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Complementary on-line preconcentration strategies for steroids by capillary electrophoresis

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Abstract

Complementary on-line preconcentration strategies are needed when analyzing different classes of solutes in real samples by capillary electrophoresis (CE) with UV detection. The performance of three different on-line preconcentration (focusing) techniques under alkaline conditions was examined in terms of their selectivity and sensitivity enhancement for a group of steroids, including classes of androgens, corticosteroids and estrogens. Electrokinetic focusing of large sample injection plugs (up to 28% of effective capillary length or 22.1 cm) directly on-capillary can be tuned for specific classes of steroids based on changes in their mobility (velocity) using a multi-section electrolyte system in CE. A dynamic pH junction was applied for the selective resolution and focusing of weakly acidic estrogens using borate, pH 11.0 and pH 8.0 in the background electrolyte and the sample, respectively. Sweeping, using an anionic bile acid surfactant and neutral γ-cyclodextrin (γ-CD) under alkaline conditions (pH 8), resulted in focusing and separation of the moderately hydrophobic (non-ionic) classes of steroids, such as androgen and corticosteroids. Optimal focusing and resolution of all test steroids under a single buffer condition was realized by a dynamic pH junction-sweeping format using borate, pH 11.0 and bile acid surfactant with γ-CD in the BGE, whereas the sample is devoid of surfactant at pH 8.0. The design of selective on-line focusing strategies in CE is highlighted by the analysis of microgram amounts of ethynyl estradiol derived from a female contraceptive pill extract using the dynamic pH junction method, which resulted in over a 100-fold enhancement in concentration sensitivity.

Keywords: Sample preparation; Sweeping; Dynamic pH; Steroids

1. Introduction

Selectivity and detector sensitivity represent two of the most important features governing the analysis of complex sample mixtures. Analyte selectivity in capillary electrophoresis (CE) is most readily modified by electrolyte properties such as buffer type, pH, ionic strength, as well as the use of additives, such as micelles, cyclodextrins (CDs) and crown ethers. Furthermore, the application of high electric fields in CE enables rapid and high resolution separations for diverse types of analytes, including colloidal microorganisms [1]. However, CE suffers from poor concentration sensitivity when using UV detection because of the small injection volumes (typically< 1% capillary length) and narrow optical pathlength. This presents a significant obstacle for routine analy-

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ses of sub-micromolar levels of a wide variety of UV-active analytes in real samples by CE. Alternative detection formats such as laser-induced fluorescence [2] and electrochemical detection [3] offer greater sensitivity than UV absorbance, but are applicable to fewer types of native analytes unless modified by chemical derivatization. Off-line preconcentration by solid-phase extraction can be used to cleanup and enrich a sample prior to CE analysis [4,5]. However, limited automation and poor sample elution properties are still major hurdles for direct coupling of solid-phase extraction methods to commercial CE instruments.

On-line sample preconcentration represents an effective and versatile way to enhance concentration sensitivity in CE when using UV detection. The high electric field and tunable electrophoretic mobility of analytes can be used to induce electrokinetic focusing within large injection volumes of sample directly on-capillary prior to detection. This is not only important for improved concentration sensitivity in CE, but also results in enhanced separation efficiency and peak capacity because of the extremely sharp analyte zones generated [6]. On-line focusing is normally performed by selecting different buffer properties to modify analyte velocity in two or more electrolyte sections in the capillary, such as sample and background electrolyte (BGE). To date, four major on-line preconcentration techniques have been reported in CE: sample stacking [7–10], transient isotachophoresis (tITP) [11–14], sweeping [15–18] and dynamic pH junction [6,19–21]. Each method relies on a distinct focusing mechanism based on different electrolyte properties between sample and BGE zones, such as conductivity (ionic strength), electrolyte co-ion mobility, additive concentration (analyte–additive interactions) and buffer pH, respectively. Accordingly, such methods are suitable for certain types of analytes based on their specific physicochemical properties in the buffer, such as charge, mobility, size, hydrophobicity and pK_a. Recently, a new hyphenated on-line focusing method by CE, referred to as dynamic pH junction-sweeping, has been introduced for enhanced focusing of flavin metabolites, resulting in over 1200-fold improvement in sensitivity relative to conventional injections [22,23]. The development of new strategies to enhance detector sensitivity in CE, that is applicable to different classes of metabolites in real samples, is required for emerging areas of biological research, such as metabolomics [24–26]. On-line preconcentration techniques in conjunction with multiplexed CE (96-capillary array) represents a promising high-throughput platform for sensitive analyses of metabolites using UV detection [27].

Steroids represent a diverse class of structurally related cyclopenta[*a*]phenanthrene compounds that serve important functions in organisms, such as hormones in chemical signaling (e.g., testosterone) and structural components in cell membranes (e.g., cholesterol). Steroid derivatives are also used widely as drugs for the treatment of a variety of disorders, as well as substances of abuse (e.g., anabolic steroids) in sports medicine [28]. Urinary estrogen metabolite levels are widely measured to monitor the stages of pregnancy [29], the impact of hormone replacement therapy on osteoporosis [30], as well as serving as potential biomarkers for early prognosis of breast cancer [31]. Previous on-line preconcentration reports in CE have generally investigated a specific class of steroid (i.e., corticosteroid or estrogen) using a single method [16,17,32]. The aim of this manuscript is to compare the selectivity and sensitivity enhancement of three on-line preconcentration techniques, namely dynamic pH junction, sweeping and dynamic pH junction-sweeping, for different types of steroids by CE. The effective focusing of large sample injection volumes and the resolution of steroid mixtures can be tuned based on specific mobility changes within a multi-section electrolyte system.

