

SEPARATION OF STEROIDS BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY. SOME PHYSICOCHEMICAL CONSIDERATIONS.

Susanne K. Wiedmer and Marja-Liisa Riekkola*

*Laboratory of Analytical Chemistry, Department of Chemistry, P.O.Box 55,
00014 University of Helsinki, Finland*

CONTENTS:

	Page
Abbreviations	68
Summary	69
1. Introduction	69
2. Selectivity and resolution in MECC	71
3. CMC measurements	74
4. Determination of distribution coefficients	74
5. Separation of steroids by MECC	78
6. Mathematical optimization procedures	78
6.1. SDS-borate electrolyte solutions	82
6.2. Mixed SDS/SC micellar electrolyte solutions.....	85
7. Analysis of corticosteroids in biological matrices	86
8. On-line partial filling MECC-ESI-MS	90
8.1. On-line partial filling MECC-ESI-MS of corticosteroids	91
9. Physicochemical studies	92
9.1. NMR studies of micellar systems	92
9.2. Mixed SDS/SC micellar solutions studied by MECC and NMR	93
10. High-molecular-mass surfactant for the separation of corticosteroids in ECC	95
11. Analysis of steroids by capillary electrochromatography	97
12. Conclusions	99
13. References	99

* To whom correspondence should be addressed

ABBREVIATIONS

AMPSO	3-[(1,2-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid
BES	<i>N,N'</i> -bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
Brij-35	polyoxyethylene 23 lauryl ether
CABRO II	computer assisted bivariate resolution optimization II
CAMOS	computer assisted multivariate optimization strategies
CCD	central composite design
β -CD	β -cyclodextrin
γ -CD	γ -cyclodextrin
CE	capillary electrophoresis
CEC	capillary electrochromatography
CHES	2-(<i>N</i> -cyclo-hexylamino)-ethanesulfonic acid
CMC	critical micelle concentration
DLS	dynamic light scattering
DTAB	dodecyltrimethylammonium bromide
EOF	electroosmotic flow
ECC	electrokinetic capillary chromatography
ESI	electrospray ionization
FUMI	function of mutual information
GC	gas chromatography
HPLC	high-performance liquid chromatography
LiDS	lithium dodecyl sulfate
LiPFOS	lithium perfluorooctane sulfonate
LOD	limit of detection
LOQ	limit of quantitation
LSER	linear solvation energy relationship
MECC	micellar electrokinetic capillary chromatography
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
MS	mass spectrometry
NMR	nuclear magnetic resonance
ORM	overlapping resolution mapping
OTAC	octyltrimethylammonium chloride
PF	partial filling
PFG	pulsed field gradient
PIPES	piperazine- <i>N,N'</i> -bis(2-ethanesulfonic acid) monosodium salt
PLS	partial least squares

RSM	response surface modeling
SB-12	<i>N</i> -dodecyl- <i>N,N</i> -dimethyl-3-ammonio-1-propanesulfonate
SC	sodium cholate
SDBS	sodium dodecyl benzenesulfonate
SDS	sodium dodecyl sulfate
SE	spin echo
SPME	solid-phase microcolumn extraction
TTAB	tetradecyltrimethylammonium bromide
TTAC	tetradecyltrimethylammonium chloride
Tween 20	polyoxyethylenesorbitan monolaurate
Tween 60	polyoxyethylenesorbitan monostearate
Tween 80	polyoxyethylenesorbitan monooleate

SUMMARY

Micellar electrokinetic capillary chromatography is nowadays a widely used analytical separation technique for a vast amount of samples. The main reason for its growing popularity are the small amounts of samples and solvents needed for the analysis, as well as in fast and efficient separations. This review summarizes studies on steroids by micellar electrokinetic capillary chromatography. Practical aspects to consider in micellar electrokinetic capillary chromatography, such as the critical micelle concentration of surfactants and optimization procedures, are discussed. Furthermore, physicochemical studies on commonly used buffers in micellar electrokinetic capillary electrophoresis are presented. Finally, steroid investigations performed with capillary electrochromatography are briefly discussed.

1. INTRODUCTION

The capillary electrophoretic (CE) separation techniques have already carved out a niche of their own in analytical separation science. The main reason for the popularity of these techniques is the potential for high selectivities in combination with short analysis times and high efficiencies. Other advantages are the small amounts of reagent and sample required. Important both from the environmental and economic aspects is the small amount of waste generated. Moreover, the range of compounds that can be separated in CE extends from

small ions to large biomolecules.

One popular CE technique is micellar electrokinetic capillary chromatography (MECC) /1/, where surfactants above their critical micelle concentration (CMC) are added to the electrolyte solution. The micelles formed act as a pseudostationary phase and the separation of compounds is based on their distribution between the micellar and aqueous phases. Differences in the distribution of the analytes, due to their charge, size, shape, and polarity make a simultaneous separation of non-ionic and ionic compounds possible. In this sense, the technique is superior to conventional CE where only charged compounds can be separated. Among the many micelles suitable for the separation of compounds in MECC, the most widely used have been low-molecular-mass surfactants, and especially anionic ones. Recently, however, high-molecular-mass surfactants have also been found suitable as pseudostationary phases in MECC /2,3/.

The MECC separation of compounds can easily be fine-tuned by changing the surfactant or by modifying the properties of the micelles, for example by the addition of organic solvent or another surfactant /4-6/. Even though several different surfactants are available, it is nevertheless seldom an easy task to find the optimal micellar solution for the separation of a particular set of compounds. In part this is due to difficulties in predicting analyte-micellar interactions. A number of mathematical optimization schemes have been developed for the determination of optimal separation conditions /7/, usually with the resolution of the system as the optimized parameter. Often just a few test runs suffice to predict the overall best running conditions, depending of course on the number of parameters included in the optimization strategy. When more than one micelle is added to the electrolyte solution the situation is complicated by the possible micelle-micelle interactions.

Corticosteroids are neutral hydrophobic compounds which, owing to their important roles in mineral and glucose equilibria, are being investigated by a number of groups. The concentration of corticosteroids in serum can serve as a direct indication of certain diseases /8/. Usually, corticosteroids in serum samples have been determined by immunological techniques, gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), or high-performance liquid chromatography (HPLC). Although the immunoassay methods are rapid and simple to perform, they suffer from cross-reactivity between the different corticosteroids. In the case of HPLC, the resolution of the method is relatively low and large volumes of samples and eluents are needed. Recently, there has been a growing interest in CE as a method of analysis. The

main problems encountered in CE analyses of body fluids are the low concentrations of the compounds of interest, which make detection difficult, and the large number of interfering compounds, such as proteins, which may adsorb on the silica walls of the capillary.

The sensitivity of MS, and the possibility it provides of obtaining molecular information on compounds, make the on-line coupling of chromatographic techniques with MS highly attractive. Several papers have already been published on the combination of CE with MS /9/. Among the MS ionization techniques, electrospray ionization is the most widely applied in on-line CE-MS /10/. Although MECC is a convenient separation technique for neutral analytes, problems are encountered in the on-line MECC-ESI-MS connection because the micelles in the electrolyte solution are non-volatile and tend to dirt the MS. Several techniques have been developed to prevent the micelles from reaching the MS /11-20/.

This review presents investigations made on corticosteroids by MECC. However, since corticosteroids very often are analysed from mixtures containing other steroids, MECC separations of other steroid mixtures will also be described. Mathematical optimization schemes are discussed and attention is also focused on surfactants commonly used in MECC separation of corticosteroids, i.e. on SDS and SC, and on physicochemical studies on the micelles. Capillary electrochromatography in the analysis of steroids will also be briefly discussed. Although it is a little apart from the rest of the discussion, it might be an important electrophoretic separation technique for the separation of steroids in the future.

2. SELECTIVITY AND RESOLUTION IN MECC

The selectivity, α , of a system in MECC is usually described by the retention factors, k /21/. In the case of a neutral analyte, the retention factor can be described as

$$k = \frac{t_r - t_0}{t_0 \left(1 - \frac{t_r}{t_{mc}}\right)} \quad (1)$$

where t_r is the migration time of the analyte, t_0 the migration time of an

unretained compound, and t_{mc} the migration time of a compound that is thought to be fully solubilized into the micelle. The selectivity can then easily be determined by the ratio of the retention factors of two compounds:

$$\alpha = \frac{k_2}{k_1} \quad (2)$$

The most effective way to alter the selectivity in MECC is to change the micellar phase by changing the type of surfactant. Since the surfactants used in MECC separations may be anionic, cationic, neutral, or zwitterionic, the possibilities are many. As demonstrated by several authors, the addition of two or more surfactants to the electrolyte solution may lead to the formation of mixed micelles with different selectivity from either of the component micelles. Various mixed micellar solutions have been tested as pseudostationary phases in MECC (Table 1) and in many cases shown to improve the selectivity of the system. In the case of cationic and zwitterionic surfactants there may be reversal of the electroosmotic flow (EOF) due to ionic adsorption on to the silica wall.

