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DETERMINATION OF CREATININE AND CREATINE
BY
CAPILLARY ELECTROPHORESIS

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ABSTRACT

The assessment of creatinine and creatine in biological fluids is important in the evaluation of renal and muscular functions. For routine creatinine determinations in the clinical laboratory, the most frequently used method is the spectrophotometric one based on the Jaffé reaction. However, this reaction is not specific for creatinine.

For this reason, several methods have been proposed, but the elimination of interferences in the determination of creatinine has still not been achieved in some of these methods; others solved this problem either with expensive equipment that does not suit routine analysis or necessitates time-waste procedures.

In this thesis capillary electrophoresis was the new tool investigated. It was applied in an attempt to achieve both the separation of creatinine from the non-creatinine 'Jaffé-reacting' chromogens and the determination of creatine in serum.

Capillary zone electrophoresis was performed with detection at wavelength 480 nm to separate creatinine from the non-creatinine 'Jaffé-reacting' chromogens in urine. The principle was based upon the different migration times due to the different molecule weights, molecular sizes and charges under the applied high voltage. The picric acid was employed as part of the running buffer to allow reaction of creatinine and picrate to take place after the sample injection. This procedure eliminated the negative influence of the reaction time that is controlled manually in the common Jaffé reaction method. Therefore, compared to the Jaffé reaction method, the new method achieved more accuracy and precision in the determination of creatinine.

Determination of creatinine in serum and urine were studied at a new wavelength 417 nm, which gave a higher sensitivity of detection than at 480 nm. This wavelength shift made the determination of creatinine in serum possible by capillary zone electrophoresis without the non-creatinine 'Jaffé-reacting' chromogens interfering. In this method, serum only needed a simple filtration before the analysis.

Creatine was discovered to have absorption at 417 nm in alkaline medium. Moreover, specific sample stacking was introduced in this method. The sample was dissolved in a mixture of two-volumes acetonitrile and one-volume 3 % ammonium chloride to give a 10-fold enhancement of detection sensitivity.

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TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	iv
Table of Contents.....	v
List of Figures.....	ix
List of Tables and Schemes.....	xiii
List of Abbreviations.....	xiv

CHAPTER ONE INTRODUCTION 1

1.1	History.....	1
1.2	Principles.....	3
1.2.1	Electroosmotic flow.....	5
1.2.2	Separation efficiency in free zone capillary electrophoresis.....	7
1.3	Instrumentation.....	9
1.4	Detection.....	10
1.5	CE reagents.....	11
1.6	Different mode of capillary electrophoresis.....	11
1.6.1.	Capillary zone electrophoresis (CZE).....	12
1.6.2	Capillary gel electrophoresis (CGE).....	14
1.6.3	Micellar electrokinetic capillary chromatography (MEKC).....	14
1.6.4	Capillary electrochromatography (CEC).....	15
1.6.5	Capillary isoelectric focusing (CIEF).....	16
1.6.6	Capillary isotachorphoresis (CITP).....	16
1.7	Capillary electrophoresis as a clinical chemistry.....	16
1.8	Summary.....	18

CHAPTER TWO CREATININE AND CREATINE 20

2.1	Introduction.....	20
2.1.1	Tests measuring glomerular filtration rate.....	23
2.1.2	Creatine and creatinine.....	24
2.2	Literature review.....	27

2.2.1	Assay of creatinine.....	27
2.2.1.1	The Jaffé reaction:.....	27
2.2.1.2	The specificity of the Jaffé reaction can be improved by:.....	33
2.2.1.3	Other method for assay of creatinine.....	36
2.2.2	Assay of creatine.....	38
2.2.2.1	A widely used method is the Jaffé reaction.....	38
2.2.2.2	The determination of creatine by enzymatic methods:.....	38
2.2.2.3	HPLC (high-performance liquid chromatography) can separates compounds before quantitation:.....	39
2.2.2.4	Biosensors: an option of determining creatine as well as creatinine.....	39
2.3	Compare CE with other techniques.....	40

CHAPTER THREE MATERIALS AND GENERAL PROCEDURES 43

3.1.	Apparatus.....	43
3.2	Chemicals.....	43
3.3	General procedures.....	44
3.3.1	Reagents.....	44
3.3.2	Apparatus.....	46
3.3.2.1	Capillary electrophoresis.....	46
3.3.2.2	UV Spectrophotometer.....	47

CHAPTER FOUR DERMINATION OF CREATININE IN URINE 48

4.1	Introduction.....	48
4.2	Methods.....	49
4.3	Results and discussion.....	50
4.3.1	Selection of the mode of capillary electrophoresis.....	50
4.3.2	Selection of capillary.....	50
4.3.3	Selection of the analysis method.....	51
4.3.3.1	CZE study one.....	51
4.3.3.2	CZE study two.....	55
4.3.3.3	CZE study three.....	62

4.3.4	Interferences.....	70
4.3.5	Reproducibility (precision).....	73
4.3.6	Calibration of the creatinine	76
4.3.7	Recovery	78
4.3.8	Sensitivity (detection limit).....	81
4.4	Conclusion.....	81

CHAPTER FIVE DETERMINATION OF CREATININE IN SERUM 82

5.1	Introduction.....	82
5.2	Methods.....	83
5.3	Results and discussion.....	84
5.3.1	Selection of the analysis method.....	84
5.3.1.1	Creatinine in serum CZE study one.....	84
5.3.1.2	Creatinine in serum CZE study two.....	86
5.3.1.3	Creatinine in serum CZE study three.....	88
5.3.1.4	Improve the resolution.....	92
5.3.2	Interferences.....	96
5.3.3	Reproducibility (precision).....	99
5.3.4	Calibration of the creatinine.....	102
5.3.5	Blank readings.....	104
5.3.6	Recovery.....	105
5.4	Determination of creatinine using the standard laboratory Jaffé reaction.....	108
5.5	Conclusion.....	110

CHAPTER SIX DETERMINATION OF CREATINE IN SERUM 111

6.1	Introduction.....	111
6.2	Methods.....	112
6.3	Results and discussion.....	113
6.3.1	Selection of the analysis method.....	113
6.3.1.1	Creatine in serum CZE study one.....	113
6.3.1.2	Creatine in serum CZE study two.....	114
6.3.2	Reproducibility (precision).....	117

6.3.3	Calibration of the creatine	120
6.3.4	Blank reading.....	122
6.3.5	Sensitivity (detection limit).....	122
6.3.6	Recovery.....	123
6.4	Conclusion.....	124

CHAPTER SEVEN	CONCLUSION AND FUTURE WORK	125
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7.1	Conclusions.....	125
7.2	Future work.....	126

REFERENCES	127
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LIST OF FIGURES

Figure 1.1	(a) A glass U tube apparatus used in early experiments with free-solution electrophoresis. Electrodes made of platinum foil were immersed in the electrolyte solution. The sample solution with indicator dye was at the bottom of the U tube. (b) An inverted U tube apparatus, which consisted of two tubes, filled with agar bridging the sample reservoir and reservoirs of distilled water.	1
Figure 1.2	Zone electrophoretic separation of dansyl amino acids.	2
Figure 1.3	Schematic representation of ions at a silica-solution interface	5
Figure 1.4	The total velocity of each molecule.	8
Figure 1.5	Basic scheme of the CE instrument.	10
Figure 1.6	Diagrammatic representation of (A) Capillary zone electrophoresis (CZE), (B) Capillary gel electrophoresis (CGE), (C) Micellar electrokinetic capillary chromatography (MEKC or MECC), and (D) Capillary electrochromatography (CEC). V_x is the linear migration velocity of the analyte X. V_{eo} is the electroosmotic velocity, V_{ep} is the electrophoretic velocity and k' is the phase capacity ratio.	13
Figure 2.1	A, Schematic drawing of right and left kidneys with respect to liver, stomach, duodenal loop, spleen, adrenal gland, and bladder, B, Diagram of a vertical section through the kidney. Nephron and blood vessels greatly enlarged.	21
Figure 2.2	Schematic drawing of the glomerulus and the tubular system.	22
Figure 2.3	Schematic presentation of the excretion of various types of substances by the nephron.	24
Figure 2.4	The pathway of synthesis for creatine in the human.	25
Figure 2.5	The pathway of the creatinine synthesised from creatine in human body.	26
Figure 2.6	Equilibrium between creatinine and creatine.	27
Figure 2.7	Structure of creatinine.	28
Figure 2.8	Identification of the 1:1 complex formed at 480 nm, by use of	

	the method of continuous variation.	30
Figure 2.9	Differential scans of the reaction of alkaline sodium picrate and creatinine with time.	30
Figure 2.10	Dependence of the observed pseudo-first-order rate constant on picric acid concentration.	31
Figure 2.11	^{13}C NMR spectrum of creatinine in $^2\text{H}_2\text{O}$.	31
Figure 2.12	^{13}C NMR spectrum of picric acid in deuterated dimethyl sulfoxide.	32
Figure 2.13	^{13}C NMR spectrum of picric acid in deuterated dimethyl sulfoxide after addition of NaO^2H .	32
Figure 2.14	^{13}C NMR spectrum of the reaction product of picric acid in deuterated dimethyl sulfoxide, and creatinine in $^2\text{H}_2\text{O}$ in the presence of NaO^2H .	32
Figure 2.15	Structure of the 1/1 complex.	33
Figure 2.16	Flow profiles in HPLC (left) and CZE (right).	41
Figure 4.1	A, 10 mM creatinine and 8 mM PA. B, 10 mM creatinine and 8 mM PA with spiked creatinine. C, 8 mM PA. Condition: Method 1 .	52
Figure 4.2	A postulated intermediate in the reaction of 1,3,5-trinitrobenzene with sodium hydroxide.	52
Figure 4.3	Peak distortion of high analyte concentration.	54
Figure 4.4	Comparison of Method 1 and Method 2c . A, 10 mM creatinine in Method 1 . B, 10 mM creatinine in Method 2c . C, 10 mM creatinine in Method 2c with spiked creatinine. D, 30mM PA in Method 2c .	57
Figure 4.5	Effect of buffer pH. Condition: Method 2a, 2b, 2c .	59
Figure 4.6	Influence of buffer pH on electroosmotic mobility. ∇ = Tris-HCl; \square = boric acid-NaOH; \circ = borax-HCl. Buffer concentration: 50 mM.	60
Figure 4.7	Migration time changed due to the affection of temperature. A, 27 °C, 10 mM creatinine. B, 30 °C, 5 mM creatinine.	61
Figure 4.8	Effect of the buffer composition.	62
Figure 4.9	Comparison of the sensitivities.	64

Figure 4.10	Plot of K^+ and Li^+ peak area as a function of sample solution resistance for both electrokinetic and hydrostatic injection. Electrokinetic injection causes a bias linear in sample solution resistance (which is inversely proportional to electrolyte concentration).	66
Figure 4.11	The allowed injection plug length as a function of the solute's diffusion coefficient. The analysis time was assumed 10 minutes. Each line corresponds to a different allowable loss in the efficiency (increasing H).	68
Figure 4.12	Comparison of the affect of migration time by difference voltages	70
Figure 4.13	Non-creatinine interferences	72
Figure 4.13a	Interferences with addition of creatinine.	72
Figure 4.14	Within run precision.	75
Figure 4.15	Differences concentration of creatinine.	79
Figure 4.16	Recovery of creatinine in Method 3f .	80
Figure 5.1	Effect of the concentration of picric acid in buffer.	85
Figure 5.2	A, 1.5 mM creatinine at the condition of Method 5a₁ . B, >1.5 mM creatinine at the condition of Method 5a₂ . C, 0.1 mM creatinine at the condition of Method 5a₃ .	87
Figure 5.3	UV spectrophotometer scans at wavelength range between 200-800 nm within 5 minutes reaction.	90
Figure 5.4	Comparison of sensitivity at difference wavelength.	91
Figure 5.5	Increase the concentration of PA in buffer.	92
Figure 5.6	Difference voltages gave difference resolution.	94
Figure 5.7	Choice of CE temperature.	95
Figure 5.8	Interferences at wavelength 417 nm.	97
Figure 5.9	Creatine at 417 nm.	99
Figure 5.10	Stability and reproducibility.	100
Figure 5.11	Differences concentration of creatinine.	103
Figure 5.12	Recovery.	106
Figure 5.13	Determination of urine sample with Method 6e .	108

Figure 6.1	Creatine has absorption at 417 nm. Condition: Method 7 .	113
Figure 6.2	Enhanced detection due to sample stacking.	115
Figure 6.3	Illustration of stacking of anions in a high-pH buffer.	116
Figure 6.4	Creatine within-run precision. Condition: Method 9 .	119
Figure 6.5	Standard creatine.	121
Figure 6.6	Creatine in serum.	123

LIST OF TABLES AND SCHEMES

Table 2.1	HPLC methods for analysis of creatinine	37
Table 4.1	Methods	49
Table 4.2	Interferences	71
Table 4.3	CV	73
Scheme 4.1	5 mM creatinine reproducibility	76
Table 4.4	Recovery	77
Scheme 4.2	Calibration of creatinine	79
Table 4.5	Standard creatinine	81
Table 5.1	Methods	83
Table 5.2	Effect of picric acid concentration on the observed forward rate constants	84
Table 5.3	Interferences	98
Scheme 5.1	Reproducibility	101
Table 5.4	CV	101
Table 5.5	Analytical range	102
Scheme 5.2	Calibration of creatinine in serum. Condition: Method 6e .	104
Table 5.6	Recovery	107
Scheme 5.3	Calibration of creatinine of the standard laboratory Jaffé reaction method	109
Table 5.7	Creatinine in serum and urine in the standard Jaffé reaction method	110
Table 6.1	Methods	112
Scheme 6.1	UV scans for creatine from 400 nm to 500 nm.	114
Table 6.2	Reproducibility	117
Table 6.3	Standard creatine	119
Scheme 6.2	Reproducibility of creatine.	120
Scheme 6.3	Calibration of creatine.	122
Table 6.4	Recovery	124

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate, disodium salt hydrate
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CI	creatine amidinohydrolase
CIEF	capillary isoelectric focusing
CITP	capillary isotachopheresis
CK	creatine kinase
CRN	creatinine
CV	coefficient variation.
CZE	capillary zone electrophoresis
EC	enzyme convention number
EOF	electroosmotic flow.
HPCE	high-performance capillary electrophoresis
HPLC	high performance liquid chromatography
HVPS	high-voltage power supply
I.D.	internal diameter
LD	lactate dehydrogenase
MECC or MEKC	micellar electrokinetic chromatography
MES	2-(N-morpholino)ethane sulfonic acid
ODS	octadecyl silica or C18 silica
PA	picric acid
PAGE	polyacrylamide gel electrophoresis
PAH	p-aminohippurate
PDA	photodiode array
PK	pyruvate kinase
Pos	positive
SDS	sodium dodecyl sulfate
SO	sarcosine oxidase
TCA	trichloroacetic acid

Tris	tris (hydroxymethyl-aminoethane)
UV	ultra violet
UV-VIS	ultraviolet-visible
WA	washing

CHAPTER ONE

INTRODUCTION

1.1 Historical background

Capillary electrophoresis (CE) is a modern analytical technique, which permits rapid and efficient separations based on the principles of charge, hydrophobicity, stereospecificity, size, or interaction with buffer additives.

The history of the development of capillary electrophoresis has been traced back to more than a century ago, as early as the late 1800s, when electrophoretic separations were attempted in free solutions as well as various gels. Many early experiments were performed using glass U tubes with electrodes connected to each of the tubes' arms as shown in **Figure 1.1**.

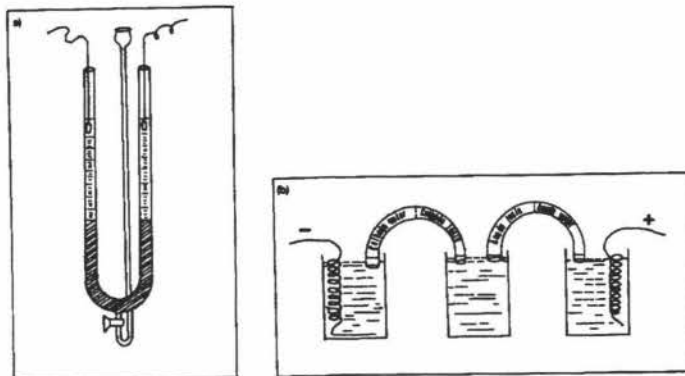


Figure 1.1 (a) a glass U tube apparatus used in early experiments with free-solution electrophoresis. Electrodes made of platinum foil were immersed in the electrolyte solution. The sample solution with indicator dye was at the bottom of the U tube. (b) an inverted U tube apparatus, which consisted of two tubes, filled with agar bridging the sample reservoir and reservoirs of distilled water (Li, 1992).

The experiments were performed using a direct current of up to several hundred volts. The separation of various types of samples, such as small ions, isotopes, toxins and proteins was investigated. In order to overcome problems of convective mixing which

were encountered in electrophoretic separations performed in free solutions, various stabilizing media have been employed, such as agar, cellulose powder, glass wool, paper, silica gel and acrylamide (Li, 1992).

An alternative approach to alleviate thermal convection problems in free solution electrophoresis was the use of tubes with small internal diameters. These small tubes or capillaries dissipate heat better and provide a more uniform thermal cross-section of the sample within the tube. Provided ideal conditions can be maintained, samples migrate rapidly as a flat plug with resolution limited only to diffusion. Hence, the technique has the potential of achieving extremely high efficiency in separations.

In the early days, tube diameters of 1-3mm, were first used and then reduced in size to 200-400 μm . In 1971, Everaerts explored the separation potential of capillary zone electrophoresis (CZE) performed in 200- μm Teflon capillaries with on-column UV detection. In 1981, Jorgenson and co-workers convincingly demonstrated the analytical potential of CZE using 75- μm fused silica capillaries. The experience of this laboratory (Jorgenson, 1981) in capillary chromatography was highly useful in designing instrumentation for this new electrophoretic system. The high-resolution capability of the method was clearly shown, as illustrated in **Figure 1.2**

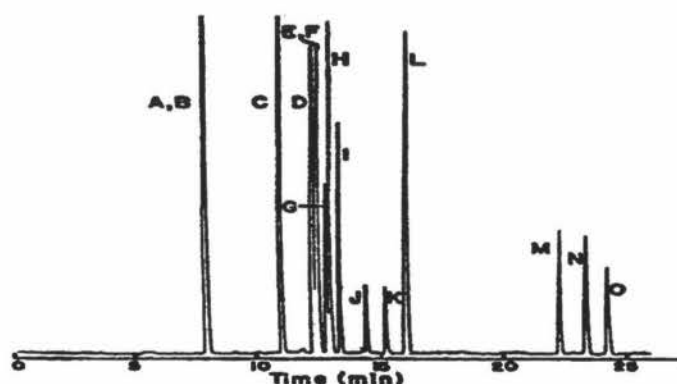


Figure 1.2. Zone electrophoretic separation of dansyl amino acids: A, unknown impurity; B, lysine; C, dilabeled lysine; D, asparagine; E, isoleucine; F, methionine; G, serine; H, alanine; I, glycine; J and K, unknown impurities; L, dilabelled cysteine; M, glutamic acid; N, aspartic acid; O, cysteic acid. The concentration of each derivative is approximately 5×10^{-4} M, dissolved in operating buffer (Guzman, 1993).

The importance of capillary operation to reduce the Joule heating effects caused by the desirable high electric fields was emphasized (Jorgenson, 1981). This work is rightly credited with the initiation of high-performance capillary electrophoresis (HPCE).

The invention of micellar electrokinetic chromatography (MECC, or MEKC), which involved adding a surfactant to the electrophoretic buffer to form micelles to enhance resolution of neutral substances (Terabe *et al*, 1984), represents another significant step forward in the development of CE. Since then, various types of modifiers for the enhancement of selectivity in CE separation have been investigated.

The developments in gel-filled capillaries and coated columns have further enhanced the scope and efficiency of capillary electrophoretic techniques (Hjerten, 1983). Theoretical plate numbers in the multimillion ranges can now be routinely achieved using gel-filled capillaries in CE separations (Cohen and Karger, 1987).

At the end of the 1980s, commercial CE instruments became available. With the rapid advances currently being made, CE is now gaining popularity as an alternative analytical tool for some routine analytical applications.

1.2 Principles of separation in capillary electrophoresis (CE)

The mechanism of separation in electrophoresis is based on the migration of charged molecules in an applied field (potential or electric field). Most electrophoretic techniques involve simple variation of the environment of the molecule to either change the charge on the molecule (i.e. isoelectric focusing) or to physically retard the movement of the molecule in the electric field (i.e. polyacrylamide gel electrophoresis, PAGE). This gives an experimenter a tremendous variety of approaches to enhance the selectivity of the separation process. The utility of the technique is so useful that it can separate anything, ranging from inorganic ions to whole cells (Camilleri, 1993).

Separations are accomplished through the movement of ions in an applied electric field. This movement is governed by the *mobility* of an ion (μ), where this mobility is defined as the average velocity with which an ion moves under the influence of an applied potential field. The two primary factors that affect the mobility include, first the applied

electric force (F_{EF}), which depends on the charge of the ion (q) and the electric field strength (E ; in V/cm), [Eq. (1.1)];

$$\overline{F}_{EF} = q\overline{E} \quad (1.1)$$

and secondly, the frictional force (F_{FR}) that a molecule encounters as it moves through solution [Eq. (1.2)]. The simplest estimation of the frictional force (which neglects the effect of other ions in solution, and merely assumes that the total retarding force is equal to the fictional drag determined by Stoke's Law) depends on a number of parameters, including the viscosity of the buffer (η) and the velocity (v ; cm/s) and size of the molecule (related to the radius of a spherical molecule, r).

$$\overline{F}_{FR} = 6\pi\eta r\overline{v} \quad (1.2)$$

At equilibrium, these two forces are directly balanced and the electrophoretic velocity [\overline{v} ; Eq. (1.3)] depends on a number of parameters involving both the analyte and the separation column.

$$\overline{v} = \frac{q\overline{E}}{6\pi\eta r} \quad (1.3)$$

More commonly, people are concerned with the electrophoretic mobility (μ), which is just the velocity normalized to the electric field strength [Eq. (1.4)].

$$\overline{\mu} = \frac{\overline{v}}{\overline{E}} = \frac{q}{6\pi\eta r} \quad (1.4)$$

The net effect of these balanced forces results in the following properties for the mobility of an ion:

1. Mobility is directly proportional to the charge of the ion
2. Mobility is inversely proportional to the viscosity of the solvent
3. Mobility is inversely proportional to the radius of particle (represented by the diffusion coefficient)

It is important to note that all of the parameters discussed here are vector quantities, not scalar numbers. This implies that each force or velocity has a directional component (either aligned with or against the electric field). This vector representation will be seen to be even more important when we consider the mobility found in capillary electrophoresis (Camilleri, 1993).

1.2.1 Electroosmotic flow (EOF)

One of the most distinguishing features of capillary electrophoresis is electroosmotic flow (EOF). This bulk movement of solvent is caused by the small zeta potential (ζ) at the silica/water interface, which induces a minute excess of anionic charge at the surface of silica (**Figure 1.3**).

(+) Anode

(-) Cathode

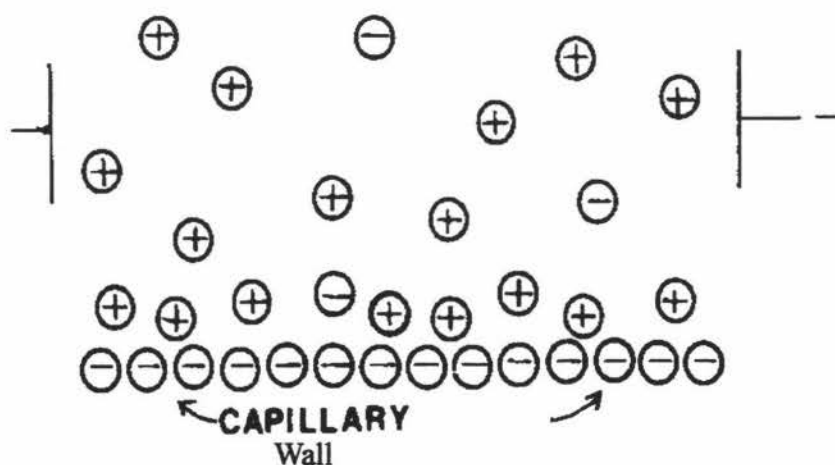
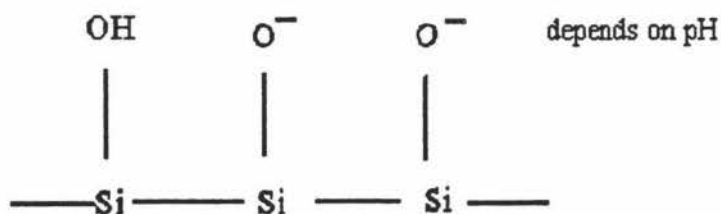


Figure 1.3

Schematic representation of ions at a silica-solution interface (Camilleri, 1993).

This charge, presumably due to the presence of dissociated -Si-OH groups on the surface of the capillary, is dependent on the pH of the buffer in the static diffuse double layer.



Titration of the silanol groups, $-\text{Si}-\text{OH} + \text{NaOH} \leftrightarrow -\text{SiO}^- + \text{Na}^+ + \text{H}_2\text{O}$ alters the degree of dissociation of the silanol (which has a pK_a of 6 to 7), thereby altering the ζ potential. This is directly related to the velocity of electroosmotic flow as shown in Eq. (1.5)

$$\bar{\mu}_{EO} = \left(\frac{\epsilon_o}{4\pi\eta} \right) E\zeta \quad (1.5)$$

For the silica surface, an excess of cationic species in bulk solution migrates toward the cathode, producing a net flow from anode to cathode. This means that the driving force of the flow originates at the walls of the capillary, producing a plug-like flow that has a flat velocity distribution across the capillary diameter, deviating only within a few nanometers of the capillary surface. This flat flow profile and its effect on net mobility (i.e., elution of positive and negatively charged species in electrophoresis) and its use as a pump in packed-column capillary chromatography were originally demonstrated by Lukacs and Jorgenson (1985). The flow velocity in a capillary previously exposed to acidic conditions was consistently lower than the flow from capillary previously exposed to alkaline solutions. For binary buffer mixtures containing water and a protic or an aprotic dipolar solvent, the pH of the solution was found to influence the electroosmotic flow (Schwer and Kenndler, 1991).

1.2.2 Separation efficiency in capillary electrophoresis

The efficiency of any chromatographic column is a function of both the retention time and the peak width of a given analyte. This function is referred to as the number of theoretical plates, (N), a name derived from the plate model of chromatography and is defined as:

$$N = \frac{16(t_R)^2}{W^2} \quad (1.6)$$

Where t_R = retention time for analyte, W = peak width.

The maximum separation efficiency that can be obtained in the most general case is that in which an analyte is introduced at one end of a tube, separated along the length of the tube, and detected as it leaves the tube. The theoretical approach to this is quite simple. Because most of the terms associated with band broadening are absent, then the problem can be avoided simply by using smaller internal diameter (<100 μ m) capillaries so that Joule heating could be virtually eliminated. In fact, the only term that remains is longitudinal diffusion. The efficiency of the separation can be represented as a number of theoretical plates (N), identical in definition to that used in chromatography. Jorenson and Lukacs (1983) demonstrated that N depends only on the total electrophoretic mobility (μ_{TOTAL} , in $\text{cm}^2\text{V}^{-1}\text{s}^{-1}$), the applied voltage (V, in volts), and the diffusion coefficient of the molecule (D_o , in cm^2s^{-1}), as shown in Eq. (1.7)

$$N = \frac{\bar{\mu}_{TOTAL} V}{2D_o} \quad (1.7)$$

The flow rate gives an additional velocity component to the ions migration in the electric field, such that the total velocity (μ_{TOTAL}) of each molecule is the vector sum of the electrophoretic velocity (v) and the velocity imparted by electroosmotic flow [v_{EO} ; Eq. (1.8)]:

$$\bar{v}_{TOTAL} = \bar{v} + \bar{v}_{EO} \quad (1.8)$$

Similarly, the total electrophoretic mobility is the vector sum of the electrophoretic mobility of the sample (μ) and the effective mobility due to electroosmotic flow (μ_{EO}), as shown in Eq. (1.9) and Figure 1.4

$$\bar{\mu}_{TOTAL} = \bar{\mu} + \bar{\mu}_{EO} \quad (1.9)$$

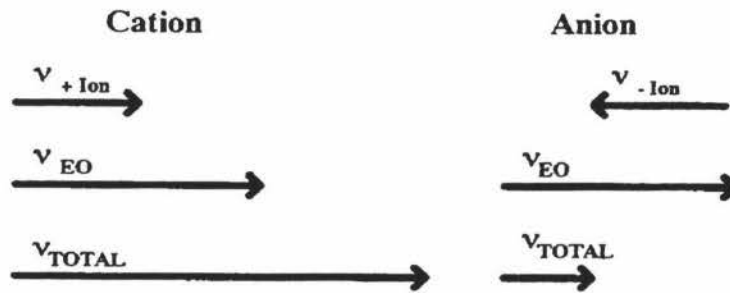


Figure 1.4 The total velocity of each molecule (Camilleri, 1993).

This can be used to great advantage in CE, because all molecules that have a net velocity toward the detector will eventually be observed. Thus, the presence of electroosmotic flow allows the separation of both negatively and positively charged species in the same run. This implies that the highest efficiency is obtained when species are migrating at the fastest velocity (i.e., have the largest value of (μ_{TOTAL}) and that we can use electroosmotic flow to help speed up the separation, and thereby increase the separation efficiency. The total number of theoretical plates is described by Eq. (1.10):

$$N = \frac{[(\bar{\mu} + \bar{\mu}_{EO})V]}{2D_o} \quad (1.10)$$

The Eq. (1.10) predicts theoretical values of the order of 10^6 for capillary electrophoresis. This compares with 5000 theoretical plates for HPLC (high

performance liquid chromatography) separations, and only 10 theoretical plates for conventional agarose gel electrophoresis (Jenkins, 1995)

The time that is required for the migration of a charged species in a separation capillary is simply the length of the capillary (L) divided by the linear velocity of the molecule [v_{TOTAL} ; Eq. (1.11)]. If we use the electrophoretic mobility (μ) instead of linear velocity in this calculation, the elution time is minimized with a high mobility.

$$t = \frac{L}{v_{TOTAL}} = \frac{L}{\mu_{TOTAL} V} \quad (1.11)$$

The resolution, R_s , between two compounds depends not only on the distance between the peak maxima, $t_{R2}-t_{R1}$, where t = time, but also on the narrowness of the peaks, i.e. the average of the inverse of the peak widths. Resolution is defined as:

$$R_s = t_{R2}-t_{R1}/1/2(W_1+W_2) \quad (1.12)$$

Resolution can be increased by increasing the length of the column.

1.3 Instrumentation

Capillary electrophoresis involves the separation of charged molecules in a buffer-filled capillary by the application of very high voltages (1 to 30 kV). The capillary, which may be either coated or fused silica, usually varies between 20 and 100 μ m in diameter and between 25 and 122cm in length. The ends of the capillary are placed in buffer vials, which also contain the electrodes. The narrow diameter of the capillary is important to permit rapid heat dissipation and to decrease analyte diffusion. A schematic representation of a capillary electrophoresis instrument is shown in **Figure 1.5**

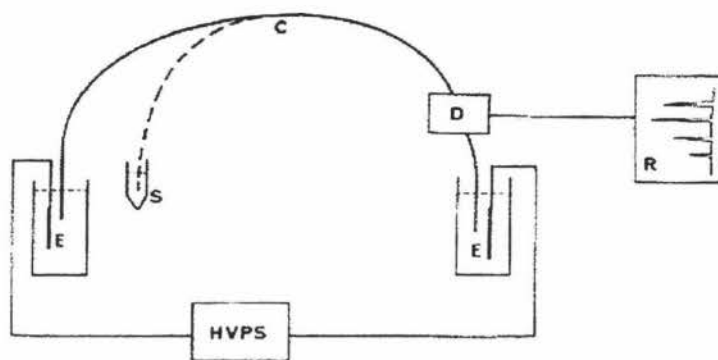


Figure 1.5 Basic scheme of the CE instrument. The separation capillary (C) is connected to the high-voltage power supply (HVPS) by electrolyte reservoirs equipped with electrode (E). The sample (S) is injected either by flow or by electromigration, and separated zones are detected on the opposite end of the capillary by a suitable detector (D). The detection signal is either recorded by a line recorder (R), or can be further evaluated by a computer (Weinberger, 1993).

Capillary columns, as indicated above, come in a wide variety of internal diameters. A polyimide outer covering makes the capillary mechanically stronger and protects the capillary from sudden angulation and breaking. Detection in CE commonly involves variable wavelength or filter UV photometry, or diode array systems. Laser induced fluorescence and mass spectrometer are available for some applications. At the detection “window” the polyimide coating of the capillary is “burnt off” to allow the light source to penetrate the capillary and for absorbency measurements to be made (Jenkins, 1995).

1.4 Detection

Capillary electrophoresis is a powerful separation technique that requires high-sensitivity detection for efficient performance. A wide range of detection techniques has been employed in CE separations. The list includes UV-absorbance, fluorescence, laser-based thermo-optical, refractive index, electrochemical, radioisotope, and mass spectrometric. Currently, the most commonly used detection systems for CE are based on UV and UV-Vis absorbance. All the standard commercial instruments are equipped with an UV detector (Li, 1992). These are similar to HPLC detectors and typically use deuterium sources with filters or a monochromator to select the detection wavelength. Source light is partitioned via a beam splitter between a reference photodiode and the

microfocusing optics of the capillary cartridge. Scanning detectors enable spectral information to be acquired as a peak moves through the detection point. Peak spectra can be used to identify the analyte or to determine if co-migrating impurities are present in the peak. Scanning detection can be accomplished by illuminating the capillary with full-spectrum white light and dispersing the transmitted light via a polychromator onto a photodiode array ("reverse optics" PDA detector). Alternatively, a conventional "forward optics" approach can be used, illuminating the capillary with a narrow spectral band isolated by a monochromator placed between the light source and the capillary. Scanning is achieved by rapid computer-controlled movement of the monochromator to sample a wide spectral range in milliseconds. This "fast-scanning" detector design produces much lower noise and, therefore, much higher sensitivity than a PDA detector (BioRad).

1.5 CE Reagents

A variety of buffers and additives can be used to achieve or enhance separations. The majority of separations use typical nontoxic biochemical buffers such as phosphate (pH 1.1 to 3.1), acetate (pH 3.8 to 5.8) borate (pH 8.1 to 10.1), and zwitterionic buffers, such as MES [2-(N-morpholino)ethane sulfonic acid] (pH 5.5 to 6.7) and Tris [(tris (hydroxymethyl-aminoethane)] (pH 7.3 to 9.3). In contrast to HPLC, capillary electrophoresis rarely requires toxic or flammable organic solvents such as acetonitrile, hexane, methanol, or tetrahydrofuran. Even when such solvents are used, only milliliter quantities are needed for many runs. The very small volumes of reagents required for CE analyses greatly minimizes the problems associated with solvent disposal and raises the possibility of using exotic solvents and additives which would be prohibitively expensive for conventional-scale separation techniques.

1.6 Different modes of CE

Different modes of capillary electrophoretic separations can be performed using a standard CE instrument. The origins of the different modes of separation may be attributed to the fact that capillary electrophoresis has developed from a combination of many electrophoretic and chromatographic techniques. In general terms, it can be

considered as the electrophoretic separation of a number of substances inside a narrow tube.

The distinct capillary electroseparation methods include: (Figure 1.6)

Capillary zone electrophoresis (CZE)

Capillary gel electrophoresis (CGE)

Micellar electrokinetic capillary chromatography (MEKC or MECC)

Capillary electrochromatography (CEC)

Capillary isoelectric focusing (CIEF)

Capillary isotachopheresis (CITP)

1.6.1. Capillary zone electrophoresis (CZE)

The separation in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the electrophoretic buffer contained in the capillary. The separation mechanism is mainly based on differences in solute size and charge at a given pH. Most capillaries used for CE today are made of fused silica, which contains surface silanol groups. These silanol groups may become ionized in the presence of the electrophoretic medium. The interface between the fused silica tube wall and the electrophoretic buffer consists of three layers: the negatively charged silica surface (at $\text{pH} > 2$), the immobile layer, and the diffuse layer of cations (and their sphere of hydration) adjacent to the surface of the silica that tend to migrate towards the cathode. This migration of cations results in a concomitant migration of fluids through the capillary. This flow of liquid through the capillary is called electroosmotic (or electroendosmotic) flow.

The electroosmotic flow in uncoated fused silica capillaries is usually significant with most commonly used buffers. In many cases, it is also significantly greater than the electrophoretic mobility of the individual ions in the injected sample. Consequently, both anions and cations can be separated in the same run. Cations are attracted towards the cathode and their speed is augmented by the electroosmotic flow.

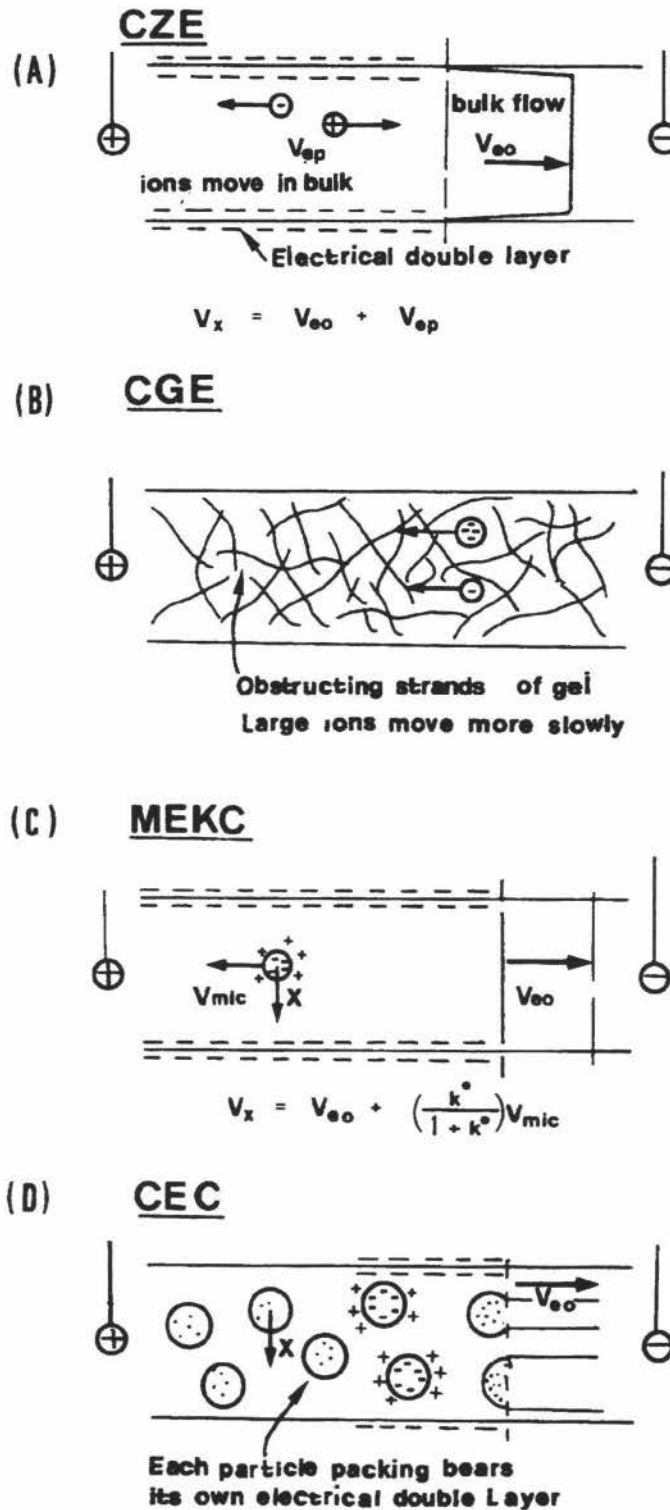


Figure 1.6 Diagrammatic representation of (A) capillary zone electrophoresis (CZE), (B) capillary gel electrophoresis (CGE), (C) micellar electrokinetic capillary chromatography (MEKC or MECC), and (D) capillary electrochromatography (CEC). V_x is the linear migration velocity of the analyte X. V_{eo} is the electroosmotic velocity, V_{ep} is the electrophoretic velocity and k' is the phase capacity ratio (Li, 1992).

