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STEROID FOCUSING BY MICELLE COLLAPSE IN CAPILLARY ELECTROPHORETIC SEPARATION

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ABSTRACT

Analyte focusing by micelle collapse (AFMC) is an on-line sample preconcentration of neutral analyte for capillary electrophoresis (CE). Study on the separation of six neutral steroids by AFMC-micellar electrokinetic chromatography (MEKC) technique was presented to investigate its efficiency, sensitivity and limits of detection. 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 is prepared using sodium dodecyl sulphate (SDS) at a concentration above the critical micelle concentration (cmc) with higher conductivity than the running buffer. The separation of selected steroids using MEKC was initially conducted as a comparison of enhancement factor to AFMC-MEKC. The use of a mixture of 20 mM 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. The same buffer condition was applied to AFMC–MEKC, whereby the selected steroids were prepared in a solution containing 7 mM SDS and 250 mM acetate buffer with conductivity ratio of 0.34. Results showed enhancement of sensitivity for each steroids with good repeatability and reproducibility. The limits of detection for the steroids ranged from 1.1-5.7 mg/L. The optimized method was successfully applied to analyse steroids in urine samples with good average recoveries ranging from 93% - 101%. AFMC-MEKC separation on selected steroids showed enhancement in sensitivity compared to MEKC.

Keywords: Analyte focusing by micelle collapse; micellar electrokinetic chromatography; on-line sample preconcentration; critical micelle concentration

INTRODUCTION

Analytical methods have been developed for the determination of some steroidal compound in different matrices which include high performance liquid chromatography (HPLC), gas chromatography (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 and 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. The capture, accumulation, transport and release 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. In order to improve the concentration sensitivity and produce lower limit of detection (LOD), AFMC is introduced. AFMC is reported by Quirino and Haddad as an 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 buffer, and the concentration of the surfactant just above the critical micelle concentration (CMC) [1]. AFMC showed a high potential for the preconcentration of large mass distribution ratio.

METHODOLOGY

Chemicals and Reagents

Steroids were obtained from Dr. Ehrenfoster GmbH (Augsburg, Germany), sodium dodecyl sulfate (Scharlau, Spain), disodium tetraborate 10-hydrate from Merck (Darmstadt, Germany), sodium acetate 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 Milipore (Bedford, MA, USA) Milli-Q water purification system with a conductivity of 18.2 MΩ cm.

Stock standard of 1000 mg/L of individual steroids was prepared by dissolving the compound in methanol. Sample solutions were prepared by diluting the stock solutions in 250 Mm acetate pH 9 and 7 Mm of SDS. The separation buffer for AFMC-MEKC was prepared by dissolving appropriate amount of SDS and methanol in 25 mM sodium acetate and adjusting the pH of the buffer to pH 9 with acetic acid. All running buffers and deionized water were filtered through 0.2 µm nylon syringe filter from Whatman (Clifton, NJ, USA) prior to use.

Urine samples were obtained from Pusat Kesihatan UTM Skudai Johor. Bond Elut NEXUS SPE cartridges 3 mL (60 mg) were obtained from Agilent Technologies (Hanover, Germany) with particle size, 70 µm; pore size 100 Å; and surface area, 575 m^2/g.

Instrumentation

All electropherograms were obtained with the Agilent capillary electrophoresis system from Agilent Technologies (Hanover, Germany), equipped with a DAD. Separations were performed using an untreated fused silica capillary of 48.5 cm x 50 µm i.d. (with an effective length of 40 cm to the detector window). Sample injections were performed hydrodynamically at a constant pressure of 40 mbar for 1s. The separation runs were done at a constant temperature of 25ºC and applied voltage of +25 kV. Data were collected and processed on computing using ChemStation software (Agilent Technologies).

A new capillary was conditioned with 0.1 M sodium hydroxide about 15 mins, followed by mili-Q water for 10 mins, and finally buffer for 10 mins by flushing at 1 bar. All injections at 40 mbar were performed at the inlet end of the capillary. The capillary was conditioned after each run with 0.1M NaOH about 1 min, followed by mili-Q water for 2 mins, methanol for 1 min, and finally buffer at about 3 mins by flushing at 1 bar. The capillary was rinsed with mili-Q water for 30 mins and followed by air about 5 mins at the end of each day. All experiments and sample injections were performed in triplicate to ensure reliability of the data presented.