When compounds occur in their neutral form, factors such as buffer and micellar concentrations, pH, voltage, and temperature usually have a relatively minor effect on the selectivity of the system. When the compounds are charged, on the other hand, pH variations may induce changes in the dissociation of the compounds, affecting their charge, and thereby the solute-micelle ionic interactions and electrophoretic mobilities /50/. Micelle-induced pK_a shifts for ionic compounds have also been shown to affect the selectivity significantly /51/. In addition, temperature variations can lead to changes in the pK_a values of charged compounds and to different micelle-solute interactions /52/.

Because of the dynamic structure of micelles, changes in temperature, pH and ionic strength of the electrolyte solution, and the addition of organic modifiers may influence micelle aggregation and size. Such variations in the structure may in turn affect the selectivity of the separation.

The resolution in MECC has usually been calculated with the following equation:

$$R_s = \frac{N^{1/2}}{4} \frac{(\alpha-1)}{\alpha} \frac{k_2}{(1+k_2)} \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + \left(\frac{t_0}{t_{mc}}\right)k_1} \quad (3)$$

Table 1
Selected mixed micellar systems used in MECC.

Mixed micellar system	Surfactants in the mixture	Ref.
anionic-non-ionic surfactants	SDS and Brij-35	22-27
	SDS and Tween 60	28
	SDBS and Brij-35	29
	SDS and Tween 20	30
	bile salts and polyoxyethylene-4-dodecyl ether	31
anionic – anionic surfactants	SDS and SC	32-37
	SDS and sodium octyl sulfate	38
	SDS and bile salts	39
	two different bile salts	40-42
	LiPFOS (fluorocarbon) and LiDS (hydrocarbon)	43
anionic – cationic surfactants	fluorosurfactants FC 128 and FC 134	44
anionic – zwitterionic surfactants	SDS and SB-12	45-46
non-ionic – non-ionic surfactants	Tween 20 and Tween 80	47
	Triton X-100 and Brij-35	47
cationic-cationic surfactants	TTAC and OTAC	48
	TTAB and DTAB	49

where N is the plate number [21]. The resolution of a system depends on the efficiency, the selectivity, the retention, and the migration time window. Hence, improving the selectivity is one way to improve the resolution of the system.

3. CMC MEASUREMENTS

The critical micelle concentration (CMC) of a surfactant is the concentration above which the surfactant starts to form micelles. The CMC decreases sharply with increasing alkyl chain length of the surfactant, but just the reverse is true for the Kraft point, which increases with the length of the chain /53/. The Kraft point is the temperature at which the solubility of the surfactant increases rapidly, and it varies widely with the surfactant. Accordingly, the formation of micelles demands surfactant concentrations above the CMC and temperature above the Kraft point. All factors that lower the electrostatic repulsion between the head groups of ionic surfactants favor micelle formation, which means that the CMC of surfactants is lower in electrolyte solutions than in pure water. Typical ways of determining the CMC of surfactants in CE buffer solutions are to measure surface tension, light scattering, refractive index, electrical conductivity, or electrophoretic mobility against increasing concentration of surfactant /53/. CMC values for the most commonly used surfactant SDS in selected electrolyte solutions are listed in Table 2. The CMC of SDS in pure water at 25°C is 8.1 mM. The use of CE for the determination of the CMC of a surfactant requires that no surfactant molecules are adsorbed onto the silica wall, and, additionally, in the case of surface tension and conductivity measurements no surfactant should be adsorbed to the wall of the beaker in which the measurements are performed. An interesting study on the effects of different organic solvents on the CMC of SDS has been published by Jacquier *et al.* /60/. Their results in Figure 1 show in an illustrative way the change in the CMC of SDS when altering the organic solvent and its concentration in the buffer.

4. DETERMINATION OF DISTRIBUTION COEFFICIENTS

The separation of neutral components in MECC is based on their partitioning between the aqueous and micellar phases /21/. The distribution or partition coefficient, P , can be described by the retention factor k and the volumes of the aqueous phase (V_{aq}) and micellar phase (V_{mc}):

$$P = k \frac{V_{aq}}{V_{mc}} \quad (4)$$

The retention factor can be calculated directly from the migration times of the electroosmotic flow marker, the separated compounds and the micelle

Table 2
CMC values of SDS in selected electrolyte solutions at 25°C.

Electrolyte solution	CMC (mM)	Method of determination	Ref.
50 mM AMPSO (pH 9.0 ¹)	3.6	conductometric titration	36
50 mM AMPSO (pH 9.0 ¹)	3.9	CE	36
50 mM AMPSO (pH 8.7 ¹)	2.7	surface tension	37
20 mM PIPES, 20 mM NaOH (pH 7.0)	3.8	conductometric titration	52
100 mM BES, 100 mM NaOH (pH 7.0)	3.1	conductometric titration	52
100 mM borate, 50 mM phosphate (pH 7.0)	2.9	conductometric titration	52
5 M urea, 100 mM borate, 50 mM phosphate (pH 7.0)	4.4	conductometric titration	52
20% DMSO (v/v), 25 mM tetraborate, 50 mM sodium dihydrogen phosphate (pH 7.0)	6	conductometric titration	54
20% acetone (v/v), 25 mM sodium tetraborate, 50 mM sodium dihydrogen phosphate (pH 7.0)	6.3	conductometric titration	54
20 mM sodium tetraborate (pH 9.2)	3.1	CE	55
20 mM sodium tetraborate (pH 8.0 ²)	5.5-9.6 ³	CE	56
5 mM sodium tetraborate – acetonitrile (85/15, v/v)	7.3	CE	55
5 mM sodium tetraborate (pH 9.2)	5.3	CE	57
100 mM sodium tetraborate, 100 mM sodium dihydrogen phosphate (pH 6.0 ⁴)	2	CE	58

Table 2 (continued)
CMC values of SDS in selected electrolyte solutions at 25°C.

Electrolyte solution	CMC (mM)	Method of determination	Ref.
100 mM sodium tetraborate, 100 mM sodium dihydrogen phosphate (pH 6.5 ⁴)	2.4	CE	58
100 mM sodium tetraborate, 100 mM sodium dihydrogen phosphate (pH 7.0 ⁴)	3.1	CE	58
100 mM sodium tetraborate, 100 mM sodium dihydrogen phosphate (pH 7.7 ⁴)	4	CE	58
50 mM CHES (pH 10.0)	2.9-5.2 mM ⁵	CE	59
50 mM CHES (pH 10.0)	2.7-5.4 mM ⁶	CE	59
80 mM CHES (pH 10.0)	1.6-2.2 mM	CE	59
100 mM CHES (pH 10.0)	1.2-2.4 mM	CE	59
50 mM ammonium acetate (pH 9.0)	1.7-2.7 mM	CE	59

¹) pH adjusted with 25% ammonia, ²) pH adjusted with 1 M acetic acid, ³) capacity factors of flavonoids used in the calculations, ⁴) pH adjusted with 0.1 M HCl or 0.1 M NaOH, ⁵) capacity factors of benzene and naphthalene derivatives used in the calculations, ⁶) capacity factors of 1,4-dihydropyridines used in the calculations

marker (eq. 1). However, there may be variations in k depending on EOF and the micelle marker. In particular, the choice of the micelle marker may have a significant effect on the value of k . Usually, a highly hydrophobic, neutral compound such as Sudan III, Sudan IV, and dodecanophenone has been chosen. All these compounds have strong interactions with the micelles and migration times are comparable with those of micelles. Since the migration time window in MECC is finite and defined by the ratio of t_0 to t_{mc} , a badly chosen marker may give a poor description of the micellar system in question.

