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Centrifuge microextraction coupled with sweeping–MEKC to analyze trace steroid hormones in urine samples

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ABSTRACT

A novel method that combines two concentration techniques, off-line centrifuge microextraction (CME) and on-line sweeping, is used to determine trace steroids in urine by micellar electrokinetic chromatography (MEKC). The CME–sweeping–MEKC is a promising technique, which has a variety of merits such as high sensitivity, rapid operation, minimal cost, and high sample throughput capability. Using CME–sweeping, over a 500-fold increase in sensitivity could be obtained as compared with the normal hydrodynamic injection without sample stacking. The linear range was 0.05–1 μg mL^{-1} with the square of the correlation coefficients ranging from 0.9998 to 0.9999. Detection limits ($S/N = 3$) were 5–15 ng mL^{-1} using a photodiode array UV detection at a wavelength of 240 nm. The relative standard deviations were between 2.8% and 3.5% ($n = 5$, progesterone as the internal standard). The diode array UV spectrum used can distinguish the analytes from the interference in the complex sample matrix, which is useful in biological and clinical sample analysis.

1. Introduction

Steroid hormones, like testosterone, methyltestosterone, and progesterone, are associated with numerous physiological developments and responses such as the development of reproductive systems, sexual differentiation, and the maintenance of secondary sexual characteristics [1]. Natural and/or synthetic steroids also have strong anabolic effects and are used as doping agents in the athletic community and illegal growth promoting agents in animal production [2,3]. Therefore, identification, quantification, and localization of steroids in biological fluids and tissues are important areas of study. Because of their close structural similarity, metabolic versatility, and their occurrence at low concentrations relative to interfering compounds in body fluids and tissues, developing reliable analytical methods for these steroids is a challenge for analytical chemists.

Numerous methods have been adopted for the characterization and determination of steroids, such as immunoassay [4,5], gas chromatography–mass spectrometry (GC–MS) [6,7], high performance liquid chromatography–ultra violet detection (HPLC–UV) [8], high performance liquid chromatography–mass spectrometry (HPLC–MS) [9–11], capillary electrophoresis–ultra violet detection (CE–UV) [12,13], and capillary electrophoresis-immunoassay [14]. However, rapid and reliable methods are still needed to simplify sample pretreatment and decrease analysis time for high-throughput detection.

Micellar electrokinetic chromatography (MEKC) separates both neutral and ionized solutes in the same system with high efficiency. Nevertheless, like other CE techniques, the concentration sensitivity of MEKC is poor due to the limited volume of sample solution injected (usually picoliters to nanoliters) and a short path length for absorbance-related detection (such as spectrophotometric detection). Injecting more analytes without sacrificing the resolution is a good strategy to lower the detection limits.

On-line sample concentration techniques give an effective and versatile way to enhance the detection sensitivity of MEKC for steroids. Among them, sample stacking [15–17] and sweeping [18–21] are most frequently used. Sweeping is defined as a phenomenon where analytes are picked up and accumulated by the pseudostationary phase (micelles or other complexation additives) that penetrate the sample zone containing no pseudostationary phase in MEKC [22]. Sweeping is applicable to all solutes irrespective of their ionic nature, which is an attractive advantage over sample stacking. Different studies using sweeping–MEKC with dodecyl sulfate (SDS) to detect testosterone in the presence of electrosmotic flow (EOF) [18] and under suppressed EOF conditions [22] have been reported with sensitivity enhancements of 200- and 1500-fold, respectively. However, because interfering compounds

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are usually present at high concentrations in body fluids and tissues, Sweeping–CE may suffer from matrix interferences when applied to the biological samples.

In order to achieve an efficient and reliable determination of steroid hormones with MEKC, appropriate sample preparation is required. Traditional sample preparations, such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE), have been used for sample cleanup and/analyte enrichment in the determination of steroid hormones [23–26]. However, these methods are tedious, time-consuming, and use relatively large amounts of sample and organic solvent. Centrifugation is usually used to remove macromolecular contaminants from the biological sample. It is also used to separate the extraction solvent/solid extractant from the sample after extraction [27]. Another important application is density gradient ultracentrifugation, which is used to separate compounds with slightly different densities [28–31] or determine the density of biological macromolecules, such as proteins [32–34].