2. Experimental

2.1. Apparatus and procedure

Separations were performed on a Beckman P/ACE 5000 (Beckman Instruments, Mississauga, Canada) and Beckman P/ACE 2000 (Beckman Instruments, Tokyo, Japan) automated CE systems. Uncoated capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 87 cm×75 μm I.D.×375 μm O.D. were used. New capillaries were first rinsed with 1.0 *M* NaOH (5 min, 20 p.s.i. or 140 kPa), followed by rinsing with the separation BGE (10 min, 20 p.s.i.).

The capillary was then left to equilibrate overnight in the separation BGE prior to use. Each separation was preceded by a 2.0-min rinse with 0.1 *M* NaOH, followed by a 4-min rinse with the separation BGE. The sample was introduced using a low pressure (0.5 p.s.i. or 3.5 kPa) injection ranging from 2 to 300 s. The average flow-rate of the low pressure injection was determined to be 4.42 cm/min using an 87 cm capillary, which was used to estimate injection bandwidth [6]. All separations were carried out at 25 °C, and absorbance detection was made with a Beckman P/ACE UV detector at 254 nm. Data were collected using System Gold software (Beckman).

2.2. Chemicals and reagents

The aqueous BGE consisted of 160 m*M* borate (Borax; Sigma, St. Louis, MO, USA). The pH of the BGE was adjusted by using 1.0 *M* NaOH (BDH, Toronto, Canada) or 1.0 *M* HCl (Fischer Scientific, Nepean, Canada) within a range of pH 8.0 to 11.0. Sodium taurodeoxycholate (STC), sodium dodecyl sulfate (SDS) and γ-cyclodextrin (γ-CD) were purchased from Sigma. Steroid standards, which include testosterone, corticosterone, prednisone, hydrocortisone (cortisol), β-estradiol and estriol were all obtained from Sigma. Stock solutions of analytes were prepared by dissolving approximately equal concentrations ($1\cdot10^{-2}$ *M*) of each steroid in 50% methanol in deionized water and stored in a refrigerator at 4 °C. These stock solutions were then further diluted with the appropriate sample matrix prior to injection into the capillary. Peaks were identified by spiking the sample solution with standard solutions of each steroid.

2.3. Preparation of female oral contraceptive pill extract

Analysis of 17α-ethynylestradiol content in the female oral contraceptive pill (Select 1/35: Searle Canada, Women's Health Care) was carried out by first grinding each tablet (48.4 mg) with a mortar and pestle, dissolving the powder in 1 ml of methanol–deionized water (50:50), mixing the solution by sonication and then vortexing for 1 min each, followed by centrifugation for 5 min at 10 000 rpm. The supernatant was then dissolved 10-fold in borate, pH 8.0 prior to analysis by CE. Each tablet contained about a 30-fold excess of norethindrone (1 mg) relative to 17α-ethynylestradiol (35 μg) as the biologically active components.

3. Results and discussion

3.1. On-line preconcentration strategies for steroids by CE

Fig. 1 shows the chemical structures of the six different test steroids investigated in this report. The androgen (testosterone) and corticosteroids (corticosterone) are neutral lipophilic solutes, whereas estrogens (β-estradiol, estriol) are weakly acidic ($pK_a \approx 10.4$) steroids because of the presence of a phenolic moiety. Borate complexion has been reported to occur with corticosteroids that possess proximal diol groups on the 17- and 21-positions (indicated by arrows), such as hydrocortisone [33]. Prednisone is a synthetic dehydrocortisone derivative used as an oral anti-inflammatory drug in the treatment of certain skin conditions [34]. Fig. 2 depicts three complementary on-line preconcentration strategies under alkaline borate conditions used to focus different classes of steroids based on their unique physicochemical properties, such as weakly acidic functional groups (pK_a), hydrophobic character and strength of micellar partitioning (retention factor, k'), as well as the presence of proximal diol groups (borate complexation).

3.2. Dynamic pH junction of estrogens

Dynamic pH junction can be used to selectively focus weakly acidic estrogens because of the changes in their mobility as a function of buffer pH. As shown in Fig. 2a, a long plug of sample prepared in borate, pH 8 is hydrodynamically injected into the capillary (estradiol is neutral), which has been previously conditioned with borate, pH 11.0. Upon application of the voltage, excess hydroxide ions rapidly migrate [35] into the sample zone, resulting in a local increase in pH at the front edge of the sample–BGE boundary. Since titration of the whole sample zone is not instantaneous, estradiol solutes residing in the front section of the sample zone are ionized

