ON-LINE PRECONCENTRATION BY ANALYTE FOCUSING MICELLE COLLAPSE IN CAPILLARY ELECTROPHORETIC SEPARATION OF STEROIDS

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In the name of Allah, the Most Merciful and the Most Beneficent.

This thesis is dedicated to my beloved mother, Noor Aidah binti Rahim and loving father, Mohamed Khir bin Abu Bakar and all family members

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ABSTRACT

Analyte focusing by micelle collapse (AFMC) is an on-line sample preconcentration of neutral analyte for capillary electrophoresis (CE). The purpose of this research is to study the separation of six neutral steroids; prednisolone, prednisone, betamethasone, testosterone, androstenedione, and methyltestosterone by AFMC-micellar electrokinetic chromatography (MEKC) technique. The separation of neutral steroids using MEKC was also conducted as a comparison of enhancement factor to AFMC-MEKC. The use of a mixture of 20 mM sodium dodecyl sulphate (SDS) and methanol 10% (v/v) in 25 mM acetate buffer (pH 9.0) with a positive applied voltage of 25 kV and pressure injection of 40 mbar for 1 sec at 25°C was able to separate the steroids by MEKC. AFMC-MEKC was employed for enhancement in terms of sensitivity. The selected steroids were prepared in a solution containing 7 mM SDS and 250 mM acetate buffer with an optimized conductivity ratio of 0.34. The separation of the six steroids by AFMC-MEKC showed enhancement of sensitivity for each steroids with 2.4 to 3-fold enhancement with good repeatability (RSD 3.5-7.1% , n = 3) and reproducibility (RSD 2.6-12.7%, n = 3). The limits of detection for the six steroids ranged from 1.1-5.7 mg/L. The optimized method was successfully applied to analyze steroids in pharmaceutical tablets and urine samples. Combination of AFMC-MEKC with solid-phase extraction was used to determine six selected steroids in spiked urine sample while liquid-liquid extraction pretreatment was used for the determination of prednisolone and betamethasone in pharmaceutical tablets. The average recoveries of the selected steroids in spiked urine samples and pharmaceutical tablets were good ranging from 93 - 101% with RSD of 0.8 - 11.2% (n = 3). AFMC-MEKC separation on selected steroids showed triple fold enhancement in sensitivity compared to MEKC.

ABSTRAK

Pemfokusan analit melalui keruntuhan misel (AFMC) adalah kaedah pemekatan talian terus bagi analit neutral dalam elektroforesis rerambut (CE). Tujuan penyelidikan ini adalah untuk mengkaji pemisahan steroid neutral; prednisolon, prednison, betametason, testosteron, androstendion, dan metiltestosteron melalui kaedah AFMC-kromatografi elektrokinetik misel (MEKC). Pemisahan steroid neutral ini turut dilakukan menggunakan MEKC sebagai perbandingan faktor peningkatan kepada AFMC-MEKC. Penggunaan campuran 20 mM natrium dodesil sulfat (SDS) dan metanol 10% (v/v) dalam 25 mM larutan elektrolit asetat (pH 9.0) dengan voltan positif 25 kV serta suntikan tekanan sebanyak 40 mbar selama 1 saat pada 25°C mampu memisahkan steroid tersebut melalui MEKC. Dalam analisis steroid tersebut, penggunaan AFMC-MEKC bertujuan meningkatkan tahap kepekaan pengesanan. Steroid terpilih disediakan dalam larutan yang mengandungi 7 mM SDS and 250 mM larutan elektrolit asetat dengan nisbah kekonduksian optimum sebanyak 0.34. Pemisahan keenam-enam steroid melalui AFMC-MEKC menunjukkan peningkatan kepekaan pengesanan bagi setiap steroid antara 2.4 hingga 3-kali ganda dengan keterulangan (RSD 3.5-7.1% , n = 3) dan kebolehulangan yang baik (RSD 2.6-12.7%, n = 3). Had pengesanan bagi keenam-enam steroid adalah dalam julat 1.1 hingga 5.7 mg/L. Kaedah optimum yang diperoleh telah berjaya digunakan untuk menganalisis steroid dalam tablet farmaseutikal dan sampel urin. Gabungan AFMC-MEKC dan pengekstrakan fasa pepejal telah digunakan bagi menentukan enam steroid terpilih dalam sampel urin. Manakala, pengekstrakan cecair-cecair dilakukan bagi mengenalpasti kandungan prednisolon dan betametason dalam tablet farmaseutikal. Purata perolehan semula steroid terpilih dalam sampel urin dan tablet farmaseutikal adalah baik, iaitu dalam julat 93 - 101% dengan RSD sebanyak 0.8 - 11.2% (n = 3). Kaedah pemisahan melalui AFMC-MEKC ke atas steroid terpilih menunjukkan tiga kali peningkatan kepekaan berbanding dengan MEKC.