Anions, although electrophoretically attracted toward the anode, are swept towards the cathode with the bulk flow of the electrophoretic medium. Under these conditions, cations with the highest charge/mass ratio migrate first, followed by cations with reduced ratios. Then the unresolved neutral components migrate as their charge/mass ratio is zero. Finally, the anions migrate. Anions with lower charge/mass ratio migrate earlier than those with greater charge/mass ratio. The anions with the greatest electrophoretic mobilities migrate last. One important point to note is that it is possible to change the charge/mass ratio of many ions by adjusting the pH of the buffer medium to affect their ionization and hence electrophoretic mobility.

CZE was used exclusively in this study; the other methods are briefly described below for completeness.

1.6.2 Capillary gel electrophoresis (CGE)

The main separation mechanism in capillary gel electrophoresis (CGE) is based on differences in solute size as analytes migrate through the pores of the gel-filled column. Gels are potentially useful for electrophoretic separations mainly because they permit separation based on "molecular sieving". Furthermore, they serve as anti-convective media, they minimize solute diffusion, which contributes to zone broadening, they prevent solute adsorption to the capillary walls and they help to eliminate electroosmosis. However, the technique is subject to the limitation that neutral molecules would not migrate through the gel, since electroosmotic flow is suppressed in this mode of operation.

1.6.3 Micellar electrokinetic capillary chromatography (MEKC)

The main separation mechanism of micellar electrokinetic capillary chromatography (MEKC) is based on solute partitioning between the micellar phase and the solution phase. The technique provides a way to resolve neutral molecules as well as charged molecules by CE.

Micelles form in solution when surfactant is added to water in concentration above its critical micelle concentration (cmc). Micelles consist of aggregation of surfactant

molecules with typical lifetimes of less than 10 μ s. MEKC is most commonly performed with anionic surfactants, especially sodium dodecyl sulfate (SDS). In the case of anionic surfactants, the hydrophobic tail groups tend to be orientated toward the center and the charged head groups along the surface of the micelle. The MEKC system contains two phases: an aqueous phase and a micellar phase. The surfaces of SDS micelles have a large net negative charge. The micelles therefore exhibit a large electrophoretic mobility (μ_{ep}) toward the anode, which is in the opposite direction to the electroosmotic mobility (μ_{eo}) towards the cathode present in most commonly used buffer systems in CE. The magnitude of μ_{eo} is slightly greater than that of μ_{ep} , resulting in a fast-moving aqueous phase and a slow-moving micellar phase. Solutes can partition between the two phases, resulting in retention based on differential solubilization by the micelles. Consequently, the MEKC technique provides a means of obtaining selective separations of neutral and ionic compounds while retaining the advantages of the capillary electrophoresis.

1.6.4 Capillary electrochromatography (CEC)

In capillary electrochromatography (CEC), the capillary is packed with a chromatographic packing which can retain solutes by the normal distribution equilibria upon which chromatography depends and is therefore an exceptional case of electrophoresis. In CEC the liquid is in contact with the silica wall, as well as the particle surfaces. Consequently, electroosmosis occurs in a similar way as in an open tube due to the presence of the fixed charges on the various surfaces. Whereas in an unpacked tube the flow is strictly plug flow, and there is no variation of flow velocity across the section of the column, the flow in a packed bed is less perfect because of the tortuous nature of the channels. Nevertheless, it approximates closely to plug flow and is substantially more uniform than a pressure-driven system. Therefore, the same column tends to provide higher efficiency when used in electrochromatography than when used in pressure-driven separations.

1.6.5 Capillary isoelectric focusing (CIEF)

Another separation method, which can be conveniently performed using a capillary electrophoresis instrument, is isoelectric focusing. Here polyionic substances (e.g. proteins) are separated on the basis of their isoelectric points or pI values. In this technique, the protein samples and a solution that forms a pH gradient are placed inside a capillary. The anodic end of the capillary is placed into an acidic solution (anolyte), and the cathodic end in a basic solution (catholyte). Under the influence of an applied electric field, charged proteins migrate through the medium until they reside in a region of pH where they become electrically neutral and therefore stop migrating. Consequently, zones are focused until a steady state condition is reached. After focusing, the zones can be migrated from the capillary by a pressurized flow, e.g. simply by lifting the height of one end of the capillary and permitting the sample to flow through the detection cell.

1.6.6 Capillary isotachopheresis (CITP)

The main feature of CITP is that it is performed in a discontinuous buffer system. Sample components condense between leading and terminating constituents, producing a steady-state migrating configuration composed of consecutive sample zones.

1.7 Capillary electrophoresis as a clinical tool

Basic knowledge in immunology, biochemistry, physiology, and pharmacology related to normal physiological activities and human diseases has expanded greatly in the past two decades. Consequently, advancements in basic scientific developments are a necessity for the analysis of biochemical substances present in biological fluids, blood cells, and tissue specimens.

Much information about a disease state has been gained by measuring a certain number of biochemical substances that are used as indicators of the disease activity, as well as providing information about the intensity of the whole pathological process. Therefore, the clinical chemistry laboratory is an important contributor to the medical team

involved in the diagnosis and treatment of disease, and physicians rely heavily on laboratory test results before making decisions (Guzman, 1993)

Clinical chemistry has been defined as "the study of the chemical aspects of human life in health and illness and the application of chemical laboratory methods to diagnosis, control of treatment, and prevention of disease" (Guzman, 1993). Thus, clinical chemistry is a fundamental science when it seeks to understand the physiological and biochemical processes operating both in the normal state and in disease. It is an applied science when analyses are performed on body fluids, or tissue specimens to provide useful information for the diagnosis or treatment of disorders.

Capillary electrophoresis (CE) is a relatively young branch of separation science. Many observations of the potential importance of capillary electrophoresis were made by analytical chemists during the last decade. Comparative studies have been carried out to learn more about the potential of high-performance capillary electrophoresis (HPCE) in the clinical laboratory.

In the modern medical environment, patient diagnosis and treatment relies heavily on investigation procedures. With the introduction of productivity-based funding of laboratories, they are now required to provide rapid, cost-effective tests. This is often best achieved by the use of automated methods.

Capillary electrophoresis (CE) involves a separation of charged molecules in a buffer-filled capillary by the application of a very high voltage. The outstanding characteristic of CE is the high separation efficiency, which can be achieved: 10^6 theoretical plates are able to be attained, at an applied voltage of 30kV. This allows an analysis time in the order of minutes (Jenkins and Guerrilla, 1996).

With commercially available CE instrumentation, automation of the assays is now available. Some instruments provide up to 50 position sample carousels, thus allowing sufficient space for a full overnight run. (Jenkins and Guerrilla, 1996).

It is now possible to determine nanoliter to picoliter quantities of material and to reach detection sensitivity levels of less than 3 zeptomoles ($1 \text{ zeptomole} = 10^{-21} \text{ mol} = 600$

molecules), and uses only nanolitres of buffer. The main consumable costs relate to the capillaries and sample cups. The automation of the system allows for considerably lower labour costs compared with other electrophoretic methods, including agarose gels.

The analytical capabilities of CE allow clinical analysts to consider automation of tests previously performed only on a manual basis. These include serum and urine protein electrophoresis, hemoglobin electrophoresis and vitamin studies. Assays for several newer drugs by CE could have significant advantages over the sometimes-difficult HPLC techniques currently used.

1.8 Summary

Capillary electrophoresis employs the separation mechanisms of conventional electrophoresis in a capillary format. Basic CE offers the ease and speed of HPLC while eliminating the problems of HPLC associated with column packing, toxic solvents and their disposal, and provides quantitative analysis and automation. Separated components are identified by on-line detection during the analysis, so, in contrast to slab gel electrophoresis, time-consuming staining and destaining steps after the separation are not required. Results are obtained in a matter of minutes, while conventional electrophoresis typically requires hours to days before results are available. (BioRad)

Rapid micro-volume analysis is one major advantage of CE over HPLC. Since only nanoliter of sample are consumed in each analysis, literally hundreds of CE runs can be performed from the microliter sample volumes typically consumed for a single HPLC injection.

The simplicity and separative power of capillary electrophoresis is another additional key advantage of the technique in contrast to HPLC. As CE separation technology evolved it became apparent that tremendous flexibility in separation selectivity can be achieved by simple changes in the composition of the electrophoresis buffer. This enables a single capillary to be used to obtain separations based on a wide range of molecular properties (e.g. size, charge, chirality, hydrophobicity) which would require thousands of dollars invested in HPLC columns. Separation methods can be quickly

developed and optimized by rapid scouting experiments with simple buffer changes, shortening method development time from a matter of weeks or months to a few hours or days.

The attributes which have made capillary electrophoresis such a successful tool in basic research are identical to those attracting clinical laboratories: speed (more efficient, less labor-intensive), low cost (minimal buffer consumption), small sample volume (reduced blood collection volume from patient), increased selectivity (determination of multiple solutes in one run), and versatility (detection of analytes over the wide range of molecular masses and chemical composition).

CHAPTER TWO

CREATININE AND CREATINE

2.1 Introduction

The human kidneys are paired, bean-shaped structures situated in the posterior part of the abdominal cavity (**Figure 2.1**). Because the liver occupies the right upper quadrant of the abdominal cavity, the right kidney, which lies behind and slightly below it, is located somewhat lower than the left kidney. Sometimes only one kidney is present and functional, or the two kidneys are fused, usually at the superior pole, to form a horseshoe kidney.

Although the kidney has multiple functions, its principal role is the regulation of extracellular fluid leading to *formation of urine*.

A nineteenth century French scientist was among the first to point out that the integrity of the body was dependent upon selective excretion of metabolites, which could not be allowed to accumulate within the body without causing harm to the individual. At the same time this excretion of metabolic products must be sufficiently selective so that substances that are utilized or required by the body are not lost. Homer Smith said, "The composition of the blood is determined not by what the mouth ingests but what the kidney keeps" (Tietz, 1976).

To accomplish its complex mission, the kidney must act first as a selective filter to remove water and filterable solutes from the blood plasma. This process occurs in the glomeruli and the fluid formed is known as the glomerular filtrate. The filtration apparatus of the kidney is built around a functional unit called the *nephron*, which consists of the *glomerulus* and the *tubule* (**Figure 2.2**). The volume of the glomerular filtrate is around 125 ml per minute and may exceed 180 liters in a single day. If the kidneys were to stop at this point and pass this filtrate to the bladder to be excreted, the

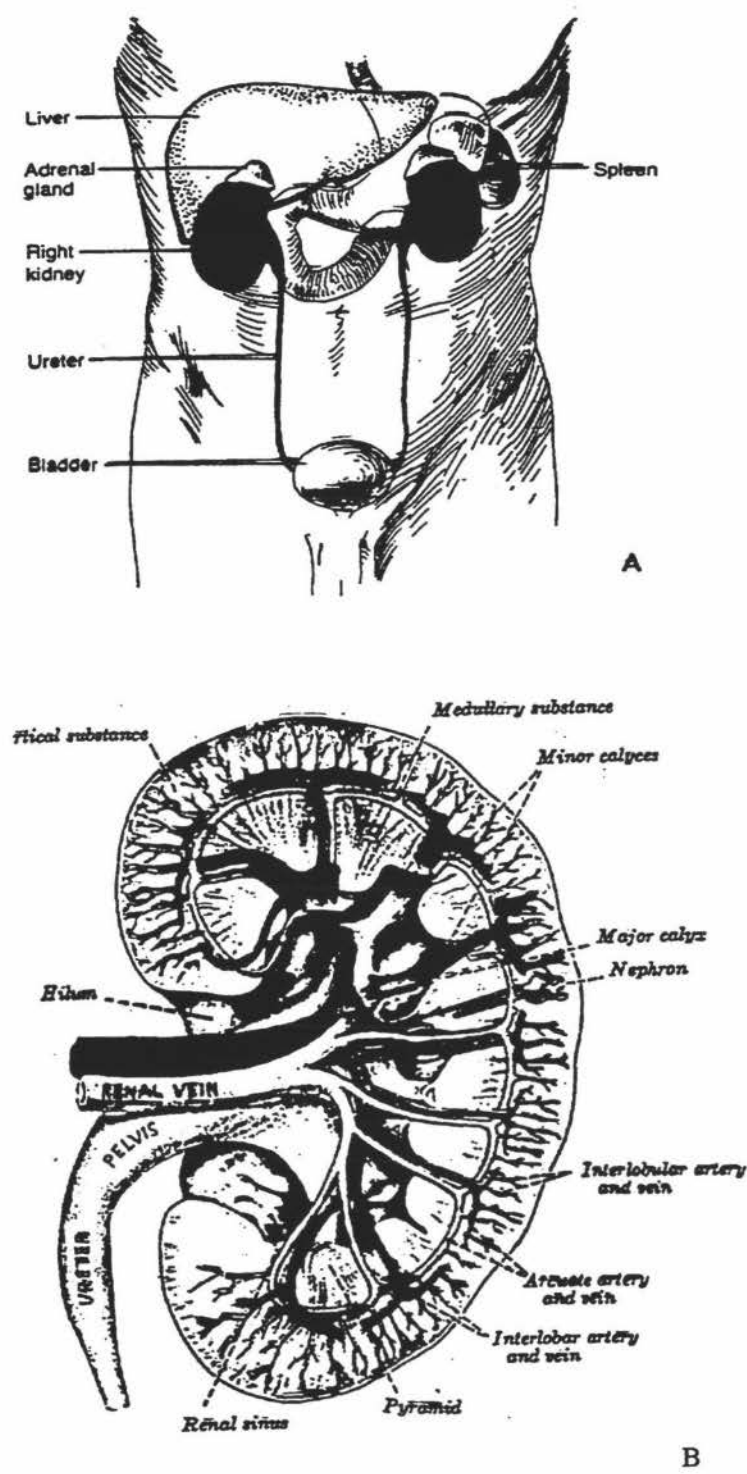


Figure 2.1 A, schematic drawing of right and left kidneys with respect to liver, stomach, duodenal loop, spleen, adrenal gland, and bladder, B, diagram of a vertical section through the kidney. Nephron and blood vessels greatly enlarged (From Gray's Anatomy, 28th Edition, Edited by C. M. Goss, Lea and Febiger, 1966).

individual could not replace the lost fluid and solutes and, therefore, life would be impossible. Among the components of the glomerular filtrate are many substances that are necessary for normal body function. To prevent their loss, the kidney is designed to reabsorb water and useful solutes selectively. By this process the glomerular filtrate is converted to urine, which contains end products of metabolism that might be injurious if accumulated. It also contains any excess of essential solutes that might be present.

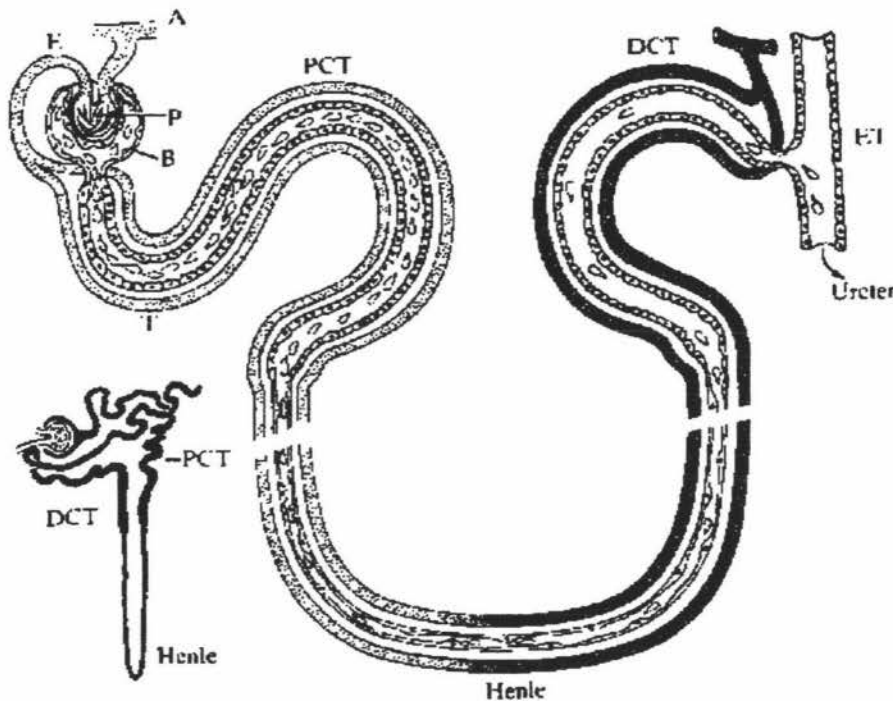


Figure 2.2 Schematic drawing of the glomerulus and the tubular system. A, afferent arteriole; E, efferent arteriole; P, plexus of capillaries (glomerular tuft); B, Bowman's capsule; T, tubular blood supply; PCT, proximal convoluted tubule; Henle, loop of Henle; DCT, distal convoluted tubule; ET, excretory tubule or duct. The blood capillaries shown along the tubular system (T) gradually change to venous capillaries as they pass down the tubular system (Tietz, 1976).

Sufficient water to keep these solutes in solution is also lost, but this volume is so small that the individual can usually replace this easily.

There are about 1.2 million nephrons in each kidney. Not all of these nephrons are working at any one time, but their very presence gives the kidney considerable reserve capacity in case of stress, disease, or injury.

Some of the substances that are present in the glomerular filtrate are known as *threshold* substances and appear in the urine only after they have reached certain minimum concentration in the blood. Glucose is such a substance and does not appear in the urine until plasma glucose level reach about 180 mg/100ml. Other substances, such as creatinine, may be excreted without appreciable reabsorption. Therefore, creatinine is useful in measuring the glomerular filtration rate.

About 20 per cent of the volume of the plasma that passes through the glomerular tuft pass into Bowman's capsule as glomerular filtrate. This filtrate has a specific gravity of 1.010 ± 0.002 , a pH of approximately 7.4, and is iso-osmotic with plasma. It contains all the filterable molecules present in plasma and at approximately the same relative concentrations (**Figure 2.2**).

2.1.1 Tests measuring glomerular filtration rate

The groups of tests generally referred to as renal clearance tests are extremely useful in measuring the actual capacity of the kidney to eliminate certain substances present in plasma. A substance may be excreted by glomerular filtration alone, or by filtration plus tubular excretion (depending on the substance, tubular excretion will more or less dominate), or it may be filtered but then subsequently reabsorbed by the tubules in whole or in part. If only a single facet of renal physiology (e.g., the glomerular filtration rate) is to be studied, a substance should be selected that is excreted (filtered) either completely or predominantly by the glomeruli without being either excreted or reabsorbed by the tubules. Inulin is such a substance; however, it is rarely employed because of the limited availability of procedures for the quantitation of inulin. Creatinine is a substance that behaves similarly to inulin (see 2.1.2 and **Figure 2.3** below). Creatinine is removed from plasma by glomerular filtration and is then excreted in the urine without being reabsorbed by the tubules to any significant extent. This results in a relatively high clearance rate for creatinine. In addition, when plasma levels increase above the normal, the kidney can also excrete creatinine through the tubules. Consequently, serum or blood creatinine levels in renal disease generally do not increase until renal function is substantially impaired. In the presence of normal renal blood flow, any increase in creatinine values above 3 to 4 mg/100ml is suggestive of moderate to severe kidney damage. This lack of sensitivity is in contrast to the

creatinine clearance test, which is one of the most sensitive tests for measuring the glomerular filtration rate.

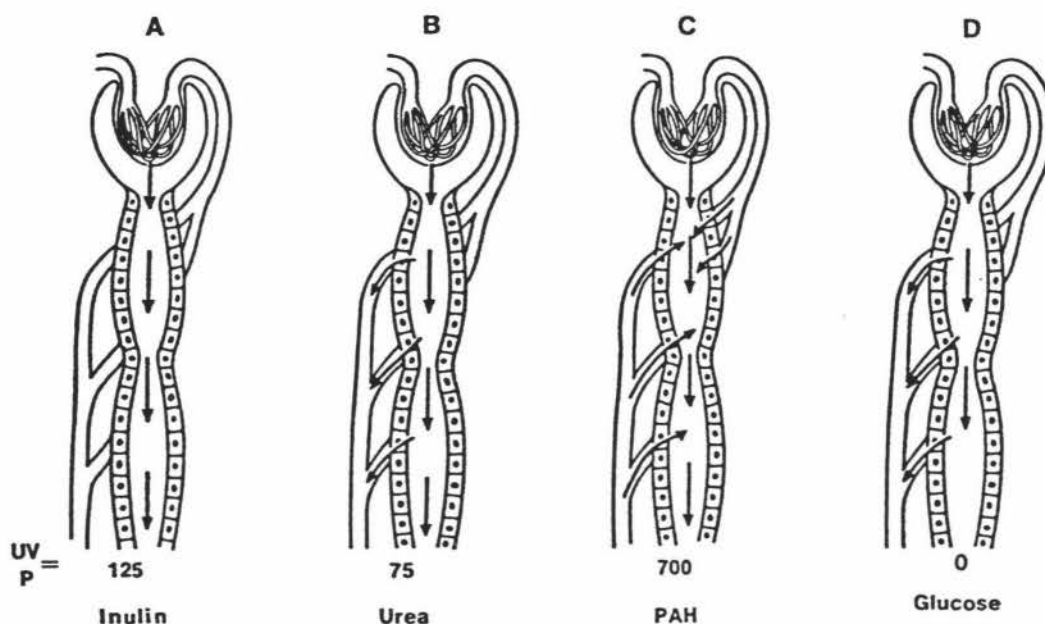


Figure 2.3 Schematic presentation of the excretion of various types of substances by the nephron. A, inulin is excreted by glomerular filtration and passes through the tubular system without re-absorption. No inulin is removed by tubular secretion. Creatinine behaves very similarly to inulin. B, urea is filtered through the glomerulus, but is subsequently partially reabsorbed by the tubular system. C, PAH (p-aminohippurate) is, to a limited extent, filtered by the glomerulus, but is mainly excreted by the tubules. Phenolsulphonphthalein behaves similarly. D, glucose is filtered and subsequently reabsorbed by the tubules (Tietz, 1976).

2.1.2 Creatine and creatinine

Creatine is synthesized in the kidney, liver, and pancreas by two enzymatically-mediated reactions from three amino acid, arginine, glycine, and methionine. In the first reaction, transamidination of arginine and glycine forms guanidinoacetic acid. In the second reaction, methylation of guanidinoacetic acid occurs with S-adenosyl methionine as methyl donor to form creatine (Figure 2.4). Creatine is thus transported in blood to other organs such as muscle and brain, where it is phosphorylated to phosphocreatine, a 'high-energy' compound. Creatine phosphate acts as a reservoir of high energy, phosphate that is readily convertible to ATP in the muscles and other tissues. Creatine diffuses into the vascular system and is thus supplied to many kinds of

cells, particularly those of muscle, where it becomes phosphorylated. Creatine and creatine phosphate total about 400 mg/100g of fresh muscle.

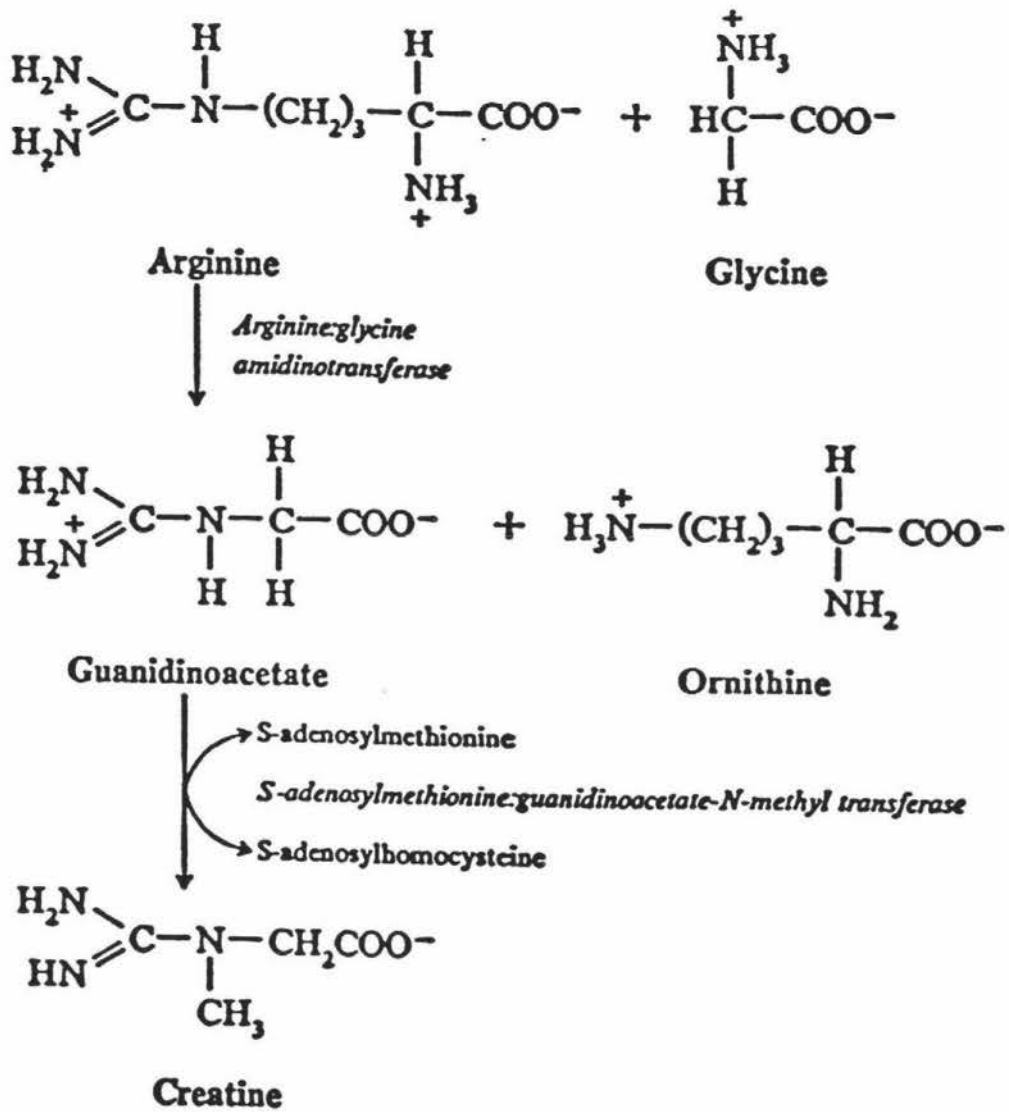


Figure 2.4 The pathway of synthesis for creatine in the human body (Tietz, 1976).

Both compounds are spontaneously converted into creatinine at the rate of about 2 % per day. Creatinine is a waste product derived from creatine and is excreted by the kidneys (**Figure 2.5**)

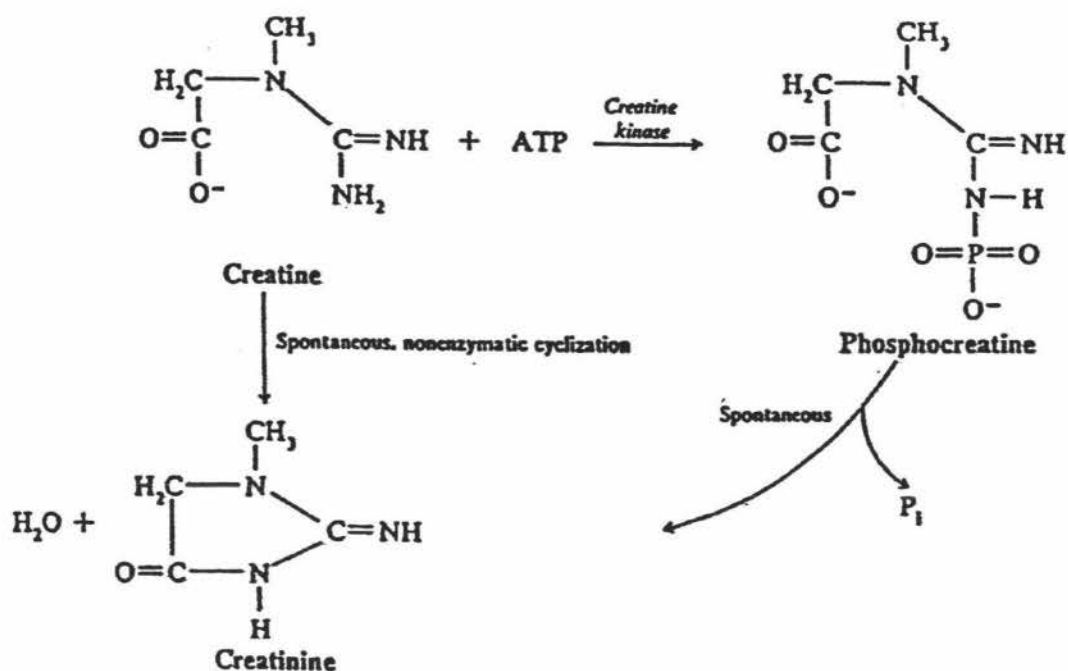


Figure 2.5 The pathway of creatinine synthesis from creatine in the human body (Tietz, 1976).

The amount of endogenous creatinine produced is proportional to muscle mass and its production varies with age and sex: non-obese adult males excrete ~1.5g/d, females ~1.2g/d, and this is not influenced by diet.

The constancy of endogenous creatinine production and its release into the body fluids at a constant rate makes creatinine a useful endogenous substance whose clearance may be measured as an indicator of glomerular filtration rate.

Creatine and creatinine may be determined in any biological fluid, but plasma, serum, and urine are the specimens most commonly employed. If kept for a few days, specimens for creatine and creatinine analysis are best stored at refrigerator temperatures or for longer periods, they should be frozen.

Aqueous solutions of creatine and creatinine very slowly approach a state of equilibrium with respect to each other, as indicated in the following: (Figure 2.6)

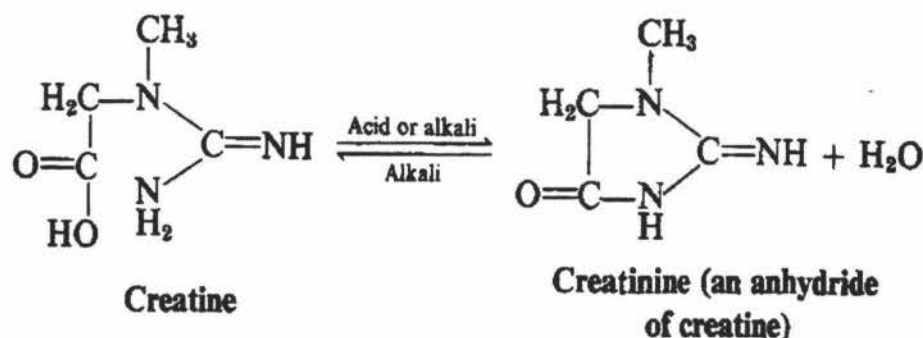


Figure 2.6 Equilibrium between creatinine and creatine (Tietz, 1976).

It probably requires days or weeks at neutral pH. Creatinine, however, is formed rather quickly from creatine in either alkaline or acid solutions. Although this reversible reaction is catalyzed in both directions by hydroxide ions, hydrogen ions promote the reaction only toward the right. Because of the lability of creatine and creatinine, it is advisable to carry out analysis for these two substances on fresh specimens. When this is not possible, adjustment of the pH to 7.0 or freezing, or both, may delay the change for indefinite periods.

2.2 Literature Review

2.2.1 Assay of Creatinine

2.2.1.1 The Jaffé reaction:

Jaffé in 1886 observed the *red* color that formed when creatinine reacted with picric acid in an alkaline medium (Jaffé, 1866). The reaction bears his name, and it has withstood the test of time. In 1904, Folin utilized the Jaffé reaction to measure creatinine in urine (Folin, 1904). Greenwald was the first to make a systematic study of the chemistry of the Jaffé reaction (Greenwald, 1925). He ascribed the red color to a salt of creatinine, picric acid, and sodium hydroxide, and noted that there were at least

tautomer (Figure 2.7): a lactam-lactim rearrangement between positions 3 and 4 (A) or a keto-enol change between positions 4 and 5 (B).

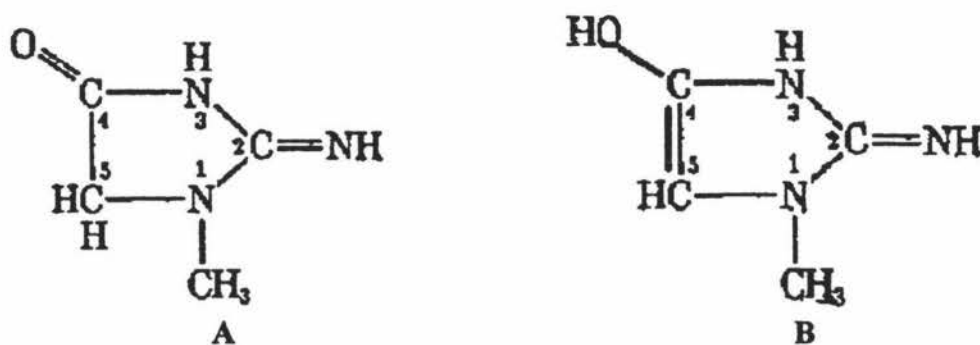


Figure 2.7 Structure of creatinine (Tietz, 1976).

2,3-dimethyl creatinine, in which a lactam-lactim rearrangement is made impossible by substitution of all the hydrogen atoms attached to nitrogen, also gives the Jaffé reaction, thus ruling out that possibility. Moreover, neither creatinine oxime, benzylideneacetyl creatinine, nor benzylidene creatinine, in all of which a keto-enol tautomerism is impossible, gives the Jaffé reaction. However, possession of a structure allowing keto-enol tautomerism is not in itself sufficient to allow the Jaffé reaction, because dimethylol creatinine does not give it. Greenwald noted that his compound could not form a salt with picric acid because the basic nitrogen had combined with a hydroxymethyl group. Thus, he concluded a molecule must also have basic properties to give the Jaffé reaction.

Greenwald and Gross (1925) also studied the structural features of the picric acid molecule. They concluded that the two-nitro groups in the *ortho* position in the picric acid molecules, or even all three nitro groups and the hydrogen in the *meta* position, were the structural components that were responsible for the Jaffé reaction. They based this conclusion on the observation that such analogs of picric acid such as 2,4-dinitrophenol or 2,4,6-trinitro-m-cresol, neither of which fulfills the above requirement, failed to give the Jaffé reaction with creatinine. Picric acid was assumed to be in the o-quinone form in picrates, and thus the yellow creatinine-picrate would have the same

quinone form in picrates, and thus the yellow creatinine-picrate would have the same configuration as trinitro-m-cresol picrate, except that the latter, which does not give the Jaffé reaction, has a methyl group instead of a hydrogen atom in the meta position. Greenwald concluded that the Jaffé reaction is a result of several events, including a keto-enol transformation in the creatinine molecule, changes in the picric acid molecule involving the hydrogen atoms in the meta positions and possibly all the three nitro groups (Greenwald, 1928).

Greenwald's work suggests that the red color formed in the Jaffé reaction is a result of 1:1 and 2:1 complexes between creatinine and picric acid.

In 1936, Bollinger isolated a red solid from the Jaffé reaction mixture and analyzed it as a complex formed from 1 mol of picric acid, 1 mol of creatinine, and 2 mol of sodium hydroxide (Bollinger, 1937). Abe pointed out, however, that picric acid itself reacts with sodium hydroxide to yield a picrate ion complex (Abe, 1960a).

Several investigators have provided evidence, based on spectroscopic and chromatographic studies, that the red color formed in the Jaffé reaction is not due to picric acid (Clarke, 1961). Depending on the concentration of sodium hydroxide used in the Jaffé reaction, two complexes of picric acid with hydroxide ions have been reported (Gold and Rochester, 1964). One prevails at low sodium hydroxide concentration, whereas the other is present if sodium hydroxide concentrations exceed 1 mol/L (Gold and Rochester, 1964). The effect of sodium hydroxide concentration on the final product formed in the Jaffé reaction has been substantiated by spectroscopic data. Increasing the hydroxide concentration shifts the absorption maxima of the product to longer wavelengths (Vasiliades, 1976). Spectrophotometric (Figures 2.8 and 2.9), kinetic (Figure 2.10), and nuclear magnetic resonance studies (Figures 2.11, 2.12, 2.13 and 2.14) indicate that alkaline sodium picrate and creatinine react first to form a 1/1 reddish-orange adduct between picric acid and creatinine, that is then slowly converted to a stable yellow compound according to first-order kinetics with a stability constant of $\log K = 4.26$. Kinetic studies indicate that the forward reaction is first order with respect to picric acid, hydroxide, and creatinine concentration. The reverse reaction, the dissociation of the 1/1 complex (Figure 2.15) shows a complex dependence on hydroxide concentration (Vasiliades, 1976).

