http://dx.doi.org/10.5935/0103-5053.20150016

J. Braz. Chem. Soc., Vol. 26, No. 3, 609-618, 2015.
Printed in Brazil - ©2015 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Optimized Separation Method for Estriol, 17-β-Estradiol and Progesterone by Capillary Electrochromatography with Monolithic Column and its Application to a Transdermal Emulsion

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A monolithic stationary phase based on 3-(methacryloxypropyl)trimethoxysilane monomer, prepared within a fused silica capillary externally coated with a UV-transparent fluoropolymer was employed for separation of estriol, 17-β-estradiol and progesterone by capillary electrochromatography in a standard mixture. A 2^3 factorial design was used to optimize the separation system. The optimized condition containing 30% (v/v) of acetonitrile and 10 mmol L^{-1} aqueous ammonium acetate presented a total run time less than 10 min by applying 25 kV. The resolution between adjacent peaks ranged from 1.8 up to 2.9 and the plate numbers *per* column meter in this condition was 1873, 3631 and 3886 for the estriol, 17-β-estradiol and progesterone peaks, respectively. The optimized method was employed in the quantitative analysis of a commercial transdermal emulsion formulation.

Keywords: capillary electrochromatography, monolithic stationary phase, fluoropolymer-coating fused-silica capillary, steroids, transdermal emulsion

Introduction

Steroids belong to a group of substances with a structural core formed by cyclopenta[a]perhydrophenanthrene and they play an important role in human physiology. Some steroids, such as estriol, 17-β-estradiol and progesterone (Figure 1), act like sex hormones, responsible for sexual characteristics and supports for reproduction.[1–3] Various pharmaceutical formulations have been developed for hormone replacement for post-menopausal women, for example, to prevent osteoporosis and relieve vasomotor symptoms and vaginal atrophy. These steroids may be used in isolation or combination of two or more into the formulation. The association between estrogens (such as estriol or 17-β-estradiol) and progestogens (such as progesterone) has been recommended for women who have gone through menopause, because the progestogen limits marked hyperplasia of the endometrium by estrogen use. In other cases, the isolated use of estrogens is recommended.[4-6]

Steroids analyses in different matrices have been possible through analytical separation techniques such as liquid chromatography (HPLC),[6,7] capillary zone electrophoresis (CZE),[8] micellar electrokinetic chromatography (MEKC),[9] and capillary electrochromatography (CEC).[10,11] However, the unavoidable use of complex electrolytes systems, surfactant addition, sample derivatization or ionization steps, high solvent consumption and functionalized stationary phase (SP) production with complex monomers mixtures seems to be not an advantage.

When two or more substances have similar chemical properties under a specific detection system, an analytical separation technique is usually required, in order to get an adequate and efficient analysis. A separation technique in liquid medium that has gained significant interest in recent years is the CEC, which combines some characteristics of both HPLC and capillary electrophoresis (CE),[12-14] techniques of high impact and acceptance. In simple terms, CEC uses the CE instrumentation and a fused-silica capillary containing a SP inside. The mobile phase transport is performed similarly to the transport in CE, by an electroosmotic flow (EOF), produced by the application

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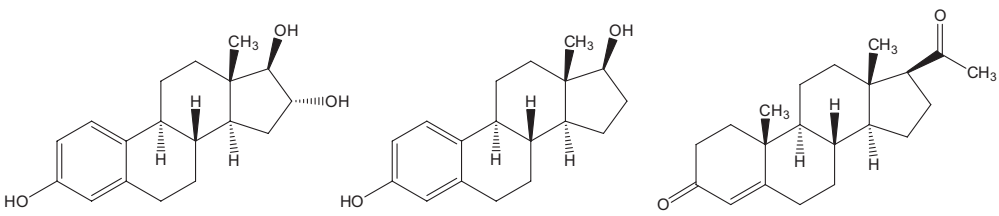

Figure 1. Structure of the three steroids studied in this work.

of an electric field through an electrolyte inside the capillary tube, without the need for high pumping pressure.[15] The EOF has a flat profile, unlike the parabolic flow profile generated by pressure, which minimizes the longitudinal diffusion effects of analytes.[16] Furthermore, the small inner diameters of capillaries reduces thermal gradients from Joule effect, thus reducing zone broadening.[17] These features allow efficient separations with a reduced solvent consumption. The separation in CEC is a result from the combination of electrophoretic mobilities dependent on the analytes charge and size with chromatographic characteristics, dependent on the distribution of analytes between mobile phase and SP. This feature allows the chromatographic separation of neutral molecules, which is not possible with conventional CE techniques without any modification, such as CZE.

