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# Anionic–zwitterionic mixed micelles in the micellar electrokinetic separation of clinically relevant steroids on a fused-silica capillary

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**Abstract**

The structural similarities of different clinically relevant steroids make their routine determination in biological samples an analytical challenge. Determination of steroidal hormones in urine and serum is of great interest in clinical endocrinology. A large number of disorders is associated with hypo- or hypersecretion of one or more steroids, for example, Addison's disease, Cushing's syndrome, hirsutism and virilism, adrenal adenomas, hypertension, congenital adrenal hyperplasia etc. A SDS/SB3-12 mixed micellar system was used to achieve baseline separation of eight steroids in less than 10 min. Five different zwitterionic surfactants were evaluated at different concentrations, pH and temperature conditions. The SB3-12/SDS mixed micellar system gave highest resolution and precision. No significant capillary wall interaction or coating effects were observed with the zwitterionic surfactants as is evident from the reproducible migration times (relative standard deviation ≤2%). A detection limit of 1–5 μg/ml was obtained for pure standards. © 1997 Elsevier Science B.V.

*Keywords:* Buffer composition; Steroids; Hormones

## 1. Introduction.

Steroidal hormones regulate many key physiological processes, such as electrolyte balance, protein, carbohydrate and lipid metabolism, growth and differentiation, reproduction, immune response and sexual characteristics. Several diseases are characterized by the imbalance of one or more of these steroids. Although each steroid elicits a specific set of biological responses, they are structurally very similar. The specificity of their activities is due to substitutions in the basic cyclopentanoperhydrophenanthrene ring (Fig. 1). Current analytical methods include colorimetric, fluorimetric, chromatographic (TLC, HPLC, GC and GC–MS) and immunoassay techniques. Of all the different meth-

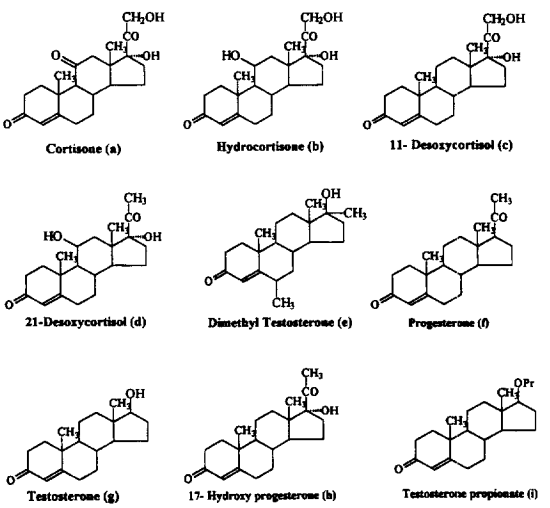


Fig. 1. Structures of steroid studied.

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ods available, immunoassays are the most sensitive and are routinely used in clinical laboratory practice. Although sensitive, the specificity of antibodies can be a problem. This can be improved by extraction of steroids from the sample matrix such as serum or urine. This, however, increases the time and cost of performing the assay. In addition, immunoassays allow determination of only one steroid at a time, thus increasing the cost if a steroid profile is desired.

Since its introduction in 1984 by Terabe et al. [1], micellar electrokinetic chromatography (MEKC) has gained wide popularity. It has been used for the separation of various hydrophobic compounds such as derivatized amino acids [2], polycyclic aromatic hydrocarbons [3], active ingredients in cold remedies [4], etc. Nishi et al. [5] were the first to demonstrate the applicability of MEKC for separating various corticosteroids on a fused-silica capillary using a sodium dodecyl sulfate (SDS)–bile salts mixed micelle system. Addition of organic modifiers such as acetonitrile [6] or bile salts [7] to SDS was found to be essential in achieving separation of steroids in a mixture. Very little resolution is achieved in the absence of organic modifiers. Due to the high lipophilicity of steroids they migrate with almost same velocity as SDS micelles. Buffers composed of cationic surfactants such as dodecyl trimethylammonium bromide also necessitated the addition of organic modifier trioctylphosphine oxide to facilitate resolution of steroids in a mixture [8]. Separation of two species in MEKC can be described by Eq. (1)