Quite often the logarithms of the octanol-water partition coefficients ($\log P_{ow}$) of compounds have been used to describe the retention of the compounds in MECC. The relationships between retention in MECC and $\log P_{ow}$ values in different micellar electrolyte solutions have been studied by a number groups

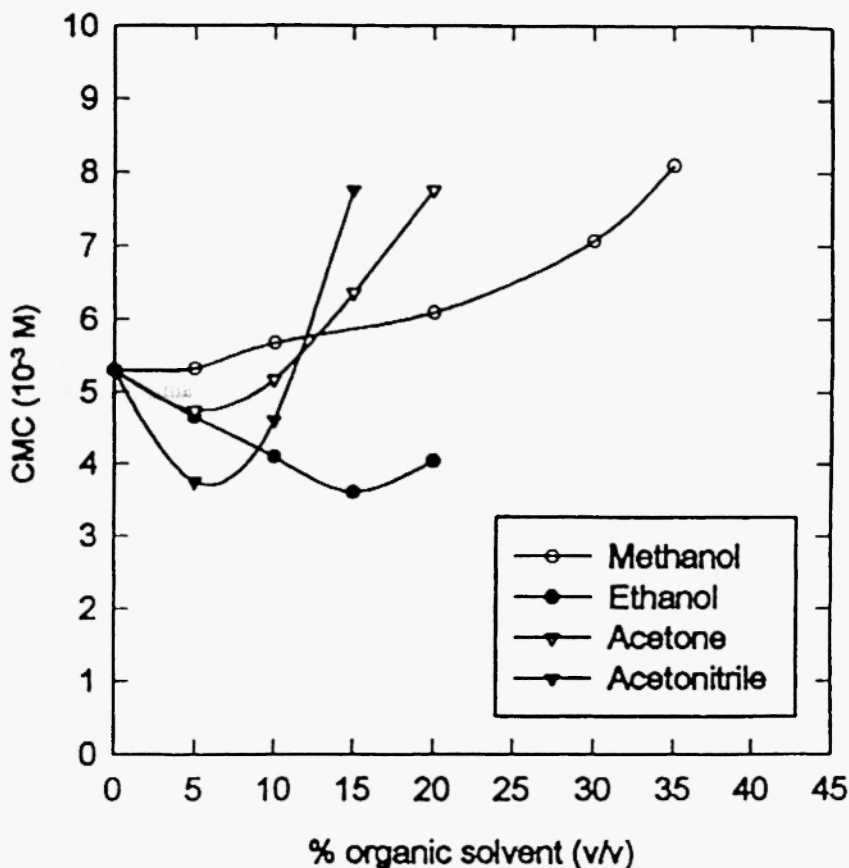


Fig. 1: Capillary electrophoretic determination of CMC of SDS as a function of added organic solvent. Naphtalene was used as a sample compound and the electrolyte solution contained 5 mM borax, pH 9.2. The temperature was 25°C. Reprinted with permission from Ref. /60/.

and in many cases the correlations have been fairly good /61-63/. However, because there are other plausible analyte-micelle interactions besides hydrophobic ones, it is not always possible to use the log P_{ow} values to describe the retention of compounds in MECC. Instead, the partition coefficients for analytes in different micellar solutions can be calculated according to eq. 4

Determination of the partition coefficient requires that the volumes of the micellar and aqueous phases are known. V_{mc} can be calculated in a rather straightforward way from the group volumes of the surfactants, which can be

found in the literature /64/. The volume of the micellar phase in the solution is

$$V_{mc} = V_{tot} - V_{free} \quad (5)$$

where V_{tot} is the total micellar volume corresponding to the total surfactant concentration in the solution and V_{free} is the volume of nonmicellized surfactant in the solution, which can be calculated from the CMC of the surfactant. Particularly in the case of neutral analytes one can assume that there are no interactions between surfactant monomers and analytes and V_{free} can be subtracted from V_{tot} as in eq. 5. In the determination of the micellar volume, the polar parts are assumed to be solvated in the aqueous phase and are not included in the micellar volume. The total aqueous solution is the rest of the solution.

5. SEPARATION OF STEROIDS BY MECC

In most of the separations of corticosteroids by MECC, samples have contained corticosteroid mixtures diluted with water or buffer solution. Usually the separations have been done under alkaline conditions, with use of anionic surfactants. Table 3 lists separations of steroid and corticosteroid mixtures with MECC reported in the literature. The parameters included (number of compounds separated, surfactant in the buffer, and pH of the buffer) were chosen as of probable interest to the reader. Although the buffer itself, especially if it is borate, may influence the selectivity of the steroids, the analyte-buffer and micelle-buffer interactions were considered negligible and neglected in the construction of the table. Various mixed micellar systems have also been applied to the separation of corticosteroids.

6. MATHEMATICAL OPTIMIZATION PROCEDURES

To find the optimal separation by trial-and-error is tedious and time-consuming. Several models for the optimization of selectivity and resolution of a system have accordingly been developed. In many of these models, first the most critical parameters for the separation are chosen and then a few of them are selected for the optimization. Thus, it is the optimization model and the analyst that decide which parameters should be included. In general, the more parameters included in the model, the more complex the

Table 3
Separation of steroids by MECC reported in the literature.

# comps.	Surfactant	pH	Comments	Ref.
7	SDS	8-10	optimization of separation	65
7	SDS + SC	8-10	optimization of separation	33
3	SDS + SC	9	determination from serum	34
3	SDS + SC	9	on-line PF-MECC-ESI-MS	35
6	SDS + SC	9	several single and mixed micellar solutions investigated	36
5	Elvacite 2669	9-10	40-80% (v/v) MeOH in the buffer	66
7	SC	7.4	20% MeOH in the buffer	67
6	DTAB	7.4	trioctylphosphine oxide in the buffer	68
17	sodium deoxycholate + sodium glycodeoxycholate	9	improved separation by the addition of sodium butane-sulfonate to the buffer	40
17	sodium glycodeoxycholate + sodium taurocholate + SDS	9	various mixtures of bile salts tested	39
17	sodium dehydrocholate + sodium taurocholate + SDS	9	various mixtures of bile salts tested	39
9	SB-12 + sodium taurocholate	9	different zwitterionic surfactants investigated	69
4	SDS + sodium taurocholate	9	direct injection of serum spiked with corticosteroids	70
8	SDS + γ -CD	9	urea added to the buffer	71
8	SDS	9	urea added to the buffer	72
3	SDS + Brij 35	7	different micellar solutions studied by LSER modeling	73

Table 3 (continued)
Separation of steroids by MECC reported in the literature.

8	SC	9	various bile salts tested	74
10	Glycodeoxycholate	6.5	comparison of micellar and microemulsion systems	75
10	SDS	9.5	comparison of micellar and microemulsion systems	75
5	SDS	9.2	10% methanol added to the buffer	76
12	SDS	8	analysis of steroids in serum samples	77
10	SDS	7	methanol or acetonitrile added to the buffer enhanced the separation	78
10	SDS + γ -CD and SDS + β -CD	9.2	better separation with the SDS + γ -CD buffer	78
4	SDS + γ -CD	2.5	sample stacking using reverse migrating micelles was introduced	79
11	SDS + β -CD	8	separation of the products of liver microsomal testosterone metabolism	80

results will be to analyze and the greater number of demands are made on the model. Approximations are always made when mathematical models are used to solve chemical problems; hence, models are always open to criticism. Examples of the statistical optimization schemes used in MECC are listed in Table 4.

Two electrolyte systems suitable for MECC runs, one containing SDS, the other SDS/SC mixtures, have mathematically been optimized for both the selectivity and resolution of some corticosteroids by Wiedmer *et al.* /65,33/. Because there were some minor but important differences in the optimization procedures as applied to the two systems, the systems will be discussed

Table 4
Statistical optimization schemes used in MECC.

Optimized parameter	Parameters varied	Modeling	Ref.
selectivity and resolution	pH, [SDS], [borate]	CCD, desirability functions	65
selectivity and resolution	pH, [SDS], [SC], [AMPSO]	CCD, desirability functions	33
resolution	[acetonitrile],[urea]	iterative regression strategy	81
resolution	9 for a stepwise screening, followed by 3: pH, [SDS], [acetonitrile]	fractional factorial design, full factorial design, RSM	82
yield for the derivatization of some dipeptides	reaction time, T, ionic strength, pH, [isopropanol]	fractional factorial design, CCD, RSM	83
selectivity	pH, [SDS]	iterative regression strategy	84
resolution	[SDS], [acetonitrile]	CABRO II	85
precision and efficiency	[SDS], V, T	FUMI	86
resolution	T, V, ionic strength, [SDS], [HPMC], [β -CD]	PLS	87
resolution	[SDS], [urea]	CABRO II	88
resolution	pH, [SDS]	CAMOS	89
resolution	pH, [buffer], [SDS], [SDS + sodium heptyl sulfate], [acetonitrile]	Plackett-Burman statistical design	90
resolution	[SDS], [<i>N,N</i> -dimethylformamide], ionic strength	ORM	91
resolution	pH, [SDS], [tetrabutylammonium salt]	ORM	92
resolution	pH, [SDS]	ORM	93
resolution	pH, [SDS]	ORM	94
resolution	pH, [SDS]	ORM	95
resolution	[SDS], [isopropanol], [β -CD]	full factorial design	96
resolution	pH, [SDS]	full factorial design	96

separately. The main differences between the optimization procedures were that, with the mixed micellar system, the selectivity was optimized in a sequential manner and the optimal selectivity was used as a starting point for the optimization of the resolution.