However, there are few reports using centrifugation as an extraction method. We developed a centrifuge microextraction (CME) technology which combines the removal of macromolecular contaminants and other interfering components, desalting, and preconcentration into a single step [35]. While this method (CME coupled with on-line back-extraction field-amplified sample stacking) proved to be an excellent method for analyzing positively charged analytes in biological samples, it could not be applied to neutral compounds such as steroid hormones.

In this study, the combination of CME and sweeping–MEKC to analyze trace testosterone and methyltestosterone is presented. Factors that influence the microextraction efficiency (e.g. centrifugation rate, extraction time, and amount of salt) and sweeping (e.g. injection time and the concentration of methanol) were optimized. The CME–sweeping–MEKC was then applied to determine trace testosterone in human urine.

2. Experimental

2.1. Apparatus

All separation experiments were performed on a Beckman P/ACETM MDQ instrument (Beckman-Coulter, Fullerton, CA, USA) equipped with a photodiode array detector (190–600 nm), automatic injector, fluid cooled cartridge (15–60 °C), and system Gold Date Station. The separations were performed in an uncoated fused silica capillary of 50.2 cm × 50 μm i.d. (effective length, 40 cm) (Yongnian Photoconductive Fiber Factory, Hebei, China).

A centrifuge model TGL-16C with a 30° fixed angle rotor (r_{min} = 25 mm, r_{max} = 65 mm) (Shanghai Anting Instrument Factory, Shanghai, China) was used to perform CME.

2.2. Reagents and solutions

All chemicals used were analytical grade or higher and used without further purification. Testosterone, methyltestosterone and progesterone were purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was obtained from AnalaR (BDH, Poole, England). Methanol was obtained from Shanghai Chemical Factory (Shanghai). All the buffers and sample solutions were prepared in doubly distilled, deionized water.

Stock solutions of 1.0 mol L^{-1} SDS were prepared every two weeks in deionized water. Nonmicellar background solutions (BGSs) were prepared by dilution of 1.5 mol L^{-1} phosphoric acid stock solution in water, with pH adjustment using 5 mol L^{-1} sodium hydroxide stock solutions. Micellar BGSs were prepared by dilution of the SDS stock solution in appropriate phosphate buffers. The BGSs were prepared every day to prevent reproducibility problems.

Standard stock solutions of steroids (testosterone, methyltestosterone and progesterone) were prepared in methanol with the concentration of 1000 μg mL^{-1}. The final solutions used in the experiment were prepared by dilution of standard stock solutions with nonmicellar BGS or water. BGSs and sample solutions were filtered through 0.45 μm filters (Xingya Purification Materials, Shanghai) and ultrasonically degassed prior to use.

2.3. Electrophoretic conditions

The new capillary was pre-conditioned prior to use by successive washings for 10 min with 0.1 mol L^{-1} sodium hydroxide, 10 min with water, and 10 min with nonmicellar BGS, followed by 3 min with micellar BGS. At the beginning of each experiment, the capillary was washed with deionized water for 3 min, and then micellar BGS for 3 min. Detection wavelength was set at 240 nm. All the experiments were run at 20 °C. The separation voltage was −16.5 kV.

2.4. Sweeping optimization procedures

The capillary was flushed with the micellar BGS before the samples were introduced into the capillary for 180 s at a constant pressure of 137.9 kPa. The sample prepared in nonmicellar BGS was then injected into the cathodic end of the capillary by pressure. Injection pressures of 3.4, 10.3, 17.2, 24.1, 31.0 kPa for 90 s approximately correspond to 2, 6, 10, 12.5, 15 cm of capillary length, respectively. After the inlet vial was changed back to the micellar BGS vials, a negative voltage of −16.5 kV was applied along the capillary. This procedure permits the SDS micelles (in the inlet reservoir) to enter the sample zone to collect hydrophobic analytes. Preconcentration terminated when the SDS micelles reached the boundary between the sample matrix and the run buffer, where the analytes were carried towards the detection window at a rate slower than the migration of the SDS micelles.

2.5. CME procedures

The 1.3 mL water/urine samples containing sodium chloride was placed into 2.0 mL polypropylene vials sealed with a cap, which prevented solvent evaporation during the extraction process. A 100 μL volume of cyclohexane was added in each sample vial after mixing. Then, twelve samples were centrifuged at a described speed for a certain time. The target compounds were extracted from water/urine samples into cyclohexane. After extracting, the upper layer was collected (50 μL), and transferred into a 1.0 mL polypropylene vial. This organic phase was evaporated to dryness in a stream of nitrogen within a 24-well format evaporator. The dried extract was then dissolved in the nonmicellar BGS (20 μL), vortexed vigorously for 1 min, and transferred to the Beckman Coulter Standard PCR vial for the subsequent sweeping–MEKC analysis.

