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STEROID FOCUSING BY MICELLE COLLAPSE IN MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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Abstract. Preliminary studies on the separation of neutral steroids through analyte focusing by micelle collapse (AFMC) are presented to investigate its efficiency, sensitivity and limit of detection (LOD). The focusing mechanism of AFMC is based on the transport, release, and accumulation of molecules bound to micelle carriers that are made to collapse into a liquid phase zone. The sample solution of the neutral analytes (S) is prepared using sodium dodecyl sulphate (SDS) at a concentration above the critical micelle concentration (cmc) with higher conductivity than the running buffer. Normal mode-micellar electrokinetic chromatography (NM-MEKC) separation was initially performed on 100 mg/L of six neutral steroids in methanol using 20 mM SDS, 10% (v/v) methanol and sodium borate buffer (pH 9.0) with positive applied voltage of 25 kV and pressure injection of 50 mbar for 1 sec at 25°C. The same buffer condition has been applied to AFMC-MEKC, whereby the mixture of six neutral steroids was dissolved in 2 mM SDS and 250 mM sodium borate buffer which gave a conductivity ratio of 0.49. Results showed a good separation of prednisolone, prednisone, betamethasone, testosterone, 17-α-methyltestosterone, and 4-androstene-3,17-dione at 240 nm with sensitivity enhancement factors of 2.08, 1.17, 0.92, 16.9, 0.8, and 1.21 respectively. AFMC-MEKC allowed several folds improvement in sensitivity compared to NM-MEKC.

Keywords: Capillary electrophoresis; analyte focusing by Micelle collapse; neutral steroid

Abstrak. Kajian permulaan bagi pemisahan steroid neutral menggunakan kaedah penumpuan analit melalui keruntuhan misel (AFMC) dilaksanakan untuk mengkaji kecekapan, kepekaan dan had pengesanan (LOD). Mekanisme penumpuan oleh AFMC adalah berdasarkan pengangkutan, pelepasan, dan pengumpulan molekul yang terikat kepada pembawa misel yang dibuat untuk runtuh ke dalam zon fasa cecair. Larutan standard yang mengandungi steroid neutral disediakan dengan menggunakan natrium dodecyl sulfat (SDS) pada kepekatan melebihi kepekatan kritikal misel (cmc) dengan kekonduksian yang melebihi larutan penimbal. Mod normal-kromatografi elektrokinetik misel (NM-MEKC) pada mulanya dijalankan ke atas 100 mg/L enam steroid neutral di dalam methanol menggunakan 20 mM SDS, 10% (v/v) metanol dan natrium borat (pH 9.0) dengan voltan positif yang digunakan 25 kV dan suntikan tekanan sebanyak 50 mbar untuk 1 saat pada 25°C. Kondisi larutan penimbal yang sama telah digunakan

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bagi AFMC-MEKC, di mana larutan campuran keenam-enam steroid neutral dilarutkan menggunakan 2 mM SDS dan 250 mM natrium borat yang memberikan nisbah kekonduksian sebanyak 0.49. Pemisahan yang baik ditunjukkan oleh prednisolon, prednison, betamethason, testosteron, 17-α-metiltestosteron, dan 4-androsten-3,17-dion pada 240 nm dengan faktor peningkatan sebanyak 2.08, 1.17, 0.92, 16.9, 0.8, dan 1.21 masing-masing. Gabungan AFMC-MEKC menunjukkan peningkatan kepekaan berbanding NM-MEKC.

Kata kunci: Elektroforesis rerambut; penumpuan analit melalui keruntuhan Misel; steroid neutral

1.0 INTRODUCTION

Steroid is a class of lipids that contains a specific arrangement of four cycloalkane rings that are joined to each other; called cyclopentanoperhydrophenanthrene ring system. Analytical methods have been developed for the determination of steroids in different matrices; among the most famous are high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE). The analysis of steroid becomes complicated due to the close structural similarities of these compounds which require a separation technique with high resolution and a sensitive detection capability. The traditional method which is based on derivative spectrophotometry is insufficient because of poor selectivity and interferences while chromatographic methods such as GC and HPLC requires further derivatization and poor separation efficiency [1].

In recent years, CE has become an analytical alternative to classical GC and HPLC methods due to its superiority as an effective separation tool and has been used in different areas of chemistry, biology, medicine and pharmaceutical. A dynamic mode of CE that has been proposed by Terabe et al. in 1984 called micellar electrokinetic chromatography (MEKC) is capable in separating both ionogenic and neutral compounds [2]. In this methodology, suitable surfactants were added in the background solution (BGS) to form micelles and the separation is based on partitioning of analytes between the micelles and BGS.

