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# Micellar electrokinetic capillary chromatographic separation of steroids in urine by trioctylphosphine oxide and cationic surfactant

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**Abstract**

Separation of the six structurally similar and hydrophobic neutral steroids, testosterone, dimethyltestosterone, testosterone propionate, cortisone, hydrocortisone and 17-deoxycorticosterone, was achieved by hydrophobic micellar electrokinetic chromatography. A triphasic separation involving micellar dodecyltrimethylammonium bromide (DTAB), a dynamic bilayer formed due to electrostatic interaction between the silica surface and DTAB, and aqueous phase is proposed to account for the observed separation of the steroids. The running buffer consisted of 0.05 *M* DTAB and 0.0052 *M* trioctylphosphine oxide in 0.01 *M* of phosphate buffer pH 7.4. A detection limit of 500 ng/ml was achieved for each steroid and the application of the method to urine samples is described.

## 1. Introduction

Steroids are compounds that contain the cyclopentanoperhydrophenanthrene ring system (Fig. 1). Substitutions in this ring system produce the different classes of steroids that have distinct physiological actions. The adrenal glands, for example, produce a variety of different structurally similar steroids including corticosteroids, androgens, progesterone, and estrogen [1]. Various clinical conditions are associated with the hypo- or hyper-secretions of these steroids, e.g. Cushings disease, Addison's disease, and Liddle and Bartter syndromes. In addition to the naturally occurring steroids, testosterone derivatives such as methyl-, dimethyl-, acetyl- and chlorotestosterone have been used as pharmaceuticals for enhancement of athletic performance. The illegal abuse of these agents far exceeds their legitimate therapeutic use. The long-term effects of anabolic steroid abuse include prostatic cancer, impotency, and acromegaly in addition to hirsutism and amenorrhea [2] in females.

The structural similarity and small concentrations 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) range, require a sensitive detection capability. The methods presently available for the quantitative determination of steroids include colorimetric, fluorimetric, chro-

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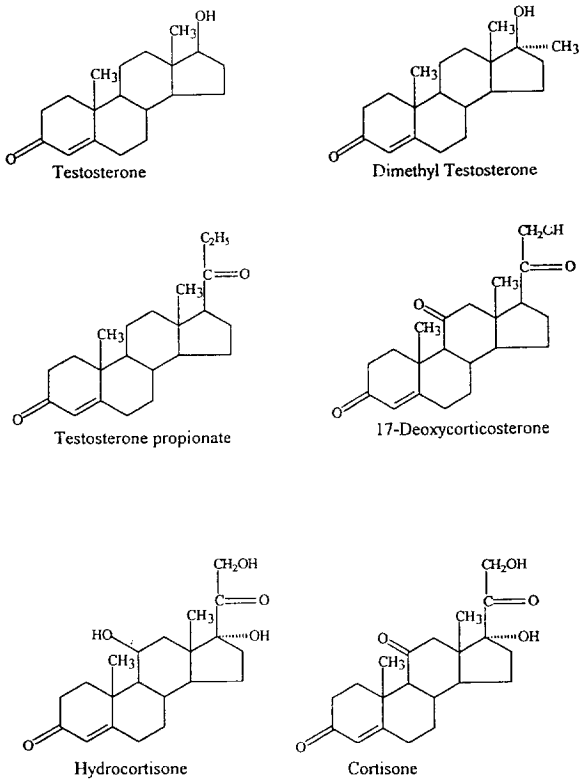


Fig. 1. Steroid structures.

matographic (TLC, HPLC, GC and GC–MS), and immunoassay [3–9]. Immunoassay techniques are by far the most sensitive and widely applied for routine determination of these steroids. Although highly sensitive, immunoassays suffer from low specificity, which is usually overcome by sample purification.