3. Results and discussion

3.1. Optimization of sweeping–MEKC

3.1.1. Influence of methanol on separation

The influence of methanol volume fraction on separation efficiency was investigated by changing its volume fraction from 0 to 35%. It can be seen that as the methanol volume fraction increased, the resolution between the analytes was improved and the migration time increased accordingly (Fig. 1). This is because organic additives in the BGS can alter hydrophobic analyte/micelle interactions by displacing the analytes from the micelle, by offering alternative hydrophobic binding sites, or by decreasing the surface tension of the separation buffer [36]. The resolution between

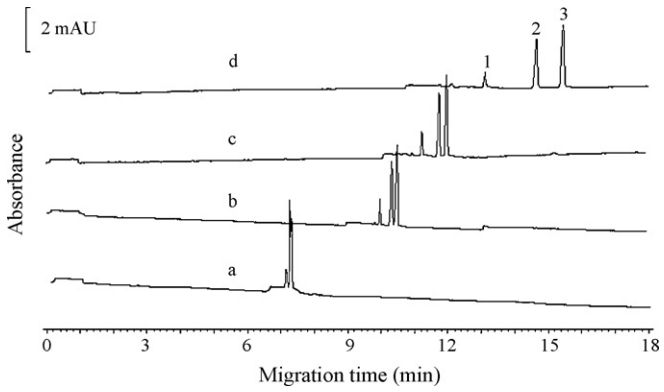

Fig. 1. Effect of methanol on the CE separation of steroids. Separation buffers with 50 mmol L^{-1} SDS and 100 mmol L^{-1} H_3PO_4 (pH = 2.5), with addition of 0% methanol (a), 10% methanol (b), 20% methanol (c), and 30% methanol (d). Sample injection, 3.4 kPa × 5 s. Analyte concentration, 100 μg mL^{-1}. Separation voltage, −16.5 kV. Peak identification: (1) progesterone, (2) methyltestosterone, and (3) testosterone.

methyltestosterone and testosterone is higher than 1.5 when the volume fraction of methanol was above 15%. A higher volume fraction of methanol allows for more samples to be injected with satisfactory resolution when sweeping is adopted, which could improve the sensitivity. However, using a methanol volume fraction higher than 30% might produce tiny bubbles due to Joule heating, thus resulting in the breakdown of the separation. The optimum methanol volume fraction for this system is 30%.

3.1.2. Effect of sample injection length on sweeping

For the sweeping technique, the injected length of an analyte plug is theoretically narrowed by a factor equal to $1/(1+k)$ where k is the retention factor [22]. To achieve a better sensitivity for the analysis, prolonging the sample injection length in sweeping–MEKC is advantageous. However, for a given length of capillary, a longer injection length reduces the separation zone. Therefore, the length of the sample plug must be optimized. In order to investigate the effects of the sample plug on the corresponding signal intensity, several different sample injection lengths (2, 6, 10, 12.5, and 15 cm) were examined (Fig. 2). It could be seen that the peak heights increased with increasing injected plug length. However, resolutions between the peaks gradually deteriorated as the injected plug length increased. Partial overlap between

