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Separation of cardiac glycosides by micellar electrokinetic capillary electrophoresis

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ABSTRACT

The separation of mixtures of primary and secondary cardiac glycosides by micellar electrokinetic capillary electrophoresis modified by cyclodextrins, urea and sodium cholate proved to be suitable for the determination of these hydrophobic compounds. It was possible to distinguish the two anomeric cardenolides glucodigifucoside and glucodigiglucomethyloside with all three buffer systems. Electropherograms of crude plant cell extracts from *Digitalis lanata* were obtained with this method.

INTRODUCTION

Cardiac glycosides isolated from ***Digitalis*** species constitute a group of pharmaceutically very important natural substances that are used for the treatment of certain cardiac diseases. Very powerful RP-HPLC methods for the determination of cardenolides have been developed and optimized [1,2]. Nonetheless, some compounds with identical polarity co-elute in HPLC even if very sophisticated solvent gradients are applied. They can only be resolved by chemical or enzymatic derivatixation and subsequent HPLC or TLC analysis, a very time-consuming procedure that is prone to artefacts and errors. For this reason we have investigated micellar electrokinetic capillary electrophoresis (MECC) [3] for the separation and determination of cardiac glycosides.

Most cardiac glycosides are very hydrophobic compounds as judged by their partition coefficients with n-octanol [4]. This can be a handicap in MECC as they tend to migrate very close to the micellar front. Several strategies have been used with other hydrophobic compounds to overcome this problem and to shift the partition equilibrium between aqueous and micellar phase towards the former. Addition of cyclodextrins [5], urea [6], bile salts [7] and organic modifiers such as 2-propanol [8] or acetonitrile [9] to the electrophoresis buffer have proved successful.

To develop a system that is applicable to analyse for digitalis glycosides, we first investi-

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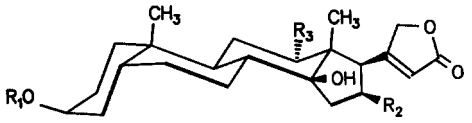

Fig. 1. Structure of the investigated cardiac glycosides. The residues are explained in Table I.

gated the migration behaviour of mixtures of primary and secondary cardiac glycosides with different electrophoresis buffers and then the resolution of a pair of cardenolides differing only in the anomeric form of their carbohydrate side-chain (Fig. 1, Table I). Finally we checked the suitability of MECC for the analysis of complex mixtures (e.g., plant extracts) containing cardenolides.

EXPERIMENTAL

Apparatus

A modular capillary electrophoresis instrument (Grom, Herrenberg, Germany) and a Chromatopac C-R6A (Shimadzu, Kyoto, Japan) for data processing were used. Polyimide-clad fused-silica capillary material of 50 μm I.D. and 360 μm O.D. was obtained from Polymicro Technology (Phoenix, AZ, USA).

Electrophoresis

Capillaries were prepared by flushing the columns for 10 min each with 1 *M* sodium hydroxide solution, water and buffer. All buffers were prepared with doubly distilled water and were degassed by evaporation in an ultrasonic bath. Sample solutions were injected by hydrostatic loading: the injection block was raised by 0.1 m for 30–60 s. Electropherograms of standard solutions were obtained by injection of 5 μl into the block. After hydrostatic loading, excess of sample solution was removed by flushing the injection block. For the injection of small amounts of sample solutions (cell extracts) we used a micro-vial with a 2-μl sample volume. Connection and disconnection of the capillary to the injection block and the micro-vial were done manually. Sample injection with the micro-vial was also done by hydrostatic loading. Between the electrophoretic runs with standard mixtures the capillary was flushed with 50 μl of buffer. Between the analyses of crude cell extracts the

