or feelings of patients such as hallucinations, delusions and thought confusions. “Negative” symptoms are those normal abilities or feelings considered reduced or absent; such are alogia (poverty of speech), affective flattening (reducing emotional expression), and avolition (social dysfunction). “Cognitive” symptoms including disorganised thinking, poor memory, poor concentration and difficulty in understanding are criteria also considered in diagnosing schizophrenia in recent years (Black and Boffeli, 1989; Capuano et al., 2002). The Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR is used in diagnosing schizophrenia.

1.2.1 Genetic and Environmental Epidemiology

The causes of schizophrenia are considered to be a combination of many elements, including environmental and genetic factors. It has been known for many years that schizophrenia runs in families. Therefore, it is not surprising such a disorder is inheritable. There is strong evidence showed two genes to schizophrenia which encod dysbinding dystrobrevin binding-protein 1 (DTNBP1) and neuregulin 1 (NRG1). The mechanisms of how these genes correlate to schizophrenia are currently unknown. Many studies significantly suggested that environmental stressors strongly contributed to the development of schizophrenia (Cardno and Gottesman, 2000). Several other factors were also reported to increase the risk of schizophrenia, such as maternal infections, influenza, genital infection, taxoplasmosis gondii parasite exposure, alcohol toxicity and stress during pregnancy. Intake of cannabis and other street drugs may also increase the risk of schizophrenia development (Arendt et al. 2005). Predisposition in one gene is insufficient to cause such a disorder; it is a complex network where

combinations of many gene interactions with the aid of environmental factors contribute to the risk of triggering schizophrenia (Tsuang, 2000). Multiple hypotheses regarding neurotransmitter levels have arisen to explain the etiology of schizophrenia, such as the dopamine (Capuano et al., 2002), glutamate (Konradi and Heckers, 2003), serotonin (Breier 1995), and the recent N-3 fatty acid hypothesis (Koichi, 2006).

1.2.2 Antipsychotic Drug Treatment for Schizophrenia

Medication therapies provide the most ameliorative properties on positive and negative symptoms in schizophrenia. There are two groups of antipsychotic drugs, “typical” antipsychotic drugs and “atypical” antipsychotic drugs. Typical antipsychotics (also referred to as conventional antipsychotics) are the “first-generation” antipsychotic drugs, which improve positive symptoms of schizophrenia. Nevertheless, 30-60% of patients given “typical” antipsychotics exhibit a wide range of side effects, often in the central nerve system, cardiovascular system, and endocrine system (Iqbal et al., 2003). These patients are grouped as “treatment resistant” schizophrenics, and eventually withdrawn from typical antipsychotic treatment. Atypical antipsychotics are the “second-generation” drugs, which are more effective than typical antipsychotic drugs. Atypical antipsychotic drugs alleviate not only positive symptoms, but also negative and cognitive symptoms. What separates atypical antipsychotics from typical antipsychotic drugs are that they do not cause extrapyramidal side effects (EPS) (movement disorder - similar to Parkinson’s disease), as well as other side effects such as tardive dyskinesia (TD) (Kapur and Remington, 2001).

1.2.2.1 Typical Antipsychotic Drugs

There are several varieties of typical antipsychotic; chlorpromazine, fluphenazine, haloperidol, molindone, thiothixene, thioridazine, trifluoperazine, loxapine, perphenazine, prochlorperazine, and pimozide. However, typical antipsychotic drugs often worsen negative symptoms and cause side effects, such are as EPS, TD, elevated prolactin, galactorrhoea in female and gynecomastia in male. Other pharmacological side effects

also include dry mouth, constipation, urinary retention, cognitive dumbness, orthostatic hypotension, and weight gain. The mechanisms of these side effects are not fully understood, however, they are probably due to the non-selective high D_2 receptors potency in mesolimbic and striatal regions of the brain. High D_2 receptor antagonism also results in reducing dopamine concentration in the mesocortical region of the brain, which leads to worsening negative and cognitive symptoms (Serretti et al., 2004).

1.2.2.2 Atypical Antipsychotic Drugs

Atypical antipsychotic drugs include amisulpride, aripiprazole, clozapine, fluoxetine, melperone, olanzapine, paliperidone, quetiapine, risperidone, sertindole, ziprasidone, and zotepine. These drugs are distinct from typical antipsychotics due to the lack of EPS and TD expression. They are classified into four groups; the serotonin-dopamine antagonists (SDA), multi-acting receptor-targeted antipsychotics (MARTA), D2/D3 receptor antagonists, and the partial dopamine receptor agonists. SDA show high selectivity for D_2 receptors and serotonin 5-TH_{2A} receptors. MARTA exhibit high affinity for D_2 receptors and 5-HT receptors, as well as other neurotransmitter receptors, such as adrenergic, muscarinic, histaminic, and cholinergic receptors (Horacek et al., 2006, and Iqbal et al., 2003). Patients who have treatment resistant schizophrenia often showed improvement after they received atypical antipsychotic drugs.

1.3 Clozapine

Out of all the atypical antipsychotic drugs, clozapine was found to be the most effective drug for alleviating both positive and negative symptoms. Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo(b,e)(1,4)diazepine) was first developed in 1961 (Hunziker et al., 1963; Iqbal et al., 2003) (Figure 1.4.1). It is a tricyclic dibenzodiazepine-derivative, atypical antipsychotic drug which exhibits low risk for EPS and TD (Kapur and Remington, 2001). The oral bioavailability of clozapine is about 60%, while 50% is excreted through urine, and 30% through faeces. Around 95% of clozapine is bound to plasma proteins. The maximum plasma concentration is reached in 1.5 – 3.6 hours, and

the steady-state plasma concentration is reached in less than 10 days. The half-life of clozapine is 14–16 hours, and total body clearance occurs at a rate of 30-57 L per hour (Wagstaff and Bryson, 1995). The plasma level of clozapine is also varied by many factors, such as smoking, gut absorption, hepatic metabolism, age, sex, and ethnic origin. These lead to difficulties in the determination of clozapine dosage. Therefore, clozapine concentrations in plasma have been used to optimise drug therapy. The optimal plasma concentration of clozapine was reported between 1070-1840 nM for clinical activity (Hiemke et al., 2000). A recent case report has found that relapses, such as increasing both positive and negative symptoms, were occurring at serum clozapine levels lower than 420 nM. The range of serum clozapine levels where schizophrenic patients were found to be most stable was between 660 nM and 2000 nM. Furthermore, increased adverse effects were found at a serum clozapine level of 3540 nM (Ulrich et al., 2003).

Clozapine is metabolised in the liver via cytochrome P450, mainly by CYP1A2, CYP3A4, and CYP2D6. Genetic variances of these enzymes within individuals can alter the pharmacokinetic properties of clozapine metabolism. Patients who are defined as slow metabolisers by the activity of these enzymes may exhibit side effects when augmentation therapy of clozapine or other medications are applied (Prior et al., 1999). It was found that clozapine can be metabolised into more than twelve different metabolites which do not appear to have any significant clinical activities. The major metabolic pathway is the phase I pathway, which results in demethylation or hydroxylation on position 7 or 8 of the benzodiazepine aromatic ring (Dain et al., 1997). The main metabolite in plasma and urine is N-desmethylclozapine, which was found to be pharmacologically active (Jann et al., 1993 and Dain et al., 1997) (Figure 1.4.1). The mean concentrations of N-desmethylclozapine in plasma are 440-1320 nM (Liu et al., 1996). The ratio of N-desmethylclozapine and clozapine has seemed to vary widely, within a range of 0.6-1.0 by Wagstaff and Bryson (1995). An investigation of 58 patients who received clozapine has shown correlation between plasma N-desmethylclozapine concentration and granulocyte counts (Comb et al., 1997). Gerson et al (1994) reported that N-desmethylclozapine may exhibit bone marrow toxicity. An *in vitro* study by Gerson et al, suggested that N-desmethylclozapine has the potential for clozapine-induced agranulocytosis. Unfortunately, the concentration of N-desmethylclozapine cannot be precisely predicted by the concentration of its parent drug, therefore

therapeutic drug monitoring for N-desmethylclozapine is relatively important to identify patients who might be at the risk of bone marrow suppression, as well as agranulocytosis development.

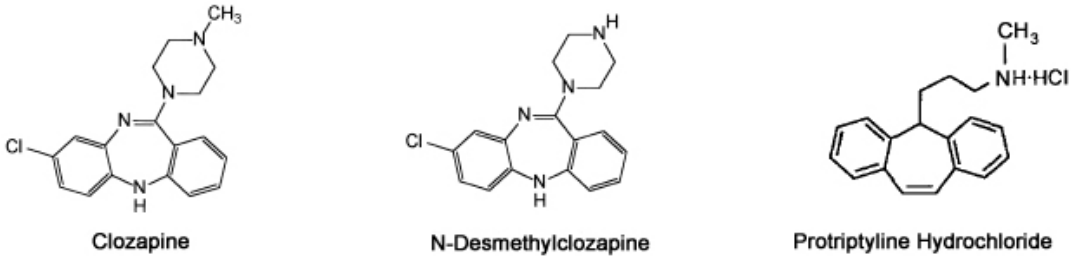


Figure 1.3.1 Molecular structure of Clozapine, N-desmethylclozapine and the internal standard protriptyline hydrochloride (Adapted from Raggi et al., 2001; Shim et al., 1997)

The fundamental mechanism of how clozapine effectively reduces both positive and negative symptoms, and lowers the propensity to produce EPS remains uncertain. However, clozapine exhibits as a MARTA-type of antipsychotic drug, which has a unique receptor binding mechanism. Clozapine shows low antagonistic effects for the dopamine D2 receptor, but high affinity for dopamine D_1 and D_4 receptors, which is in contrast to typical antipsychotic drugs. Clozapine also shows high antagonistic effects on the serotonin 5-HT_2 receptors, which resulted in increasing dopamine level in the stratum and reduced EPS. Clozapine also has an antagonistic effect toward histamine H_1, muscarinic M_1 and adrenergic α_1- and α_2-receptor. (Wagstaff and Bryson, 1995 ; Horacek et al., 2006 ; Iqbal et al., 2003 ; Gerlach and Peacock, 1995).

Out of all atypical antipsychotic drugs, clozapine demonstrated the most promising results. Clozapine has showed efficacy toward treatment-resistant schizophrenia by reducing positive, negative, and cognitive symptoms. A research study has demonstrated 30-60% of treatment-resistant schizophrenic patients respond to clozapine (Iqbal et al., 2003). A non-blinded study also showed that 81% of patients respond to clozapine within 6 months of commencement of treatment (Meltzer et al.,

1989). A study from Reid et al (1998) has demonstrated that the suicide rate was reduced by 80% when compared with patients receiving any antipsychotic drug. Even though the mechanism of preventing suicide incidents by clozapine is currently unknown, the beneficial outcomes from clozapine prove it to be the first-line therapeutic drug out of all atypical antipsychotic.

It was reported in the 1970's that approximately 1-2% of patients who received clozapine developed agranulocytosis (granulocyte count < 500/mm^3), a potentially fatal blood disorder which results in low white blood cell count with a mortality rate of 3-4% (Amsler et al., 1977 ; Gerson, 1994). Therefore, clozapine was removed from the market in 1975. However, the benefits and efficacy provided by clozapine far outweigh its risks, clozapine was reintroduced in 1990's. Accepting that the use of clozapine exposes schizophrenic patients to certain risks, regular therapeutic drug monitoring and white blood cell count monitoring is necessary for those who receive clozapine.

1.4 Capillary Electrophoresis

Electrophoresis is a technique for charged molecules to be separated based on their charge to mass ratio by attraction or repulsion in an electric field. That is, cations move toward the negative electrode (cathode) due to its positively charged property, and anions move toward the positive electrode (anode) due to its negatively charged property. This process occurs in a medium such as paper, cellulose acetate or agarose gels. Conventional electrophoresis is a simple, low cost and widely used technique. However, quantification of electrophoresis is often difficult and imprecise. Furthermore, electrophoresis is often slow in process, and exhibits indirect detection only. Capillary Electrophoresis (CE) is a merged technique derived from traditional electrophoresis and high performance liquid chromatography (HPLC). CE was first developed by Hjērten in 1967 as a simple homemade system using a 3 mm internal diameter (i.d.) capillary. Fourteen years later, Jorgenson and Lukacs developed the first instrument resembling modern CE. Jorgenson and Lukacs had demonstrated the potential of CE as an analytical technique by efficient separations of amino acids, peptides and urinary amines using a 75 μm (i.d.) open tube glass capillary (Jorgenson and Lukacs,

1981). The advantage of a CE system compared to conventional electrophoresis is that it provides an on-line detection system and the result output appears as a chromatograph, similar to HPLC. Other advantages for CE are the minute amounts of sample required (estimated 1 to 50 nL injected), faster separation, high efficiency, reusable capillary, and less labour-intensive compared to conventional electrophoresis. The main advantages of CE over HPLC are the inexpensive capillaries compared to expensive HPLC columns and reduced organic solvent use, which is a considerable save in cost.

A typical commercial CE instrument consists of the following main components; a high voltage power supply (up to 30 kV), a vial carousel, a capillary, a capillary cartridge, a capillary thermostat, a diode-array detector, electrodes, and a computer which connects to the CE (Figure 1.5.1). The separation takes place in a narrow internal diameter (i.d.) capillary (usually 25 to 100 μm) in a CE system. A strong electrical field is applied to the capillary (up to 500 V cm^{-1}) which inevitably generates heat. Therefore, the use of narrow i.d. capillaries is relatively important in CE, as they have large surface area-to-volume ratios which provide constant heat dissipation in order to suppress undesirable temperature gradients throughout the capillary (Jorgenson and Lukacs, 1981). Capillary thermostatting is used to controlling the temperature of a capillary. The run buffer, also known as the background electrolyte (BGE), provides stability of pH variations during analysis. When both ends of a capillary are placed in two separate BGE reservoirs, along with the electrodes, the passage of electric current (ions) of BGE provides sufficient electrical conductivity. The sample is injected into the capillary by simply replacing the BGE reservoir at the inlet (anode) with a sample reservoir, and applying either electric potential (electrokinetic injection) or external pressure (hydrodynamic injection) across the capillary. After replacing the BGE reservoir, a voltage is applied, and the separation performed based on the difference in analyte velocity in an electric field. Optical on-line UV detection is performed directly through the capillary wall near the cathode at the detection window.