The monolithic stationary phases (MSP) are considered to represent a good choice to work with in CEC.[18–20] MSP are a continuous, unitary and highly porous structure, generally based on organic polymer or silica, with high surface areas that allows for mass transfer of the analytes from the mobile phase to the chromatographic active sites for separation. The MSP can be generally prepared by *in situ* polymerization of organic monomers and crosslinkers initially solubilized in porogenic solvents, or via a sol-gel approach after chemical modifications of the capillary wall with silylation reagents.[21] Comparing to the open-tubular columns, MSP does not require the creation of a thick stationary phase inner coating which is associated with practical difficulties and reproducibility problems.[22] Moreover, unlike the conventional packed columns, the chemical bonding of the MSP on the capillary inner surface enhances thermal and mechanic stability and dispenses the use of frits, required to retain the packed material inside a capillary.[23,24]

Recently, our research group published an article concerning the optimization of a MSP preparation from the 3-(methacryloxypropyl)trimethoxysilane (MPTMS) monomer inside fused-silica capillaries with polyacrylate coating, showing its morphological and spectroscopic characterizations.[25] This MSP showed to be useful for electrochromatographic separations of neutral molecules, such as polycyclic aromatic hydrocarbons. The successful use of this coating allowed visual control of the capillary filling with the polymer mixture, the polymerization *in situ* and the evaluation of macroscopic homogeneity of the dry monolith. Nevertheless, the polyacrylate does not have enough transmittance of the radiation at wavelengths shorter than 300 nm.[26] Thus, its removal in a short section of the capillary is necessary to create a UV detection window (as made with polyimide capillaries), making this region fragile and prone to breakage. A good alternative to overcome this problem is to use fused-silica capillaries coated externally with a fluoropolymer (Teflon® AF), which is transparent in a wide range of ultraviolet (over 90% at 214 nm).

In this scenario, this paper aims to show an application of the photopolymerized sol-gel MSP in CEC analysis of the steroids estriol and 17-β-estradiol. The mobile phase and some instrumental parameters were optimized through separation of a standard mixture of these steroids in presence of progesterone in less than 10 min of total running time. Additionally, a commercial transdermal emulsion containing a combination of estriol and 17-β-estradiol was assayed using the optimized method.

Experimental

Materials, reagents and chemicals

Fused-silica capillaries externally coated with a fluoropolymer (TSU series) with dimensions of 100 μm i.d. and 360 μm o.d. were purchased from Polymicro Technologies (Phoenix, USA).

Sodium hydroxide was obtained from Labsynth (Diadema, Brazil); hydrochloric acid, ammonium acetate and acetonitrile (ACN) were obtained from Vetec (Rio de Janeiro, Brazil); methanol was obtained from Quimex (Tubarão, Brazil); toluene was obtained from Beckman (Fullerton, USA); 3-(methacryloxypropyl)trimethoxysilane (MPTMS) from Acros Organics (New Jersey, USA); and phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (photoinitiator) was obtained from Sigma-Aldrich

(Steinheim, Germany). Estriol (99.61%), 17-β-estradiol (100.23%) and progesterone (99.32%) standards, United States Pharmacopeia grade, were cordially donated by Ortofarma (Matias Barbosa, Brazil). A commercial transdermal emulsion sample containing estriol (4.0 mg) and 17-β-estradiol (1.0 mg) was used to verify the applicability of the method.

Instrumentation

A BioSan MSC-6000 centrifuge/vortex (Riga, Latvia) was used to accelerate the separation between the organic and aqueous phases in the sol. A labmade high pressurization injection device (HPID) was used for controlled injection of solvents, aqueous solutions and photopolymerizable solution (sol) into the capillaries on preparation steps of the MSP;[25] 25 μL and 50 μL Gastight® microsyringes from Hamilton Company (Reno, USA) were used with the HPID for controlled injection of sol solutions and to wash monoliths, respectively; 1 mL plastic syringes were used for pre-treatment of the capillaries; and MicroTight® unions from Upchurch Scientific (Oak Harbor, USA) were used to connect capillaries to the syringes.

A labmade UV photo-chemical reactor equipped with six 15 W black-light lamps of predominantly 375 nm wavelength was used to irradiate the polymerization mixture.[27]

An Agilent 7100 capillary electrophoresis instrument (Palo Alto, USA) equipped with diode array detector, temperature control device in cartridge, external pressure by ultra-pure nitrogen cylinder from White Martins (Juiz de Fora, Brazil) and HP 3D-CE ChemStation acquisition/treatment data software (Rev. B.04.03) was used to carry out all CEC experiments.

The sample solutions were sonicated in Unique Ultrasonic Cleaner USC 2800A model (Indaiatuba, Brazil).

All aqueous solutions were prepared with water treated by reverse osmosis system using the Osmose Reversa-Q842 equipment from Quimis (Diadema, Brazil).

Standard and Sample Preparation

Transdermal formulation samples were weighed into a beaker (about 1 g), solubilized in methanol and sonicated for 1 h. The beaker contents were transferred to a 25.0 mL volumetric flask, which was completed with methanol to obtain a sample solution. The sample solution was filtered through a 0.45 μm filter membrane Millex™ (Barueri, Brazil), before analysis.

