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Microemulsion and micellar electrokinetic chromatography of steroids

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Abstract

A mixture of ten steroids was separated by microemulsion and micellar (SDS and glycodeoxycholate) electrokinetic chromatography systems. Separations were done on a 50 cm (to the detector) × 50 μm I.D. fused-silica capillary. Complete separation of all the test compounds in the micellar mode was obtained with glycodeoxycholate (50 m*M*) in 25 m*M* borate buffer, pH 6.5, as the micelle-forming agent. The best results, however, were obtained using microemulsion electrokinetic chromatography in which higher aliphatic alcohols were used as the microemulsion-forming modifiers. The system consisted of *n*-hexanol (0.81%), SDS (3.31%) and *n*-butanol (6.61%) in 20 m*M* phosphate buffer, pH 10.0 (89.28%, w/w). In the microemulsion mode, linear calibration for steroid standards was obtained in the concentration range 3×10^{-4}–3×10^{-5} mol l^{-1} with a detection limit of 1 pmol. The method was validated and applied to an 11β-hydroxysteroid dehydrogenase assay in tissues.

Keywords: Steroids

1. Introduction

Steroids, i.e. steroid hormones and steroid drugs, represent a category of compounds that have a crucial influence on the morphological formation of living organisms and on their physiological functions. Their toxicology and forensic properties also attract considerable attention. All these aspects stimulate interest in the analytical properties of these compounds, particularly in the assay of steroids in body fluids.

There is a plethora of methods for the separation, identification and quantitation of steroids in biological samples (for review see Ref. [1]), including spectrophotometry [2], fluorimetry [3], paper chromatography [4], thin-layer chromatography [5], CPBA (competitive protein binding assay) [6], RIA (radioimmunoassay) [7], GC (gas chromatography) [8], GC–MS [9] and particularly HPLC [10], that can be used for estimating both endogenous and exogenous steroids in tissues. Recently, methods using micellar electrokinetic chromatography (MEKC) have been added to the available methodologies.

Steroid separations by MEKC can be materialised with different tensides. Nishi et al. [11] reported that separation with SDS is impossible because of the high lipophilicity of the solutes to be separated, as the equilibrium distribution is shifted very much in favour of the micellar phase and little, if any,

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partition between the background electrolyte and the micelle occurs. The separation, however, can be successful when bile acids, i.e. cholate and deoxycholate, are used as the micellar phase. The results of Bumgarner and Khaledi [12] confirm these assumptions. These authors were able to separate a mixture of seventeen corticosteroids using glycodeoxycholate and taurocholate and their mixed micelles as the pseudostationary phase. While the separations with SDS micelles were generally poor, best results were obtained with mixed micelles of 50 m*M* glycodeoxycholate and 50 m*M* taurocholate (in 50 m*M* phosphate–borate buffer, pH 9.0). On the contrary Jumppanen et al. [13] successfully separated a mixture of seven corticosteroids by using SDS micelles. This separation was achieved at low SDS concentration (10 m*M*) in 60 m*M* borate buffer, pH 9.2. Terabe et al. [14] reported the separation of a mixture containing six corticosteroids with SDS, however, 6 *M* urea must have been present in the background electrolyte in order to decrease the polarity of the aqueous phase. Rigorous comparison of these methods is complicated by the fact that different authors have tested different mixtures of steroids.

Microemulsion electrokinetic chromatography (MEEKC) has emerged only recently. It was used for the first time by Watarai [15] in 1991, for the separation of fluorescent aromatic compounds. Terabe et al. [16] used it for the separation of test mixtures of some aromatics and drugs and they compared this mode with MEKC (using SDS). Recently Boso et al. [17] used this system for the separation of fat- and water-soluble vitamins. In general, the microemulsions (oil/water) are formed from oil, water, surfactant and co-surfactant (medium alkyl-chain alcohol). It appears that the structure of the microemulsion is similar to the structure of the micelle – the oil droplet is stabilised by the surfactant and co-surfactant, located on the surface of the droplet [16].

The aim of this work was to compare the separation of a steroid test mixture by MEKC using both micellar (SDS and glycodeoxycholate) and microemulsion pseudostationary phases and to develop a rugged method for the separation and quantitation of these compounds.