Several advantages of CE over other separation techniques which are high separation efficiency and minimum requirement of sample and chemical amounts make it increasing popular among academia. Unfortunately, similar to other modes of CE, MEKC suffers from poor concentration sensitivity of photometric detectors which are the most popular CE detectors [3]. The problem of low concentration sensitivity can be solved with the use of on-line sample

preconcentration such as sample stacking, sweeping and a relatively new approach known as analyte focusing by micelle collapse (AFMC) [4,5].

AFMC was first introduced by Quirino as a recently new dimension for on-line preconcentration [6]. Through this technique, micelles leave the sample zone. The focusing steps of AFMC involve transport, release and accumulation of analytes that are bound to the micelles, which are made to collapse into liquid phase [7,8]. The accumulated analytes were subsequently separated by MEKC or capillary zone electrophoresis (CZE). AFMC should be applicable to neutral and charged analytes based on their degree of interaction with the micelles [9].

Preparation of the sample solution of neutral analytes (S) is different from other on-line sample preconcentration in which S is prepared using sample matrix in BGS. The conductivity value of S prepared should be greater than BGS. The conductivity of S can be increased by the addition of electrolyte salt used to prepare the BGS [10]. Operating parameters such as the injection length of the sample, conductivity ratio between the BGS and S, and surfactant concentration has been reported to affect the performance of AFMC [11].

In this paper, a comparison between AFMC-MEKC and NM-MEKC has been studied primarily for the separation of six neutral steroids, in terms of sensitivity enhancement. Figure 1 shows the chemical structures of the studied steroids.

2.0 EXPERIMENTAL

2.1 Chemical and Reagents

Steroids; prednisone, prednisolone, betamethasone, 17-α-methyltestosterone, 4-androstene-3,17-dione and testosterone were obtained from Dr. Ehrenfoster GmbH (Augsburg, Germany), sodium dodecyl sulfate (SDS) from Scharlau (Spain), disodium tetraborate 10-hydrate from Merck (Darmstadt, Germany) and sodium hydroxide pellets (Kanto Chemical Co. Inc., Tokyo, Japan). All other chemicals and solvents were common brands of analytical-reagent grade or better, and were used as received. Deionized water was collected from a Water Purification System from Millipore (Bedford, MA, USA) Milli-Q water purification system with a conductivity of 18.2 MΩ cm.

