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THE DEVELOPMENT OF A METHOD TO DETERMINE FELININE IN BODY FLUIDS BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Ion-exchange, paper-chromatography and high performance liquid chromatography were used in earlier studies for the determination of felinine in biological fluids. These methods were either inadequate and/or need laborious sample pre-treatments.

A new method for the determination of felinine by capillary zone electrophoresis has been developed. Preliminary investigations were carried out to address the conditions required for the separation of felinine. The separation of felinine can be performed on a fused-silica capillary with a 20 mM phosphate buffer (pH 2.0) and detection wavelength 200 nm. The separation principle was based on the different migration times due to the different molecular weights, molecular sizes and charges under an applied potential field.

The quantitative determination of felinine levels in cat urine has been achieved. The cat urine analysis was performed directly on the capillary electrophoresis without making any felinine derivative(s). The levels of felinine in different cat genders are reported. The results were compared with the results of an HPLC felinine derivatization method. Felinine levels in entire male cat urine were much higher than those in female and castrated male cat urine. A synthetic felinine was employed as standard felinine. Linear relationships between peak area and concentration of synthetic felinine calibrations are reported. Mean felinine recovery in cat urine was 95.9%. Taurine, urea, creatine and creatinine, which exist in large amounts in cat urine, showed no interference with the analysis of felinine by this method.

The new capillary zone electrophoresis method was then applied to the study of felinine stability. Conditions reported to influence the stability of felinine were investigated. These conditions included oxidation, storage temperatures and times, heating, acidic and alkaline solutions. Both synthetic felinine and felinine in cat urine were investigated. Storage temperature (-20°C to 20°C) had no significant influence on the stability of felinine while higher temperatures increased the decomposition of felinine. Felinine degraded at strong

acid and base conditions but was relatively stable under mild acid and base conditions. A similar stability of felinine in human urine is also reported.

The capillary zone electrophoresis method was also employed to study felinine in plasma and serum. Plasma and serum as well as urine can be analysed directly on the capillary electrophoresis after sufficient dilution. Conditions (eg. protein clean up, changing of injection time, 37°C heating) that might influence of felinine behaviour in plasma and serum are discussed. This study indicated that no traces felinine be found in cat plasma, within the detection limits of this new capillary electrophoresis method.

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LIST OF ABBREVIATIONS

AU	absorbance unit
AUFS	absorbance unit full scale
β-ME	β-mercapto-ethanol
BOC	tert-butyloxycarbonyl
BSA	bovine serum albumin
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CMC	critical micelle concentration
CV	coefficient of variation
CZE	capillary zone electrophoresis
D.C.	direct current
DTBD	di-tert-butyloxy-dicarbonate
EDTA	ethylenediaminetetraacetic acid disodium salt dihydrate
EOF	electroosmotic flow
ESTD	external standard
fel	felinine
FSCE	free solution capillary electrophoresis
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
ISTD	internal standard
I. D.(or i.d.)	internal diameter
MEKC	micellar electrokinetic capillary
MS	mass spectrometry
N-(NO_2)arg	Nω-nitro-L-arginine
OPA	O-phthalaldehyde
Pos	positive
R.S.D.	relative standard deviation
S.D.	standard deviation

SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UV/Vis	ultraviolet-visible
WA	washing

CHAPTER ONE

INTRODUCTION

PART I FELININE: A URINARY AMINO ACID OF FELIDAE

1.1 Discovery, isolation and characterization of felinine

Felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid) is a urinary amino acid excreted in large quantities by domestic cats and several other Felidae. It was first discovered by Datta and Harris in 1951. At that time they were examing amino acids in the urine and plasma of various animals by two dimensional paper chromatography. Datta and Harris (1951) found a previously unknown amino acid that was frequently present in considerable quantity in the urine of domestic cats (Felis catus) and ocelots (Felis pardinus). They found that this material moved to a position similar to leucine in the two-dimensional paper chromatograms, when phenol and collidine-lutidine were used as solvents. They tentatively named this amino acid "cat spot". Soon after R.G. Westall (1951) became interested in this "cat spot" and isolated it as an amorphous solid from cat urine using large ion-exchange columns and renamed the amino acid, felinine. The simplest empirical formula that could be derived from degradation experiments was $C_8H_{17}O_3NS$, and the structure of this sulphur amino acid was provisionally determined as 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid **(Figure 1.1)**.

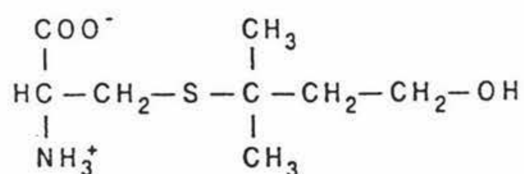

Figure 1.1. Molecular structure of felinine, 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (Westall, 1953)

Following these events, experiments by Datta and Harris (1953) showed felinine to be present in the urine of the leopard (Felis pardus) and the Indian leopard cat (Felis bengalensis). It was not found in the urine of any of the other animals investigated: lions, tigers, pumas, genets, dog, rabbit, rat, mouse, guinea pig, horse, cow or human. Roberts

(1963) found felinine in cat and bobcat urine but not in that of the leopard. **Table 1.1** shows their investigation for the presence of felinine in many mammals:

Table 1.1 Reported occurrence of felinine in the urine of different mammalian species (Hendriks, et al., 1995)

Species	Reference*		
	1	2	3
Cat (*Felis catus*)	Yes	Yes	Yes
Ocelot (*Felis pardalis*)	Yes	Yes	-
Leopard (*Panthera pardus*)	-	Yes	No
Indian leopard cat (*Felis bengalensis*)	-	Yes	-
Bobcat (*Felis rufus*)	-	-	Yes
Cheetah (*Acinonyx jubatus*)	-	-	No
Puma (*Felis concolor*)	No	No	No
Tiger (*Panthera tigris*)	No	No	No
Lion (*Panthera leo*)	No	No	No
Serval (*Felis serval*)	-	No	-
Fishing cat (*Felis viverrina*)	-	No	-
Dog (*Canis familiaris*)	No	No	-
Binturong (*Arctictis binturong*)	No	No	-
Genet (*Genetta tigrina erlangeri*)	No	No	-
Pole cat (*Putorius putorius*)	-	No	-
Bear (*Ursus ?*)	-	-	No
Rat (*Rattus norvegicus*)	No	No	-
Mouse (*Mus musculus*)	No	No	-
Guinea-pig (*Cavia porcellus*)	No	No	-
Golden hamster (*Mesocricetus auratus*)	-	No	-
Rabbit (*Oryctolagus cuniculus*)	No	No	-
Rhesus monkey (*Macacus rhesus*)	-	No	-
Horse (*Equus caballus*)	No	No	-
Cow (*Bos taurus*)	No	No	-
Goat (*Capra hircus*)	-	No	-