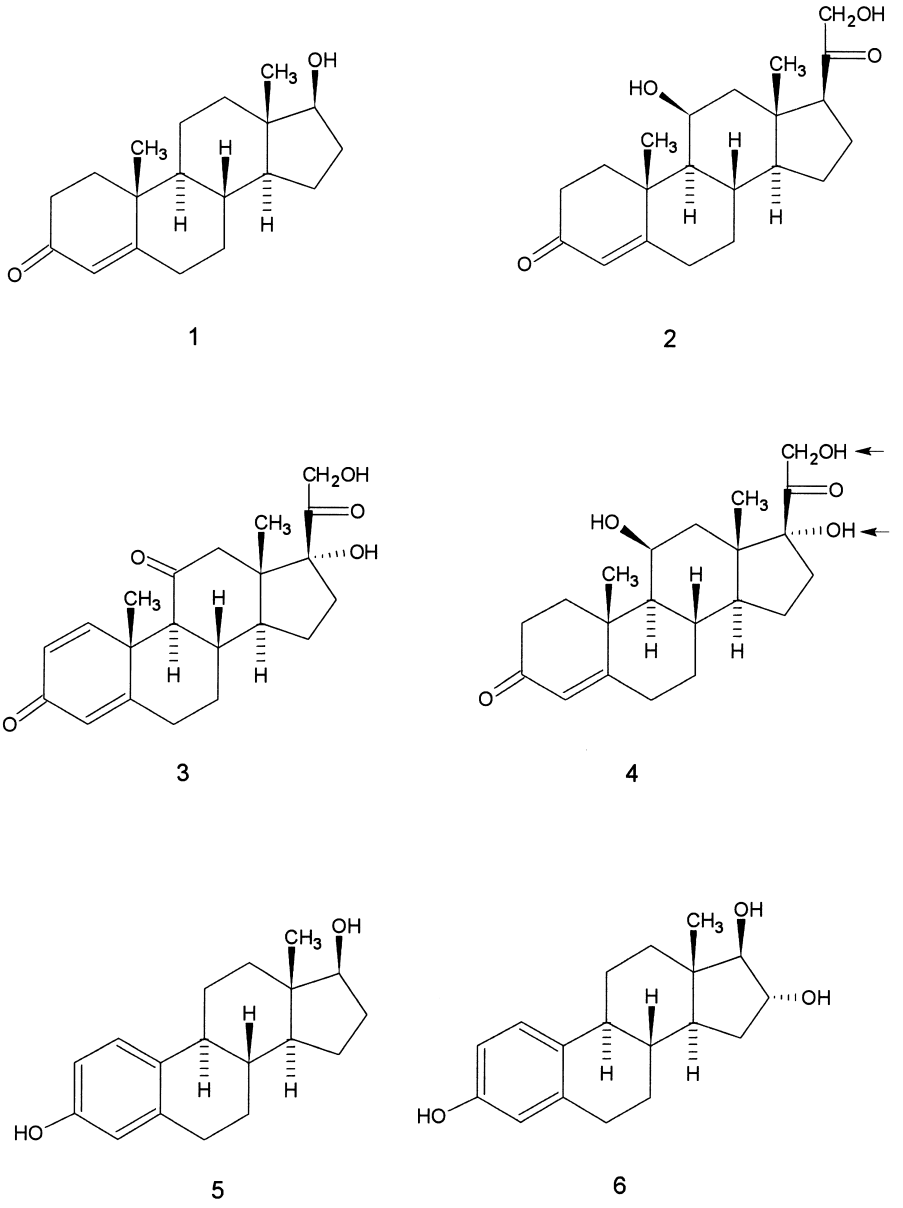

Fig. 1. Chemical structures of six different test steroids examined in this report, including classes of androgens, corticosteroids and estrogens. Selective borate complexation to 17/21-proximal diols is denoted by arrows. Analyte peak numbering corresponds to: 1—testosterone, 2—corticosterone, 3—prednisone, 4—hydrocortisone, 5—estradiol and 6—estriol.

and migrate with a large negative mobility counter to the electroosmotic flow (EOF) (lower velocity), whereas neutral estradiol in the remaining sample zone co-migrates with the EOF (higher velocity). This results in an electrokinetic band narrowing mechanism in which the original large sample plug is focused into a sharp zone as the higher velocity estradiol in the back section of the sample compresses into the slower front section. Other non-acidic steroids whose mobility is independent on buffer pH

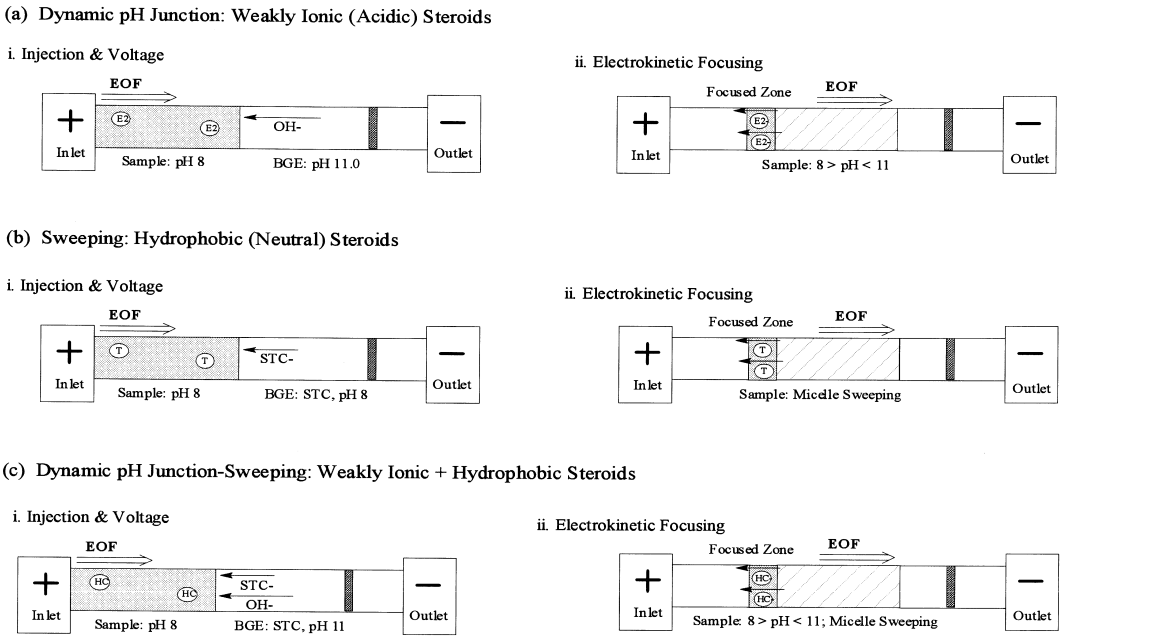

Fig. 2. A schematic depicting three complementary on-line preconcentration strategies by CE under alkaline conditions used for electrokinetic focusing of large sample volumes by CE: (a) dynamic pH junction, (b) sweeping and (c) dynamic pH junction-sweeping. Focusing and resolution of specific analytes can be designed based on selective velocity changes within a multi-section electrolyte system based on buffer pH (weakly ionic estrogens), micelle partitioning (hydrophobic steroids) and borate complexation (proximal diols). Abbreviations refer to: E2—estradiol, T—testosterone, HC—hydrocortisone, STC—sodium taurodeoxycholate and OH—hydroxide ion.

are not focused by the dynamic pH junction and continue to migrate as a broad analyte zone undergoing normal band dispersion.