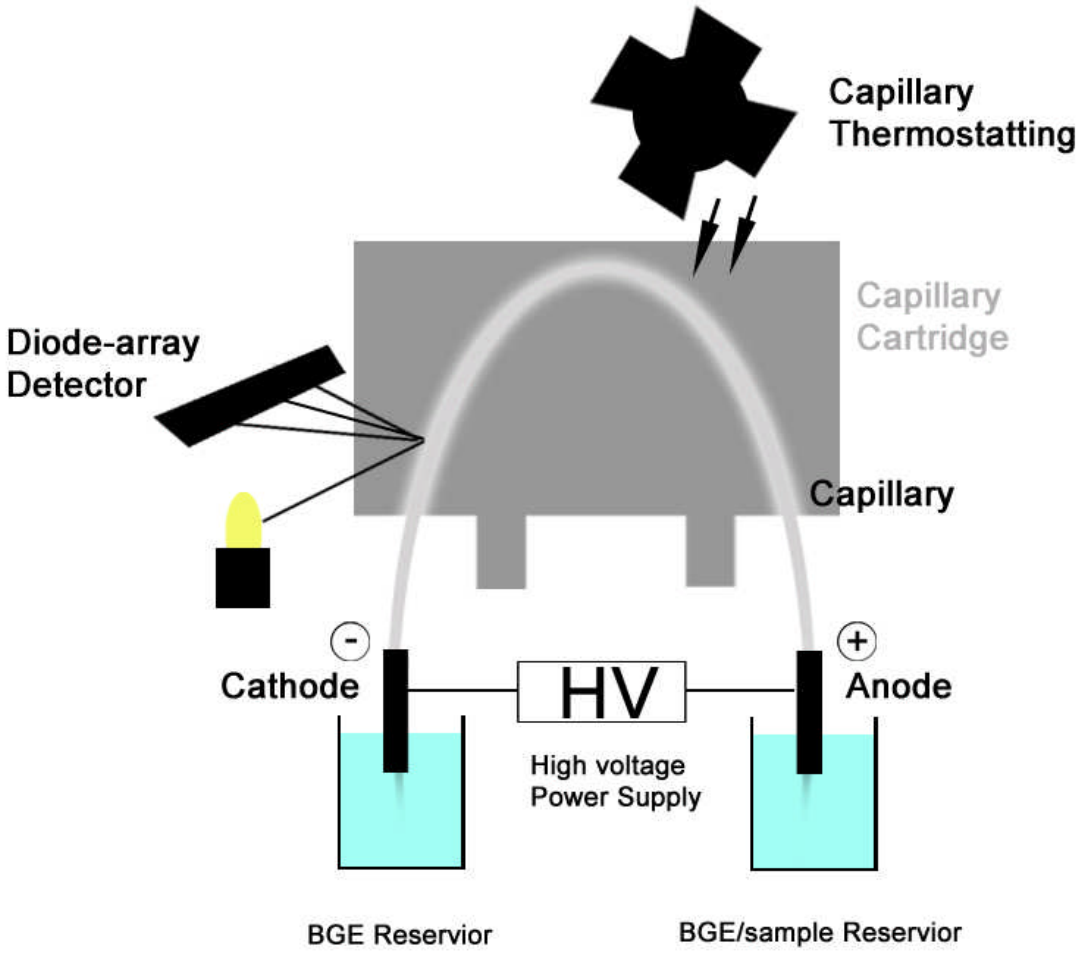


Figure 1.4.1 Schematic of a capillary electrophoresis instrument

1.4.1 Operation Modes of Capillary Electrophoresis

There are several major operation modes for CE systems. These modes are; capillary zone electrophoresis (CZE), micellar electrical chromatography (MEKC), and capillary gel electrophoresis (CGE). CZE is the principle mode for CE. It is widely used for amino acid, peptides, ions, enantiomers and protein separation. CZE is a simple method where the capillary is filled with BGE. Analytes separate into different zones based on their charge to mass ratio. Cationic species will elute first, followed by neutral species, and anionic species will elute last. The addition of cyclodextrins (CD) and macrocyclic oligosaccharides to the BGE was found to enhance enantiomer separation; α-CD is

useful for a single ring aromatic molecule separation, β-CD is useful for one or two ring aromatic molecule separation, and γ- CD is used for larger and more complex molecules. Kvasniˇcka et al, (2005) compared α-CD, β-CD and highly sulphated γ-CD (HS-γ-CD) for separation of butorphanol and cycloamine. It was found the cavities of α- and β-CD are too small for butorphanol and cyclozmine, whereas HS-γ-CD has a larger inner cavity for analytes binding. Even though the use of α-CD and β-CD exhibit longer migration time for separation, the enantiomers of these analytes were successfully separated. The separation efficiency was further enhanced by introducing HS-γ-CD.

MEKC and CGE are the separation techniques under the concept of CZE. If the separation is not possible by varying the BGE pH or concentration, MEKC is an alternative technique for CE. MEKC is a useful operation mode for neutral and charged species separation by addition of various surfactants. Anionic surfactants such as sodium dodecyl sulphate (SDS) have successfully been introduced for various anti-inflammatory drug separations, for example paracetamol and phenacetin (Terabe, 1989). Le et al (2005) have demonstrated separation of twelve phenothiazines and their metabolites using glycol monododecyl ether as a non-ionic surfactant with the MEKC technique. The addition of crown ethers to the BGE have shown efficient separation for inorganic cationic species such as sodium, calcium and magnesium ions (Riviello and Harold, 1993). A mixture of bile salt, phosphatidylcholine and fatty acid micelles were successfully introduced for various drug separations e.g. ibuprofen, and diclofenac (Schwarza et al., 1998). CGE is a technique similar to conventional gel electrophoresis but using a capillary as the medium. CGE has a polymer matrix in the capillary. Examples of the polymer matrix are polyacrylamide, agarose, and methylcellulose. CGE is widely used for oligonucleotides (Szilagyi et al., 2007), DNA fragments (Végvári and Hjertén, 2002), and proteins separation (Hjertén et al., 2004).

There are several other operation modes for CE; capillary isotachophoresis (CITP) (Kvasnička et al., 2007), capillary isoelectric focusing (CIEF) (Wu and Huang, 2006), microchip capillary gel electrophoresis (MCGE) (Kim et al., 2005), microemulsion electrokinetic chromatography (MEEKC) (Zhou et al., 1999), high performance frontal analysis-capillary electrophoresis (HPFA-CE) (Wan et al., 2005), and capillary electrophoresis – mass spectrometry (CE-MS) (Lagarrigue et al., 2008) for example.

Each operation mode is uniquely designed depending on the properties of the desired analysis.

1.4.2 Basic Theory of Electrophoresis

The separation process by electrophoresis is based on the analytes movement through a fluid solution in an electric field. In the solution, the current is carried by ions. The conductivity of this solution depends on the concentration and the mobility of the ionic species in an electric field. Therefore, the conductivity of a solution is described by the Ohm's Law.

$$E = I\,R \tag{1}$$

$$W = I^2 R \tag{2}$$

where E is the applied electric field, I is the current in a solution, R is the resistance of the solution, and W is power measured is watts. Equations (1) and (2) demonstrate the electric field is proportional to the current, and heat can be generated by current flows.

The steady state of the electrophoresis process is defined by the equilibrium of applied electric force and the frictional force for an ion (Stokes's Law). The electrophoretic mobility (μ_e) is the velocity of an ion moving in an applied electric field. By combining these two characteristics for an electrophoreticprocess, the equation for describing electrophoretic mobility (μ_e) is given as;

$$\mu_e = \frac{\nu}{E} = \frac{q}{6\pi r\eta} \tag{3}$$

where v is the ion velocity, E is the applied electric field, q is the ion charge, r is the ion radius, and η is the viscosity of a solution. Equation (3) shows that the electrophoretic mobility is governed by the charge to size ratio of an ion, that is, large lower charged ions have low mobility and small highly charged ion have high mobility.

1.4.3 Electro-Osmotic Flow

In theory, only cationic species are able to migrate towards the cathode and travel through the detector. However, in practice, non-ionic and anionic species are also able to migrate toward to the cathode with the aid of strong electro-osmotic flow (EOF). The EOF, also called the electroendosmotic flow, is the fundamental component of a CE system. EOF is the bulk of liquid (ie. BGE) which moves toward the cathode under the application of an electric field (Figure 1.5.3). An uncoated fused-silica capillary is made of highly cross-linked polymers of silicon dioxide. The silanol groups of these polymers are readily dissociated and become party deprotonated at the capillary wall by flushing with strong base, such as 1M of sodium hydroxide. Filling the capillary with BGE will attract positively charged ions of the BGE to the negatively charged capillary wall. This generates an electrical double layer and creates a potential difference close to the capillary wall. The potential difference of the capillary wall to the EOF is called the zeta potential (ζ). The inner layer is the rigid layer called the Helmholtz layer. This rigid layer consists of cations which are tightly bound and adsorb to the negatively charged capillary wall. The diffuse layer, also called the Debye-Hückel layer, is a mobile region affluent in cations (Wätzig and Günter 2003). This layer is free to migrate and drag the bulk solution, including analytes, towards the cathode (Figure 1.5.3). The centre region of a capillary is electrically neutral since the zeta potential is no longer applied in this section. The flat flow of EOF is evenly distributed throughout the capillary, which is a unique feature of CZE.

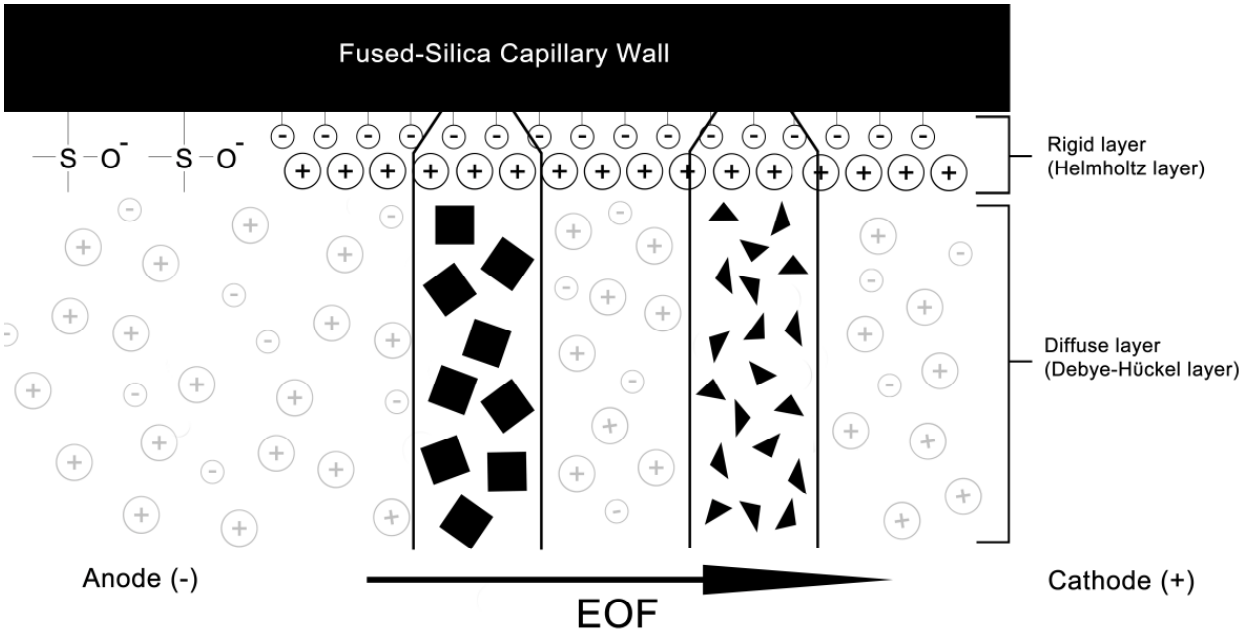


Figure 1.4.2 Dissection of a capillary under the influence of an electric field.

The EOF mobility is dependent on the zeta potential of the capillary wall, the dielectric constant and the viscosity of BGE. This phenomenon is described by the Helmholtz-Smoluchowski formula;

$$\mu_{EOF} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad (4)$$

where ε is the dielectric constant of the BGE, ζ is the zeta potential, and η is the viscosity of the BGE. Note that the EOF mobility is proportional to the zeta potential and inversely proportional to the viscosity of the BGE (Wang et al., 2007).

The total mobility (μ_{tot}) of a solution in a capillary is the sum of the electrophoretic mobility of its component analytes (μ_e) and the EOF mobility (μ_{EOF}) .

The relationship of an analytes migration time (t) to the mobility and capillary length can be described by the following equation(Huang et al., 1989);

$$t = \frac{\ell L}{(\mu_e + \mu_{EOF})V} \quad (5)$$

where ℓ is the effective capillary length, L is the total capillary length, V is the applied voltage, μ_e is the electrophoretic mobility and μ_{EOF} is the EOF mobility. The effective capillary length is the distance from the injection end of a capillary to the detection window (Figure 1.5.1). An equation (5) show the use of short capillary length and high voltage application is the key to a short analysis. There are several factors that can influence the EOF and, consequently, the analysis time. These include the BGE concentration, the applied voltage, and the temperature. The analysis time decreased with increasing voltage and temperature. Since the viscosity of the BGE is dependent on temperature, increasing temperature will result in reducing the viscosity of BGE. This results in greater total mobility and reduction in the analysis time (Equation 3, 4, and 5). The analysis time is also effected by the BGE concentration. Dilute BGE is less viscous, therefore, the use of dilute BGE will result in decreasing the analysis time. However, these parameters can influence the separation itself, and therefore the resolution and precision of the analysis. Although short analysis time was one of the primary goals for this study, the resolution and precision should also be carefully take into an account.

1.4.4 Separation Efficiency

The efficiency in CE system is calculated in theoretical plates (N). In order to improve the separation, maximum efficiency must be achieved. Zone length in CZE is dependent on the dispersion, thus the dispersion must be controlled. In a perfect system, analyte zone broadening is regard as longitudinal diffusion. There are many variances of dispersion involved in longitudinal diffusion. These include molecular diffusion, injection, temperature gradients, wall absorption, detection, and

electrodispersion. Assuming the molecular diffusion is the only source of zone broadening, the efficiency was described by Jorgenson and Lukacs (1981);

$$N = \frac{\mu_{tot} V}{2D} \quad (6)$$

Where N is the maximum theoretical plate, μ_{tot} is the total mobility, V is the applied voltage, ℓ is the effective capillary length, D is the diffusion coefficient of the analyte, and L is the total capillary length. Separation efficiency is independent of the capillary length, in theory. This is because the length of analyte zone is short compared to the total capillary length. However, the injected amount can be affected by the capillary length. It is difficult to quantify the amount of analyte injected, because it depends on the μ_{tot}, injection voltage, injection time, capillary radius, sample concentration, and capillary length. The injection amount is estimated to be from 1 to 50 nL in a CE system.

1.5 Objective of Study

As mentioned earlier, high concentrations of clozapine and N-desmethylclozapine in plasma may lead to the development of agranulocytosis or other side effects in the schizophrenic patient. Agranulocytosis is a potentially fatal blood disorder with a mortality rate of 3-4% (Gerson et al., 1994; Ulrich et al., 2003). Due to this possible life threatening side effect, regular therapeutic drug monitoring is important for patients who receive clozapine as their primary antipsychotic drug.

HPLC is the current technique performed at Canterbury Health Laboratories for therapeutic drug monitoring of clozapine. This method was developed from a combination of two research works by Volpicelli et al, (1993) and Lovdahl et al, (1991). Large volumes of mobile phase are needed for this HPLC method. The mobile phase

includes the use of acetonitrile, which is expensive in cost, and heptane-sulphonic acid, is also expensive. C8 HPLC columns as used in this assay are also relatively expensive.

This study will explore an alternative method by using CE for clozapine analysis. The use of the CE technique aims to reduce the cost of solvent use, as well as replacing expensive HPLC column with inexpensive capillaries. Small volumes of phosphate buffer are the major solutions required for CE. A new protocol for CZE will be developed in this study by adjusting various parameters that may influence the EOF of a capillary. These parameters include BGE pH, BGE concentration, temperature, and applied voltage. Serum spiked with clozapine and N-desmethylclozapine will be analysed by CZE using the developed optimal conditions. Patient samples will also be analysed by CZE in this study, and compared with their HPLC results.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All reagents used in this study were of analytical grade or better.

Acetonitrile	Mallinckrodt Chemicals
Clozapine	Sigma
n-Desmethylclozapine	Sigma
Dichloromethane	Mallinckrodt Chemicals
Ethyl acetate	Merck
1-Heptane sulphonic acid	Sigma Chemical Co.
n-Hexane	Merck
Isopropanol alcohol	Merck
Methanol	Merck
3-Methyl-1-butanol	Sigma-Aldrich
Phosphoric acid	Merck
Potassium dihydrogen phosphate	Merck
Protriptyline hydrochloride	Sigma
Sodium carbonate	Anala-R®
Sodium dihydrogen phosphate	Baker Chemical Co.
Sodium hydroxide	Merck
Trichloroacetic Acid	Merck

2.1.2 Solutions and Buffers

A. Capillary Electrophoresis Reagents

Sodium Dihydrogen Phosphate Solution ($NaH_2PO_4 \cdot H_2O$)

A stock solution of 1 M $NaH_2PO_4 \cdot H_2O$ solution was prepared by dissolving 13.799g of $NaH_2PO_4 \cdot H_2O$ in 100 mL of deionised water. This was stored at room temperature.

Phosphoric Acid ($H_3PO_4^-$)

A stock solution 1 M of $H_3PO_4^-$ was prepared by adding 5 mL of concentrated phosphoric acid into 100 mL of deionised water. This solution was stored at room temperature.

Sodium Hydroxide (NaOH)

A stock solution of 1 M NaOH was prepared by dissolving 4 g of NaOH into 100 mL of deionised water. A 0.1 M NaOH solution was prepared by diluting 10 mL of 1 M NaOH into 100 mL of deionised water. These solutions were stored in room temperature.

B. High Performance Liquid Chromatography Reagents

Sodium Hydroxide (NaOH)

A 0.4 M of NaOH solution was prepared by dissolving 1.6 g of NaOH in 100 mL of deionised water. This solution was stored at room temperature.