A standard addition curve was constructed, in triplicate, by diluting the stock solutions (estriol: 11.9 mmol L^{-1} and 17-β-estradiol: 10.4 mmol L^{-1}) in flasks containing a fixed amount of sample solution to obtain the final concentrations 0, 1.2, 2.4, 3.6, 4.8 and 5.9 mmol L^{-1} of estriol and 5.2, 4.2, 3.1, 2.1, 1.0 and 0 mmol L^{-1} of 17-β-estradiol, respectively. The procedure adopted was to increase the concentration of estriol while was to decreased the concentration of 17-β-estradiol.

Monolithic stationary phase preparation

The monolithic column was prepared according to the procedure previously described.[25] Firstly, a hydrolysis and condensation step was carried out to obtain MPTMS oligomers by mixing 1.537 mL of monomer with 0.463 mL of HCl (0.12 mol L^{-1}) for 10 min with magnetic stirring (solution A). Meanwhile, a solution containing 0.0559 g of photoinitiator in 8 mL of toluene was prepared by magnetic stirring for 5 min (solution B). The two solutions above were joined and stirred for 30 min to obtain a sol. These steps were performed in the dark environment. To allow the polymer attaching, a step of silanization of the capillary inner wall was carried out with flushes of NaOH (1.0 mol L^{-1}), HCl (0.1 mol L^{-1}), methanol and air for 30 min each, in that order. The sol was placed into the silanized capillary as the HPID pushed the syringe plunger. Just 8.0 cm of a capillary end segment was filled and the other 28 cm was kept empty. The capillary was placed in the photoreactor for 20 min with irradiation at 375 nm. After this polymerization step the residues were removed with methanol also through HPID and a 50 μL syringe.

Analytical procedures

The analytes were injected in the capillary containing the MSP via the end near to the detector (8.5 cm effective length), i.e., through short-end injection.[28,29] The column occupies the initial 8.0 cm.

External pressure of 5 bar was applied into the inlet vial for promoting the column preconditioning before the first run of the day (flush with methanol for 5 min and with mobile phase for 20 min); the conditioning between each run (flush with mobile phase for 5 min); and the cleanup after the last run of the day (flush with methanol for 10 min).

Results and Discussion

Preliminary considerations over MSP in the fluoropolymer

A common challenge related to the use of MSP in CEC with optical methods of detection performed in the capillary itself (like UV detection), is the difficulty to obtain

a detection window, since the MSP blocks the radiation. In this case, the window can be obtained in different ways. In one mode, two pieces of capillary (one containing the SP and other not) are joined through a specific detection cell, which features complicated handling and maintenance or through special connectors, which can generate bubbles in the mobile phase or increase the analytes diffusion.[16] Another way is to obtain the window during the photopolymerized MSP preparation. The capillary is full filled with the sol solution and a small segment is covered with a bulkhead (such as an opaque tape) to block the UV light used to form the photopolymer.[30] However, this control can be no efficient, because the polymerization reaction can propagate even onto regions that were not radiated.

An interesting alternative is the controlled introduction of sol solution up to a desired point in the capillary (in this case just before the detection window), leaving the rest of the capillary (including the window) empty and free of polymer formation. This is only possible thanks to the use of capillaries with transparent coatings, such as fluoropolymer, which allow visual control of sol-air interface; the use of HPID, which has short screw advancement; and the use of microsyringes, which release small volumes as the HPID is slowly rotated. Furthermore, the fluoropolymer-coating is transparent to UV radiation and its removal (as must be done with polyimide coatings) is unnecessary, eliminating the serious problem of the loss of mechanical strength, both in the MSP and the detection window regions.[16] The photoinitiated polymerization (20 min of polymerization in our case) is usually much faster than thermoinitiated,[31] and just requires the use of a photochemical reactor.[27] Another great advantage observed with this type of capillary coating is the chemical resistance against organic solvents such as toluene (used as the porogen) and ACN (used in the mobile phase), which are frequently used in CEC analysis.[32] All these fluoropolymer characteristics simplify the whole procedure from preparation of MSP until its analytical use. Therefore, UV-transparent capillaries associated to MPTMS are being widely used in the production of photopolymerized monolithic columns.[33–36]

Optimization of the separation system

A 2^3 factorial design for the optimization of the three analyte standards separation was carried out, taking into account three variables: percentage of ACN in the mobile phase; the concentration of ammonium acetate in the aqueous portion of the mobile phase; and applied voltage during runs. The variation in the first variable was selected due to ACN capability to alter the mobile phase polarity and consequently influence the interactions mobile phase-analyte-MSP. The other two variables have the potential to influence the EOF rate, the analysis time, the separation profile and electric current. It is noteworthy that there is a limitation with respect to the electric current. In CE, high electrical currents can increase the temperature (Joule effect) hard to be dissipated. The damage to the monolith and bubble formation on the mobile phase leads to electric current disruption. Nonetheless, these problems can be avoided adjusting these factors.