The close structural similarities between different steroids demands a technique with a high resolving power. Currently, capillary electrophoresis (CE) is the only technique that is capable of attaining a resolution greater than 250 000 theoretical plates. It has been used for the determination of a variety of compounds such as amino acids [10,11], oligonucleotides [12,13], carbohydrates [14,15], organic acids [16,17], and catecholamines [18,19]. Electrokinetic chromatography (EKC) is the general name given to the family of electrophoretic techniques based on the deferential migration caused by electrokinesis phenomena and differential partitioning. Micellar electrokinetic capillary chromatography (MEKC), first introduced by Terabe et al. in 1984 [20] to facilitate the separation of neutral analytes, is a mode of EKC in which an ionic surfactant at a concentration greater than its critical micelle concentration is added to the buffer solution.

Although MEKC has been used for the determination of a wide array of biologically important molecules, its applications in the field of steroid analysis are relatively few. Our literature search revealed only one application of CE for the determination of corticosteroids by MEKC with bile salts [21]. MEKC with SDS (sodium dodecyl sulfate) was unsuccessful in separating these steroids, since owing to their high lipophilicity, they migrated with almost the same velocity as that of the SDS micelle. By using a system consisting of dodecyltrimethylammonium bromide (DTAB) and trioctylphosphine oxide (TOPO), we have for the first time separated six structurally similar steroids on a fused-silica capillary.

## 2. Experimental

### 2.1. Apparatus

CE analysis was performed on a Beckman P/ACE 5010 (Beckman Instruments, Fullerton, CA, USA) equipped with system Gold software for data analysis. A fixed wavelength UV detection at 254 nm was employed for all separations performed in this study. MEKC was performed in a 50 cm (injection to detection) × 50 μm I.D. × 375 μm O.D. fused-silica capillary tube (Beckman Instruments). The total capillary length was 57 cm. High-pressure injections were made for 5 s and the temperature of the capillary was maintained at 15 ± 0.1°C. The applied voltage was 15 kV with operating currents of less than 30 μA.

### 2.2. Materials and method

Testosterone, dimethyltestosterone, 17-deoxycorticosterone, cortisone, hydrocortisone and testosterone propionate (all 99.9% pure) were purchased from Sigma Chemicals (St. Louis,

MO, USA). TOPO, mesityl oxide, HPLC grade acetonitrile and DTAB were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade or better. Phosphate (0.1 *M*) and citrate (0.1 *M*) stock buffer solutions were prepared by dissolving appropriate amounts of $NaH_2PO_4 \cdot H_2O$ and citric acid in deionized water and adjusting the pH to 7.4 with NaOH. Phosphate (0.01 *M*) or citrate (0.01 *M*) buffers were prepared by dilution of the appropriate stock solution and were used in all the experiments. DTAB (0.1 *M*) and TOPO (0.1 *M*) stock solutions were prepared by dissolving appropriate amounts of both reagents in deionized water and acetonitrile, respectively. The run buffer comprised of 0.05 *M* DTAB, 0.0052 *M* TOPO and 0.01 *M* phosphate or 0.02 *M* citrate, pH 7.4. It was prepared fresh every day and filtered through 0.45-$\mu$m Nylon syringe filters (Waters, Milford, MA, USA). Steroid stock solutions (10 mg/ml) were made by dissolving the respective steroids in acetone and were used to prepare 0.05, 0.01, 0.005, 0.001, and 0.0005 mg/ml standards in electrophoresis buffer.

Each day the capillary was activated by rinsing it with 0.5 *M* NaOH for 30 min followed by distilled water for an equal period of time. Before each injection the capillary was equilibrated with run buffer for 3 min. At the end of each run the capillary was washed for 3 min with 0.1 *M* HCl followed by 0.5 *M* NaOH and then distilled water. All runs were done in sets of five in order to monitor the precision of the migration times for each analyte. Mesityl oxide was used as an internal reference.

### *2.3. Analysis of steroids added to urine*

A 5-ml aliquot of spiked urine sample was first acidified to pH 6, then extracted twice with 2 ml of methylene chloride. The solvent was evaporated under vacuum and the extracted steroids were reconstituted with 0.5 ml of electrophoresis buffer. The dissolution of the extracted steroids in the buffer was ensured by sonicating the solution for 5 min. As a control, unspiked urine was also carried through the extraction procedure.