*Reference: 1, Datta and Harris (1951); 2, Datta and Harris (1953); 3, Roberts (1963).
-, not determined

The investigation of felinine in cat blood plasma was carried on as well. Datta and Harris (1953), Westall (1953) failed to show the presence of felinine in cat blood by using paper chromatography, Tallan et al., (1954) found traces of felinine in the plasma. Felinine was not found in tom cat plasma using modern HPLC analysis (Hendriks, et al., 1995).

The different quantitative data on levels of felinine in fluid and tissues of the cat have been published. Dietary factors influence the excretion of felinine, and the amount of felinine excreted in the urine of the cat is sex dependent. Westall (1953) estimated that felinine occurs in cat urine at a level of 1.0-1.2 mg/ml. Tallan et al. (1954) found levels of felinine in cat urine as high as 1.85 mg/ml, but with minimal quantities observable in

extracts of various tissues **(Table 1.2).** Acid-hydrolyzed extracts of tissues and fluids contained no felinine whatsoever. Excretion of more than 8mg felinine/milliliter (8 mg/ml) have been reported by Avizonis and Wriston (1959) in a cat fed a cystine enriched diet for 3 days. The lowest urinary concentration reported by them was 1.5 mg/ml by using ion exchange chromatography and Westall's (1953) "standard". Shapiro (1962) reported urinary excretion rates of 1.7 and 1.0 mg/ml for a male and female cat, respectively. More recently data is reported by Hendriks et al (1995b), who assayed 24-hr urinary felinine excretions in a total of 28 male (entire and castrated) and female (entire and spayed) cats. By using HPLC analysis and a synthetic felinine standard of known purity, the average felinine concentration in the urine of entire male cats was found to be 2.0 mg/ml (range 0.4-3.6 mg/ml) with entire females excreting on average 0.3 mg/ml. The higher concentration of felinine in the urine of entire male cats as compared with entire female cats was found to result in higher amounts of felinine excreted per day for the male cat. The average amount of felinine excreted (mg/24 hr) was 95 for entire male cats, 29 for castrated males, 19 for entire females and 13 for spayed females (Hendriks, et al., 1995).

Table 1.2 Reported extracts of felinine in various tissues (Tallan et al, 1954)

Tissues	felinine (per 100 g)
Bladder	3.4 mg (per 100mg)
Liver	<1.2 mg
Kidney	<1.0 mg
Brain	<0.6 mg
Plasma	0.4 mg
Gastrocnemius muscle	<0.3 mg

Felinine could not be detected in the urine of kittens of either sex by using paper chromatography (Roberts, 1963). The age of attaining sexual maturity is variable in female kittens but can be expected to occur around nine months (Beaver, 1992).

It was suggested by Tallan et al (1954), that felinine may be synthesised in the kidney and excreted directly into the urine or that it may be present in a bound form in the plasma.

Roberts (1963) detected felinine in tissue extracts of the liver, kidney and skin of the cat and suggested that felinine is carried in the blood, at low concentrations, and that it appears in many tissues and that the low blood levels too low to be detected would be maintained by a rapid excretion through the kidney.

1.2 Synthesis of felinine

Several chemical synthesis procedures of felinine were reported since 1957. It was first achieved by Trippett (1957) by the reductive S-debenzylation of 3-methyl-3-thiobenzylutan-1-ol in the presence of sodium in liquid amonia and β-chloroalanine. The synthetic material behaved exactly as a sample of "natural" felinine obtained from Westall (1953). In 1962, Eggerer (1962) synthesised felinine by using a phosphate derivative of isopentenol in a reaction with cysteine. He also found two other routes, using isopentenol and cysteine in the presence of pyridine, yielded felinine. Schoberl et al. (1966,1968) reported a synthesis procedure yielding felinine up to 80%. More recently a new procedure for the synthesis of felinine leading to high yields of this amino acid was published by Hendriks et al. (1995b). To obtain a standard for high-performance liquid chromatograph (HPLC), Hendriks et al, also followed the synthesis procedures of Trippett (1957), Eggerer (1962), Schoberl et al. (1966, 1968), using nuclear magnetic resonance, mass spectrometry and an HPLC procedure.

1.3 Biosynthesis of felinine

Avizonis and Wriston (1959) studied the biosynthesis of felinine in the cat and found that cystine, leucine and mevalonic acid can contribute to the formation of felinine in the cat. They injected C^{14}-labeled compounds into a cat and isolated radioactive felinine from the urine. Felinine concentration increased when cystine and leucine were fed to the cats. Shapiro (1962) and Wang (1964) found similar rates of incorporation of radioactivity into felinine and cholesterol when 2-[^{14}C]acetate was used. Shapiro (1962) suggested that mevalonic acid is diverted to the metabolic pathways for cholesterol and felinine and that these pathways use the same isoprenoid pool. Radioactivity from injected D,L-[^{14}C]leucine was also incorporated into felinine (Shapiro, 1962). This supports the observations made by Avizonis and Wriston (1959) that leucine is a precursor to felinine (Hendriks, et al., 1995). Roberts (1963) indicated that cystine and methionine are not

immediate precursors to felinine, as he failed to detect any radioactivity in felinine after cats were injected intravenously with [^{35}S]cystine and he was also unable to incorporate any 2-[^{14}C]mevalonic acid into felinine. Support for Roberts (1963) was provided by the experiments of Wang (1964). **Figure 1.2** illustrates the proposed metabolic pathway (Hendriks, et al., 1995) according to which felinine is believed to be synthesized in the cat (*F. catus*).