Potassium Dihydrogen Phosphate Buffer ($KH_2PO_4 \cdot H_2O$)

The phosphate buffer was made up by dissolving 4.08 g of $KH_2PO_4 \cdot H_2O$ in 600 mL of deionised water. The solution was adjusted to pH 2.7 with 2 M of phosphoric acid. This solution was then diluted to 1 L with deionised water. The phosphate buffer was stored in room temperature.

Mobile Phase

The mobile phase for HPLC in this assay contained 310 mL of phosphate buffer, 190 mL of acetonitrile, and 1 g of 1-heptanesulphonic acid. The solution was made up in a 500 mL beaker, and was dissolved completely using a magnetic stirrer. The solvent was filtered through a 0.5 µm micro filter, and degassed under vacuum.

C. Clozapine / N-Desmethylclozapine / Protriptyline Hydrochloride Preparation

Clozapine Standard (Stock)

A 1 mM clozapine standard solution was made up by dissolving 32.7 mg of clozapine in 100 mL of methanol. This stock clozapine solution was stored in the freezer at -20 ^{0}C.

N-Desmethylclozapine Standard (Stock)

A 1 mM N-desmethylclozapine stock solution was made by dissolving 31.8 mg of N-desmethylclozapine in 100 mL of methanol. The stock solution was stored in the freezer at -20 ^{0}C.

Clozapine / N-Desmethylclozapine Intermediate Standard

A 100 µM intermediate clozapine / N-desmethylclozapine standard was prepared by diluting 1 mL of each stock solution up to 10 mL of methanol. This intermediate standard solution was not stored.

Clozapine / N-Desmethylclozapine Working Standard (in drug free serum)

A set of 1000, 2000 and 4000 nM working clozapine / N-desmethylclozapine standards were prepared by pipette 500 µL, 1 mL and 2 mL of intermediate standard and made up to 50 mL with human drug free serum. The serum sample was mixed completely by the use of a magnetic stirrer, and was stirred overnight. These working standards were divided into aliquots and stored in the freezer at -20 ^{0}C.

Clozapine Standard / N-Desmethylclozapine Working Solutions

A 120 µM intermediate clozapine / N-desmethylclozapine standard was prepared by diluting 1.2 mL of each stock solution to 10 mL with methanol. A series of 3000, 6000, and 12000 nM working solutions were made by diluting 300 µL, 600 µL, and 1.2 mL of the 120 µM intermediate standard to 10 mL with 50% methanol. These solutions were stored in the fridge at 5 ^{0}C.

The concentrations of these working solutions are equivalent to the concentrations of the working standards (in drug free serum) taking into account the concentration effect of three during extraction.

Protriptyline Hydrochloride (stock- internal standard)

A 250 mg/L of protriptyline hydrochloride solution was prepared by dissolving 25 mg of protriptyline hydrochloride into 100 mL of deionised water. This stock solution was stored in the fridge at 5 ^{0}C.

Protriptyline Hydrochloride Working Solution (internal standard)

A 25 mg/L working internal protriptyline hydrochloride solution was prepared by diluting 10 mL of the stock internal standard to 100 mL with deionised water. This internal standard was stored in the fridge at 5 ^{0}C. An 8.33 mg/L solution of protriptyline hydrochloride was also prepared by diluting the 25 mg/L internal standard one in three.

D. Reagents used for serum extraction process

Sodium Carbonate Solution (Na_2CO_3)

A 100 mL solution of Na_2CO_3 was prepared by dissolving 1.06 g of anhydrous Na_2CO_3 in deionised water. This solution was stored in the fridge at 5 ^{0}C.

Potassium Dihydrogen Phosphate Buffer Solution (KH_2PO_4)

A 45 mM buffer solution was prepared by dissolving 0.61 g of KH_2PO_4 in 100 mL of deionised water. The pH was adjusted to 2.8 with concentrated phosphoric acid, and was stored in the fridge at 5 ^{0}C.

2.2 Method

2.2.1 Blood Collection

Drug free serum was collected from hemachromatosis patients and was stored at -20 ^{0}C until used in experiments for working standards. Patient samples (either serum or plasma) were obtained from the Toxicology department laboratory at Canterbury Health Laboratories. Serum was obtained from coagulated gel tubes and plasma was obtained from anti-coagulated heparin tubes. Patient samples were stored at 5 ^{0}C until used in the analysis (maximum storage time was one week). External quality control samples were stored at -20 ^{0}C.

2.2.2 Clozapine and N-desmethylclozapine Extraction Methods for Human Serum and Plasma

A. <u>Clozapine Extraction Method A (Toxicology lab menu)</u>

A 900 µL of working standard or patient's sample was aliquoted into a 10 mL plastic centrifuge tube, followed by addition of 50 µL of the working internal standard solution (protriptyline hydrochloride). Ethyl acetate (4 mL) was added as an extraction solvent and shaken vigorously for one minute. The mixture was centrifuged for five minutes at 3500 rpm before transferring the top organic phase into a clean 10 mL plastic centrifuge tube. This organic phase solvent was evaporated to dryness under a soft stream of dry nitrogen gas in the heating block. Following this, the residual working standard or patient's sample was reconstituted with 300 µL of 50% methanol. The sample was vortex mixed for 30 seconds and then transferred to an eppendorf microfuge tube,

followed by centrifugation for 5 minutes at 14,000 rpm in a microfuge. 200 µL of each sample was transferred into an autosampler vial, which contained 20 µL of buffer.

B. Clozapine Extraction Method B (Dusci et al., 2002)

This assay was employed by Dusci et al., (2002) to extract Olanzapine from human plasma samples. N-desmethylclozapine was used as an internal standard in this assay for determining olanzapine by HPLC. Olanzapine is an atypical antipsychotic drug that has a very similar molecular structure to clozapine. In contrast to method A, hexane:dicloromethane was used as an extraction solvent in this assay.

Working standard (600 µL) and 50 µL of working internal standard (protriptyline hydrochloride) were pipetted into a 10 mL screw-capped glass tube containing 8 mL of extraction solvent (85:15 v,v- hexane:dicloromethane) and 500 µL of 0.1M sodium carbonate. The mixture was shaken vigorously for five minutes and centrifuged for 5 minutes at 3500 rpm. Organic phase was then transferred to another clean 10 mL screw-capped glass tube and back extracted into 200 µL of potassium dihydrogen phosphate buffer (45 mM, pH 2.8). The mixture was then centrifuge for five minutes before the majority of the top organic phase was discarded. The rest of the organic layer was evaporated under a soft stream of nitrogen gas, leaving aqueous phase at the bottom of the glass tube. Phosphate buffer (20 µL) was added to the aqueous sample before transferring into an autosampler vial.

C. Clozapine Extraction Method C (Edno et al., 1997)

Extraction method C was performed by Edno et al., (1997) to quantify clozapine and N-desmethylclozapine in human plasma by HPLC with ultraviolet detection. Loxapine was added as internal standard. This method differs from the previous methods by using hexane-isoamyl alcohol mixture as the extraction solvent. This assay, with a few modifications, was performed in the study in comparison with other clozapine extraction methods.

Working standard, 600 µL and working internal standard (protriptyline hydrochloride), 50 µL, were pipetted into 10 mL screw-capped glass tube, followed by addition of 200 µL of 0.33 M sodium hydroxide. This mixture was extracted with 6 mL of hexane-isoamyl

alcohol (985:15 v,v) and shaken vigorously for two minutes. The preparation was centrifuged for 5 minutes at 3500 rpm. Organic phase was then transferred to a clean 10 mL screw-capped glass tube and back extracted into 200 µL of potassium dihydrogen phosphate buffer (45 mM, pH 2.8). Centrifuge for 5 minutes before the majority top organic phase was discarded. The remaining organic layer was evaporated under a soft stream of nitrogen gas. The aqueous phase was transferred into an autosampler vial.

D. Clozapine Extraction Method D (D'Arrigo et al., 2006)

This liquid-liquid extraction method was employed by D'Arrigo et al., (2006) for determining olanzapine in human plasma by reversed-phase HPLC with ultraviolet detection. Clozapine was used in this assay as an internal standard. Ten percent isoamyl alcohol in hexane (10:90 v,v) was used in this method, whereas 1.5% isoamyl alcohol in hexane- was used in clozapine extraction method C.

Working standard (600µL) and 50 µL of working internal standard (protriptyline hydrochloride) were aliquoted into a 10 mL screw-capped glass tube and 200 µL of 0.33 M sodium hydroxide was added. The solution was extracted by shaking vigorously with 6 mL of hexane-isoamyl alcohol (90:10 v,v) for two minutes. This mixture was centrifuged for 5 minutes at 3500 rpm. The organic phase was transferred to another clean 10 mL screw-capped glass tube and then back extracted into 200 µL of potassium dihydrogen phosphate buffer (45 mM, pH 2.8). The mixture was centrifuged for 5 minutes and then the top organic layer was discard. The remaining organic layer was evaporated under a soft stream of nitrogen gas, leaving the aqueous phase in the bottom of the glass tube. The aqueous phase with 200 µL of phosphate buffer was then transferred into an autosampler vial.

2.2.3 Determination of Clozapine and N-desmethylclozapine by Capillary Electrophoresis

The capillary zone elctrophoresis (CZE) analysis was performed using a 3D Capillary Electrophoresis System, Model G1602A from Agilent Technologies (Palo Alto, CA, USA)

with a diode array detector. Analysis was carried out by using a homemade fused-silica capillary (polymicro technologies L.L.C.), total length of 33 cm (effective length 24.5 cm), and internal diameter of 50 µm or 75 µm. The electrophoretic data were acquired with a 3D capillary electrophoresis (CE) ChemStation (Rev. A.10.02[1757]) equipped with a HP workstation controlled by windows software.

The capillary electrophoresis machine was initialised once before the analysis. The initialisation turns on the ultraviolet light, calibrates the wavelength and checks the vial lifts.

The capillary was preconditioned by flushing with 0.1 M NaOH for 30 minutes, deionised water for 20 minutes and phosphate buffer for 10 minutes before starting the analysis. The capillary was also preconditioned by flushing 0.1 M of NaOH for 1 minute, deionised water for 1 minute, and phosphate buffer for 1 minute before each run. For a new capillary, 1 M of NaOH was flushed through for 30 minutes prior to the capillary preconditioning process. The capillary was washed with isopropanol for 20 minutes at the end of analysis.

The CE tray was setup with eight standard vials (contained 700 µL of liquid) from position one to position eight in the following sequence; 1 M NaOH, 0.1 M NaOH, phosphate buffer (positions three to five), IPA, waste vial, and deionised water. Samples were placed from position nine onward. The sample tray was equilibrated at room temperature. The detection was carried out by the on-capillary measurement of UV absorption at 210 nm.

2.2.4 Determination of Clozapine and N-desmethylclozapine by High Performance Liquid Chromatography

Analysis of clozapine was performed using the method published by Volpicelli et al., (1993) and Lovdahl et al., (1991). A reversed-phase liquid chromatographic C8 column with photodiode array detection at 254 nm was used in this assay. The dimension of the column is 150x4.6 mm. The mobile phase contained 310 mL of potassium dihydrogen phosphate buffer, 190 mL of acetonitrile and 1 g of 1-heptanesulphonic acid. Extracted sample (50 µL) was injected into the HPLC and the mobile phase was pumped through the column at a flow rate of 1.5 mL per minute. A mixture of methanol: water (50:50 v,v)

was pumped through the column for 5 minutes at end of analysis to clean the column, followed by methanol for 10 minutes.

2.3 Statistical Analysis

Results present in this thesis were acquired from single experiments representing a minimum of two experiments (unless otherwise stated). The mean and standard deviation of each data point were calculated from triplicate analysis. Statistical analyses were carried out by using PRISM software (version 4.00, GraphPad Software, Inc). The comparison between treatments of significance was calculated by one-way analysis of variance (ANOVA), where $p < 0.05$ indicated as not significant, and $p < 0.01$ indicated significant difference between two comparisons. Method validator (version 1.1.10.0) was used to performed linear regression plots and difference plots for comparisons between HPLC and CE.

RESULTS

3.1 Optimizing clozapine separation for capillary electrophoresis by adjusting the following conditions:

3.1.1 BGE Concentration

An initial study by Fujiwara and Honda (1986), had demonstrated that different salt concentration of a back ground electrolyte (BGE) will affect the migration time of a solute, as well as the chromatography resolution of peaks. This indicated that the velocity of the EOF can be affected by the ionic strength of a BGE. Since the solute's mobility and the EOF are sensitive to pH changes, Buffer is an important parameter in CZE. It provides the stability for minor pH changes, and also provides the ionic strength for electrical conductivity to occur. This study was carried out to compare the effectiveness of different phosphate buffer concentrations to the clozapine separation by CZE. A 4000 nM clozapine working solution was made with crystallised clozapine in 50% methanol. The working solution of this concentration is equivalent to the maximum clozapine concentration in human plasma. Phosphate buffer ranging from 20 to 100 mM were compared, and the buffer was prepared of one part of sodium dihydrogen phosphate solution with one part of phosphoric acid in distilled water. The phosphate buffer will be address as BGE from now on.

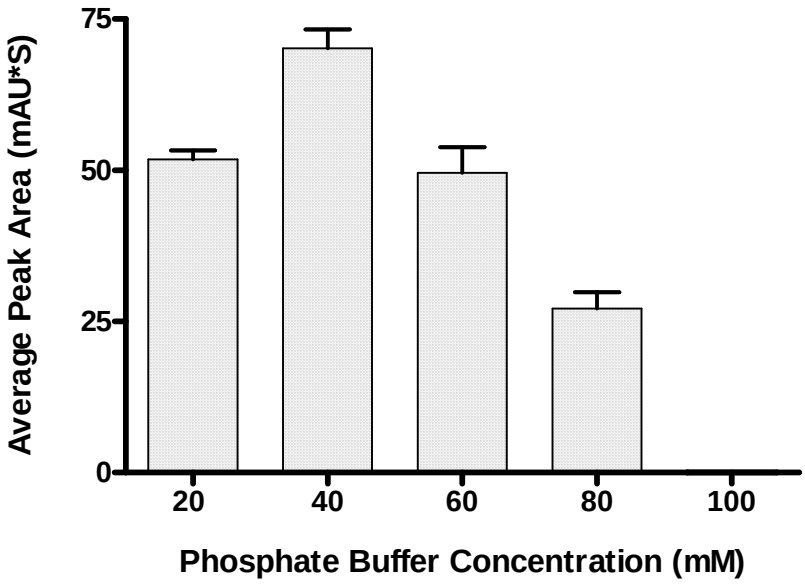


Figure 3.1.1 The effectiveness of different BGE concentration to the clozapine separation by CZE

Different BGE concentrations were compared ranging from 20 to 100 mM. A number of conditions were kept constant in this experiment; 50 µm (i.d) fused-silica capillary was used, BGE pH of 2.5, temperature at 20 ^{o}C, apply voltage at 10 kV, sample injection of 20 kV per 2 seconds, operating at a wavelength of 210 nm. The average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

The BGE at 40 mM exhibited an average peak area of 70.17 mAU*s (CV= 4%) for clozapine separation. Diluted and concentrated BGE demonstrated decreases in the peak area (Figure 3.1.1). Band broadening was observed for clozapine separation as BGE concentrations of 80 mM or above were used (data not shown). This was possibly due to the increase in BGE viscosity, which resulted in decreasing the EOF. There was no result obtained from the BGE concentration of 100 mM. This study demonstrated that BGE at 40 mM is the best condition for clozapine separation using a 50 µm (i.d) fused-silica capillary. Therefore, BGE concentration of 40 mM was used for all subsequent experiments in determining the optimal condition for clozapine separation.

3.1.2 BGE pH

The EOF in CZE is extremely sensitive to pH, consequently, the BGE pH is the most important experimental parameter. BGE pHs 1.5 and 3.0 showed dramatic decreases in average peak area with a value of 24.25 mAU*s (CV= 58%), and 24.16 mAU*s (CV= 87%) respectively. BGE pH 2.5 demonstrated an average peak area of 70.17 mAU*s

(CV= 4%), whereas BGE pH 2.0 showed an average peak area of 62.86 mAU*s (CV= 11%) (Figure 3.1.2). ANOVA analysis showed no significant difference between BGE pH 2.0 and pH 2.5 ($p > 0.05$), however, the use of BGE at pH 2.0 demonstrated a high value of CV (CV= 11%). This indicated that the EOF and zeta potential was unstable with the BGE at pH 2.0, which means the reproducibility using BGE pH 2.0 is relatively low. Therefore, BGE pH 2.5 was chosen for use in all following experiments determining the optimal condition for clozapine separation using CZE.