AFMC-MEKC Separation of Selected Steroids

Separation using MEKC was initially done by adding micelles to the running buffer. In this initial study, running buffer containing 25 mM sodium borate (pH 9), was based on previous research [2,3], the addition 20 mM SDS to form micellar phase, and 10% (v/v) methanol was added as organic modifier.

The optimized separation buffer (BGS) obtained from MEKC method was then used as the separation buffer for AFMC-MEKC method. In order to perform AFMC-MEKC, addition of SDS and acetate buffer was done into the sample solution. The conductivity of both sample solution and the BGS was monitored which the conductivity of the sample should be at least 2.2 greater than the conductivity of the BGS to make sure that the micelles collapse in micellar dilution zone (MDZ). Each set of results was evaluated for migration time (t) and peak height (h) obtained from ChemStation software.

The conductivity of buffer solution containing 25 mM acetate, 10% of methanol with various concentration of SDS was measured. SDS concentration was chosen from the range of 0 mM to 10 mM. A plot of conductivity against SDS concentration was plotted. The intersection of the straight lines from the plot was taken as the cmc value of SDS. All measurements and steps were performed in triplicate.

Extraction Procedure

Urine Samples

All extraction procedures follow reference method [4]. Urine samples were collected from UTM’s student volunteer who came for medical checkup. Steroid-free urine sample (SFUS) were prepared by percolating urine samples through Bond Elut NEXUS SPE cartridges. In this way, urinary steroids and other potential interfering compounds are retained in the cartridge. After this, the eluates were collected.

SFUS (3 mL) was placed in a stoppered centrifuge tube and then spiked with 300 µL of 1000 mg/L steroids. Prior to CE analysis, extraction of steroids from urine solutions was performed with a vacuum manifold system through Bond Elut NEXUS SPE cartridges which has been conditioned with 5 mL of methanol followed by 2x5 mL of water. The cartridges were washed with 5 mL of a mixture of water/acetone (4:1, v/v) close to dryness and 1 mL of n-hexane. The elution of steroids was performed with 2x2 mL of diethyl ether. Then, the eluate was evaporated to dryness by passing a stream of nitrogen over the surface of the solution. The residue obtained was reconstituted using 1 mL of aqueous solution containing 250 mM of acetate buffer and 7 mM of SDS. All sample preparations and sample injections were performed in triplicate.

RESULTS AND DISCUSSION

In order to perform AFMC-MEKC, the cmc value of SDS was determined by conductometry method. AFMC-MEKC was then applied to the simultaneous separation of the six steroids. Separation of selected steroids was studied based on two different parameters; conductivity ratio and SDS concentration in the sample solution.

Determination of Critical Micelle Concentration

A plot of conductivity versus SDS concentrations was plotted as in Figure 1. As the concentration of SDS in buffer solution increases, the measured conductivity of the solutions also increases.

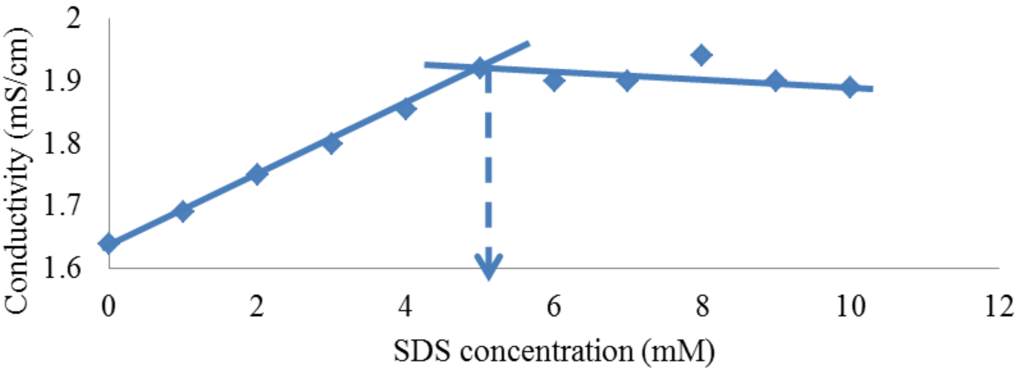

Figure 1: Variation of conductivity versus surfactant concentration for conductometric determination of the cmc

From Figure 1, two straight lines with different slope were observed. The cmc value of 5 mM was determined from the interception of the two straight lines. The first one corresponds to the concentration range below the cmc value (5 mM) when only monomers of SDS exist in solution. At higher concentrations of SDS, micelles start to form and a change of slope occurred due to the increasing in conductivity value in a different manner.