As possible interactions between these variables are probable to occur, a multivariate approach is desired. All possible combinations between the three factors (variables) in two levels, generating a total of eight experiments, were randomly performed in duplicate, according to the presented matrix (Table 1). As this factorial design had just a character of screening, the separation profiles were qualitatively evaluated.

Table 1. Factorial design

Experiment	Factor		
	Acetonitrile / %[a]	NH_4Ac / (mmol L^{-1})[b]	Voltage / kV
A	50	20	25
B	50	20	20
C	50	10	25
D	50	10	20
E	30	20	25
F	30	20	20
G	30	10	25
H	30	10	20

[a] Percentage relative to the total volume of mobile phase; [b] concentration in the aqueous fraction.

The percentage of ACN is the factor that most influences the separation profile. With 50% ACN there is not an adequate separation as the three analytes coelute with the electroosmotic flow (EOF). By a visual examination of electrochromatograms of Figure 2, it is possible to see that with 30% it is possible to obtain a good resolution separation between all peaks. Taking into account that steroids have a nonpolar character, the increasing of the water fraction in the mobile phase elevates the interactions between analytes and MSP (hydrophobic) slowing down its migration.

The elution order, confirmed by individual analysis of each steroid standard, indicates a reversed-phase aspect of the hydrophobic MSP, in which the less polar analyte (in this case progesterone) elutes last. The presence of an OH group in the estriol structure makes it interact more with the hydrophilic mobile phase leading to a fast elution. It is