$$R = (N)^{1/2}/4[(\alpha - 1)/\alpha][(k_2'/k_2' + 1)][(1 - t_0/t_{mc})/(1 + t_0/t_{mc})k_1'] \quad (1)$$

where is $R$ resolution, $N$ the theoretical plate number, $t_0$ and $t_{mc}$ are the migration times of an unretained component or electroosmotic flow (EOF) marker and the micelle, respectively, $\alpha$ is the separation factor equal to $k_2'/k_1'$, and $k_1'$ and $k_2'$ are the capacity factors of analytes 1 and 2, respectively. From this equation it is apparent that resolution can be improved by optimizing efficiency, selectivity and/or the capacity factor. The capacity factor is proportional to the concentration of surfactant and ionic strength of the run buffer. Thus, the higher the ionic strength and the surfactant concentration, the better the resolution due to the increase in theoretical plates and capacity factor. For separation of steroids by MEKC, the optimum SDS concentration reported so far is between 0.075 $M$–0.1 $M$ with the addition of buffers and organic modifiers. This can result in currents greater than 100 μA, for voltages in the range of 25–30 kV on fused-silica capillaries. This results in Joule heating giving current errors on the Beckman P/ACE system. This problem can be circumvented either by using low ionic strength buffers, or neutral capillaries. In a clinical laboratory neither of these options are appropriate since low concentrations of SDS decreases resolution and lot-to-lot inconsistencies limit the use of neutral capillaries in routine applications.

Our overall objective was to develop a buffer system that provides high resolution of clinically important steroids, uses commercially available detergents, has a relatively low ionic strength and does not necessitate the use of neutral capillaries. Ahuja et al. [9] reported the use of anionic–zwitterionic mixed micelles in MEKC. Several advantages such as a 2–5 fold increase in column efficiency, ability to change selectivity by modifying the zwitterionic/SDS ratio, resolution of structural isomers, excellent precision and low Joule heating were observed by these researchers. These qualities led us to investigate five zwitterionic surfactants at different conditions of zwitterionic/anionic surfactant ratio, pH and temperature as a potential buffer system for robust clinical assay for steroid determinations.

## 2. Experimental

### 2.1. Apparatus

A Beckman P/ACE 5010 (Beckman Instruments, Fullerton, CA, USA) instrument equipped with system Gold software for data analysis was used for capillary electrophoresis (CE). A fixed wavelength UV detection at 254 nm was employed for all separations. MEKC was performed in a 60 cm (injection to detection)×50 μm I.D.×375 μm O.D. fused-silica capillary tube. The total capillary length was 67 cm. High-pressure injections were made for 5 s and the temperature of the capillary was maintained at 25±0.1°C in all experiments except two in which

30°C and 16°C were tried. The applied voltage was 30 kV with operating current of less than 40 μA.

### 2.2. Materials and method

SDS, N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3- 10), N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-12), N-hexadecyl – N,N-dimethyl -3- ammonio -1- propanesulfonate (SB3-16), N-octadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-18), N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-8), N,N-bis(2-hydroxyethyl)glycine (Bicine), piperazine-N,N′-bis[2-ethanesulfonic acid]; 1,4-piperazine diethanesulfonic acid (PIPES), testosterone, 7α,17-dimethyltestosterone, cortisone, cortisol, testosterone propionate, 17-OH-progesterone, progesterone, 4-pregnene-17α,21-diol-3,20 dione (21-desoxycortisol), 4-pregnene-17α,11β-diol-3,20-dione (11-desoxycortisol) were purchased from Sigma (St. Louis, MO, USA). Steroid stock solutions (10 mg/ml) were made by dissolving the respective steroids in acetone and were used to prepare the standards in 4 m*M* SDS. Deionized water treated with a 0.22 μm Millipore filter system to 18 MΩ resistivity was used throughout. Stock solution (0.1 *M*) of SB3-8, SB3-10, SB3-12 and SDS were made by dissolving appropriate amounts of these detergents in deionized water. Due to low solubility of SB3-16 and SB3-18 detergents they were used at concentrations close to their critical micelle concentrations (CMCs). Stock solutions (0.1 *M*) of PIPES (pH 6) and Bicine (pH 8–9) buffers were used to prepare run buffers for pH studies. In order to prevent excessive Joule heating, individual buffers concentrations in final run buffers for all experiments did not exceed 0.025 *M*. Run buffers were made fresh for each set of experiments and were filtered through 0.45 μm nylon syringe filter (Waters, Milford, MA, USA). Acetone was used to mark the EOF ($t_0$) and Sudan III ($t_{mc}$) was used to track the micelle migration.