6.1. SDS-borate electrolyte solutions

In the optimization of the selectivity and resolution of the corticosteroids the aim has been to investigate the suitability of simple equations for the calculations. The three parameters varied in the calculations were pH and borate- and SDS concentrations /65/. The experimental design was similar to the central composite design (CCD). Models for the analyte migration time and band broadening were selected on the basis of a step-wise regression where the selection or dropping criterion for the terms was given by the 'leave one out' cross-validation. The R² values, which measure the goodness of fit of the regression model, are computed by comparing the regression model and the primary data. The Q² values, on the other hand, measure the goodness of prediction of the model, and are computed by comparing the values of each datapoint with the value predicted by the regression model. At each step the terms included in the model (forward stepwise regression) or left out (backward stepwise regression) were selected so as to maximize the Q² values. Both linear and quadratic regression models were studied for the analyte migration time and band broadening, and the quadratic models turned out to yield better fit to the data. In an evaluation of the six most important terms giving the highest value of Q² for the model of the analyte migration time, the most critical parameters were SDS and borate concentrations. Seven terms were included in the models of band broadening, and those terms including the migration time of the analyte seemed to dominate.

Optimization of resolution with the SDS-borate system. After a sufficient model had been found by cross-validation, the resolution was calculated for all possible pairs of corticosteroids. For a rapid and approximate calculation of the resolution, the simple well-known chromatographic equation was used. Desirability functions were applied to deal with the multi-criterion optimization /97/. The optimum resolution was found at pH 9.2 with 60 mM borate and 10 mM SDS. The peaks in the electropherogram were evenly distributed but rather broad, perhaps due to the relatively high borate concentration, which may lead to Joule heating effects.

A poor desirability at pH values 8 to 9 was observed, which was due to a surprising change in the migration order of the first two migrating species, *i.e.* 1-dehydroaldosterone and 17-isoaldosterone. Figure 2 shows the relative migration times of these first two compounds as a function of the buffer. Since the analytes are neutral in the pH range studied, the change in the migration

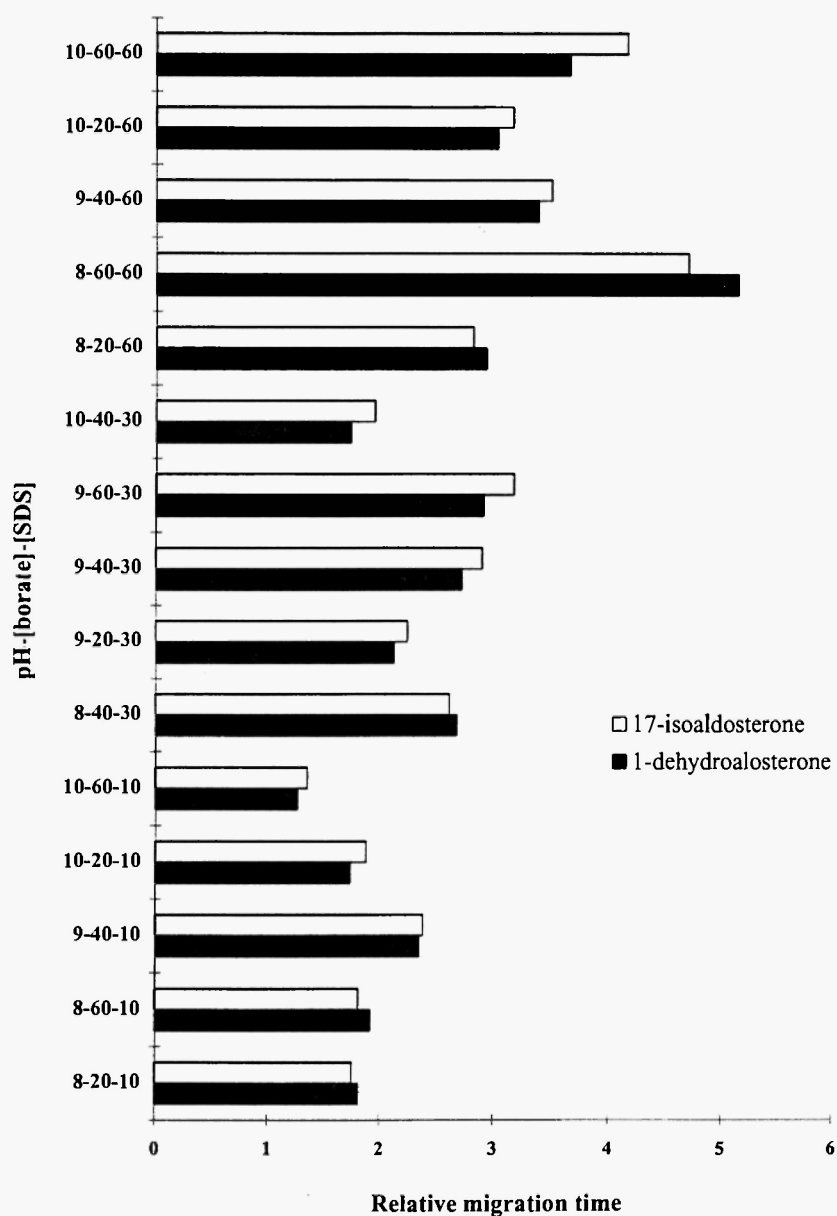


Fig. 2: Relative migration times of 1-dehydroaldosterone and 17-isoaldosterone as a function of the electrolyte solution.

order was probably due to some specific interactions between the analytes and buffer components. There should not be any changes in SDS in the pH range in question. As to the borate, it is well-known that borate forms complexes with analytes with vicinal hydroxy groups /38/. C. Fernandez *et al.* suggested in their analysis of mixtures of steroids that some degree of complexation between the borate and the steroids occurs /80/.

Optimization of selectivity with the SDS-borate system. The corticosteroids are neutral in the pH values studied and accordingly have zero electrophoretic mobilities in the electrolyte solution. The normalized velocities (v_n) of the compounds and the normalized velocity ratio ($v_{nr(ij)}$) have been used to describe the selectivity of the system /65/:

$$v_{nr(i,j)} = \frac{v_n(i)}{v_n(j)} = \frac{v_{tot(i)} - v_{eo}}{v_{tot(j)} - v_{eo}} \quad \text{for } i = (1,2,...,6) \text{ and } j = (i+1,...,7) \quad (6)$$

where v_{tot} is the total velocity of a compound and v_{eo} is the velocity of the electroosmotic flow. The electroosmotic flow was approximated to remain constant within one run. Both linear and quadratic regression models were investigated for the normalized velocities of the analytes, and as for the analyte migration time and band broadening, the quadratic models were found to be satisfactory. The models were cross-validated to yield a maximum value for Q2. Six terms were included in the model and, as in the modeling of the migration time of the analyte, the SDS concentration was found to be the most important factor for the normalized velocity of the analyte. The pH also played an important role. For the v_{eo} the most critical parameter was the borate concentration and to a lesser extent the pH.

After a good model was found the normalized velocity ratios were calculated for all 21 pairs of corticosteroids. The desirability function was set to be selective for the range of normalized velocity ratios typical to pairs formed by successive peaks. The optimum was found at pH 10 with 10 mM SDS and 60 mM borate.

Best resolution and selectivity were both obtained at the lowest concentration of SDS tested, but at different pH values. Thus, in this case, optimal resolution and optimal selectivity were achieved under different conditions. The similar partition coefficients for the corticosteroids between the SDS micellar phase and the aqueous phase made the separation difficult. A number of ways exist to decrease the partitioning of hydrophobic compounds into micelles, among them

the addition of an organic solvent or a different surfactant to the buffer solution. Organic solvents modify the micellar phase and/or increase the solubility of hydrophobic compounds in the aqueous phase and in that way change the partitioning. Addition of a different surfactant to the micellar phase may cause mixed micelles to form, leading to altered partitioning of hydrophobic compounds and thereby to selectivity changes.

6.2. Mixed SDS/SC micellar electrolyte solutions

The results from the optimization study of the separation of corticosteroids with an SDS-borate system /65/ suggest that an improved separation might be obtained by decreasing the hydrophobicity of the micellar phase. Wiedmer *et al.* have also investigated the separation of the same compounds with a mixed micellar system of SDS and SC with AMPSO as the buffer /33/.

Optimization of selectivity with the SDS/SC system. As with the SDS-borate system, empirical regression models and desirability functions were used in the optimization of the mixed micellar system /33/. Four variables were chosen for the optimization: pH and SDS, SC, and buffer concentrations. The experimental design was similar to the central composite design.

The selectivity was optimized by following the normalized velocity ratios of the corticosteroid pairs. Eight terms were included in the cross-validated model for normalized velocities and the parameter identified as the most important was the concentration of SDS. The concentration of AMPSO and, for the last analyte, the concentration of SC also were identified as important.