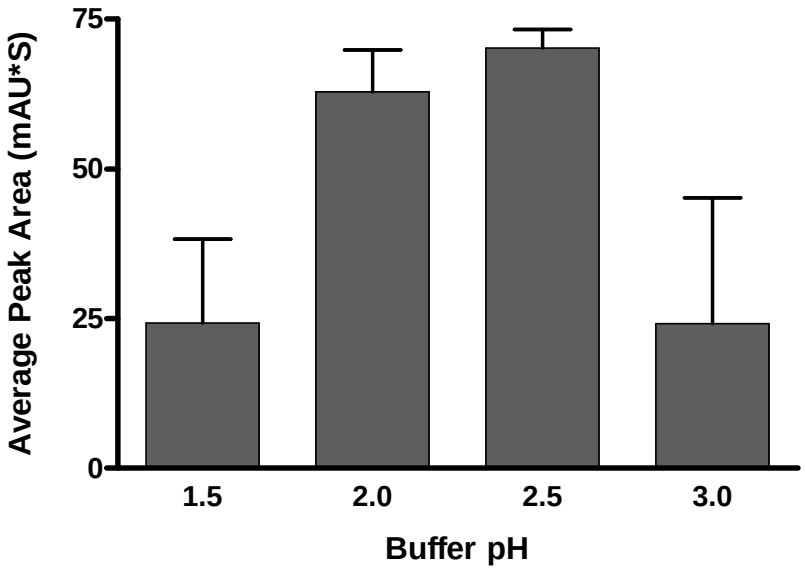


Figure 3.1.2 The effectiveness of different BGE pHs in the clozapine separation by CZE

Different BGE pHs were compared, ranging from pH 1.5 to 3.0. The clozapine concentration was 4000 nM. A number of conditions were kept constant in this experiment; 50 µm (i.d) fused-silica capillary was used, BGE concentration of 40 mM, temperature at 20 ^{o}C, voltage at 10 kV, sample injection of 20 kV per 2 seconds, and operating at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

3.1.3 Temperature

The temperature of a capillary during CZE analysis is also an important factor that can have an influence on the EOF. This is because the temperature is inversely proportional to the viscosity of the BGE, and it is the major parameter that controls a solute's migration time (Equation 5). A temperature of 22 ^{o}C for clozapine separation showed an average peak are of 70.13 mAU*s (CV= 6%), whereas 24 ^{o}C exhibited an average peak area of 60.05 mAU*s (CV= 14%) (Figure 3.1.3). ANOVA analysis showed that there is

no significant difference between temperatures of 22 ^{o}C and 24 ^{o}C ($p < 0.05$). However, as the temperature elevated, the viscosity of the BGE reduced, and the current increased. This disrupted the EOF, and may result in an inconstant thermal gradient through the capillary, consequently, band broadening occurred at high temperatures (data not shown) of 24 ^{0}C and above. In contrast, low BGE temperatures increased the viscosity and reduced the injection volume compared to high temperatures. This might be the reason for lower peak areas obtained from temperatures of 20 ^{0}C and below. Therefore, a temperature of 22 ^{0}C was used for all subsequent experiments in determining the optimal condition for clozapine separation by CZE.

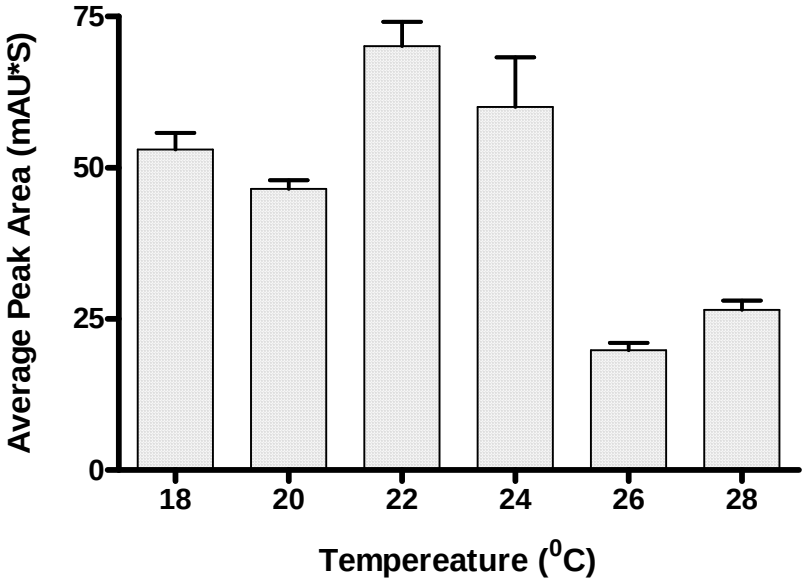


Figure 3.1.3 The effectiveness of different temperatures on the clozapine separation by CZE

Different temperatures from 18 to 28 ^{o}C were compared. The clozapine concentration was 4000 nM. A number of conditions were kept constant in this experiment; 50 um (i.d) fused-silica capillary was used, BGE concentration of 40 mM (pH 2.5), voltage at 10 kV, sample injection of 20 kV per 2 seconds, and operating at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

3.1.4 Applied Voltage

When voltage is applied to CZE, current will be generated through the free ions of BGE. This separates the analytes by their charge to mass ratio. Therefore, the control of applied voltage is also an important parameter in CE. In theory, maximum theoretical plates can be achieved by applying the voltage as high as possible (Equation 6). In practice, disruption of EOF occurs when high voltage is applied to CE.

Five different voltages were applied and compared for clozapine separation. There was no separation at 5 kV, and the maximum separation was achieved when 10 kV was applied with an average peak area of 45.53 mAU*s (CV= 2%). As the applied voltage increased, the average peak area decreased (Figure 3.1.4). Although the migration time was reduced by the use of higher voltage, unstable current, disrupted baseline and low signal to noise ratio were observed when 20 kV and 25 kV were applied to the CE (data not shown).

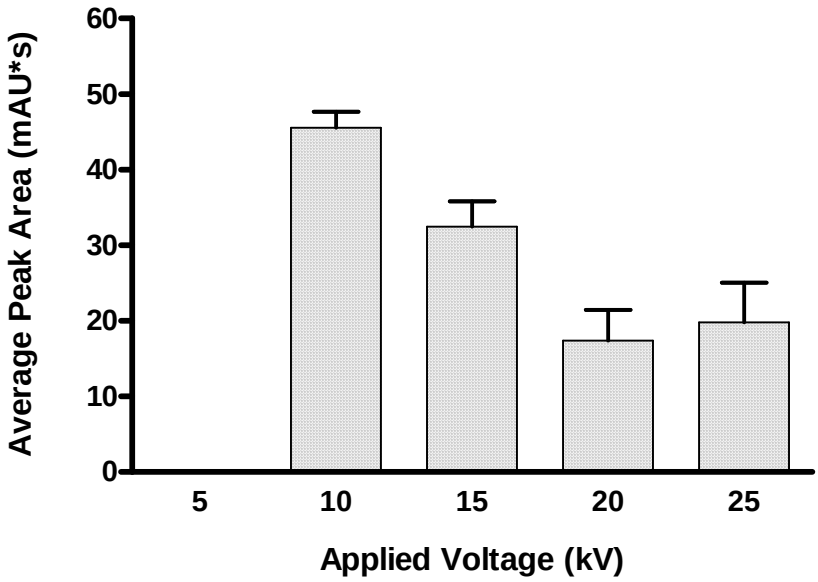


Figure 3.1.4 The comparison between different applied voltages to the clozapine separation by CZE

Different applied voltages were adjusted from 5 kV to 25 kV. The clozapine concentration was 4000 nM. The following conditions were kept constant in this experiment; 50 um (i.d) fused-silica capillary was used, BGE concentration of 40 mM (pH 2.5), sample injection of 20 kV per 2 seconds, and operating at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

The final optimal conditions for clozapine separation were determined from the above studies. These include, the BGE concentration, BGE pH, temperature, and applied voltage. Table 3.2.1 is a summary of the optimal condition for clozapine separation using a 50 µm (i.d) fused-silica capillary.

BGE Concentration	40 mM
BGE pH	2.5
Temperature	22 ^{0}C
Applied Voltage	10 kV

Table 3.1.1 A summary of the optimal CZE condition for clozapine separation

A summary of the optimal CZE condition for clozapine separation by using a 50 µm fused-silica capillary. Clozapine concentration of 4000 nM was used for these studies. Each sample was injected by electrokinetic injection at 20 kV per two seconds. The on-line UV detection wavelength was at 210 nm.

3.2 Clozapine and N-desmethylclozapine separation using the optimal conditions

To confirm the optimal conditions for clozapine and N-desmethylclozapine separation by CZE, the following experiments were carried out by using the optimal conditions determined from the previous studies (Figure 3.2.1). Three different concentrations of clozapine and N-desmethylclozapine were analysed; 1000, 2000, and 4000 nM. Figure 3.2.1 showed a linear increment in the peak area as the concentration increased. This indicated that the optimal conditions were giving a proportional response to concentration.

N-desmethylclozapine displayed slightly higher peak area values for the three concentrations than the values obtained for clozapine. However, ANOVA analysis showed no significant difference between clozapine and N-desmethylclozapine average peak areas for each concentration ($p < 0.05$). The migration times for clozapine and N-desmethylclozapine were 4 and 3.8 minutes respectively. Protriptyline hydrochloride, as an ISD, was introduced in this study. The migration time for protriptyline was 6 minutes.

A linear relationship was found for both clozapine and N-desmethylclozapine, with an r^2 value of 0.979 and 0.989 respectively (Figure 3.2.1).

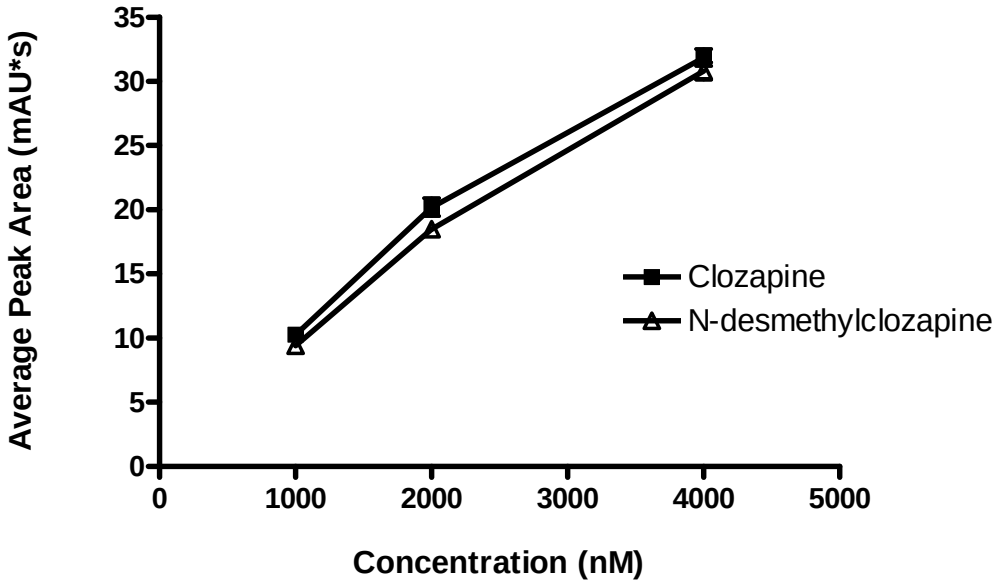


Figure 3.2.1 The standard curve for clozapine and N-desmethylclozapine separation using CZE

Three different clozapine and N-desmethylclozapine concentrations were analysed, 1000, 2000 and 4000 nM. The optimal conditions were applied in this experiment; 50 µm (i.d) fused-silica capillary was used, BGE concentration of 40 mM, BGE pH 2.5, voltage at 10 kV, sample injection of 20 kV per 2 seconds, and operating at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

3.3 A comparison of protocols for plasma clozapine and N-desmethylclozapine extraction

Clinical drug monitoring for schizophrenic patients is relatively important as clozapine has been reported to induce side effects, such as agranulocytosis and hypotension (Amsler et al., 1977). The method of CZE was developed for the clinical measurement of clozapine and N-desmethylclozapine levels in human plasma. Serum spiked with clozapine was used for the following studies to confirm the effectiveness of the above method. Drug free serum was donated from hemachromatosis patients as the blank serum sample. The optimal plasma level of clozapine was determined from many studies, ranging from 1000-4000 nM (Hiemke et al., 2000 and Ulrich et al., 2003). Therefore, serum spiked with clozapine was made in three concentrations, 1000, 2000,

and 4000 nM as a standard. Six hundred µL of each sample was extracted by extraction method A. The samples were injected into CE by electrokinetic injection at 20 kV 2 s^{-1}. The result showed a linear relationship; as the clozapine concentration increased, the average peak area also increased. However, the sensitivities were reduced two to six folds when compared with he results obtained from the working solution, which was clozapine in 50% methanol (data not shown). It was not certain whether the loss of sensitivity was due to the serum extraction method or the detection of CE. Ninety percent of clozapine is protein bound, therefore, there is a possibility that only partial clozapine was extracted during the process.

To investigate this further, three separate experiments were carried out to confirm whether the loss of sensitivity was due to the protein bound clozapine. Serum samples were exposed to acidic or alkaline solution in order to adjust the serum pH. This was carried out to break the hydrocarbon and nitrogen bonding between clozapine and proteins prior to the extraction process. Ten µL of 15% trichloroacetic acid (TCA) and 0.1 M sodium bicarbonate was used to adjust the pH to extremely acidic (pH1.7) or extremely alkaline (pH 8.7). The use of TCA was also to precipitate serum proteins before the extraction. Acetonitrile was introduced in this study to precipitate proteins as well, and the result was compared with the use of TCA. However, the outcome of this experiment showed no significant improvement to the sensitivity of clozapine separation by CZE (data not shown). The electropherogram exhibited low sensitivity and resolution. An extra peak was observed ten seconds before clozapine by using acetonitrile for protein precipitation during the extraction. This observation indicated that acetonitrile may have discharged a methyl group from the benzodiazepine ring of clozapine during the extraction process, which resulted in the formation of N-desmethylclozapine. Therefore, four different types of liquid-liquid extraction protocol using different extraction solvents were investigated and compared.

Clozapine extraction methods A, B, C, and D were carried out using ethyl acetate, dicloromethane, 1.5% and 10% hexane-isoamylid alcohol as extraction solvents. The results showed a linear relationship between peak area and clozapine concentration (Figure 3.3.1). However, there were no significant differences found between the four methods at each data point ($p < 0.05$). This indicated that the loss of sensitivity during clozapine separation using CZE may not be due to partial clozapine being extracted.