Each day the fused-silica capillary was activated by rinsing it with 0.1 *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 0.1 *M* NaOH and then distilled water. All runs were done in triplicate in order to monitor the precision of the migration times for each analyte.

## 3. Results and discussion

CE in general has found relatively few applications in clinical laboratory practice when compared to other chromatographic techniques. Although a very powerful separation technique, it is plagued with problems such as low sensitivity for absorbance detection, clogging of capillaries, short shelf life of run buffers and more importantly, current errors. This increases hands on time and diminishes the walk-away capabilities that other chromatographic techniques offer. Even with these limitations the clinical applications of CE have increased over the last five years [10–12]. Some of the inherent disadvantages of CE, such as low sensitivity, can be offset either by using sensitive detections systems, on-line preconcentration or sample stacking by isotachophoresis [13–15]. Capillary clogging can be prevented by filtering sample and run buffers through 0.45 μm filters before each run. However, current errors that arise due to overheating and deposition of water vapors around the sample vial, can only be avoided by using buffers with a low ionic strength and a minimum amount of organic solvents. This may not be a serious problem for research laboratories but special considerations must be given if the assay is to be used routinely in a clinical laboratory.

Ahuja et al. [9] reported low currents and excellent resolution for a series of neutral aromatic compounds such as benzyl alcohol, nitrobenzene, anisole etc., with run buffers comprised of zwitterionic and anionic surfactants on fused-silica capillaries. To the best of our knowledge no one has described this system for the separation of clinically important steroids. Zwitterionic surfactants, like other surfactants, form micelles at concentrations greater than the CMC. Due to the electrical neutrality of these micelles they do not move in the electrical field and therefore their transition in a capillary is solely determined by the EOF. When used in combination with an anionic surfactant such as SDS, zwitterionic surfactants constitute the pseudo-stationary phase, thus allowing the partitioning of

Table 1
Critical micelle concentration of detergents

| Name of steroid | Zwitterionic–SDS system | | SDS–$CH_3CN$ system | |
|---|---|---|---|---|
| | $N=2\pi(t_r h/A)^2$ | Detection limit (μg/ml) | $N=2\pi(t_r h/A)^2$ | Detection limit (μg/ml) |
| Cortisone | 102 322 | 5 | 216 390 | 0.5 |
| Hydrocortisone | 100 561 | 5 | 216 072 | 0.5 |
| 11-Desoxycortisol | 82 591 | 5 | 218 370 | 0.5 |
| 21-Desoxycortisol | 121 223 | 3 | 220 465 | 0.5 |
| Dimethyltestosterone | 101 367 | 1 | – | – |
| Progesterone | 143 071 | 3 | 199 737 | 0.5 |
| Testosterone | 131 387 | 2 | 214 106 | 0.5 |
| 17-Hydroxyprogesterone | 94 840 | 5 | 207 220 | 0.5 |
| Testosterone propionate | 74 709 | 5 | 183 068 | 0.5 |

neutral molecules, resulting in their separation based on individual partition coefficients. Addition of a zwitterionic surfactant to SDS did resolved various steroids at considerably lower ionic strengths. Some of the theoretical aspects of this system are discussed below.