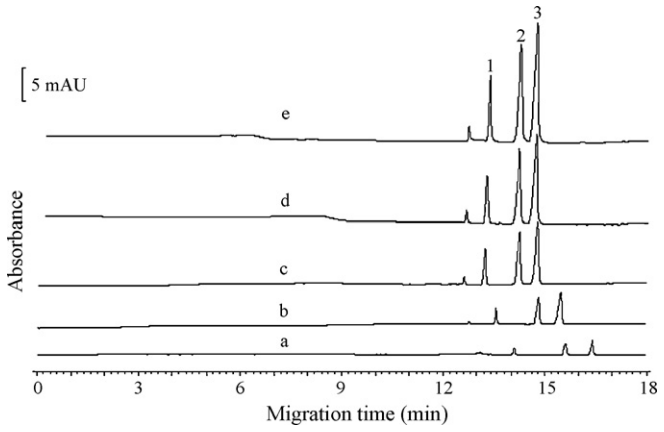

Fig. 2. MEKC electropherograms obtained at different sample injection lengths with the sweeping method. Concentrations of analytes, 0.50 μg mL^{-1} in buffer without SDS. Sample injection plug: (a) 2 cm, (b) 6 cm, (c) 10.0 cm, (d) 12.5 cm, and (e) 15 cm. Separation buffers with 50 mmol L^{-1} SDS and 100 mmol L^{-1} H_3PO_4 (pH = 2.5), with addition of 30% methanol; separation voltage, −16.5 kV. Peak identification as in Fig. 1.

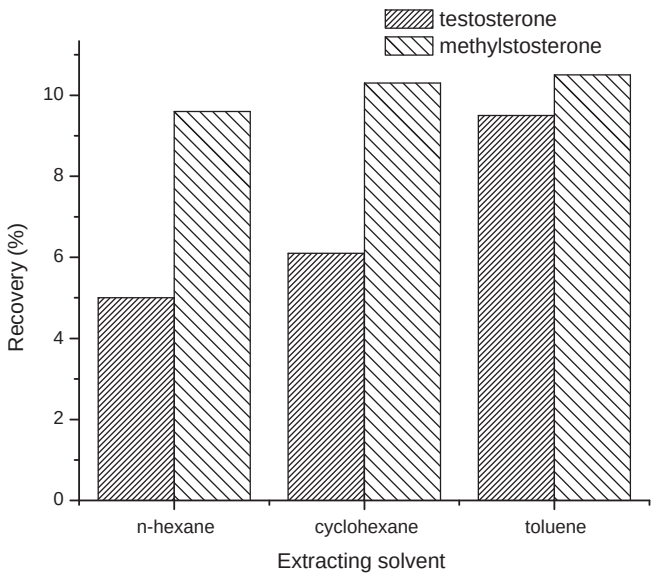

Fig. 3. Extraction efficiency of steroids with different solvents (n = 3). CME conditions: centrifugation rotor speed, 10,000 rpm; mass concentrations, methyltestosterone, 3.5 μg mL^{-1}, testosterone, 1 μg mL^{-1}; sample volume, 1.3 mL; the extracting solvent volume, 100 μL. The 50 μL extractants was dried and dissolved in 20 μL buffer (without SDS). Sample injection, 31.0 kPa × 90 s. MEKC conditions were as in Fig. 2.

testosterone and methyltestosterone was observed when the injection length was 16 cm (data not shown). Therefore, as a compromise between resolution and peak height, a 15 cm injection length was chosen as the optimized condition.

3.2. Optimization for CME

Several parameters influencing the CME efficiency were investigated, including the extraction profile (extracting solvent, extraction time, and rotor speed) and the aqueous medium characteristics (ionic strength).

3.2.1. Consideration of extracting solvents

The extracting solvent should be immiscible with the sample solution, have acceptable analyte recovery and minimal interference from matrix components, as well as moderate volatility if removal of the extracting solvent is needed after extraction. The steroids, with a density lower than that of water, will float onto the top of the solution during centrifugation. So, the ideal extracting solvent should have a density lower than that of water but higher than that of steroids, which can separate the analytes from the sample solution after centrifugation. In this study, three different extracting solvents, n-hexane, cyclohexane, and toluene, were evaluated. Recovery, which is the ratio of extracted analytes to the total amount of analytes in sample, was used to evaluate the extraction efficiency. The better results were obtained with cyclohexane and toluene (Fig. 3)). However, considering the relatively low volatility of toluene (the vapor pressure of toluene is 3.1 kPa at 21 °C) [37], we selected cyclohexane (the vapor pressure of cyclohexane is 10.5 kPa at 21 °C [37]) as the extracting solvent. The volume of cyclohexane was tested between 40 μL and 150 μL. When using low volumes of cyclohexane (40 μL), the dispersion of cyclohexane into solution made it difficult to collect the cleaning extractant. It is important to avoid sampling the aqueous phase because it would interfere with detection and make evaporation time-consuming. The amount of steroids extracted increased with the increasing volume of cyclohexane. When 100 μL of cyclohexane was adopted, the recovery was 6.1% and 10.3% for testosterone and methyltestosterone,