Fig. 3 shows two electropherograms demonstrating the use of dynamic pH junction for selective focusing of weakly acidic estrogens. A long sample plug (5.5 cm or about 7% of effective capillary length) was injected containing 10 μ*M* of steroids in a sample matrix of 160 m*M* borate, pH 8.0, whereas the pH of BGE is 11.0. Optimization of the dynamic pH junction method was carried out by incrementally changing the pH of the BGE from 8.0 to 11.0, while the sample pH was fixed at pH 8.0. When both the sample and BGE have a pH of 8.0, the injection of the long sample plug resulted in a single broad zone (co-migrates with the EOF) since all steroids are neutral. However, when the pH of BGE was >9.5, estradiol and estriol zones were observed to separate and undergo band narrowing in contrast to other neutral steroids. A greater fraction of estrogen solutes are ionized (negative mobility) when the pH of BGE approaches its pK_a, resulting in improved electrokinetic band compression, as well as resolution from other neutral steroids in the sample. A dramatic enhancement in both resolution and focusing performance was achieved when using borate pH 11.0, however estradiol and estriol peaks overlap, as depicted in Fig. 3a. Interestingly, hydrocortisone was observed to be resolved from the other steroids due to weak borate complexation [33], yet the zone is not significantly narrowed. When using conventional small injection lengths (0.20 cm), it was observed that the hydrocortisone peak was unusually broad in comparison to estrogens under pH 11.0. This may be indicative of slow borate complexation kinetics relative to the separation time used in CE, which is commonly observed with sugar–borate interactions [36]. Higher operating temperatures during CE separations can be used to enhance borate complexation kinetics and peak shape, but this also results in larger electric currents and possible Joule heating. Fig. 3b shows optimum resolution and focusing of estrogens

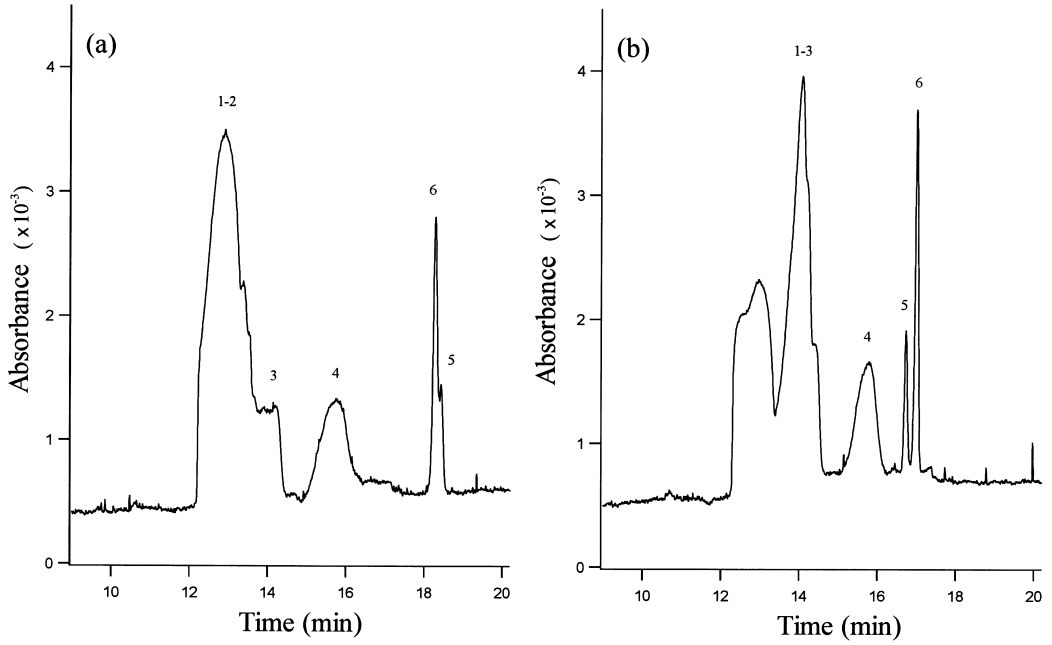

Fig. 3. Electropherograms showing optimization of on-line focusing for weakly acidic steroids (estrogens) by dynamic pH junction. The sample contained 10 μ*M* of steroids in 160 m*M* borate, pH 8.0, which was injected for a length of 5.5 cm or about 7% capillary length to detector. Electropherograms highlight the influence of CD in the BGE on estrogen resolution: (a) 0 m*M* and (b) 0.5 m*M* γ-CD. Conditions: aqueous 160 m*M* borate, pH 11.0; voltage, 22 kV; capillary length, 87 cm; injection, 75 s. Analyte peak numbering as depicted in Fig. 1.

with the addition of 0.5 m*M* γ-CD to borate, pH 11.0. Estrogen peaks are baseline resolved with sharp detector bandwidths (w_{det}) of 0.88 and 1.03 cm for estradiol and estriol, respectively. In contrast, when both the sample and BGE are borate, pH 11.0, estrogens co-migrate as a single broad zone with a w_{det} of over 6.70 cm. The addition of a neutral cyclodextrin additive to the BGE results in the formation of an estrogen–cyclodextrin inclusion complex of lower mobility (increased size of complex), thus estrogen peaks migrate with shorter migration times. Based on the reversal in migration order of estrogens in Fig. 3a and b upon γ-CD addition, it is apparent that estradiol has a greater affinity for the cyclodextrin host relative to estriol. Thus, low concentrations of neutral additives can be used in the BGE to improve resolution without significant loss in focusing performance. It was observed that the use of 160 m*M* 3-cyclohexylamino-1-propanesulfonic acid (CAPS), pH 11.0 buffer (sample: borate, pH 8.0) also resulted in estrogen focusing by dynamic pH junction. Hence, other buffer types can also be used to perform on-line focusing of weakly acidic analytes by dynamic pH junction, unless borate complexation is vital for mobility change, such as vicinal diols groups on catecholamines [6] or nucleosides with neutral purine and pyrimidine bases [19].