TABLE I

INVESTIGATED CARDIAC GLYCOSIDES

Compound[a]	Abbreviation	R_1 [b]	R_2	R_3
Primary glycosides				
Lanatoside A	LA	Glc-β1–4-α-AcDox-β1–4-Dox-β1–4-Dox-β1–	H	H
Purpureaglycoside A	PA	Glc-β1–4-Dox-β1–4-Dox-β1–4-Dox-β1–	H	H
Lanatoside C	LC	Glc-β1–4-α-AcDox-β1–4-Dox-β1–4-Dox-β1–	H	OH
Desacetyllanatoside C	DC	Glc-β1–4-Dox-β1–4-Dox-β1–4-Dox-β1–	H	OH
Glucoevatromonoside	Gev	Glc-β1–4-Dox-β1–	H	H
Glucogitoroside	Ggr	Glc-β1–4-Dox-β1–	OH	H
Anomeric primary glycosides				
Glucodigifucoside	Gdf	Glc-β1–4-Fuc-β1–	H	H
Glucodigiglucomethyloside	Gdm	Glc-β1–4-Glm-β1–	H	H
Secondary glycosides				
Evatromonoside	Ev	Dox-β1–	H	H
Digitoxin	Dt	Dox-β1–4-Dox-β1–4-Dox-β1–	H	H
α-Methyldigitoxin	a-MDt	α-H_3C-Dox-β1–4-Dox-β1–4-Dox-β1–	H	H
Digoxin	Dg	Dox-β1–4-Dox-β1–4-Dox-β1–	H	OH
a-Acetyldigoxin	α-AcDg	α-AcDox-β1–4-Dox-β1–4-Dox-β1–	H	OH
β-Methyldiginatin	β-MDn	β-H_3C-Dox-β1–4-Dox-β1–4-Dox-β1–	OH	OH

[a] See Fig. 1 for general structure.
[b] Glc = Glucose; α-AcDox = a-acetyldigitoxose; Dox = digitoxose; Fuc = fucose; Glm = glucomethylose.

capillary was rinsed subsequently with 50 μl each of 0.1 A4 sodium hydroxide solution, water and buffer. Sample mixtures containing 80 μM of each glycoside were prepared by mixing standard solutions in water-dimethyl sulphoxide (DMSO).

Pseudo-effective mobilities, $m_{eff,S}$, according to Ackermans et *al.* [10] were determined:

$$m_{eff,S} = m_{app,S} - m_{EOF} = \frac{l_c l_d}{V t_s} - \frac{l_c l_d}{V t_{EOF}}$$

where l_c and l_d are the total length of the capillary and the length from injection to detection, respectively, V is the applied voltage and t_s and t_{EOF} are the observed migration times of the sample and an unretarded neutral molecule, respectively.

Chemicals

Sodium cholate (NaC) for biochemical use was obtained from Merck (Darmstadt, Germany) and analytical-reagent grade sodium dodecyl sulphate (SDS) and α-, β- and y-cyclodextrins (CDs) from Serva (Heidelberg, Germany). All other chemicals for buffer preparation were of research grade (Merck).

Digitoxin (Dt) , evatromonoside (Ev) and lanatoside C (LC) were obtained from Roth (Karsruhe, Germany), lanatoside A (LA) from Arzneimittelherstellung (Dresden, Germany) and a-acetyldigoxin (α-AcDg) and α-methyldigitoxin (α-MDt) from Boehringer (Mannheim, Germany). Desacetyllanatoside C (DC), glucoevatromonoside (Gev) , purpureaglycoside A (PA) and β-methyldiginatin (β-MDn) were isolated in our laboratories. The purity (>95%) of these cardiac glycosides were confirmed by HPLC. Glucogitoroside (Ggr) was a gift from Professor M. Wichtl (Marburg, Germany).

RESULTS

As electrically neutral compounds, cardiac glycosides migrated with the electroosmotic flow in capillary zone electrophoresis (CZE) with an alkaline phosphate buffer (data not shown). Interestingly, when CZE is carried out with a borate buffer, electrophoretic mobility and a slight separation can be observed. The electrophoretic mobilities of all cardiac glycosides were about (-2.75 ± 0.3) $\cdot 10^{-9}$ m^2/V$\cdot$ s in 150 mM borate buffer (pH 9.3) at an electric field strength of 18 500 V/m. This is probably due to the formation of borate complexes with the carbohydrate side-chains of the cardenolides [11]. It seems that these borate complexes are not only formed with *cis*-diol [12], as both primary and secondary glycosides without a *cis*-diol structure also show slight electrophoretic mobility (data not shown). Because of the formation of the complexes and the better solubility of the hydrophobic compounds, we decided to prepare the buffers for all the subsequent experiments with borate. Cyclodextrins have been successfully used to increase the solubility of cardiac glycosides in aqueous systems [13]. It therefore appeared reasonable that they could also be suitable for MECC analysis of these compounds.