Conductivity Ratio

The basic requirements for AFMC are to have the conductivity of the micellar sample solution higher than that of the BGS, and the concentration of the surfactant just above the cmc. The effect of conductivity ratio was studied by using various concentration of acetate (200 mM, 225 mM and 250 mM) in the sample solution. In this study, the concentration of SDS in the sample solution was fixed at 7 mM. The concentration of surfactant at MDZ is expected equal to:

Concentration of surfactant at MDZ = (BGS/S) × Concentration of surfactant in S (1)

The effect of lowering conductivity ratio (BGS/S) from 0.66 (a), 0.51 (b), to 0.34 (c) on AFMC-MEKC is summarized in Table 1. With all these sample conditions, the micelles from the sample solutions collapse at the MDZ. According to Eq 1, the expected concentrations of SDS at the MDZ are 4.62 mM (a), 3.57 mM (b) and 2.38 mM (c) correspondingly [5].

Table 1: SEF of AFMC-MEKC at SDS concentration of (a) 7 mM, (b) 8 mM and (c) 9 mM in sample solution

Analyte	Peak Height (mAu)				SEF = $\frac{Peak\ Height_{AFMC}}{Peak\ Height_{MEKC}}$		
	AFMC			MEKC			
	BGS/S						
	a	b	c		a	b	c
1	1.53	1.99	2.30	0.76	2.01	2.62	3.03
2	1.66	1.83	2.04	0.79	2.1	2.32	3.04
3	1.51	2.2	2.50	0.86	1.76	2.56	2.91
4	0.95	1.44	1.70	0.64	1.48	2.25	2.66
5	2.15	3.58	3.07	1.56	1.38	2.29	2.37
6	3.20	4.73	5.00	1.89	1.69	2.50	2.65

SDS Concentration

Effect of concentration of SDS in sample solution on AFMC-MEKC separation of the six steroids was studied. Increasing concentration of SDS in sample solution causes in increasing of conductivity in the sample solution. However, the increasing concentration of SDS in sample solution causes the increasing of SDS concentration at the MDZ which might affect the collapse of the micelle. Thus, less micelle may collapse followed by decreasing in releasing of analyte from micelle [5]. A slight decreasing in peak height can be observed from the electropherogram as increasing the SDS concentration in the sample. From Table 2, the peak height of each steroids decreases as increasing the concentration of SDS in the sample from 7 mM to 10 mM. Among the values of SDS concentration in the sample (7 mM, 8 mM and 10 mM) that were optimized, 7 mM of SDS gave the best value of SEF as stated in Table 2 which make it was chosen to be the optimized SDS concentration in the sample for the separation of the six steroids by AFMC-MEKC as the concentration is closer to CMC.

Table 2 SEF of AFMC-MEKC at SDS concentration of (a) 7 mM, (b) 8 mM and (c) 9 mM in sample solution

Analyte	Peak Height (mAu)				SEF		
	AFMC			MEKC			
	SDS Concentration						
	a	b	c		a	b	c
1	2.3	2.10	1.42	0.76	3.03	2.76	1.87
2	2.4	2.06	1.49	0.79	3.04	2.61	1.89
3	2.5	2.23	1.59	0.86	2.91	2.59	1.85
4	1.7	1.26	0.79	0.64	2.66	1.97	1.23
5	3.7	3.36	2.42	1.56	2.37	2.15	1.55
6	5	4.83	3.94	1.89	2.65	2.56	2.08

From the studied parameters, 25mM of acetate buffer (pH 9) with addition of 20mM SDS and 10% of methanol was chosen as the optimized condition of the BGS with shorter capillary length of 48.5. Typical electropherogram of selected steroids employing AFMC-MEKC at conductivity ratio of 0.34 are shown in Figure 2.