3.3. Micellar sweeping of hydrophobic steroids

Dynamic pH junction is not an effective on-line preconcentration technique for many classes of steroids that are neutral since their electrophoretic mobility is independent of buffer pH. In this case, the velocity of neutral steroids can be readily modified by partitioning within the hydrophobic core of ionic micelles (analyte–additive complex is charged), which are added to the BGE. This principle is not only useful for separation selectivity in micellar electrokinetic chromatography (MEKC) [37], but also can be used for electrokinetic focusing of large sample volumes by sweeping [15–17]. Fig. 2b depicts the general format of sweeping of hydro-

phobic steroids, such as testosterone, when a large sample plug (devoid of micelle), is injected into a capillary that is previously filled with a buffer containing micellar solution. Both sweeping and dynamic pH junction operate under non-electric field-enhanced conditions, since the conductivity of the sample and BGE segments are normally of the similar magnitude. Application of the voltage results in the infiltration of anionic micelles (e.g., sodium taurodeoxycholate) within the sample zone counter to the EOF. Hydrophobic steroids that partition within the micelles are effectively ''swept'' up by the micelle front, resulting in focusing of the original sample zone, as determined by the magnitude of the retention factor [15]. A recent review of the mechanism and application of sweeping has been reported [38].

Enhanced sweeping performance has been reported to occur when using acidic conditions to suppress the EOF [16], however this limits CE selectivity from the use of other types of buffer (i.e., neutral, alkaline) or micelles (e.g., weakly acidic bile salts are insoluble), unless covalently coated neutral capillaries are employed [39]. In this study, the bile salt surfactant, STC was selected as the micelle for sweeping under alkaline conditions, which has been used previously for enhanced resolution of chiral drugs [40] and mixtures of polycyclic aromatic hydrocarbons [41] by MEKC. Bile salt surfactants form large helical micelle aggregates [42], which can be expected to offer different selectivities than conventional aliphatic surfactants (e.g., SDS). Optimization of sweeping conditions was performed by adding increasing concentrations of STC to the BGE (borate, pH 8.0), while the sample is devoid of micelle. Among the different classes of steroids, it was observed that the estrogens had the strongest interaction for STC, as reflected by their large mobility shift (longer migration times) even when using 5 m*M* of the surfactant. When using 10 m*M* STC in the BGE, both estradiol and estriol were observed not to elute within 50 min because of their high retention factor for the anionic micelle (long migration window, t_m). Fig. 4a shows optimum focusing and resolution of androgen and corticosteroids using 30 m*M* STC in borate, pH 8.0. Hydrocortisone and prednisone have significantly weaker affinity for STC, as reflected by their shorter migration times and broader peaks, however improved band narrowing was observed for the more apolar steroid, testosterone. The addition of organic solvent or neutral cyclodextrin (as a competitive ligand) has been used to improve resolution by lowering the magnitude of k' [15,16]. Fig. 4b depicts optimum focusing by sweeping using a combination of 80 m*M* STC and 10 m*M* γ-CD in the BGE. It is apparent that improved focusing of the weakly interacting steroids (i.e., prednisone, hydrocortisone and corticosterone) is achieved when using higher concentrations of STC, while the overall migration time is similar because of the stronger interaction of testosterone with the neutral γ-CD. The focusing efficiency increases for hydrophobic steroids with stronger interaction to the micelle, reflected by w_{det} values of 2.63, 2.20, 1.88 and 1.31 cm for prednisone, hydrocortisone, corticosterone and testosterone, respectively. The addition of higher concentrations of γ-CD did not sufficiently lower the retention factor (i.e., reduce the migration time) of estrogens to bile salt surfactant without compromising the resolution of the other steroids. In this study, about a 50-fold enhancement in sensitivity relative to a conventional injection (0.20 cm) was realized for testosterone while maintaining baseline resolution with other steroids. Although sweeping is generally favored for analytes with a large retention factor, it is also dependent on the buffer conditions (magnitude and direction of EOF), as well as the physicochemical properties of other analytes present in the sample. For complex sample mixtures, separate optimization conditions are needed when optimizing sweeping for both weakly (low k') and strongly (high k') interacting analytes using micelles under alkaline buffer conditions.

3.4. Dynamic pH junction-sweeping of weakly ionic and hydrophobic steroids

In conventional sweeping methods, the buffer pH in the sample and BGE segments are normally the same [15]. Hydrophilic (weakly ionic/polar) analytes generally undergo poor band narrowing using micelles because of their low retention factor. As depicted in Fig. 2c, dynamic pH junction-sweeping is defined when the large sample injection plug is devoid of micelle (or other additives) and has a