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LIST OF ABBREVIATIONS

AFMC	-	Analyte Focusing by Micelle Collapse
BGS	-	Background Solution
CE	-	Capillary Electrophoresis
CGE	-	Capillary Gel Electrophoresis
CIEF	-	Capillary Isoelectric Focusing
CITP	-	Capillary Electrochromatography
CMC	-	Critical Micelle Concentration
CZE	-	Capillary Zone Electrophoresis
EOF	-	Electroosmotic Flow
HPLC	-	High-Performance Liquid Chromatography
LIF	-	Laser Induced Flourescence
LOD	-	Limit of Detection
MEECK	-	Micro-Emulsion Electrokinetic Chromatography
MEKC	-	Micellar Electrokinetic Chromatography
NaOH	-	Sodium Hydroxide
S	-	Neutral Analytes
SC	-	Sodium Cholate
SDS	-	Sodium Dodecyl Sulphate
UV	-	Ultraviolet

LIST OF SYMBOLS

cm	-	Centimeter
ºC	-	Degree Celcius
µm	-	Micrometer
µA	-	Micro Ampere
µL	-	Micro Litre
nL	-	Nano Litre
i.d	-	Inner Diameter
l	-	Effective Capillary Length
M	-	Molarity
N	-	Efficiency
pI	-	Isoelectric Point
ppm	-	Part Per Million
T	-	Temperature
t_m	-	Migration Time
V	-	Volt
σ	-	Standard deviation

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Steroid is an organic compound that contains a specific arrangement of four cycloalkane rings that are joined to each other. Examples of steroids include the dietary fat cholesterol, the sex hormones estradiol and tetstosterone, and the anti-inflammatory drug dexamethasone. In addition to the naturally occurring steroids, testosterone derivatives such as methyltestosterone and androstenedione have been used as pharmaceuticals for enhancement of athletic performance. The illegal abuse of these agents may give bad effects on consumers such as prostatic cancer, impotency, and acromegaly in addition to hirsutism and amenorrhea in females (Makin *et al*., 1995).

The most useful techniques for drug and steroid separation are chromatography and electrophoresis such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). HPLC is one of the most commonly used separation technique for steroid analysis. However, HPLC uses larger volume of mobile phase resulted on more waste disposal. CE is a relatively modern analytical technique which permits rapid and efficient separations of charged components present in small sample volumes. Separations are based on the differences in electrophoretic mobilities of ions in electrophoretic media inside a small capillary (Jorgenson and Lukacs, 1981). It is extensively ilized in biological,

environmental, pharmaceutical, toxicological, forensic, clinical, and food-analytical fields (Aranas *et al.*, 2009).

HPLC has different modes of chromatography, including reversed phase, normal phase, ion exchange, size exclusion, chiral, hydrophobic interaction, and affinity. Similar to HPLC, CE also has different modes which attribute to the versatility of this method.