### 3.1. Resolution

One of the main objectives of this research was to achieve the optimum resolution in a minimum amount of time. For this purpose we examined five different zwitterionic surfactants and SDS combinations. The preliminary separations were performed in solutions containing detergents just above their respective CMCs which are listed in Table 1. None of the anionic/zwitterionic buffer compositions gave a baseline separation for all of the nine steroids studied. The buffer composed of 0.005 *M* of SB3-12, 0.01 *M* SDS and 0.025 *M* Bicine pH 9 gave the best resolution for all nine steroids, as shown in Fig. 2, with cortisone migrating first and testosterone propionate migrating last. Baseline separation was not achieved for testosterone and progesterone with any combination of zwitterionic and SDS mixed micellar systems studied. The SB3-10/SDS system was able

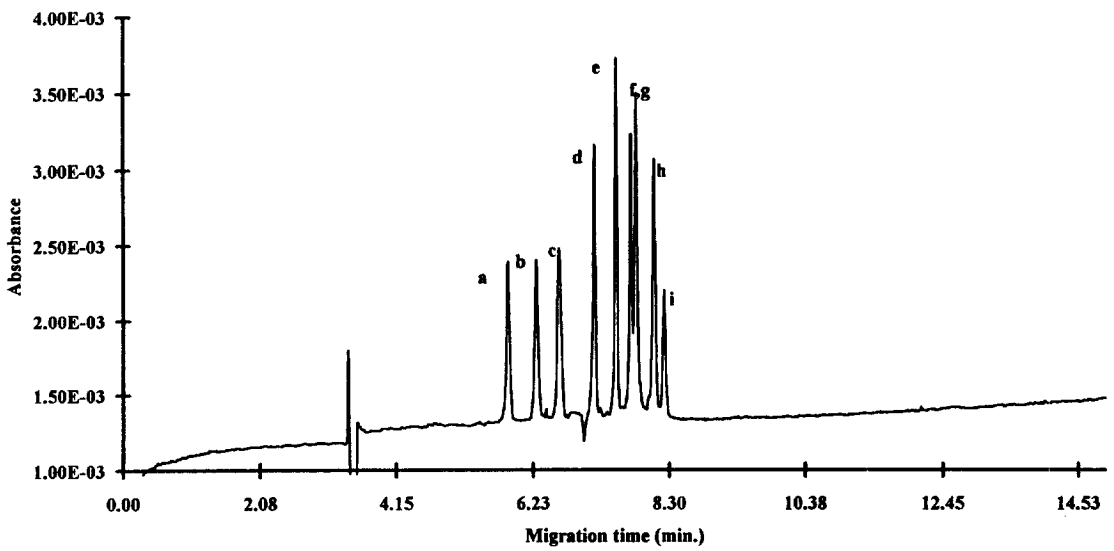


Fig. 2. Separation of steroid on fused-silica capillary using SB3-12/SDS system. Run buffer 5 m*M* SB3-12, 10 m*M* SDS and 25 m*M* Bicine, pH adjusted to 9.