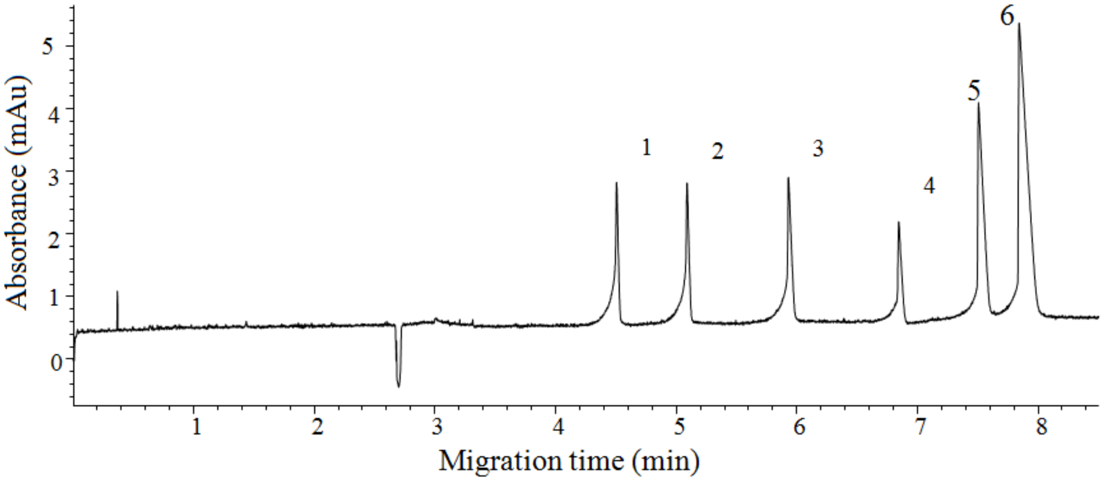

Figure 2: AFMC-MEKC separation of a mixture of (1) 100 mg/L prednisone, (2) 100 mg/L prednisolone, (3) 100 mg/L betamethasone, (4) 25 mg/L 4-androstene-3,17-dione, (5) 100 mg/L testosterone and (6) 150 mg/L 17-α-methyltestosterone.

Conditions: BGS: 25 mM sodium acetate (pH 9.0), micellar solution: 20 mM SDS and 10 % v/v methanol, sample matrix 250 mM acetate and 7 mM SDS, capillary 48.5 cm (40.0 cm to detector), +25 kV, temperature 25ºC, detection at 240 nm and pressure injection at 40 mbar for 1 second.

Application of AFMC-MEKC to Samples

Urine is a sample that exhibits high chemical complexity, resulting in increased matrix effects. As a consequence, the analysis of urine is a particularly challenging task. Thus, AFMC-MEKC on the separation of the six steroids in urine matrix was studied. The electropherogram of the separation of the steroids by AFMC-MEKC were shown in Figure 3.

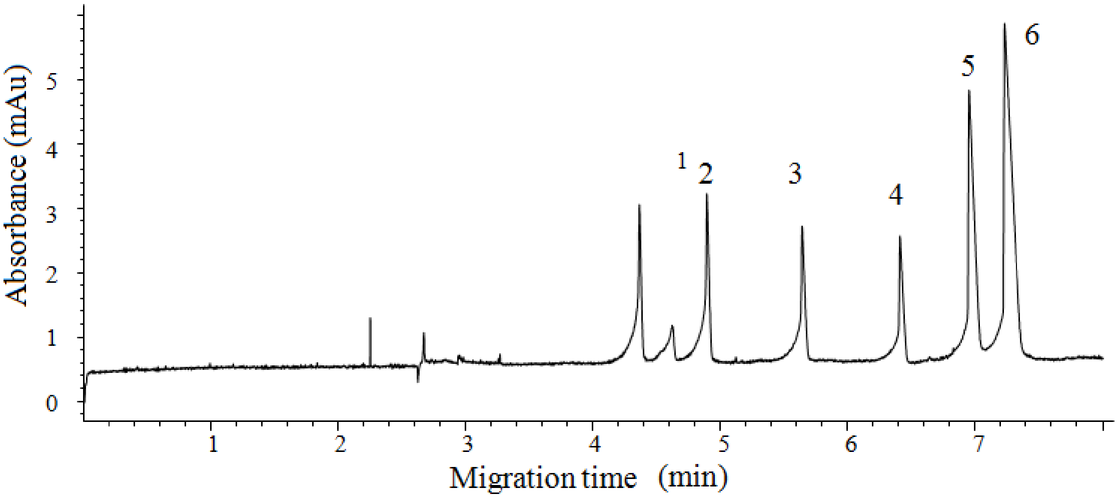

Figure 3: AFMC-MEKC separation of a mixture of (1) 100 mg/L prednisone, (2) 100 mg/L prednisolone, (3) 100 mg/L betamethasone, (4) 25 mg/L 4-androstene-3,17-dione, (5) 150 mg/L testosterone and (6) 100 mg/L 17-α-methyltestosterone in urine matrix. Other conditions were the same as Figure 2.