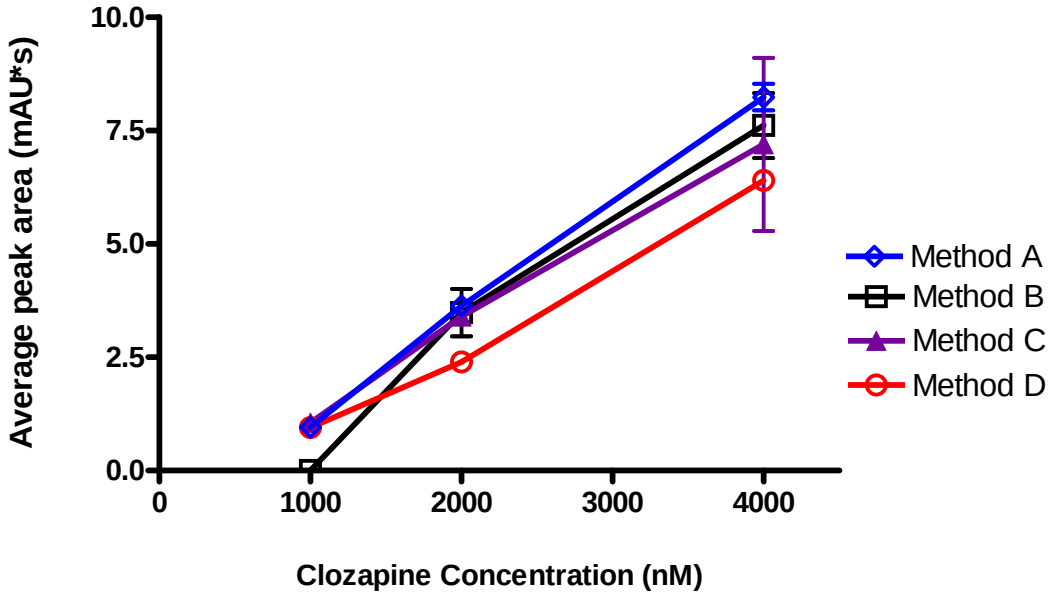


Figure 3.3.1 The comparison of four different extraction methods for clozapine separation by CZE

Four different extraction methods were compared. Clozapine concentrations of 1000, 2000 and 4000 nM were analysed. Thirty µL of protriptyline hydrochloride (internal standard - 25 mg/L) was used in this experiment. The following conditions were kept constant; 50 µm (i.d) fused-silica capillary was used, BGE concentration of 40 mM (pH 2.5), voltage at 10 kV, sample injection of 20 kV for 2 seconds, and operating at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

3.4 Increasing the sensitivity of capillary electrophoresis for clozapine detection

It was found the extraction method was not responsible for the low sensitivity detection for CZE from the previous study. Therefore, in order to increase the sensitivity, several methods can be investigated. These include adjusting the sample injection value and increasing the optical pathlength of a capillary by either using a high sensitivity cell or a capillary wider in internal diameter. The use of a high sensitivity cell is further discussed in section 4.3.

Sample loading of CE was performed by electrokinetic injection in this study. The amount of sample injected was controlled by applying high voltage for a certain time, which supported the elctroosmotic migration of the BGE. In order to increase the

amount of sample injected into the capillary, the voltage was raised from 20 kV $2s^{-1}$ to 25 kV $2s^{-1}$. The results exhibited band broadening and tailing (data not shown). The reason for this observation was possibly due to sample overloading. Therefore, the sample injection was fine tuned to 23 kV $1.5s^{-1}$. The outcome of this adjustment did not resolve the low sensitivity problem, nevertheless, the resolution of the peaks was significantly increased.

It was therefore decided to increase the optical pathlength of the capillary by using a 75 µm (i.d.) fused-silica capillary in order to increase the detection sensitivity. A capillary with a wider internal diameter not only increases the optical pathlength, but also increases the loading capacity of a capillary. The result shows a linear relationship between the average peak area and clozapine concentration (Figure 3.4.1). By using a 75 µm (i.d.) fused-silica capillary for clozapine separation a two to six fold improvement of the detection limit was exhibited when compared to a standard 50 µm (i.d) capillary ($p < 0.01$). The electropherogram showed improvement in the sensitivity and resolution and the migration time was also shortened to 3.68 minutes (Figure 3.4.2). Regression analysis of 0.983 when using the 75 µm (i.d.) capillary, and 0.947 using the 50 µm (i.d.) capillary were obtained. Therefore, a 75 µm (i.d.) fused-silica capillary was used for all subsequent studies.

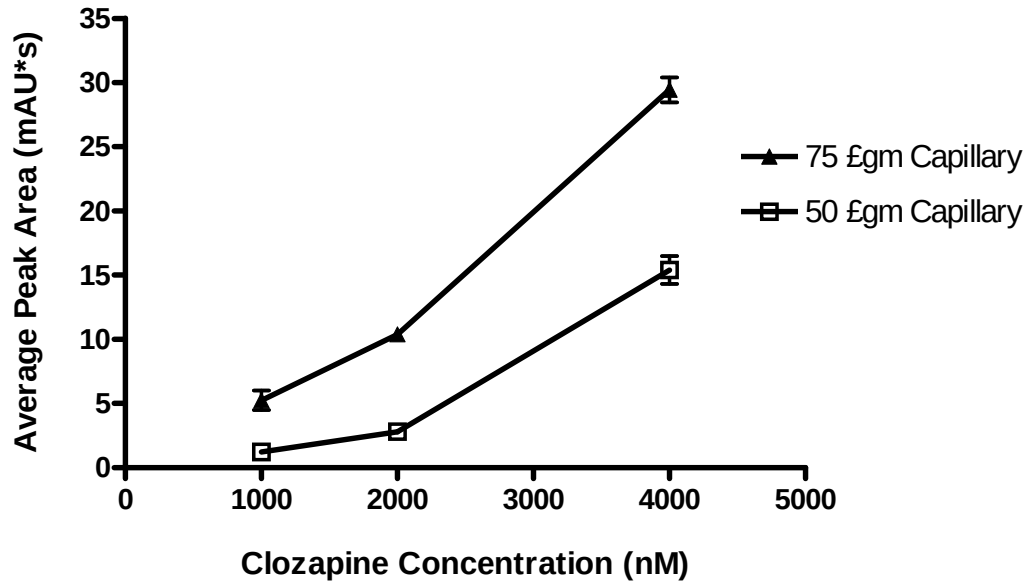


Figure 3.4.1 The comparison between the use of two separate capillaries different in internal diameter

Two separate capillaries were compared, one is a standard capillary with 50 µm internal diameter, and a 75 µm in internal diameter. Clozapine concentrations of 1000, 2000 and 4000 nM were analysed. Fifty µL of protriptyline hydrochloride (internal standard - 25 mg/L) was used in this experiment. The following conditions were kept constant; BGE of 40 mM (pH 2.5), apply voltage at 10 kV, sample injection of 23 kV for 1.5 seconds, and operate at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

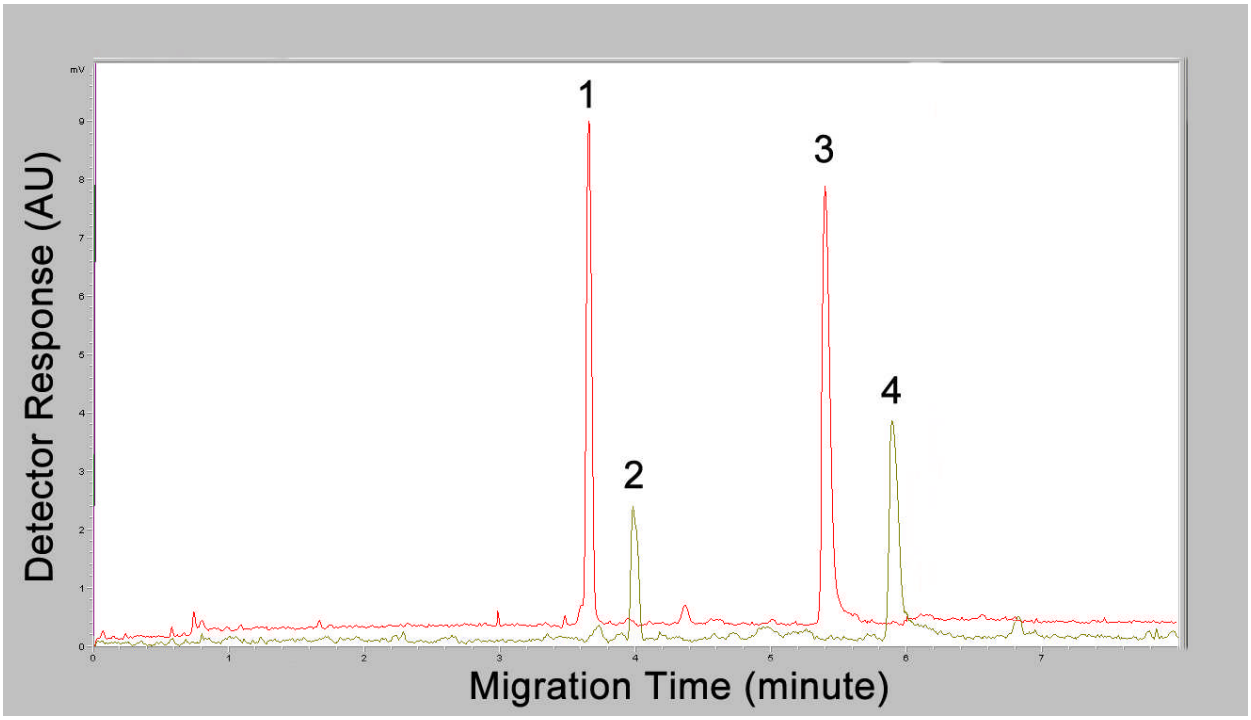


Figure 3.4.2 The electropherogram for the comparison between two different types of capillary for clozapine separation

Clozapine concentrations of 4000 nM were analysed. Internal standard, protriptyline, analysed by 75 µm (peaks 1 & 3) and 50 µm (peaks 2 & 4) capillary. The following conditions were kept constant; BGE concentration of 40 mM, BGE pH 2.5, apply voltage at 10 kV, sample injection of 23 kV for 1.5 seconds, and operate at a wavelength of 210 nm.

The use of a 75 µm (i.d.) capillary not only increased the internal diameter but the EOF was also different compared to the standard capillary (50 µm i.d.). The BGE concentration, hence the ionic strength, influences the EOF, and also the viscosity of the medium inside a capillary (Equation 4). The viscosity of the medium also depends on the capillary diameter, therefore, an experiment was carried out to compare the effect of using different BGE concentrations with a 75 µm (i.d.) capillary. BGE concentrations of 40, 60 and 80 mM were compared. Serum spiked with clozapine was extracted by method A. The result showed a linear correlation between the peak area and clozapine concentration (Figure 3.4.3); as the BGE concentration increased, the average peak area also increased. The ANOVA analysis showed that there was no significant difference between the use of 60 and 80 mM BGE for each data point ($p < 0.05$). However, there are significant differences between 40 and 60 mM BGEs used for clozapine concentrations of 2000 and 4000 nM ($p < 0.01$). Furthermore, the arrow in figure 3.4.4 indicates peak tailing for the use of 80 mM, therefore, it was decided to use a BGE at a concentration of 60 mM for all later experiments using a 75 µm (i.d.) capillary.

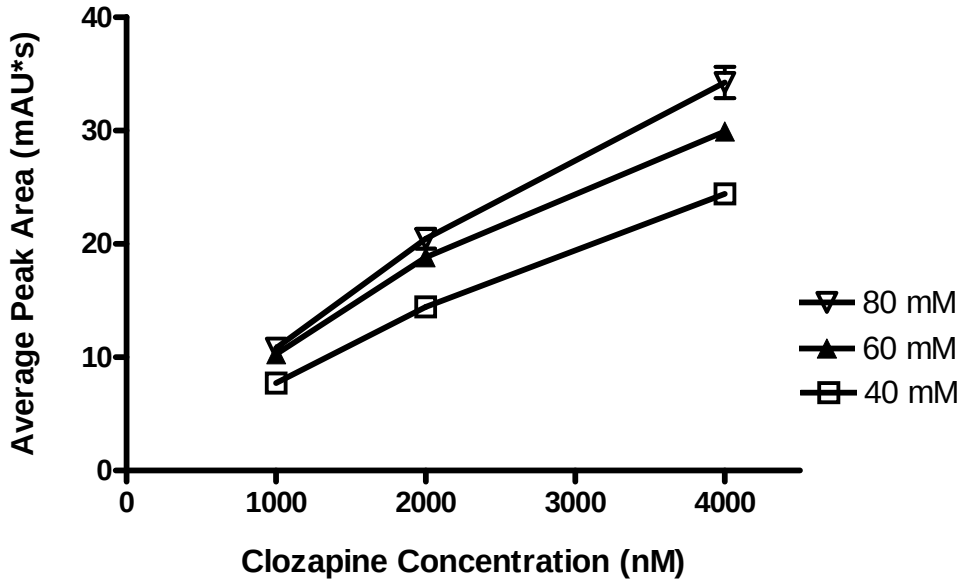


Figure 3.4.3 The effectiveness of different BGE concentrations to the clozapine separation by using a 75 µm (i.d.) capillary

Different BGE concentrations were compared ranging from 40 to 80 mM. Clozapine concentration of 1000, 2000, 4000 nM were analysed. Thirty µL of protriptyline hydrochloride (internal standard - 25 mg/L) was used in this experiment. A number of conditions were kept constant in this experiment; 75 µm (i.d) fused-silica capillary was used, BGE pH of 2.5, temperature at 22 ^{o}C, apply voltage at 10 kV, sample injection of 23 kV per 1.5 seconds, and operate at a wavelength of 210 nm. Average peak area was calculated as absorbance multiplied by the clozapine migration time. Each data point represents the mean ± SE of triplicate analysis.

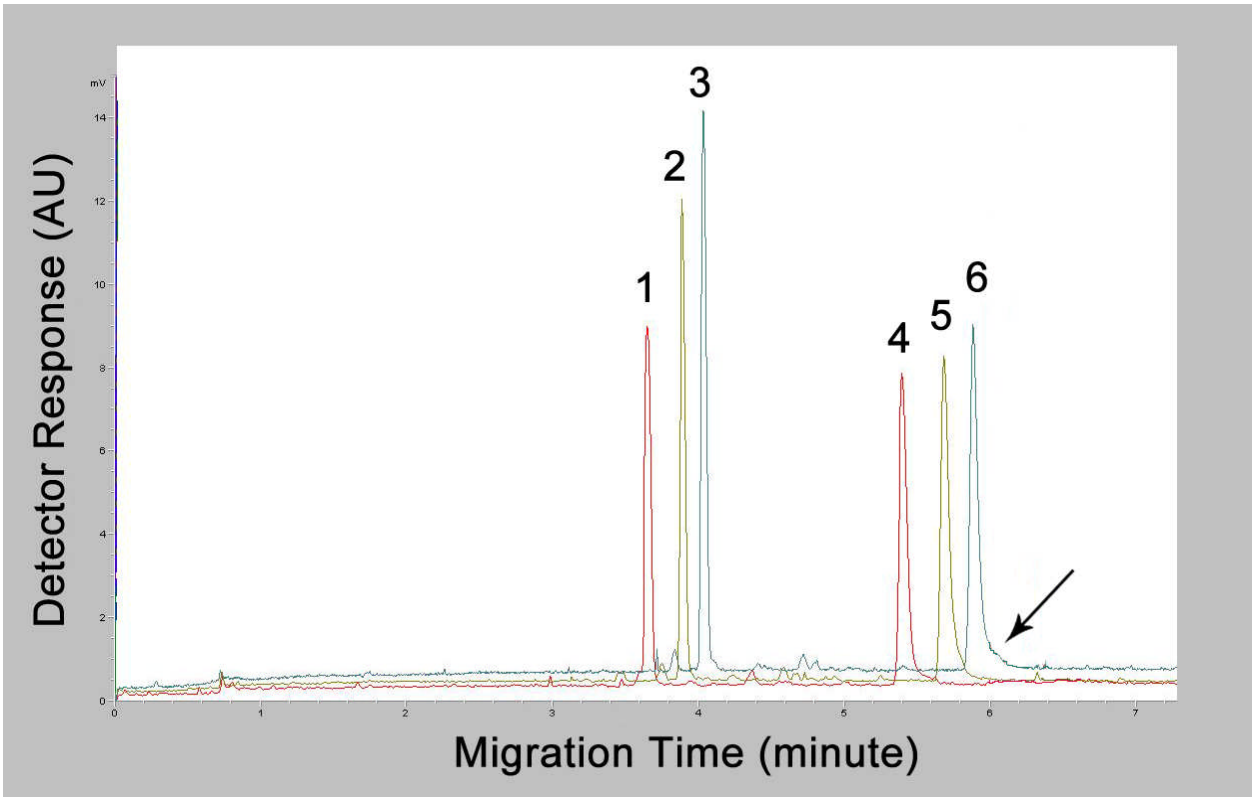


Figure 3.4.4 The electropherogram for the comparison between three different BGE concentrations

The electropherogram for clozapine separation by using a 75 µm (i.d.) fused-silica capillary with BGE concentration of 40 mM (peaks 1 & 4), 60 mM (peaks 2 & 5), and 80 mM (peaks 3 & 6). Peak 1-3 are clozapine, and peak 4-6 are the internal standard. Clozapine concentrations of 4000 nM were analysed. The following conditions were kept constant; BGE pH 2.5, apply voltage at 10 kV, sample injection of 23 kV for 1.5 seconds, and operate at a wavelength of 210 nm.