## 3. Results and discussion

An interesting feature of cationic surfactants is the reversal in direction of the electroosmotic flow (EOF) at concentrations dependent upon the surfactant. This phenomenon was first described by Tsuda [22] and is due to reversal in sign of the zeta potential at the fused-silica wall when DTAB is substituted for sodium dodecyl sulfate (SDS) as shown schematically in Fig. 2. It involves the formation of a bilayer due to electrostatic attraction between the positively charged amine head group of the cationic surfactant and the negatively charged fused-silica capillary surface. We call the bilayer formed with the cationic surfactant a dynamic bilayer phase because unlike the $C_{18}$ bonded phase in reversed-phase high-performance liquid chromatography (RP-HPLC), this bilayer is held by electrostatic attraction and is continuously regenerated. Fig. 3 shows a typical electropherogram depicting the separation of the six electrically neutral steroids extracted from a spiked urine sample. Superimposed is the electropherogram of an unspiked urine sample. A peak present in the unspiked urine, eluting at 30.8 min, is probably hydrocortisone, a normal constituent of urine present at a concentration that can be detected by this method. As seen a baseline separation was obtained for six structurally similar steroids. The more polar cortisone, having a lower distribution coefficient than the testosterone derivatives for the micellar and dynamic bilayer phase, is eluted first, whereas testosterone propionate, being most lipophilic, is eluted last. No separation whatsoever was achieved by the buffer system when 0.05 *M* SDS replaced DTAB.

In order to gain a better understanding about the role of the dynamic bilayer in facilitating separation, a buffer composed of 0.005 *M* DTAB, 0.02 *M* phosphate buffer pH 7.0 and 5% acetonitrile, was used to separate the various steroids. TOPO was not added in this experiment since it is insoluble at DTAB concentrations less than the critical micelle concentration (CMC) which is approximately 0.015 *M*. The detergent concentration in this buffer system is one third the CMC. Even though there is no micelle