2. Experimental

2.1. Capillary electrophoresis

Fused-silica capillaries, untreated, UV transparent, 60 cm long (50 cm to the detector)×50 μm I.D., purchased from Supelco (Bellefonte, PA, USA) were mounted into a home-made capillary electrophoresis system [18]. Stock solutions of standards were prepared as 1.4×10^{-2} mol l^{-1} solutions in methanol, except for triamcinolone which was used at a concentration of 0.7×10^{-2} mol l^{-1}. The test mixture was prepared by mixing 10 μl of all stock solutions (except triamcinolone - 20 μl) with 0.8 ml of the selected background buffer. Injections were made electrokinetically (15 kV, 10 s). Separations were routinely run at 15 kV and monitored at 220 nm, at ambient temperature. In between runs, the capillary was washed using 0.5 *M* NaOH for 4 min.

2.1.1. SDS MEKC

Separations were carried out in borate buffer made 25 m*M* with respect to SDS, with an optimized buffer concentration of 12.5–50 m*M* and with a pH of 8.5–11 (adjusted to the desired value with 1 *M* NaOH or 3 *M* HCl).

2.1.2. Glycodeoxycholate MEKC

Separations were carried out in borate buffer with 50 m*M* glycodeoxycholate, with an optimum buffer concentration of 12.5–50 m*M* and a pH of 5.5–9.5 (adjusted to the desired value with 1 *M* NaOH or 3 *M* HCl).

2.1.3. MEEKC

Microemulsions were prepared according to the method of Boso et al. [17] by mixing microemulsion-forming organic solutes (0.81%, w/w), SDS (3.31%, w/w), *n*-butanol (6.61%, w/w) with 20 m*M* phosphate buffer (89.28%, w/w) that was optimized with respect to pH (7–11, adjusted to the desired value with 1 *M* NaOH or 3 *M* HCl).

n-Hexane, cyclohexane, diethyl ether, *n*-amylalcohol, *n*-hexanol and *n*-octanol were tested as emulsion-forming solvents.

2.2. Chemicals

SDS and cyclohexane were purchased from Merck (Darmstadt, Germany), glycodeoxycholic acid, sodium salt was from Sigma (St. Louis, MO, USA) and *n*-hexane was from Aldrich (Milwaukee, WI, USA). Steroid standards (aldosterone, dexamethasone, triamcinolone, cortexolone, cortisone, cortisol, corticosterone, 11-dehydrocorticosterone, deoxycorticosterone and deoxycorticosterone acetate) were purchased from Sigma, Serva (Heidelberg, Germany) and Koch-Light (Colnbrook, UK). All other chemicals were obtained from Lachema (Brno, Czech Republic) and were of the highest purity available. All buffers were prepared in Milli-Q water.

2.3. Application

Practical applicability of the separation method was shown with 11β-hydroxysteroid dehydrogenase activity estimation in rat intestine (the procedure is described in Ref. [19]).

2.4. Calculation of coefficients

The number of theoretical plates N was calculated according to the equation $N = 5.545\ (t_m/\sigma)$, where t_m is the migration time and σ is the peak width at half the peak height; resolution R was calculated using the equation $R = 2\ \Delta t \cdot (W_1 + W_2)^{-1}$, where Δt is the difference in migration times of the two solutes involved and W_1 and W_2 are the peak widths at the baseline.

3. Results

3.1. SDS micellar electrokinetic chromatography

Optimalization of the system was made in the pH range 8.5–11.0 and with a buffer concentration of 12.5–50 m*M*. Best results were obtained using 25 m*M* SDS in 25 m*M* borate buffer, pH 9.5. A typical separation of the standard test mixture is shown in Fig. 1. The number of theoretical plates was low and ranged from 4×10^3 (triamcinolone) to 45×10^3 (dexamethasone). Resolution in this artificial test

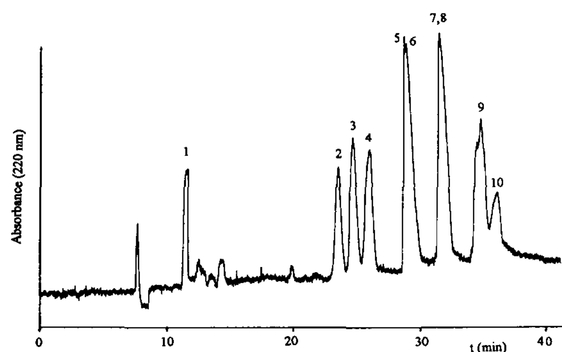

Fig. 1. Separation of the test mixture of steroids by SDS micellar electrokinetic chromatography. Separation conditions: 25 m*M* SDS, 25 m*M* borate buffer, pH 9.5 at 15 kV. Identification of peaks: 1 = triamcinolone, 2 = aldosterone, 3 = cortisone, 4 = cortisol, 5 = dexamethasone, 6 = 11-dehydrocorticosterone, 7 = corticosterone, 8 = cortexolone, 9 = deoxycorticosterone and 10 = deoxycorticosterone acetate.