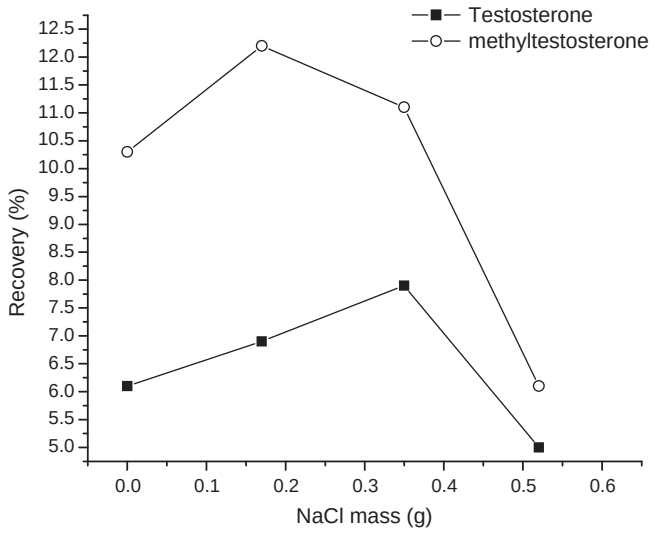

Fig. 4. Effect of NaCl on the extraction efficiency of steroids ($n = 3$). CME conditions: centrifugation rotor speed, 10,000 rpm; extraction time, 10 min; mass concentration, methyltestosterone, 3.5 μg mL^{-1} and testosterone, 1 μg mL^{-1}; sample volume, 1.3 mL; the cyclohexane volume, 100 μL. The 50 μL of extractants was dried and dissolved in 20 μL buffer (without SDS). Sample injection, 31.0 kPa × 90 s. MEKC conditions were as in Fig. 2.

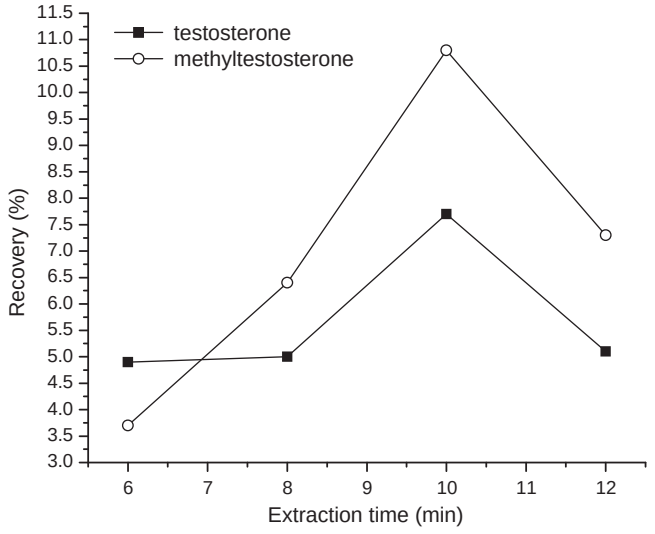

Fig. 5. Effect of extraction time on the extraction efficiency of steroids ($n = 3$). CME conditions: centrifugation rotor speed, 10,000 rpm; mass concentration, methyltestosterone, 3.5 μg mL^{-1} and testosterone, 1 μg mL^{-1}; sample volume, 1.3 mL; NaCl mass in sample, 0.35 g; the cyclohexane volume, 100 μL. The 50 μL of extractants was dried and dissolved in 20 μL buffer (without SDS). Sample injection, 31.0 kPa × 90 s. MEKC conditions were as in Fig. 2.