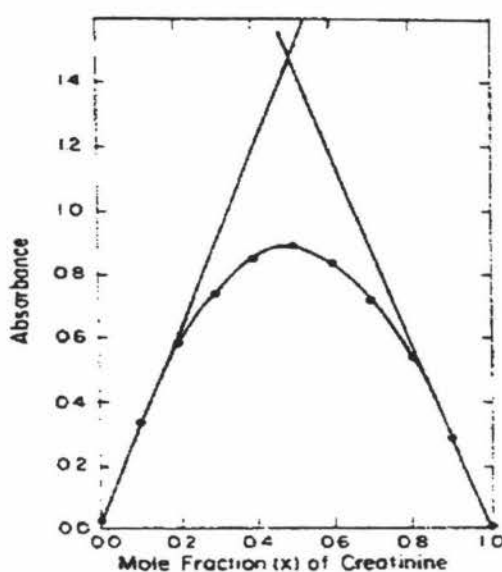


Figure 2.8 Identification of the 1:1 complex formed at 480 nm, by use of the method of continuous variation. Conditions: 480 nm, picric acid = 10 mmol/l, creatinine = 10 mmol/l (Vasiliades, 1976).

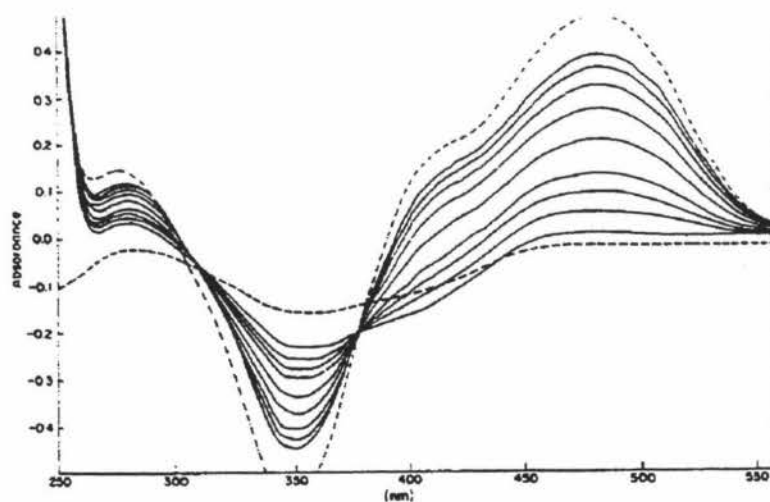


Figure 2.9 Differential scans of the reaction of alkaline sodium picrate and creatinine with time. Time between scans is 5 min. Two isosbestic points are observed. Sample cell: picric acid 0.149 mmol/l, creatinine 0.298 mmol/l, and sodium hydroxide 37 mmol/l. Reference cell: picric acid 0.149 mmol/l, sodium hydroxide 37 mmol/l. (---) initial spectrum at time zero, (- . - . -) final spectrum (Vasiliades, 1976).

Kinetic study:

$$K_{\text{obsd}} = k_{\text{forward}} [\text{P}] [\text{OH}] + k_{\text{rev}} [\text{OH}]$$

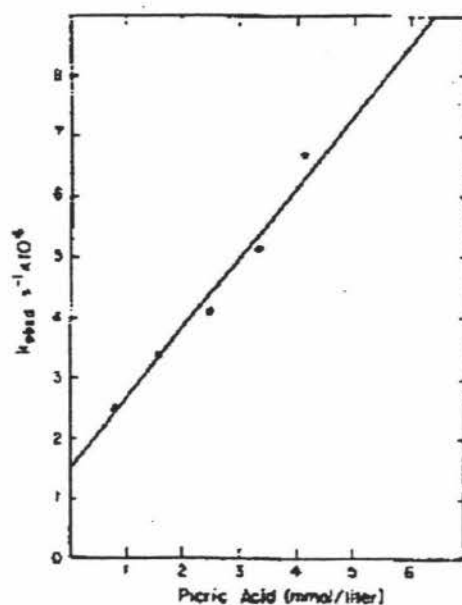


Figure 2.10 Dependence of the observed pseudo-first-order rates constant on picric acid concentration. Conditions: creatinine 8.2×10^{-5} mol/L, 0.1 mol/L KNO_3 (ionic strength adjustment), 25°C , 0.33 mol/L sodium hydroxide (Vasiliades, 1976).

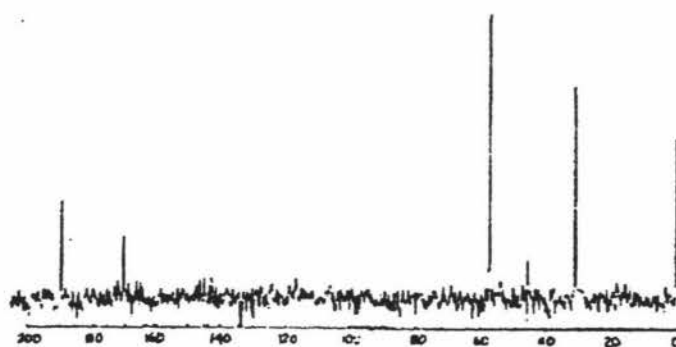


Figure 2.11 ^{13}C NMR spectrum of creatinine in $^2\text{H}_2\text{O}$. Creatinine concentration: 0.7 mol/L (Vasiliades, 1976).

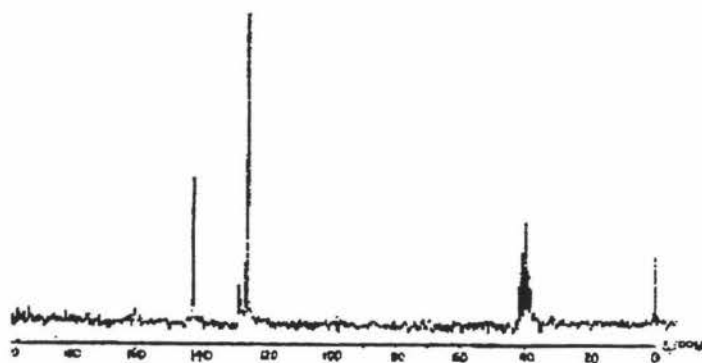


Figure 2.12 ^{13}C NMR spectrum of picric acid in deuterated dimethyl sulfoxide. Picric acid concentration: 0.5 mol/L (Vasiliades, 1976).

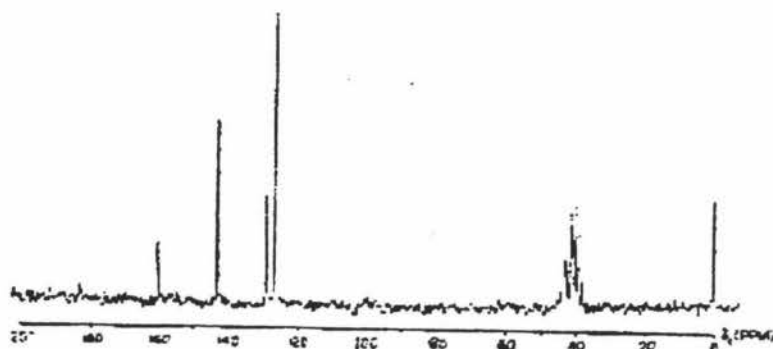


Figure 2.13 ^{13}C NMR spectrum of picric acid in deuterated dimethyl sulfoxide after addition of NaOH . Picric acid concentration is 0.5 mol/L. NaOH concentration is 0.5 mol/L (Vasiliades, 1976).

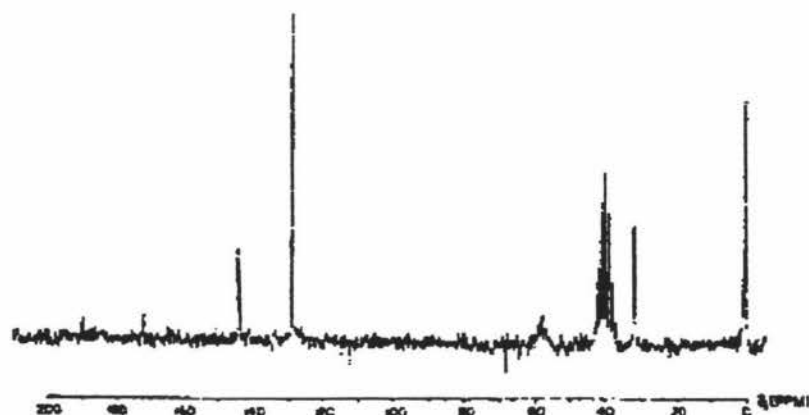


Figure 2.14 ^{13}C NMR spectrum of the reaction product of picric acid in deuterated dimethyl sulfoxide, and creatinine in $^2\text{H}_2\text{O}$ in the presence of NaOH . Final concentrations: picric acid 0.25 mol/L, creatinine 0.25 mol/L, NaOH 0.3 mol/L (Vasiliades, 1976).

Alkaline sodium picrate and creatinine react to form a product that absorbs maximally at 480nm (Vasiliades, 1976).

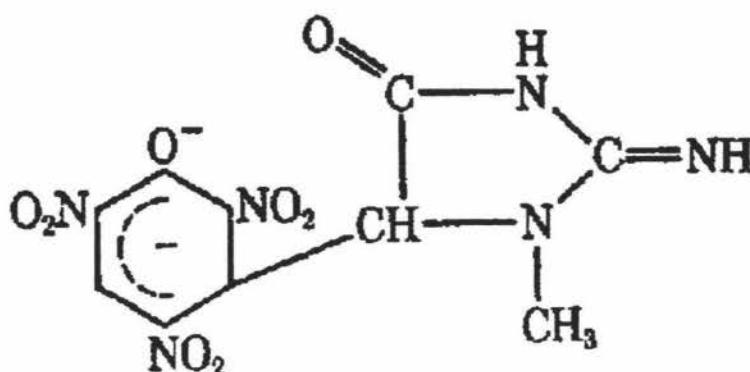


Figure 2.15 Structure of the 1/1 complex.

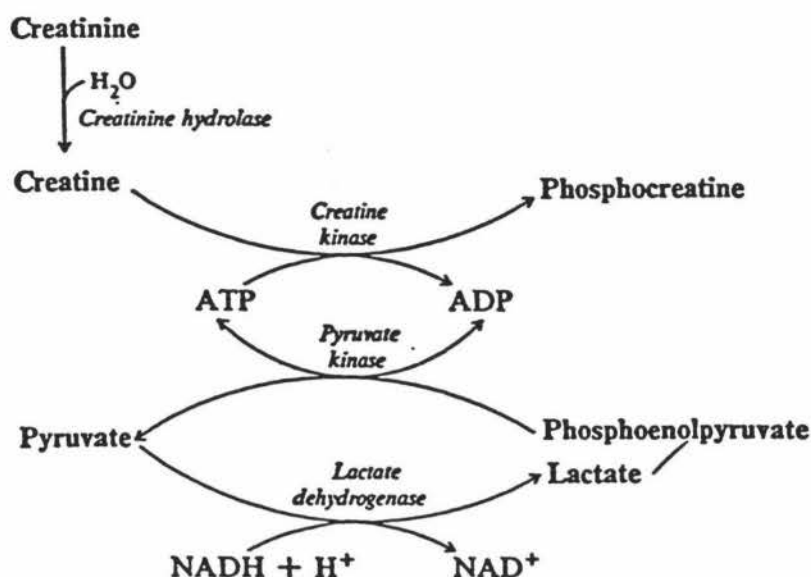
The Jaffé reaction has long been known to be non-specific when applied to the measurement of creatinine in plasma. Among the non-creatinine 'Jaffé-reacting' chromogens are proteins, glucose, ascorbic acid, guanidine, acetone, cephalosporins and α -ketoacids such as acetoacetate and pyruvate.

2.2.1.2 The specificity of the Jaffé reaction can be improved by:

- The use of Lloyd's reagent, an aluminium silicate that selectively adsorbs creatinine, leaving the creatinine chromogens in solution. However, it was found that Lloyd's reagent absorbs several Jaffé-positive compounds such as keto acids, indole, glycohydrazide, various steroids, and pigments. Its effectiveness has been questioned (Owen *et al*, 1954).
- The Jaffé reaction modified to include an iodine oxidation and an ether extraction (Taussky, 1956). Extraction with ether reportedly eliminates interference from acetone, acetoacetic acid, fructose, and glucose. A preliminary oxidation step with iodine and treatment with zinc before extraction is claimed to increase specificity. Nevertheless, the specificity of the iodine-oxidation/ether-extraction step and its advantage over the Lloyd's reagent modification has been questioned (Slot, 1965).

- Introduction of a dialysis step with a continuous-flow procedure that reportedly improves specificity (Chasson *et al*, 1961). The difference in behavior of creatinine-picric acid and noncreatinine chromogens subsequent to acidification has been exploited (Slot, 1965). The difference in absorbance obtained before and 5min after acidification reportedly represents “true” creatinine absorbance. Apparently, the color due to the creatinine-picric acid complex fades rapidly, whereas the color resulting from noncreatinine chromogens is either stable or fades slowly. This method is satisfactory for estimating creatinine values in the normal range, but is less sensitive at high creatinine concentrations. To correct this limitation the ratios of the tungstic acid filtrate, picric acid, and sodium hydroxide can be readjusted to provide optimum alkalinity for color development (Grafnetter *et al*, 1967).
- The measurement of absorbance at 500nm with two alkaline picric acid reagents buffered at pH 9.65 and 11.5. This has also been reported to give “true” creatinine values. Creatinine-picric acid does not absorb at pH 9.65, whereas the absorbance at pH 11.5 is due to both creatinine and protein. Thus, without deproteinization, the measurement of absorbance of the reaction mixture at pH 11.5 minus the absorbance of the pH 9.65 reactants reflects the “true” creatinine value because the other commonly encountered pseudocreatinine chromogens do not react under the conditions of the assay (Yatzidis, 1974).
- Using the kinetic assay. Creatinine can be assayed by the Jaffé reaction without deproteinization, by monitoring initial reaction rates (Lustgarten, and Wenk, 1972; Romer, 1975). However, this process is not entirely free of interference from Jaffé-reactive pseudo-creatinine chromogens. Although some have reported that bilirubin does not interfere in the kinetic assay (Lustgarten, and Wenk, 1972; Romer, 1975), others have noted a significant decrease in creatinine values when high concentrations of bilirubin are present (Daugherty *et al*, 1978).
- Incubation with the enzyme creatininase to improve the specificity of the Jaffé reaction. The creatinine is hydrolyzed to form creatine, which can be measured using creatine kinase. The indicator reaction in this coupled-enzyme system is

monitored spectrophotometrically at 340 nm in either an endpoint or a kinetic mode (Tietz, 1976). The reactions are:



This method is highly specific and has been adapted for both manual and automated use.

- Employing other enzymatic methods using creatinine-decomposing enzymes. Treatment of one portion of the sample with the enzyme systems and then comparing the difference in color produced by the Jaffé reaction in the enzyme-treated and untreated samples, one can attribute the difference to the true creatinine content of the sample. Successful application of these methods, however, depends in the availability and purity of these creatinine-decomposing enzymes (Cook, 1975; Spencer, 1986).
- Differential elution of creatinine and non-creatinine chromogens using high-performance liquid chromatography with pellicular cation-exchange resins and on-line application of the Jaffé reaction. These approaches have been reported for improving specificity (Brown *et al*, 1977). Non-creatinine chromogens are eluted with a sodium citrate buffer (pH 4.25) before creatinine. In other procedures involving high-performance liquid chromatography, the absorbance of creatinine is

measured in the ultraviolet region (Soldin and Hill, 1978). In such procedures, reported to be relatively rapid and specific and to have good precision, creatinine is separated by paired-ion chromatography and its absorbance is measured at 200 nm.

2.2.1.3 Other methods for assaying creatinine

- Reaction with *3,5-dinitrobenzoic acid*: Creatinine reacts with *3,5-dinitrobenzoic acid* at alkaline pH to give a purple-rose color (Langley and Evans, 1936), but the stability of the color has been questioned, and the superiority of this procedure over the Jaffé reaction without modification to include the use of Lloyd's reagent is doubtful (Sirota *et al*, 1950).
- Derivatives of *3,5-dinitrobenzoic acid* have been used in two procedures for assay of serum creatinine. In one, creatinine is reacted with 3,5-dinitrobenzoyl chloride (Parekh *et al*, 1976); in the other, with methyl-3, 5-dinitrobenzoate in a mixture of dimethyl sulfoxide, methanol, and tetramethyl ammonium hydroxide (Sims and Parekh, 1977). The latter procedure was reported to have good precision and correlates well with alkaline picrate procedures. Protein precipitation to yield neutral supernates reportedly eliminates interference by cephalothin (an antibacterial agent), and thus increases specificity of the 3,5-dinitrobenzoate procedures over the picric acid procedure (Sims and Parekh, 1977; Parekh and Sims 1977).
- Reaction with *1,4-naphthoquinone-2-sulfonate*: Measurement of the reaction product of creatinine and *1,4-naphthoquinone-2-sulfonate* (potassium salt) has been attempted (Sullivan and Irreverre, 1958), but reportedly gives highly spurious results (Cooper and Biggs, 1961).
- Sakaguchi color reaction: Reaction of creatinine with o-methylguanidine, is followed subsequently by conversion of that product to methylguanidine by heating after neutralization. The latter gives the Sakaguchi color reaction for monsubstituted guanidines by formation of a red color with α -naphthol, thymine, sodium hydroxide, sodium hypochlorite, and sodium thiosulfate (Van Pilsum, 1956). Use of the above assay mixture for the Sakaguchi color reaction increases the stability and the intensity of the color formed with creatinine. This reaction

presumably does not measure the nonspecific chromogens encountered in the Jaffé reaction (Van Pilsum, 1959).

- Chromatographic methods: Recognizing the non-specificity associated with the alkaline picrate methods, many investigators have developed HPLC procedures for determination of creatinine levels. These methods, in general, offer improved specificity and sensitivity.

Table 2.1 HPLC METHODS FOR ANALYSIS OF CREATININE

Reference	Column	Composition of mobile phase	Detection	Sample pretreatment
Soldin and Hill, 1978	RP-C ₁₈ (paired ion, 30°C)	0.02M potassium phosphate (pH 5.1) containing 80mg of sodium lauryl sulfate-methanol (76:24, v/v), isocratic	200 nm	methanol
Chiou and coworkers, 1978, 1979, 1983	cation exchanger	0.1 M ammonium phosphate (pH 2.6) 0.012~0.035M ammonium phosphate (pH 4.8), isocratic	215 nm 254 nm or 235nm	acetonitrile acetonitrile then methylene chloride
Lim et al, 1978	RP-C ₁₈	0.01M ammonium acetate isocratic	235nm	cation exchange column
Krsulovic et al, 1979	RP-C ₁₈	0.01M monopotassium phosphate (pH 6.5-6.6) acetonitrile-water (3:2, v/v) gradient	220nm 254nm	simple filtration or dilution
Spierto et al, 1980	RP-C ₁₈	0.01M ammonium acetate (pH 6.5-6.6) isocratic	236nm	ultra-filtration
Ambrose et al, 1983	cation exchanger	0.02 M lithium acetate (pH 4.68) 0.075 M lithium acetate (pH 7.1) step gradient	234nm	10% TCA
Patel and George, 1981	silica	200ml methanol, 800ml acetonitrile, 10ml concentrated ammonium hydroxide, isocratic	254nm	acetonitrile

- Miniaturized disposable amperometric biosensors for determination of creatinine in human serum (Mádâras and Buck, 1996). The base electrodes are fabricated using microelectronic techniques to build a multilayer film structure on a polyimide foil. By using a thin electropolymerized film of poly(1,3-diaminobezene), the

electrochemical interferences from ascorbate, urate, acetaminophen, and other oxidizable species are greatly diminished.

- **Determination of creatinine by capillary electrophoresis:** Since the new technique-HPCE is established and quickly developed during the last decade, it has attracted the attention of many scientists trying to solve some of the problems that had previously been very difficult to solve by other than current techniques.

A method for the rapid analysis of creatinine in urine has been developed using capillary zone electrophoresis (CZE) without sample pretreatment other than dilution. An indirect ultraviolet detection mode was employed at 255 nm (Xu *et al*, 1994).

Another method of determination of creatinine by CE was the application of micellar electrokinetic capillary electrophoresis (MEKC, Tran *et al*, 1997). This method was using an sodium dodecyl sulfate-borate buffer system at pH 9.0 to determine creatinine levels in human serum.

2.2.2 Assay of Creatine

2.2.2.1 A widely used method is the Jaffé reaction:

Here the determination involved is the difference between the preformed creatinine and the total that results after the creatine present has been converted to creatinine by heating at acid pH (Tietz, 1976). However, the amount of creatine in urine is quite small compared with the amount of creatinine, which can result in large errors (Tietz, 1976).

2.2.2.2 The determination of creatine by enzymatic methods:

The determination of creatine by enzymatic methods with the use of creatine kinase (CK; EC 2.7.3.2), pyruvate kinase (PK; EC 2.1.7.40), and lactate dehydrogenase (LD; EC 1.1.1.27) after sample pretreatment is cumbersome and likely to result in error. The enzymatic serum creatine assay (Suzuki and Yoshida, 1984), in which creatinase (EC 3.5.5.3), sarcosine oxidase (EC 1.5.3.1), and peroxidase (EC 1.11.1.7) are used in the

color-indicator reaction, is subject to interference by reducing substances such as bilirubin (a common endogenous serum compound), dopamine, and ascorbic acid (Weber and Van Zanten, 1991). In recent years, this serum creatine assay has been automated by using a commercially available creatinine kit (Oversteegen *et al.*, 1987) but omitting the starting reagent creatininase. The same kit is used in a similar way for creatine measurement in urine (Delanghe *et al.* 1988; Poortman and Diaz, 1986). However, a detailed description of its analytical performance is still lacking (Beyer, 1993).

2.2.2.3 HPLC (high-performance liquid chromatography):

- An ODS reversed-phase ion-pair column has been used (Murakita, 1988). The mobile phase was 100mM sodium phosphate solution containing 30 mM sodium lauryl sulphate-acetonitrile (3:1,v/v) and the pH was adjusted to 2.1 with phosphoric acid. The column temperature was 55°C and flow-rate was 2.0 ml/min. The absorbance was monitored at 210 nm. BondElut C₁₈ columns were used for sample pretreatment. Serum was pretreated as follows: 100 µl of 1 M perchloric acid were added to 200 µl of serum, and the sample was shaken then centrifuged at 16000 g for 2 minutes. The supernatant was placed on a BondElut C18 column, and creatine and creatinine were eluted with 400mM sodium phosphate solution (pH 2.0), then analyzed by HPLC.
- Determination of serum creatinine, and creatine in human serum and urine using liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (Yasuda *et al.*, 1997): The determinations of creatinine, creatine in the samples of human urine and serum were carried out by scanning the $[M + H]^+$ ions of each compound.

2.2.2.4 Biosensors: an option for determining creatine as well as creatinine:

The creatine sensor is based on a bienzyme sequence, involving creatine amidinohydrolase (CI) and sarcosine oxidase (SO). The signals of the sensor are based on the oxidation of the hydrogen peroxide formed in the enzymatic layer to yield currents proportional to the concentration of creatine in the sample solution. For the

optimization of enzyme loading, crosslinking with glutaraldehyde was selected as the immobilization method for the enzymatic system. However bienzyme potentiometric biosensors so far described in the literature usually had worse parameters and response time (10-15 minutes) and short lifetime (few days to one week) (Mâdâras and Buck, 1996).

2.3 Comparison of CE with other techniques

Although kinetic methods and different pretreatments of the sample were developed to address the interference problem, the determination of the “true” creatinine concentration in serum remains a problem. Enzymatic methods are time-consuming and rather costly and the elimination of interferences in the determination of creatinine in serum has still not been achieved. That is why so far the Jaffé reaction is still the most widely used method in clinical laboratory. Now it is time to have something that can be used instead of the Jaffé reaction method and can offer a more accurate, convenient service for people. Beside the well-established spectrophotometric procedure, separation methods such as HPLC have been described (Table 2.1) for the creatinine determination that seem to have achieved more than the other techniques. However, these methods are not well suited for large-scale routine clinical analysis but are useful as reference techniques. One of the major problems appears to be the sample preparation time.

Capillary electrophoresis (CE) has frequently been compared with HPLC. Bearing in mind the relatively recent development of CE as a separation technique, it should be recognized that there would probably be more rapid growth in CE than in the other more mature techniques. In fact, currently there is such a tremendous interest in CE that rapid progress is constantly being made, both in instrumentation and separation methodologies. There will certainly be immense scope for further advances in the development of new applications for CE. The comparison made here is therefore partly intended to serve as an indicator of the areas where efforts can be made to achieve significant future developments in CE.

CE has attracted considerable attention in recent years because of its potential to achieve very high efficiency. The main reason for the extraordinary high efficiency in

CE is attributable to its characteristically flat flow profile. In **Figure 2.16**, flow profiles in HPLC and CE is shown. In general, the flow of mobile phase in HPLC is maintained by a pump and therefore under normal operating conditions, a parabolic flow profile is observed. As a result of its contributions to peak broadening, the flow profile inherently limits the separation efficiency theoretically achievable in HPLC separations. In the case of CE, charged species migrate under the influence of an applied voltage, resulting in a practically flat flow profile. This fundamental difference in flow profile is the main reason for the extremely high efficiencies achievable using CE. Here narrower peaks and potentially better resolution can be readily obtained, especially when selectivity is also optimized for the separation.



Figure 2.16 Flow profiles in HPLC (left) and CZE (right).

In terms of instrumentation, HPLC and CE are similar in some respects but different in others. It is simpler for CE due to the absence of an injector, a pump (one or more) a solvent mixer for gradient HPLC and a special detection cell. For CE, injection is usually performed using one end of the separation capillary, and on-column detection is accomplished with part of the column forming the detection cell. The solutions are most commonly introduced into the capillary either by the electrokinetic or by the hydrodynamic mode.

Compared to the other techniques, **Capillary Electrophoresis (CE)**, the relatively new technique, described in Chapter One, provides a viable option for people who work in the clinical lab for determination of creatinine and creatine. Speed (more efficient, less

labor-intensive), low costs (minimal buffer consumption), small sample volume (reduced blood collection volume from patient), increased selectivity (determination of multiple solutes in one run), and versatility (detection of analytes over the wide range of molecular masses and chemical composition) are among the advantages offered by CE.

Creatinine, the most widely used clinical marker for renal dysfunction, is zwitterionic at pH 9.0 and therefore co-elutes with neutral serum components when free solution CE is performed at this pH. Creatinine has pK_a values at 4.8 and at 9.2 (Dawson *et al.*, 1986). In principle, creatinine can be separated from other serum components using free solution CE at a pH below 4.8 (where it is cationic) or at a pH above 9.2 (where it is anionic). The former has been reported (Xu *et al.*, 1994) and is useful for creatinine determinations, but results in the coelution of many acidic metabolites. The latter has proven ineffective for separating creatinine from other neutral components, including nicotinamide and caffeine, at least with borate buffer systems with pH values as high as 10.00 (Tran *et al.*, 1997). Alternatively, zwitterionic creatinine can be separated from neutrals by the addition of a micellar pseudo-stationary phase to the CE buffer. However, sometimes it is difficult to wash away the micellar pseudo-stationary phase from the capillary. In addition, it easily flows into the inside of the CE equipment.

Although the Jaffé reaction has proven to be a non-specific reaction and many methods already have been tried to improve the specificity of the Jaffé reaction, it does not mean however that there is no more room for improvement. First, the Jaffé reaction has not yet been combined with CE, which has totally different separation principles from the other techniques such as HPLC, and should have more chance of separating the creatinine from interfering compounds. Secondly, CE, which is a multi-principle technique, has more chances of determination and separating the creatine as well. So far, there has been no report of creatine determination by CE. Even if there were some methods for determining creatinine by CE, probably little work have been achieved yet for both creatinine and creatine. Therefore, CE, the newer technique that has fast developed in recent years, was the main tool in my research for determination creatinine and creatine.

CHAPTER THREE

MATERIALS AND GENERAL PROCEDURES

3.1. Apparatus

An Applied Biosystems Model 270 capillary electrophoresis system was used throughout this study. The buffer volumes of the vials at the injection end and at the detector end were 4 and 12.5 ml respectively. Eppendorf sample vials (0.5 ml) were used in all experiments.

The polyimide-coated fused-silica capillary (I.D. 75 μm) was obtained from Phenomenex. The total length of the capillary was 50cm and the effective length was 25 cm. The window for the on-column detector cell was created by burning off a small section (~ 0.4 cm) of the polyimide-coating, and the excess residue was then wiped off with methanol. The electropherogram was recorded and evaluated on a Kipp and Zonen DB 40 recorder.

The TTT80 titrator, the PHM 82 standard pH meter and the ABU80 autoburette (all have Radiometer) were used to measure the pH of the picric acid solution and the buffers.

A centrifuge was used for sample clarification.

3.2 Chemicals

Sodium hydroxide, monosodium phosphate, picric acid, borax (sodium tetraborate decahydrate, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) were from BDH. The CAPS, creatinine, acetoacetic acid, L-ascorbic acid, glucose, guanidine, α -ketoglutarate, creatine, bovine serum albumin, sodium chloride, ammonium chloride were from SIGMA. The acetone was from AJAX.

Biological samples (urine and serum) were obtained from healthy laboratory colleagues.

3.3 General procedures

3.3.1 Reagents

Standard creatinine:

Standard creatinine was prepared by dissolving creatinine in Milli-Q water to make a 20mM solution and diluted to other concentrations as necessary. The standard solutions were filtered through 0.2 μ m filter before running on the CE. The solution was stored in the refrigerator.

Standard creatine:

Standard creatine was prepared by dissolving creatine in a buffer, which contained two-volumes of acetonitrile and one-volume of 3 % ammonium chloride to make a 10 mM creatine solution and diluted to other concentrations as necessary. The standard solutions were filtered through 0.2 μ m filter before running on the CE. The standard solution was stored in the refrigerator.

Saturated picric acid (PA) solution:

Saturated solution of picric acid was prepared by adding an excess solid picric acid into Milli-Q water at room temperature and allowing the mixture to stir overnight. The concentration of the saturated picric acid was measured by titrating with 1 M NaOH using the PHM 82 standard pH meter. This solution was put into a bottle covered by aluminum foil to protect it from the light.

Sodium hydroxide (NaOH) solutions:

Sodium hydroxide were dissolved in Milli-Q water, cooled and diluted to 2 M, 1 M, and 0.1 M, respectively, and stored in plastic bottles after filtration through a 0.2 μ m filter.

30 mM phosphate buffers: (pH 8.0, pH 9.0, pH 9.5, pH 10.0, pH 10.5)

3.6 g NaH_2PO_4 were dissolved in 1L of Milli-Q water and filtered through a 0.2 μ m filter, then adjusted with 2 M NaOH to the desired pH.

30 mM phosphate buffers (NaH_2PO_4) + 1 mM, 5 mM, 8 mM picrate:

In 1L volumetric flasks, 3.6 g NaH_2PO_4 were dissolved in 500 ml Milli-Q water with addition of 20 ml, 101 ml, and 161 ml saturated PA, respectively, then made up to 1 L with Milli-Q water and filtered through a 0.2 μm filter. Then the solutions were adjusted with 2 M NaOH to the desired pH. The bottles were totally covered by aluminum foil to protect them from the light.

30 mM CAPS + 5 mM picrate:

A similar procedure as that above was followed: 30 mM phosphate (NaH_2PO_4) + 1 mM picric acid.

30 mM borax + 5 mM picrate:

A similar procedure as that above was followed: 30 mM phosphate (NaH_2PO_4) + 1 mM picric acid.

Alkaline picrate solution:

Saturated PA was diluted to 8mM with distilled water, filtered and then adjusted to pH 8, 9, 9.5, 10, respectively with 2M NaOH.

Creatinine in buffer and 8mM picrate solution:

Creatinine was dissolved in phosphate buffer to produce 20 mM creatinine solution at the desired pH and with addition of 8mM picrate (1/1 volume) immediately prior to analysis on CE.

Interference standards:

20 %	Acetone
25 mg/dl	Acetoacetic acid
25 mg/dl	Guanidine
25 mg/dl	α -Ketoglutamate
25 mg/dl	Cephalosporin
400 mg/dl	Glucose
25 mg/dl	Bovine serum albumin

Urine samples:

1. Fresh urine samples were collected, and filtered through a 0.2 μm nylon filter just before the analysis.
2. Fresh urine samples were collected and diluted to 5-times, 100-times, respectively, and then filtered through a 0.2 nylon filter just before the analysis.

Serum samples:

1. Serum samples were stored frozen, thawed and were filtered through a 0.2 μm nylon filter immediately prior to the analysis (**Method 6e**).
2. The serum samples were diluted in acetonitrile and 3 % NH_4Cl (2:1 v/v) buffer, which contained one volume serum and two volumes buffer. After centrifugation at 6000 r.p.m. for 10 minutes, then were filtered through a 0.2 μm nylon filter (**Method 9**).

3.3.2 Apparatus**3.3.2.1 Capillary electrophoresis**

Routine operation of the Applied Biosystems 270A capillary electrophoresis system was as follows:

Cycle: 0.1 M NaOH WASH

Time: 2 minutes

Vial: WA

Pause: No

Cycle: BUFFER

Time: 4 minutes

Vial: B1

Pause: No

Cycle: SAMPLE

Time: sec

Vial: S

Mode: Vacuum

Polarity: Pos

Level: 5

Pause: No

Cycle: DETECTOR

Risetime: 2.00

Range: 0.020

Autozero: Yes

Change: Yes

Cycle: Time

Time:	min	Buffer: B1	Wavelength:	nm
Voltage:	kV	Temperature:	°C	Polarity: Pos

The peak absorption (peak height) was measured in mm.

The new capillaries were washed with 1 M NaOH for 10 minutes, then distilled water for 5 minutes.

3.3.2.2 UV Spectrophotometer

1. The 30 mM borax + 1 mM PA (pH 10) buffer was used for the blank. Creatinine was then added to this solution for the measurements. The sample was then scanned against the blank from 200 nm-600nm at room temperature in order to find out the maximum absorption of the final 1/1 complex under the condition used.
2. The 30 mM phosphate buffer (pH 10) was used for the blank. Creatinine was then added to this solution for the measurements. The sample was scanned against the blank buffer from 400 nm to 500 nm at room temperature.

CHAPTER FOUR

DERMINATION OF CREATININE IN URINE

4.1 Introduction

Creatinine occurring in urine has long been recognized as an important metabolic product of diagnostic significance. Urinary creatinine is not only used as an index of renal function (Tietz, 1986) but also as a reference compound for the comparison of excretion rates of other metabolites (Free, A. and Free, H., 1975).

Creatinine determinations in most clinical laboratories still rely on the Jaffé reaction. The critical problem is the interferences from non-creatinine chromogens, which are extremely difficult to avoid or separate. Even though there are some methods that have been designed to solve this problem, either they questioned or have had other disadvantages such as high cost, time consuming procedures not suited for routine operations or short life times.

Capillary zone electrophoresis (CZE) is a popular mode of capillary electrophoresis because it is relatively simple. Most separations can be carried out using an untreated fused silica capillary. Aqueous buffers are usually used in CZE.

The separation principle in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the electrophoretic buffer contained in the capillary. The separation mechanism is mainly based on differences in **solute size and charge** at a given pH. In addition, for the Jaffé reaction the creatinine reacts with picric acid (yellow colored solution) to form a **negatively charged 1/1 complex** (reddish-orange color) in alkaline conditions.

Even these non-creatinine chromogens can be separated from creatinine / picric acid (1/1 complex) by HPLC. CZE has some advantages over HPLC both in principle and instrumentation (see Chapter Two). There is certainly more scope for CZE to achieve the better separation of chromogens from the 1/1 complex.

4.2 Methods

Capillary	75 μ m ID \times 25 cm
Injection type	vacuum
Injection time	1 second
Wavelength	480 nm
Running time	3 minutes
Detector Rise time	2 second

Table 4.1 Methods

method	buffer	buffer pH	sample	Voltage (kV)	Temperature (°C)	detector range
1a	30 mM phosphate	10	CRN & 8 mM PA in buffer (1/1)	20	30	0.02
1b	30 mM phosphate	10	1a + spiked CRN	20	30	0.02
1c	30 mM phosphate	10	8 mM PA	20	30	0.02
2a	30 mM phosphate + 1 mM PA	7	CRN	20	30	0.02
2b	30 mM phosphate + 1 mM PA	8	CRN	20	30	0.02
2c	30 mM phosphate + 1 mM PA	10	CRN	20	30	0.02
2d	30 mM phosphate + 1 mM PA	10	CRN	20	27	0.02
3a	30 mM CAPS+1mM PA	10	CRN	20	30	0.02
3b	30 mM phosphate + 1 mM PA	10	CRN	20	30	0.01
3c	30 mM phosphate + 1 mM PA	10	CRN	15	30	0.02
3d	30 mM phosphate + 1 mM PA	10	CRN	30	30	0.02
3f	30 mM phosphate + 1 mM PA	10	CRN	20	30	0.02

4.3. Results and discussion

4.3.1 Selection of the mode of capillary electrophoresis

The separation principle in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migrating ionic species in the electrophoretic buffer contained in the capillary. The separation mechanism is mainly based on differences in **solute size** and **charge** at a given pH.

The Jaffé reaction is a reaction where creatinine reacts with picric acid (yellow color solution) to form a **negatively charged** 1/1 complex (reddish-orange color) under alkaline conditions. Moreover, based on the difference of the charge/size ratios of the solutes, apparently there is some opportunity for CZE to separate the interfering solutes from the creatinine-picric acid 1/1 complex.

4.3.2 Selection of capillary

There are three types capillaries have been introduced, TeflonTM, PyrexTM, and fused silica capillaries. Fused silica capillaries are the most popular because they are transparent to a wide range of the UV wavelengths, flexible and easy to handle and cut. Untreated fused silica capillaries were chosen as the best option for this study.

Capillaries are available in a variety of internal diameters, but the most commonly used are 50-100 μm I.D. More Joule heat is dissipated quickly in a capillary with a smaller internal diameter. Capillary length affects both peak spacing and separation time. The longer the capillary, the longer the separation time and the wider the spacing between peaks, with all other conditions being equal. Migration times are proportional to capillary length and resolution is proportional to $(l/L)^{1/2}$ where l is the capillary length to the detector (effective length) and L is the total capillary length. In order to have the solutes separated in the fastest time, the shortest capillary that gives the required resolution should be used (Baker, 1995).

In this study, capillaries with 75 μm I.D and 25 cm effective length (from injection end to the detector window) with total length 50 cm length were used.

4.3.3 Selection of analysis method

4.3.3.1 CZE study one

A. Sample: 20 mM creatinine mixed with 8 mM picrate.

After 5 minutes running on CE, two peaks appeared with migration times at 1.1 minutes and 2.0 minutes, respectively (**Figure 4.1a**). In order to identify these two peaks, further experiments were carried on:

B. Sample: 20 mM creatinine mixed with 8 mM picrate spiked with creatinine.

The result (**Figure 4.1b**) showed that the only difference from the very first run was at migration time 1.1 minutes, a much higher peak appeared than in the unspiked run.