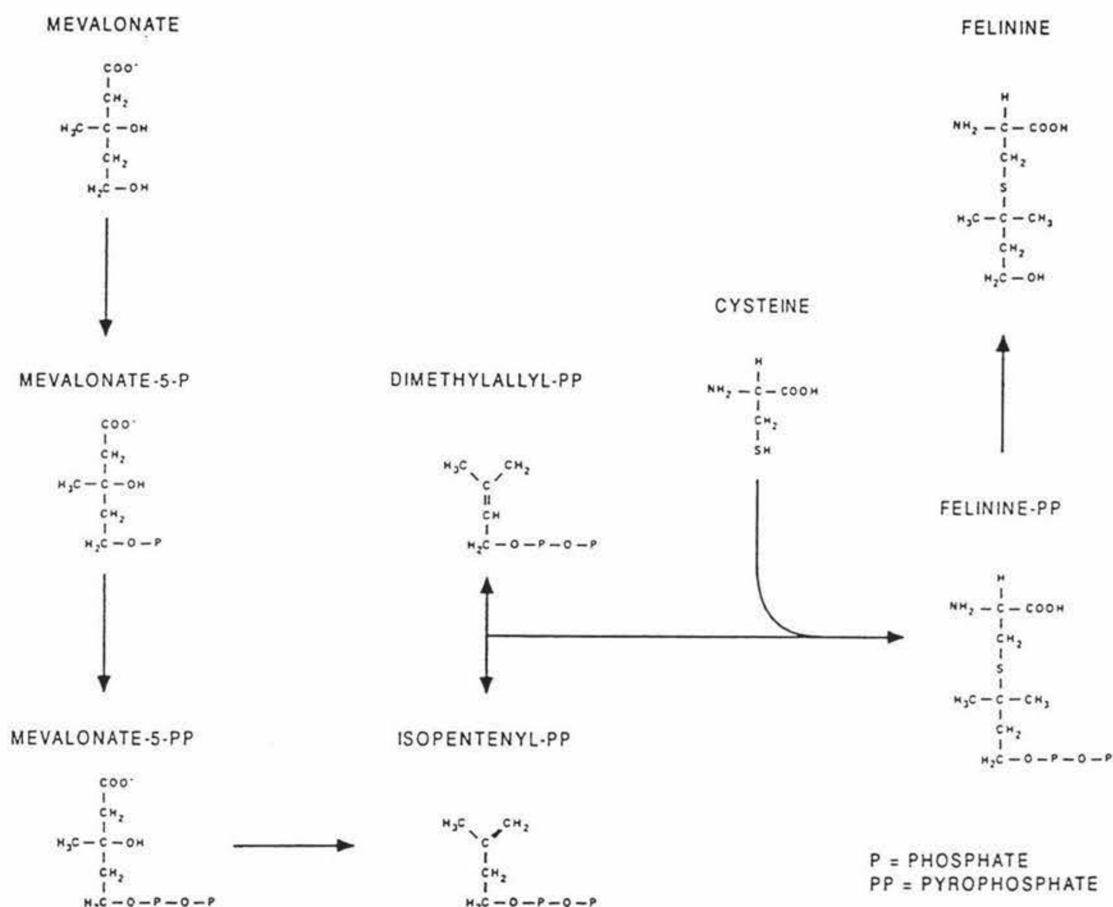

Figure 1.2 Proposed metabolic pathway for the biosynthesis of felinine in the domestic cat (Herdriks, 1995)

Felinine is believed to be synthesized from the same isoprennoid units as cholesterol in the cat. The major sites of synthesis of cholesterol in the rat, squirrel monkey, guinea pig, rabbit and hamster are the liver, skin, gastrointesterinal tract and carcass muscles (Spady and Dietschy, 1983). Similar studies in the cat have not been reported, but it may be assumed that cholesterol synthesis occurs in the same tissues (Hendriks, et al., 1995). The question of how felinine is transported from the site of synthesis to the kidney is raised. A

possible solution to the question concerning the transportation of felinine in the blood was provided by Kuwaki et al. (1963). Using guinea pig liver homogenates, they showed that isovalthine could be formed *in vitro* as part of a glutathione-isovaleric acid conjugate (GSIV).

In *vitro* synthesis of felinine in liver homogenates of the cat has largely been unsuccessful. Roberts (1963) and Wang (1964) failed to find any felinine produced in their experiments. Wang (1964) also attempted to demonstrate the presence of a glutathione conjugates, but none could be detected and no glutathionase activity could be demonstrated in the kidney. If felinine is transported in the blood as a glutathione conjugate, it would be expected that hydrolysis of the blood would yield felinine. However, it has been observed that synthetic felinine is an acid-labile amino acid that is destroyed under the conditions of 2 and 6 M HCl hydrolysis at 110°C for 24 hr (Hendriks, et al., 1995).

1.4 Biological significance of felinine

The biological significance of felinine to the animal is still unknown. Although felinine and cholesterol seem to be synthesized from the same isoprenoid pool, no direct evidence for a regulatory effect of felinine on cholesterol or steroid metabolism has been found (Hendriks, et al., 1995). A more likely role for felinine, as a urinary component for territorial marking, was suggested by MacDonald et al., (1984). The observed differences in urinary felinine excretion rates between male and female cats (Roberts, 1963; Hendriks et al., 1995a) are in accordance with this role. Further evidence to support this hypothesis was published by Joulain and Laurent (1989). They observed that "fresh" cat (F. catus) urine was mostly odourless, but upon "aging" an odour developed reaching a peak at ~12-24hr. The extent of the odour depended on the diet and the period within the sexual cycle of the cats. Two volatile components, 3-mercapto-3-methyl-1-butanol (I) and 3-methyl-3-methylthio-1-butanol (II) **(Figure1.3)** (Hendriks, et al., 1995), which may be formed on degradation of felinine, were found in the urine of the tom cat (Joulain and Laurent, 1989) and bobcat (Mattina et al., 1991). Other components (disulfides and trisulfides) in the urine of these animals that are derivatives of compounds I and II were also found by these authors. Hendriks et al., (1995b) noted that odourless synthetic

felinine stored as a lyophilisate at -20°C and room temperature developed an odour that was similar to the odour of tom cat urine. They suggested that felinine degrades to another compound or other compounds to give rise to the distinct tom cat urine smell.