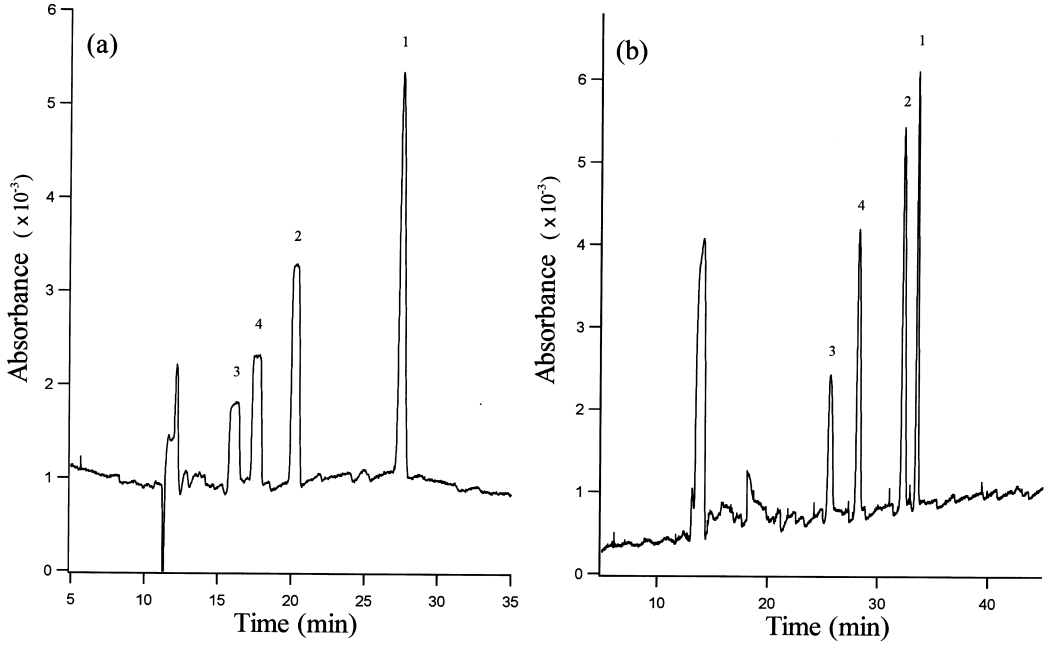

Fig. 4. Electropherograms showing optimization of on-line focusing for moderately hydrophobic steroids by sweeping under alkaline conditions. The sample contained 10 μ*M* of steroids in 160 m*M* borate, pH 8.0, which was devoid of micelle. Electropherograms show the influence of changes in the concentration of STC and CD in BGE (pH 8.0) on steroid focusing: (a) 30 m*M* and (b) 80 m*M* with 10 m*M* γ-CD. Note that estrogens with strong partitioning to STC micelles were not eluted within 50 min. Conditions: aqueous 160 m*M* borate, pH 8.0; voltage, 22 kV; capillary length, 87 cm; injection, 75 s. Analyte peak numbering as in Fig. 1.

different buffer pH relative to BGE. A wider variety of analytes can be focused by dynamic pH junction-sweeping, since analyte velocity (e.g., hydrocortisone) is modified by three discrete electrokinetic mechanisms, including buffer pH, micelle partitioning and borate complexation. Dynamic pH junction-sweeping experiments were carried out using borate, pH 11.0 in the BGE in order to take advantage of the unique properties of estrogens (weakly acidic) compared to other steroids, as well as reducing estrogen affinity to STC micelles, thereby reducing total analysis time. Furthermore, estrogens have higher absorbance in strongly alkaline buffer conditions (pH 11.0) than under neutral or acidic buffers, as reflected by about a fivefold improvement in detector sensitivity (in terms of peak height) relative to pH 8.0. Optimization of steroid focusing by dynamic pH junction-sweeping was carried out by adding increasing concentrations of STC micelles to the BGE, where the pH values of the sample and the BGE segments are 8.0 and 11.0, respectively. Under these conditions, partially ionized estrogen anions in the BGE have a lower affinity for STC micelles, resulting in a slower change in migration time as a function of surfactant concentration. Fig. 5a depicts optimum focusing and resolution of all six steroids by dynamic pH junction-sweeping using 32 m*M* STC and 5 m*M* γ-CD in borate, pH 11.0. It is apparent that both estradiol and estriol have significantly lower k', allowing simultaneous analysis of different classes of steroids under a single buffer condition, as compared to either sweeping (pH 8.0) or dynamic pH junction methods. Despite using highly alkaline conditions, significant band narrowing of peaks is still preserved. For example, the peak width for hydrocortisone was in fact sharper when using dynamic pH junction-sweeping method compared to sweeping (32 m*M* STC, pH 8.0), with w_{det} values of 3.45 and 4.26 cm, respectively. However, the w_{det} of estriol was slightly broader using opti-