C. Sample: 8 mM picrate solution only without creatinine:

The result was that only one peak appeared at migration time 2.0 minutes (**Figure 4.1c**)

From the above experiments, apparently, the second peak, which was at migration time at 2.0 minutes, represents an intermediate reactive species (picric acid in alkaline condition) at wavelength 480 nm. A postulated intermediate in the reaction of 1,3,5-trinitrobenzene with sodium hydroxide to give a red color having structure **Figure 4.2** has been reported (Abe, 1960b), with its wavelength maximum reported at 480 nm. That picric acid in the presence of hydroxide can form such an intermediate species therefore seems likely. Tetryl (2,4,6-trinitrophenylmethylnitramine) in the presence of sodium hydroxide also forms a red color, which slowly changes to yellow. The final spectrum is identical to the spectrum of picric acid (Abe, 1959). ¹³C NMR data are consistent with the above findings and strongly support the fact that hydroxide interacts with picric acid to form some intermediate reactive species (**Figure 2.12** and **Figure 2.13**, Chapter Two). The first peak that appeared at 1.1 minutes represents the final complex of 1/1 between creatinine and picric acid.

Absorption
(mm)

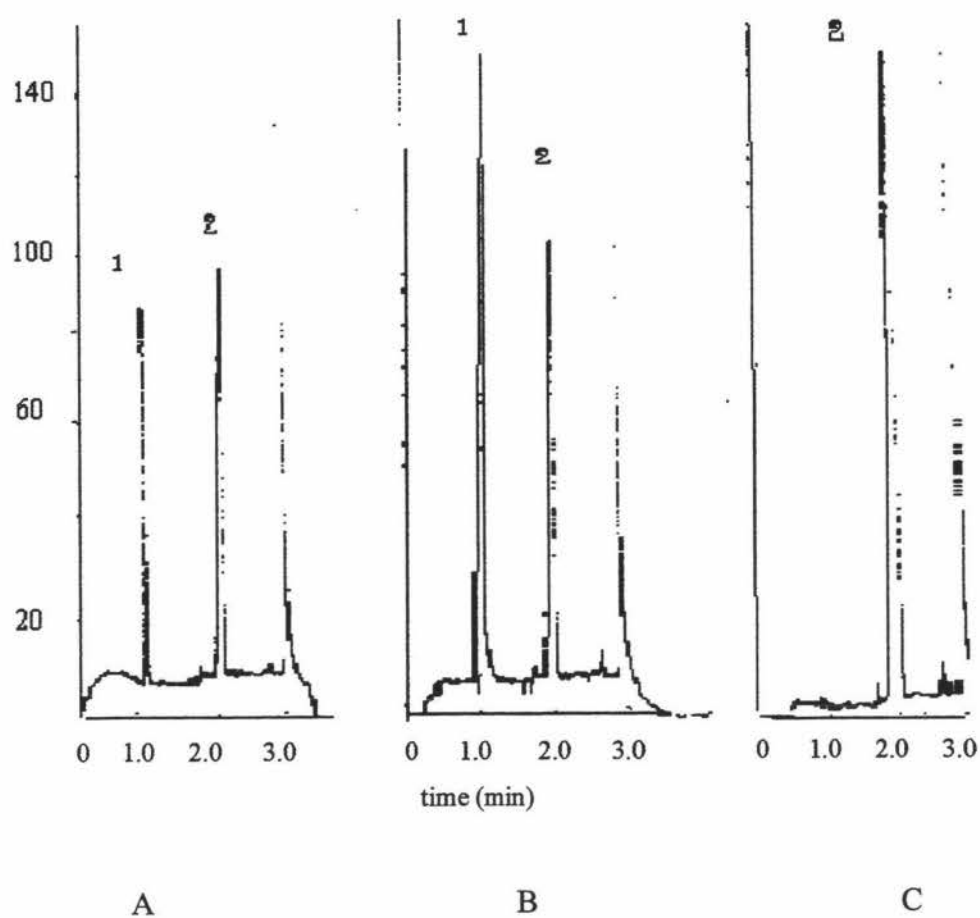


Figure 4.1 A, 10 mM creatinine and 8 mM PA (**Method 1a**). B, 10 mM creatinine and 8 mM PA with spiked creatinine (**Method 1b**). C, 8 mM PA (**Method 1c**).

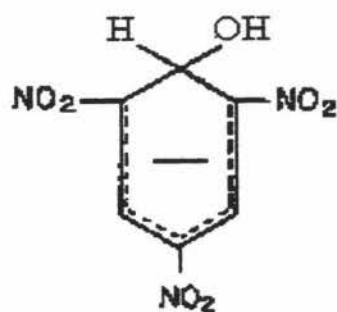


Figure 4.2 A postulated intermediate in the reaction of 1,3,5-trinitrobenzene with sodium hydroxide (Abe, 196b).

Reaction time and Analysis time:

However, there were some problems inherent in **Method 1**. Note from the differential scan of the creatinine complex from alkaline sodium picrate and creatinine with time (**Figure 2.9** Chapter Two), the absorbance at 480 nm increased from 5 minutes to 50 minutes. The reaction time becomes a factor of the Jaffé reaction; therefore controlling of the Jaffé reaction time is a matter of measuring the correct, accurate absorbance of the 1/1 complex. Nevertheless, in **Method 1**, the Jaffé reaction takes place as soon as the creatinine encounters the PA, that is the reaction time is controlled manually by the operator. This gives no guarantee of accuracy of the reaction time at all. Hence, the absorbance of the 1/1 complex could vary with each individual run.

Another problem was the slight peak broadening due to the smaller ratio of ionic strengths between the running buffer and the sample. Analytes that have higher mobilities than the separation buffer ions will generate peaks that demonstrate fronting (peak A in **Figure 4.3**, Li, 1992). This is because the high-mobility solute ions tend to migrate ahead of the sample zone. Analytes that have the same ionic strength as the buffer will produce a symmetrically broadened peak shape (peak B in **Figure 4.3**). Analytes with lower mobilities than that of the buffer complements will demonstrate tailing (peak C in **Figure 4.3**).

In this case, the sample contained both creatinine and PA that gave higher ionic strength. In order to prevent the peak broadening, the buffer's concentration must be much higher than that of sample. The consequences of increasing the concentration of the running buffer would generate a higher current during sample analysis, hence, decreasing the dispersion of the Joule heating. Therefore this results in viscosity changes and convection may result in a loss of resolution.

The conduction of electric current through an electrolytic solution results in the generation of Joule heat because of frictional collisions between mobile ions and buffer molecules. Since high field strengths are employed in CE, this Joule heating can be substantial. There are two problems that can result from Joule heating: (1) temperature changes due to ineffective heat dissipation; (2) development of thermal gradients across the capillary.

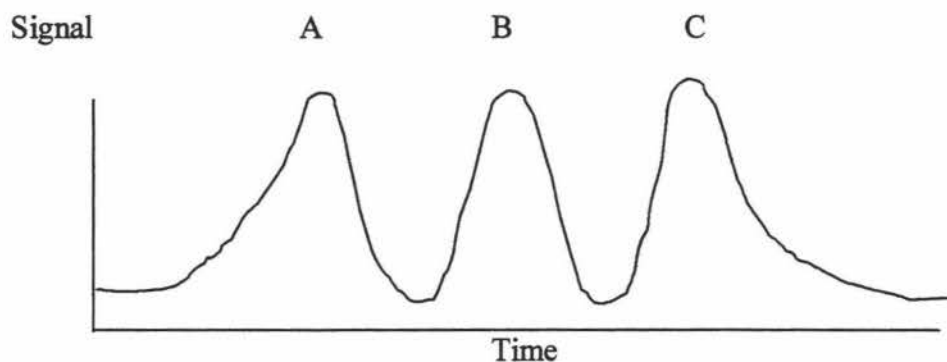


Figure 4.3 Peak distortion at high analyte concentration (Li, 1992).

If heat is not dissipated at a rate equal to its production, the temperature inside the capillary will rise, and eventually the buffer solution will degas or boil. Even a small bubble inside of the capillary will disrupt electrical continuity.

The rate of heat production inside the capillary can be estimated by

$$dH / dt = IV / LA, \quad (4.1)$$

where L = capillary length and A = the cross-sectional area, V = voltage applied, I = current. By rearranging this equation using $I = V/R$, where R the resistance = L/kA , and k = the conductivity, then

$$dH / dt = kV^2 / L^2 \quad (4.2)$$

The amount of heat that must be removed is proportional to the conductivity of the buffer, and to the square of the field strength. Higher concentration buffers are more conductive, draw higher currents, and produce more heat than more diluted solutions. Consequently, the internal temperature rises, reducing the viscosity of the buffer. Since Eq. (4.3), the basic expression for electroosmotic velocity (v_{eo}), contains a viscosity parameter in the denominator, v_{eo} increases with decreasing buffer viscosity (Weinberger, 1993).

$$v_{eo} = \frac{\epsilon \zeta}{\eta} E \quad (4.3)$$

In any electrophoretic system, the current carried by a single ion in the running buffer is proportional to the product of the concentration of the ion, its charge, and its velocity. Thus, at any given voltage, the current will be a function of the ionic strength of the running buffer. This relationship, however, becomes more complicated as the internal heat becomes less efficiently dissipated eventually leading to significant temperature gradients within the capillary. When this occurs, the viscosity of the buffer decreases, resulting in an increase in ion velocity and therefore current (Colburn, *et al*, 1990). The current generated in **Method 1** was 33 μA .

Based on the **Method 1**, one must bear in mind that the reaction time was difficult to control manually under each individual sample running. Allowing the reaction to take place automatically improve methods. There were some adjustments to **Method 2c** compared to **Method 1**. Instead of mixing creatinine with picrate solution before the analysis takes place, the PA has been dissolved into the running buffer. This change allows the creatinine to encounter the PA right after the sample injection at the beginning of each sample run, so that the reaction time was under the program's control. On the other hand, the ionic strength ratio between running buffer and sample was increased so that shaper peaks were produced and lower current was being generated (22 μA) in **Method 2c**. In addition, picrate solution in **Method 2c** became part of the absorbency background, which simplified the analysis (**Figure 4.4**).

4.3.3.2. CZE study two

A. Buffer: 30 mM phosphate, pH = 10

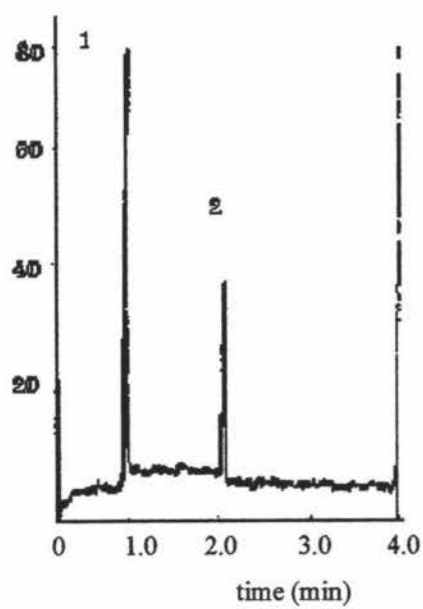
Sample: 20 mM creatinine mixed with 8 mM picrate.

Result: After 3 minutes running on CE, there were two peaks appeared on the recorder, with migration times at 1.1 minutes and 2.0 minutes, respectively (**Figure 4.4A**).

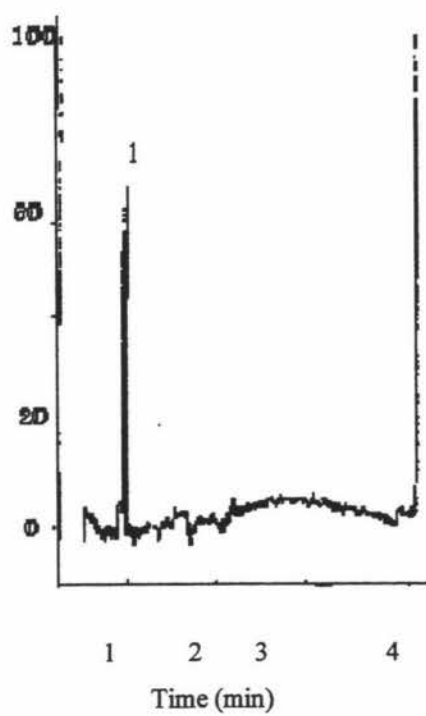
B. Buffer: 30 mM phosphate and 1 mM PA, pH = 10.

Sample: 10 mM creatinine.

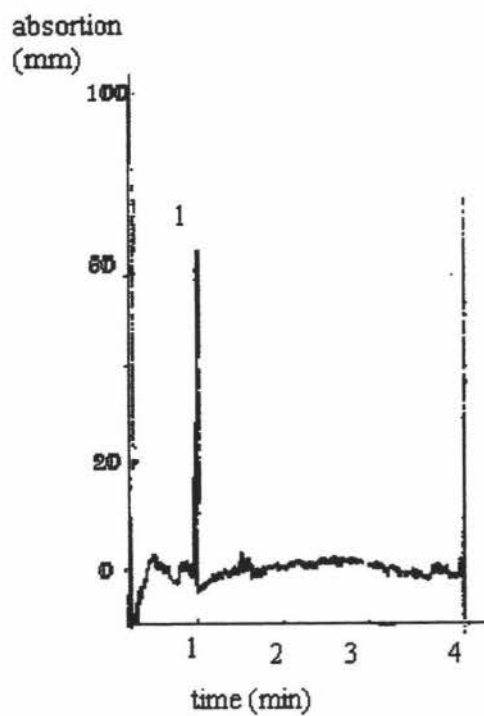
Absorption (mm)



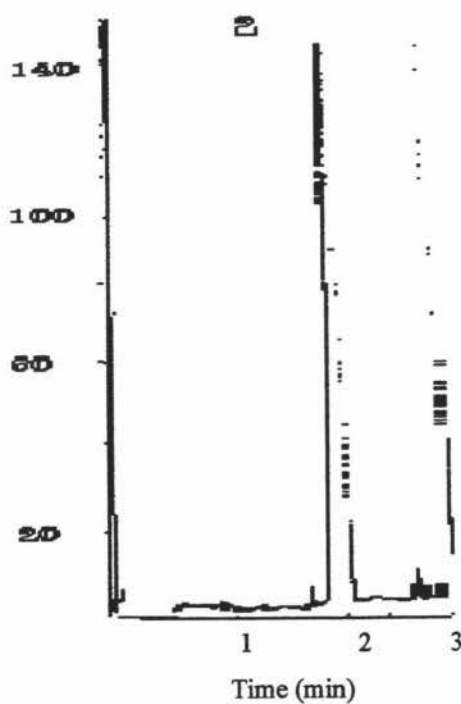
A



B



C



D

Figure 4.4

Comparison of **Method 1** and **Method 2c**. A, 10 mM creatinine in **Method 1**. B, 10 mM creatinine in **Method 2c**. C, 10 mM creatinine in **Method 2c** with spiked creatinine. D, 30mM PA in **Method 2c**.

Result: only one peak appeared at migration time 1.1 minutes (**Figure 4.4B**).

C. Buffer: 30 mM phosphate and 1 mM PA, pH = 10.

Sample: 10 mM creatinine with spiked creatinine.

Result: one peak appeared at same migration time (1.1 minutes), but much bigger (**Figure 4.4c**).

D. Buffer: 30 mM phosphate and 1 mM PA, pH = 10.

Sample: 30 mM picrate solution.

Result: one peak appeared at migration time 2.0 minutes. (**Figure 4.4d**).

Selection of buffer pH:

The Jaffé reaction occurs in alkaline condition where creatinine reacts with picric acid to form a 1/1 complex, which has a maximum absorbance at wavelength 480 nm. The rate of development of color in the Jaffé reaction is a function of pH (Owen, 1954). The concentration of sodium hydroxide is first order for the forward Jaffé reaction. I have examined this point by changing the pH of the buffer from 7 to 10 in **Method 2a – 2c** (**Figure 4.5**).

The result showed that the running buffer at relatively higher pH 10 produced more 1/1 complex of creatinine-picrate at slightly slower migration time than that at lower pH 7.

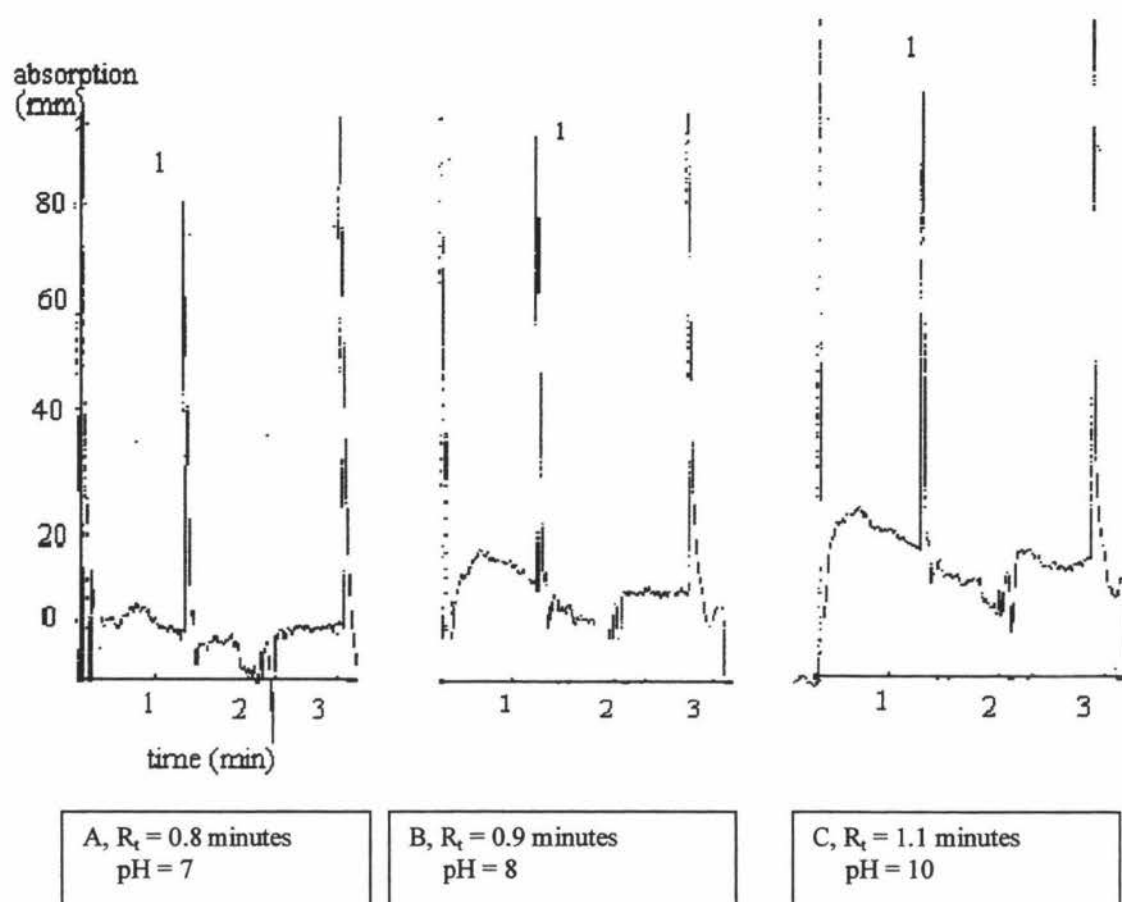


Figure 4.5 Effect of buffer pH. Condition: **Method 2a, 2b, 2c.**

The degree of ionization of species present in an electrolyte system depends on the pH of the solution. Differences in the degree of ionization give rise to differences in electrophoretic and electroosmotic mobilities. Consequently, both the separation efficiency and the flow velocities may be affected by the buffer pH (**Figure 4.6**).

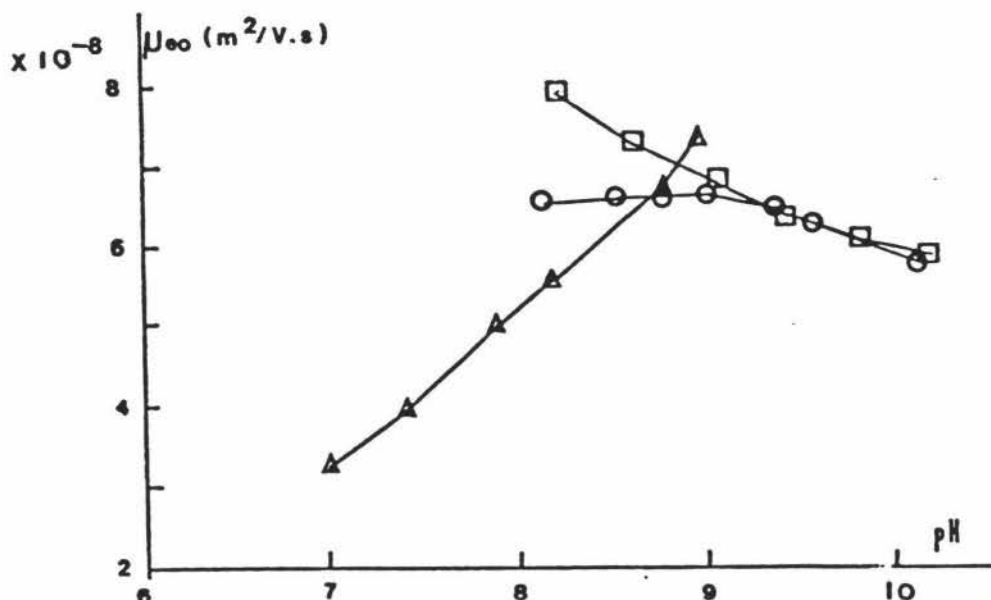


Figure 4.6 Influence of buffer pH on electroosmotic mobility. ∇ = Tris - HCl; \square = boric acid - NaOH; o = borax - HCl. Buffer concentration: 50 mM (Li, 1992).

When constant-concentration buffers are used, μ_{eo} increases with pH for a weak base-strong acid-type buffer, and decreases with pH for a strong base-weak acid type buffer (Figure 4.6). In my experiments, the phosphate buffer is a strong base-weak acid type buffer, and hence belonged to the latter situation; which has its mobility decreased when the pH was increased.

Choice of temperature:

The color developed in the Jaffé reaction has been attributed to the formation of a red tautomeric form of creatinine at temperatures lower than 30° C and a reaction time not exceeding 15 minutes (Archibald, 1962). On longer reaction, methylguanidine and presumably picramate, a reduction product of picrate, are produced, this effect being pronounced at temperatures exceeding 30 °C. Temperatures between 15° C and 25° C apparently has little effect upon color development, but the temperature at which absorbance is measured appears to influence assay results (Owen, *et al*, 1954). In this case, for CE analysis, temperatures of less than 30° C were chosen to avoid further product formation.

Furthermore, precise temperature control offers a selectivity parameter in separation by capillary electrophoresis. The mobility equation (Eq. 4.4) includes a viscosity function,

which is affected by temperature changes. As viscosity (η) decreases, mobility (μ) increases (Colburn, 1990).

$$\mu = C\varepsilon\zeta / \eta \quad (4.4)$$

Therefore, in order to have a stable migration time for identification, the temperature must be constant during the analysis, and the CE apparatus can only keep the temperature steady when the run temperature is 5° C higher than the room temperature. Because there was no air conditioner in our laboratory, it was very difficult to lower the run temperatures to below 25° C and maintain constancy. Thus, the temperature was limited to run between 25° C and 30° C. However, higher temperature leads to faster migration times. The running temperature could be set at a relatively higher setting (30° C), so that the analysis could be achieved faster due to the decreased of viscosity, hence, decreased the migration time. (Figure 4.7).

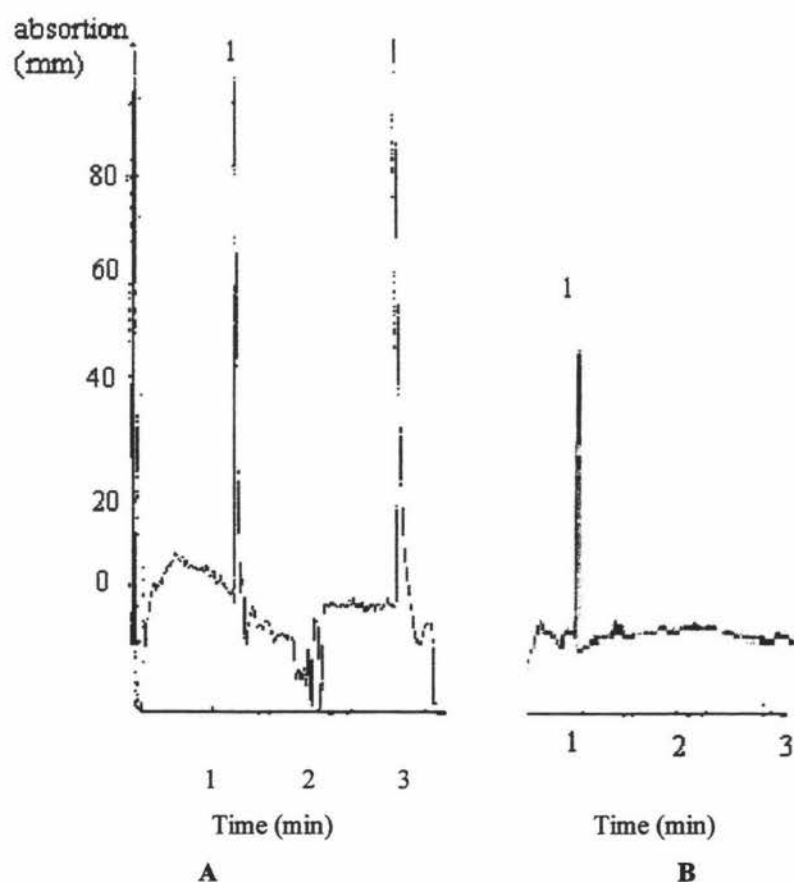


Figure 4.7 Migration time changed due to the affection of temperature. A, 27 °C, 10 mM creatinine.. B, 30 °C, 5 mM creatinine.

4.3.3.3 CZE study three

A. Buffer: 30 mM phosphate (NaH_2SO_4) + 1mM PA, pH = 10.

Sample: 10 mM creatinine.

Result: (Method 2d, Figure 4.8 effect of the buffer)

B. Buffer: 30 Mm CAPS + 1 mM PA, pH = 10.

Sample: 10 mM creatinine.

Result: (Method 3a,)

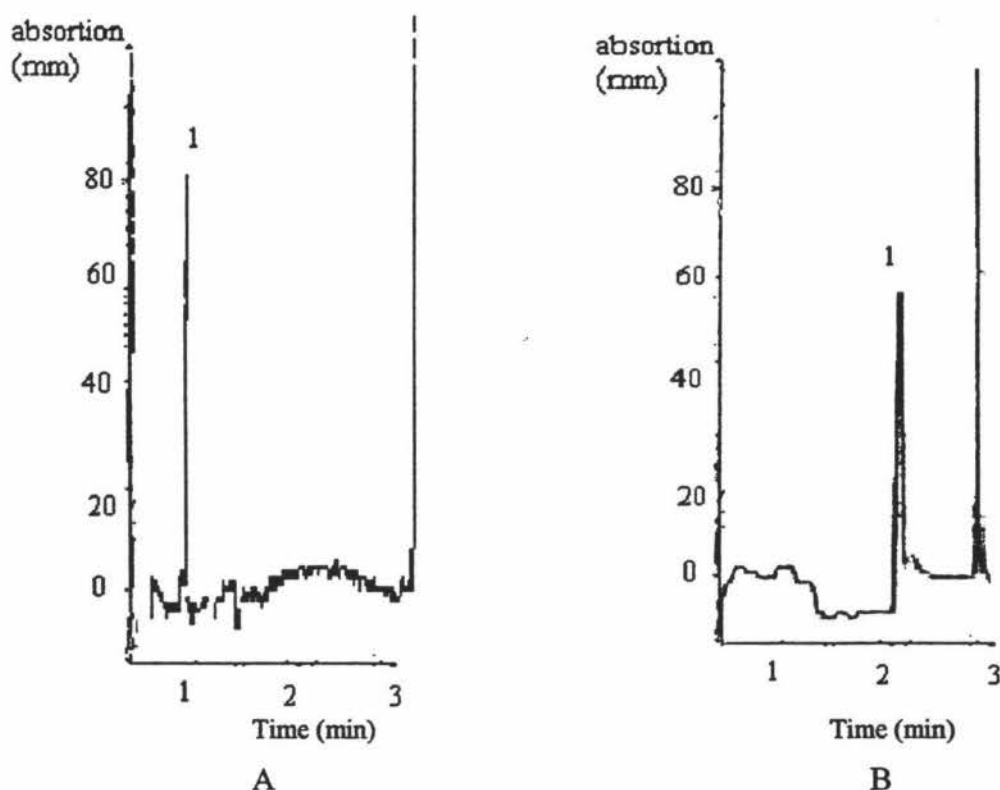


Figure 4.8 Effect of the buffer composition. A, phosphate buffer, $R_t = 1.1$ minutes. B, CAPS buffer, $R_t = 2.1$ minutes. Condition: A, Method 2d. B, Method 3a.

Selection of the buffer composition:

The composition, pH, and concentration of the run buffer are among the most important parameters for adjusting retention, selectivity, and peak shape in CZE.

The buffer pH and concentration were already discussed above. Buffer composition can also alter retention and selectivity. Different ions in the run buffer will interact differently with the capillary wall and can change the electroosmotic flow. Also, the run buffer ions may interact with solute ions, and changing the buffer composition may change the selectivity (Baker, 1995).

If the voltage is kept constant, the heat generated inside the capillary is directly proportional to the current ($P = VI$, where P is the Power, I is the current and V is the applied voltage). It is therefore an important consideration to select the type of buffer, which would give the lowest current at the same applied voltage under constant experimental conditions. In practice, this can be achieved by selecting buffers which have low conductance, since the power dissipated per unit length (P) of the capillary is given by Eq. 4.5

$$P = \frac{kCr^2V^2}{L} \quad (4.5)$$

where k , C , r , and L are the conductance, concentration of the buffer, radius and length of capillary, respectively (Li, 1992).

The results of the **Method 3** showed that when using CAPS as the running buffer, the migration time shifted to a longer retention time (2.1 minutes), compare to the phosphate buffer (1.1 minutes). That is due to the decreased ionic strength of the CAPS buffer under the constant buffer concentration study.

Therefore, phosphate buffer became the running buffer used to analysis the creatinine in urine. Its lower conductance compared to the CAPS buffer made it capable of analysis creatinine in a minimum retention time.

Selection of the sensitivity of the recorder:

The sensitivity of the recorder can be adjusted via the programmer of the CE apparatus. Sensitivities of 0.020 and 0.010 were examined on CE (**Method 2c** and **Method 3b**). The result showed that sensitivity at 0.010 increased the ratio between the peak signal and the baseline noise. On the other hand, sensitivity that at 0.020 gave a relatively smoother baseline rather than that of 0.010 (**Figure 4.9**).

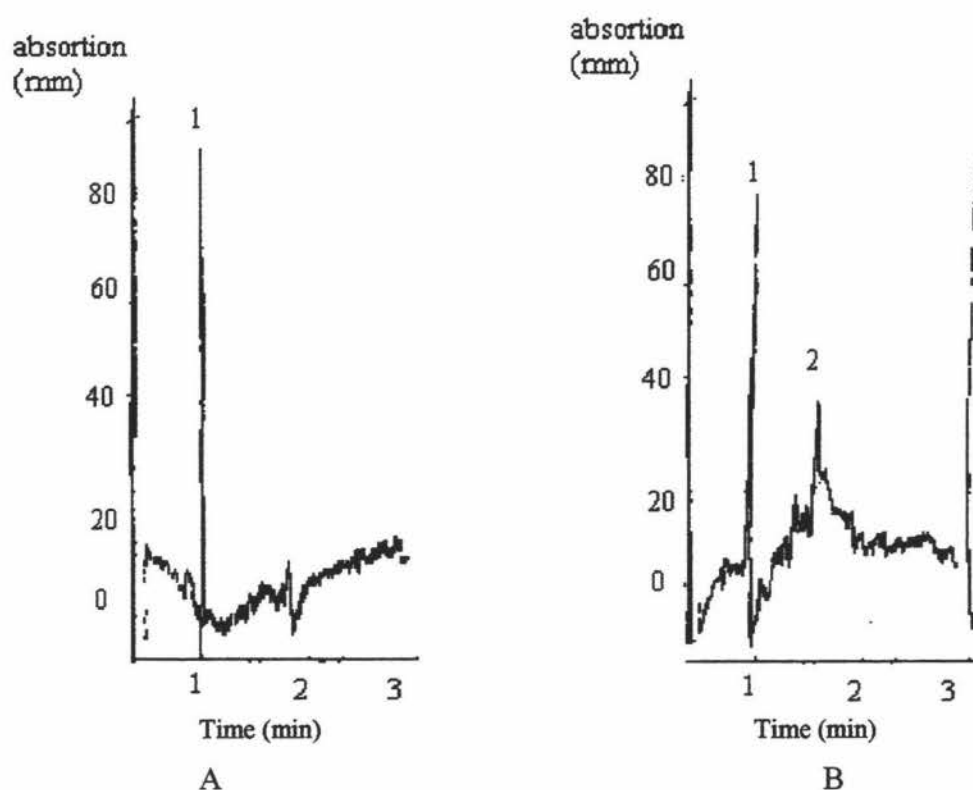


Figure 4.9 Comparison of the sensitivities. A, 10 mM creatinine at sensitivity 0.020 (**Method 2c**). B, 10 mM creatinine at sensitivity 0.010 (**Method 3b**).

The creatinine concentration in human urine is ~10 mM, and with the current methods, there were a strong signal for the 10 mM creatinine. Therefore it was not necessary to use an extremely high sensitivity to identify the creatinine in urine. Therefore, the sensitivity of the current method has been fixed to 0.020.

Selection of the injection type

To preserve the high efficiency capabilities of capillary electrophoresis, the injection system must not introduce significant zone broadening. It is important to ensure that the sample injection method employed is capable of delivering small volumes of sample (typically several nanolitres) onto the column efficiently and reproducibly.

Consequently, the most commonly employed injection methods for CE are direct on-column methods such as electromigration and hydrodynamic flow. In both of these types of injection methods, one end of the capillary is used as the sample injector directly, thereby eliminating the zone broadening due to connection with sample injection valves.

I. Electrokinetic injection:

Electrokinetic injection is also called electromigration injection. To perform electrokinetic injection, the electrode is removed from the buffer vial and placed into the sample vial. The buffer reservoir at the high-voltage electrode is replaced with the sample vial such that the capillary and electrode dip into the sample solution. An injection voltage is then applied for a brief period of time, causing the sample to enter the end of the capillary by electromigration. Electromigration injection includes a contribution from both electrophoretic migration of charged sample ions and electroosmotic flow of the sample solution.

II. Hydrodynamic injection

Hydrodynamic injection, also referred to as hydrostatic injection in some cases, can be performed by gravity flow, pressure or vacuum suction. The main advantage of this type of injection method is that unlike electrokinetic injection, there is no inherent discrimination of the sample injected. In **Figure 4.10**, a comparison of electrokinetic injection and hydrodynamic injection is shown. The peak area is plotted as a function of the sample solution resistance. For hydrodynamic injection, the peak area remained constant, whereas in the case of electrokinetic injection, the peak area increases with solution resistance despite the fact that the injection voltage and duration were kept constant (Li, 1992).

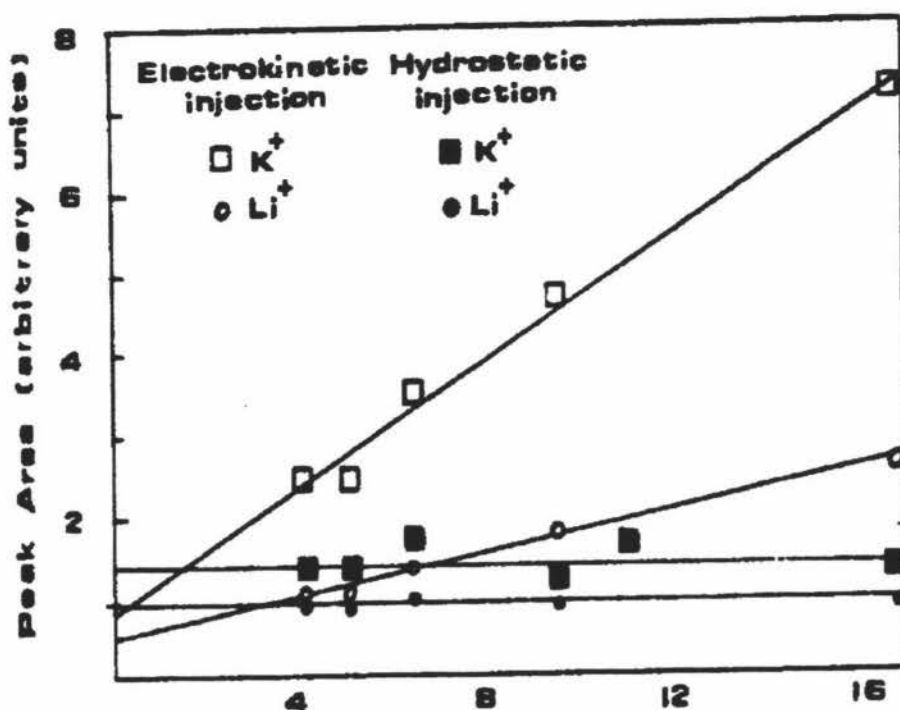


Figure 4.10 Plot of K^+ and Li^+ peak area as a function of sample solution resistance for both electrokinetic and hydrostatic injection. Electrokinetic injection causes a bias linear in sample solution resistance (which is inversely proportional to electrolyte concentration, Li, 1992).

For the 270A mode CE, there are two injection types available, electrokinetic injection and vacuum injection. From the above reasoning, vacuum injection has been chosen to be the way of sample injection. During vacuum injection, a sample is placed at the opposite end of the capillary, drawing the sample into the capillary.

Selection of the injection time:

Capillary electrophoresis systems are easily overloaded by large sample volumes. Sample overload can affect system efficiency by two distinct mechanisms. One mechanism relates to the volume of the sample injected (q_{inj}) relative to the total volume of the capillary (q_c). The standard deviation of the injection plug in volume units is:

$$\sigma_{q_{inj}} = \frac{q_{inj}}{\sqrt{12}} \quad (4.6)$$

and

$$N_{\max} = 12 \left(\frac{q_c}{q_{inj}} \right)^2 \quad (4.7)$$

From Eq. (4.7), it can be seen that the maximum number of theoretical plates (N_{\max}) of the overall system is constrained to a value proportional to the square of the ratio of the volume of the injected sample to the volume of the column.