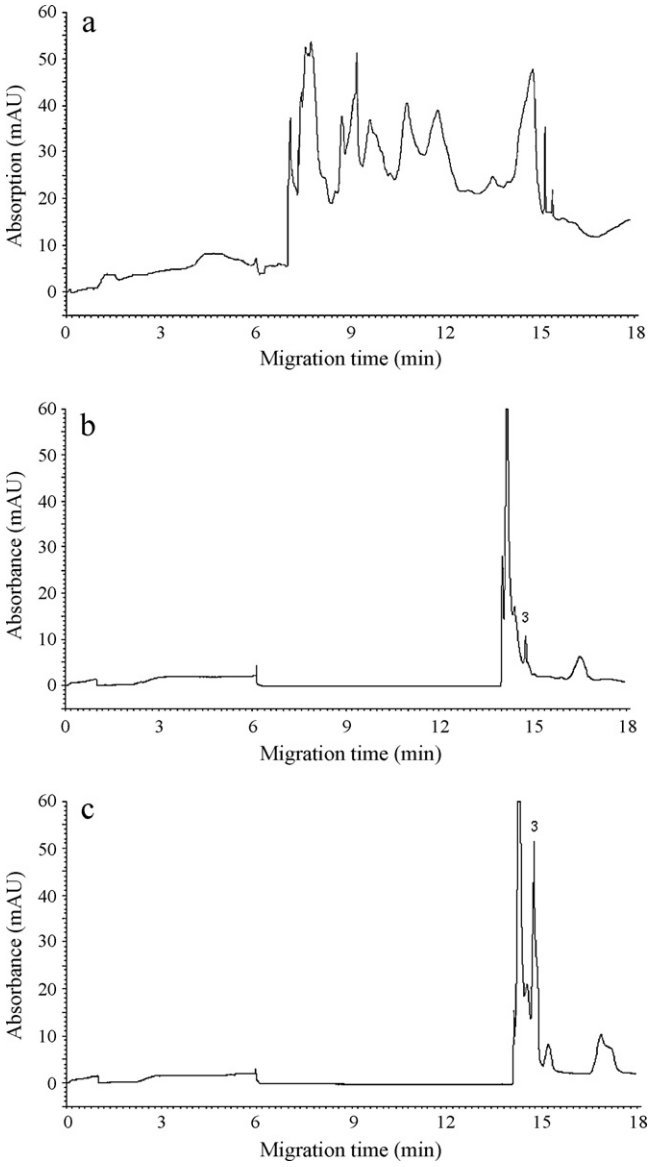

Fig. 6. Separation of blank and spiked urine sample. Electropherogram of blank urine sample with direct injection (a) and after CME (b); urine sample spiked with 1.5 μg mL^{-1} testosterone after CME (c). CME conditions: centrifugation rotor speed, 10,000 rpm; extraction time, 10 min; 1.3 mL urine sample volume adjusted with 0.35 g NaCl; the cyclohexane volume, 100 μL. The 50 μL extractants of CME was dried and dissolved in 20 μL buffer (without SDS). (a–c) Sample injection, 31.0 kPa × 90 s. MEKC conditions were as in Fig. 2. Peak identification as in Fig. 1.

Table 1
Linear correlation, linear range, correlation coefficients (R^2), limits of detection, RSD, recovery, and EF_{area} of the analytes with CME–sweeping–MEKC–UV.[a]

Analyte	Linear correlation	Linear range (μg mL^{-1})	Correlation coefficient (R^2)	LOD (ng mL^{-1})[b]	RSD (%)		Relative recovery	EF_{area}[f]
					No IS	With IS[c]		
MTS	$Y = 149{,}031x - 317$	0.05–1	0.9998	15	8.9	3.5	98.4[d], 103.2[e]	1278
TS	$Y = 138{,}538x + 339$	0.05–1	0.9999	5	7.4	2.8	96.3[d], 99.4[e]	513

MTS, methyltestosterone; TS, testosterone.

[a] CME conditions: centrifugation rotor speed, 10,000 rpm; extraction time, 10 min; 1.3 mL sample adjusted with 0.35 g NaCl; the cyclohexane volume, 100 μL. The 50 μL extractants of CME was dried and dissolved in 20 μL buffer (without SDS). Sample injection, 31.0 kPa × 90 s. MEKC conditions were as Fig. 2 ($n = 6$).

[b] S/N = 3.

[c] Progestogene as internal standard.

[d] The relative recovery of MTS and TS at 0.25 μg mL^{-1} ($n = 4$).

[e] The relative recovery of MTS and TS at 1.0 μg mL^{-1} ($n = 4$).

[f] Sample concentration for hydrodynamic injection, 100 μg mL^{-1} (3.4 kPa × 5 s), and for CME–sweeping, 0.5 μg mL^{-1}. $EF = area_{CME-sweeping}/area_{HI} \times 200$.

respectively (Fig. 3). The recovery of testosterone was 7.2% using 150 μL of cyclohexane (data not shown). However, larger volumes of cyclohexane take longer to evaporate, increasing sample preparation time and causing more environmental contamination. A 100 μL solution of cyclohexane was used for the following experiments.