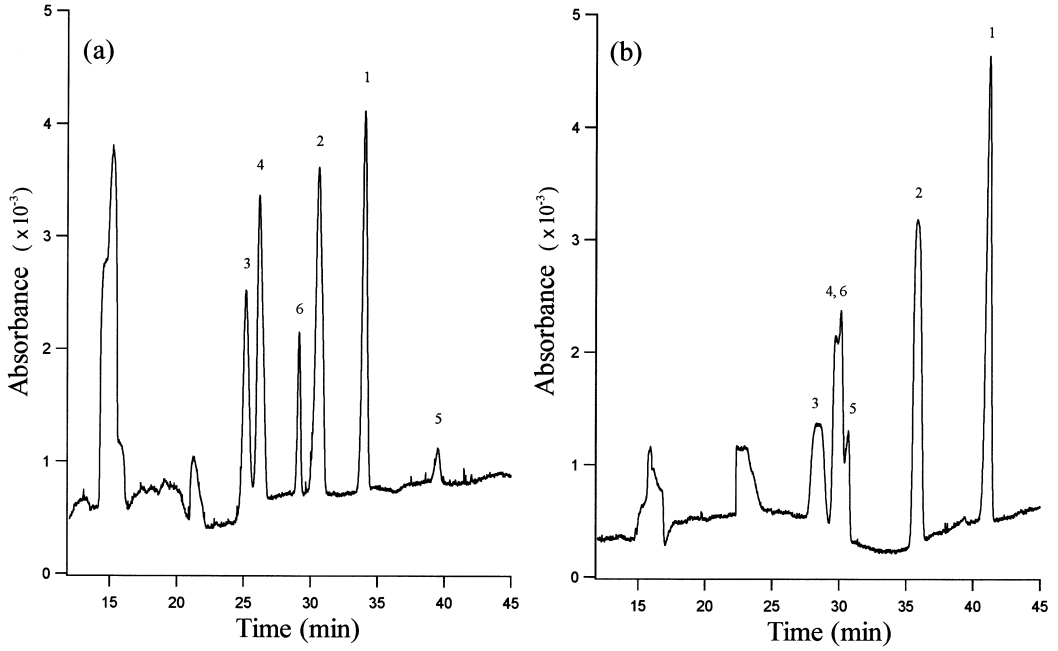

Fig. 5. Electropherograms comparing the focusing efficiency and selectivity of steroids using (a) dynamic pH junction-sweeping and (b) sweeping under alkaline conditions. The sample contained 10 μ*M* of steroids in (a) 160 m*M* borate, pH 8.0, and (b) 160 m*M* borate, pH 11.0. Optimum focusing and resolution of all six steroids was realized with dynamic pH junction-sweeping method using 160 m*M* borate, pH 11.0, 32 m*M* STC and 5 m*M* γ-CD as BGE. Note the differences in the focusing and selectivity for polar corticosteroids and weakly acidic estrogens. Conditions and analyte peak numbering as depicted in Fig. 4.

mized dynamic pH junction-sweeping (1.67 cm) relative to dynamic pH junction (1.03 cm) because of increased band diffusion due to the longer migration time. In this study, although modest gains in focusing efficiency were achieved by the dynamic pH junction-sweeping format, the selectivity for different classes of steroids was far superior to either dynamic pH junction or sweeping techniques used alone. In order to demonstrate the importance of the pH 8.0 sample segment, Fig. 5b compares conventional sweeping under 32 m*M* STC, 5 m*M* γ-CD, borate pH 11.0, where both BGE and sample have the same pH. Although the selectivity and focusing efficiency of hydrophobic steroids (i.e., testosterone and corticosterone) in Fig. 5a and b are similar, there are considerable differences for polar and weakly ionic steroids (e.g., predinsone, hydrocortisone, estradiol, estriol). In general, dynamic pH junction-sweeping format can provide much better selectivity than conventional sweeping for different classes of analytes in complex mixtures. For example, estrogens have a much longer migration time and stronger retention when using dynamic pH junction-sweeping format, since micelles within the borate, pH 8.0 sample zone have a higher local k' (estrogens are neutral) than the BGE at pH 11.0. In effect, the pH junction between sample and BGE segments serves as a convenient way to tune k' for weakly ionic analytes, similar to organic solvent or cyclodextrin addition in MEKC and sweeping. In addition, the w_{det} values for polar corticosteroid peaks are focused sharper in Fig. 5a than Fig. 5b. For instance, the w_{det} values for hydrocortisone are 3.45 and 4.67 cm for dynamic pH junction-sweeping and sweeping (32 m*M* STC, pH 11.0), respectively. Overall, about a 30-fold enhancement in concentration sensitivity for all six steroids relative to a conventional injection can be realized by dynamic pH junction-sweeping under alkaline conditions. The resolution required for the separation places an upper limit on the sensitivity

enhancement possible when using large hydrodynamic injections since the effective separation length is reduced.

3.5. Selective analysis of ethynyl estradiol in oral female contraceptive pill

In some cases, on-line preconcentration techniques in CE that offer high selectivity and focusing efficiency for specific classes of analytes are advantageous. This property is particularly useful in situations where the analysis of a single minor component of interest is required in the presence of high concentrations of other species. Many pharmaceutical formulations consist of low concentrations of one or more biologically active components, compared to higher concentrations of other drugs and excipients [21,43]. For example, current versions of the oral female contraceptive pill contain low amounts (35 μg) of the potent synthetic female estrogen, 17α-ethynylestradiol, in comparison to a 30-fold excess of the progestin analogue, norethindrone (1 mg). Also, there is growing interest in measuring trace levels of ethynyl estradiol in sewage effluents, since it has been identified as a possible endocrine-disruptor on exposed marine organisms [44]. A methanol extract of each oral female contraceptive tablet was prepared (see Experimental section), which was then diluted 10-fold in borate, pH 8 buffer prior to injection. Fig. 6a depicts an electropherogram of the female contraceptive pill extract using a conventional small volume injection (3 s, 0.22 cm) by CE with borate, pH 11.0 as the BGE. Although CE with UV detection can readily measure the major neutral species, norethindrone (335 μ*M*), there is poor detector sensitivity for the minor anionic component, ethynyl estradiol (12.0 μ*M*) under these conditions. Fig. 6b shows the application of a dynamic pH junction for the selective on-line preconcentration of ethynyl estradiol using a 300 s hydrodynamic injection, which corresponds to an injection plug of 22.1 cm or 28% of capillary length. Despite the long sample injection used in Fig. 6b, the w_{det} for ethynyl estradiol is very sharp at 0.82 cm, which is nearly the same bandwidth (0.75 cm) when using a small injection depicted in Fig. 6a. This represents more than a 27-fold band narrowing of the initial sample injection plug. In contrast, the neutral norethindrone migrates as an extremely broad zone, reflected by a w_{det} of 30.7 cm. The use of larger injection plugs (>300 s) eventually resulted in the co-migration of norethindrone and ethynyl estradiol peaks. The reproducibility of the method based on triplicate measurements was excellent based on RSDs of 0.7 and 1.4% for migration time and peak area of ethynyl estradiol, respectively. Thus, about a 100-fold enhancement in sensitivity was realized by dynamic pH junction for the selective analysis of ethynyl estradiol in female contraceptive pill extract. Higher enrichment factors were obtained in this case since the sample consisted of only two major UV-active components and the resolution requirements for the separation were less significant.