Micellar electrokinetic chromatography (MEKC) is a dynamic mode of CE since it can be used for charged and uncharged analytes and for a wide range of substance with hydrophobic characteristics. It is a modification of CE, where the samples are separated by differential partitioning between micelles as pseudo-stationary phase and a surrounding aqueous buffer solution as mobile phase. Thus, for the separation of steroids, MEKC is chosen as the hydrophobicity characteristics of steroids that take part in the interaction between the steroid and micelle as the micelle will carry the analyte through the detector. The basic set-up and detection methods used for MEKC are the same as those used in CE. The difference is that the solution contains a surfactant at a concentration that is greater than the critical micelle concentration (CMC). Above this concentration, surfactants monomers are in equilibrium with micelles (Abubaker *et al.*, 1995).

MEKC is performed in open capillaries under alkaline conditions to generate a strong electroosmotic flow. Sodium dodecyl sulfate (SDS) is the most commonly used surfactant in MEKC applications. The anionic character of the sulfate groups of SDS cause the surfactant and micelles to have electrophoretic mobility that is counter to the direction of the strong electroosmotic flow. As a result, the surfactant monomers and micelles migrate quite slowly, though their net movement is still toward the cathode. During a MEKC separation, analytes distribute themselves between the hydrophobic interior of the micelle and hydrophilic buffer solution (Abubaker *et al.*, 1995).

UV absorbance detectors are the most popular among the CE detectors. Since the volume or length of the sample zone is very small (nanoliters or millimeters), it is essential to employ on-capillary detection methods in order to not deteriorate separation efficiency. However, the path length of the light is nearly equal to the diameter of the capillary (50–100 mm), <1/100 of the conventional detection cell used in HPLC. Therefore, the concentration sensitivity of the UV detector in CE is much lower than that in HPLC. To improve the concentration sensitivity, several techniques have been developed. Among the techniques is the use of high sensitivity detectors such as laser-induced fluorescence (LIF), chemiluminescence or electrochemical detectors in addition to the sample preconcentration (Skoog, *et al.*, 2007).

Online sample preconcentration is a unique characteristic of CE, where a large volume of sample is introduced into the capillary by pressurized or electrokinetic methods and the analytes in the sample solution are concentrated into a narrow zone before separation. The principle of most techniques is based on the velocity change of the analytes during electrophoresis. Instead of using high sensitivity detectors, several on-line preconcentration techniques like sweeping, stacking and pH-junction are employed in CE to enhance the concentration sensitivity and to reduce the limits of detection (LODs) due to its low cost (Simpson *et al.*, 2008).

More recently, the CE phenomenon of analyte focusing by micelle collapse (AFMC) was introduced as an on-line sample preconcentration of neutral and charged analytes. This unique technique relies on the use of a micellar carrier phase in the sample that can collapse into a micellar dilution zone found in between the sample and background solution. The sample is prepared in a matrix containing the micelles and an electrolyte anion of high mobility, while the conductivity of background solution is lower compared to the sample (Quirino, 2008).

In analyte focusing by micelle collapse (AFMC), the analytes that have a reasonable affinity for the pseudostationary phase (PS) are carried through the sample. Upon application of the voltage, and reaching the sample/background electrolyte (BGE) boundary, the micelle collapse as their concentration is adjusted below the critical micelle concentration. As a result, the analytes are released. As the analytes cannot move unless associated with the micelle, they remain on the boundary while the remaining analytes are deposited and thus their concentration is increased.

The steps of AFMC, namely transport, release and accumulation of analytes with the aid of micelles are investigated by looking at the effect under various parameters including retention factor of analytes, concentration of micelle forming agent in the sample, conductivity ratio of BGE to the sample, conductivity or concentration of the electrolyte salt in the background electrolyte, as well as organic modifier content in the BGE.

1.2 Steroid Analysis

Steroids consist of an essentially lipophilic or hydrophobic, non-polar cyclopentanoperhydrophenanthrene nucleus modified on the periphery of the nucleus or on the side chain by the addition of hydrophilic and polar groups. These additional groups are mainly hydroxyl and oxo or carboxylic acid groups. Despite the addition of these polar groups, the essential non-polarity of the steroids means that they are all too varying degrees soluble in organic solvents and can thus be extracted from aqueous media by a solvent or solvent mixture of suitable polarity (Makin *et al.*, 1995).