3.2.2. Effect of ionic strength

The effect of ionic strength on extraction efficiency was studied by adding NaCl from 0 to 0.52 g to 1.3 mL of sample solutions. The results showed an initial increase in the extraction efficiency with the increments of salt mass. A maximum was reached at 0.30 g, followed by a decrease in extraction efficiency with further increase in salt mass (Fig. 4. The phenomenon can be explained by two simultaneously occurring processes [38]. Initially, the extraction efficiency was increased due to the salting out effect. However, in competition with this process, electrostatic interactions between polar molecules of the analyte and the salt ions in solution begin to predominate, thus reducing their ability to move into the extraction phase. Moreover, ionic strength may influence the physical properties of the Nernst diffusion film, which reduces the diffusion rate of the analytes into the organic phase [39,40]. 0.3 g of NaCl was selected since it provided the best extraction efficiency for all of the analytes.

3.2.3. Effect of rotor speed

As pointed out in our previous report [35], the rotor speed has an important influence on the extraction efficiency. Various centrifugation rotor speeds were studied to determine the optimal conditions for CME. The extraction efficiency of the steroids improved with the increase of rotor speed in the range of 4000–10,000 rpm and then slightly decreased at 12,000 rpm. So, a rotor speed of 10,000 rpm was chosen.

3.2.4. Effect of extraction time

Fig. 5 shows the influence of extraction time on the extraction efficiency. As it can be seen, the recovery increased with extraction time from 3 to 10 min for steroid hormones and decreased when time was longer than 10 min. Therefore, an extraction time of 10 min was selected.

3.3. Analytical performance of the CME–sweeping MEKC

The CME–sweeping MEKC was performed to analyze trace testosterone and methyltestosterone in aqueous standard solutions. Data for linear correlation, linear range, correlation coefficient, limit of detection, relative standard deviation (RSD) of peak area, and recovery of the method are summarized in Table 1. For testosterone and methyltestosterone, linearity was obtained in the range of 50–1000 ng mL^{-1} with correlation coefficients (R^2) ranging from 0.9998 to 0.9999. The RSDs for six replicate runs were <9.0% for all analytes and were <3.6% taking progesterone as an internal standard. The limits of detection (LOD) for methyltestosterone and testosterone were 15 and 5 ng mL^{-1}, respectively, which were evaluated on the basis of a signal-to-noise ratio of 3 ($S/N = 3$) (Table 1). Compared with solid phase extraction coupled with GC–MS [7], whose LODs are about 1 ng mL^{-1}, this method is less sensitive. However, as testosterone and methyltestosterone are of low volatility and moderate polarity, these compounds must be chemically modified by derivatization before GC–MS analysis. This additional step is often time-consuming and labor intensive, which leads to the limitation of its wide utilization. The sensitivity of this method is comparable to or even better than these measured with solid phase extraction–HPLC–MS [10,11]. Moreover, HPLC–MS needs expensive instrumentation and consume high amount of solvent/sample, which limits its application in routine analysis.

In this paper, the reliability of the analysis of steroids in the urine by standard addition was evaluated. Relative recovery has been estimated as:

$$\frac{\text{the mass of concentration found in the spiked sample} - \text{the mass concentration found in the sample}}{\text{the amount added}} \times 100$$

The relative recovery of methyltestosterone and testosterone was between 96.3% and 103.2% for 0.2 μg mL^{-1} and 1 μg mL^{-1} of analytes (Table 1). The enrichment factor (EF), defined as the ratio of analyte concentration in redissolving nonmicellar BGS to its initial concentration in sample, was used to evaluate the enrichment efficiency. The maximum attainable enrichment factor (EF_{max}) for CME in this experiment is the ratio of sample volume to the volume of nonmicellar BGS to redissolve the dry extract. The EF_{max} here is 65 (1300 μL/20 μL), and 5- to 7-fold enrichment was achieved for CME in 10 min. The low EF may result from the dispersion of extractant (only 50 μL from 100 μL extraction solvent can be collected), which decreases the analytes recovery. Decreasing the interface between the sample and the extraction solvent should decrease the dispersion, thus allowing more extractant to be collected [27]. Increasing the sample volume and decreasing the volume of nonmicellar BGS to redissolve the extract could be adopted if a larger preconcentration factor is desired. Sweeping provided a further on-line concentration of the analytes. Using CME coupled with sweeping, a 513- to 1278-fold increase in sensitivity was obtained as compared with the normal hydrodynamic injection without sample stacking (analytes dissolved in separation buffer and injected by 3.4 kPa for 5 s) (Table 1).