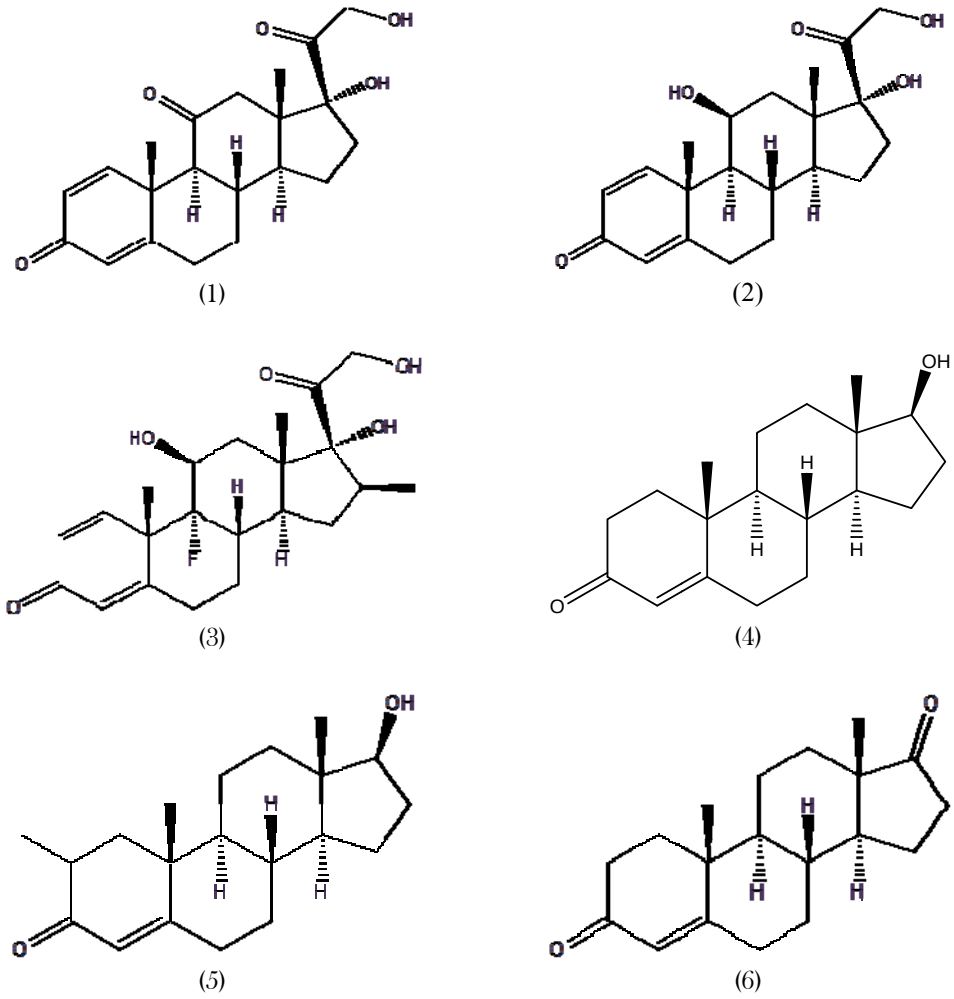

Figure 1 Chemical structure of studied steroid; (1) prednisone, (2) prednisolone, (3) betamethasone, (4) testosterone, (5) 17-α-methyltestosterone, and (6) 4-androstene-3,17-dione

Stock standard of 1000 mg/L of individual steroids were prepared by dissolving the compound in methanol. Final dilutions were prepared by diluting the stock solution with methanol to 100 mg/L. The separation buffer for NM-MEKC was prepared by dissolving appropriate amount of SDS and methanol in 25 mM sodium borate and adjusting the pH of the buffer with boric acid. All running buffers and deionized water were filtered through 0.2 μm nylon syringe filter from Whatman (Clifton, NJ, USA) prior to use.

To induce AFMC, the standard solution of the neutral steroid was prepared in a solution containing SDS at a concentration slightly above the critical micelle concentration (cmc) with conductivity at least twice that of the separation buffer, which can be achieved by adding more borate buffer to the sample. All these solutions were filtered with 0.2 μm filters prior to use. Sample solutions were prepared by appropriate dilution with the stock micellar and electrolyte solutions.

2.2 CE Apparatus

An Agilent capillary electrophoresis system (220 Agilent Capillary Electrophoresis System, Hanover, Germany) was performed for all experiments. A UV diode-array detection (DAD) system was operated at 240 nm. Data acquisition and system control was carried out by the 3D-ChemStation Software by Agilent Technologies. Separations were performed using a 50 μm i.d. fused silica capillary with 46.4 cm total length, 37.9 cm effective length. New capillary was conditioned with 0.1 M sodium hydroxide for about 15 min, followed by Milli-Q water for 10 min, and finally BGS for 10 min by flushing at 1 bar. The capillary was thermostated at 25°C in all experiments.

2.3 General CE Procedure

Sample injections were performed hydrodynamically at a constant pressure of 50 mbar for 1s. The separation runs were done at a constant temperature of 25°C and applied voltage of 25 kV. Separation voltage applied was at positive polarity with the buffer at both ends of the capillary, until all peaks are detected. The capillary was conditioned after each run with 0.1 M NaOH about 1 min, followed by Milli-Q water for 2 min, methanol for 1 min, and finally BGS for 3 min by flushing at 1 bar. The capillary was rinsed with Milli-Q water for 30 min and followed by air about 5 min at the end of each day. All experiments were performed at least in triplicate to ensure reliability of the data presented.

2.4 NM-MEKC Separation of Selected Steroids

Separation using NM-MEKC was initially done by adding micelles to the running buffer. In this initial study, running buffer containing 25 mM sodium borate (pH 9), the addition 20 mM SDS to form micellar phase, and 10% (v/v) methanol was added as organic modifier [5]. Each set of results was evaluated for peak migration time (t_R) and height (h).

2.5 AFMC Separation of Selected Steroid

In AFMC, the running buffer was prepared using 25 mM sodium borate, 20 mM SDS and 10% (v/v) of methanol. Sample solutions were prepared by appropriate dilution with the stock SDS and sodium borate solutions. To induce AFMC, the conductivity of sample solution of neutral analyte must be greater by at least 2 times than the separation buffer. This was achieved by the addition of electrolyte salt to the sample solution. The sample solution of the neutral steroid was prepared using SDS at a concentration slightly above the cmc (~2 mM). All these solutions were filtered with 0.2 μm filters prior to use.