There are many situations in clinical and forensic toxicology when the solid form of a drug is encountered. It is advantageous to be able to identify the steroid directly from this evidence without recourse to analysis, although the suite of analytical techniques available to and used by toxicologists is extensive.

The methods presently available for the quantitative determination of steroids include colorimetric, fluorimetric, chromatography, and immunoassays. Colour tests and thin layer chromatography (TLC) and immunoassays are typically used for screening purposes to direct the choice of analyses used for identification and quantification. Spectroscopic techniques such as UV, visible and fluorescence may be used as stand-alone techniques for screening or quantification, but are also often hyphenated with chromatographic techniques such as HPLC or CE. Gas chromatography (GC) is another important technique used in toxicology and typically coupled with mass spectrometry similar to HPLC and CE. This technique provides what is often considered to be the best combination technique for identification and quantification (Makin *et al.,* 1995).

1.3 Problem Statement

Analytical methods have been developed for the separation of steroids; these include HPLC, GC, and CE. However, only a few of these methods were designed for the separation of a mixture of steroids due to the close structural similarities between such steroids. The structural similarity and small concentration of these steroids in urine, serum and saliva samples makes their determination an analytical challenge. The challenges are two-fold: first the close structural similarity of these steroids demands a separation technique with high resolution, and secondly the low concentrations in the parts per billion (ppb) ranges, require a sensitive detection capability. It is an important need for analytical methods for the determination of these steroids.

Despite their relatively simple chemical structure, steroids occur in a wide variety of biologically active forms. This variety is not only due to the large range of compounds secreted by steroid-synthesizing tissues, but also to the fact that circulating steroids are extensively metabolized peripherally, notably in the liver, and in their target tissues, where conversion to an active form is sometimes required before they can elicit their biological responses. Steroid metabolism is therefore important not only for the production of these hormones, but also for the regulation of their cellular and physiological actions.

For certain classes of hormones and particular target tissues, steroids must be converted in situ to an active form before they can interact with their specific receptors. This metabolic activation step is either an absolute prerequisite or a way of achieving a range of complex effects which involve interaction with more than one type of receptor. The metabolism of steroid may cause the level of the steroids in real sample such as human blood or urine in low trace level or amount. Therefore, separation analytical method that is efficient and effective would be preferred. Thus, CE gives a better choice for the separation since it is a green technology which requires smaller amount of reagents and samples.

HPLC is one of the most commonly used separation technique for steroid analysis. Detection limit obtained with HPLC for analysis of steroid was reported in the range of 64-70 ng/L (Amundsen and Siren, 2006). However, HPLC uses larger volume of reagents as mobile phases resulted on more waste disposal. In HPLC, flow rates are typically 1-2 mL/min, requiring 500-1000 mL of mobile phase for a day of operation, whereas in CE, only a few mL of buffer are required for a day of analyses. Thus, CE is preferred.

For the separation of steroids using CE, MEKC mode was chosen since it provided a method for separation of electrically neutral compounds as neutral analytes are not influenced by electrophoretic mobility, and therefore move through the capillary at the same rate of EOF. Thus, the development of MEKC as a dynamic

mode of CE was a major advancement. Using MEKC, even extremely hydrophobic compounds such as polycyclic aromatic hydrocarbons have been separated (Terabe *et al.*, 1984).

Similar to other CE modes, MEKC suffers from poor detection sensitivity due to the limited amount of sample that can be introduced into the capillary (typically <1 µL) and short optical path length for on-line spectrophotometric detection (typically 50-100 µm) necessitates a new on-line sample preconcentration study. Based on previous study for the analysis of anabolic steroids, under optimal conditions, anabolic steroids; androstenedione, methandrostenolone, methyltestosterone, 19-androstenedione, 1,4-androstadiene-3,17-dione were completely separated within 12 min with the detection limits ranged from 0.20 to 0.51 µg/mL (Zhang *et al.*, 2009).