mixture was also poor; corticosterone and cortexolone were not separated and the resolution of the dexamethasone–11-dehydrocorticosterone pair was insufficient (except at pH 9.5 where some resolution is indicated, at all other pHs, this pair is not resolved at all). It is to be borne in mind that many of these steroids would never have to be analyzed in the same sample and that, in other cases, their levels could be different by several orders of magnitude.

3.2. Glycodeoxycholate MEKC

This system was also examined within the pH range 5.5–9.5 and with a buffer concentration of 12.5–50 m*M*. The optimized background electrolyte consisted of 50 m*M* glycodeoxycholate and 25 m*M* borate buffer, pH 6.5. Separation of standards in this buffer is presented in Fig. 2. The glycodeoxycholic acid-containing system is capable of separating all of the steroid standards examined, however, peaks showed a high tendency to tailing, that can be partially prevented by dissolving the solutes in methanol prior to injection. The number of theoretical plates was higher than with SDS and ranged from 31×10^3 (cortisone) to 83×10^3 (deoxycorticosterone). Resolution for all consequent peaks was higher than 1.00 (at least 1.05 for the aldosterone–cortisone critical pair).

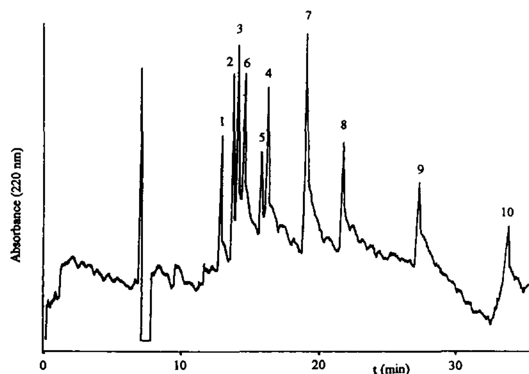

Fig. 2. Separation of the test mixture of steroids by glycodeoxycholate micellar electrokinetic chromatography. Separation conditions: 50 m*M* glycodeoxycholate, 25 m*M* borate buffer, pH 6.5, 15 kV. Identification of peaks is the same as for Fig. 1.

Table 1
Influence of the organic modifier on resolution in microemulsion electrokinetic chromatography

Organic modifier	Resolution
n-Hexane	0.00
Cyclohexane	0.00
Diethyl ether	0.00
n-Amyl alcohol	0.50
n-Hexanol	0.67
n-Octanol	0.81

Composition of the background electrolyte was the same in all cases [i.e., 0.81% organic modifier, 3.31% SDS, 6.61% *n*-butanol and 89.28% (in all cases w/w) 20 m*M* phosphate buffer pH 10.0] and differed in the nature of the organic modifier only.

3.3. MEEKC

Optimization of the buffer pH was carried out within the range 7.0–11.0; separation at pHs of 7 and 8 was not satisfactory, at pH 9 and beyond the separation was successful and was practically pH-independent. Separation of standards by MEEKC, with *n*-hexane as the organic solvent, is shown in Fig. 3. The number of theoretical plates is high, ranging from 18×10^3 (triamcinolone) to 278×10^3 (deoxycorticosterone). Also, the resolution was high (at least 2.5) except for the critical pair of aldosterone–cortisone. Attempts to resolve this pair are summarised in Table 1. *n*-Hexane, cyclohexane, diethylether, *n*-amylalcohol, *n*-hexanol and *n*-octanol (in all cases at a concentration of 0.81%, w/w) were tested as microemulsion-forming solvents. It is evident that the critical pair was resolved when using higher alcohols only and that better resolutions were obtained with alcohols possessing higher carbon numbers in the molecule. Separation obtained with an *n*-hexanol-containing emulsion is shown in Fig. 4. In this context, it is necessary to mention that the run times increased when alcohols with increasing carbon numbers were used. For this reason, *n*-hexanol