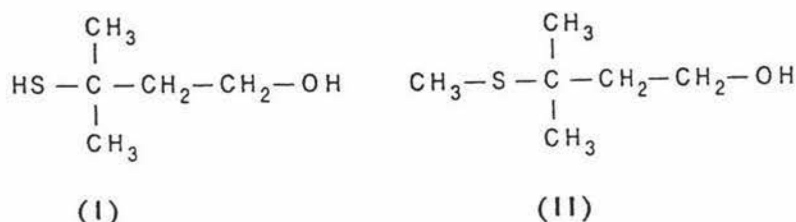

Figure 1.3 Molecular structure of 3-mercapto-3-methyl-1-butanol (I) and 3-methyl-3-methylthio-1-butanol (II) (Hendriks et al., 1995)

Although bobcat urine has been shown to have a repellent effect on the snowshoe hare (Sullivan et al., 1985a), the black-tailed deer (Sullivan et al., 1985b) and the white-tailed deer (Swihart, 1991), there is no direct evidence that felinine degrades to volatile compounds I and/or II whether any of these compounds or derivatives of these compounds actually have a repellent effect. Related to the possible repellent effect and territorial marking roles of felinine, is its possible role as a pheromone to attract the opposite sex (Hendriks, et al., 1995). Although no direct evidence is known to substantiate this hypothesis, Joulain and Laurent (1989) noted that the sexual cycle has some effect on the occurrence of the odour of tom cat urine. Further research into the function of felinine as a pheromone is warranted.

PART II CAPILLARY ELECTROPHORESIS

1.5 Background and advantages

Capillary electrophoresis is usually said to date from 1981, when Jorgenson and Lukacs (1981) described spectacular separations of peptides using free zone electrophoresis in glass capillaries of 75 μm i.d.; however, due to the limitations of light transmission through glass, fluorescence detection was needed. This capillary format enabled the Joule heating, which occurs in all electrophoretic separations and degrades the resolution by thermal mixing, to be efficiently dissipated. It was thereby possible to use much higher voltages (up to 30 kV) for electrophoretic separations and so shorten the analysis times. Later, glass capillaries were superseded by polyimide-coated silica capillaries which are much more robust in use and, more importantly, transmit ultraviolet light (Perrett, 1999).

Today, capillary electrophoresis has become a modern analytical technique for the separation and analysis of chemical compounds. Separations are based on the differences in electrophoretic mobilities of ions in electrophoretic media inside small capillaries (Jorgenson and Lukacs, 1981, 1983 and Karger *et al.*, 1989). Many methods of analysis that currently use high-performance liquid chromatography or slab gel electrophoresis will probably be converted to capillary electrophoresis because it offers highly efficient and fast separations, relatively inexpensive and long lasting capillary columns, small sample size requirements, and low reagent consumption (Wiktorowicz and Colburn, 1990; Novotny *et al.*, 1990 and Jorgenson, 1983). CE is characterized by its ability to resolve, using a high applied D.C. voltage (field strengths up to 500 V/cm), the components of complex aqueous samples with very high resolution (N > 200000 plates/m), analysing with analytical precision of less than 10 nL of sample (Perrett, 1999). The separated components are identified by on-line detection during the analysis, and the results are obtained in a matter of minutes, sometimes seconds. High-resolution separations of a wide variety of sample types can be done by capillary electrophoresis. It can be used for analysis of polar ionic, polar nonionic, and nonpolar nonionic compounds, as well as high molecular weight biomolecules (Lauer and McManigill, 1986; McCormick, 1988; Messana et al., 1997 and Dolnik, 1997), and chiral compounds (Nishi, 1997; Hage, 1997 and Fanali, 1997).

The same capillary can be used for separations and analyses of polar compounds, nonpolar compounds, chiral compounds, and large biomolecules at ambient temperature and low pressure without the need for a liquid pump. Separation can be performed on the basis of the size of the compounds in a mixture through the use of a gel-filled capillary. Compared to high-performance liquid chromatography columns, capillaries are relatively inexpensive, easy to use, and last a long time. Now, capillary electrophoresis is being used by more and more analysts in the fields of analytical chemistry and biochemistry.

1.6 Capillary electrophoresis system

The main components of a capillary electrophoresis system, shown in **Figure 1.4,** are a sample vial, source and destination vials, capillary, detector, high-voltage power supply, and a data output and handling device, such as an integrator or computer (Baker, 1995).

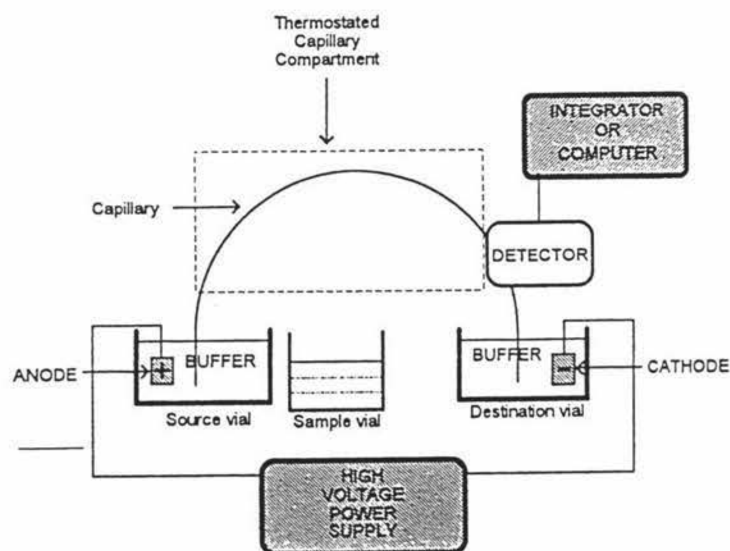

Figure 1.4 Schematic representation of a capillary electrophoresis system (Baker, 1995)

Electrophoresis is performed by filling the source vial, capillary, and destination vial with an electrolyte, usually an aqueous buffer solution. The capillary inlet is placed into a sample vial, the sample is introduced, then the capillary inlet is placed back into the source vial, and an electric field is applied between the source and destination vials. As the solutes

migrate through the capillary, they are detected by the detector and its output is sent to an integrator or computer. Since different solutes pass through the detector at different times, the separated compounds appear as peaks with different *migration times* in the electropherogram.