Once a good model was found, the normalized velocity ratios were calculated for all 21 possible pairs and the overall desirability function was determined. The separations of the first and third analyte pairs showed to be problematic: Even though the R² and Q² values were good for the individual velocities, for these first and third pairs the model sometimes predicted a different migration order than the experimental results. In both pairs the analytes were migrating close to each other, sometimes overlapping and sometimes even reversing migration order.

Because the differences in the values of the experimental velocities might be smaller than the prediction error of the model, the goodness profile of the respective desirability function should take into account not only the desired separation but also the prediction error of the model. The model employed did not realistically predict the separation at the optimal point suggested, but

considering the complexity of the electrolyte solution this was not very surprising. A step-wise sequential design was subsequently tested and the optimum selectivity point was found at pH 9.0 with 49 mM AMPSO, 18 mM SDS, and 55 mM SC. As compared with the separation of the compounds with the SDS-borate systems, the peak shapes were now considerably improved.

Optimization of resolution with the SDS/SC system. The next step in their investigation was to optimize the resolution of the separation. This time the optimization of the resolution was performed with the best overall conditions for selectivity as the starting point. One way to enhance the resolution in CE is to control the length of the capillary and the total analysis time independently. The analysis time can be controlled by adding to the electrolyte solution a modifier that has a pronounced effect on the electroosmotic flow but negligible effect on the selectivity. Such a modifier should be effective even at very low concentration. Compounds that have been found suitable for this purpose include metal-ammonia complexes, which are effective under basic conditions /98-99/, and divalent amines /100-102/. In the work of Wiedmer *et al.* 1,3-diaminopropane was used as EOF modifier /33/. Capillaries with total lengths of 37 cm to 77 cm (in steps of 10 cm) were tested, using various amounts of 1,3-diaminopropane.

The resolution was calculated and regression models for the separation and band broadening were validated separately for each analyte pair. Four desirability functions were used to determine the resolution optimum. This time there was no exact optimum point, but as the analysis time increased so did the overall desirability. For any specific analysis time the overall desirability decreased with increasing capillary length. Figure 3 shows the electropherograms of the separation with different capillary lengths and amounts of modifier. This approach provides a convenient way to determine either the optimal analysis time when the capillary length is known, or the optimal capillary length when the analysis time is known.

7. ANALYSIS OF CORTICOSTEROIDS IN BIOLOGICAL MATRICES

Recently interest in applying MECC to the analysis of corticosteroids in biological matrices has slowly started to grow. The major advantages, in comparison with other chromatographic techniques, are the short analysis times,

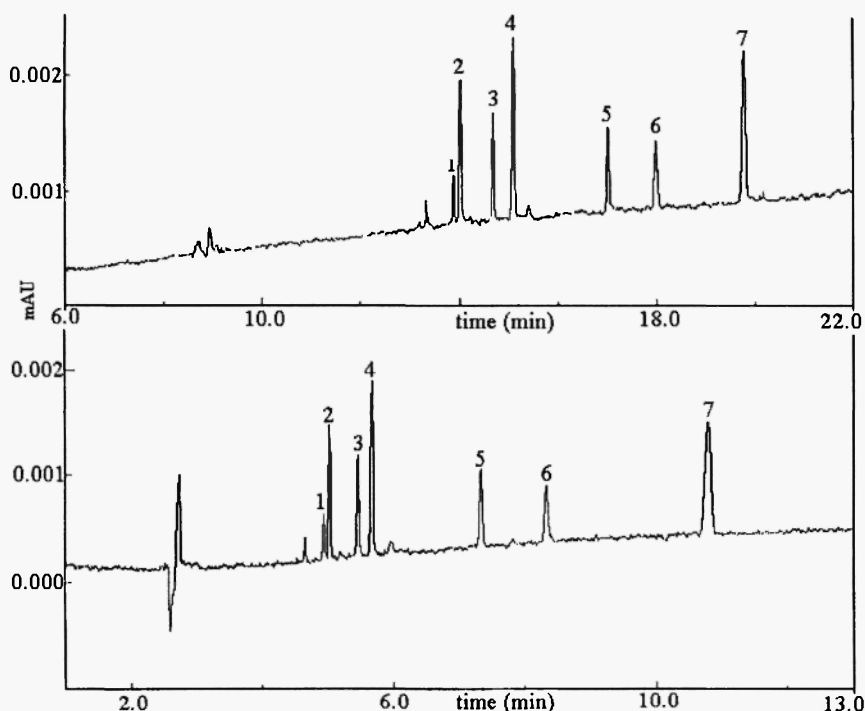


Fig. 3: Separation of corticosteroids with capillaries of different length and different amounts of 1,3-diaminopropane added to the electrolyte solution. A) 77 cm capillary, no modifier added, B) 37 cm capillary, 5 mM modifier added. Running conditions: UV at 260 nm, 20 kV, liquid cooling at 25°C. Injection at 35 mbar for 4.0 s (A) and 1.9 s (B). The migration order was 1) 1-dehydroaldosterone, 2) 17-isoaldosterone, 3) cortisone, 4) *d*-aldosterone, 5) cortisol, 6) 21-deoxycortisol, 7) corticosterone.

the small amount of sample and the relatively low cost of analysis. However, the problem with low sensitivity of conventional CE techniques still needs to be solved before the techniques can commonly be applied to routine use in industry.

Wiedmer *et al.* have investigated the applicability of a mathematically optimized SDS/SC mixed micellar electrolyte solution (49 mM AMPSO, 18 mM SDS, and 55 mM SC at pH 9.0) to the analysis of three serum corticosteroids: cortisone, cortisol, and dexamethasone [34]. The concentration of cortisol in serum serves as an indicator of illnesses such as Addison's disease and Cushing's

syndrome /8/. Dexamethasone is of interest because of its usefulness in confirming Cushing's syndrome. Before the MECC run the serum samples were enzymatically hydrolyzed, precipitated, and solid-phase extracted. Five blank serum samples were spiked with corticosteroids in concentrations between 0.1 and 0.7 $\mu\text{g/ml}$. The linearity (expressed as correlation factor R^2), the limit of quantitation (LOQ), the limit of detection (LOD), and the recoveries (calculated for two concentrations, 0.1 and 0.7 $\mu\text{g/ml}$) were determined. The concentrations of cortisol and cortisone were measured in several patient samples, but cortisone was present in low concentration and could only be measured in two of the samples. To increase the sensitivity of the method some preconcentration technique, such as solid-phase extraction or alternatively bubble or Z-shaped capillaries, could be employed. Fernandez *et al.* have used a Z-shaped capillary to increase the sensitivity of testosterone metabolites /80/. Quirion *et al.* have recently presented a very interesting and efficient on-line concentration method for neutral analytes in MECC /79/. The electroosmotic flow was reduced by employing low-pH buffer solutions, leading to a reversed direction of the negatively charged micelles. Because the mobility of the micelles was higher than the EOF, a negative voltage, i.e. from cathode to anode, was used in order to detect the compounds. Very long injection times could be used, and with an injection for 350 s. at 50 mbar progesterone, cortisol, cortisone, and testosterone with concentrations of 1 ppm gave very intense sharp peaks (5 mAU), Fig. 4. The separation of the steroids was achieved using a phosphate buffer (pH 2.5) containing SDS and γ -CD.

Usually human fluids have been pre-treated before the MECC run, mainly to get rid of proteins which may adsorb onto the capillary walls under basic conditions (note that the separation of corticosteroids has mainly been performed with alkaline buffers). However, Noe *et al.* injected directly serum samples spiked with prednisone, cortisone, cortisol and prednisolone into uncoated fused silica capillaries. /71/. In the separation they used phosphate/borate buffers containing mixtures of SDS and bile salts as micelle forming agents (pH 9). Before each run the capillary was conditioned for 30 min with 0.1 M NaOH, 10 min with water, and 10 min with the running buffer, and in such a way the authors achieved reproducible migration times.

The simultaneous separation of free and conjugated steroids by MECC has been demonstrated, using an SDS micellar solution containing 16% of acetonitrile /78/. The applicability of the technique was demonstrated by the determination of steroids in serum samples (from a patient with Cushing's syndrome and from a premature infant).