The hydrodynamic mechanism imposes a limit on the concentration of the sample injected and is related to the difference in electrical conductivity of the sample and the electrophoretic medium. At high sample concentrations, system efficiency can be degraded due to perturbations in the potential field gradient by the sample within the column. Several distorted peaks may result. On the other hand, if the sample injected has a slightly lower conductivity than the electrophoretic buffer, sample stacking can be achieved which improves peak shape and hence increases efficiency.

Grushka and McCormick (1989) approximated the maximum allowable injection plug length as

$$l_{inj} = (24De_h t)^{1/2} \quad (4.8)$$

$$H = \frac{2D}{v} \quad (4.9)$$

where D is the solute's diffusion coefficient, v is the solute's velocity, t is the migration time, and e_h is the acceptable increase in plate height (H) relative to the theoretical minimum HETP of the system. They calculated the allowable injection plug length as a function of the diffusion coefficient (or molecular mass) of the solute, and of the migration time from the system. **Figure 4.11** shows the allowed injection plug length as a function of the solute's diffusion coefficient at a constant analysis time of 10 minutes. Three different cases are shown, which correspond to 5, 10 and 20 % loss in

efficiency. Based on **Figure 4.11**, it can be seen that the restriction on the injection plug length could be rather stringent.

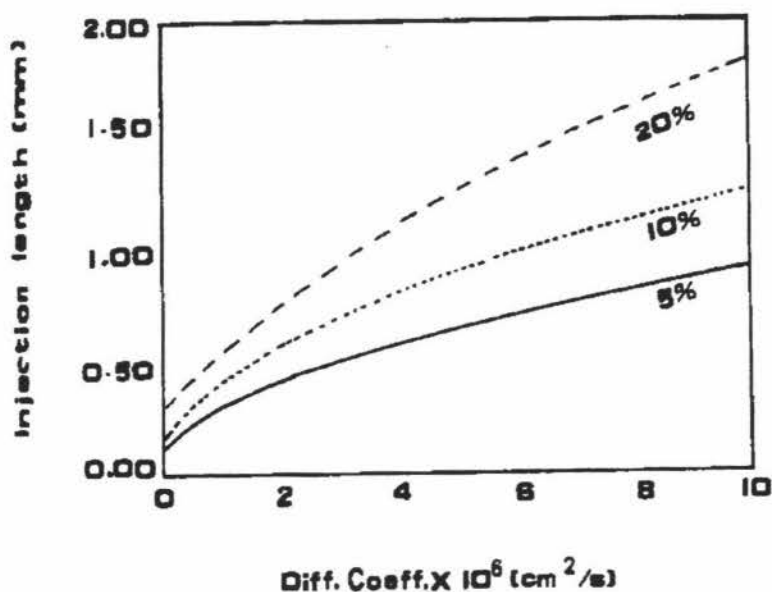


Figure 4.11 The allowed injection plug length as a function of the solute's diffusion coefficient. The analysis time was assumed 10 minutes. Each line corresponds to a different allowable loss in the efficiency (increasing H , Grushka and McCormick, 1989).

In my methods, one-second vacuum injection was enough to satisfy the efficient identification of creatinine in urine.

Selection of the voltage:

High voltages will give faster separations with higher efficiencies and resolution. As voltage is increased, there is more Joule heat generated in the capillary, which can be detrimental to the separation because it causes broader peaks, the possibility of sample decomposition, or formation of bubbles in the capillary, which may result in electrical discontinuity causing the instrument to shut down. In other words, the better the heat is dissipated, the higher the voltage that can be used.

Voltage control in capillary electrophoresis offers an important parameter affecting sample migration time. The relationship is described in the electrophoretic mobility (Eq. 4.10).

$$\mu = v / E \quad (4.10)$$

As field strength, E , is increased, migration velocity, v , increases, and therefore the migration time decreases for a solute with electrophoretic mobility, μ .

Voltage control is effective in controlling the sample migration times in CE analysis. Increasing voltages decrease sample migration times. The use of programmed voltage changes offers the advantage of speeding up CE analysis.

Different voltages were examined while other conditions were kept constant. When voltage was at 15 kV (**Method 3c**), the retention time of the 1/1 complex between creatinine and picrate was 2.2 minutes, and the current generated was 21 μA . When voltage was at 30 kV (**Method 3d**), the retentive was 0.6 minutes, and the current generated was 55 μA . Although the 30 kV voltage gave the shortest retention time for identification, the higher current was accompanied by the disadvantage of more Joule heat generated. Moreover, the sensitivity decreased by increasing the voltage. In order to avoid more Joule heat being generated, the current method used the voltage at 20 kV, which generated a reasonable current (27 μA) and a relatively shorter migration time ($R_t = 1.1$, for creatinine identification, **Figure 4.12**).

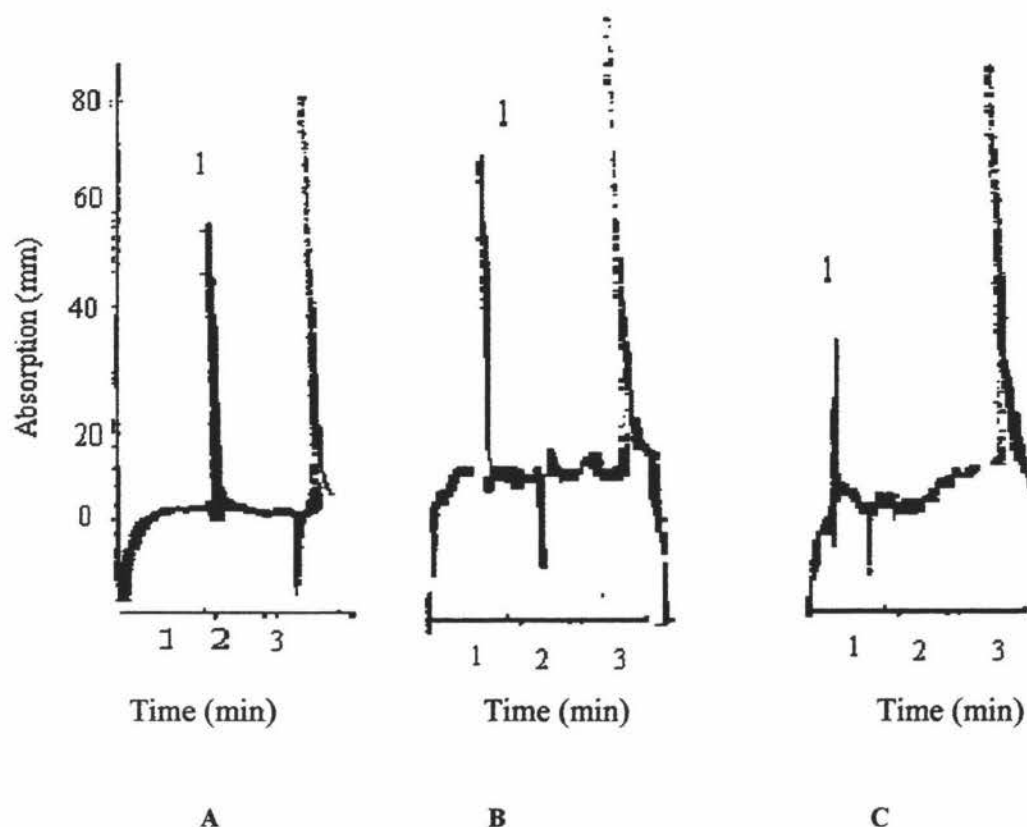


Figure 4.12 Comparison of the affect of migration time by difference voltages. A, 15 kV (Method 3c). B, 20 kV (Method 3b). C, 30 kV (Method 3d).

4.3.4 Interferences

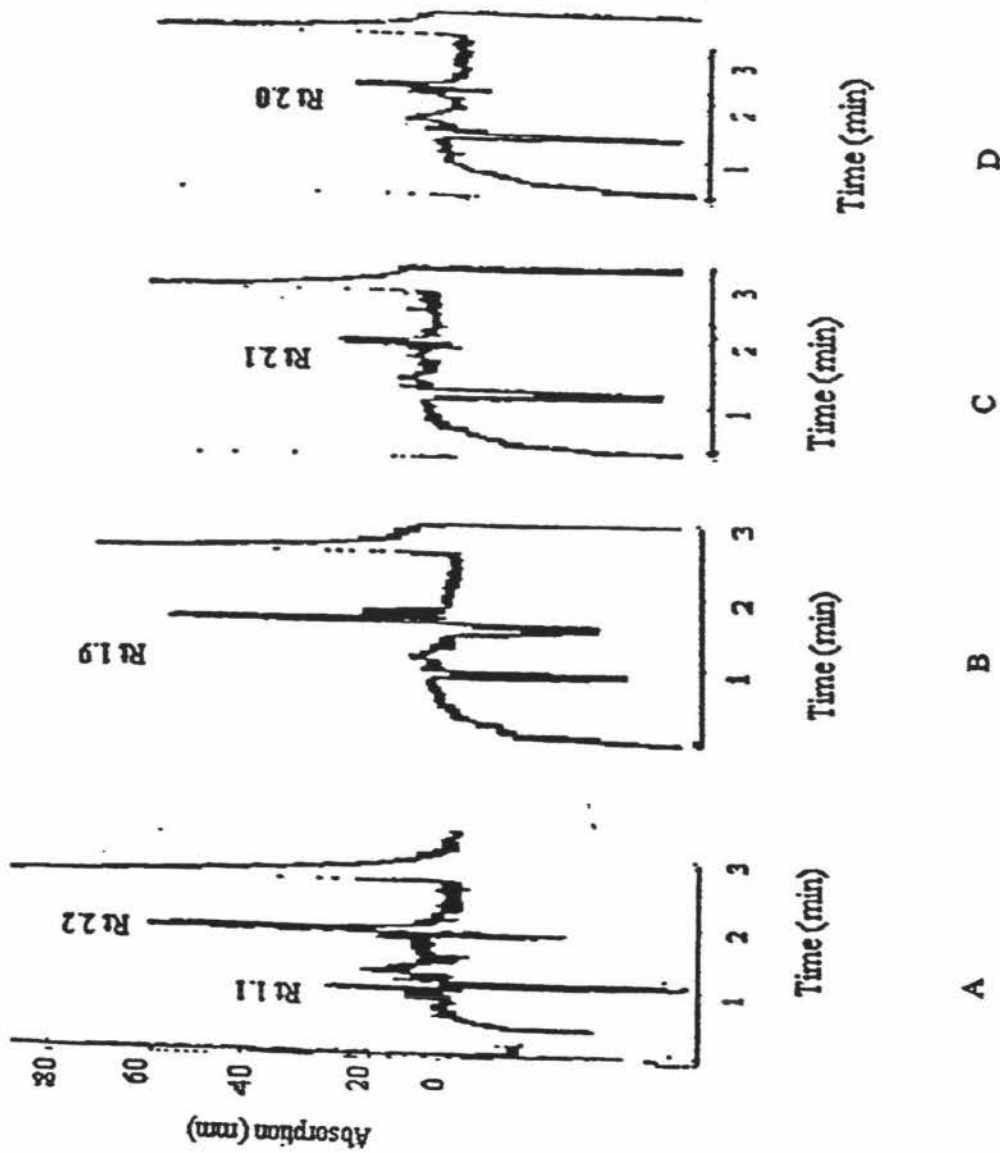
The Jaffé reaction has long been known to be non-specific. Among the non-creatinine Jaffé-reacting chromogens are protein, glucose, ascorbic acid, guanidine, acetone and α -ketoacids. Although negligible amounts of Jaffé-positive non-creatinine chromogens were found in urine (Narayanan and Appleton, 1980), it is necessary to examine these possible interferences in my method.

In principle, these interferences should be separated from the creatinine-picrate complex, due to the differences in **molecular weight, compounds size and charge** at a given pH.

The non-creatinine interferences that are listed in **Table 4.1** were examined under the conditions of **Method 3f** (**Figure 4.13**).

Table 4.2 Interferences

interferences	concentration	retention time (min)	peak height (mm)
albumin	25mg/dl	2.0	30
acetone	20%	1.1, 2.2	5, 29
L-ascorbic acid	25mg/dl	1.9	11
glucose	25mg/dl	2.1	27
guanidine	25mg/dl	1.2	34
α -ketoglutarate	25mg/dl	1.9	13



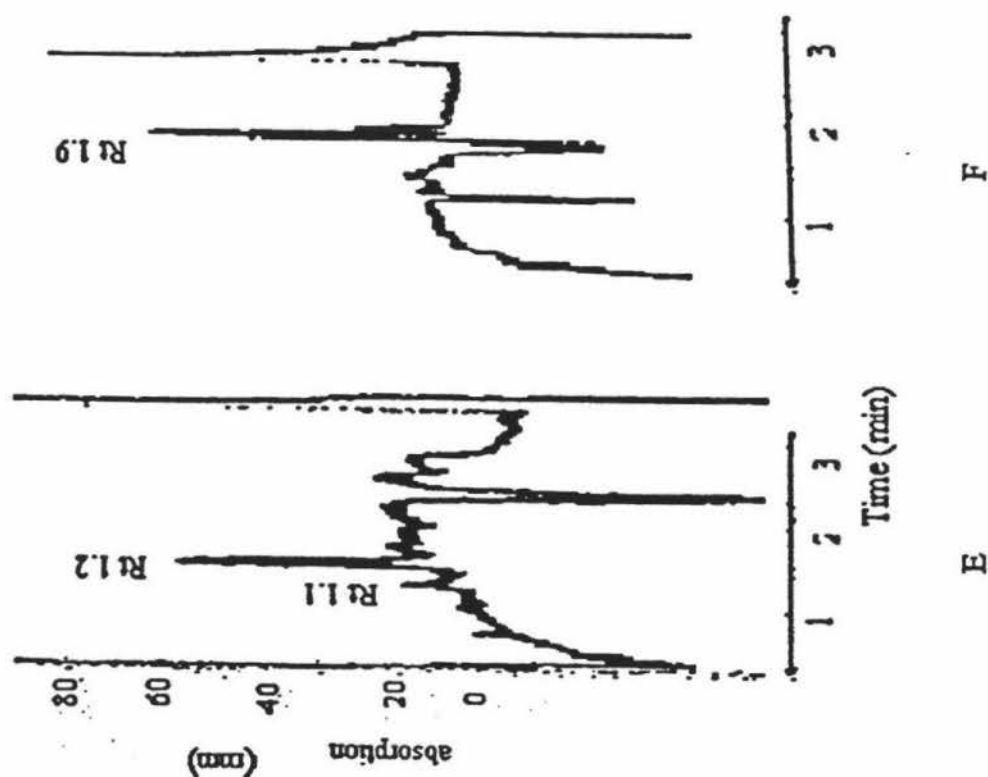


Figure 4.13 Non-creatinine interferences. A, acetone, B, ascorbic acid, C, glucose, D, albumin, E, guanidine, F, a-ketoglutarate. Condition: **Method 3f**.

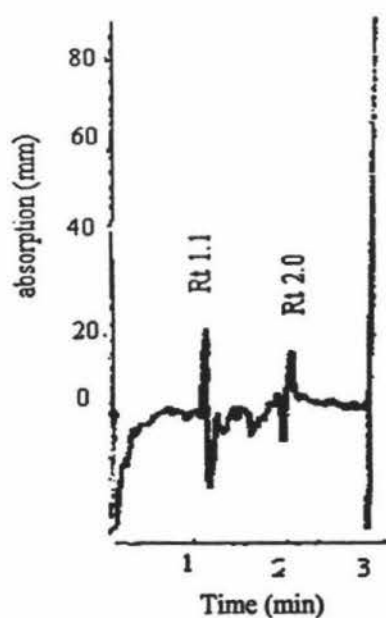


Figure 4.13a Interferences with addition of creatinine. Condition: **Method 3f**.

A spiking experiment was carried out with creatinine in the interferences showed only one more peak at retention time 1.1 minutes (**Figure 4.13a**).

The results showed that within 3 minutes running time, no interferences showed peak absorption at migration time 1.1 minutes, except acetone who has a minor peak appeared at migration time 1.1 minutes when its concentration was 20 %. **Glucose** has one peak at retention time 2.1 minutes. **Albumin** has a peak at retention time 2.0 minutes, which was suspected to be a contaminant peak. Because of the protein's size and its pK_a , albumin was negatively charged species at pH 10, it migrates much slower than the other smaller species. Therefore, there was no albumin absorption within 3 minutes running. **Ascorbic acid** has one peak appeared at retention time 1.9 minutes. **α -ketoglutarate** has one peak appearing at retention time 1.9 minutes. **Guanidine** has one peak appearing at retention time 1.2 minutes.

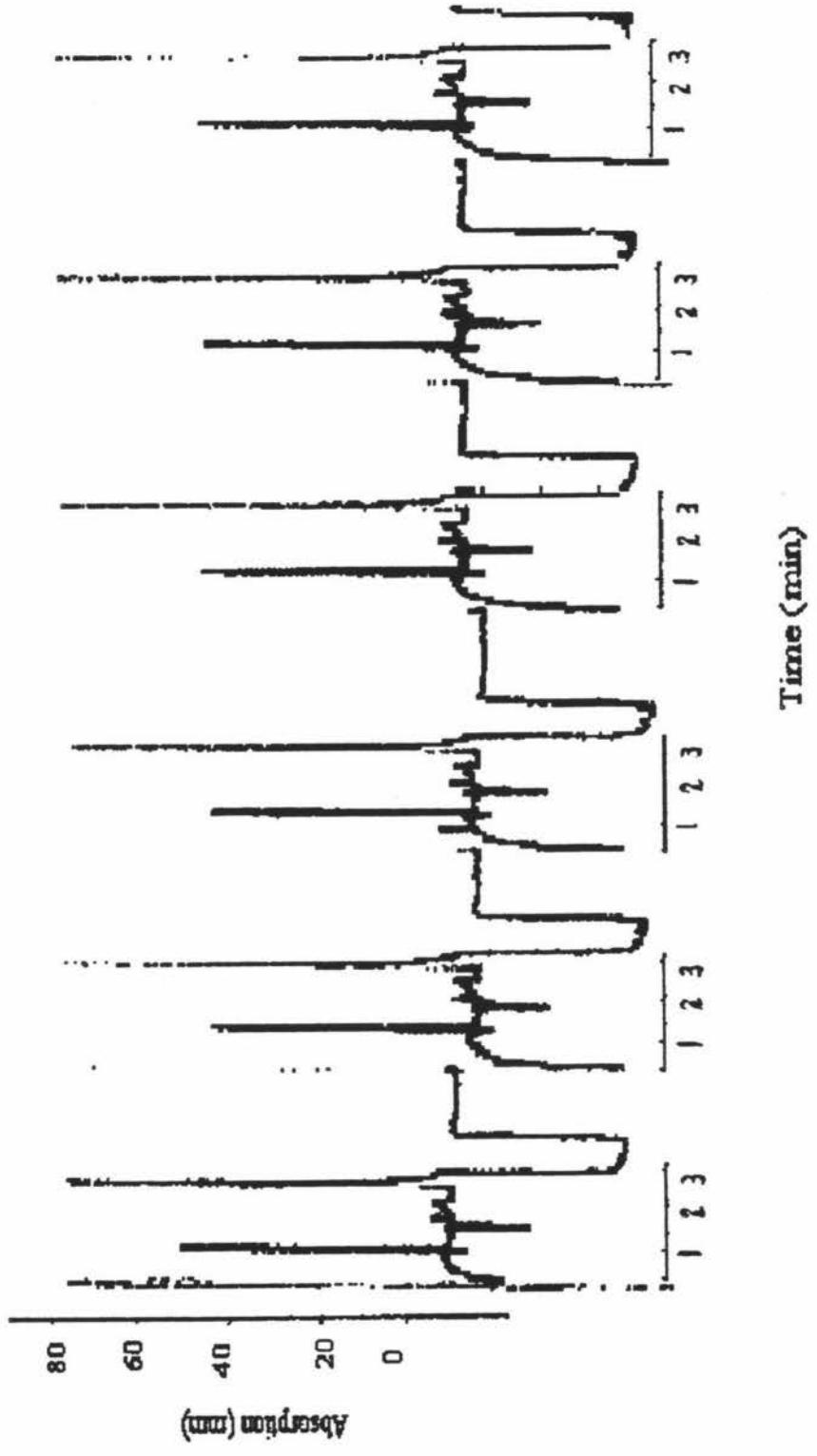
4.3.5 Reproducibility (precision)

A 5 mM creatinine sample was used to examine the reproducibility of the **Method 3f** (Figure 4.14).

Table 4.3 CV

creatinine	migration time	reproducibility	mean	deviation	CV
5 mM	1.1	31.46	31.00	0.46	1.47%
5 mM	1.1	31.26	31.00	0.26	0.83%
5 mM	1.1	31.36	31.00	0.36	1.15%
5 mM	1.0	31.06	31.00	0.06	0.18%
5 mM	1.0	30.66	31.00	-0.34	-1.11%
5 mM	1.1	31.06	31.00	0.06	0.18%
5 mM	0.9	30.66	31.00	-0.34	-1.11%
5 mM	1.0	30.56	31.00	-0.44	-1.43%
5 mM	1.1	30.66	31.00	-0.34	-1.11%
5 mM	1.1	31.16	31.00	0.16	0.51%
5 mM	1.1	30.86	31.00	-0.14	-0.46%
5 mM	1.1	31.06	31.00	0.06	0.18%
5 mM	1.0	31.26	31.00	0.26	0.83%
5 mM	1.1	30.96	31.00	-0.04	-0.14%

$$\text{Coefficient of variation CV} = [\text{Standard deviation/mean}] \times 100$$



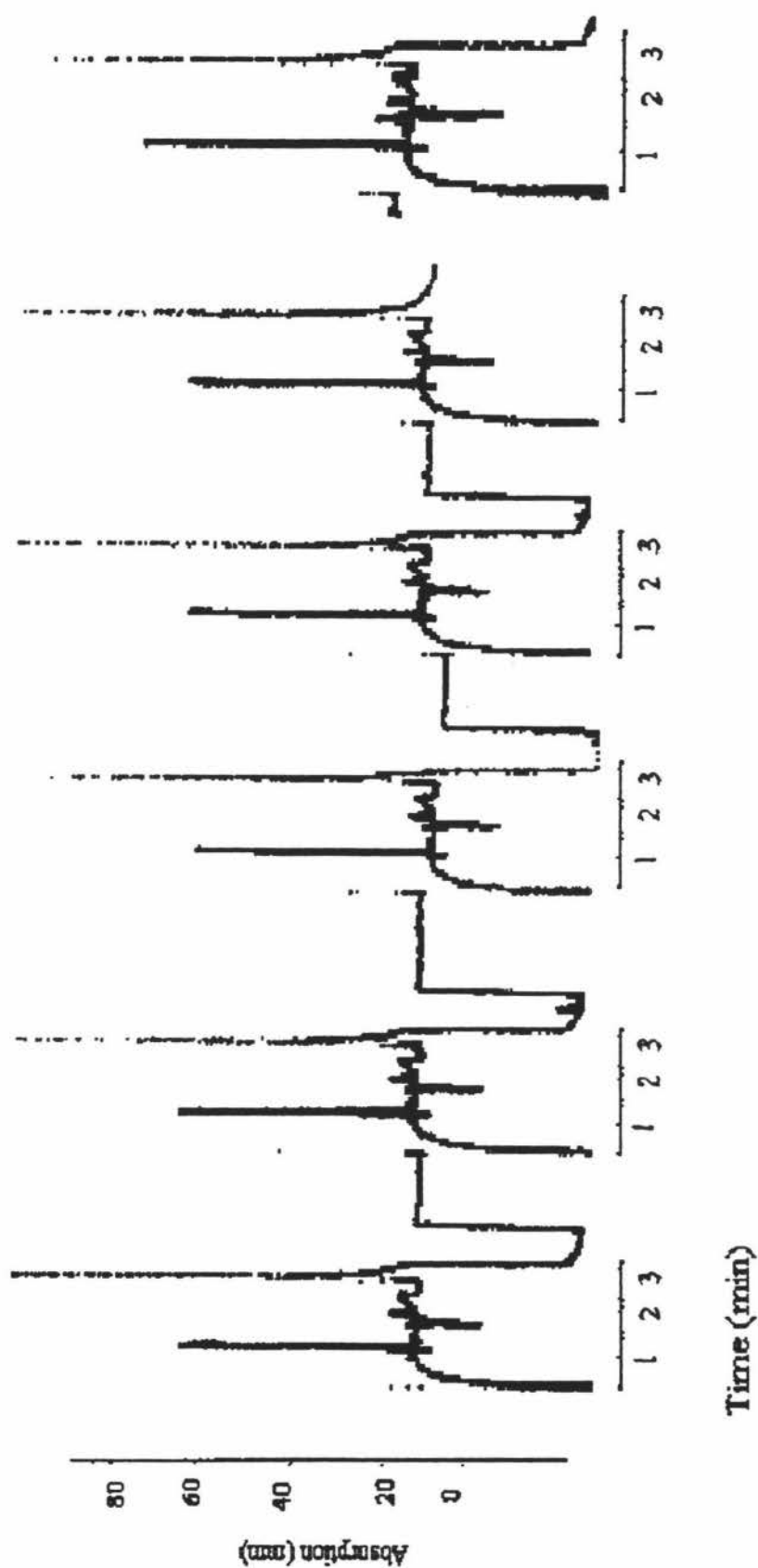
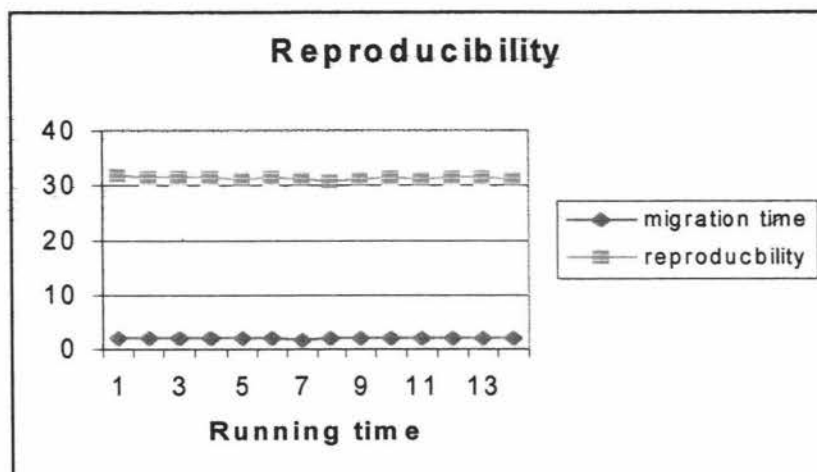


Figure 4.14 Within-run precision (reproducibility). Condition: **Method 3f**.



Scheme 4.1 5 mM creatinine reproducibility. Condition: **Method 3f**.

The differences in migration time were caused by changes in the temperature. This was due to the variable temperature in our laboratory that made the temperature of CE inconstant (the temperature of CE is only constant when it is at least 5°C higher than the room temperature).

Constant temperature is most important to obtain reproducible retention times for the analyte. The viscosity of aqueous solutions decreases by about 2.5% for each degree of temperature rise because electrophoretic mobility is inversely proportional to viscosity. Variations in temperature lead to variations in separation time for analyte (Camilleri, 1993).

4.3.6 Calibration of the CZE apparatus for assaying creatinine

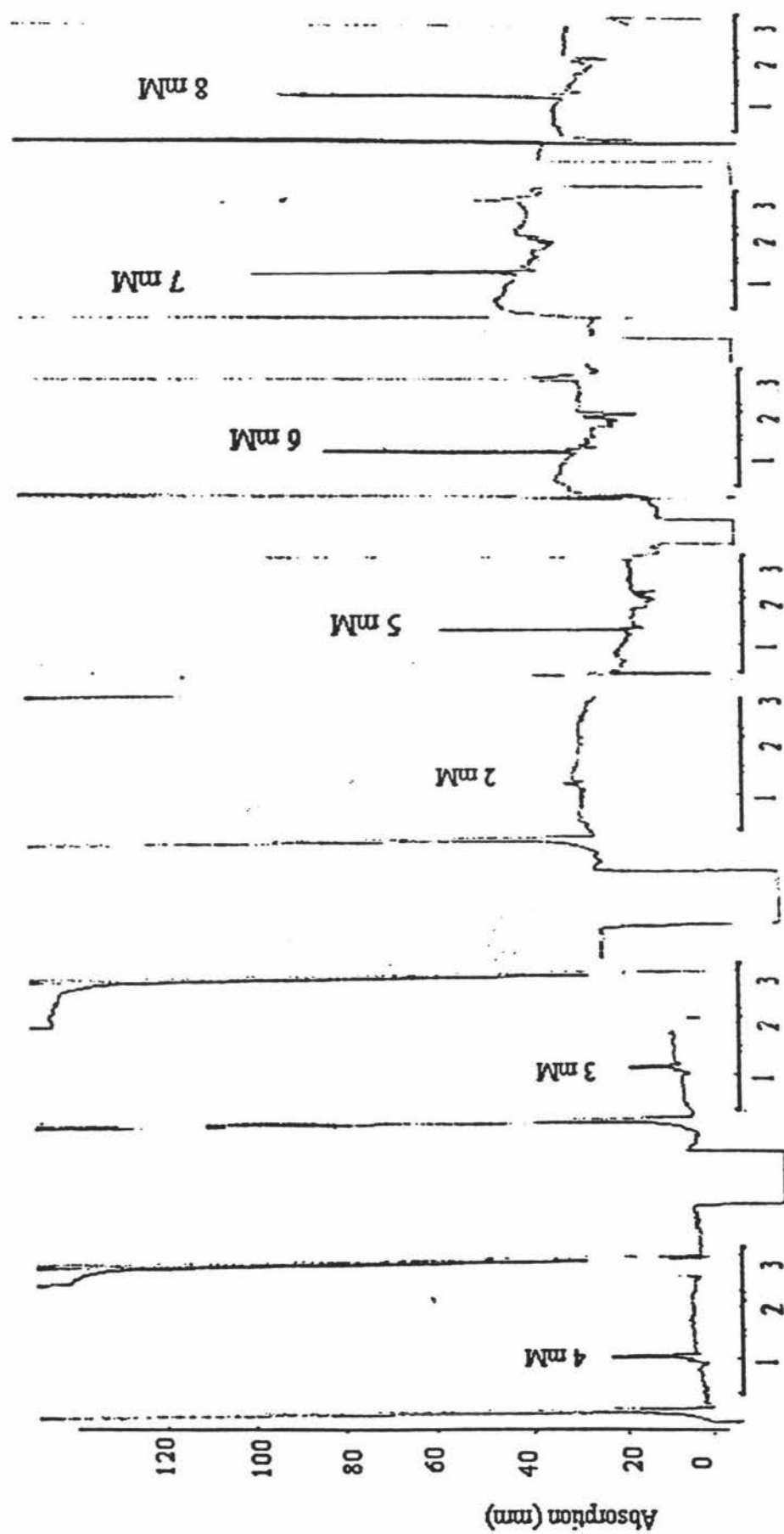
Conditions:

Capillary:	25 cm length × 75 μm I.D.
Buffer:	30 mM phosphate + 1 mM PA, pH = 10
Injection:	Vacuum
	1 second
Voltage:	20 kV
Temperature:	30 °C

Temperate: 30 °C
Wavelength: 480 nm
Detector:
Rise time: 2.00
Range: 0.02
Current: 22 μ A

Table 4.4 Standard creatinine

creatinine (mM)	peak height(mm)
2	5
3	14
4	24
5	31
6	43
7	52
8	58
9	68
10	78
12	95
16	122
18	138



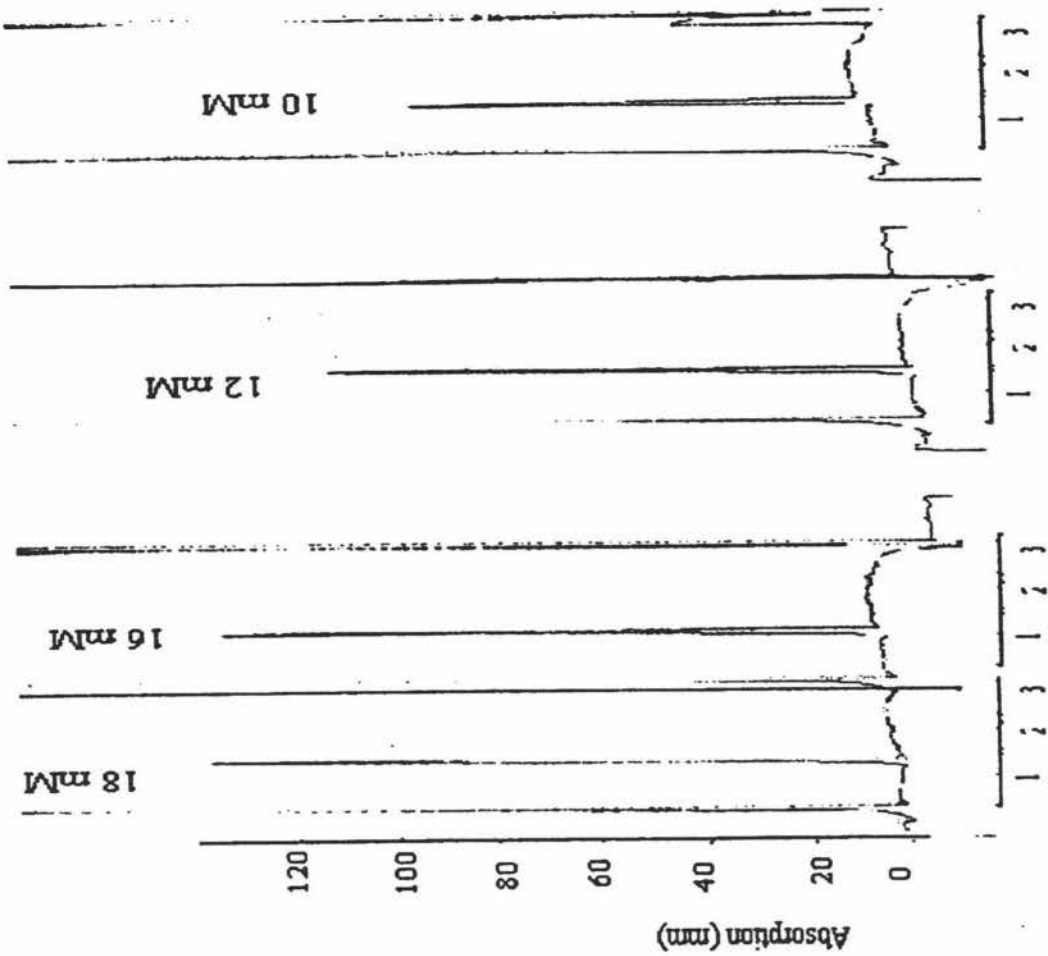
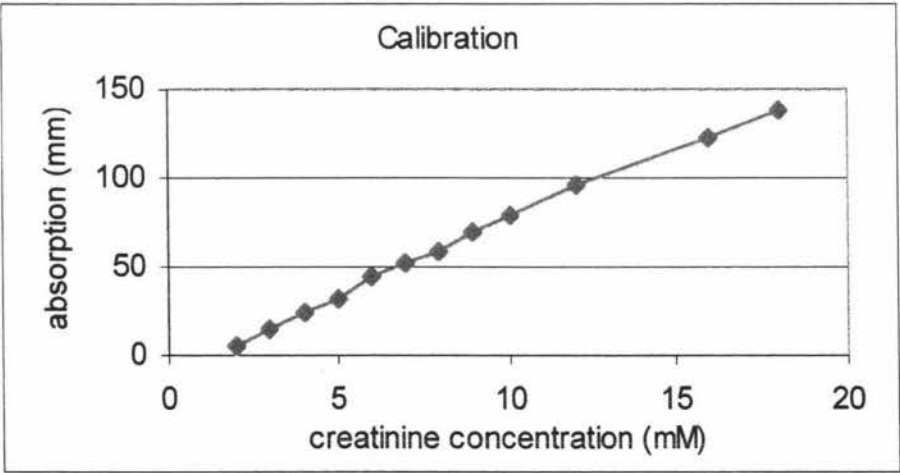


Figure 4.15 Differences concentration of creatinine (2 mM–18 mM).



Scheme 4.2 Calibration of creatinine

4.3.7 Recovery

Recovery is related to specificity and is determined by adding known amounts of an analyte to a real specimen and determining what percentage of the added analyte is detected (recovered). This should be close to 100 % (King, 1998).

A fresh urine sample was injected directly into the CE after filtration, with a comparison of spiked 10 mM creatinine in the urine (**Figure 4.15**). Since the urine sample was not a 24-hour collection, the concentration of the creatinine in urine did not match exactly the 10 mM value which is the normal concentration of creatinine in a 24 hour pooled urine sample.

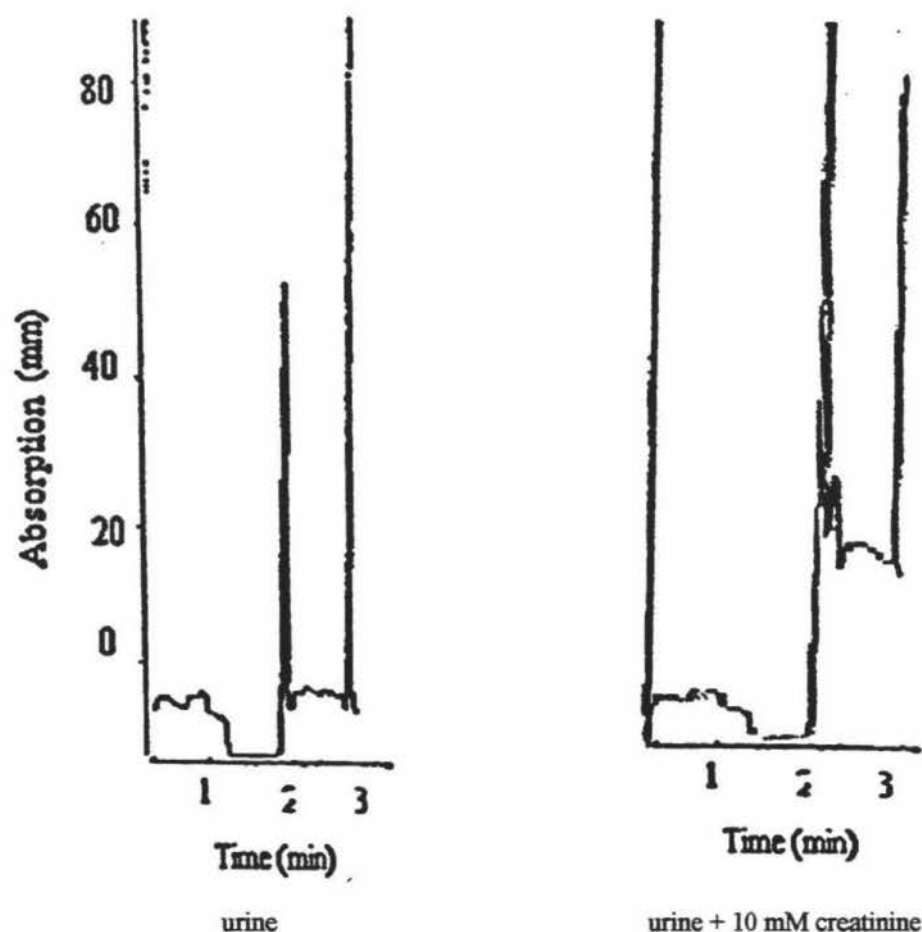


Figure 4.16 Recovery of creatinine in Method 3f.