3.4. Analysis of urine sample

To evaluate the practical applicability of the CME–sweeping–MEKC method, the developed system was applied to analyze testosterone in urine samples. Fig. 6 illustrates the analysis of urine sample from a healthy man (aged 24) by direct injection (Fig. 6a), before (Fig. 6b) and after the addition of the testosterone (Fig. 6c) using CME. The electropherogram for the extracts is cleaner than that of direct injection which indicates that most of the matrix in urine was removed after CME. According to the migration time, the peak detected at 14.2 min was suspected to be methyltestosterone. However, the diode array UV spectrum of it was quite different from that of methyltestosterone (Fig. 7a), therefore methyltestosterone was not present in the urine sample. This is consistent with the fact that methyltestosterone is a synthetic steroid and the man had not taken any medications containing steroids in the past year. Thus, a UV spectrum of a peak is used to verify the identification of the peak in addition to migration time. The peak detected at 14.8 min was clearly identified to be testosterone by comparing migration time and the UV spectrum with that of the standard (Fig. 7b). Moreover, spiked urine samples were employed to further confirm the identities of the testosterone (Fig. 6c). The concentration of testosterone was determined to be 55 ng mL^{-1} using calibration curves, which was in agreement with the value of 60 ng mL^{-1} as measured by the standard addition method.

In this experiment, twelve samples (the maximum sample capacity of the centrifuge used) were extracted within 10 min by a centrifuge and dried under a stream of nitrogen with a 24-well format evaporator in 3 min. The throughput of the CME is remarkably

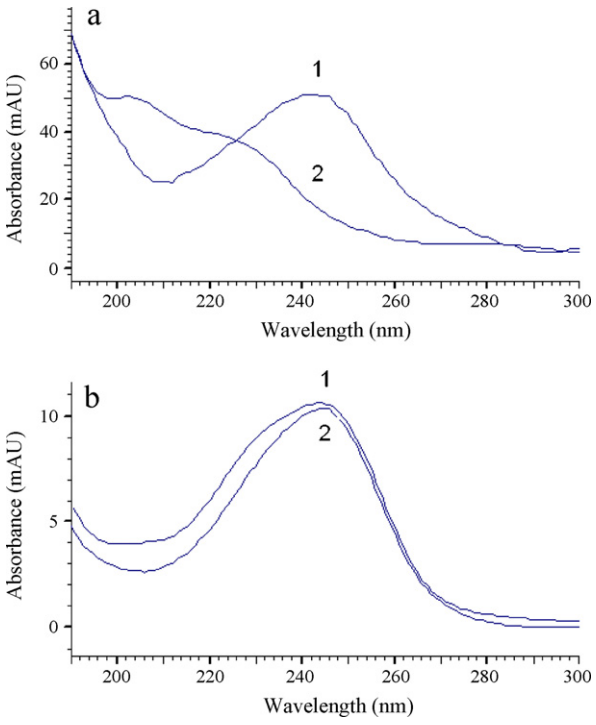

Fig. 7. Comparison of diode array UV spectrum (190–300 nm) at migration time of testosterone and methyltestosterone. (a) Reference methyltestosterone (1) and UV spectrum at migration time of 14.2 min in urine sample (2); (b) reference testosterone (1) and UV spectrum at migration time of 14.8 min in urine sample (2).

high considering that 48 samples were prepared in only 1 h. Combined with the short MEKC runs, a large number of samples could be analyzed within a short time. This method is high-throughput and cost-effective, and, therefore, would greatly benefit the studies in which many samples need to be analyzed in a short time.

4. Conclusion

In the present study, CME coupled with sweeping–MEKC was used to detect trace testosterone and methyltestosterone in water/urine samples. The analytes could be reliably analyzed by this rapid and selective method, making it an attractive alternative to GC–MS and HPLC–MS for the qualitative and quantitative determination of steroids in urine samples. In view of the high sample throughput, the method developed may find applications in the clinic field and doping control where large amount of samples are to be analyzed. Combining CME with solid phase extraction, which can provide a further concentration of analytes as well as sample cleanup, is under consideration.

Acknowledgements

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