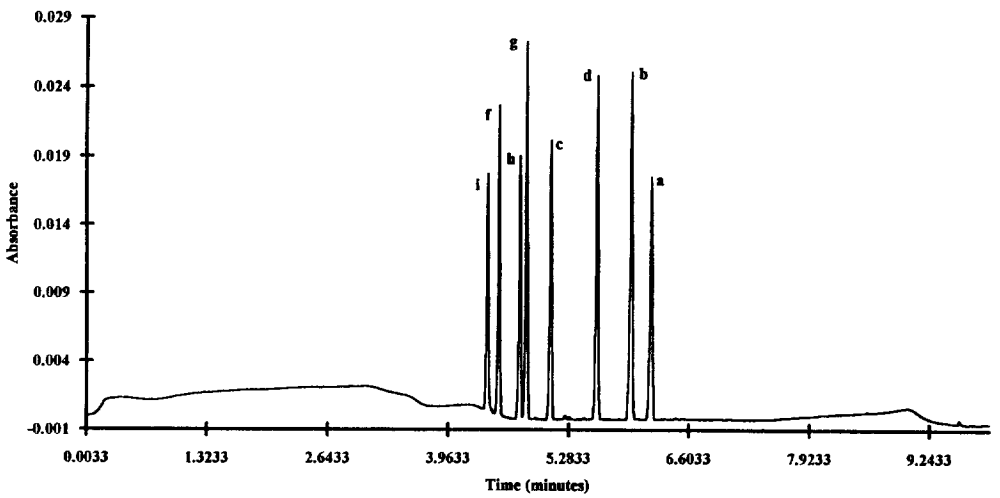


Fig. 3. Separation of steroids on neutral eCAP using SDS/$CH_3CN$ system. Run buffer, 100 m*M* SDS, 20% $CH_3CN$ and 20 m*M* MES with pH adjusted to 6.

to resolve five of nine steroids, whereas SB3-8, SB3-16 and SB3-18 gave very poor resolution. Fig. 3 shows a comparative separation achieved with a run buffer composed of 0.1 *M* SDS, 20% acetonitrile and 0.02 *M* MES buffer pH 6 using a coated neutral eCAP capillary (Beckman Instruments). Baseline resolution for testosterone and progesterone was achieved, however dimethyltestosterone comigrated with progesterone. The migration order of the steroids in this system is reversed with the hydrophobic steroids such as testosterone propionate migrating first and cortisone migrating last. The SDS/$CH_3CN$ system also required voltages of less than 15 kV and capillary cooled at 16°C. The resolutions obtained by both systems were comparable, although a slightly better peak shape was obtained using SDS/$CH_3CN$.

### *3.2. Effect of anionic/zwitterionic ratio on currents and capacity factor*

One of the main problems of using the SDS/$CH_3CN$ buffer system is the high current involved. Ohm plot analyses of this system gave a very narrow working range for applicable voltages extending to 15 kV. Even at 15 kV currents greater than 80 μA were observed, which resulted in rapid generation of heat inside the capillary due to the high resistance of narrow bore capillaries. The cartridge used for temperature control in the Beckman P/ACE system does not allow for dissipation of heat at the two ends of the capillary that are inserted into the inlet and outlet reservoirs. The Joule heat generated in these regions is lost to the surrounding run buffer, resulting in the localized heating of buffer solution and the formation of water vapor which is deposited on the sides of the buffer reservoir within five runs; this is further accentuated by the generation of hydrogen and oxygen gas in the reservoir due to the hydrolysis of water. This deposition of water vapors results in current thereby stopping the electrophoresis. Zwitterionic surfactants, being electrically neutral, do not cause an increase in the current; this in contrast to $CH_3CN$ which is known to cause a significant increase in operating currents [16]. Increasing the SB3-12 concentration from 2.5 to 20 m*M* gave no significant increase in current; however, a steady increase in current from 18 to 36 μA is observed when the SDS concentration is increased from 2.5 to 20 m*M*, as expected. The buffer composition that gave best resolution had currents of less than 40 μA for voltages as high as 30 kV. The capacity factor ($k'$) and selectivity ($\alpha$) were calculated by using Eqs. (2) and (3) for varying concentrations of SB3-12 and SDS (Figs. 4 and 5, respectively).