Table 4.5 Recovery

Sample	peak height(cm)
Urine	46.2
Urine + 10 mM creatinine	123.5
10 mM creatinine(Average)	78

$$recovery = \frac{123.8 - 46.2}{78} 100\% = 99.10\%$$

4.3.8 Sensitivity (detection limit)

Preparing a series of dilutions of a standard which when measured will determine the smallest amount of analyte that can be distinguished from the blank will determine the analytical sensitivity i.e. the **detection limit**

The **detection limit** of this method was at the creatinine concentration of 2 mM (see **Figure 4.16**).

4.4 Conclusion

Using the CZE method developed in this work, creatinine in urine can be determined simultaneously in the presence of other analytes in a simple and rapid manner. Sample preparation involved only filtration of urine, no other treatment. Selecting CE conditions that allowed creatinine to migrate away from these other interferences solved the problem of non-creatinine chromogens. This quantitative study for creatinine indicates that this method is well suited to the clinical range. The good reproducibility and recovery allows the analysis of urine samples to be performed automatically on a large scale.

CHAPTER FIVE

DETERMINATION OF CREATININE IN SERUM

5.1 Introduction

Measurement of the creatinine levels in serum and determination of the renal clearance of creatinine are widely used for laboratory diagnosis of renal diseases. The assay of creatinine in human serum samples is a challenging analytical problem, due to possible interferences and the fact that creatine is also present in the serum at approximately the same concentration levels as creatinine. The creatinine reference range in serum for adults is between 0.6 and 1.2 mg/dL (0.53 – 1.06 mM); men have slightly higher values than women (Whelton *et al*, 1994). In clinical practice, there are two significant decision levels. Values above 1.4 mM indicate the necessity of performing other tests for assessment of renal function such as the creatinine clearance test. Values above 5.3 mM are almost invariably associated with severe renal impairment.

Most creatinine determinations in the clinical laboratory and almost all the commercially available analysers use spectrophotometric procedures (either end point or kinetic) based on the Jaffé reaction. The method monitors the absorbance of the addition complex resulting from the reaction between creatinine and picrate in alkaline solution. However, this reaction is not specific for creatinine because many substances could interfere in this assay. The long list of these so-called “non-creatinine chromogens” includes acetoacetate, pyruvate, glucose, cephalosporines, and bilirubin and other (Mădăras and Buck, 1996).

In **Chapter 4**, a method was designed using CE to determine creatinine levels in urine. This CE method showed advantages over the other methods (efficiency, specification). But it has the higher *determination limit* that could not be used to determine creatinine in serum. That has been solved at this chapter.

5.2 Methods

Condition:

Capillary	75 μ m ID \times 25 cm
Buffer pH	10
Injection type	vacuum
Injection time (second)	2
Detector Rise time (second)	2.00
Detector range	0.01

Table 5.1

method	buffer	sample	wavelength (nm)	running time(min)	voltage (kV)	temperature (°C)
4b	30 mM phosphate + 1 mM PA	5 mM creatinine	480	5	20	30
4c	30 mM phosphate + 5 mM PA	5 mM creatinine	480	5	20	30
4d	30 mM phosphate + 8 mM PA	5 mM creatinine	480	5	20	30
5a ₁	30 mM borax + 1 mM PA	1.5 mM creatinine	480	5	20	30
5a ₂	30 mM borax + 1 mM PA	>1.5 mM creatinine	480	5	20	30
5a ₃	30 mM borax + 1 mM PA	0.1 mM creatinine	480	5	20	30
6a	30 mM borax + 1mM PA	0.1 mM creatinine	417	5	20	30
6b ₁	30 mM borax + 1mM PA	0.5 mM creatinine	417	5	20	30
6b ₂	30 mM borax + 5 mM PA	0.5 mM creatinine	417	5	20	30
6c	30 mM borax + 5mM PA	0.1 mM creatinine	417	7	15	30
6d	30 mM borax + 5mM PA	0.1 mM creatinine	417	7	10	30
6e	30 mM borax + 5mM PA	0.1 mM creatinine	417	7	10	27
6f	30 mM borax + 5mM PA	0.1 mM creatinine	417	7	10	24.4

Bold entries: indicated changes down the table.

5.3 Results and Discussion

5.3.1 Selection of the analysis method

5.3.1.1 Creatinine in serum CZE study one

The normal concentration of creatinine in serum or plasma is $\sim 0.1\text{mM}$, and **Method 2c** has the detection limit at 2 mM . This of course is not sensitive enough to detect the creatinine in serum. Therefore, further experiments were carried out to increase the sensitivity in an attempt to be able to measure creatinine in serum.

Concentration of the Picric acid in buffer:

Kinetic studies indicated that the reaction of alkaline sodium picrate and creatinine is first order in creatinine and first order in picric acid concentration (**Figure 2.10**, Chapter Two and **Table 5.2**).

Table 5.2 Effect of Picric Acid Concentration on the Observed Forward Rate Constants

$[P]_T, \text{M}$	$k_{\text{obs}}, \text{s}^{-1} \times 10^3$
Creatinine concentration, $0.164\text{ }\mu\text{M}$	
9.85×10^{-4}	0.289 ± 0.01
1.64×10^{-4}	0.410 ± 0.008
3.28×10^{-3}	0.631 ± 0.006
4.92×10^{-4}	0.706 ± 0.007
8.20×10^{-4}	1.260 ± 0.130
Creatinine concentration, $82\text{ }\mu\text{M}$	
1.64×10^{-3}	0.346 ± 0.010
2.46×10^{-3}	0.402 ± 0.012
3.28×10^{-3}	0.493 ± 0.050
4.1×10^{-3}	0.636 ± 0.120

*Conditions: $25 \pm 0.1^\circ\text{C}$, 0.1 M KNO_3 (ionic strength adjustment), 33 mM NaOH , and 480 nm . $[]_T$ represents "total analytical concentration of substance in solution", (Vasiliades, 1976).

A, Method 4b:

Sample: 5 mM creatinine

Buffer: 30 mM phosphate + 1 mM PA, pH =10

B, Method 4c

Sample: 5 mM creatinine

Buffer: 30 mM phosphate + 5 mM PA, pH =10

C, Method 4d:

Sample: 5 mM creatinine

Buffer: 30 mM phosphate + 8 mM PA, pH =10

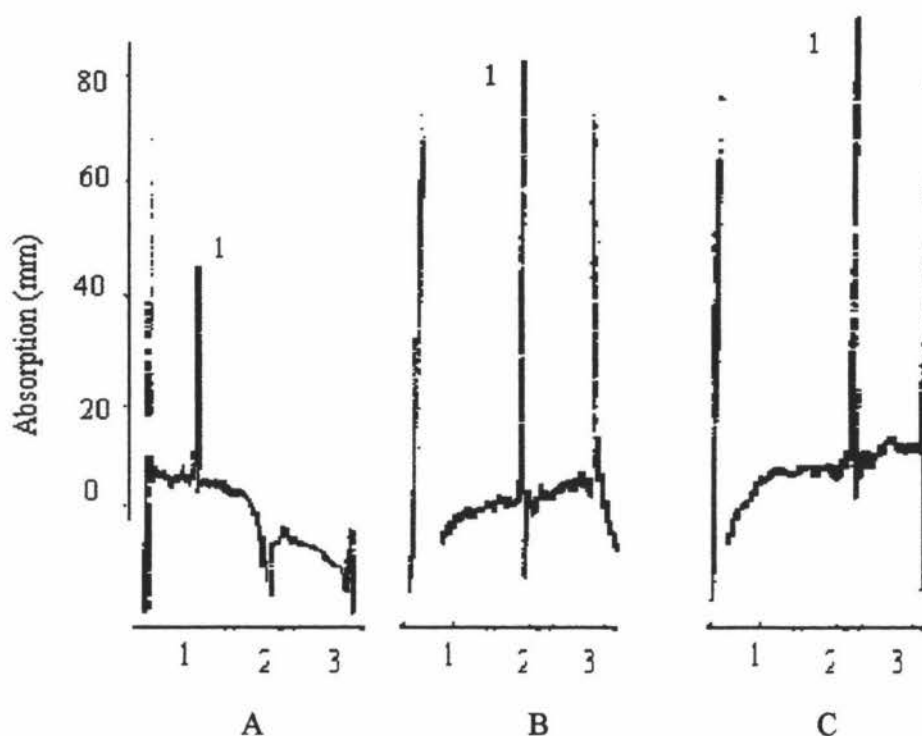


Figure 5.1 Effect of the concentration of picric acid in buffer. A, 1 mM (**Method 4b**). B, 5 mM (**Method 4b**). C, 8 mM (**Method 4b**).

Results: Different concentrations of picric acid in buffer have been examined in **Method 4b, 4c, and 4d** on CE (**Figure 5.1**). Normally, it takes about 15 - 20 minutes to reach the maximum development of the creatinine-picric acid complex in the Jaffé reaction (Narayanan, 1980). CE has the advantage of speed that can identify or separate compounds within few minutes or even less than one minute. Therefore, a high Jaffé

reaction rate is preferred on the CE to identify the creatinine-picric acid complex in the minimum migration time possible. Principally, higher concentration of the picric acid helps produce higher reaction rate to the Jaffé reaction, and the 8 mM PA seems to be the best choice. However, when we look back on the CE, the ionic strength of the buffer needs to be taken into account as well. As discussed above, if the ionic strength of the buffer was at a higher condition, the current being generated during the analysis is going to be higher as well (43 μ A, 8 mM PA). Consequently, the steady constant temperature may be disturbed. Bearing this in mind, 5 mM became the best option for the picric acid concentration in running buffer (27 μ A).

5.3.1.2 Creatinine in serum CZE study two

Buffer composition:

Anions present in the electrolyte system may affect the current and hence the electroosmotic flow, the heat generated, the interaction of analytes with the wall of the capillary, as well as the mobilities of the ions. The relative importance of each of these effects would depend on the system under investigation (Li, 1992). Therefore, change of buffer composition could give dramatic influences on the determination.

A, Method 5a₁

Sample: 1.5 mM creatinine
 Buffer: 30 mM borax + 1 mM PA, pH = 10
 Results: Two positive peaks appeared right after a negative peak at retention time 3.9 minutes and 4.0 minutes.

B, Method 5a₂

Sample: >1,5 mM creatinine
 Buffer: 30 mM borax + 1 mM PA, pH = 10
 Results: The first positive peak (peak 1), which appeared at 3.9 minutes coming up that was much higher than the one in **Method 5a₁**

C, Method 5a₃

Sample: 0.1 mM creatinine
 Buffer: 30 mM borax + 1 mM PA, pH = 10

Results: One small positive peak (peak 1) appeared at retention time 3.9 minutes.

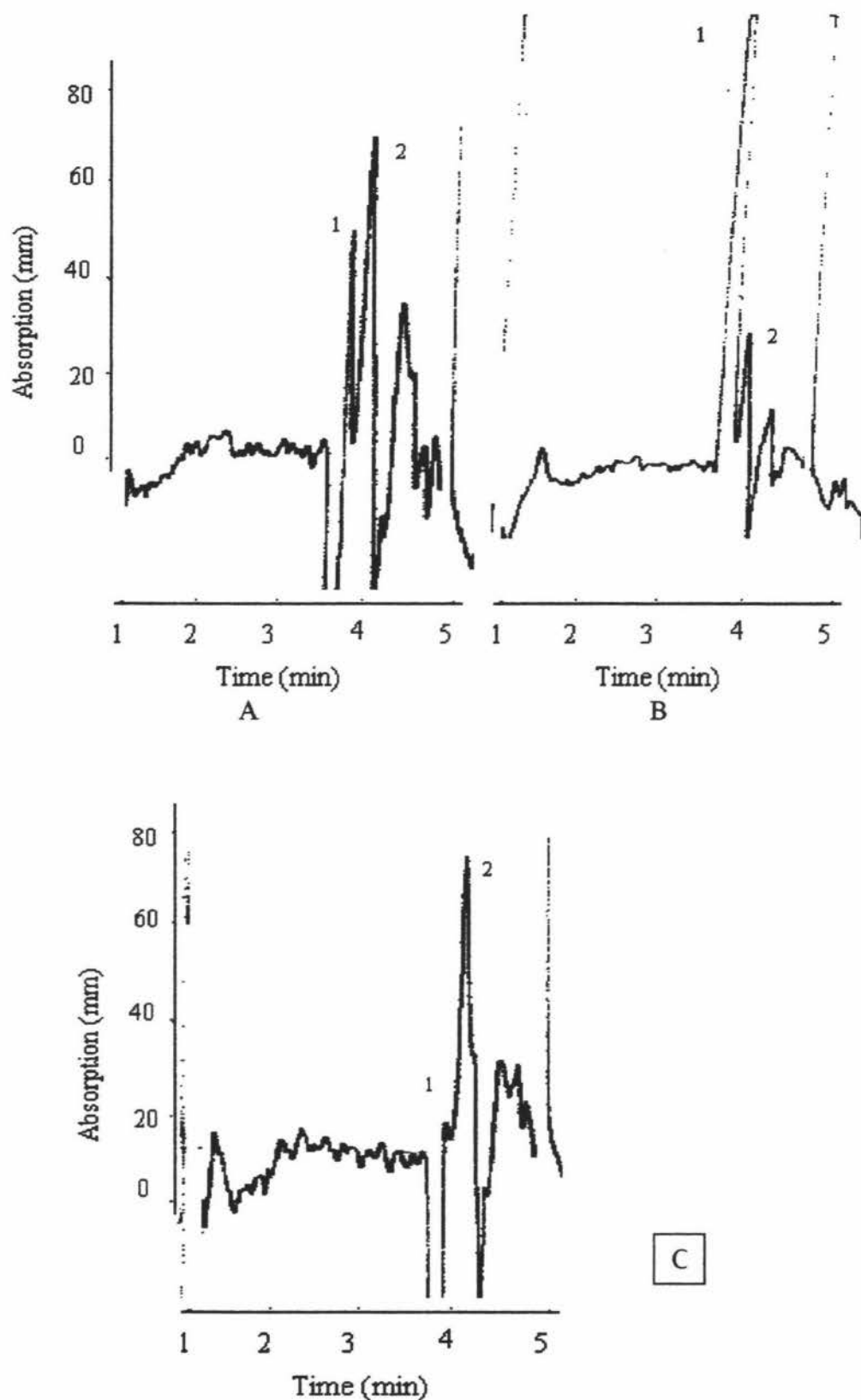


Figure 5.2 A, 1.5 mM creatinine at the condition of **Method 5a₁**. B, >1.5 mM creatinine at the condition of **Method 5a₂**. C, 0.1 mM creatinine at the condition of **Method 5a₃**.

Results: The only difference was the peak height that appeared at retention time 3.9 min increased with the increasing of the creatinine concentration (**Figure 5.2**). That gave the identification of this peak to be the creatinine-picrate 1/1 complex.

From the results of above, we can see that the borax buffer offered better determination of creatinine at 480 nm rather than the phosphate buffer for determining creatinine at lower creatinine concentrations. Nevertheless, the sensitivity was not high enough to determine the creatinine concentration at or lower than 0.1 mM.

5.3.1.3 Creatinine in serum CZE study three

Wavelength change:

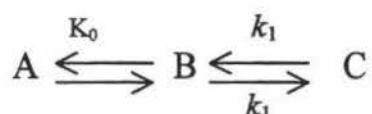
The Applied Biosystems CE instrument employs an ultraviolet-visible (UV-VIS) absorbance detector. Sensitivity of UV detection depends on the path length of the detection cell; hence, detection limits in CE tend to be lower than those of other specific-property detectors. The principle of UV/vis absorbance detection is based on Beer's law where absorbance, A , is given by

$$A = \log 1/T = abc \quad (5.1)$$

where T is the transmittance, a is the absorptivity (this used to be referred to as the extinction coefficient), b is the path length of light in the sample compartment in cm (the capillary I.D.), and C is the solute concentration. From Beer's law, (Eq. 5.1), absorbance is proportional to path length, which is equal to the inner diameter of the capillary. Sensitivity can be thus enhanced with larger inner diameter capillaries. This is not a good approach though, because large-diameter capillaries produce high currents that cause Joule heating increased. Because of problems associated with large inner diameter capillaries, most approaches to enhancing sensitivity have been directed toward increasing the path length without increasing the inner diameter of the entire capillary. The path length can be increased by bending the capillary into a "Z" shape with the light parallel to the capillary and through a section of the "Z" instead of perpendicular to it (Moring *et al*, 1993), or use of *rectangular capillaries* (Tsuda *et al*, 1990).

Since the “z” shape and *rectangular capillaries* are not commonly used capillaries, they were not used in my experiments. Bear in mind, the UV detector has less sensitivity at longer wavelength than at shorter wavelength, and the final product of creatinine-picrate 1/1 complex has absorption not only at 480 nm but also at 417 nm.

A spectrophotometric study indicated that alkaline sodium picrate and creatinine react to form a product that absorbs at a maximum of 480 nm. The final product is a 1/1 complex of picrate and creatinine with an effective stability constant of $\log K = 4.26$. A differential scan of the reaction of picric acid and creatinine with time in the presence of 37 mM sodium hydroxide is shown in **Figure 2.9** (see Chapter Two). The first lower dashed line at zero absorbance is at time zeroing (no creatinine added). On addition of creatinine, there is an increase in absorbance at 280 nm and 480 nm and a decrease in absorbance at 350 nm. Two isobestic points are observed, 310 nm and 378 nm, indicating that at least three substances must be in equilibrium during the course of the reaction. The final spectrum (dotted upper line) shows that the substance absorbing at 280 nm is completely converted to the substance absorbing negatively at 350 nm, which is still in equilibrium with the substance absorbing at 480 nm, and that the reaction can be represented by the following equilibria:



with the conversion of B to C, the final product. Increasing the sodium hydroxide concentration to 0.62 mM results in the separation of the absorption bands at 400-500 nm into two distinct absorption bands. Again, three species are in equilibrium during the time of the reaction, with isobestic points observed at 305 and 370 nm. In this case there is a rapid equilibrium between A and B, first scan, with B finally being in equilibrium with C. The final product has absorption maxima at **390 nm** and **480 nm**. Thus, the sodium hydroxide concentration influences the final product that is formed (Vasiliades J, 1976).

UV spectrophotometer scanning of the absorption of creatinine has been examined under the conditions of **Method 5**. The results show that within 5 minutes after the

combination of creatinine and picrate there were four peaks appearing at wavelengths 278 nm, 357 nm, 417 nm, and 492 nm (Figure 5.3).

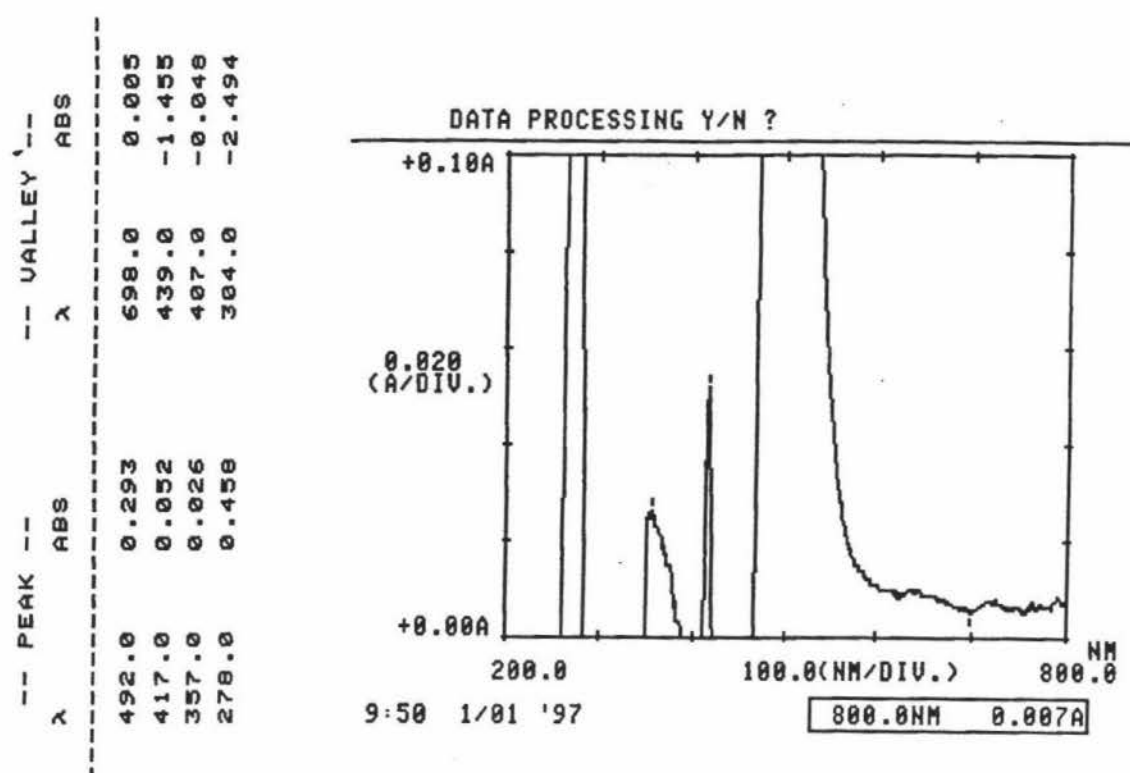


Figure 5.3 UV spectrophotometer scans at wavelength range between 200-800 nm within 5 minutes reaction.

Apparently, in my UV spectrophotometric experiments, the two absorptions at wavelength 417 nm, and 492 nm correspond to the final product that has absorption maxima at 390 nm and 480 nm in the literature (Vasiliades J, 1976). Therefore, **Method 6** was designed to detect the creatinine at shorter wavelength 417 nm (**Method 6a**, Figure 5.4).

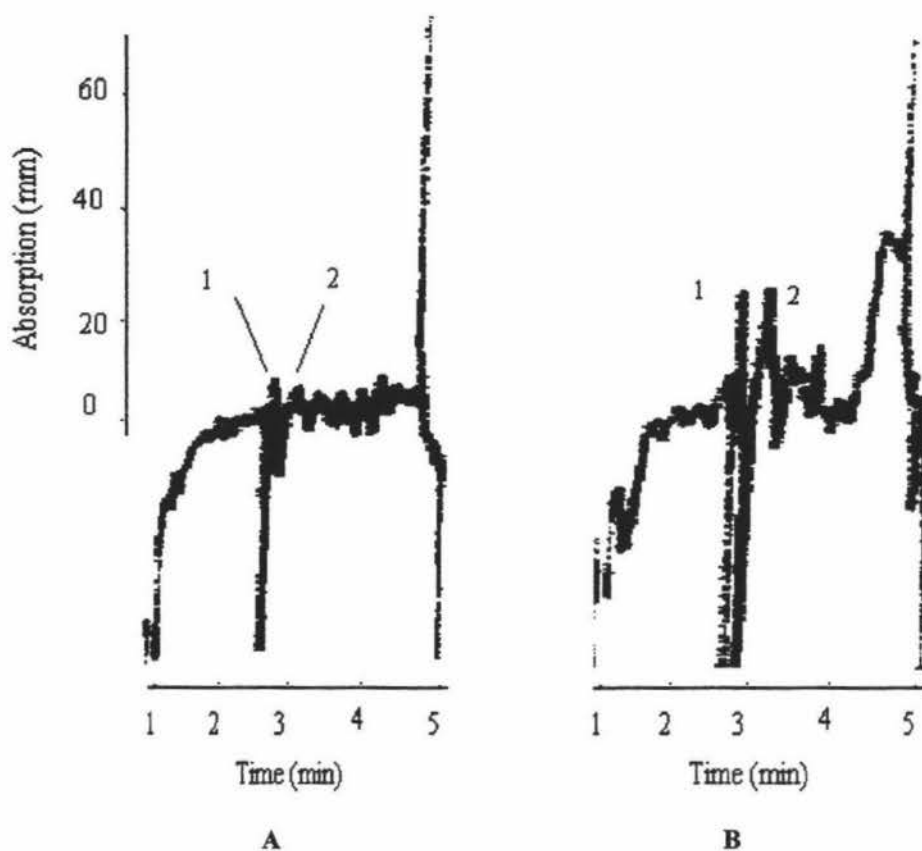


Figure 5.4 Comparison of absorptivity at different wavelength. A, 480 nm (**Method 5a₃**) B, 417 nm (**Method 6a**).

The results of changing the wavelength for the detection of the complex showed that the first positive peak had more absorption at wavelength 417 nm than that at 480 nm.

PA concentration in borax buffer:

Again, increasing the PA concentration from 1 mM to 5 mM gave more absorption that enhanced the peak height at lower creatinine concentration (**Method 6b, Figure 5.5**).

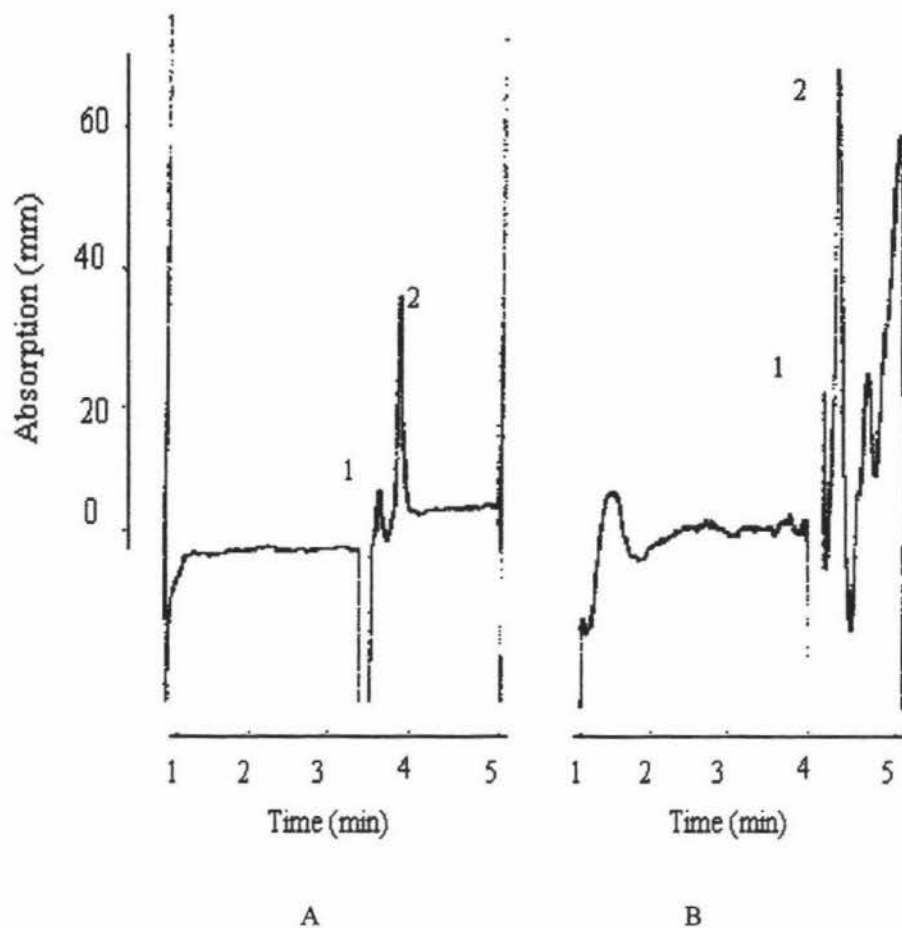


Figure 5.5 Increased concentration of PA in buffer. A, 1 mM PA in buffer (**Method 6b₁**). B, 5 mM PA in buffer (**Method 6b₂**).

5.3.1.4 Improving the resolution

Although changing the wavelength increased the absorption dramatically, another problem arose. This was the poor resolution between the two-closely-eluting species. The first peak has been confirmed as the 1/1 complex, while the second one remained unknown.

The resolution, R , can be calculated from an electropherogram using

$$R = \Delta t / W_{AVE} \quad (5.2)$$

$$R = 2(t_2 - t_1)/(w_1 + w_2) \quad (5.3)$$

where w_1 and w_2 are peak widths of adjacent peaks. Resolution between two adjacent peaks can also be expressed as

$$R = \frac{1}{4}(N)^{1/2}(\Delta v / v_{AVE}) \quad (5.4)$$

and

$$N = \frac{(\mu_{EP} + \mu_{EOF})V}{2D} \quad (5.5)$$

where N is the number of theoretical plates, V is the voltage applied across the capillary, D is the diffusion coefficient of the solute, Δv is the velocity difference between two solutes and v_{AVE} is their average velocity. The term $\Delta v / v_{AVE}$ is the relative velocity difference and is given by

$$\Delta v / v_{AVE} = \frac{\mu_2 - \mu_1}{\mu_{AVE} - \mu_{EOF}} \quad (5.6)$$

where μ_{AVE} is the average electrophoretic mobility of the solutes. Substituting Eq. 5.5 and 5.6 into Eq. 5.4 gives

$$R = \frac{1}{4} \left[\frac{(\mu_{EP} + \mu_{EOF})V}{2D} \right]^{1/2} \left[\frac{(\mu_2 - \mu_1)}{(\mu_{AVE} - \mu_{EOF})} \right] \quad (5.7)$$

The resolution of two zones in electrophoresis is given by the equation 5.8 (Baker, 1995):

$$R = \frac{1}{4(2)^{1/2}} (\mu_2 - \mu_1) \left[\frac{V}{D(\mu_{AVE} + \mu_{EOF})} \right]^{1/2} \quad (5.8)$$

This equation states that the resolution between two adjacent eluting species is directly proportional to the difference in their mobilities and inversely proportional to the square root of their average total mobility. It points out the limitations imposed on the electrophoretic separation. As the speed of migration increases ($\mu_{ep} + \mu_{eo}$ large), resolution decreases, simply because there is not enough time for the components to physically separate from one another. Thus, there are physical limitations to how fast we can perform the separation and maintain the better resolution in the separation (Camilleri, 1993).

Voltage control

Although in general, peak efficiency and therefore resolution increases as a function of voltage up to a point under a given set of run conditions; then efficiency may diminish beyond that point when the speed of migration is too fast to permit enough time for analytes to separate from one another. Using **Method 6b₂**, **6c**, **6d**, , difference voltages were examined to discover the balance between the voltage and the speed of migration to achieve the best resolution in a reasonable time period. The result showed that the better resolution achieved at voltage 10 kV (**Figure 5.6**).

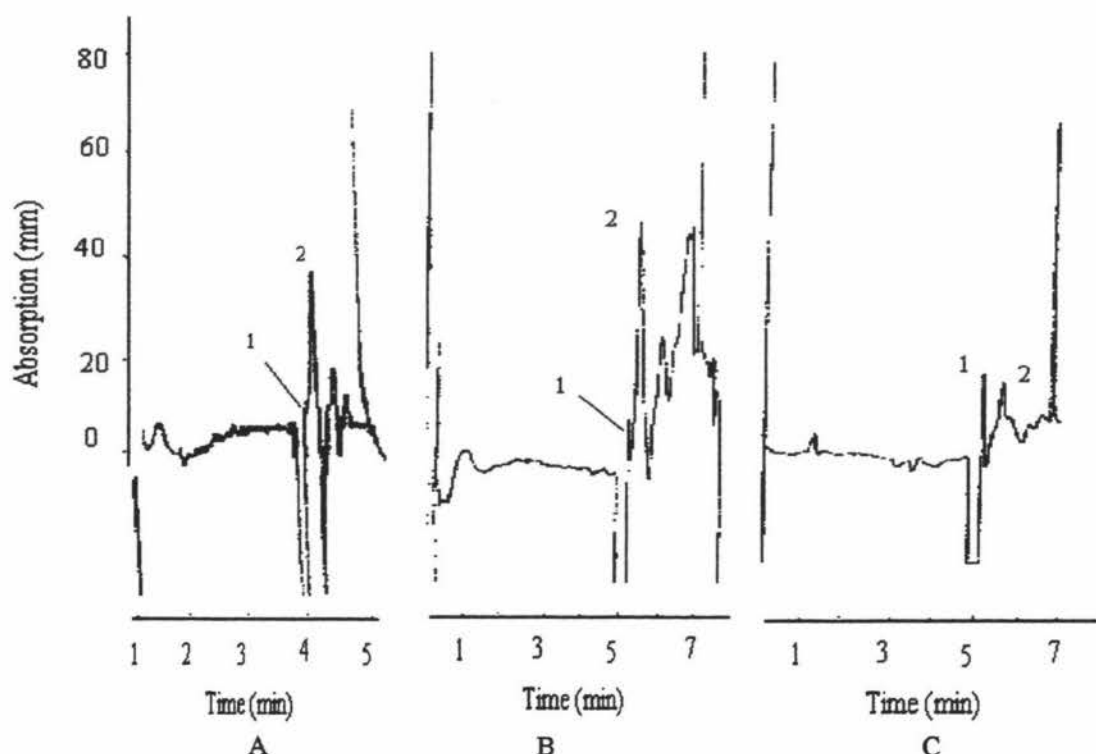


Figure 5.6 Difference voltages gave difference resolution. A, 20 kV at the condition of **Method 6b₂**. B, 15 kV at the condition of **Method 6c**. C, 10 kV at the condition of **Method 6d**.

However, the retention times were shift forward from 3.9 minutes (20 kV), to 4.5 minutes (15 kV), to 5.3 minutes (10 kV).

Choice of Temperature

Decrease of temperature also could help decrease the mobility that gave more separation time to the adjacent two species, see Eq. 3.4.

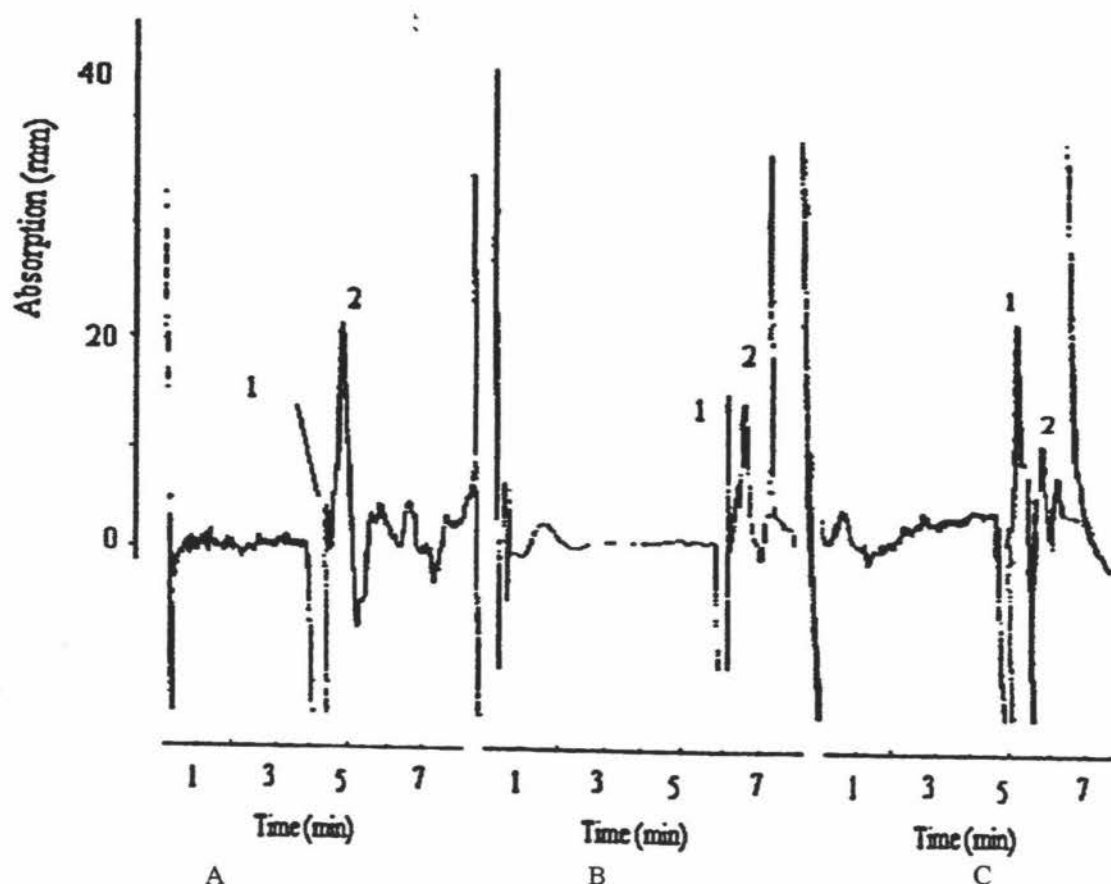
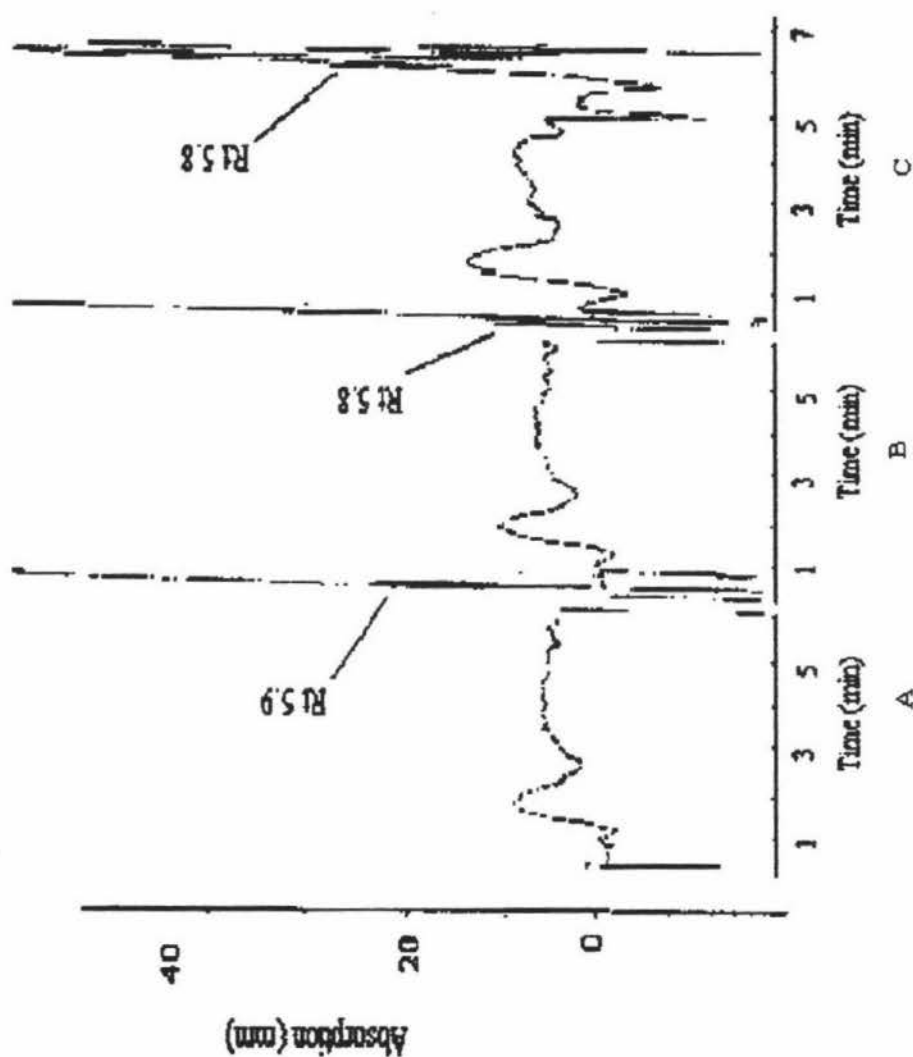


Figure 5.7 Choice of CE temperature. A, **Method 6d**: 30° C. B, **Method 6e**: 27° C. C, **Method 6f**: 24.4° C.