Table 3.2.1 is a summary of clozapine and N-desmethylclozapine separations using a 75 µm (i.d.) fused-silica capillary. Two sets of serum spiked samples were analysed with three different concentrations, 1000, 2000, and 4000 nM. The recovery percentage was calculated by dividing the observed concentration with the spiked concentration and multiplied by one hundred. The intra-assay CV % was calculated as the ratio of the standard deviation and the mean, multiplied by one hundred. The recovery and intra-assay represent one set of samples, each with triplicate runs, whereas the inter-assay was calculated by combining two sets of samples.

	Concentration (nM)	Recovery ± SD (%)	Intra-assay CV (%) (n=3)	Inter-assay CV (%) (n=9)
Clozapine	1000	97 ± 1.1	0.9	3.1
	2000	107 ± 1.6	0.7	1.2
	4000	100 ± 0.8	1.2	1.4
N-desmethylclozapine	1000	89 ± 1.2	1.9	4.7
	2000	100 ± 4.6	0.8	1.7
	4000	98 ± 2.0	2.5	2.0

Table 3.4.1 A summary from the determination of clozapine level from spiked standard by using the optimal conditions for CZE

A summary of the final CZE condition for clozapine separation by using a 75 µm fused-silica capillary. Clozapine concentration of 1000, 2000 and 4000 nM was used for this study. Each result represents a triplicate analysis. BGE of 60 mM (pH 2.5), and apply voltage at 10 kV was used in this study. Each sample was injected by electrokinetic injection at 23 kV per 1.5 seconds. The on-line UV detection wavelength was at 210 nm.

3.5 The comparison of high performance liquid chromatography and capillary electrophoresis for clozapine and N-desmethylclozapine analysis

Patients' samples were investigated using the final method developed from the previous experiments.CZE was performed using a 75 µm (i.d.) fused-silica capillary, BGE concentration of 60 mM and pH of 2.5 at a temperature of 22 ^{o}C. 10 kV was applied during the separation, and each sample was injected at 23 kV per 1.5 seconds. One hundred and eight patients' samples were analysed by CZE using these parameters. Nine hundred µL of each sample was extracted by extraction method A. The clozapine concentration was calculated as the ratio of the clozapine to the internal standard peak area, and compared to the standard ratio stored in the integrator. The results gained by

CZE were compared to the HPLC analysis. N-desmethylclozapine was not analysed here because analyses of this metabolite are not performed in the Canterbury Health Laboratory. A regression line of 1.100 was obtained from a linear graph of the results gained from CE versus the results analysed by HPLC (Figure 3.5.1). The X axis is the clozapine concentration gained by HPLC method, and the Y axis is the result gained from CE analysis. The equality line is demonstrated in a dashed line (x=y), and the regression line shown here as a solid line. The graph showed regression line bias to the left of the graph.

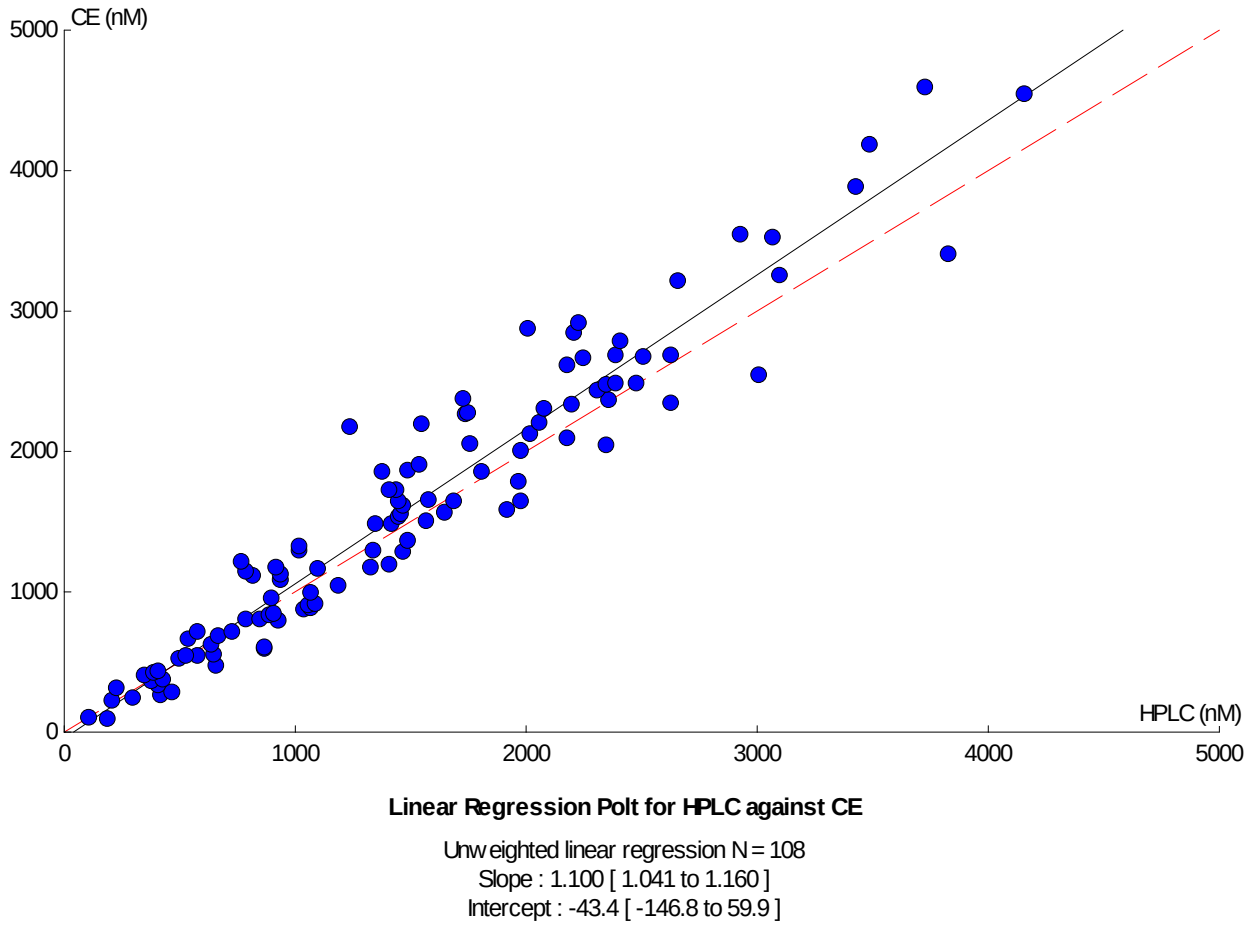


Figure 3.5.1 The comparison between clozapine separation by CZE and HPLC

A 75 µm (i.d.) fused-silica capillary was used for the CZE study. Fifty µL of protriptyline hydrochloride (internal standard - 25 mg/L) was used. The following conditions were kept constant for CZE method; BGE concentration of 60 mM, BGE pH 2.5, apply voltage at 10 kV, sample injection of 23 kV for 1.5 seconds, and operated at a wavelength of 210 nm. Brownless RP-8 5 micron column were used for HPLC. The flow rate of mobile phase was 1.5 mL per minute, and operated at a wavelength of 254 nm.

A difference plot was performed by method validator (Figure 3.5.1). Each point indicates the difference between the two analytical methods, HPLC and CE. The X axis indicates the mean result, and the Y axis is the differences between these two methods. This graph shows the majority of differences between the two methods lie between +800 and -400 nM. It also shows a trend for the difference to increase with the magnitude of the measurement. This indicates that larger discrepancies between these two methods occur at higher clozapine concentrations.

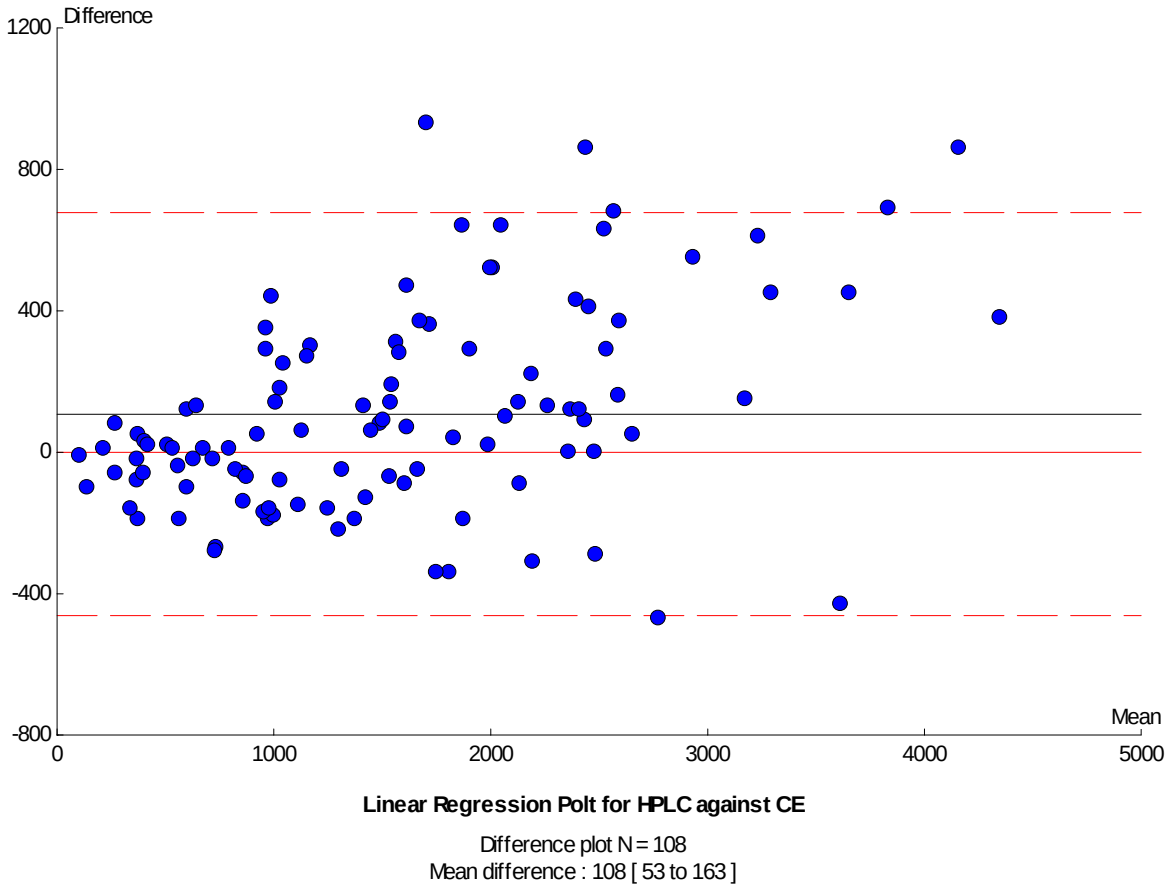


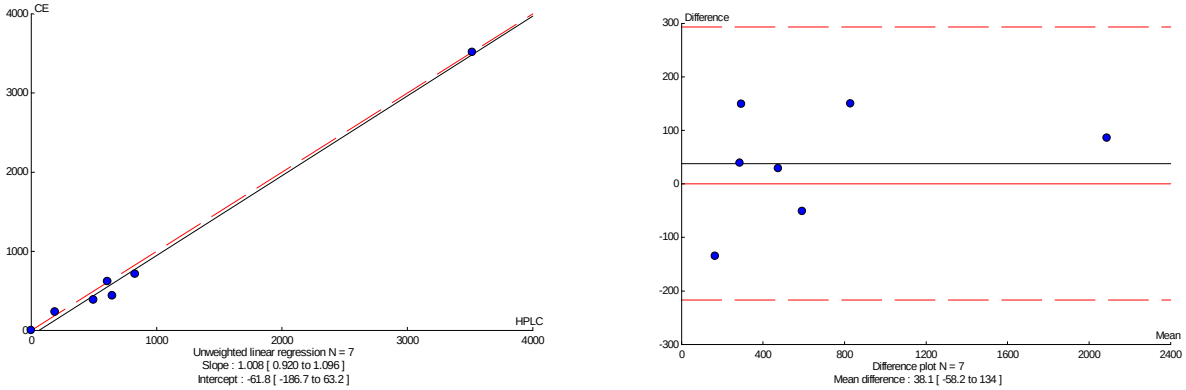
Figure 3.5.2 The difference plot for the comparison between clozapine separation by CZE and HPLC

A difference plot with 95% limits of agreement (dash line) was plotted with the same experimental conditions as above. Each point is the difference between concentrations for HPLC and CE analysis, and was plotted against the mean of each patient's sample.

Seven separate external quality control (EQC) samples from the Cardiff Bioanalytical services were analysed by CE for both clozapine and N-desmethylclozapine. The CE

results were compared with HPLC. Figure 3.5.3 shows the liner regression graph and difference graph for clozapine and N-desmethylclozapine comparisons between CE and HPLC. The linear regression graph (a) for clozapine analysis shows bias toward the right of the equality line, indicating the result obtained from HPLC is slightly higher than CE, whereas the linear regression line bias is towards the left of the equality line for N-desmethylclozapine (b). The difference plot for clozapine showed small discrepancies throughout all concentrations. The differences lie between +200 and -200 nM for clozapine, but between +100 and -300 nM for N-desmethyclozapine. The difference plot (b) shows that large discrepancies between HPLC and CE occurred at low concentration levels.

(a) Clozapine



(b) N-desmethylclozapine

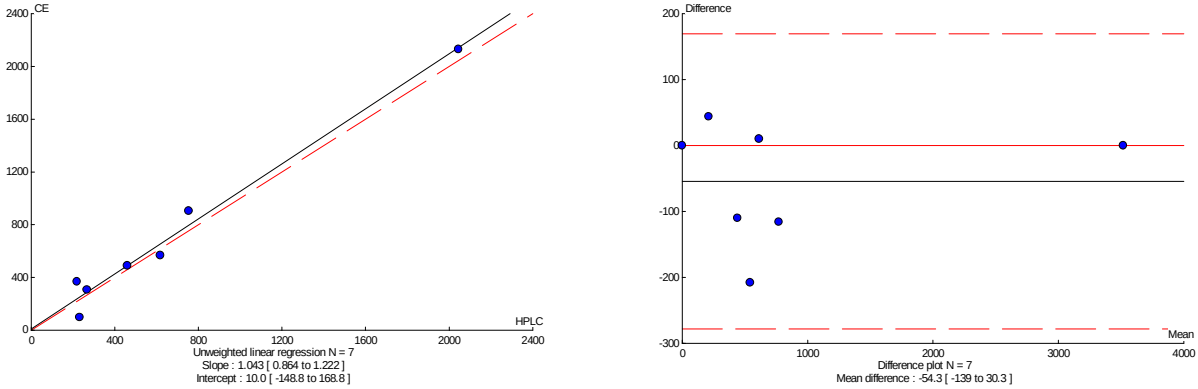


Figure 3.5.3 The linear regression plots and difference plots for external quality control samples

Experimental conditions were same as above. Seven external quality control samples were analysed by CE and compared to HPLC results. The linear regression plot and difference plot for clozapine (a) and N-desmethylclozapine (b) are performed with 95% limits of agreement.

DISCUSSION

4.1 The optimal condition for clozapine separation by capillary electrophoresis

4.1.1 Overview of capillary electrophoresis

CE is a fast and powerful separation technique for the analysis of charge molecules, amino acid, peptides, proteins, and compounds. It is a simple automatic technique that has many advantages over the traditional gel electrophoresis. Analytes are separated by the strong force of EOF based on the differences of their charge to mass ratios. The on-line electronic sample detection of CE is similar to HPLC, where the output results are represent as chromatography. However, CE is fundamentally based on electrophoresis.