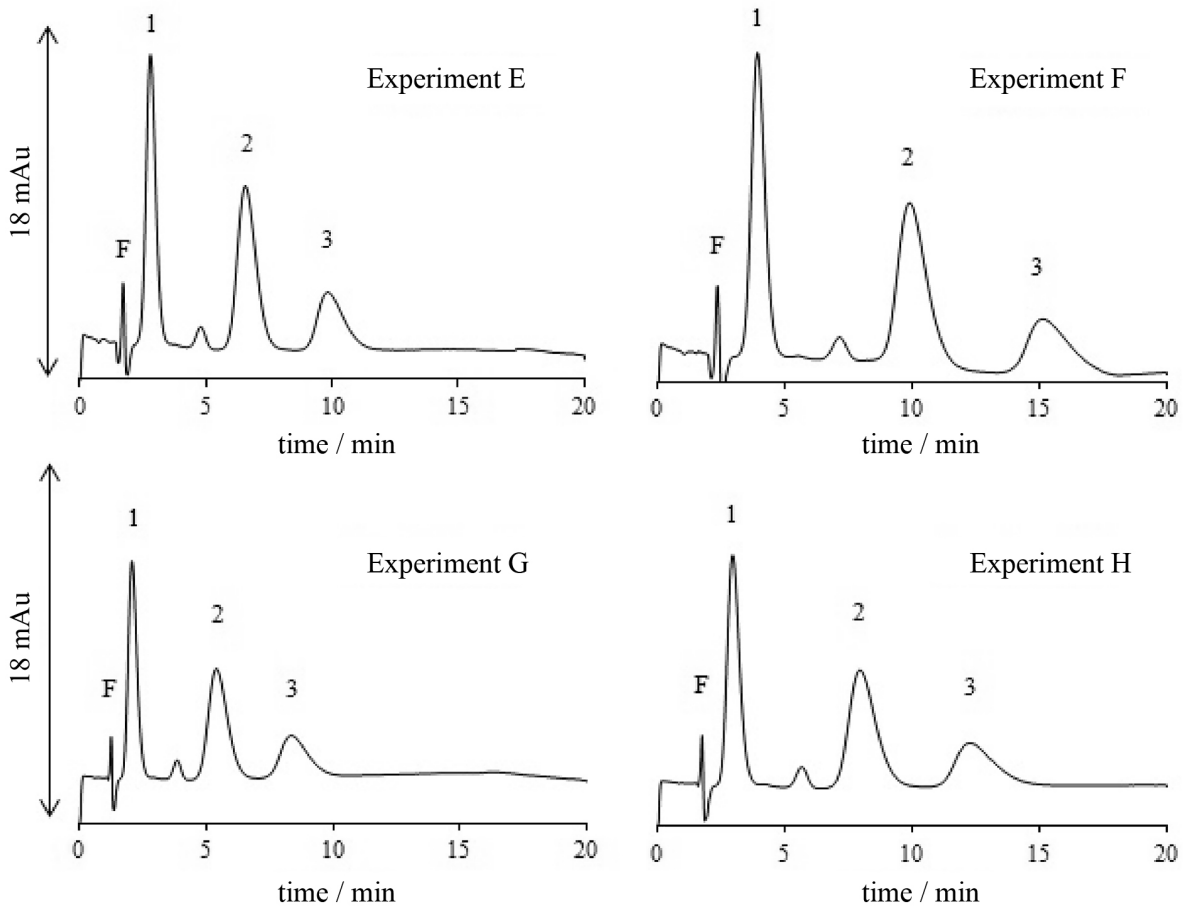

Figure 2. Electrochromatograms of one of the replicates for the experiments E, F, G and H of factorial design according to Table 1; peaks: (F) EOF; (1) estriol; (2) 17-β-estradiol; and (3) progesterone; analytes at 4.5 mmol L^{-1} in methanol; injection: 25 mbar × 15 s; detection at 214 nm; cartridge temperature: 20 ºC; dimensions of the capillary: 36 cm (8.5 cm effective length and 8.0 cm column length) × 100 μm i.d. × 360 μm o.d.

noteworthy that, as the mobile phase pH (7.0) is lower than estriol, 17-β-estradiol and progesterone pKa (10.4, 10.7 and 18.9, respectively),[37] these substances are encountered in a non-ionized forms. Thus, the separation occurs strictly by the interaction of these compounds with the MSP and not by differences in electromigration.

Faster analysis were obtained with voltage at 25 kV, but with currents slightly higher than those obtained at 20 kV (experiments E and G of Table 2). This is a fast analysis time, compared to HPLC methods.[6,38–41] On the other hand, the higher electrolyte concentration did not show significant influence on the electrochromatogram profiles, although it had contributed a lot on the generated current (experiments E and F). Lower currents were obtained with lower electrolyte concentration (experiments G and H), allowing that Joule effect limitations could be avoided. In other experiments some damage in the column was observed when currents higher than 100 μA were employed (not shown). Thus, undesirable higher currents can be avoided with the use of more diluted electrolyte, permitting higher voltages application.

Table 2. Mean values of average currents obtained for the experimental design and values of resolution between the adjacent peaks for the experiments with 30% of ACN

Experiment	Current / μA[a]	$Rs_{F,1}$[b]	$Rs_{1,U}$	$Rs_{U,2}$	$Rs_{2,3}$
E	84	2.2	2.7	1.9	2.3
F	61	2.4	3.2	1.9	2.3
G	38	2.0	2.9	1.8	1.8
H	29	2.2	3.1	2.2	2.6

[a]Average current over the total running time; [b]$Rs_{ij} = 1.175(t_{RB} - t_{RA})/(w_A + w_B)$, where Rs_{ij}: resolution between peaks i and j; t_R: retention time; w: peak width at half height; A: less retained analyte and B: more retained analyte; peaks: (F): electroosmotic flow, (U): unknown peak; (1), (2) and (3): analytes.

Considering these previous discussions, the factorial design points to the condition G as the best choice for providing the fastest analysis with a satisfactory separation between peaks and low values of electric currents. This condition provided resolution values between adjacent peaks higher or equal to 1.8 (Table 2) and plate numbers *per* meter of column from 1873 to 3886 (Table 3).

Table 3. Mean values (n = 2) for retention time (t_R) and the plate number *per* meter (N/m)

Peaks	E		F		G		H	
	t_R[b]	N/m[a]	t_R	N/m	t_R	N/m	t_R	N/m
Estriol	2.7	2771	3.5	2770	2.1	1873	2.8	1925
17-β-estradiol	6.6	5316	9.1	4903	5.5	3631	8.0	5965
Progesterone	10.0	6550	14.1	6942	8.5	3886	12.6	7312

[a]Plate number *per* meter = $[5.54(t_R/w_h)^2]/0.08$; [b]t_R in min.

Preventing instability and disruption of the electrical current

Over several runs, it was observed a gradual decrease of the current, associated with a shift of the peaks onto lower retention times in the electrochromatograms. As it can be seen in the graphic (c) of Figure 3, there is not a tendency for current stabilization even after fourteen consecutive runs.

In CE analyses, it is known that the application of voltage causes the electrolysis of the electrolytic solution, producing changes in pH, reduction of electric current and hence variations in the EOF speed, leading to analyses with large variations in the analyte retention time.[42,43] In CEC this problem is aggravated because organic solvents are generally employed and they also suffer electrolysis, such as acetonitrile.[44]

A solution to solve this problem was the renewal of mobile phase before each CEC run. For this, the replenishment system on the CE equipment, where it is possible to replace the mobile phase recently used in a run with fresh mobile phase from a reservoir, was used. The graphic (d) of Figure 3 shows how the electric current behavior became more stable and constant, using this system.