Result: **Method 6f** achieved better separation for the two adjacent species than the **Method 6e** and **Method 6d** (Figure 5.7). In **Method 6d**, the migration time of the 1/1 complex peak was 5.3 minutes; in **Method 6e**, the migration time of the 1/1 complex peak was 5.5 minutes; in **Method 6f**, the migration time of the 1/1 complex peak was 5.8 minutes. Since in our laboratory, it was difficult to keep the CE's temperature at 24.4° C constantly, the 27° C figure was chosen to be the separation temperature.

5.3.2 Interferences

Determination of serum creatinine by the Jaffé reaction may give erroneous results because of non-creatinine chromogens interfering at wavelength 480 nm. Now, the wavelength has been changed from 480 nm to 417 nm in order to get a lower determination limit for the creatinine in serum. In this case, the interferences need to be reexamined at wavelength 417 nm under exactly the same conditions as the creatinine was at 480 nm (Method 6e, Figure 5.8).



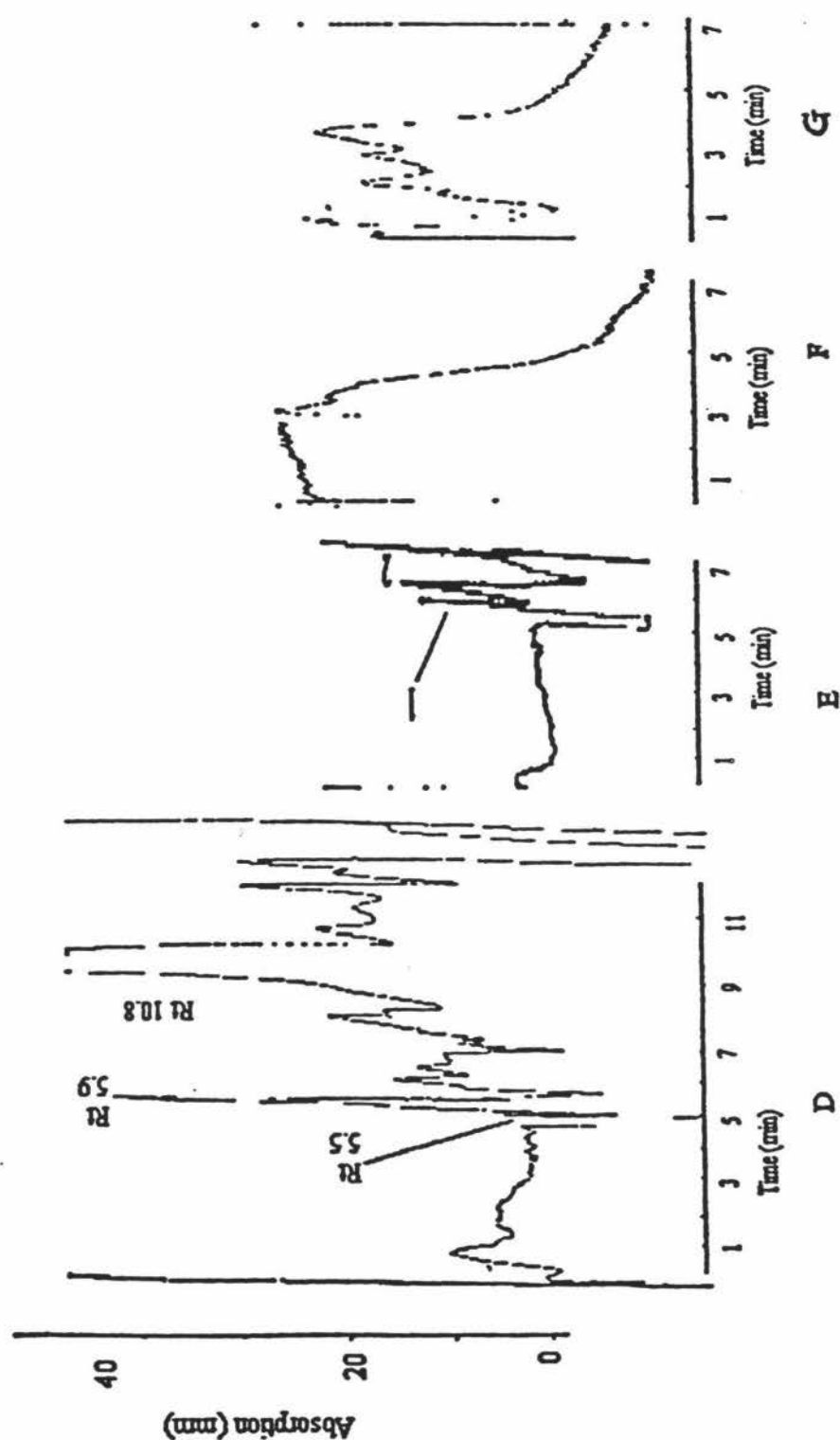


Figure 5.8

Interferences at wavelength 417 nm. A, guanine, B, L-ascorbic acid, C, α -ketoglutarate, D, albumin, E, acetone, F, acetoacetic acid, G, glucose.

Table 5.3 Interferences

interferences	concentration	retention time (min)	peak height (mm)
H ₂ O		5.6	11
albumin	concentrated	10.8	180
acetone	25mg/dl	5.6	11
acetoacetic acid	25mg/dl	not detected	
L-ascorbic acid	25mg/dl	5.8	11
glucose	25mg/dl	not detected	
guanidine	25mg/dl	5.9	19
creatine	25mg/dl	5.7	14
a-ketoglutamate	25mg/dl	5.8	135

The result indicated that the “non-creatinine chromogens” at wavelength 417 nm have no interference affects on creatinine. One major suspicious possibility was creatine which has a absorbance at retention time 5.7 minutes when its concentration was at 1.9 mM (25 mg/dL), which the normal creatine concentration in serum is ~ 0.1 mM (~ 1.31 mg/dL). When 0.1 mM creatine examined at 417 nm, there was no absorbance at retention time 5.7 higher than the water peak (**Figure 5.9**). Further discussions of creatine at 417 nm are to be found in **Chapter 6**.

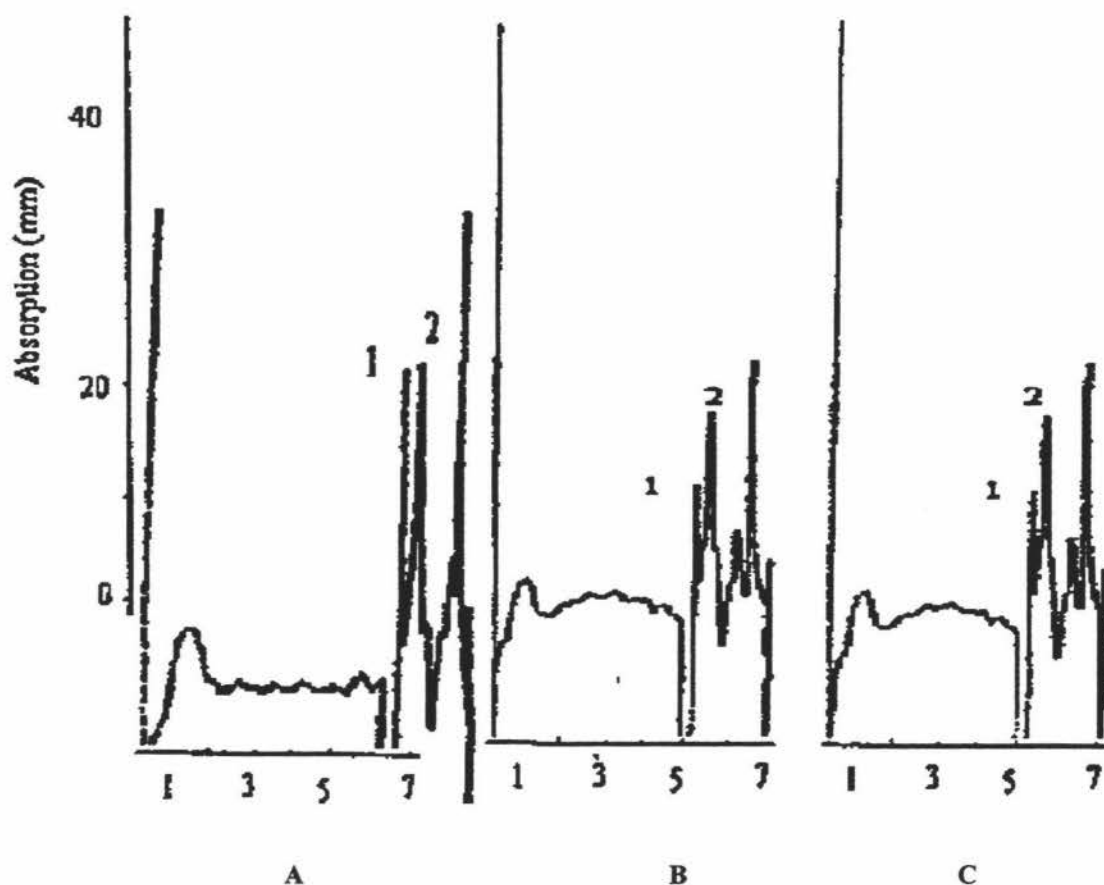


Figure 5.9 Creatine at 417 nm. A, 25 mg/dL creatine. B, 0.1 mM creatine. C, water. Condition: **Method 6e**.

Where peak 1 = creatinine-picrate complex, peak 2 = an unknown followed behind the peak 1.

5.3.3 Reproducibility (precision)

This was the agreement between replicate samples. Precision was determined by performing a replication experiment and determining the mean, standard deviation and coefficient of variation from results obtained. A variety of specimens were tested including those which have high levels and those which have low levels of analyte (King, 1998).

The reproducibility of **Method e** has been examined by running the same sample (2 mM creatinine) seven times automatically under identical conditions, which were designed from the CE program (**Figure 5.10**).

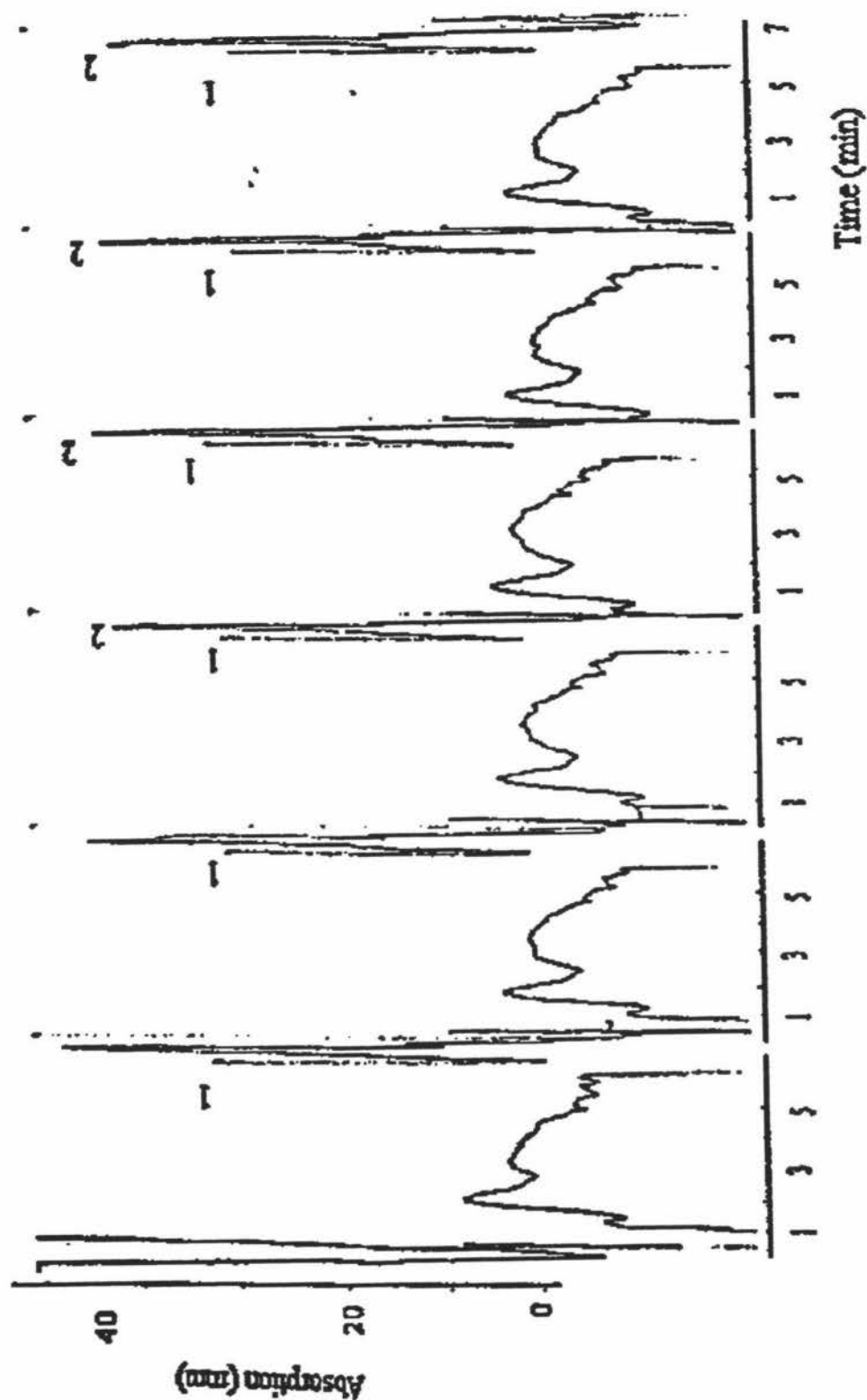
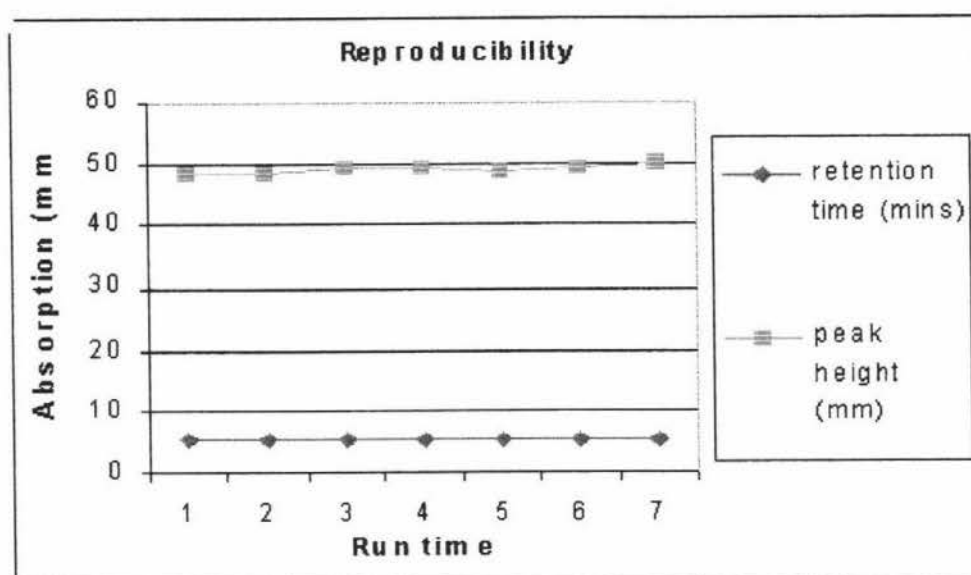


Figure 5.10 Stability and reproducibility.



Scheme 5.1 Reproducibility

Table 5.4 CV

sample	retention time (minutes)	peak height (mm)	mean (mm)	deviation	CV
2.0 mM creatinine	5.5	48	48.8	-0.8	-1.61%
2.0 mM creatinine	5.5	48	48.8	-0.8	-1.61%
2.0 mM creatinine	5.5	49	48.8	0.2	0.44%
2.0 mM creatinine	5.5	49	48.8	0.2	0.44%
2.0 mM creatinine	5.5	48.5	48.8	-0.3	-0.59%
2.0 mM creatinine	5.5	49	48.8	0.2	0.44%
2.0 mM creatinine	5.5	50	48.8	1.2	2.49%

Coefficient of variation CV = [Standard deviation/Mean] \times 100

Because each run time was 10 minutes (1 minutes 0.1 NaOH washing, 2 minutes buffer reequilibrium, and 7 minutes sample running times), the total run time was 70 minutes. From the results of **Table 5.4**, we can see that within 70 minutes, the sample was in a stable condition and each migration time of the peak identified matched well.

5.3.4 Calibration of the creatinine

Condition: Method 6e (Figure 5.11)

Table 5.5 Analytical range

creatinine (mM)	peak height(mm)	retention time (min)
H ₂ O	11	5.5
0.1	14	5.5
0.5	21	5.5
1.0	32	5.5
1.5	42	5.5
2.0	49	5.5
3.0	72	5.5
4.0	93	5.5

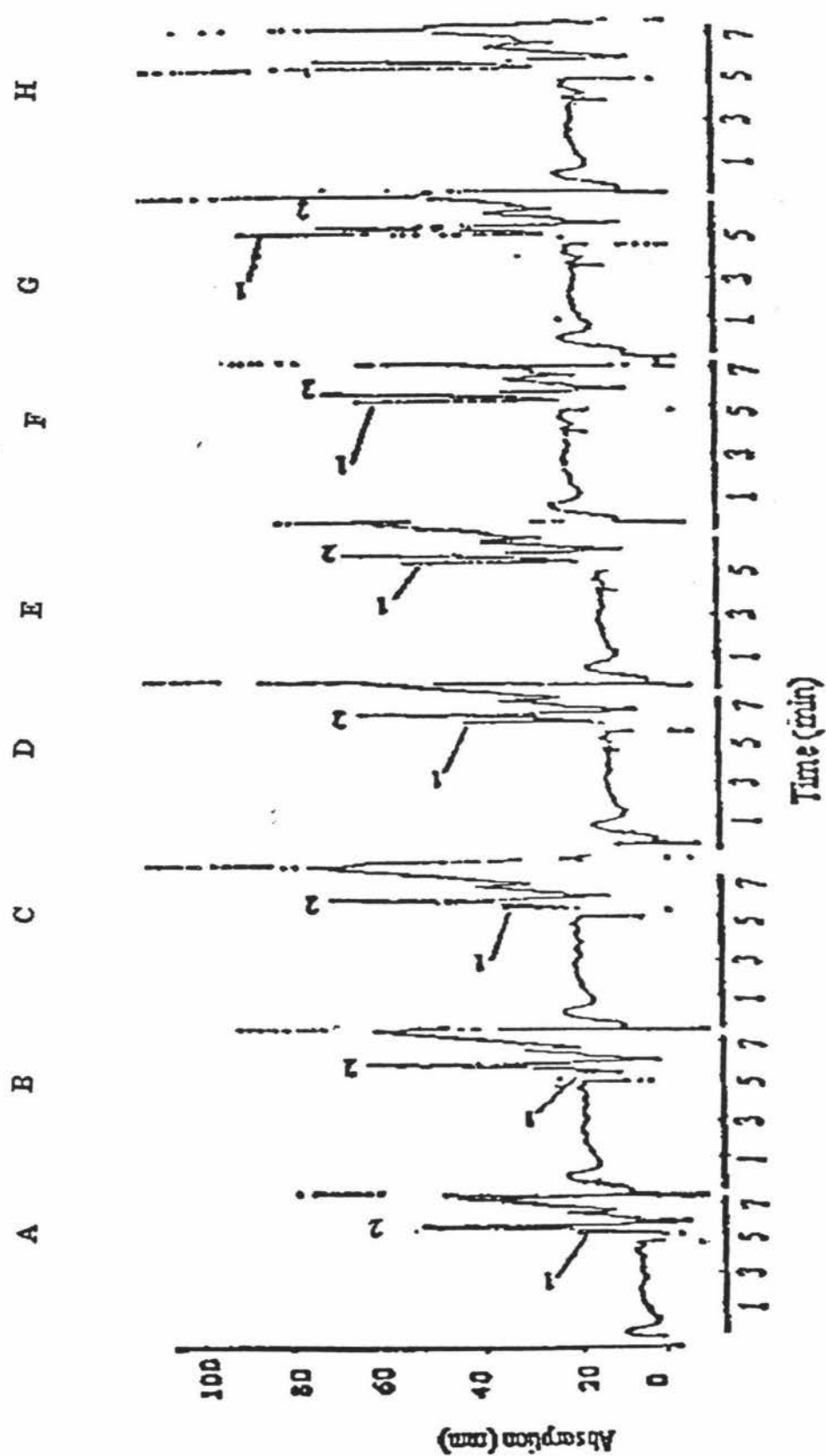
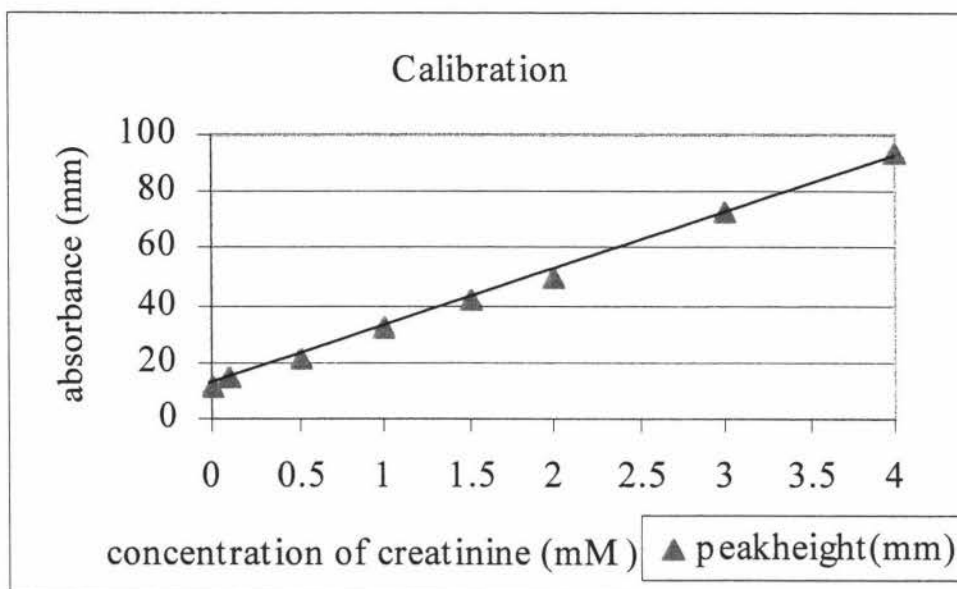


Figure 5.11 Differences concentration of creatinine. A, 0.5mM, B, H_2O , C, 0.1 mM, D, 1.0 mM, E, 1.5 mM, F, 2.0 mM, G, 3.0 mM, H, 4.0 mM.



Scheme 5.2 Calibration of creatinine in serum. Condition: **Method 6e**.

The linear calibration equation can be calculated as

$$y = 22.98x + 11 \quad (5.10)$$

where x is the creatinine concentration, y is the related absorbance.

5.3.5 Blank readings

Distilled water was used as blank sample (**Figure 5.11**). There was a positive peak (the peak height was 11 mm) at the retention time 5.5 minutes where creatinine absorbs. That means the blank reading is not exactly at the zero point when there is no creatinine in the sample.

5.3.6 Recovery

Recovery is related to specificity and is determined by adding known amounts of an analyte to a real specimen and determining what percentage of the added analyte is detected (recovered). This should be close to 100 % (King, 1998).

Frozen serum samples from healthy individuals were obtained has been melted at room temperature, and filtered before injection onto the CE. There was one small sharp peak appearing directly after the negative peak at retention time 5.7 minutes. Since this retention compared closely with creatinine (retention time 5.5 minutes) but had shifted to a later elution time; more experiments were carried out to identify the 1/1 complex peak. The same amounts of creatinine at concentration of 1.5 mM and 3.5 mM have been spiked into filtered serum, respectively, in order to examine the recovery as well. The results showed that the peak which appeared at retention time 5.7 minutes in serum has increased in height from 13.5 mm to 30.8 mm and 53.4 mm, respectively (**Figure 5.12**).

These results indicated that the peak of creatinine-picrate 1/1 complex had a delayed elution shift 0.2 minutes in the real specimen compared to the standard creatinine solutes. The reason of retention time shift can be explained by the influences of other compounds that exist in serum that could change the electroosmotic flow (μ_{os}).

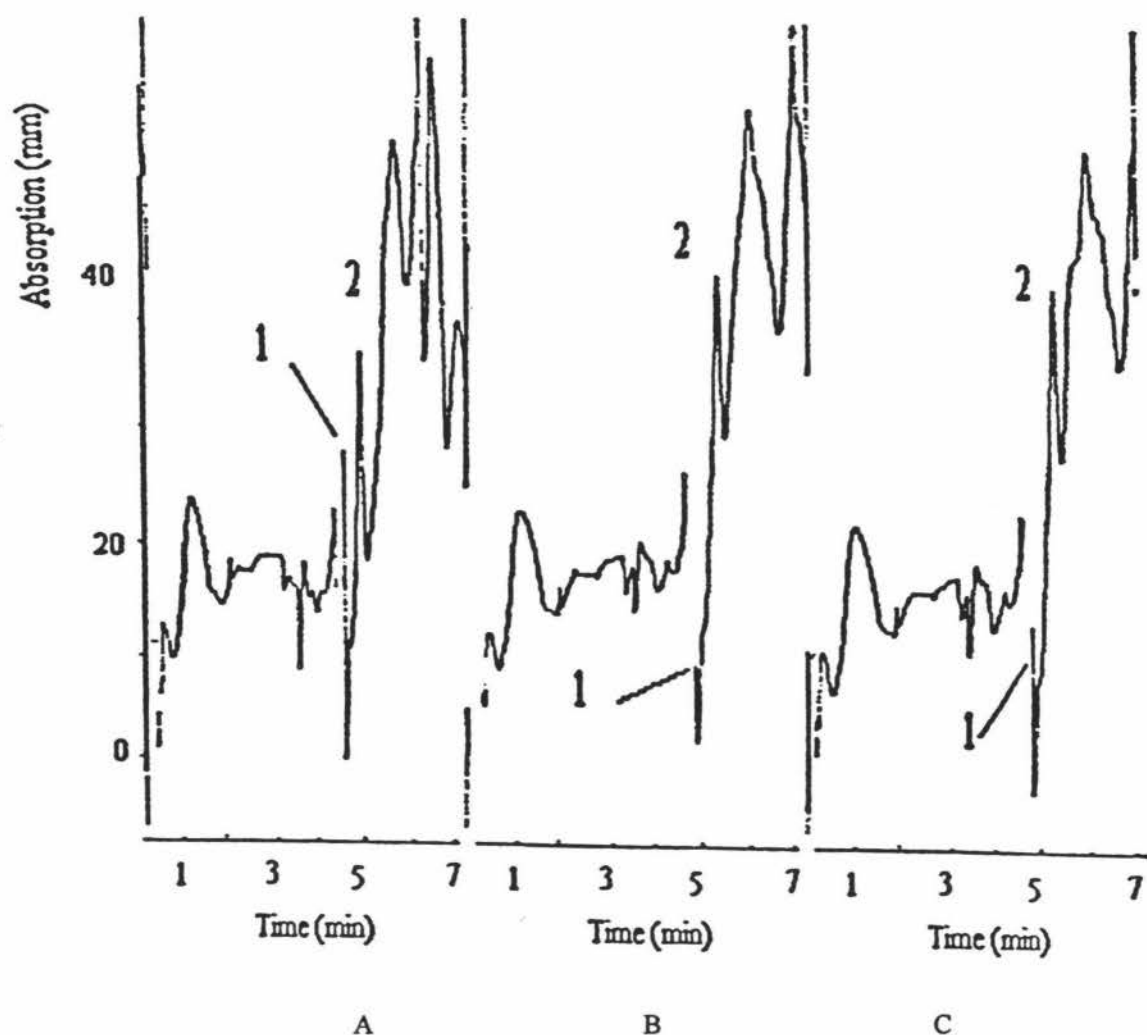


Figure 5.12 Recovery. A, serum spiked with 3.5 mM creatinine. B, serum. C, serum spiked with 1.5 mM. Condition: **Method 6e**.

Electroosmosis (EO) is the bulk flow of liquid due to the effect of the electric field on counterions adjacent to the negatively charged capillary wall. The wall of the fused-silica capillary is negatively charged at most pH conditions; there is a build-up of positive counterions in the solution adjacent to the capillary wall. When an electric field is applied, this layer of positively charged ions is drawn toward the negative electrode, resulting in the bulk flow of liquid toward that electrode.

The electroosmosis can be changed by either the charge density on the capillary wall, the double-layer thickness, or the viscosity of the solution adjacent to the capillary wall (Grossman and Colburn, 1992)

$$\mu_{os} = \frac{\sigma^* k^{-1}(\varepsilon, C_i)}{\eta} \quad (5.9)$$

where σ^* is the capillary inner surface charge density, k is the Boltzmann's constant, ε is the electrical permittivity of the solvent, C_i is ionic concentration in moles per liter, and η is the viscosity of the solution. From Eq. 5.9, we can see electroosmotic flow is inversely proportional to the viscosity of the solution. Since the serum sample injected on CE was only filtered without being deproteinized prior to analysis by CE, the viscosity of the serum sample was higher compared to the standard creatinine solutions measured previously thus causing a decrease in the electroosmotic flow. Therefore, the migration time of the identified peak shifted forward.

After calibration the concentration of creatinine in serum sample has been examined can be calculated as 0.108mM.

Table 5.6 Recovery

sample	peak height (mm)	absolute absorption	recovery(%)
H ₂ O	11		
serum	14.1		
serum + 1.5 mM CRN (1:1v/v)	30.8	30.8-14.1=16.7	97.09%
0.75 mM CRN (standard)	28.2	28.2-11=17.2	
serum + 3.5 mM CRN (1:1v/v)	53.4	53.4-14.1=39.3	97.76%
1.75 mM CRN (standard)	51.2	51.2-11=40.2	

$$\text{Recovery} = \frac{\text{spikedserum} - \text{serum}}{\text{relativeCRN}} \times 100$$

A sample of fresh urine from an healthy colleague was obtained (not 24 hour pooled). The urine sample was diluted five-times with distilled water and filtered before injection onto the CE. The result showed below (Figure 5.12).

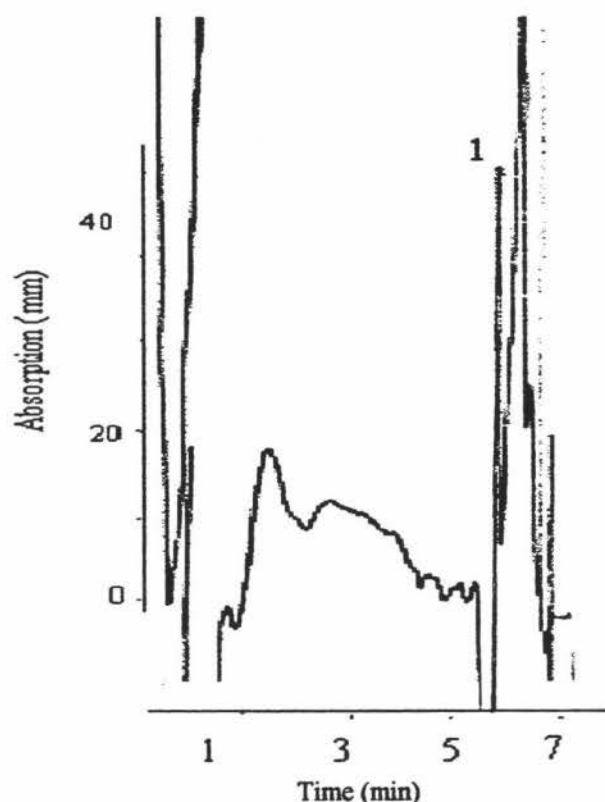


Figure 5.12 Determination of urine sample with Method 6e.

The absorbance of the five-times diluted urine was 50.1 mm which related to creatinine concentration was 1.7 mM according to the standard creatinine calibration equation. That gave the total creatinine concentration of 8.5 mM in a urine sample.

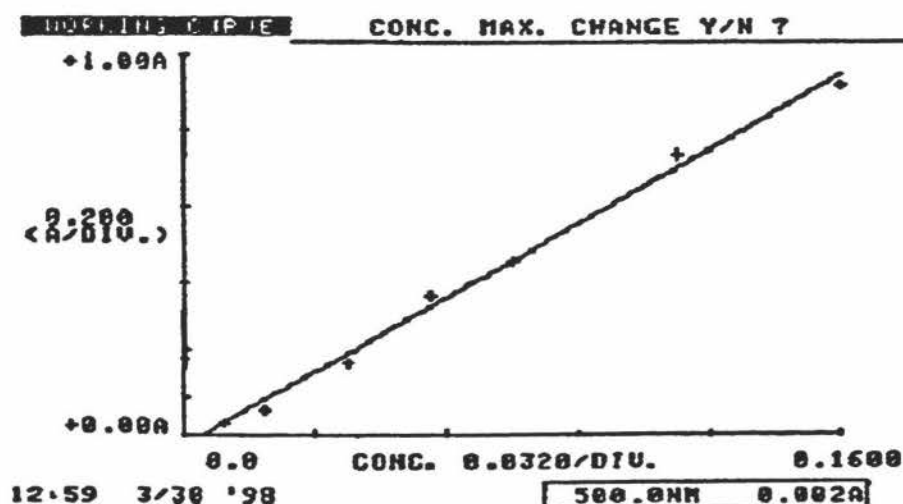
5.4 Determination of creatinine using the standard laboratory Jaffé reaction

Procedure

1. Deproteinize serum and controls by placing 4.5 ml tungstic acid in a 16 × 125 mm tube and adding 0.5 ml sample. Mix thoroughly for 10 s and centrifuge at 1500 × g for 10 min.

2. Into a series of appropriately labeled tubes pipet 3.0 ml deproteinized supernatant, or standard. Set up also a reagent bland of 3.0 ml water.
3. Add 1.0 ml picric acid to each tube and mix thoroughly.
4. At accurately timed 30 s intervals add 0.5 ml of NaOH to each tube and mix thoroughly.
5. Exactly 15 ml after adding NaOH, read the absorbance of each tube at 500 nm against the reagent bland set to zero absorbance.
6. Plot absorbance versus concentration for standards and read concentrations of unknowns and controls from the curve (Scheme 5.3).

CALIBRATION		WORKING CURVE DISPLAY Y/N ?	
STD. N°.	CONC.	ABS.	
1	0.0000	0.000	
2	0.0100	0.026	
3	0.0200	0.052	
4	0.0400	0.106	
5	0.0600	0.159	
6	0.0800	0.215	
7	0.1200	0.336	
8	0.1600	0.452	



Scheme 5.3 Calibration of creatinine of the standard laboratory Jaffé reaction method.

The same serum and urine samples (100-times diluted) were examined by the standard laboratory Jaffé reaction method (Table 5.7).

Table 5.7 Creatinine in serum and urine in the standard Jaffé reaction method

sample	absorption	concentration (mM)
serum	0.130	0.2784
100-times diluted urine	0.623	0.1050

The result showed that the creatinine level (**Scheme 5.4**) were much higher than using CE method (**0.108mM** in serum and **8.5 mM** in urine in **Method 6e**). The differences of the concentration measured by the two different methods indicated that due to the non-creatinine interferences and menu operation error in the standard laboratory method based on the Jaffé reaction

5.5 Conclusion

Determination of creatinine in on CE at 417 nm offered a better sensitivity than at 480nm. The wavelength shift also eliminated some of the non-creatinine interferences and the employment of the new technique-CE, achieved a better separation of creatinine from the possible non-creatinine chromogens and offered an efficient, cheaper, automatable routine creatinine analysis.

CHAPTER SIX

DETERMINATION OF CREATINE IN SERUM

6.1 Introduction

The presence of creatine in urine has been suggested as a biochemical marker for evaluating painful vasoocclusive sickle cell crises with muscular involvement (Beyer, 1985) and as a sensitive indicator of active myositis in patients with dermatomyositis (Rowell, 1986). In addition, creatine in urine and serum has been suggested as an early marker for the diagnosis of acute myocardial infarction (DeLange, 1988).

Several methods to quantitate creatine in serum and urine have been described. A widely used method is the Jaffé reaction before and after conversion of creatine to creatinine (Faulkner, 1976). Other methods such as HPLC, enzymatic and others have been described in **Chapter Two** already with their disadvantages and advantages.

Literature searches revealed that creatine has not been determined by any form of CE, and there is thus the possibility of using the principles of CE for the determination of creatine as well as creatinine.

In **Chapter Five**, creatine was shown to have an absorbance in **Method e** when its concentration was much higher than that in serum. Moreover, creatine does not react with picric acid under alkaline conditions (Yatzidis, 1974). There are therefore two possibilities for the peak observed:

- Creatinine existed in the creatine sample due to the equilibrium established between creatine and creatinine (**Figure 2.6**, Chapter Two).
- Creatine itself shows absorbance at wavelength 417 nm under the conditions of **Method e**.

This suggested further experiments needed to be done in this chapter to determine creatine levels in serum. These studies are reported in this chapter.