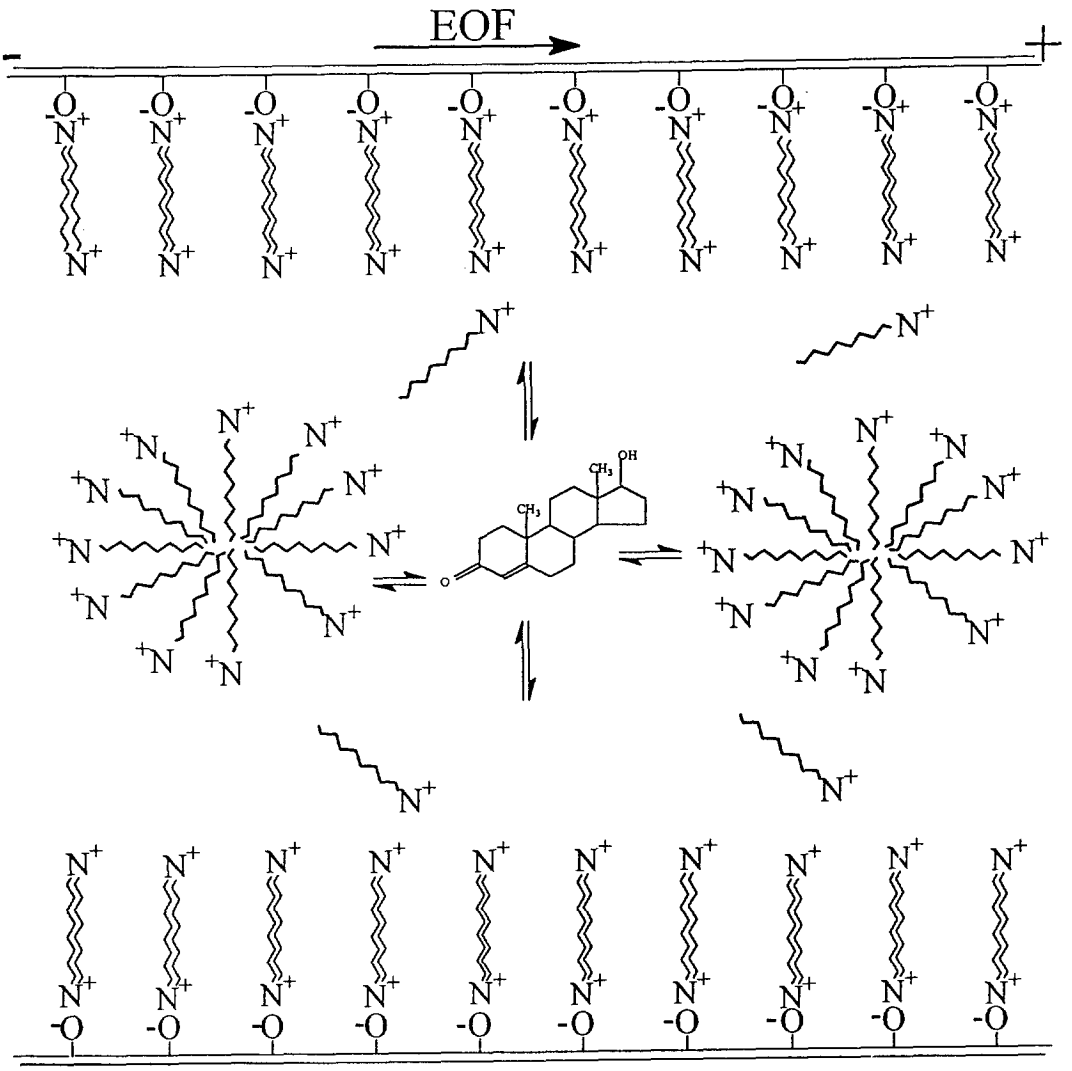


Fig. 2. Steroid separation via electrophoretically mediated partitioning. Run buffer: 0.05 *M* DTAB, 0.0052 *M* TOPO, 0.01 *M* phosphate, pH 7.0.

formation, a bilayer is formed as confirmed by the reversal of EOF. As seen in Fig. 4 a baseline separation was achieved for testosterone, dimethyltestosterone and testosterone propionate. The other three steroids were not resolved and eluted as one peak. Close inspection of this electropherogram in terms of order of elution in addition to peak shape and width reveals similarities to an HPLC profile, suggesting a similar separation mechanism. The separation of these closely related steroids in this buffer can only be explained by the differential partitioning of these steroids into the dynamic bilayer. The migration order of steroids was found to be consistent with that seen when the DTAB concentration was above the CMC, i.e. the polar cortisone eluting first and the non-polar testosterone propionate eluting last. This migration order is also similar to that seen in RP-HPLC or micellar RP-HPLC using $C_{18}$ coated silica columns [23]. Based on the above observations it is reasonable to conclude that when MEKC is using a cationic