Sample injection

The most commonly employed injection methods for CE are direct on-column methods such as hydrodynamic (Huang *et al.*, 1989; Rose and Jorgenson, 1988 and Honda *et al.*, 1987) and electrokinetic (Jorgenson and Lukacs, 1981 and Rose and Jorgenson, 1988) injection. Hydrodynamic injection can be performed by pressure or siphoning. In electrokinetic injection, an electric field is applied to the sample vial, causing the sample components to migrate into the capillary.

Capillaries

Fused silica capillaries, which are externally coated with a polymer such as polyimide to improve their mechanical strength, that are 30-100 cm long with inner diameters of 50-75 μm and outer diameters of 375 μm are typically used. The cell window of a capillary can be made by simply burning or scraping off a small section of the polyimide outer coating of the capillary. This section of the capillary is then placed in the light path of the detector.

Detectors

A variety of detectors have been used in capillary electrophoresis, including: UV/Vis absorbance, fluorescence, laser-induced fluorescence, mass spectrometric, conductivity, amperometric, radiometric, and refractive index. The most widely used are UV/vis absorbance detectors.

Power supply

The purpose of the power supply is to provide an electric field across the capillary. It can be operated in either the constant voltage, constant current, or constant power mode and

have the ability to reverse the polarity. Voltages up to 30 kV, currents up to 300 μA, and power up to 6 W are used.

Data Handling

An electropherogram is a plot of detector response versus time, as shown in **Figure 1.5**. This is the same way data are displayed in a chromatogram obtained from a high-performance liquid chromatograph or a gas chromatograph, so the same type of data handling that is used in these techniques can be used in capillary electrophoresis.

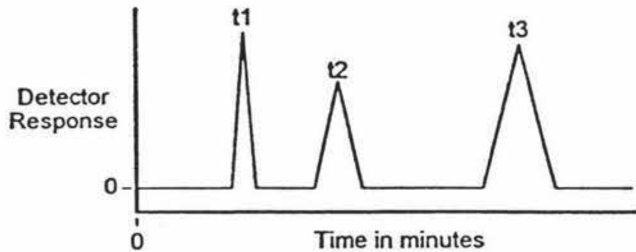

Figure 1.5 Drawing of a capillary electropherogram. t1, t2, and t3 represent the migration times of sample components, measured at the apices of the peaks (Baker, 1995).

Qualitative analysis could be done by measuring the migration time of a compound of unknown identity and matching its migration time to that of a compound whose identity is known. By comparing migration times of unknowns and standards, we can determine which peak corresponds to which compound. Quantitative analysis is done by injecting a standard containing known concentrations of each of the components of interest, and measuring either the heights or areas of each of the resultant peaks. Then, a sample containing unknown concentrations of the sample components is injected and its peak heights or areas are measured. By comparing areas or heights obtained from the standard to those obtained from the sample, the concentrations of components in the sample can be determined.

1.7 Modes of capillary electrophoresis

Different modes of capillary electrophoresis separations can be performed using a standard CE instrument. The most often used modes of CE are capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). The mechanisms by which solutes separate in the six techniques are illustrated in **Figure 1.6** and **Figure 1.7** (Li, 1992). The migration of each type of charged species under the influence of the applied voltage is represented by an arrow in **Figure 1.6**, and the distribution of electrolytes and a two-component sample are shown at three different times in **Figure 1.7**.

1.7.1 Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis (CZE) is also referred to as free solution capillary electrophoresis (FSCE). Currently CZE is the most commonly used separation mode available within CE. Many compounds can be separated rapidly and easily. It can be used to separate almost any ionized compounds that are soluble in a buffer. Samples as diverse as small inorganic anions (Harrold, et al., 1993 and Jandik and Jones, 1991) and large biomolecules (Knox, 1988) have been separated by CZE. The separation in CZE is based on the differences in the electrophoretic mobilities of the ions. The mobility differences result in different velocities of migration of the ionic species in the electrophoretic buffer contained in the capillary.