The use of bare glass capillary in CE was found to have a high potential for analyte and protein absorption to the capillary wall. This solute-wall interaction results in dissentious zeta potential across the capillary, and consequently alters the EOF (Towns 1992). Binding of solute to the capillary wall may lead to band broadening, band tailing and irreproducible results. Furthermore, bare glass capillary is extremely fractured, hence difficult to install. Other types of capillary such as, pyrex borosilicate, fused silica, and teflon capillary were investigated in detail. Lukacs and Jorgenson (1985) demonstrated pyrex borosilicate capillary could not be use in an analysis at a wavelength shorter than 280 nm. The use of teflon capillary resulted in serious absorption of proteins, as well as hydroxyl group and other anions from the medium. Moreover, teflon capillary also exhibited poor thermal conductivity. The author concluded that fused silica capillary is preferable for heat dissipation, and it is the better choice for short wavelength analysis in CE. Fused silica capillary is now widely used in CE, and is the most preferable capillary material, although it exhibits some solute-wall absorption. Capillary surface modifiers, such as trimethylsilane, and cross linked methylcellulose were used in conjunction with protein separation in order to reduce protein and solutes binding to the fused silica capillary wall. However, in the case of clozapine separation, the additional of a modifier is unnecessary, because clozapine has a low molecular weight (326.823 g

Mol^{-1}). Furthermore, the addition of a surface modifier in BGE has the potential to reduce the separation migration time, due to the interaction with analytes.

A total capillary length of 33 cm with an effective length of 24.5 cm was used in this study. The effective length is the distance from the injection end of capillary to the detection window (Figure 1.5.1). For an efficient, fast separation, it is best to use a capillary that is as short as possible (Equation 5). Since the molecular weight of clozapine is small, 33 cm of capillary is sufficient for the separation. This is the minimum length for the CE instrument used in this study.

The total mobility in a capillary is the sum of the electrophoretic mobility and the EOF mobility. Several factors can influence the total mobility, which is the dielectric constant of BGE, zeta potential, and the viscosity of the BGE (Equation 4). Thus, these factors can be influenced by various parameters, such as the BGE concentration, BGE pH, temperature, and applied voltage (Section 3.1). These parameters easily affect on the analyte's migration time and the efficiency of the separation (Equation 5 and 6). Therefore, an optimal condition was determined in this study as the primary goal for the clozapine and N-desmethylclozapine separation. Once the optimal condition was confirmed, serum spiked with clozapine was analysed. Patients samples were analysed using the final optimal condition for CZE and compared with the result obtained from HPLC (Section 3.5).

4.1.2 BGE Concentration

An early study was done by Fujiwara and Honda in 1986, examining the effects of different BGE concentration and pH on cinnamic acid and its analogues separation by CE. It was found that as the salt concentration of BGE increased, the migration time of analyte also increased. The separation of clozapine in this study also exhibited the same observation seen in Fujiwara and Honda's study (data not shown). The migration time of clozapine for 40 mM and 60 mM BGE was 5 and 5.3 minutes respectively. As the BGE concentration increased, the velocity of EOF decreased and hence increased the migration time (Equation 5). This study also showed the ionic strength of BGE is also correlating with the current. The current increased significantly as the BGE concentration increased above 80 mM. This resulted in excessive background noise, reducing the signal to noise ratio, low resolution and eventually band broadening (data

not shown). This observation was possibly due to the Joule heating effect (further discuss on section 4.1.5 and section 4.4).

There was no peak obtained by using 100 mM of BGE. The current was increased to 100 µA at this concentration. This resulted in increasing the viscosity of BGE, and decreasing the EOF (Equation 3 and 4). Putting all these factors together, this suggested that clozapine was either not separated during the run time course (10 minutes), or the BGE was too viscous for clozapine separation to occur. A paper published by Gao et al, (2006) showed an example of phospholipids separation using a high concentration of BGE. By taking the migration time and resolution into account, the author decided to use the BGE concentration as low as 5 mM. In the case of clozapine separation in this study, diluted BGE (ie. 20 mM) resulted in low conductivity, and less viscosity, consequently the loading capacity was also reduced. Although the migration time was reduced with 20 mM BGE, considering the peak area and efficiency of the separation, it was decided to use 40 mM of BGE as the final condition (Figure 3.1.1).

4.1.3 pH of BGE

BGE pH is the most important parameter for selectivity in CZE. Since the EOF is related to the dielectric constant of the BGE, the zeta potential and the viscosity of the BGE is relatively important as well (Equation 4). Thus variation of the pH may lead to differences of the EOF because the charged capillary surface wall is extremely sensitive to pH changes. The EOF in a fused-silica capillary was found to exhibit a sigmoid relationship with pH (Lukacs and Jorgenson, 1985; Lambert and Middleton, 1990). That is, as the pH of BGE increases, the electroosmotic mobility also increased. Phosphate buffer has a pK_a of 2.1, therefore, BGE was prepared within its buffering range. pH of 1.5 to 3.0 was investigated in this study.

It was found pH between 2.0 and 2.5 demonstrated the best condition for clozapine separation. However, pH at 2.0 demonstrated variable and irreproducible results. Therefore it was decided to use pH 2.5 as the final condition. The peak area and resolution was small and low for the use of BGE at pH 1.5. This is due to the suppressed silanol group at the capillary wall, resulting in reducing the EOF approximate to zero. The current was drawn up to 150 µA, and irreproducible separation was seen in this study.

At high pH, the silanol groups at the surface of capillary are fully ionised. This generated strong zeta potential, which resulted from the dense electrical double layer (Figure 1.5.3). Consequently, the EOF increased as the pH of BGE increased. In the end, the migration time also increased with pH, and band broadening was seen as the pH elevated (Fujiwara and Honda, 1986; Gao et al., 2006). The same effect was also observed in this study for the BGE pH at 3.0 (Figure 3.1.2). However, this result was contradictory to the result obtained from a clozapine separation study by Hillaert et al (2004). Hillaert et al has successfully separated clozapine by using BGE pH 3.5. This was probably due to the high concentration of BGE which was used in their study. Hillaert et al used BGE at a concentration 80 mM which reduced the EOF in theory, and eventually the EOF was increased to maintain the EOF mobility since BGE pH 3.5 was used. This could explain the reason why BGE pH 3.0 demonstrated a low peak area, because a low concentration of BGE (40 mM) was used in this study.

4.1.4 Temperature

Temperature in CZE is an important parameter which is inversely proportional to the viscosity of BGE, thus influences the total mobility of BGE in a capillary (Equation 3 and 4). When high voltage is applied to the CE, heat will be generated as a function of current. It was estimated the electrophoretic mobility increases by two percent per one degree in temperature. The temperature in the center can be predicted to be higher than at both ends of the capillary. This creates an axial temperature gradient during the separation process. Since the BGE in the middle region is less viscous than the outer region near to the wall, a viscosity gradient will also result from a radial temperature gradient. Therefore, the BGE is sensitive to the temperature, and it can influence the migration time of an analyte, as well as the selectivity and efficiency of the analysis (Grushka et al., 1989; Jorgenson and Lukacs, 1983; Xuan and Li, 2005).

Research work from the early stage of CE development has suggested that capillary should be thermostated. The temperature effect was investigated and cautiously controlled in this study by adjusting the thermostat from 18 to 28 ^{0}C. The result showed both 22 and 24 ^{0}C demonstrated the best condition for clozapine separation. However, due to the large variations seen for 24 ^{0}C (CV = 14%) the temperature of 22 ^{0}C was used for the final optimal condition (Figure 3.1.3).

Reijenga et al (2007) have demonstrated the use of phosphate BGE at different temperatures for *p*-aminobenzonic and *o*-aminobenzonic enantiomers separation. It was found the migration time roughly decreased 0.7 second when the temperature increased from 15 ^{0}C to 25 ^{0}C. In the case of this study, the migration time was only decreased 0.3 second with the 10 ^{0}C difference. The peak shapes and baseline noise showed no differences for Reijenga's aminobenzonic separations when the temperature was elevated, even up to 45 ^{0}C, whereas in this study, when the temperature was elevated above 24 ^{0}C, the background noise increased, and band broadening was observed. The observations seen in this study were somehow different compared to Rejienga's result. This might possibly be due to the differences of analytes and BGE's p*K*a value. Moreover, the temperature gradients model proposed by Grushka et al (1989) showed that the radial temperature effects are negligible for narrow capillaries, (i.e. 50 µm i.d.) therefore, the band broadening observed at higher temperature in this study might be due to the development of axial temperature gradients, generated by nonuniform electric field across the capillary. A pressure gradient might also be developed during the process in order to maintain the EOF. This axial thermal gradient effect is often seen in CZE with thermostatting due to the use of short capillary, where both ends of the capillary were left outside of the cartridge for sample injection and maintaining the EOF flow (Xuan and Li, 2005).

4.1.5 Applied Voltage

Assuming the molecular diffusion is the only source of zone broadening in a capillary, equation 6 has demonstrated by applying the highest voltage, maximum efficiency of separation can be achieved. According to equation 5, the capillary length does not directly influence the efficiency of a separation but it does affects the migration time of the analyte. Therefore, in theory, it is expected to apply the highest voltage to the shortest capillary during method development. In reality, it is impractical to use high voltage because of the thermal effects. It is because the shorter capillary has less surface area for heat dissipation. As a result, band broadening, disrupted baseline, and nonuniform EOF will occur due to Joule heating effect (Jorgenson and Lukacs, 1983). When an electric field is applied, current will be generated by the flow of ions from BGE. Thus Joule heating is the by product of current flowing through the resistant BGE inside the capillary. As a consequence, the internal temperature will increase, and the viscosity

of the BGE will decrease. Therefore, Joule heating effects can interfere with the total mobility and affect the efficiency of the separation (Equation 3, 4, and 6).

Joule heating effects were observed in this study when 20 kV or above was applied to the CZE system. When the applied voltage increased to 20 kV, the migration time decreased to 2.1 seconds. The current raised over 70 μA and the peak area decreased dramatically (Figure 3.1.4). Unstable current, disrupted baseline and low signal to noise ratio were observed throughout the analysis. The same effect was also seen for 25 kV as applied voltage with the addition of band broadening. Moreover, the migration time was decreased to 1.6 seconds, and the current was raised to 90 μA. The reason for decreasing migration time is because the electro-osmotic mobilities were increased as the applied voltage elevated (Cross and Gao, 1999). There was no peak obtained by using 5 kV (Figure 3.1.4). This is probably due to the low electric field applied which resulted in low current. As a consequence, the ion flow of BGE was not rapid enough for analytes zone broadening inside the capillary. Thus, the separation did not occur.

4.2 A comparison of protocols for plasma clozapine and N-desmethylclozapine extraction

The optimal condition for a working solution of clozapine separation was determined from the previous experiments by using a 50 μm (i.d.) fused silica capillary, as described in Table 3.1.1. To confirm this optimal condition for clozapine and N-desmethyclozapine separation by CE, three different concentrations of working solutions were investigated, 1000, 2000 and 4000 nM. The result showed a r^2 value of 0.979 and 0.989 for clozapine and N-desmethylclozapine respectively (Figure 3.2.1). This indicates the average peak area increased as a function of the drug concentration. When serum spiked with clozapine and N-desmethylclozapine was separated by this optimal condition, the result demonstrated two to six fold decrement in sensitivity when compared to the working solutions (data not shown). To investigate further whether the lost of sensitivity was due to the extraction process, four extraction protocols were compared (Section 2.2.2). Method A is the current clozapine extraction method carried out in the Canterbury Health Laboratory, whereas extraction methods B, C, and D were from literature. Serum spiked with clozapine was analysed in three concentrations, 1000, 2000 and 4000 nM. The results showed no significant differences ($p < 0.05$) for each

data point when these four methods were compared. This indicated the lost of sensitivity may be due to other reasons, possibly the limit of detection of CE.

CE is a highly efficient separation technique, and it is very sensitive to even a slight change of BGE or analyte composition. Serum sample was extracted and reconstituted in 50% methanol. In theory, the result of serum spiked sample would recover from the standard solution which only consists of clozapine with 50% methanol. However, in practical analysis, the analyte injected into CE was more viscous than the standard solution. This was possibly due to lipids and impurities carried over during the extraction process, resulting in a different matrix than standard solution. Therefore, capillary blockage was often seen when a 50 µm (i.d.) capillary was used. The sudden drop of current to zero was a sign of capillary blockage.

4.3 Increasing the sensitivity of capillary electrophoresis for clozapine detection

The sample volume injected onto CE was estimated as 1000 fold smaller than HPLC. Moreover, the use of narrow capillary has 100 times less detection pathlength in comparison to a HPLC column (Hempel, 2000). Therefore, the sensitivity of detection has always been the greatest barrier for the development of CE. There are various other reasons for the low sensitivity and low resolution. These may include the binding of solutes to the capillary wall, which resulted in band broadening, limited loading capacity or short optical pathlength of the capillary.

In order to investigate whether the loss of sensitivity was due to the solute-wall interaction, 1M NaOH and IPA was introduced to flush the capillary between each run to diminish possible precipitate formation at the side of capillary wall. However, the result showed no significant improvement in the clozapine separation ($p>0.05$). Furthermore, flushing strong NaOH and IPA between runs resulted in irreproducible resolution and efficiency, and variable migration time (data not shown). This may be due to the disrupted EOF by strong solvent during the analysis.

Beer-Lambert's law stated that the absorbance is directly proportional to the molar extinction coefficient, the pathlength of the capillary, and the concentration of the

solution. According to the theory, the absorbance will increase with the increment of optical pathlength of a capillary. To improve the detection limits of the CE, a high sensitivity cell (Agilent Technologies) was introduced in this study to increase the sensitivity for clozapine separation by CZE. Mrestani and Neubert (2000) have demonstrated thiamine separation using a high sensitivity cell. The results showed the detection limit was improved up to nine fold compared to a standard capillary. In this study, the result showed an improvement of twenty fold when a high sensitivity cell was introduced to the CZE. However, irreproducible and variable results were obtained (data not shown). Longer migration time was also required for the use of a high sensitivity cell for clozapine separation.

It was finally decided to use a 75 µm (i.d.) capillary to increase the limits of detection. The use of a wider capillary provided a longer detection pathlength, and permited a larger sample size. Furthermore, a wider capillary has less potential for capillary blockage, and it is easier to wash out any impurities left over in the capillary during the analysis. Furthermore, it was suggested that a wider capillary has smaller surface to volume ratio, thus solute-wall interaction may be reduced. Loading capacity will also be increased by using a wider capillary (Grushka et al., 1989). The results showed a two to six fold increase in the detection limit compared to the 50 µm (i.d.) capillary (Figure 3.4.1; Figure 3.4.2).

4.4 Increasing the sensitivity of capillary electrophoresis using a 75 µm (i.d.) capillary

As discussed in section 4.1, temperature and applied voltage affected the migration time and resolution of a CZE system, whereas BGE pH affects the selectivity of a separation analysis. The BGE concentration is the major parameter that would influence many factors, such as the migration time, the resolution and the efficiency. Possible Joule heating may have occurred when the applied voltage was above 20 kV. Axial temperature gradient generation might be the reason for band broadening and reducing signal to noise ratio at high temperature. Figure 3.1.2 showed a distinct value of BGE at pH 2.5 is the best condition for clozapine selectivity. Therefore, when the narrow capillary was replaced with a wider internal diameter capillary (75 µm), the BGE pH, temperature and the applied voltage were kept constant but three different BGE concentrations were compared for the use of a new wider capillary.

The result showed both 60 mM and 80 mM of BGE concentration demonstrated the best condition for clozapine separation using a 75 µm (i.d.) capillary (Figure 3.4.3). However, the electropherogram exhibited peak tailing and band broadening when the 80 mM of BGE was used (Figure 3.4.4).

Joule heating is a function of time, voltage applied, cross section area of a capillary, and the total length of a capillary (Rathore, 2004). Thus there is a potential to increase the joule heating effect by increasing the capillary inner diameter. Furthermore, Joule heating effect was also found to be a function of phosphate BGE concentration from 100 to 210 mM (Cross and Cao, 1999). The author has also mentioned that Joule heating is negligible for low BGE concentrations (ie. 25 to 75 mM). Their experiments were done using a 50 µm (i.d.) capillary. However, possible Joule heating effect was found in this study using 75µm (i.d.) capillary with BGE concentration of 80 mM. This finding is some what contradictory to the results obtained from Cross and Cao's study. A high concentration of BGE has more conductivity than dilute BGE, which draws higher current and produces more heat. Consequently, band broadening by Joule heating effect possibly occurred in this study.