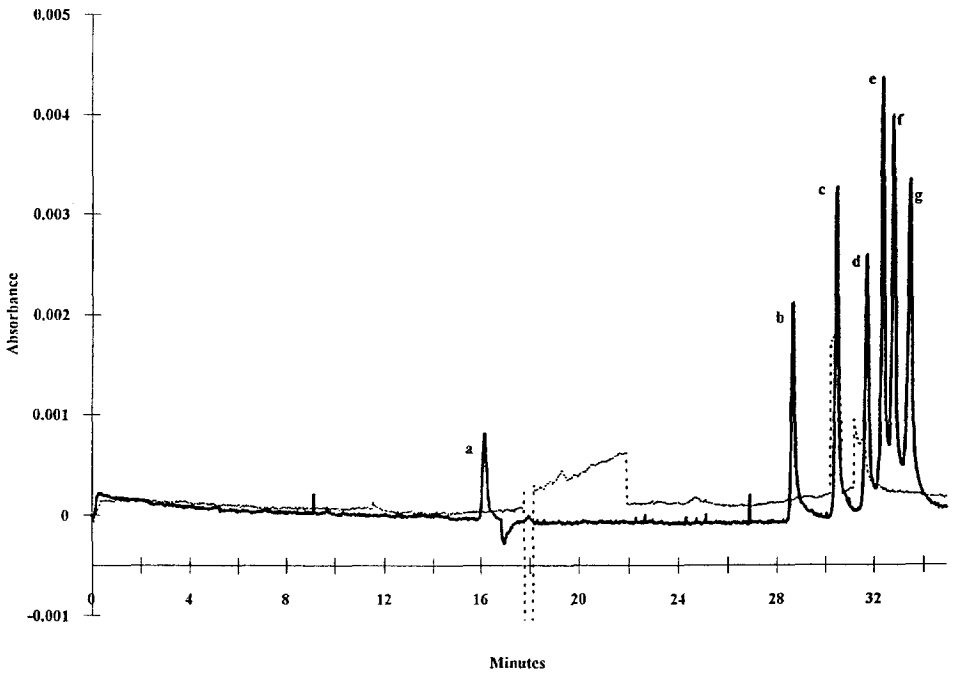


Fig. 3. Electropherogram of a normal extracted urine (dashed line) urine spiked (solid line) with the following compounds and then extracted: a, mesityl oxide; b, cortisone; c, hydrocortisone; d, 17-deoxycorticosterone; e, testosterone; f, dimethyltestosterone; and g, testosterone propionate. The running buffer was 0.05 *M* DTAB, 0.0052 *M* TOPO and 0.01 *M* phosphate buffer pH 7.4; detection with UV at 254 nm and a temperature of 15°C.

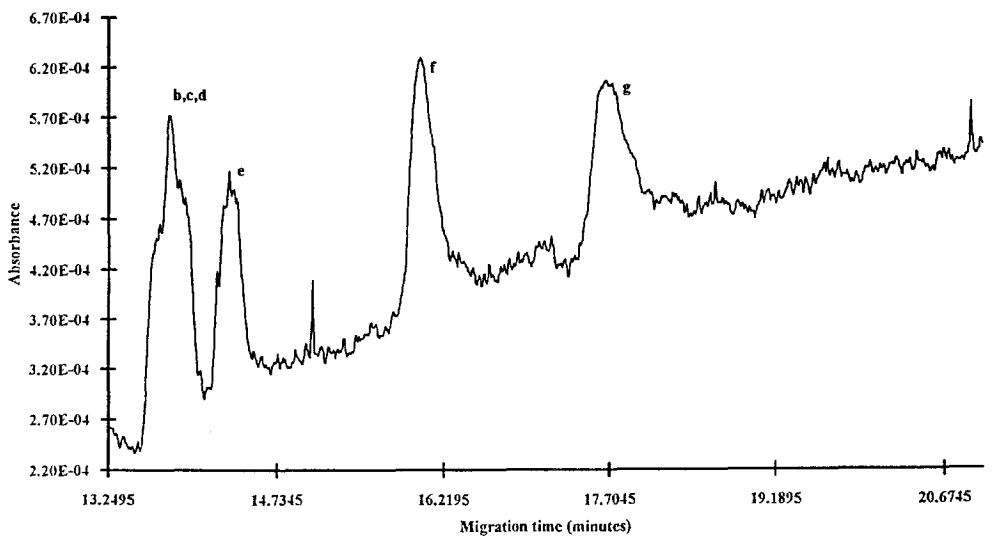


Fig. 4. Electropherogram of six steroids in running buffer consisting of 0.005 *M* DTAB, 0.02 *M* phosphate buffer pH 7.0 and 5% acetonitrile, detection UV 254 nm, temperature 15°C. Refer to Fig. 3 for individual steroid identification.

detergent such as DTAB, the separation of neutral analytes is determined by three partition equilibria: (i) the micellar and aqueous phase; (ii) micellar and dynamic bilayer phase; and (iii) the aqueous phase and the dynamic bilayer phase. The bilayer formed by the cationic surfactant, not only reverses the EOF but also behaves in a manner similar to a $C_{18}$ stationary phase in RP-HPLC. This is further supported by the fact that a significant reduction in the theoretical plates is observed at DTAB concentrations less than its CMC. As shown in Table 1 the theoretical plates calculated by the equation $N = 2\pi (t_r h/A)^2$ [24], are very similar to those expected for RP-HPLC.