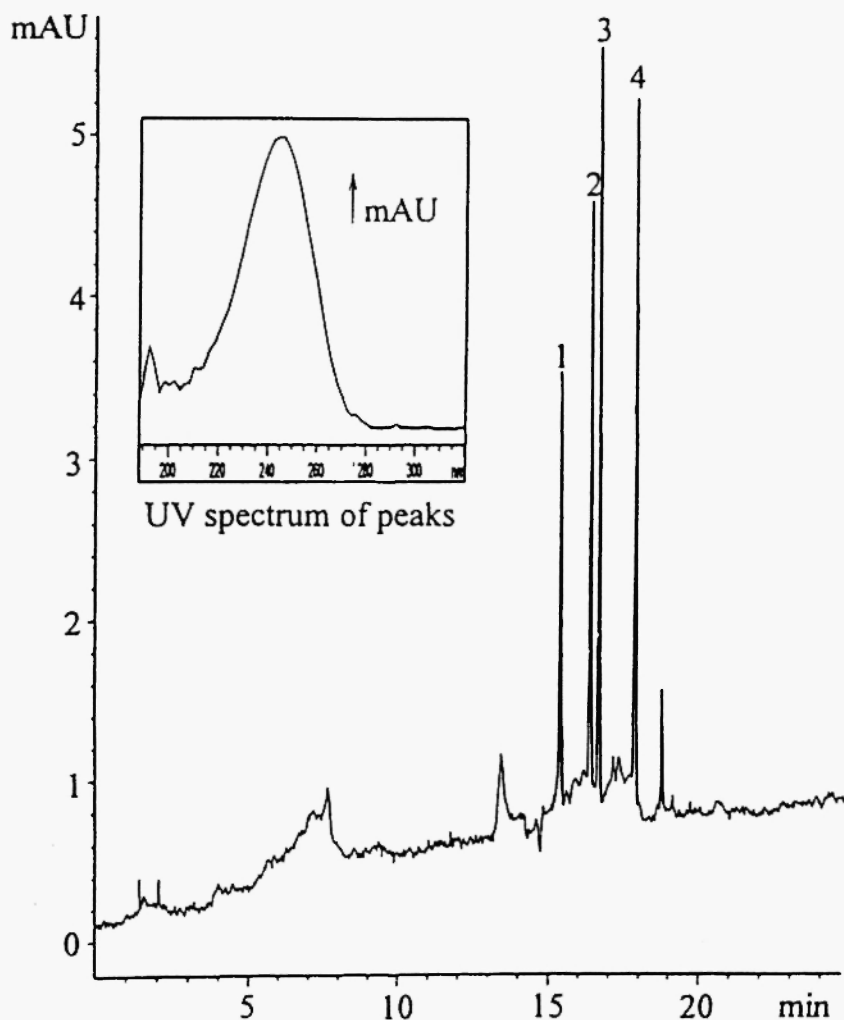


Fig. 4: Stacking with reverse migrating micelles. Separation of 1) progesterone, 2) hydrocortisone, 3) cortisone and 4) testosterone. The concentration of the compounds was $\sim 1 \mu\text{g/mL}$. Electrolyte solution: 100 mM SDS and 40 mM γ -cyclodextrin in 50 mM phosphate buffer (pH 2.5). Running conditions: -20 kV, 247 nm, sample injection for 350 s. Reprinted with permission from Ref. /79/.

MECC has also been applied to the separation of steroids in urine samples using SDS /76/ or DTAB /68/ as micellar solutions. In the latter case, the use of the cationic surfactant leads to a reversal of the EOF.

Recently solid-phase micro extraction (SPME) has been growing in popularity for pre-treatment of samples and an increasing amount of selective fiber coatings have been introduced. As long as the sensitivity in CE remains a problem it will take a long time before SPME can be considered for routine use. In addition, the long extraction times needed with several fiber materials makes it less attractive than solid-phase extractions which are already known to work well. However, in combination with on-column stacking techniques in CE the SPME may be an alternative for future analysis of human fluids. Alternatively easy-to-use on-line SPME-CE techniques should further be developed.

8. ON-LINE PARTIAL FILLING MECC-ESI-MS

On-line combination of CE with mass spectrometry (MS) is attractive because it allows simultaneous separation and identification of compounds in a single run. Since its introduction about 15 years ago, electrospray ionization has been one of the most popular ionization techniques in coupled CE-MS /10,103/. The most widely applied interfacing techniques with electrospray ionization (ESI) sources are the sheath flow technique, the liquid-liquid junction, and the sheathless technique. In the sheath flow technique the liquid from the CE capillary is mixed with the sheath liquid at the tip of the ESI needle. In the liquid-liquid junction the CE capillary and a separate transfer capillary are placed in a liquid reservoir containing the sheath liquid. The sheathless technique differs from the first two approaches in that no sheath liquid is needed. To maintain the electrical contact between the ESI needle and the CE electrolyte solution, the tip of the CE capillary is coated with a thin layer of metal. All the interfaces mentioned have their own advantages and disadvantages.

Electrospray is a technique that allows ions to be transferred from solution to the gas phase and subjected to mass spectrometric analysis. The major processes in ESMS are the production of charged droplets from compounds in solution, the shrinkage of these charged droplets by solvent evaporation and repeated droplet disintegrations, leading to very small highly charged droplets capable of producing ions in gas phase. Volatile buffers of low ionic strength are the preferred means of ensuring a stable electrospray and preserving the sensitivity of ESMS.

Because the micelles present in the electrolyte solution in MECC are non-volatile, several approaches have been developed to prevent the micelles from reaching the MS. These include the heart-cut technique /11/,

high-molecular-mass surfactants /12,13/, a semipermeable membrane interface /14/, anodically migrating micelles /15/, and the partial filling (PF) technique /16-20/. Among these the PF-MECC technique has been most commonly used. In PF-MECC the capillary, which has been filled with electrolyte solution, is only partially filled with the micellar solution. After introduction of the micelles, the sample mixture is injected and the voltage applied. The sample compounds partition into the micelles according to their partition coefficients, separate, and migrate out of the capillary. In the case of neutral analytes and negatively charged micelles, the analytes, after their migration through the micellar phase, move with the velocity of the EOF and enter the MS well before the retarded micelles. Immediately after the detection of the analytes in the MS, the CE run is interrupted, the CE and ES voltages are switched off, and the micelles are rinsed out of the capillary.

8.1. On-line partial filling MECC-ESI-MS of corticosteroids

Considering the potential applicability of MECC to the analysis of serum corticosteroids, it would be an obvious advantage if the compounds could be separated and identified simultaneously. The possibility of on-line coupling between MECC and MS for the investigation of corticosteroids has recently been published /35/. Two corticosteroid mixtures was studied, each containing three compounds. Because it is usually better that the micelles not reach the MS, the partial filling technique was applied. The compounds were ionized by electrospray ionization, using the sheath flow approach.

Several CE and MS parameters had to be optimized in order to achieve good separations as well as a stable electrospray /35/. Different SDS, SC, and SDS/SC solutions were tested as micellar phases for the PF-technique. Because all ions influence the stability of the electrospray in some degree, the ionic strength of the buffer and the micellar solutions was minimized. A solution containing 10 mM ammonium acetate, adjusted to pH 9 with ammonia, was applied as the electrolyte solution. For the first mixture tested (cortisone, cortisol, and corticosterone), the smallest amount of micelles that still gave good separation was a solution containing 10 mM SDS, 10 mM SC, and 8.5 mM ammonium acetate, pH 9. Both hydrodynamic and electrokinetic injections of the micellar plugs were investigated, and the latter was preferred because the time needed was less. For the other corticosteroid mixture (cortisone, cortisol, and 1-dehydroaldosterone) the optimal micellar solution was 15 mM SDS, 15 mM SC, and 7.7 mM ammonium acetate, pH 9.

Since the corticosteroids were detected in the positive ionization mode, an acidic sheath liquid was required. Several sheath liquids were tested and a relatively stable spray was achieved with a 0.5/50/49.5 (v/v) solution of formic acid, methanol, and water. Even though the on-line partial filling MECC-ESI-MS study of the corticosteroid mixtures was successful, the results have shown that improvements will be needed to give a wider migration time window and more stable electrospray before the technique can be applied to routine analysis.

9. PHYSICOCHEMICAL STUDIES

9.1. NMR studies of micellar systems

NMR self-diffusion studies. The pulsed field gradient spin echo (PFG SE) NMR method has become an important tool in the characterization of surfactant systems /104/, as it offers a convenient way to determine self-diffusion coefficients in microheterogeneous systems. Not only is the method fast and accurate but any component with a distinct NMR signal can be studied. Indeed, from a single experiment, which often takes less than 20 minutes, the self-diffusion coefficients can be determined for several components of a solution. One of the advantages of the technique is that no isotopic labeling of compounds is needed. Furthermore, a wide range of diffusion coefficients can be measured, that is, from small molecules in solutions with diffusion coefficients around $10^{-9} \text{ m}^2\text{s}^{-1}$ to large polymers with diffusion coefficients as low as $10^{-16} \text{ m}^2\text{s}^{-1}$.