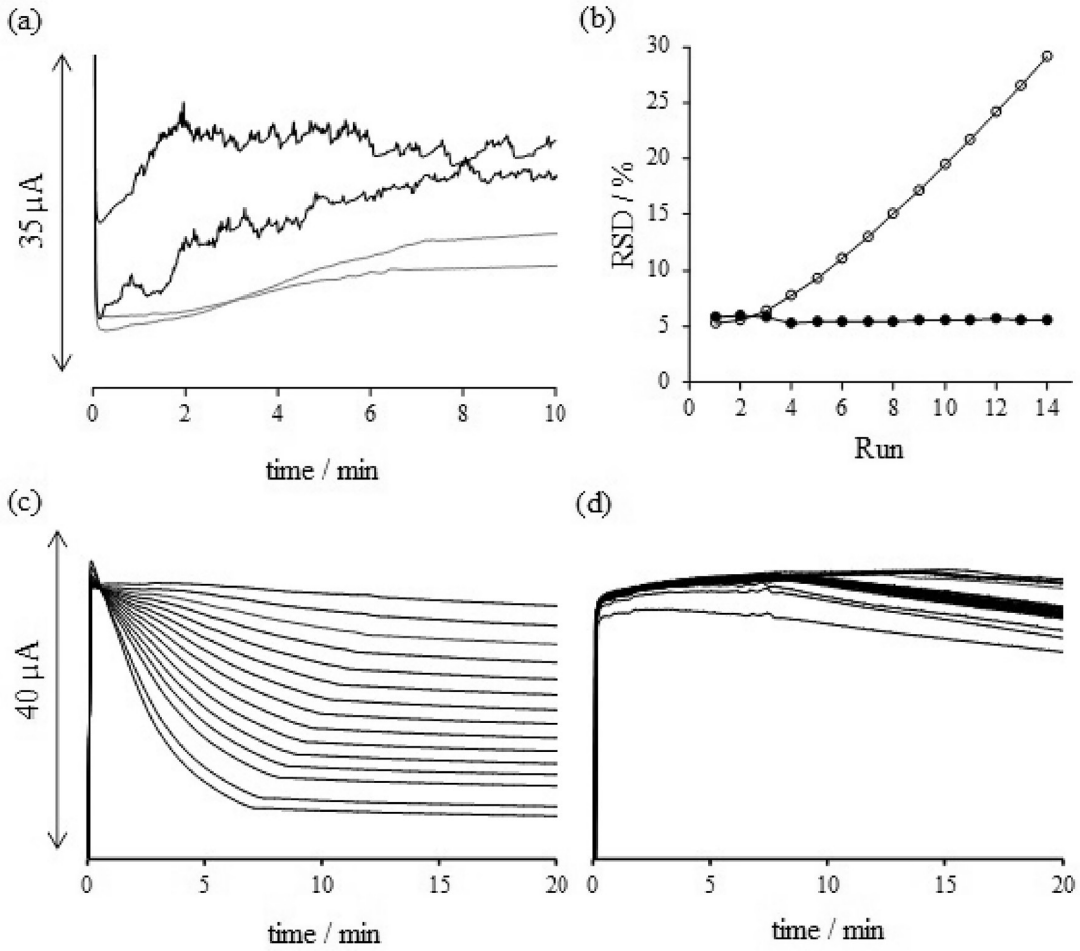

Figure 3. Electric current profiles: (a) (grey line) with and (black line) without vials pressurization; (b) relative standard deviation for current values of 14 consecutive runs (●) with and (○) without renewal of mobile phase; electric current profiles: (c) without and (d) with renewal of mobile phase.

These results are more evident when the relative standard deviations (RSD) for the current of fourteen consecutive runs with and without mobile phase renewal are compared (Figure 3b). In the first case, there is no significant variation between the RSD (5.30-5.89%). However, the variation in RSD without mobile phase renewal was considerable (5.32-29.11%).

As regarding CEC analysis, the tendency of bubble formation in mobile phase due to the presence of dissolved gases in mobile phase among other factors,[45] which may generate instability and disruptions on the current as well as analytical signal interferences, is a common challenge to be overcome. The graphic (a) of Figure 3 shows how a simultaneous pressurization on both vials during the application of running voltage yielded more stable currents, probably due to the suppression of bubble formation, which leads to a more uniform movement of the electric charges.

Repeatability in preparing the MSP applied to steroids

After having the mentioned problems were solved, two MSP in fluoropolymer-coating capillaries were made from sol solutions prepared independently. These columns were used in the separation of a mixture of steroids standards under the condition G in order to verify the repeatability of MSP preparation. The retention time and the plate numbers for each peak were evaluated (Table 4).

With a box-plot graph (Figure 4) obtained with SPSS software (Release 8.0), it is possible to observe that the boxes location, the median values (horizontal lines within the boxes) and absence of outlier points indicate a similarity between retention times and plate numbers, both in comparisons between replicates on a same column as on different columns. In addition, the boxes sizes indicate that variabilities are also similar. From a qualitative point of view, these results point to good run-to-run and column-to-column repeatabilities.