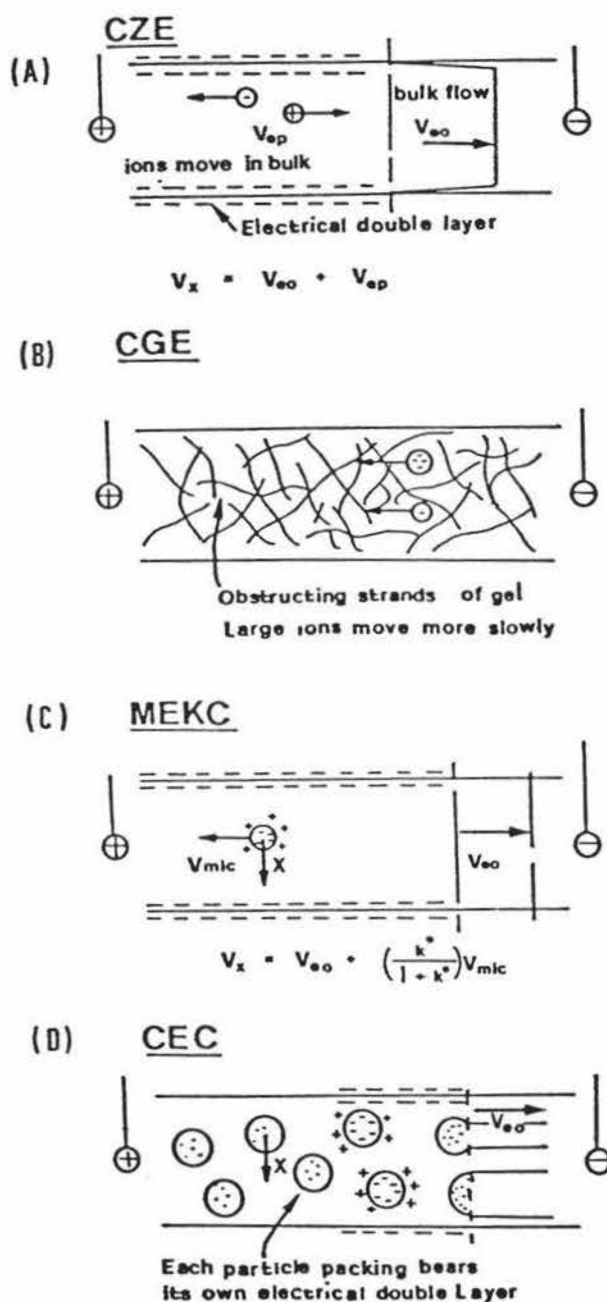

Figure 1.6 Diagramatic representation of (A) capillary zone electrophoresis (CZE), (B) capillary gel electrophoresis (CGE), (C) micellar electrokinetic chromatography (MEKC), and (D) capillary electrokinetic chromatography. v_x is the linear migration velocity of the analyte X. v_{eo} is the electroosmotic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

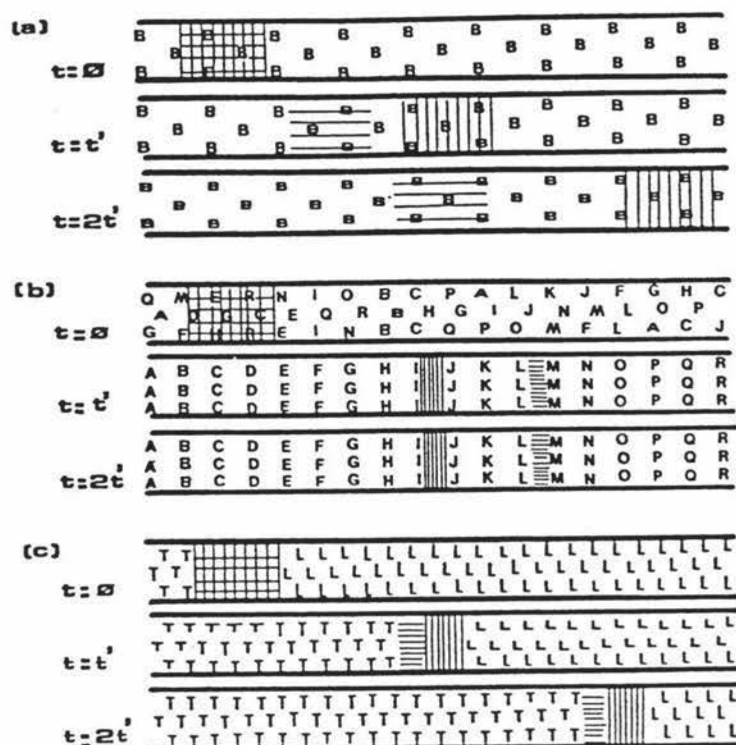

Figure 1.7 Schemes of electrophoretic techniques: (a) zone electrophoresis, (b) isoelectric focusing, and (c) isotachophoresis. The distribution of electrolytes and a two-component sample are shown at three different times: the start of the analysis (t = 0), the time interval t′ after the start (t = t′), and the double time interval after the start (t = 2t′) (Li, 1992).

The separation mechanism is mainly based on differences in solute size and charge at a given pH. **Figure 1.8** represents a CZE separation. A sample is injected into the capillary, which is filled with a buffer, and when a voltage is applied, the solutes migrate through the capillary in zones. Solutes are separated as they move through the capillary due to differences in their rates of migration, which are dependent on their electrophoretic mobilities. Electroosmotic flow moves the solutes through the capillary from the anode to the cathode. The order of elution in CZE is cations, neutrals, then anions. Neutral compounds are not separated in CZE, whereas ions are separated on the basis of their charge-to-size ratios. Cations are attracted towards the cathode and their speed is augmented by the electroosmotic flow, with small, highly charged cations eluting first, follow by cations with reduced charge/size ratio. Neutral molecules, which move through the capillary under the influence of only the electroosmotic flow and are not separated

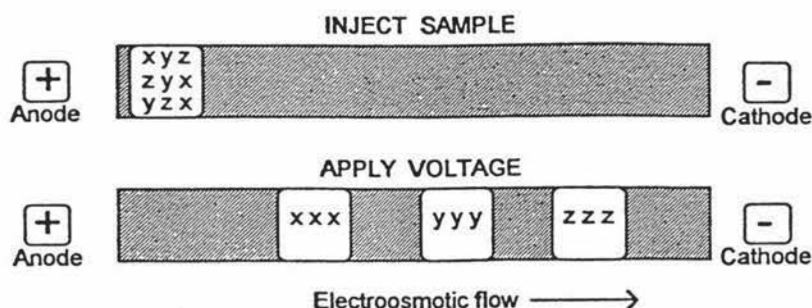

Figure 1.8 Separation by capillary zone electrophoresis with "normal" electroosmotic flow and polarity. The *x*'s, *y*'s, and *z*'s represent ionic solutes with different charge-to-size ratios, with *z* having the highest ratio and *x* the lowest (Baker, 1995).

from each other, elute after the cations. Anions, which are attracted to the positive electrode and consequently tend to migrate in the opposite direction to the electroosmotic flow, elute last. Anions with lower charge/size ratio migrate earlier than those with greater charge/size ratio. The anions with the greatest electrophoretic mobilities migrate last. **Figure 1.9** is a representation of the elution order in CZE.

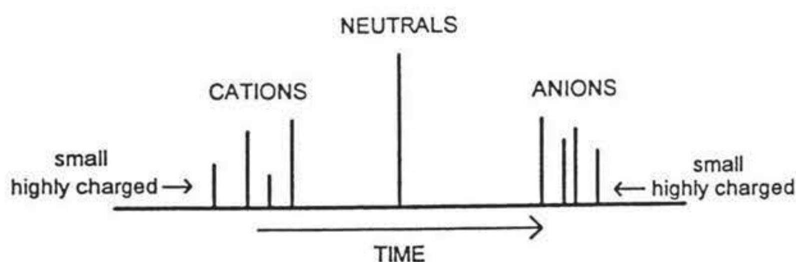

Figure 1.9 Drawing of a capillary zone electropherogram indicating elution order. Small, highly charged cations elute first (Baker, 1995).