Increasing the DTAB concentration to 50 m*M* also increases the theoretical plates (Table 1) which appears to be in contradiction to the proposed mechanism for the separation of the steroids. One would expect a decrease in the theoretical plates since the number of micelles and the size would increase, allowing the micelle to compete more effectively with the dynamic phase. This anomaly can be explained by considering the time spent by each steroid molecule in either the DTAB micelle or the bilayer. This in turn is directly proportional to the lifetime of the micelle and bilayer. The DTAB molecules in the micelle are held together by Van der Waals interactions. The dynamic bilayer, on the other hand, involves both electrostatic and hydrophobic interactions; therefore it is reasonable to assume that the lifetime of the bilayer will be greater relative to the micelles. The greater longevity of the bilayer in comparison to the micelles is important because it permits the partitioning of the neutral analytes between the different phases. In addition, the bilayer formed in CE, unlike the $C_{18}$ phase in RP-HPLC, is continuously disrupted by EOF turbulence and then regenerated. This results in a decreased resident time of the steroid molecules in the bilayer. In other words, the mass transfer from the bilayer to micellar phase is much more efficient in CE than in RP-HPLC. The net result is decreased band broadening, faster electrophoretic mobilities and higher efficiencies. In buffers having detergent concentrations less than the CMC, separation is primarily dictated by a two-way partitioning, between the dynamic bilayer and water–DTAB–acetonitrile phase. When the detergent concentration is greater than the CMC, there is an increase in both micelle concentration and the bilayer permitting efficient partitioning due the formation of three distinct phases, i.e. the micellar phase, dynamic bilayer phase and aqueous phase. This not only improves resolution but also enhances efficiency by promoting increased mass transfer to the mobile phase composed primarily of DTAB micelles.

Table 1
Calculated theoretical plates

| Steroid | *N* (50 m*M* DTAB) | *N* (5 m*M* DTAB) |
|---|---|---|
| Testosterone | 185 006 | 20 403 |
| Dimethyltestosterone | 220 010 | 26 139 |
| Testosterone propionate | 215 067 | 17 447 |
| 17-Deoxycorticosterone | 194 500 | * |
| Cortisone | 216 003 | * |
| Hydrocortisone | 218 560 | * |

* Unable to calculate due to co-elution of the peaks.

MEKC with an anionic detergent is a different situation. Here no formation of the dynamic bilayer phase is found. On the contrary, efforts are made to prevent the adsorption of detergent onto the capillary surface. The separation of the neutral analyte in this two-way partitioning system is mainly dictated by partitioning between the aqueous and micellar phases. In MEKC using SDS and Brij 35 to form the micelles, the non-ionic Brij 35 surfactant coats the capillary wall by adsorption. However, analytical precision is poor due to the fact that the Brij coating increases in thickness with repeated injections, causing irreproducible retention times [25]. In contrast, when a cationic detergent such as DTAB is used, the dynamic bilayer coating the silica capillary is regenerated with high reproducibility after each run. The reproducibility of this coating is substantiated by the highly reproducible migration times for all the 6 steroids with R.S.D. $\leq 1.2\%$ over 18 injections.