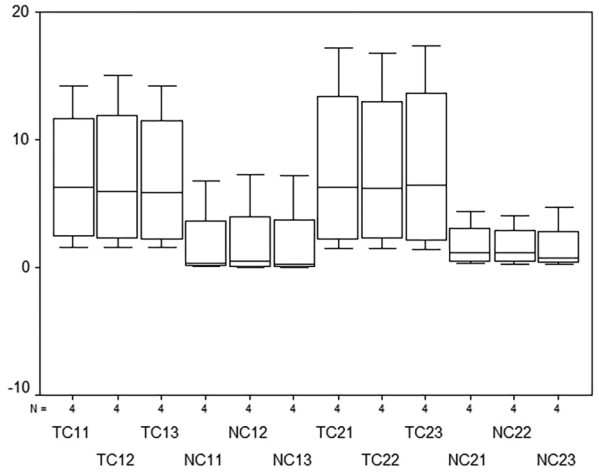

Figure 4. Box-plot graph for the variables: retention time and plate number of two monolithic columns prepared independently. The values of plate number were divided by 10^4 just to adjust in the scale. TCij: retention time for the i column and j replicate; NCij: plate number for the i column and j replicate.

Application in transdermal emulsion assay

Once the method was proven to be adequate to separate the hormones in a single-run analysis, a commercial sample containing two of them in combination was assayed to verify its practical application. The analysis of the transdermal emulsion sample using the optimized conditions showed that it is possible to separate the active ingredients from the other matrix constituents (excipients/vehicles) which could remain in the sample solution after the preparation process. Anyway, a standard addition curve

Table 4. Retention time and plate number of two monolithic columns prepared independently

Peaks	Column 1			Column 2		
	Retention time / min					
	TC_{11} [a]	TC_{12}	TC_{13}	TC_{21}	TC_{22}	TC_{23}
EOF	1.6	1.6	1.6	1.5	1.5	1.4
Estriol	3.4	3.1	3.0	3.0	3.2	3.0
17-β-estradiol	9.2	8.8	8.8	9.6	9.3	9.9
Progesterone	14.2	15.0	14.2	17.2	16.8	17.4
	Plate number / (N/m)					
	NC_{11} [b]	NC_{12}	NC_{13}	NC_{21}	NC_{22}	NC_{23}
Estriol	1375	730	678	3059	2872	2632
17-β-estradiol	3776	2481	2165	7441	7443	6347
Progesterone	2897	8116	3146	17307	17098	8684

[a]TC_{ij}: retention time for the i column and j replicate; [b]NC_{ij}: plate number *per* meter for the i column and j replicate.

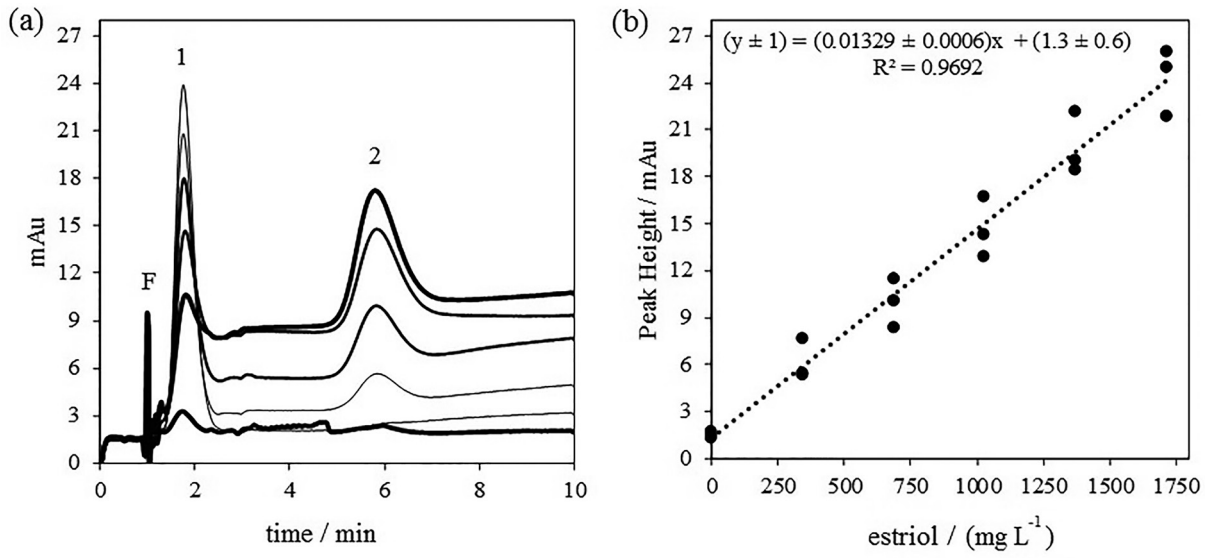

Figure 5. (a) Electrochromatograms of one of the replicates for the standard addition method through condition G; Peaks: (F): EOF, (1): estriol (sample + 0, 1.2, 2.4, 3.6, 4.8 and 5.9 mmol L^{-1}), (2):17-β-estradiol (sample + 5.2, 4.2, 3.1, 2.1, 1.0 and 0 mmol L^{-1}); (b) Standard addition curve (genuine triplicate).