NMR relaxation studies. NMR relaxation is a useful technique for studying surfactant aggregation and has often been applied to the investigation of micellar properties such as the size of surfactant aggregates /105/. Some surfactants contain phosphate or ammonium groups, which offer suitable nuclei for relaxation studies. However, the best nucleus for relaxation studies of surfactant systems is deuterium (^2H), which is a $I=1$ nucleus (I is the spin quantum number), whose dominating relaxation is the strong quadrupolar interaction. ^2H can synthetically be incorporated into the hydrocarbon chain of the surfactant molecule. Usually the interpretation of relaxation data is more complex than the analysis of self-diffusion data and requires the use of a special model to explain the dynamical processes that bring about relaxation. One common model is the two-step model /104/. The model makes certain assumptions: one that the system

is isotropic and another that the dynamic processes that cause the relaxation occur on two time scales. These processes are the longitudinal (R_1) and the transverse (R_2) relaxation rates. For a $I=1$ nucleus in an isotropic solution the R_1 and R_2 relaxation rates due to a quadrupolar interaction, are

$$R_1 = \frac{3\pi^2}{40} \chi^2 [2j(\omega_0) + 8j(2\omega_0)] \quad (7)$$

$$R_2 = \frac{3\pi^2}{40} \chi^2 [3j(0) + 5j(\omega_0) + 2j(2\omega_0)] \quad (8)$$

where χ is the quadrupolar coupling constant and $j(\omega_0)$ the reduced spectral density function evaluated at the Larmor frequency ω_0 .

9.2. Mixed SDS/SC micellar solutions studied by MECC and NMR

To obtain a deeper understanding of the mixed micellar system of SDS and SC as a pseudostationary phase in the analysis of corticosteroids by MECC, the micelle-micelle and micelle-buffer interactions, have been studied by CE and NMR techniques [36,37]. Because of the low concentrations of analytes relative to the surfactants and buffer in MECC separations, no analyte-micelle or analyte-buffer interactions were investigated.

The partition coefficients of corticosteroids in several different surfactant solutions have been determined by calculation of the retention factor k and the phase ratio V_{aq}/V_{mc} according to equation 4 [36]. Methanol and Sudan III were chosen as t_0 and t_{mc} markers. For the determination of the phase ratio, the volume of the micellar phase in the solution was calculated by subtracting the free surfactant volume from the total micellar volume (eq. 5). The group volumes of the surfactants, excluding the polar groups, were 351 \AA^3 for SDS and 450 \AA^3 for SC. The polar groups were excluded since these were considered to be solvated in the aqueous phase. The densities of the surfactants (SDS: 0.801 g/ml and SC: 1.154 g/ml) were calculated on the basis of the group volumes. The CMC value of the mixed micellar solution (of ratio 3.06) was 5.0 mM and this value was used in the calculation of the different surfactant solutions.

Calculation of the partition coefficients of the compounds (eq. 4) revealed marked changes in selectivity on going from the SDS to the mixed SDS/SC micellar system. In this last case, with the SDS concentration constant and the

SC concentration increasing, there was an overall decrease in the partition coefficients of the corticosteroids. Comparison of the logarithms of the partition coefficients of the corticosteroids between 1-octanol and water with the determined log P values revealed wide differences and no correlation was evident. This suggests that the P_{ow} values may not always explain the behavior of the analytes in micellar media suitable for MECC analyses.

An NMR study has been carried out to clarify the properties of the mixed micellar system /36,37/. NMR self-diffusion and relaxation studies were done on SDS (15 mM)-AMPSO (50 mM) solutions with varying amounts of SC (0-100 mM). The results showed the diffusion of AMPSO to remain fairly constant as SC was added. In contrast, the self-diffusion of SDS decreased markedly upon addition of 15 mM SC, before becoming constant with further addition of SC. The addition of cholate to the SDS solution decreased the fraction of monomeric SDS. From the diffusion coefficients of SC it was evident that the first added cholate was solubilized into the SDS micelles. The NMR parameters were further predicted by means of a theoretical model, the Bragg-Williams model /37/. The interaction parameter χ of the model describes the interaction between surfactants in mixed micellar systems: the more negative the value of χ the stronger the attractive interaction. For ideal mixing ($\chi = 0$) of surfactants in the micelle, the mixed CMC is easily calculated as a function of the overall composition of the mixture and the CMC values of the individual surfactants. In the case of the mixed SDS/SC micelles a value of -2 was chosen for the interaction parameter, which means that the behavior of the SDS/SC mixture clearly deviated from ideal mixing behavior. The value was higher (less negative) than a reported value of -3.7 for mixtures of a non-ionic and an anionic surfactant, but this was as expected since both SDS and SC are anionic surfactants. Deviations between the predicted and observed NMR values of the diffusion and relaxation coefficients are probably explained by varying micellar size and deficiencies in the predicting model. Since there were micelles present with varying molar ratios of SDS to SC, it is indeed possible that the micelles were of different sizes.

As mentioned earlier, the NMR study was made only on buffer-micelle or micelle-micelle interactions. No studies on analyte-micelle interactions were carried out. However, corticosteroid-micelle interactions have been investigated using linear solvation energy relationships (LSER) /40/. The variations in selectivity of corticosteroids using different molar ratios of sodium doexychole to sodium cholate in electrolyte solution were explained by differences in the

hydrogen bonding characteristics of the micellar systems. To achieve a deeper knowledge of the LSER modeling of analyte-micelle interactions in MECC, using various anionic, cationic or mixed micellar phases, the reader is referred to the publications in references /106,107,73/.

10. HIGH-MOLECULAR-MASS SURFACTANT FOR THE SEPARATION OF CORTICOSTEROIDS IN ECC

In addition to low-molecular-mass surfactants such as SDS and SC, surfactants with higher molecular masses have also been applied as pseudostationary phases in MECC. These are either oligomers of monomeric surfactants /108-111/ or block copolymers with surface-active properties /12,13,112-117/. In high-molecular-mass surfactants, the micelle is considered to comprise a single molecule, which is termed a molecular micelle. Since their CMCs are close to zero, molecular micelles are considered to be highly stable irrespective of the experimental conditions. The usefulness of high-molecular-mass surfactants as pseudostationary phases in MECC for on-line ESI-MS studies has been demonstrated by Ozaki and co-workers /12,13/, who also suggested that the high-molecular-mass surfactant micelles are stable in the ESI system because each micelle consists of a single covalently bonded molecule. Use of such a molecular micelle in on-line MECC-ESI-MS, it was surmised, might overcome the problem of strong background ions in the mass spectra such as encountered with low-molecular-mass surfactants. Recently Elvacite 2669, an anionic polyelectrolyte, has been investigated as pseudostationary phase for the separation of corticosteroids by electrokinetic capillary chromatography (ECC) /66/. The polyelectrolyte selected have earlier been used in the separation of other hydrophobic compounds /115-117/. In the study by Wiedmer *et al.* buffer solutions containing Elvacite 2669 (in the range 1.5-4.3 wt%), MeOH (40-80 v/v), ammonium acetate (45 mM), and ammonia (0.5-3%) were used /66/. Variations in the MeOH/water ratio had a dramatic effect on the relative migration times of the compounds. In subsequent studies on the effect of polymer concentration on the EOF it was found that when the concentration of polymer in the buffer was 0.5% or higher, there was a 6-10% decrease in t_0 (Fig. 5). A fresh capillary was rinsed for ½ hour with a buffer solution not containing the polymer, and the EOF was measured in three runs. The capillary was again rinsed for ½ hour with a buffer solution, now containing a certain amount of the polymer and the EOF was measured in three consecutive runs.