6.2 Methods

Conditions:

Buffer	30 mM phosphate
Buffer pH	10
Injection type	vacuum
Detector Rise time(second)	2.00
Detector range	0.01
Wavelength(nm)	417
Temperature (°C)	30.0

Table 6.1

Method	Capillary	Sample	Running time(min)	Voltage(kV)	Injection time (sec)
7a	75 μ m ID \times 25cm	3 mM creatine	7	10	2
7b	75 μ m ID \times 50cm	1 mM creatine	10	15	10
7c	75 μ m ID \times 50cm	0.1 mM creatine	10	15	10
8	75 μ m ID \times 50cm	0.01 mM creatine	10	15	10
9	75 μ m ID \times 50cm	0.01 mM creatine in (3 % NH₄Cl + Acetonitrile1:2 v/v)	10	15	10

Bold entries: indicate changes shown in the table.

6.3 Results and discussion

6.3.1 Selection of the analysis method

6.3.1.1 Creatine in serum CZE study one

In order to find out that if the creatine itself has absorption at 417 nm, a phosphate buffer at pH 10 without picrate was used instead of the buffer with picrate in **Method 6e**. The result showed that creatine had absorption at 417 nm without reaction with picrate (**Figure 6.1**). The retention time was 5.6 minutes. That confirmed the absorption at 417 nm is creatine itself, because creatine is a negatively charged molecule at pH 10 according to creatine structure (**Figure 2.4**). Under the conditions of **Method 7**, 0.1 mM creatine was examined. The result showed that there was only a negative peak (**Figure 6.1c**). Apparently, the current sensitivity is not high enough to determine the creatine in serum. (The maximum concentration of creatine in serum is 0.1 mM).

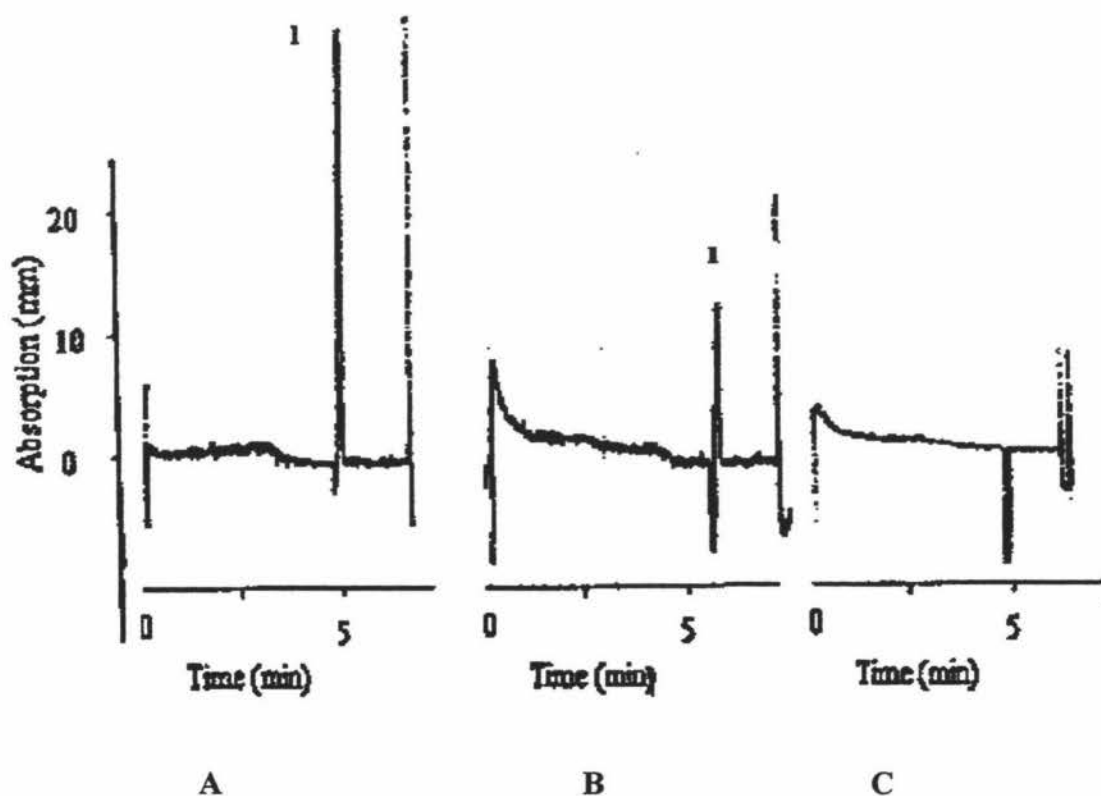
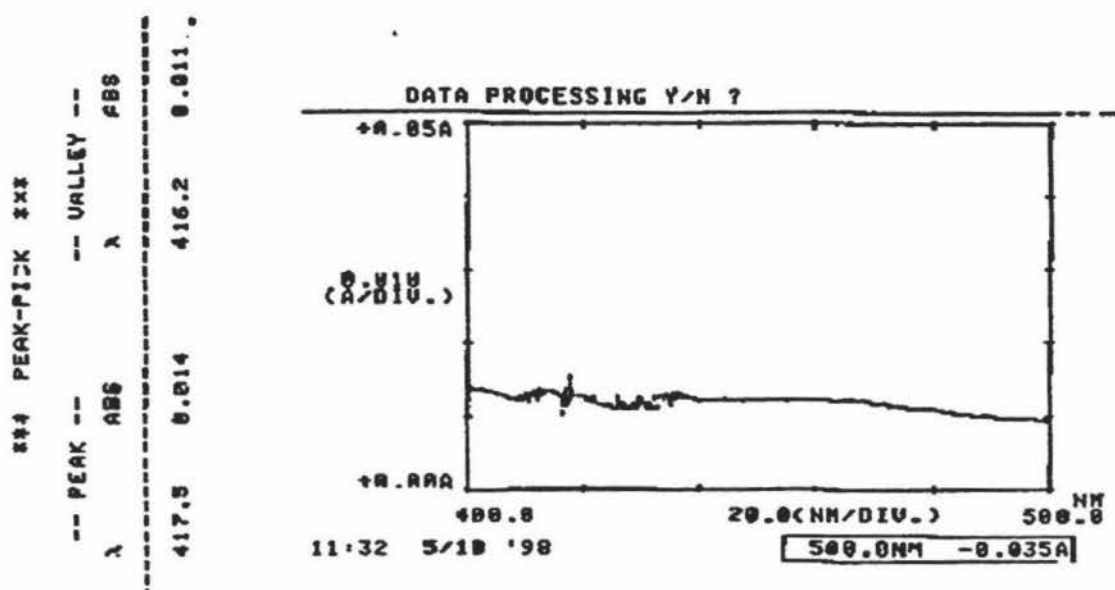


Figure 6.1 Creatine has absorption at 417 nm. A, 3 mM creatine. B, 1mM creatine. C, 0.1 mM creatine. Condition: **Method 7**.

UV spectrophotometry confirmed that under alkaline conditions, there was a small absorption for creatine at 417.5 nm (Scheme 6.1).



Scheme 6.1 UV scans for creatine from 400nm to 500nm.

6.3.1.2 Creatine in serum CZE study two

Sample stacking

In order to achieve a high plate number in CZE, the sample size has to be very small, preferably less than 1% of the capillary length (Shihabi and Garcia, 1994). Unfortunately, the sensitivity is very low which makes analysis of 0.1 mM creatine by CZE very difficult under the condition of **Method 7**. Sample volume increased to 30% of the capillary volume had been reported under a specific circumstance, which give a much higher sensitivity due to sample stacking for the determination of peptides (Shihabi, 1993 and 1996).

Bearing in mind, in order to improve the sensitivity by increasing the sample volume, longer capillary (75 μ m I.D. \times 50 cm effective length) was used (**Method 8a**) instead of the shorter capillary (75 μ m I.D. \times 25 cm effective length) use in early methods. Also, instead of dissolving the creatine into distilled water only, creatine dissolved in a mixture of two-volume acetonitrile and one volume of 3% ammonium chloride, and 10

second injections of the sample were performed. The result showed that 10-fold enhancement of peak height has achieved (**Figure 6.2A**). As a result of sample stacking, the retention time increased from 7.8 minutes to 8.2 minutes.

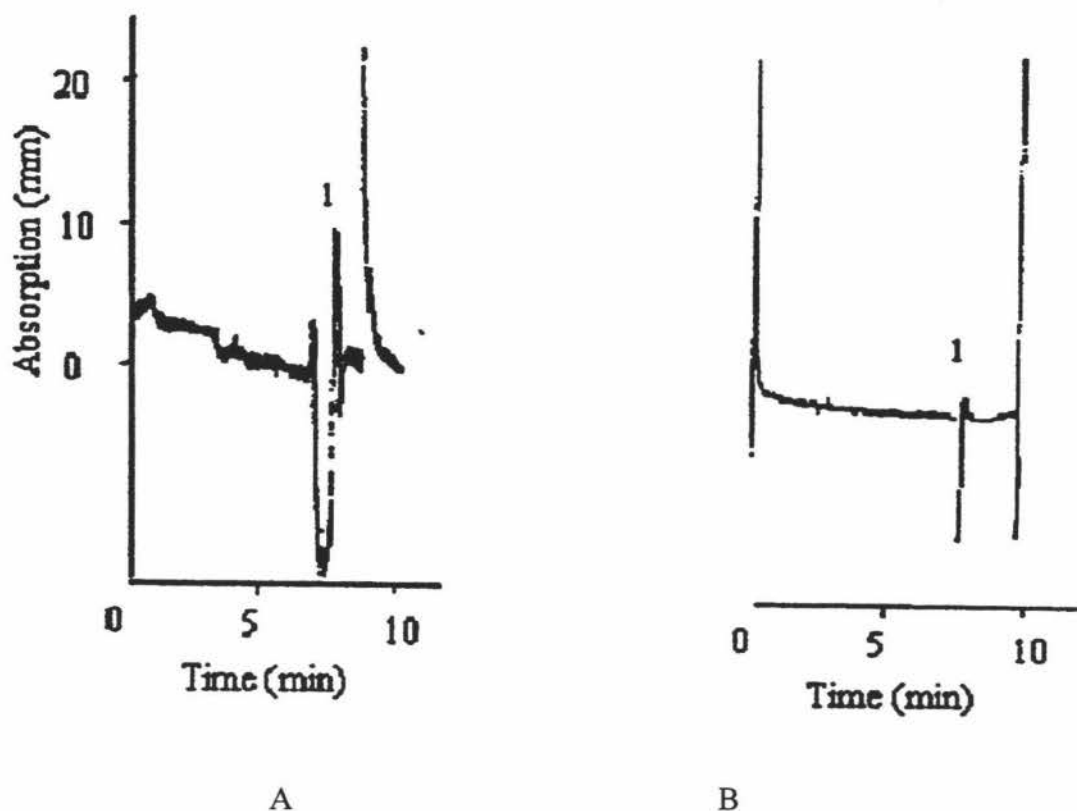


Figure 6.2 Enhanced detection due to sample stacking. A, 0.01 mM creatine in a mixture of two volumes acetonitrile and one volume of 3 % ammonium chloride. Condition: **Method 9**. B, 0.01 mM creatine in water. Condition: **Method 8**.

The stacking effect produced here by the acetonitrile shares some similarities and some differences with the traditional stacking produced at lower concentrations of aqueous buffers. The traditional stacking is based on differences between the conductivity of the injection zone and that of the carrier electrolyte, which can have an impact on the field strength that is expressed over each zone. This is simply a consequence of Ohm's Law. Since a solute's electrophoretic velocity is proportional to the field strength, differing velocities can be realised within each compartment. The basis of stacking is to provide a high field strength over the injection zone. At high pHs, as shown in **Figure 6.3**, a complex situation occurs because of the presence of the strong EOF. Anionic solutes now compress or stack up at the rear of the injection zone (Weinberger, 1993).

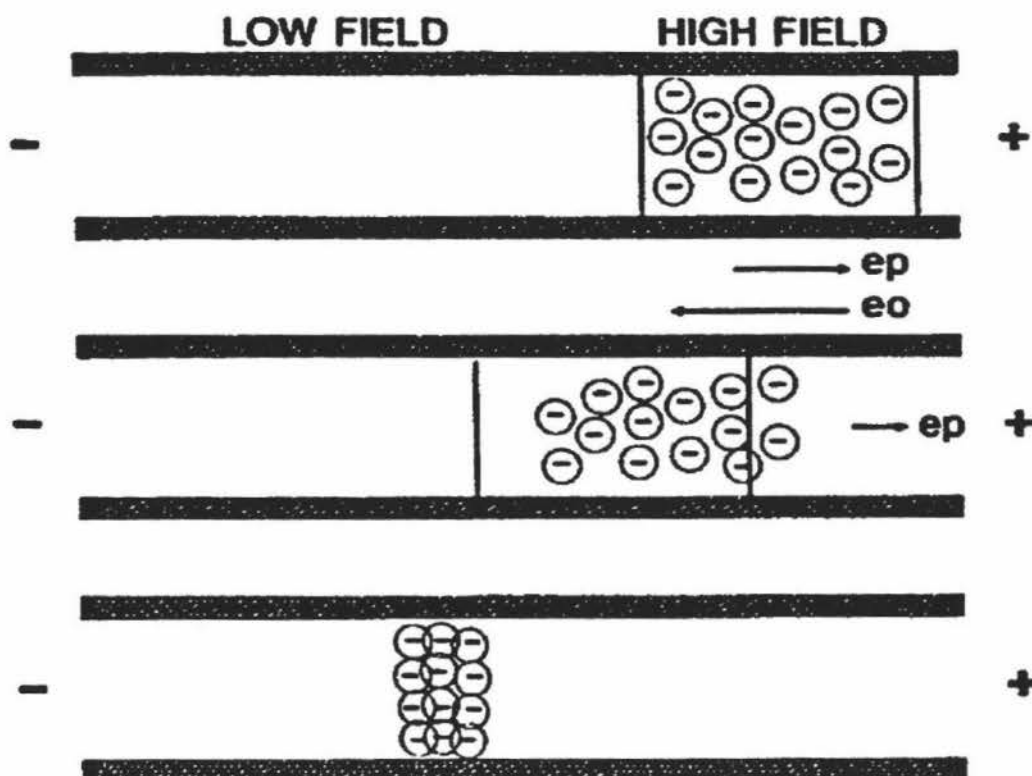


Figure 6.3 Illustration of stacking of anions in a high pH buffer (Weinberger, 1993).

In both cases, the stacking is produced by the difference in the resistivity of the sample and the running buffer and it allows a larger sample volume to be introduced into the capillary. However, the stacking in acetonitrile is different because the high resistivity of the sample results from introduction of an organic solvent into the sample. In addition, acetonitrile also offers an effective method for the removal of high concentrations of proteins by precipitation (Shihabi, 1993).

When salts are added to the sample, a further stacking occurs due to a different and more complex mechanism. Because of the limited solubility of inorganic ions in acetonitrile, they migrate rapidly with some water, leaving behind a more concentrated and narrower segment of acetonitrile. Weakly ionized organic compounds migrate behind in the acetonitrile zone. This is reminiscent of the “salting effect” in liquid extraction (Shihabi, 1996).

6.3.2 Reproducibility (precision)

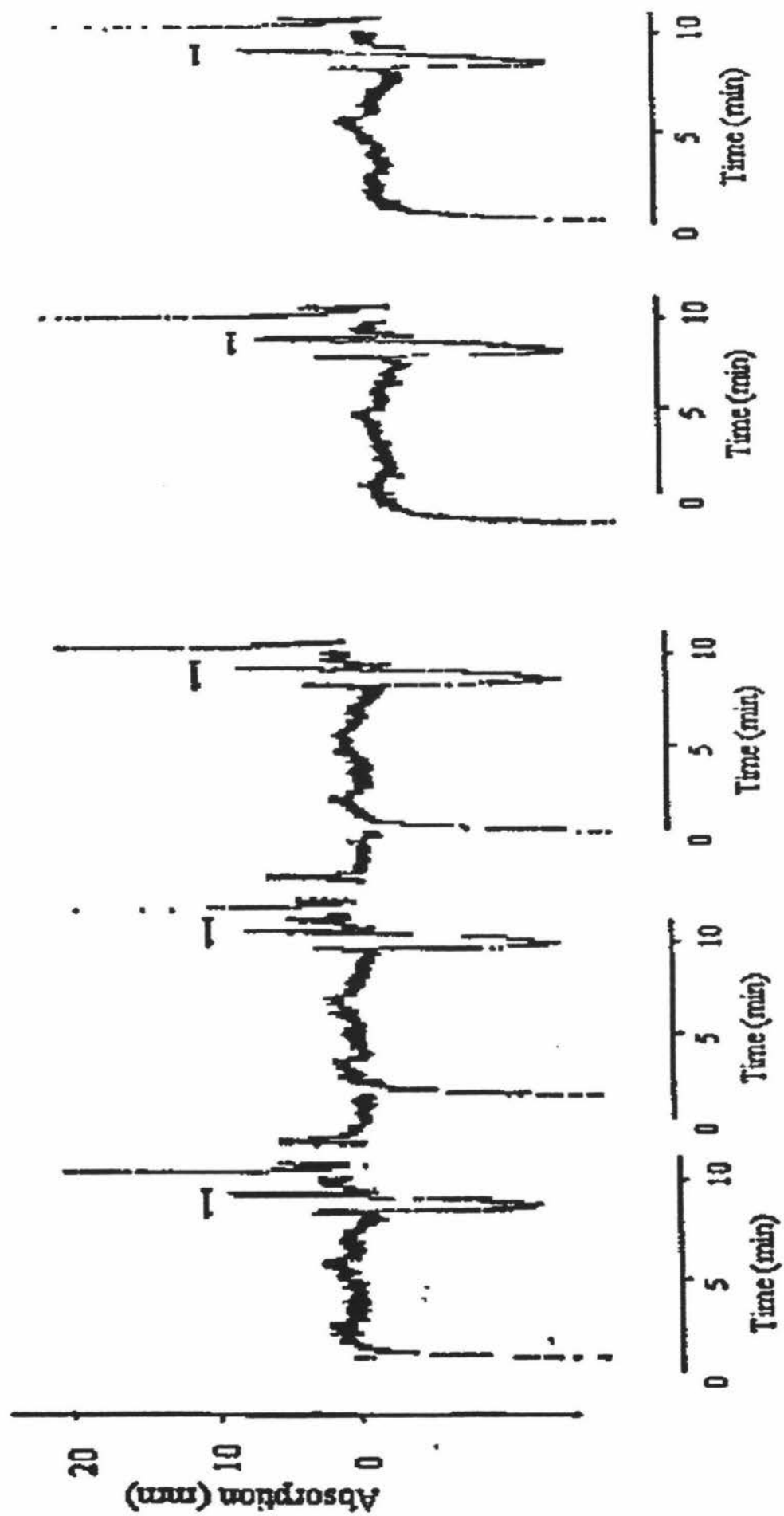
A 0.2 mM creatine in 3 % NH₄Cl + acetonitrile (1:2 v/v) sample was running repeatedly nine times to examine the reproducibility of the **Method 9**.

Conditions:

Capillary	75 μ m \times 50 cm
Buffer	30 mM phosphate, pH = 10
Wavelength	417 nm
Temperature	30° C
Voltage	15 kV
Current	43 μ A
Injection time	10 sec
Run time	10 min
Sample	0.02 mM creatine in 3 % NH ₄ Cl + acetonitrile (1:2 v/v)

Table 6.2 Reproducibility

conc. of creatine (mM)	peak height (mm)	mean (mm)	deviation	CV
0.02	9.8	10.6	-0.80	-7.55%
0.02	10.5	10.6	-0.10	-0.94%
0.02	9.8	10.6	-0.80	-7.55%
0.02	10.5	10.6	-0.10	-0.94%
0.02	10.0	10.6	-0.60	-5.66%
0.02	10.0	10.6	-0.60	-5.66%
0.02	11.8	10.6	1.20	11.32%
0.02	11.2	10.6	0.60	5.66%
0.02	11.8	10.6	1.20	11.32%



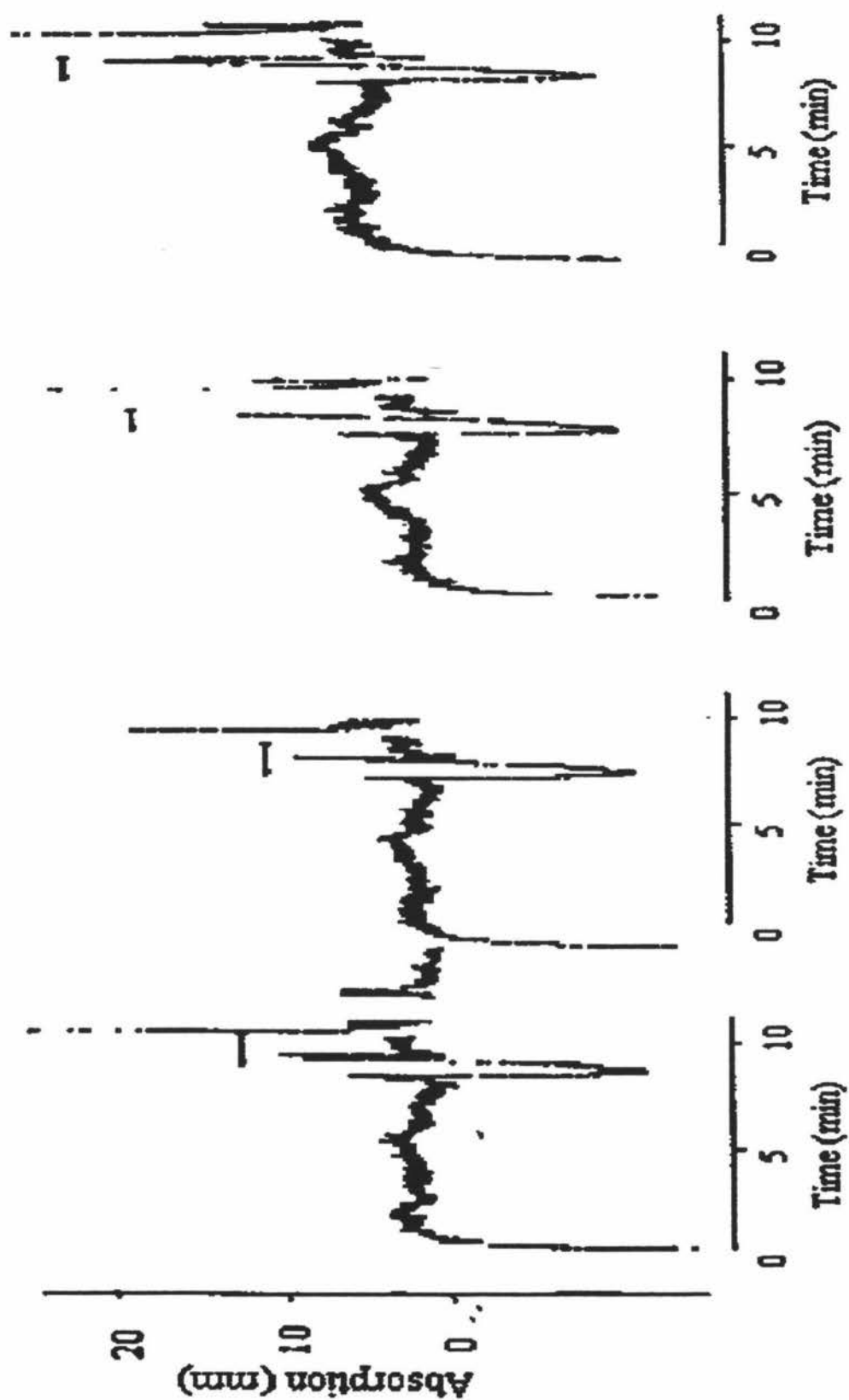
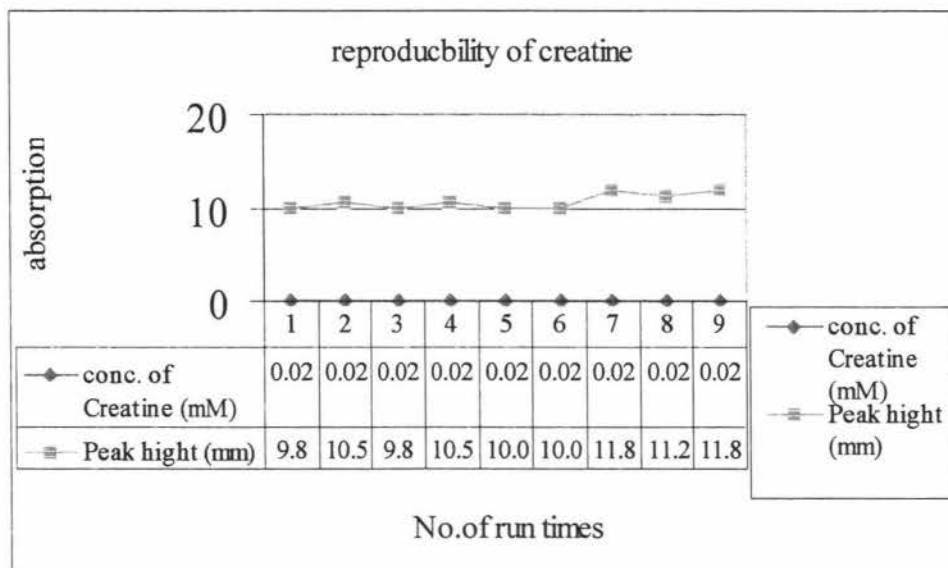


Figure 6.4 Creatine within-run precision. Condition: Method 9.

The reproducibility of 0.02 mM creatine has showed increasing absorption after sixth time repeat runs (~ 90 minutes) of the same sample. That was due to the evaporation of water within the sample that eventually concentrated the sample.



Scheme 6.2 Reproducibility of creatine.

6.3.3 Calibration of the creatine

Different concentrations of standard creatine dissolved in the buffer of 3% NH_4Cl + acetonitrile (1:2 v/v), then examined under the **Method 9** for calibration.

Table 6.3 Standard creatine (Method 9)

Conc. of creatine (mM)	Peak height (mm)
0.00	4.0
0.005	6.0
0.010	9.0
0.015	
0.020	10.8
0.025	
0.030	14.7
0.044	20.0

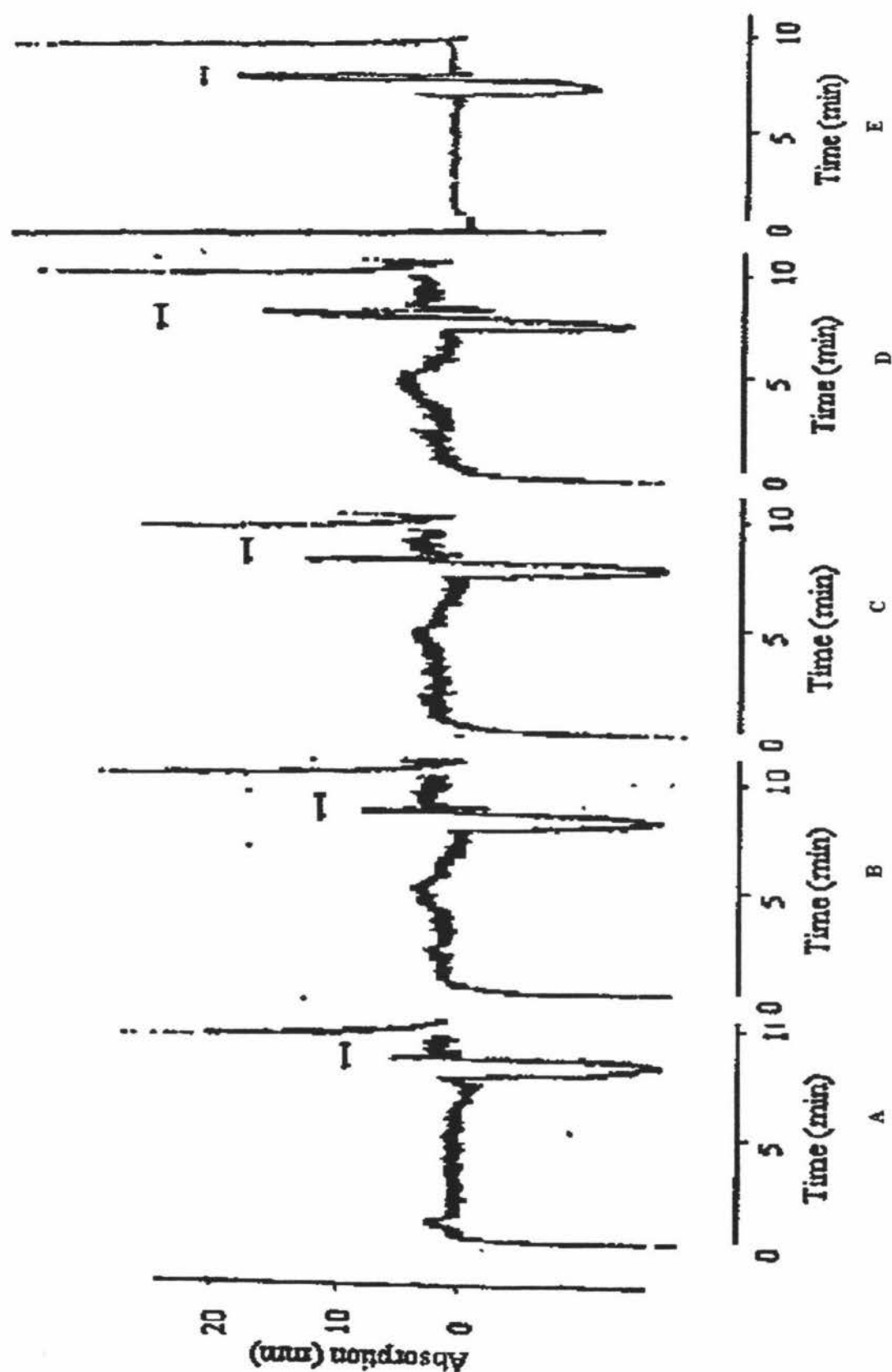
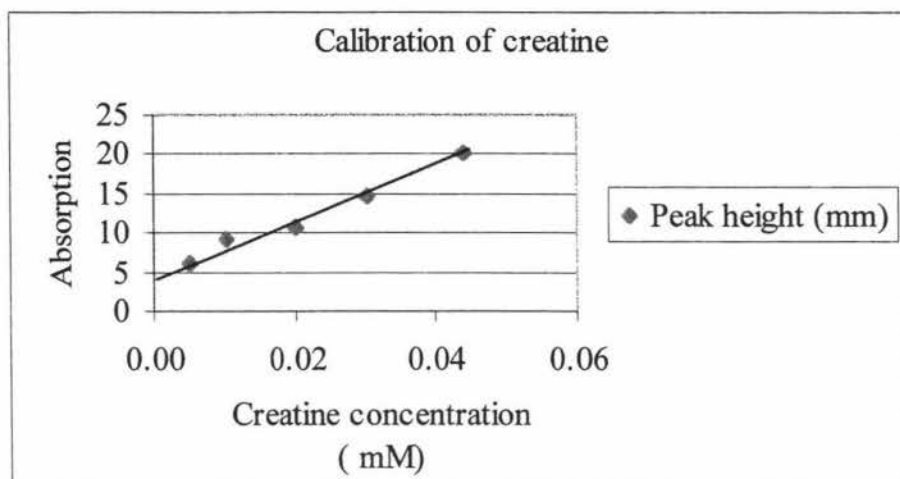


Figure 6.5

Standard creatine. A, 0.005 mM, B, 0.01 mM, C, 0.02 mM, D, 0.03 mM, E, 0.044 mM.
Condition: Method 9.



Scheme 6.3 Calibration of creatine.

The linear calibration of creatine can be calculated as:

$$y = 365x + 4$$

where x is the creatine concentration, y is the related absorbance.

6.3.4 Blank reading

In this method, when the buffer only (acetonitrile and 3% ammonium chloride) was injected, there was a minor positive peak (4 mm) at 8.2 min.

6.3.5 Sensitivity (detection limit)

The detection limit for this method was 0.005 mM creatine, which is about 10-times diluted than that in serum (males: 0.02-0.05 mM, females: 0.04-0.09 mM).

6.3.6 Recovery

Three-times diluted serum (1:2 v/v in buffer) was examined (**Figure 6.6A**). Peak 1 that at 8.2 min was the creatine absorbance peak at 417 nm, peaks 2-6 were the other compounds in serum. The three-times diluted serum had shown that creatine absorbance was 9.2 mm. The creatine concentration was 0.014 mM calculated by the standard calibration equation, and the total creatine concentration in serum was 0.042 mM.

In addition, with spiked creatine (0.03 mM) into the diluted serum (1:1 v/v), the creatine peak height increased to 9.8 mm (**Figure 6.6B**), that means the total creatine concentration was 0.15 mM more than before. Nevertheless, the two small peaks of number 2 and 3 followed behind the creatine peak almost disappeared due to the further dilution.

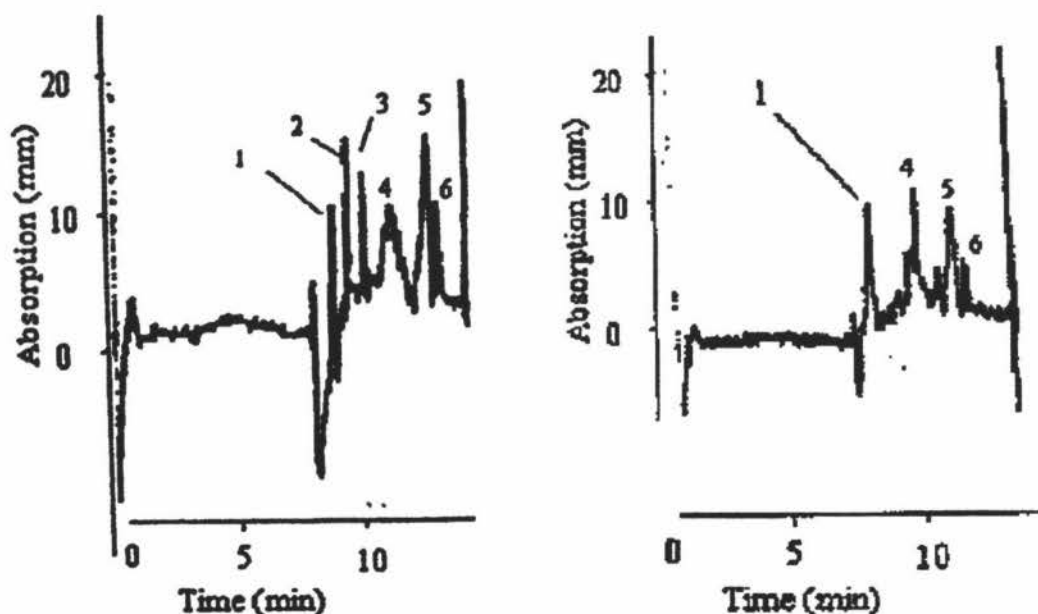


Figure 6.6 Creatine in serum. A, three times diluted serum. B, A dissolved in 0.03mM standard creatine (1:1 v/v). Condition: **Method 9**.

Table 6.4 **Recovery**

Sample	Peak height (mm)	Recovery(%)
A: creatine in serum	9.2	
B: A + 0.03 mM creatine (1:1 v/v)	9.8	96.8%
C: 0.015 mM standard creatine	9.5	

6.4 Conclusion

Creatine has an absorbance at 417 nm under alkaline condition thereby allowing the possibility for CZE to determination. The specific sample stacking of the mixture of acetonitrile and salts overcame the disadvantage of the limited sensitivity of standard CE due to the narrow capillary. That meant that the much-diluted creatine in serum could be determined on CE directly.

Compare this Method (9) with the common Jaffé reaction method:

- For the common Jaffé reaction there is a need to convert creatine to creatinine and measure the difference between the creatinine and both creatinine and converted creatine. Since the Jaffé reaction has long been known as a non-specific method, it makes the creatine determination unreliable as well. In addition, the traditional method is a complicated and time wasting procedure.
- In **Method 9**, pre-treatment of serum was simple. It took only 10 minutes centrifugation and a filtration before the analysis. The analysis time was 10 minutes as well, the advantage of the CE offers automation, and hence a walk-away procedure that allows batches of sample can be analysed and calculated under the designed computer controlled programme. No Jaffé reaction involvement means no interferences of chromogens exist. This makes this method more precise and accurate than the standard Jaffé reaction.

CHAPTER SEVEN

CONCLUSION AND FUTURE WORK

7.1 Conclusions

The study reported in Chapter 4-6 investigated that the use of a relatively new technique – CZE – as a clinical tool to analyse creatinine and creatine in serum and creatinine in urine.

The common used Jaffé reaction method is based on the reaction between creatinine and picric acid under alkaline conditions. The final product is a red-orange 1/1 complex, which has an absorbance at 480nm. The analysis is carried out by measuring the absorbance at 480nm with an UV spectrophotometer. Nevertheless, this method is far well known to be a non-specific method that is due to the non-creatinine chromogen interferences.

In Chapter Four, an Applied Biosystem Electrophoresis system was used to instead of the UV spectrophotometer to analyse creatinine in urine. A fused silica capillary that was 75 μm I.D. \times 25 cm effective length (total length 50 cm) was used. Picric acid was in the phosphate buffer and creatinine was analysed under the principles of CZE and identified at 480 nm. CE allowed the non-creatinine chromogen interferences in urine to be separated away from creatinine to give a more accurate result for the creatinine concentration in urine than that in the common Jaffé reaction method.

Similarly in Chapter Five, the separation principles used were the same with the only difference being that creatinine in serum and urine were identified at 417 nm in order to increase the detection sensitivity. The non-creatinine chromogen interferences did not show absorption at 417 nm. This made the Jaffé reaction under CE conditions more specific to creatinine once again.

In the common Jaffé reaction method, creatine in serum is measured by heating up the serum to convert creatine to creatinine, then the measurement taken to get the creatine

concentration indirectly. If the non-specificity of the standard Jaffé reaction method for the determination of creatinine is in question, then the accuracy for creatine must also be questioned.

In Chapter Six, creatine absorption at 417 nm was discovered. This meat creatine could be determined by CZE directly. Furthermore, the use of the acetonitrile and the salt NH_4OH in the sample buffer produced sample stacking and therefore increased 10-fold the detection sensitivity. Because creatine was determined by CZE directly, there was no participation of the Jaffé reaction. In this case, therefore non-creatinine chromogens were not a problem in this method at all.

Apparently, using capillary electrophoresis was a better way to determine creatinine and creatine. These new methods in Chapter 4-6 showed more accurate, time saving and automatic determination of creatinine and creatine over the common Jaffé reaction that determines creatinine and creatine by the standard procedure which requires an UV spectrophotometer.

7.2 Future work

In Chapter Four, the limitation of detection made the method can only useful for the determination of creatinine in urine but it was not sensitive enough for determination of creatinine in serum. The idea of sample stacking that increased the sensitivity in Chapter Six needs to be investigated in Chapter Four. If it achieved the same result as in Chapter Six, then the method in Chapter Four can determine creatinine in both urine and serum.

Further study can focus on developing a method that is able to determine creatinine and creatine in one method by CE.

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