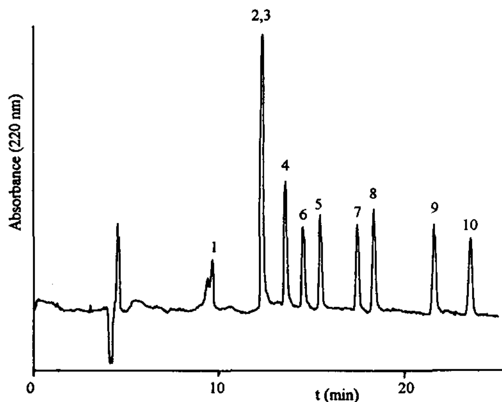

Fig. 3. Separation of the test mixture of steroids by microemulsion electrokinetic chromatography. Separation conditions: *n*-hexane (0.81%), SDS (3.31%), *n*-butanol (6.61%) with 20 m*M* phosphate buffer, pH 10.0 (89.28%, w/w), 15 kV. Identification of peaks is the same as for Fig. 1.

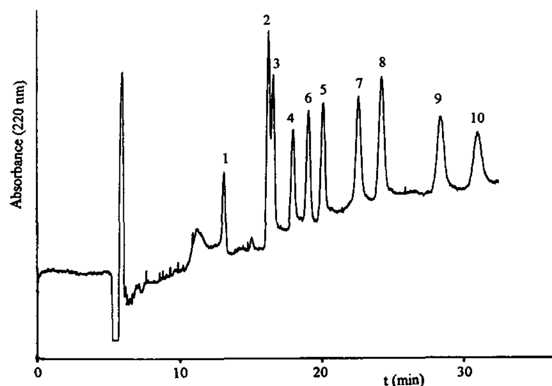

Fig. 4. Separation of the test mixture of steroids by microemulsion electrokinetic chromatography. Separation conditions: *n*-hexanol (0.81%), SDS (3.31%), *n*-butanol (6.61%) with 20 m*M* phosphate buffer, pH 10.0 (89.28%, w/w), 15 kV. Identification of peaks is the same as for Fig. 1.

was preferred to n-octanol (running times for corticosterone were 22.8 and 30.2 min, respectively).

3.4. Application

Applicability of the examined methods for biologically relevant samples was shown with the 11β-hydroxysteroid dehydrogenase activity assay in rat intestine. Two separation systems were tested in this assay; separation in the micellar mode with glycodeoxycholate and with the microemulsion-containing system in the optimized version, as described above. In the case of glycodeoxycholate micelles, calibrations for 11-dehydrocorticosterone and corticosterone were linear in the range 3×10^{-4}–1×10^{-5} mol l^{-1}, with a detection limit of 5×10^{-6} mol l^{-1} (with regression coefficients of 0.9993 and 0.9997, respectively; S.D. = 0.24 and 0.12, respectively). In the microemulsion system, calibrations were made for all separated steroids; the linearity range was from 3×10^{-4} to 3×10^{-5} mol l^{-1}, with a detection limit of 1×10^{-6} mol l^{-1} (ca. 1 pmol per injection); the regression coefficient was in the range 0.9993–0.9998 (S.D. = 0.21–1.36) (see Table 2). Both the microemulsion and micellar electrokinetic separations were applicable to the enzyme activity assay and the results obtained were in good agreement with data obtained by the HPLC method (compare with Ref. [19]) (see Table 3).

Table 2
Linear regression calibration data for steroids in the microemulsion system ($y = a + bx$, where y is the peak height and x is the amount in μmol l^{-1})

Steroid	a	b	Regression coefficient
Triamcinolone	1.736	0.218	0.9997
Aldosterone	1.945	0.205	0.9993
Cortisone	2.244	0.216	0.9994
Cortisol	2.110	0.198	0.9996
11-Dehydrocorticosterone	2.490	0.118	0.9994
Dexamethasone	0.026	0.245	0.9995
Corticosterone	0.812	0.196	0.9986
Cortexolone	1.108	0.238	0.9994
Deoxycorticosterone	1.697	0.211	0.9998
Deoxycorticosterone acetate	0.402	0.187	0.9998

Calibration for aldosterone and cortisone was carried out separately because these two compounds are incompletely separated and cannot be quantitated if present in the sample simultaneously.

Table 3
Comparison of the measurement of 11β-hydroxysteroid dehydrogenase activity by HPLC, MEKC and MEEKC (expressed as DCS/(DCS+CS), where DCS is 11-dehydrocorticosterone and CS is corticosterone).