was plotted to eliminate possible matrix effects (Figure 5). Equation (1) describes the curve, in genuine triplicate, wich showed no lack of fit in 95% of confidence interval ($F_{calculated} = 0.12 < F_{0.05;\,4;\,12} = 3.26$).[46]

$$(y \pm 1) = (0.01329 \pm 0.0006)x + (1.3 \pm 0.6) \quad (1)$$

The limits of quantitation (LOQ) and detection (LOD) for estriol were 7 mg L^{-1} e 2 mg L^{-1}, respectively, calculated by equations (2) and (3):[47]

$$LOQ = 10 \times \left(\frac{S_n \times C_S}{H_{max} - H_{min}} \right) \quad (2)$$

$$LOD = 3 \times \left(\frac{S_n \times C_S}{H_{max} - H_{min}} \right) \quad (3)$$

where S_n is the standard deviation of the baseline noise; C_s is the concentration of added standard; and $H_{max} - H_{min}$ is the height of the peak from the baseline.

The observed concentration of estriol was 99.1 mg L^{-1}, amount equivalent to 5.0 mg g^{-1} in transdermal emulsion. This value differs from the labeled value, (4.0 mg g^{-1}). On the other hand, the 17-β-estradiol peak was not observed in the sample electrochromatograms, so it was not possible to quantifie this steroid in the emulsion. However, with standard addition, this peak appears separated from the other ones, as expected. Is noteworthy that, the reported concentration of 17-β-estradiol in the sample is 1.0 mg g^{-1}, i.e., the lack of 17-β-estradiol and the excess of estriol are exactly the same ammount.

This was an interesting finding for this work. On the one hand, the analysis with standard addition proved that the method was adequate to assay real samples, with adequate specificity (no coelution or resolution smaller than 1.5 was observed). On the other hand, the commercial sample, expected to meet the quality criteria defined within the pharmacopeias, failed its assay. This put, one may consider two hypothesis for the situation: (*i*) the sample did not meet effectively the quality criteria, whether by a bad practice of production or by adulteration; or (*ii*) the sample was produced with the labeled amount of active pharmaceutical ingredients, but a conversion between the two steroids may have occurred. This last hypothesis is plausible to occur once the aqueous media is propitious to reactions that reduce the stability of the products. It is known, yet, that estradiol can be reversely converted into estrone, and that these two can be converted into estriol.[48] In fact, the official method of the United States Pharmacopeia is able to identify these metabolites, and a certain amount of estrone is tolerable within the products.[49] Thus, this is coherent with a higher amount of estriol and a smaller amount of estradiol in the sample. In this case, another good perspective for the method can be seen: it can also be used as to indicate the pharmaceutical stability, which extends its applicability for pharmaceutical industries.

Conclusions

With the experimental design applied to the optimization of the mobile phase composition and the run applied voltage, it was possible to discover an analysis condition that allowed satisfactory CEC separation of a standard mixture of estriol, 17-β-estradiol and progesterone as uncharged species in a relatively short time and with

good resolution between peaks. After optimization of the analytical conditions, the MSP was successfully applied in a sample of transdermal emulsion for female hormone replacement. Considering that the progesterone can also be included in this matrix, as an "all-in-one" future product, the proposed method is also promising for analysis of this steroid.

The fused-silica capillary externally coated with fluoropolymer proved to be chemically stable in the presence of the solvents used, such as ACN, water and methanol; mechanically strong and flexible during handling; and sufficiently transparent to UV-visible radiation. This latter feature enabled the injection of sol solution through the HPID with the aid of visual control; the use of UV radiation for the MSP photopolymerization; and the detection of proposed analytes with CEC analysis without the need of a detection window.

Finally, the electric current stability was achieved with external pressure applied on both vials during runs and with renewal of mobile phase between each run. With the understanding of this important variable, it was possible to improve the repeatability between columns prepared independently since the sol solution preparation until the final analyses.

Acknowledgments

The authors wish to acknowledge the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) (Project CAPES–PNPD 23038.007000/2011-70), The Brazilian National Council for Technological and Scientific Development (CNPq) (Project CNPq: 475055/2011-0 and 301689/2011-3) and Minas Gerais Research Foundation (FAPEMIG) (Process FAPEMIG: CEX-APQ 02420/11 and CEX-PPM 00205/11) for fellowships and financial support, as well as the Ortofarma Laboratório de Controle da Qualidade (Matias Barbosa, MG, Brazil) for the provision material.

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Submitted: October 10, 2014
Published online: February 3, 2015