The exact role of TOPO used in this system is uncertain. We believe it functions in a manner similar to the organic modifiers routinely used to

improve resolution. DTAB by itself was able to resolve only one of the six steroids studied as indicated by baseline separation. However, a baseline resolution was achieved when 0.0052 *M* of TOPO was added to the run buffer. It can be speculated that TOPO being electrically neutral and composed of three highly hydrophobic octyl chains, partitions in the micelles and dynamic bilayer. By doing so, it reduces the overall charge on the micelle thereby reducing its electrophoretic mobility and increasing the hydrophobic interactions between steroid molecules and the micellar medium. Interestingly, it was observed that the Sudan III which is routinely used as a micellar phase marker, migrated faster than testosterone propionate. A possible explanation is that the electrophoretic mobilities of individual solutes in this three-way partitioning system are dependent on the partition coefficient of individual analytes and thus are no longer limited by the separation time window. This time window is simply the difference between the $t_o$ and $t_m$, where $t_o$ is the retention time of the unretained solute moving with the EOF rate (or dead time) and $t_m$ is the micelle retention time. Thus by forming a dynamic partitioning of the neutral solutes between the three phases, we have eliminated the restriction of fixed time window that is available for separation. By doing so we have achieved a near infinite elution range, i.e. $t_m/t_o = 0$ for the steroids studied. Recently, Ahuja et al. [26] reported an infinite elution range for aromatic ketones in MEKC by using a non-ionic (Brij 35) and anionic (SDS) mixed micellar system. The decrease in electrophoretic mobility in this system is attributed to the combination of increased solution viscosity and adsorption of Brij 35 to the capillary surface.

Increasing the surfactant concentration beyond 50 m*M* resulted in decreased resolution. Temperature also affected the separation. Electrophoresis at 25°C caused band broadening resulting in no separation of the testosterone derivatives. This improved remarkably when the temperature was lowered to 15°C, in agreement with the earlier reports that showed better resolution when the temperature was reduced [27]. Increasing the buffer concentrations to greater than 0.02 *M* resulted in higher currents and reduced EOF, increasing the migration time of the steroids with no significant enhancement in resolution.

Direct injection of an untreated urine resulted in multiple peaks, one of which co-eluted with the testosterone derivatives. Hence, it was deemed necessary to clean up the sample prior to the injection. This was achieved by extracting the steroids in dichloromethane followed by reconstitution in run buffer prior to injection. For all six steroids the corrected peak area vs concentration calibration curve was linear between 0.05 and 0.0005 mg/ml with correlation coefficients ranging from 0.9996 to 0.9978 for triplicate injections. The sensitivity for each steroid was between $2.5 \cdot 10^{-14}$ and $3.47 \cdot 10^{-14}$ mol/injection corresponding to a concentration of 500 ng/ml in the sample solution. The injection volume was calculated by using the Hagen–Poiseuille equation [24]. We believe that better sensitivity can be achieved if the absorbance maximum of these steroids which occurs at approximately 240 nm is used. The use of 214 nm wavelength resulted in high background noise, possibly due to the impurities present either in TOPO or DTAB.

## 4. Conclusions

The method described here is an example of MEKC chromatography using the cationic detergent, DTAB. For the first time we have demonstrated that excellent separation can be achieved for structurally similar neutral steroid molecules such as testosterone, dimethyltestosterone and testosterone propionate on a fused-silica capillary with a cationic surfactant. The system described here mimics the micellar RP-HPLC with the more polar steroids being weakly retained on the dynamic bilayer phase and thus migrating faster than the relatively lipophilic testosterone derivatives. The possibility of achieving an infinite elution range in combination with cationic surfactants and a highly hydrophobic micellar phase modifier such as TOPO or its analogues are under investigation in this laboratory. The obvious advantages of such a

modifier will be the reduced viscosity of run buffer, better efficiency and faster migration times for highly hydrophobic analytes. The potential application of such a system in clinical laboratories is of great importance since the determination of steroids in neonates has major clinical importance in the diagnosis of certain inborn errors of steroid metabolism such as congenital adrenal hyperplasia (CAH). Throughput and small sample size necessitate a fast, selective and sensitive analytical method with a sensitivity in ppb range. The RIA method currently used for these analytes although sensitive is extremely laborious and has low specificity. We are currently developing a method for the separation and quantitative determination of the steroids involved in CAH using the system described above.

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