Sample	HPLC	MEKC	MEEKC
1	0.00	0.00	0.00
2	0.42	0.45	0.44
3	0.96	0.92	0.94

4. Discussion

Comparison of the different micellar electrokinetic separations with the microemulsion procedure can be demonstrated best by comparing the number of theoretical plate numbers obtained with various solutes in different separation modes (Table 4). From this comparison, it is evident that the highest efficiency was obtained with the microemulsion system (with the exception of triamcinolone and to a lesser extent aldosterone and cortisone). The lowest separation efficiency was obtained with the SDS-containing system. Also, resolution was poor with the SDS-containing system, in which two pairs of steroids, namely dexamethasone–cortexolone and dexamethasone–11-dehydrocorticosterone were either poorly resolved or not resolved at all. On the other hand, good resolution was obtained with the glycodeoxycholate-containing system in which the resolution of all examined steroids was better than 1.0. These findings are in good agreement with previously published results [12,13].

MEEKC seems to be an appropriate method for the separation of various compounds, although the actual conditions have been elaborated in only a few cases to date. Watarai [15], who introduced microemulsion separations, used heptane as the microemulsion-forming agent. Later, the effect of different agents on the resolution of some solutes was rather well documented [17]. It was also shown that better separations could be obtained when alcohol-containing emulsions were used (shown with a mixture of fat- and water-soluble vitamins). These results were confirmed in this report, although it is feasible to expect that optimum composition of the organic phase will depend on the polarity of the solutes to be separated (partitioned between the

Table 4
Number of theoretical plates in MEKC systems (SDS and glycodeoxycholate) and MEEKC with *n*-hexane as the organic component of the microemulsion system

Steroid	SDS	Glycodeoxycholate	Microemulsion
Triamcinolone	4042	40 063	18 016
Aldosterone	10 829	35 982	28 347
Cortisone	9515	30 775	28 347
11-Dehydrocorticosterone	31 889	40 589	76 091
Dexamethasone	44 915	39 126	115 247
Corticosterone	30 775	57 690	139 010
Cortexolone	37 994	74 614	84 916
Deoxycorticosterone	7364	82 643	278 223
Deoxycorticosterone acetate	13 003	45 918	121 663

aqueous and the emulsion phases). When hexane was used as the microemulsion-forming agent, good resolution was obtained for eight steroids (with a resolution of better than 2.5), but the aldosterone–cortisone pair remained unresolved. When we used alcohol, separation of this pair was possible and increased as the carbon chain length of the alcohol used was increased (resolution of 0.81 for *n*-octanol). The highest number of theoretical plates obtained with this system is in agreement with the data of Terabe et al. [16] who compared MEEKC and MEKC.

Also, the migration order of steroids changed in the systems examined. In all cases, triamcinolone emerged first, followed by aldosterone and cortisone. The three steroids that eluted after the first triad, i.e. cortisol, 11-dehydrocorticosterone and dexamethasone changed their migration order depending on the system used. The last four steroids (corticosterone, cortexolone, deoxycorticosterone and deoxycorticosterone acetate) were eluted in same order, regardless of the system examined. It can be assumed that while triamcinolone, aldosterone and cortisone were sufficiently polar to shift the partition in favour of the aqueous phase, corticosterone, cortexolone and deoxycorticosterone exhibited just the opposite behaviour and were imbedded readily into the micellar (microemulsion) phase. The intermediate three members of the mixture were in a situation in which changes in the pseudostationary phase could bring about discernible changes in the affinity of the solutes (similar effects were demonstrated in the paper of Boso et al. [17]).

It appears that such changes are typical for the application of a microemulsion system. Alterations in a rather narrow polarity range usually affect only a few members of a test mixture, the reason being that with most test mixtures the span of the polarity range is rather large. On the other hand, this behaviour can be purposefully exploited for separating the critical pairs, however more knowledge is needed about the behaviour of different solvents and, perhaps, of their mixtures, as microemulsion-forming components.

5. Conclusions

In this work we demonstrated the possibility of using microemulsion systems for the separation of selected steroids. The studied mixture of steroids showed good separation characteristics in MEEKC, particularly with alcohols as the microemulsion-forming solutes. These characteristics were generally better than those obtained with the MEKC systems that were used in comparison (with the exception of aldosterone and cortisone which were incompletely separated).

Quantitation of steroids was possible both in the micellar system, with glycodeoxycholate, and in all the microemulsion systems used; the linearity range was from 3×10^{-4} to 3×10^{-5} mol l^{-1}. With SDS as the micellar phase, quantitation of all members of the test mixture was precluded by incomplete resolution of some steroids. The separation procedure was used for the 11β-hydroxysteroid dehydrogenase activity assay.

Acknowledgments

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