1.7.2 Capillary gel electrophoresis (CGE)

Capillary gel electrophoresis (CGE), which combines the principles of slab gel electrophoresis with the instrumentation and small diameter capillaries of CZE, was introduced by Cohen and Karger (1987). The separation efficiency which they achieved was extremly high using gel-filled capillary columns. The capillaries were filled with

polyacrylamide gels which contained sodium dodecyl sulfate. This technique is referred to as a capillary SDS-PAGE separation and has been used for the separation of proteins, polynucleotides and DNA fragments (Hjerten, et al., 1987).

In CGE, the capillary is filled with a gel, which is usually either a polyacrylamide/bisacrylamide crosslinked polymer or a linear, noncrosslinked polyacrylamide polymer. There are pores within these gels, and as charged solutes migrate through a gel-filled capillary, they are separated by a molecular sieving mechanism on the basis of their sizes. Small molecules are able to pass through the pores and elute first, whereas larger molecules are retarded by the gel and elute later. In CGE, the solutes are separated on the basis of size, so it is well suited for the analysis of charged molecules that vary in size, but not in their charge-to-size ratios, regardless of their chain lengths, such as oligonucleotides, DNA restriction fragment, or proteins.

1.7.3 Micellar electrokinetic capillary chromatography (MEKC)

Micellar electrokinetic capillary chromatography was introducted by Terabe and co-workers (1984, 1989). It is the most popular of the electrokinetic chromatographic techniques (Terabe, et al., 1991). Separation in MEKC is based on a combination of electrophoretic mobility and chromatographic partitioning between the slower moving micellar "pseudo stationary phase" and the solution phase. The separation mechanics have been illustrated by Dulffer (1990). The technique provides a way to separate both ionic and neutral analytes. Also, large peptides have been separated by MEKC (Yashima, et al., 1992).

MEKC is most commonly performed with anionic surfactants, especially sodium dodecyl sulfate (SDS). Micelles form in solution when a surfactant is added to water in concentrations above its critical micelle concentration (CMC). In the case of anionic surfactants, the hydrophobic hydrocarbon molecules are in the centre of the micelle and the charged head groups around the surface of the spheres. The micelles can be considered as small droplets of oil with a highly polar surface which is negatively charged.

They are used with ionic substances as well as organic modifiers with can lead to significant changes in resolution. The micelles of MEKC can also be replaced with any

material that reacts differentially with the analytes of separation and affects their velocity through the capillary.

1.7.4 Capillary isoelectric focusing (CIEF)

Capillary isoelectric focusing (CIEF) is a relatively new mode of capillary electrophoresis introduced by Hjerten and Zhu (1985). CIEF is a focusing type of capillary electrophoresis in which the solutes, usually proteins, are separated on the basis of their isoelectric points or pI values (Hjerten and Zhu, 1985). In CIEF, the protein samples and a solution that forms a pH gradient are placed inside a capillary. The anodic end of the column is placed into an acidic solution (anolyte), and the cathodic end in a basic solution (catholyte). When an electric field is applied, charged proteins migrate through the capillary until the pI is reached (where they become electrically neutral) and therefore stop migrating. At this time, a steady state is attained. Sharp peaks with good resolution are obtained, and a large peak capacity is observed.

1.7.5 Capillary isotachorphoresis (CITP)

Capillary isotachorphoresis is a moving boundary technique in which the sample is sandwiched between two buffers. The main feature of CITP is that it is performed in a discontinuous buffer system with "leading" and "terminating" electrolytes. The leading electrolyte (or buffer) has the ion with the highest mobility whilst the terminating electrolyte (or buffer) has the ion with the lowest mobility (Schwer and Kenndler, 1990; Jougenson, 1986). These leading and terminating buffers, act to separate the anions of the sample from each other. Although CITP separates analytes on the basis of their electrophoretic mobilities, the means of separation and the output derived from the separation are significantly different from other electrophoretic techniques. In CITP, the isotachopherogram obtained contains a series of steps, with each step representing an analyte zone. The quantitation in CITP is mainly based on the measured zone length which is proportional to the amount of sample present. CITP is useful only for the separation of ionic materials, and it is not possible to separate anions and cations in the same run.

1.8 Principles of separation in capillary zone electrophoresis (CZE)

1.8.1 Electroosmosis

An important phenomenon in capillary electrophoresis is electroosmosis, which refers to the flow of solvent in an applied potential field. At pH values above 3, the fused silica capillary has negative surface charges due to ionization of silanol groups. Positive ions in the buffer will be attracted to these fixed anionic sites, forming an electrical double layer. When high voltage is applied, migration of hydrated cations in this double layer causes movement of bulk fluid in the direction of the cathode. This phenomenon is called eletroosmosis, as represented in **Figure 1.10**. The magnitude of electroosmotic flow (EOF) increases as a function of pH at a rate much greater than the migration speed of sample ions. This can be an advantage when analyzing mixtures of anionic and catonic species, since all analytes will eventually swept past the detection point by EOF.

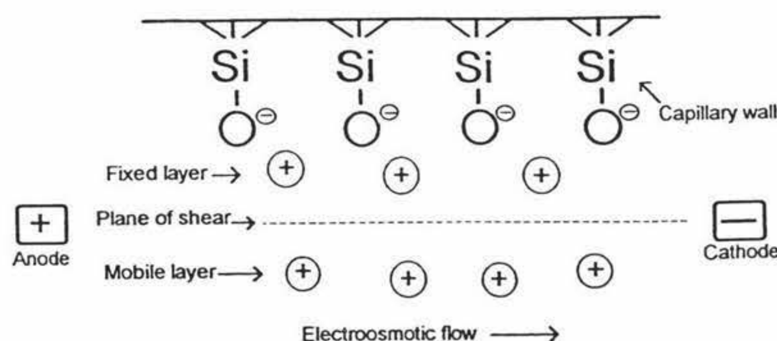

Figure 1.10 Representation of electroosmotic flow in a capillary. Electroosmotic flow is caused by the negatively charged Si-O⁻ groups on the inner wall of the capillary attracting the positively charged cations, represented by the circled +'s, forming the fixed layer. The mobile layer of cations is pulled toward the cathode, dragging the bulk buffer solution with it. The anions and the solvation of the cation are not shown (Baker, 1995)

The potential across the layers is called the zeta potential, denoted by ζ, which is given by the Helmholtz equation:

$$\zeta = 4\,\pi\,\eta\,\mu_{eo} / \varepsilon \qquad (1.1)$$

where η is the viscosity, ε is the dielectric constant of the solution, and μ_{eo} is the coefficient for electroosmotic flow (Li, 1992). It is important that electroosmotic flow be

consistent from run to run, and control of EOF often requires extensive capillary conditioning before and between analyses. If the flow varies, the migration times of the solutes will change, which may cause peaks to be identified incorrectly or errors in quantitation.