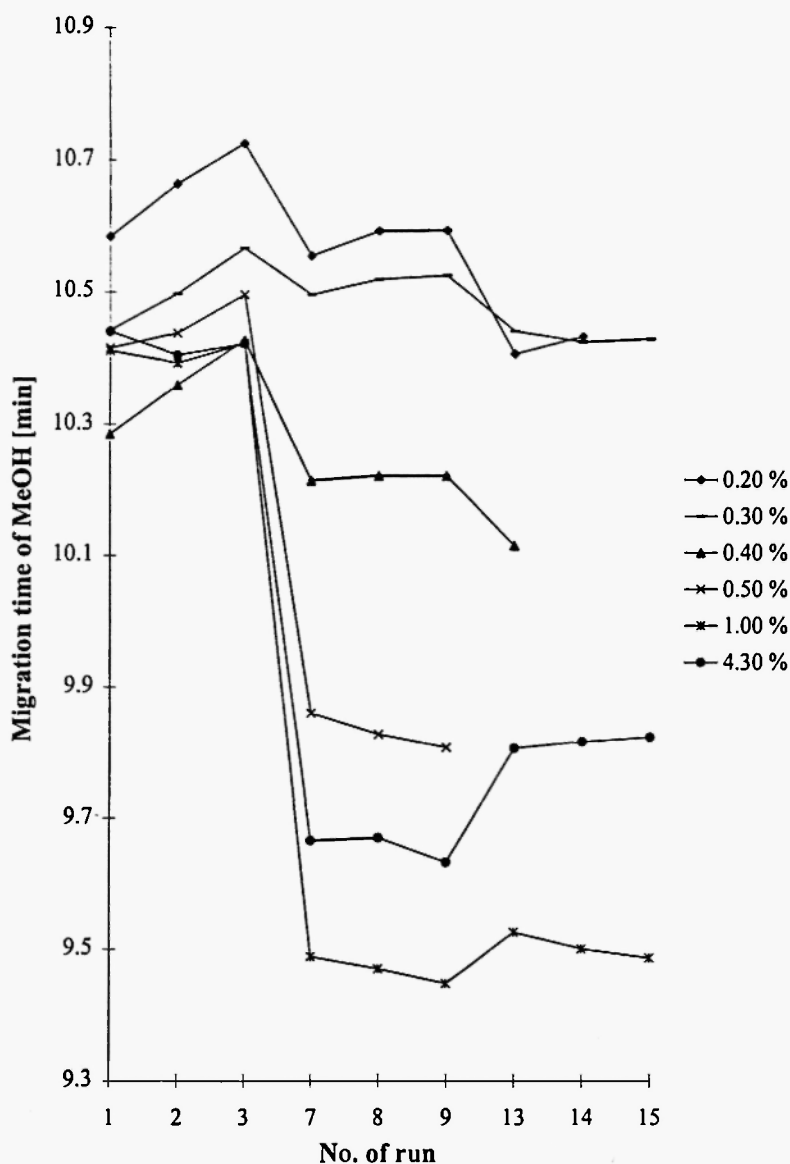


Fig. 5: EOF plotted against number of injections. The electrolyte solutions consisted of 0-4.3% Elvacite 2669, 45 mM ammonium acetate, 3% ammonia, and 50/50 (v/v) MeOH/water. Methanol was used as EOF marker. The runs were performed with the Beckman 2050 P/ACE instrument. Running conditions: capillary (50 μ m ID, 360 μ m OD) 50/57 cm (length to detector / total length), 30 kV, 25°C, 260 nm, inj. 3 s at 35 mbar. The reader is referred to Ref. /66/ for details.

The first step was then repeated. The authors suggested that the increase in the EOF was probably due to the adsorption of Elvacite 2669 on the silica wall, leading to an increase in the zeta potential.

To determine whether there is any correlation between the hydrodynamic volume of the polymer and the relative migration times, various polymer solutions have been studied by dynamic light scattering (DLS) /66/. Several observations were made: 1) increasing the pH of the solution caused the degree of dissociation of the carboxylic acids to increase and the polymer coil to expand; 2) in the buffered methanol solution the polymer tended to aggregate; 3) the size of the polymer aggregates was largest with the solvent ratio 60/40 (MeOH/water, v/v).

The original plan was to use Elvacite for on-line MECC-ESI-MS studies, which explains the choice of the buffer. However, owing to the adsorption of the polyelectrolyte onto the silica walls and the varying size of the polymer with changes in the buffer composition, the plan was not followed through. In addition, mixing of the CE liquid and the sheath liquid might have led to rather drastic changes in the properties of the polyelectrolyte.

11. ANALYSIS OF STEROIDS BY CAPILLARY ELECTROCHROMATOGRAPHY

Capillary electrochromatography (CEC) is a capillary electrophoretic separation technique using capillaries packed with HPLC stationary phases. The technique has gained a lot of attention during the last years, but due to complications with the technique, such as the formation of bubbles, great improvements are still needed before it finds its own niche among the capillary electrophoretic separation techniques. One advantage with CEC is the possibility of directly transferring already optimized HPLC analysis to a CEC system. This has recently been successfully demonstrated for a mixture of steroids by the group of Stead /118/. They made a comparison between CEC and HPLC using the same mobile phase and the results showed that the same factors that control the separation of neutral analytes in HPLC are also valid in CEC. The higher efficiency of CEC was obvious. In addition, they analysed some solid-phase extracted (SPE) human plasma samples by CEC. Another investigation of corticosteroids in biofluids has been performed by Taylor *et al.* using CEC with gradient elution /119/. The matrices were extracts of equine urine and plasma.

A two-stage SPE procedure was used to purify the samples and prevent contamination of the column. Seifar *et al.* have demonstrated the feasibility of capillary electrochromatography using capillaries packed with 1.5 μm ODS-modified non-porous silica spheres /120/ or with 1.8 μm ODS-modified porous Zorbax particles /121/. As test compounds they used, among other compounds, mixtures of steroids (Fig. 6).

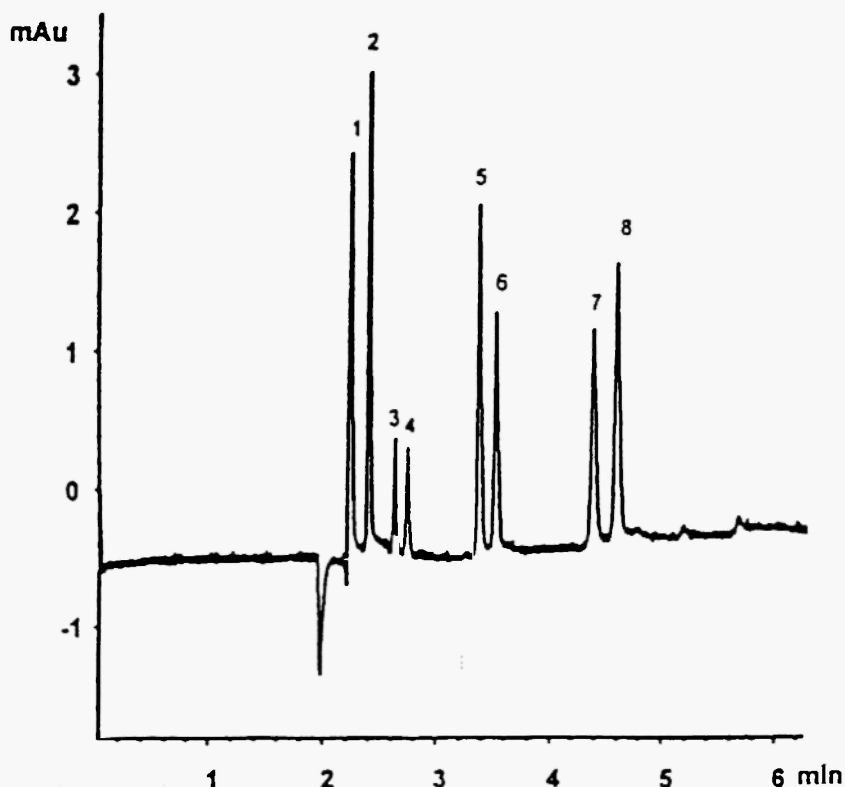


Fig. 6: CEC chromatogram of 1) estriol, 2) hydrocortisone, 3) estradiol, 4) estrone, 5) testosterone, 6) 17 α -methyltestosterone, 7) 4-pregnen-20 α -ol-3-one and 8) progesterone. Mobile phase: 0.8 mM sodium tetraborate, 80% acetonitrile, 5 mM SDS. Running voltage 25 kV, detection at 254 nm. Reprinted with permission from Ref. /121/.

12. CONCLUSIONS

During the last fifteen years the study of several groups has been focused on separations of corticosteroids and other steroids by micellar electrokinetic capillary chromatography. Among a large number of surfactants, SDS is still the most popular in the analysis of these rather hydrophobic neutral compounds. However, lately various mixed SDS micellar solutions have gained popularity, which is not very surprising considering the high solubilizing power of SDS, leading to long migration times and insufficient resolution. It seems that, in order to optimize separations, the use of systematic, mathematical optimization procedures is increasing. In the analysis of corticosteroids in biological matrices by MECC some major improvements of the sensitivity of CE are still needed before the technique can commonly be applied to routine use in industry. Several on-line sample concentration methods have recently been developed and a very efficient one, suitable for MECC, is the stacking method using reverse migrating micelles. More and more people are starting to realize the importance of understanding the mechanisms hiding behind MECC separations. Physicochemical studies on mixed micellar SDS/SC solutions have shown that the first added SC to an SDS solution is solubilized into the SDS micelles and, hence, competes with the structurally very similar steroids. This explains the better separation ability of mixed SDS/SC micelles than that of the pure component micelles. Future trends are probably the preparation of new pseudostationary phases for MECC that will work for all kind of analytes, including moderate hydrophobic analytes like corticosteroids. In addition, the development of CEC, by means of technical improvements as well as preparation of new stationary phases, will possibly make it more attractive in the future also for the separation of steroids.

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