4. Conclusion

The use of complementary on-line preconcentration methods in CE was demonstrated using a mixture of different classes of steroids under alkaline buffer conditions. Both the sensitivity enhancement and selectivity requirements need to be considered when selecting an appropriate on-line preconcentration method for a sample of interest. Electrokinetic focusing of large sample injections on-capillary is based on specific analyte velocity changes in a multi-section electrolyte system as a result of buffer pH, micelle partitioning and borate complexation. The selective focusing and resolution of weakly acidic estrogens was achieved using a dynamic pH junction format, where the pH values of the sample and BGE are 8.0 and 11.0, respectively. Under alkaline (pH 8.0) conditions, sweeping using a mixture of bile salt surfactant and cyclodextrin in the BGE was effective for the enrichment and resolution of the moderately hydrophobic corticosteroids and androgens. Simultaneous focusing and resolution of all test steroids under a single buffer condition was demonstrated by a hyphenated dynamic pH junction-sweeping method, where the sample is devoid of micelle and dissolved in a lower buffer pH (pH 8.0) relative to the BGE (pH 11.0). Dynamic pH junction-sweeping offers unique advantages in terms of enhanced selectivity and focusing for certain analyte mixtures, in cases where conventional sweeping or dynamic pH junction are less effective. About a

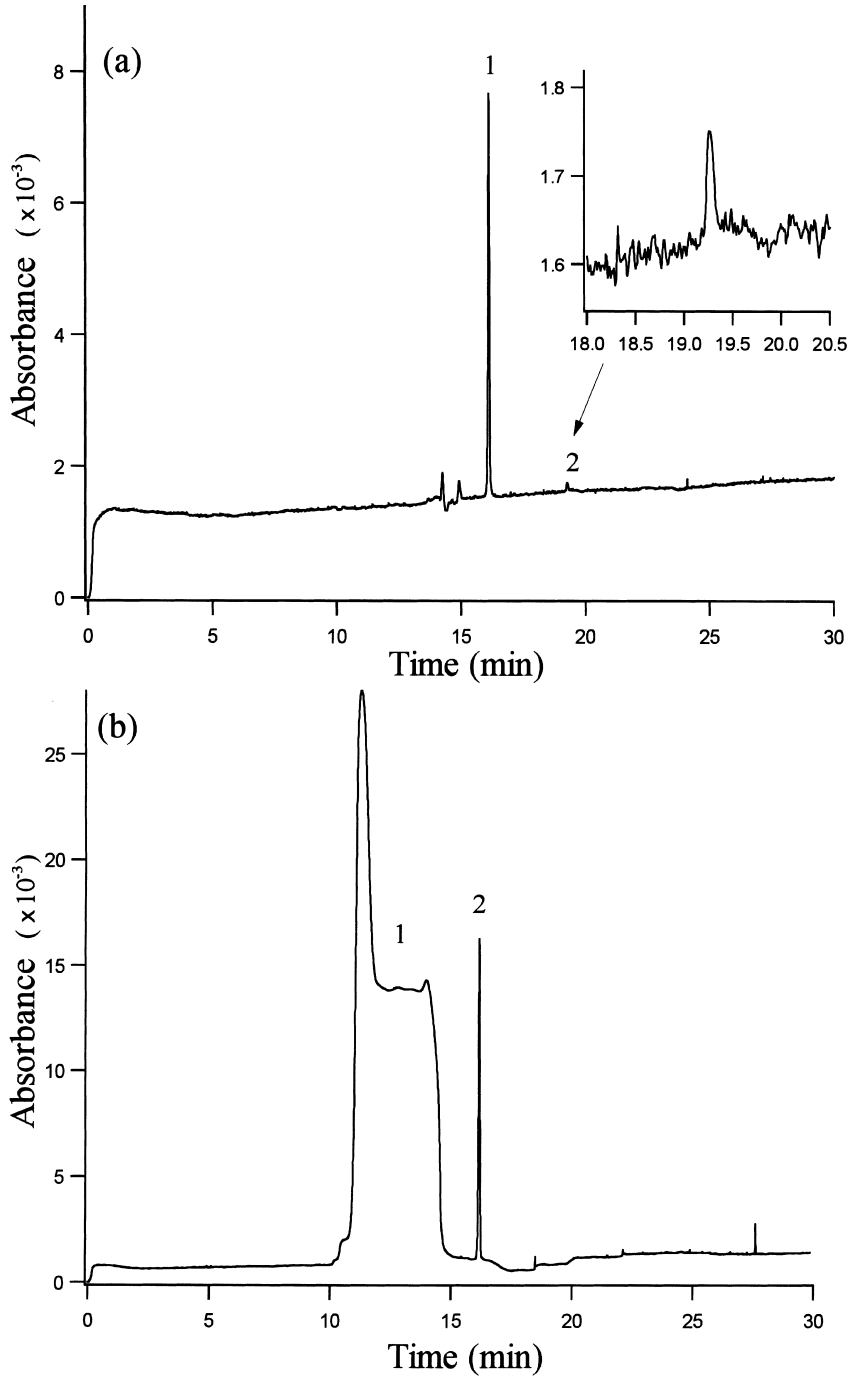

Fig. 6. Selective focusing of micromolar levels of ethynyl estradiol from a 30-fold excess of norethindrone in a female oral contraceptive pill extract using dynamic pH junction. Electropherogram (a) represents a conventional small volume injection (3 s or 0.22 cm), whereas (b) depicts a large volume injection (300 s or 22.1 cm). Note that norethindrone is a neutral steroid analogue whose velocity is independent of buffer pH. Over a 100-fold enhancement in concentration sensitivity is attained while retaining extremely sharp bandwidths (0.82 cm). Conditions as in Fig. 3. Analyte peak numbering corresponds to: 1—norethindrone (EOF) and 2—ethynyl estradiol.

100-fold enhancement in concentration sensitivity was demonstrated for minor levels of ethynyl estradiol in a female contraceptive pill extract using a dynamic pH junction. Further studies are needed to examine the usefulness of different on-line preconcentration strategies for complex sample mixtures by CE.

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