Table 3.4.1 is a summary of clozapine and N-desmethylclozapine separation results using the optimal condition for CZE analysis with a 75 (i.d.) capillary. Drug free serums were spiked with clozapine and N-desmethylclozapine in three concentrations, 1000, 2000, and 4000 nM. The recovery percentages ranged from 89 to 107 percent. The intra-essay coefficient of variation percentage (CV%) represent in triplicate analysis of the same sample and the inter-essay represent in three different samples with the same concentration. The CV% for both clozapine and N-desmethylclozapine are 0.8-2.5% for the intra-essay, and 1.2–4.7% for the inter-essay. These results are acceptable as the CV% are less than 10%. This indicates the developed CE essay is validated for clozapine and N-desmethylclozapine separation.

4.5 The comparison of high performance liquid chromatography and capillary electrophoresis for clozapine and N-desmethylclozapine analysis

One hundred and eight patients' samples were analysed by CE and compared with the HPLC results. Due to time constraints and sample quantity, the HPLC results for comparison were obtained from the Canterbury Heath Laboratory toxicology's routine work. The clozapine separation results were compared and plotted into a linear regression plot (Figure 3.5.1). The graph demonstrated a straight line with a r^2 value of 1.100 and slightly biased to the left of the equality line. This indicated the results gained from CE are generally higher in value compared to HPLC. The difference plot (Figure 3.5.2) indicated a larger discrepancy occurred at high clozapine concentration. There were three samples which had a result of +800 nM difference compared to HPLC. Roughly 85% of the samples were under 2500 nM for clozapine, and more than 90% of the results were within +700 nM and -400 nM differences.

The External Quality Control (EQC) program involves various clinical laboratories world wide. There are roughly 70 and 60 laboratories which contribute to the EQC program for clinical clozapine and N-desmethylclozapine measurement respectively every month. Roughly 70% of these laboratories are using HPLC as their primary method. The objective of the EQC program is to strengthen the performance of each laboratory involved in this program, and ensure the validity of the results obtained by their methods. Seven EQC samples from 2007 to 2008 (stored at -20 ^{0}C) were analysed by CE, and compared with the HPLC results gathered worldwide. The results were compared by a linear regression plot and a difference plot for clozapine (a) and N-desmethylclozapine (b) (Figure 3.5.3). The linear regression graph shows a r^2 value of 1.008 for clozapine and 1.043 for N-desmethylclozapine. The data are within 95% of agreement. The difference plot for clozapine demonstrated a difference between +200 and -200 nM compared to HPLC, and N-desmethylclozapine had a difference between +100 and -300 nM. The results obtained from EQC demonstrated a small discrepancy between CE and HPLC, whereas the patients' sample comparison demonstrated a larger discrepancy at high concentration. This is due to the small sample size from ECQ program resulting in small variations. However, combining these two comparisons, it has been shown that clozapine and N-desmethylclozapine separation by CE is a valid method that can replace HPLC.

4.6 Instrumental Issues

CE has shown to be a more cost effective technique in terms of the solvent use for clozapine and N-desmethylclozapine essay compared to HPLC. Although CE is performed in a simple system which does not encounter sophisticated problems in this study, there are still some disadvantages involved with CE. Although the capillary was flushed with strong NaOH before each experiment, it was found solute-wall absorption within the capillary can still build up over time. Solute-wall absorption is commonly found with fused-silica capillary, and may result in band broadening. Therefore, a new capillary each month is recommended for daily analysis. Furthermore, the use of 75 μm (i.d.) capillary generates more waste due to the greater amount of analyte and reagents going through the capillary. It is recommended that a new waste vial is necessary every 10 runs, since the capacity of a standard vial is only 800 μL. A new deionised water vial, 0.1M NaOH vial, and one of the BGE vial (for flushing between runs) are also needed to after 30 runs.

4.7 Summary

An assay for clinical clozapine and N-desmethylclozapine was developed in this study using CE as a more cost effective technique compared to HPLC. The optimal condition for CZE was found to use a 75 μm (i.d.) fused-silica capillary, which provides a longer detection pathlength and permits a larger sample size. This improves the detection limit of the CE. The final CE separation technique was compared with HPLC over 108 patient's samples, and seven EQC samples. The results demonstrated clozapine and N-desmethylclozapine separation by CE is a validated method.

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Alan B, Robert WB, Brian K, Orlando RD, Irish D, Summerfelt A, Carpenter WJ (1994) Effects of Clozapine on Positive and Negative Symptoms in Outpatients With Schizophrenia. The American Journal of Psychiatry 151(1):20

Alastair GC, Gottesman II (2000) Twin studies of schizophrenia: From bow-and-arrow concordances to Star Wars Mx and functional genomics. American Journal of Medical Genetics 97(1):12-17

Alessandro S, Diana DR, Cristina L, Domenico B (2004) New Antipsychotics and Schizophrenia: A Review on Efficacy and Side Effects. Current Medicinal Chemistry 11(3):343

Amsler HA, Teerenhovi L, Barth E, Harjula K, Vuopio P (1977) Agranulocytosis in patients treated with clozapine. A study of the finnish epidemic. Acta Psychiatrica Scandinavica 56(4):241-248

Arendt M, Rosenberg R, Foldager L, Perto G, Munk-Jorgensen P (2005) Cannabis-induced psychosis and subsequent schizophrenia-spectrum disorders: follow-up study of 535 incident cases. The British Journal of Psychiatry 187(6):510-515

Black DW, Boffeli TJ (1989) Simple schizophrenia: Past, present, and future. The American Journal of Psychiatry 146(10):1261

Breier A (1995) Serotonin, schizophrenia and antipsychotic drug action. Schizophrenia Research 14(3):187-202

Capuano B, Crosby IT, Lloyd EJ (2002) Schizophrenia: Genesis, Receptorology and Current Therapeutics. Current Medicinal Chemistry 9(5):521

Combs MD, Perry PJ, Bever KA (1997) Norclozapine as a marker of Clozapine induced agranulocytosis and granulocytopeni. Pharmacotherapy 17:1300-1304

Cross RF, Cao J (1998) Salt effects in capillary zone electrophoresis: II. Mechanisms of electrophoretic mobility modification due to Joule heating at high buffer concentrations. Journal of Chromatography A 809(1-2):159-171

Dain JG, Nicoletti J, Ballard F (1997) Biotransformation of Clozapine in Humans. Drug Metab Dispos 25(5):603-609

Fujiwara S, Honda S (1986) Determination of cinnamic acid and its analogous by electrophoresis in a fused silica capillary tube. American Chemical Society 58:1811-1814

Gao F, Dong J, Li W, Wang T, Liao J, Liao Y, Liu H (2006) Separation of phospholipids by capillary zone electrophoresis with indirect ultraviolet detection. Journal of Chromatography A 1130(2):259-264

Gerlach J, and Peacock, L. (1995) Antipsychotics: the present status. International Clinical Psychopharmacology 10(Suppliment 3):39-48

Gerson SL (1994) G-CSF and the management ofclozapine induced agranulocytosis. Journal of Clinical Psychiatry 55(suppl B):139-142

Gerson SL, Arce C, Meltzer HY (1994) N-desmethylclozapine: a clozapine metabolite that suppresses haemopoiesis. British Journal of Haematology 86(3):555-561

Grushka E, McCormick RM, Kirkland JJ (1989) Effect of temperature gradients on the efficiency of capillary zone electrophoresis separations. Analytical Chemistry 61(3):241-246

Hempel G (2000) Strategies to improve the sensitivity in capillary electrophoresis for the analysis of drugs in biological fluids. Electrophoresis 21:691-698

Hiemke C, Dragicevic AG, Gerhard, Hätter S, Sachse J, Vernaleken I, Müller MJ (2004) Therapeutic Monitoring of New Antipsychotic Drugs. Therapeutic Drug Monitoring 26(2):156-160

Hillaert S, Snoeck L, Van den Bossche W (2004) Optimization and validation of a capillary zone electrophoretic method for the simultaneous analysis of four atypical antipsychotics. Journal of Chromatography A 1033(2):357-362

Hjertén S, Mohabbati S, Westerlund D (2004) Influence of ignored and well-known zone distortions on the separation performance of proteins in capillary free zone electrophoresis with special reference to analysis in polyacrylamide-coated fused silica capillaries in various buffers: I. Theoretical studies. Journal of Chromatography A 1053(1-2):181-199

Horacek J, Bubenikova-Valesova V, Kopecek M, Palenicek T, Dockery C, Mohr P, Hoschl C (2006) Mechanism of action of atypical antipsychotic drugs and the neurobiology of schizophrenia. CNS Drugs 20(5):389

Hunziker F, Kunzte F, Schmutz J (1963) Ube nein 5-stellung basisch substituente 5H Dibenzo (b,e) -1-4 diazepine. Helvetica Chimica Acta 46:2337-2347

Jann MW,Grimsley SR,Gray EC, Chang WH (1993) Pharmacokinetics and pharmacodynamics of clozapine. Clinical Pharmacokinetic 24:161-176

Jorgenson JW, Lukacs KD (1981) Free-zone electrophoresis in glass capillaries. Clinical Chemistry 27(9):1551-1553

Jorgenson JW, Lukacs KD (1983) Capillary zone electrophoresis. Science 222:266-272

Kim TM, Susan RM (2004) Schizophrenia. The Lancet 363(9426):2063

Kim Y-J, Chae J-S, Chang JK, Kang SH (2005) Microchip capillary gel electrophoresis using programmed field strength gradients for the ultra-fast analysis of genetically modified organisms in soybeans. Journal of Chromatography A 1083(1-2):179-184

Koichi O (2006) The n-3 fatty acid/dopamine hypothesis in schizophrenia. Japanese Journal of Psychopharmacology 26(4):149-153

Konradi C, Heckers S (2003) Molecular aspects of glutamate dysregulation: implications for schizophrenia and its treatment. Pharmacology & Therapeutics 97(2):153-179

Kvasnicka F, Biba B, Cvak L, Kratka J, Voldrich M (2005) Separation of enantiomers of butorphanol and cycloamine by capillary zone electrophoresis. Journal of Chromatography A 1081(1):87-91

Kvasnicka F, Voldrich M, Vyhnalek J (2007) Determination of glycyrrhizin in liqueurs by on-line coupled capillary isotachophoresis with capillary zone electrophoresis. Journal of Chromatography A 1169(1-2):239-242

Lagarrigue M, Bossée A, Bégos A, Delaunay N, Varenne A, Gareil P, Bellier B (2008) Field-amplified sample stacking for the detection of chemical warfare agent degradation products in low-conductivity matrices by capillary electrophoresis-mass spectrometry. Journal of Chromatography A 1178(1-2):239-247

Lambert WJ, Middleton DL (1990) pH hysteresis effect with silica capillary in capillary zone electrophoresis. Analytical Chemistry 62:1585-1587

Le DC, Morinb CJ, Beljeanc M, Siouffia AM, Desbèneb PL (2005) Electrophoretic separations of twelve phenothiazines and N-demethyl derivatives by using capillary zone electrophoresis and micellar electrokinetic chromatography with non ionic surfactant. Journal of Chromatography A 1063(1-2):235-240

Liu HC, Chang WH, Wei FC, Lin SK, Lin SK, Jann MW (1996) Monitoring of plasma clpzapine level and its metabolites in refractory schizophrenic patients. Therapeutic Drug Monitoring 18(2):200-207

Lovdahl MJ, Perry PJ, Miller DD (1991) The assay of Clozapine and N-desmethylclozapine in human plasma by HPLC. Therapeutic drug monitoring 13(1):69-72

Lukacs KD, Jorgenson JW (1985) Capillary zone electrophoresis: Effect of physical parameters on separation efficiency and quantitation. Journal of High Resolution Chromatography & Chromatography Communications 8:407-411

Meltzer HY, Alphs L, Green AI, Altamura AC, Anand R, Bertoldi A, Bourgeois M, Chouinard G, Islam MZ, Kane J, Krishnan R, Lindenmayer J-P, Potkin S (2003) Clozapine Treatment for Suicidality in Schizophrenia: International Suicide Prevention Trial (InterSePT). Archives of General Psychiatry 60(1):82-91

Meltzer HY, Bastani B, Ramirez L, Matsubara S (1989) Clozapine: New research on efficacy and mechanism of action. European Archives of Psychiatry and Clinical Neuroscience 238(5):332-339

Mohammad Masud I, Atiq R, Zahid H, Syed Zaber M, William GR, Jacqueline MF (2003) Clozapine: A clinical review of adverse effects and management. Annals of Clinical Psychiatry 15(1):33

Mrestani Y, Neubert RHH (2000) Thiamine analysis in biological media by capillary zone electrophoresis with a high-sensitivity cell. Journal of Chromatography A 871(1-2):351-356

Prior TI, Chue PS, Tibbo P, Baker GB (1999) Drug metabolism and atypical antipsychotics. European Neuropsychopharmacology 9(4):301-309

Rathore AS (2004) Joule heating and determination of temperature in capillary electrophoresis and capillary electrochromatography columns. Journal of Chromatography A 1037(1-2):431-443

Reid WH, Mason M, Hogan T (1998) Suicide Prevention Effects Associated With Clozapine Therapy in Schizophrenia and Schizoaffective Disorder. Psychiatric Services 49(8):1029-1033

Reijenga JC, Gagliardi LG, Kenndler E (2007) Temperature dependence of acidity constants, a tool to affect separation selectivity in capillary electrophoresis. Journal of Chromatography A 1155(2):142-145

Riviello JM, Harrold MP (1993) Capillary electrophoresis of inorganic cations and low-molecular-mass amines using a copper-based electrolyte with indirect UV detection. Journal of Chromatography A 652(2):385-392

Schwarz MA, Raith K, Dongowski G, Neubert RHH (1998) Effect on the partition equilibrium of various drugs by the formation of mixed bile salt/phosphatidylcholine/fatty acid micelles: A characterization by micellar affinity capillary electrophoresis. Part IV. Journal of Chromatography A 809(1-2):219-229

Shitij K, Gary R (2001) Atypical antipsychotics: New directions and new challenges in the treatment of schizophrenia. Annual Review of Medicine 52:503

Szilagyi A, Bonn GK, Guttman A (2007) Capillary gel electrophoresis analysis of G-quartet forming oligonucleotides used in DNA-protein interaction studies. Journal of Chromatography A 1161(1-2):15-21

Terabe S (1989) Electrokinetic chromatography: an interface between electrophoresis and chromatography, trends analytical chemistry. Trends in Analytical Chemistry 8:129-134

Towns JK (1992) Impact of polycation adsorption on efficiency and electroosmotically driven transport in capillary electrophoresis. Analytical Chemistry 62:2473-2478

Tsuang M (2000) Schizophrenia: genes and environment - One process or more? Biological Psychiatry 47:210-220

Ulrich S, Wolf R, Staedt J (2003) Case report; Serum level of Clozapine and relapse Therapeutic drug monitoring 25(3):252-255

Végvári Á, Hjertén S (2002) Stable homogeneous gel for molecular-sieving of DNA fragments in capillary electrophoresis. Journal of Chromatography A 960 (1-2):221-227

Wagstaff AJ, Bryson HM (1995) Clozapine; a review of its pharmacological properties and therapeutic use in patients with schizophrenia who are unresponsive to or intolerant of classical antipsychotic agents. . CNS Drugs 4(5):370-400

Wan H, Östlund Å, Jönsson S, Lindberg W (2005) Single run measurements of drug-protein binding by high-performance frontal analysis capillary electrophoresis and mass spectrometry. Rapid Communications in Mass Spectrometry 19(12):1603-1610

Wang W, Zhou F, Zhao L, Zhang J-R, Zhu J-J (2007) Measurement of electroosmotic flow in capillary and microchip electrophoresis. Journal of Chromatography A 1170(1-2):1-8

Wätzig H, Günter S (2003) Capillary Electrophoresis -- A High Performance Analytical Separation Technique. Clinical Chemistry & Laboratory Medicine 41(6):724-738

Wu J, Huang T (2006) Peak identification in capillary isoelectric focusing using the concept of relative peak position as determined by two isoelectric point markers. Electrophoresis 27(18):3584-3590

Xuan X, Li D (2005) Band broadening in CZE with axial temperature gradients. Electrophoresis 26:166-175

Zhou G-H, Luo G-A, Zhang X-D (1999) Microemulsion electrokinetic chromatography of proteins. Journal of Chromatography A 853(1-2):277-284