$$k' = t_r - t_{eo}/t_{eo}(1 - t_r/t_{mc}) \quad (2)$$

$$\alpha = k_2'/k_1' \quad (3)$$

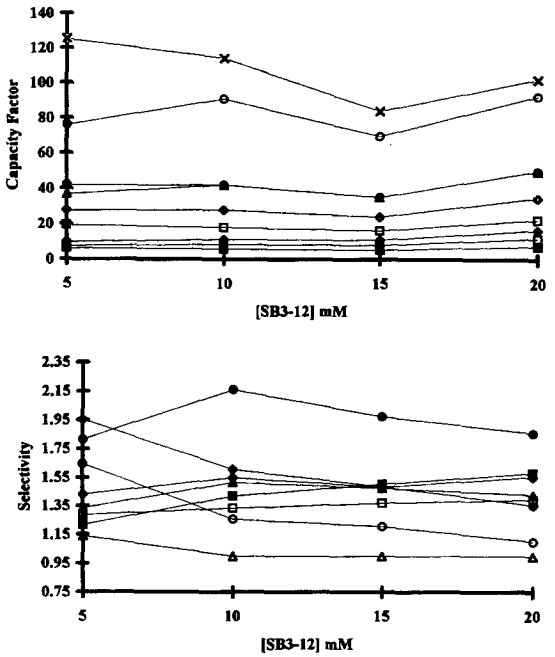


Fig. 4. Effect of SB3-12 concentration on capacity factor and selectivity. (△) Progesterone and testosterone, (◇) 11-desoxycortisol and dimethyltestosterone, (□) hydrocortisone and 11-desoxycortisol, (♦) 11-desoxycortisol and 21-desoxycortisol, (▲) dimethyltestosterone and progesterone, (■) cortisone and hydrocortisone, (●) testosterone and 17-hydroxyprogesterone and (○) 17-OH-progesterone and testosterone propionate.

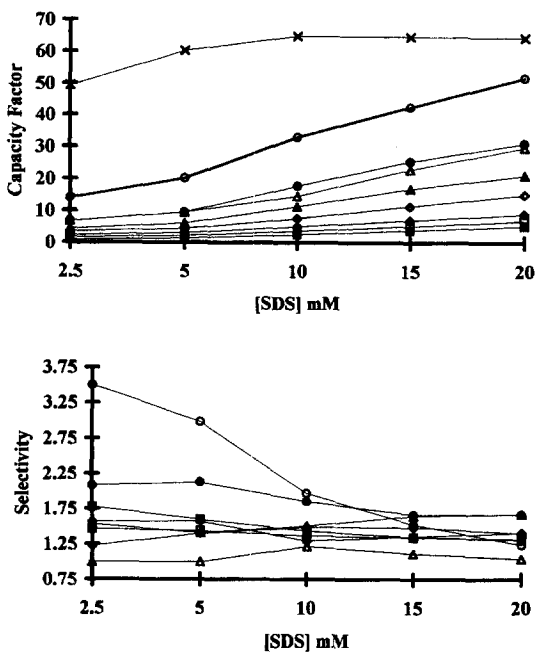


Fig. 5. Effect of SDS concentration on capacity factor and selectivity. (△) Progesterone and testosterone, (◇) 11-desoxycortisol and dimethyltestosterone, (□) hydrocortisone and 11-desoxycortisol, (♦) 11-desoxycortisol and 21-desoxycortisol, (▲) dimethyltestosterone and progesterone, (■) cortisone and hydrocortisone, (●) testosterone and 17-hydroxyprogesterone and (○) 17-OH-progesterone and testosterone propionate.

where $t_r$, $t_{eo}$ and $t_{mc}$ are the migration times of the analyte of interest, an unretained analyte or EOF marker and the micelle, respectively. As shown in Fig. 4, the capacity factors for the more hydrophilic steroids such as cortisone, hydrocortisone, 4-pregnene-17α,11β-diol-3,20-dione and 4-pregnene-17α,21-diol-3,20-dione showed little change with increasing concentration of SB3-12. The remaining hydrophobic steroids, specifically testosterone propionate, showed a decrease in $k'$ values up to 0.015 *M* of SB3-12, then increasing slightly at 0.020 *M* SB3-12 concentration. This was not found when the SDS concentration was increased; in these cases the $k'$ values increased steadily with increasing SDS concentrations (Fig. 5). However the rate of change of $k'$ values is steeper for hydrophobic steroids in comparison to hydrophilic ones. The $\alpha$ values are highly dependent on both SB3-12 and SDS concentrations and can change rather drastically with increasing concentrations of either of two surfactants, as seen in Figs. 4 and 5. This is a significant advantage of this system, as the resolution can be manipulated by just changing the molar ratio of either of the two surfactant. The data further suggest that the hydrophilic steroids, being less soluble in SDS–zwitterionic mixed micelles, have lower capacity factors and are detected first, whereas hydrophobic steroids, being more soluble in SDS–zwitterionic mixed micelles, are migrating towards the anode and therefore have greater capacity factors and are detected last. This holds true till the concentrations of the individual surfactants are close or slightly greater than their respective CMC. Increasing the concentration of both SB3-12 and SDS at a constant molar ratio of 1:2, considerably increased the migration times and decreased resolution. It may be speculated that the formation of a greater proportion of mixed micelles resulted in loss of selectivity.