3.0 RESULTS AND DISCUSSION

3.1 NM-MEKC

The separation of steroids would not be possible with the use of SDS alone. Thus, the use of organic modifiers such as methanol in BGS has been reported to be effective for the separation of such compounds [12]. The addition of methanol as organic modifier dramatically improved the separation as shown in Figure 2. Six peaks of each analyte can be seen although peak 4 showed very low response while the other five peaks of compounds 1, 2, 3, 5, and 6 are asymmetrical and appeared as doublet or "split" peaks.

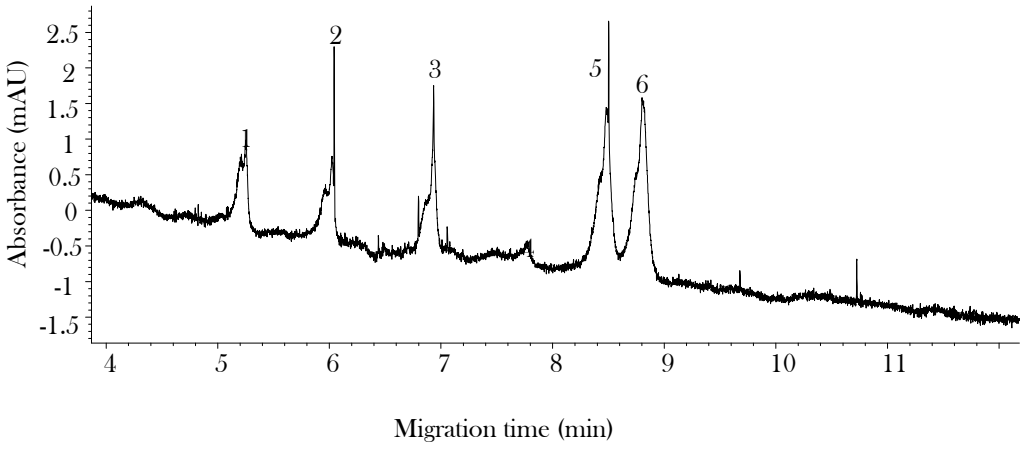

Figure 2 NM-MEKC separation of a mixture of 100 mg/L (1) prednisone, (2) prednisolone, (3) betamethasone, (4) testosterone, (5) 17-α-methyltestosterone, and (6) 4-androstene-3,17-dione. Conditions: buffer 25 mM sodium borate (pH 9.0), micellar solution: 20 mM SDS and 10 % (v/v) methanol, capillary 46.4 cm (37.9 cm to detector), +25 kV, temperature 25°C, detection at 240 nm and pressure injection at 50 mbar for 1 second

To ascertain the presence of peak 4 in the mixture, spiked mixture with testosterone was injected. Since peak 4 increased in size as illustrated in Figure 3, this confirmed the identity of peak 4.

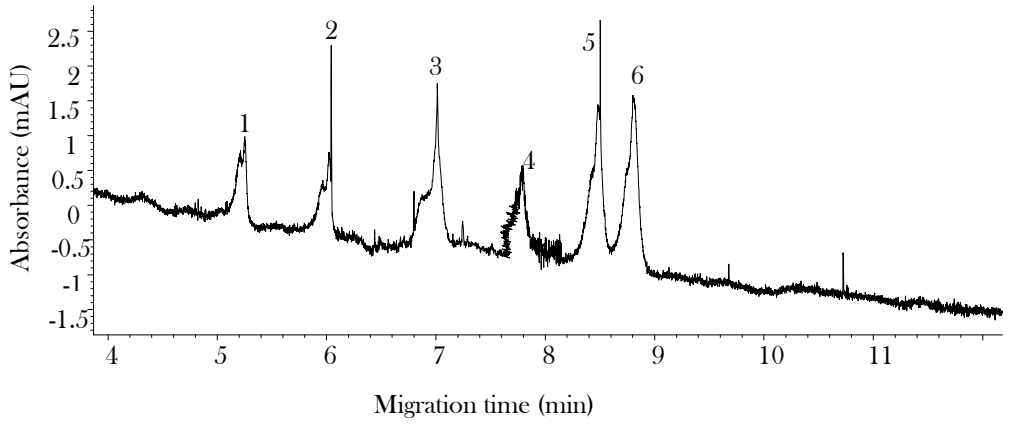

Figure 3 NM-MEKC separation of the mixture spiked with 300 mg/L testosterone. The same condition as in Figure 1 was employed

3.2 AFMC-MEKC

AFMC technique was coupled with MEKC for the separation of six neutral steroids. For AFMC, the analytes were prepared in a solution containing 2 mM SDS with conductivity at least 2.2 times higher than the separation BGS, which was achieved by adding more sodium borate buffer to the sample. The decrease in conductivity ratio (BGS/S) reduces the electric field strength in the S zone that causes the micelles to move slowly to the micellar dilution zone [10].