The performance of the separation of each steroid in urine matrix by AFMC-MEKC was studied based on linearity, repeatability, LODs and SEF which has been summarized in Table 3. The repeatability in the AFMC-MEKC system in urine matrix in terms of RSD for migration time, peak area, peak height and SEF for each steroids was briefly examined (n = 3). The obtained repeatability in the migration time, peak area and peak height were good ranging from 0.9% to 5.3%, 0.9% to 7.4%, and 0.0% to 3.3% respectively. The increase in detection sensitivity for each steroid is about triple fold in AFMC-MEKC at conductivity of 0.34 compared to the conventional MEKC. Limit of detections for selected steroids ranged from 0.49-18.13 mg/L.

Table 3: The performance of the separation of each steroid in urine matrix by AFMC-MEKC

Analyte	Curve Calibration		RSD (%)			LOD (mg/L)	SEF
	Equation	r^2	Migration Time	Area	Height		
1[a]	y=0.0221x+0.2982	0.97	0.96	7.41	2.37	18.13	3.20
2[a]	y=0.0197x+0.2933	0.95	5.30	0.92	0.00	6.81	3.04
3[a]	y=0.0185x+0.4327	0.95	1.32	1.52	2.19	5.43	3.06
4[b]	y=0.0676x-0.0615	0.97	1.43	3.98	3.33	4.46	2.70
5[a]	y=0.0295x+0.832	0.97	1.65	4.44	1.51	2.81	2.46
6[c]	y=0.0249x+1.1157	0.96	1.83	1.79	2.96	0.49	2.74

Table 3 Linearity, repeatability, LODs (S/N = 3) and SEF of the optimized AFMC-MEKC in urine matrix.Conditions as in Figure 3.

[a] Linear range: 12.5 - 120 mg/L

[b] Linear range: 3.125 - 30 mg/L

[c] Linear range: 18.75 - 180 mg/L

In order to verify the reliability of the method, the recoveries of these compounds in the urine samples were also investigated and the repeatability was determined by repeated injection of solution (n = 3). The results showed that no interference was observed when the steroids were coexisted in urine samples. The method recoveries of the six analytes ranged from 93% to 102% as in Table 4.

Table 4 Percent recovery and repeatability of all analytes in urine sample by SPE-AFMC-MEKC.

Analyte	Added Concentration (mg/L)	Average Recovery (%)	RSD (%, n = 3)
1	75	93.36	2.65
2	75	96.86	2.76
3	75	95.08	1.52
4	20	97.78	2.06
5	75	98.71	0.83
6	100	93.07	2.44

CONCLUSION

In order to perform AFMC-MEKC separation, CMC value of surfactant has to be determined. In this study, the CMC value of SDS was 5 mM in 25 mM acetate pH 9.0. A method for the AFMC-MEKC was performed and investigated. Conductivity ratio and SDS concentrations in sample solution were optimized for the separation. Optimized condition of AFMC-MEKC method at conductivity ratio of 0.34 and 7 mM of SDS slightly enhanced the detection sensitivity of the six steroids 3-fold with repeatability (RSD 3.5-7.1% , n = 3) and reproducibility (RSD 2.6-12.7%, n=3). This is due to more analytes being transported and focused at the boundary of sample zone and the adjacent solution zone as continued application of voltage is applied which led to more micelle collapse and analytes released. The SEF is very low compared to report in literatures and other findings with hundred folds of enhancement.

The LODs for the six steroids ranged from 1.14-5.61 mg/L. Combination of solid-phase extraction (SPE) pretreatment and AFMC-MEKC procedure was applied to the determination of six neutral steroids in spiked urine samples at various concentrations as summarized in Table 3**.** The average recoveries of the selected steroids in spiked urine samples were good ranging from 93% - 101% with RSD of 0.8% - 11.2% (n = 3). AFMC-MEKC separation on selected steroids showed enhancement in sensitivity compared to MEKC.

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