### 3.3. Efficiency

Efficiencies were calculated by using Eq. (4):

$$N = 2\pi(t_r h/A)^2 \quad (4)$$

(where $t_r$, $h$ and $A$ are migration time, peak height and area, respectively) and are listed in Table 2. For purpose of comparison, the theoretical plates achieved with the buffer system consisting of 100 m*M* SDS and 20% acetonitrile are also listed. As can be seen, the number of theoretical plates is half of that obtained with the SDS/$CH_3CN$ buffer system. Efficiencies were found to be highly dependent on the solvents used to dissolve the steroids. Dissolving the steroids in organic solvents such as ethanol, methanol and acetonitrile affected resolution severely. Using 5 m*M* of SB3-12 solution only resolved cortisone, hydrocortisone and 11-desoxycortisol with theoretical plates ranging from 180 000 to 250 000 for these three steroids, with the rest of the steroids eluting as one broad peak. The SDS concentration in the sample buffer was also found to be very critical: concentrations greater than 4 m*M* of SDS in sample buffer gave no separation. The lower number of theoretical plates for the SB3-12/SDS mixed micellar system also effected the detection limits ranging from 1 to 5 μg/ml ($S/N \geq 3$) for the different steroids, as shown in Table 2. In comparison, the SDS/$CH_3CN$ buffer system gave a sensitivity of 500 ng/ml.

### 3.4. Effect of pH and temperature

In theory, the separation of steroids, being neutral molecules, should be independent of pH. However reduced EOF under acidic conditions allows partitioning for a longer time period, thereby resulting in baseline separation of all steroids except for 17-OH-progesterone and testosterone propionate which co-migrated. At basic pH the increased EOF reduced migration times considerably with some loss in resolution for the testosterone and progesterone pair but gave better efficiencies for resolved steroids when compared to the ones obtained at pH 6. The migration times reached a minimum at pH 8 and then started to increase at more alkaline pH values with a significant loss in resolution at pH greater than 10. One possible explanation is the increased deprotonation of the quaternary ammonium group on SB3-12 in alkaline conditions, thereby causing it to behave more like an anionic surfactant. Decreasing the capillary temperature to 16°C or increasing it to 30°C deteriorated resolution. Best resolution was achieved at 25°C.

## 4. Conclusions

Run buffers composed of zwitterionic and anionic surfactants can be used to separate steroids on a bare fused-silica capillary. Best resolution is achieved with SB3-12/SDS, when used at concentrations close to their CMCs. The low ionic strength of this buffer system prevents efficient sample stacking, thus decreasing sensitivity. The selectivity can be changed by varying the mole ratio of SB3-12 and SDS. No significant capillary wall interaction or coating effects were observed with the zwitterionic surfactants, as is evident from the reproducible migration times having R.S.D.s of ≤2% for triplicate runs. The sensitivity for different steroids ranged from 1 to 5 μg/ml. This means that at least a 200-fold preconcentration is needed to determine

Table 2
Efficiencies and detection limits

| Detergent | CMC |
|---|---|
| Sodium dodecyl sulfate (SDS) | 0.008 *M* |
| N-Octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-8) | – |
| N-Decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-10) | 0.025–0.040 *M* |
| N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-12) | 0.002–0.004 *M* |
| N-Hexadecyl–N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-16) | 0.00001–0.00004 *M* |
| N-Octadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-18) | – |

these steroids at physiological levels. This is the main drawback of this buffer system; nevertheless it is very promising in the development of a robust assay in situations where sensitivity is not a key issue. Especially since it is able to resolve steroids on bare fused-silica capillaries, does not require stringent temperature control, and gives good resolution at relatively low concentrations of SDS.

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