Electropherogram of the separation of the steroid by AFMC-MEKC can be observed in Figure 4. Each analytes can be seen to be well separated with symmetrical peak shapes. Compared to the separation of the steroid using NM-MEKC as in Figure 2, slight improvement in peak heights and migration times can be seen. Peak 4 showed drastic improvement in sensitivity.

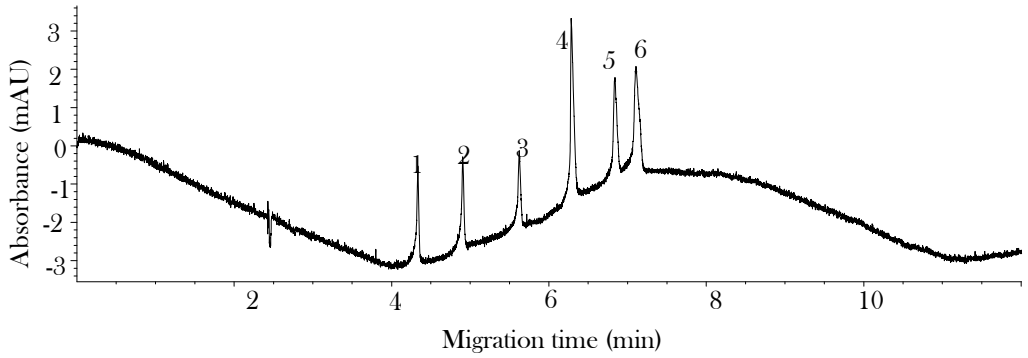

Figure 4 Electropherogram of AFMC separation of a 100 mg/L mixture of (1) prednisone, (2) prednisolone, (3) betamethasone, (4) testosterone, (5) 17-α-methyltestosterone, and (6) 4-androstene-3,17-dione in 2 mM SDS with 250 mM sodium borate, pH 9.0. Conditions: buffer 25 mM sodium borate (pH 9.0), micellar solution: 20 mM SDS and 10 % (v/v) methanol, conductivity ratio (BGS/S) : 0.49, capillary 46.4 cm (37.9 cm to detector), +25 kV, temperature 25°C, detection at 240 nm and pressure injection at 50 mbar for 1 second

3.3 Comparison between NM-MEKC and AFMC

Peak heights of the selected steroids using NM-MEKC and AFMC are compared as shown in Table 1 and sensitivity enhancement factor (SEF) was calculated by equation (1) where h refers to the peak height.

$$\text{SEF} = \frac{h_{\text{NM-MEKC}}\,(\text{mAU})}{h_{\text{AFMC}}\,(\text{mAU})} \times \text{Dilution Factor} \qquad (1)$$

There are slight improvements in sensitivity enhancement factor in the range of 1.2-16.9 folds. Testosterone which was difficult to be detected by NM-MEKC showed marked improvement in sensitivity by AFMC. This improvement in term of sensitivity is due to the accumulation of analyte in AFMC in which the analytes are focused. Betamethasone and 17-α-methyltestosterone gave SEF lower than 1. This may be caused by the condition of some parameters such as conductivity ratio that should be improved to increase the SEF and maintain good separation using AFMC.

Table 1 Sensitivity enhancement factor of AFMC compared to NM-MEKC of six selected steroids; (1) prednisone, (2) prednisolone, (3) betamethasone, (4) testosterone, (5) 17-α-methyltestosterone, and (6) 4-androstene-3,17-dione

Analyte (Steroid)	1	2	3	4	5	6
$h_{\text{NM-MEKC}}$ (mAU)	1.19	2.00	2.18	0.29	3.29	2.34
h_{AFMC} (mAU)	2.48	2.33	2.01	4.89	2.64	2.82
SEF	2.08	1.17	0.92	16.9	0.8	1.21

4.0 CONCLUSION

A NM-MEKC method has been studied for the determination of six neutral steroids. It was found that the neutral steroids can be successfully separated by MEKC using methanol as organic modifier. AFMC in MEKC allowed improvement for the six steroids in terms of peak height sensitivity of several folds. Further studies are in progress to understand the mechanism of AFMC-MEKC separation of these neutral steroids.

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