In order to achieve lower LOD, an on-line preconcentration which is known as AFMC was recently introduced in the mode of MEKC as an alternative approach. The focusing mechanism is based on the transport, release, and accumulation of molecules bound to micelle carriers that are made to collapse into a liquid phase zone. Then, the analytes will be released from the micelle and passed through to the detector. Thus, only analyte will be highly focused. This relatively new technique has potentials to be developed for the separation of neutral drugs and steroids as they are neutral analytes.

1.4 Objectives of Study

The aims of this study are as follows:

a) to separate six neutral steroids; prednisolone, prednisone, betamethasone, testosterone, 4-androstene-3,17-dione, and 17-α-methyltestosterone by MEKC.
b) to employ AFMC-MEKC technique in steroid analysis
c) to apply the optimized AFMC-MEKC method for steroid analysis in urine samples and pharmaceutical tablets.

1.5 Scope of Study

On-line preconcentration technique in CE known as AFMC was employed in this research. This technique was studied by looking on several parameters such as conductivity ratio between the sample and background solution and SDS concentration in the sample that may affect the performance of AFMC.

This technique was carried out using neutral steroids. Steroids were chosen from steroid hormones group which are corticosteroids (prednisolone, prednisone and betamethasone) and anabolic steroids (4-androstene-3,17-dione, testosterone, and 17-α-methyltestosterone). These steroids were chosen as they are commonly abused and widely used in pharmaceutical applications. The optimized conditions were applied to analyse real samples of pharmaceutical tablets and spiked urine samples.

1.6 Significance of Study

Analytical methods have been developed for the determination of some steroidal compound in different matrices includes HPLC, GC and CE. Interestingly, only a few of these methods were designed for the separation of a mixture of steroids due to the close structural similarities between such steroids. Thus, there is an important need for analytical methods for the determination of mixtures of these steroids. Under several considerations as stated previously; CE gives a better option for the separation method.

A dimension for on-line preconcentration in CE without the modification of current CE commercial instrumentation known as AFMC is introduced in order to improve the concentration sensitivity and produce lower limit of detection (LOD). The capture, accumulation, transport and releaser of molecules with carriers are highly important processes with vast practical applications. Introduction of new mechanisms on the micellar transport, release and accumulation of molecules will transform the use of these carrier-based processes in chemistry and related fields. AFMC was reported by Quirino and Haddad (2008) as the newest approach for on-line preconcentration in MEKC where the basic requirements of AFMC are to have the conductivity of the micellar sample solution higher than that of the BGE, and the concentration of the surfactant just above the CMC. Based on the mechanism of action, AFMC should be applicable to neutral and charged analytes, based on their degree of interaction with the micelles.

Although this technique is still in infancy, it is capable of producing improvement in detection sensitivity of more than two orders of magnitude. AFMC showed a high potential for the preconcentration of large mass distribution ratio (k_{MEKC}). On-line preconcentration by AFMC gives better sensitivity enhancement factor in focusing the analyte because the micelles leave the sample zone before reaching the detector. So, the analyte will be released from the micelle and only analyte will be highly focused. However, AFMC was only suitable for preconcentration of analytes that interacted strongly with the SDS. Since the steroids chosen are neutral analytes, therefore combination of AFMC with MEKC were employed.

AFMC is an alternative approach of on-line preconcentration that still in progress of study. Based on the literature, it can be concluded that the development of AFMC as an on-line preconcentration improves the detection sensitivity at least an order of magnitude (Dawod *et al.,* 2010). Since the common method for steroid analysis that are used in industry such as HPLC is not an environmental friendly method, the instrumentation using CE is recommended to be used instead of HPLC for environmental profits. AFMC should also be employed for the on-line preconcentration for better quantitation. Besides, improvement in detection

sensitivity for model steroidal compounds using sodium dodecyl sulfate as surfactants was achieved.

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