The velocity of the electroosmotic flow, v_{EOF} is given by

$$v_{EOF} = \varepsilon \zeta E / 4 \pi \eta \tag{1.2}$$

where E is the applied electric field in vols/cm.

The electroosmotic mobility, μ_{EOF}, of the buffer is given by

$$\mu_{EOF} = \varepsilon \zeta / 4 \pi \eta \tag{1.3}$$

Note that electroosmotic mobility is dependent solely on buffer characteristics, that is, dielectric constant, viscosity, pH, and concentration (which influence the zeta potential) and is independent of the applied electric field.

There are several approaches to alter electroosmotic flow. The methods used involve changing the zeta potential across the solution-solid interfaces; increasing the viscosity at the interface; adjusting the pH and the ionic composition of the buffer and introducing additives to the buffer to alter the zeta potential developed across the capillary solution interface. Other approaches of varying or elimination electroosmotic flow include covalently bonding γ-methacryloxylpropyltrimethysilane to the glass surface or coating the capillary wall with a polymer such as methylcellulose (Li, 1992).

1.8.2 Electrophoretic mobility

An electrically charged solute will migrate through a buffer under the influence of an electric field with an electrophoretic velocity, v_{EP}, given by

$$v_{EP} = \mu_{EP} E \tag{1.4}$$

where μ_{EP} is the electrophoretic mobility and E the applied electric field. Separation is achieved because solutes migrate through the capillary at different velocities. Electrophoretic mobility is related to its charge-to-size ratio as given by

$$\mu_{EP} = q / 6 \pi \eta r \quad (1.5)$$

where q is the charge of the ionized solute, η the buffer viscosity, and r the solute radius. Anything that affects the charge or size of a solute or the viscosity of the buffer will cause changes in the electrophoretic mobility. For a given applied electric field and buffer, the greater the charge-to-size ratio (q/r), the higher the electrophoretic mobility and velocity. Small, highly charged molecules move through the capillary the fastest, and large molecules with a lower charge move slower. Neutral molecules have an electrophoretic mobility of zero.

1.8.3 Factors affecting performance in capillary electrophoresis

Joule heating is an important factor to consider when performing any electrophoretic separation. A major limitation on the speed, resolution, and scale of electrophoretic separations is the ability to dissipate the Joule heat that is generated as a result of the electric current passing through the electrophoresis buffer. This Joule heating and the resulting temperature gradient can negatively affect the quality of the separation.

Migration time, efficiency, selectivity and resolution are the parameters of an electrophoretic separation. They are influenced by one or more of the electrophoretic parameters, including voltage, electrophotetic mobilities, electroosmotic flow, and capillary length.

The migration time of a solute, t_m, is the time it takes it to migrate the effective capillary length, l, from the inlet to the detector. Higher voltages, shorter capillaries, and high electroosmotic flows give shorter migration times and, therefore, faster analysis times. Efficiency, N, is expressed as the number of theoretical plates, and can be calculated by measuring the migration time and the peak width. If the peak width is measured at half the peak height, $w_{1/2}$, N can be calculated by

$$N = 5.54\,(t_m / w_{1/2})^2 \tag{1.6}$$

It can be seen that the narrower a peak and the longer its migration time, the higher its efficiency.

Selectivity, α, has to do with how far apart adjacent solutes are when they pass through the detector, that is, the distance between the apices of adjacent peaks in an electropherogram. Selectivity is dependent on differences in electrophoretic mobilities. The most effective way to affect a change in selectivity is to change the pH of the buffer.

Resolution, R, is the most important separation parameter. It shows how well the components in a mixture are separated. Resolution is influenced by the applied voltage, buffer pH and composition, capillary length and electroosmotic flow. It is increased by increasing the applied voltage, by optimizing the buffer pH and composition, increasing capillary length, and by optimizing the electroosmotic flow.

1.9 Applications of capillary electrophoresis in biological fluids

The application of CE methodology to the analysis of biological fluids has provided great promise for its use in clinical chemistry. Recently, a few studies employing CE for the rapid analysis of biological fluids have been reported. Analytical methods based on CE for diagnosis and studies of several types of human diseases have been developed (Jellum, et al., 1991). Separation of urinary porphyrins by MEKC has been performed (Weinberger, et al., 1990) and amino acids in human urine were separated by CE (Yu and Dovichi, 1989). Schoots et al. (1990) used CE to identify hippuric acid, 2-hydroxyhippuric acid, and uric acid in the blood serum of chronic renal-failure patients. Miyake et al. (1991) used MEKC to quantify the levels of creatinine and uric acid in human plasma and urine. Guzman et al. (1990), Lee et al. (1992) and Guo (1998) used CZE to quantify creatinine and creatine in serum and urine. Barbas, et al. (1998) developed a CE method to separate, identify, and measure the short-chain organic acids in urine. Petucci et al. (1995) reported a CZE method for the analysis of biological fluids for low-molecular-mass species. CE is suitable

for detecting important changes in the metabolic profiles of body fluids and provides a rapid and simple alternative to other techniques in routine analysis.

Since capillary electrophoresis has been widely and successfully applied for the analysis of various biological fluids, it should be possible to analyse the amino acid, felinine, by this modern method. The aim of my study is to develop a capillary electrophoresis method for simple, rapid and accurate determination of felinine in biological fluids.