a-Cyclodextrin had no effect on the migration behaviour of cardenolides compared with the electropherogram with SDS alone. The pseudo-effective mobilities, $m_{eff,S}$, were nearly identical [(4.275 ± 0.025). 10^{-8} m^2/V$\cdot$ s in 30 mM borate buffer (pH 9.3)–50 mM SDS-10 mM α-CD; all other conditions as in Fig. 2] for all the investigated cardiac glycosides. β-CD, however, significantly increased the resolution power (Fig. 2). It is interesting that primary glycosides of the C-series (DC, LC) did not yield peaks that could be evaluated but eluted as broad bands. Secondary glycosides of the C-series (Dg, α-AcDg, β-MDg) showed normal peaks although the separation efficiency in general was lower than for the primary glycosides (except of DC and LC).

With γ-CD a very good separation could be achieved for both primary and secondary glycosides (Fig. 3, Tables II and III). The separation efficiency was higher than with β-CD, although again lower theoretical plate numbers for the secondary glycosides were observed. Increasing concentrations of y-CD induced a reduction in the pseudo-effective mobility, $m_{eff,S}$, as they increased the partitioning of the cardenolides in the aqueous buffer phase (Fig. 4). It was not possible to find an optimum concentration of γ-CD. Ggr and Gev were better separated at low

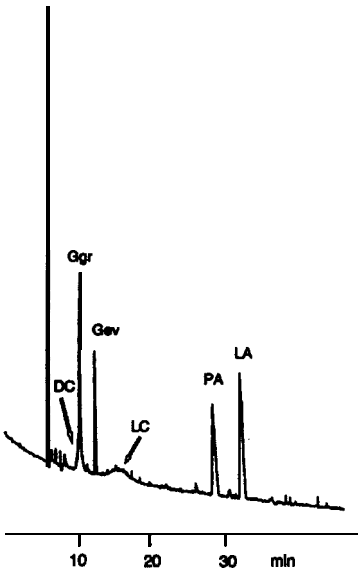

Fig. 2. Electropherogram of six primary cardiac glycosides. Buffer, 30 m*M* $Na_2B_4O_7$–50 m*M* SDS-10 m*M* β-CD (pH 9.3); capillary length, 0.8 m (0.5 m to the detector); detection at 225 nm, range 0.005 (attenuation 4); voltage, 20 kV; current, 33 μA; 30-s hydrostatic injection (0.1 m high).

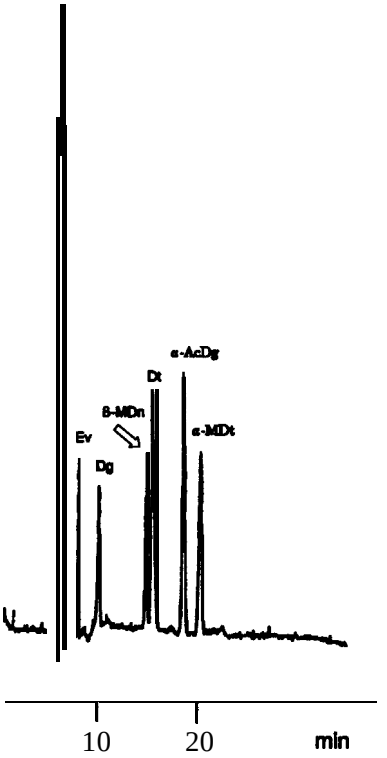

Fig. 3. Electropherogram of six secondary cardiac glycosides. Buffer, 30 m*M* $Na_2B_4O_7$–50 m*M* SDS-10 m*M* γ-CD (pH 9.3); other conditions as in Fig. 2 except attenuation 2.

γ-CD-concentrations whereas the resolution of the secondary cardiac glycosides increased with increasing CD concentration.

With 7 *M* urea-SDS-borate electrophoresis buffer, good separation of the cardenolides investigated could also be achieved (Fig. 5, Tables II and III). Because of the high viscosity of the 7 *M* urea buffer, the time required for hydrostatic injection was longer than with γ-CD-buffer. The borate and SDS concentrations were decreased and the voltage was increased in order to reduce the analysis times, which would otherwise have been unacceptably long (more than 50 min, data not shown). The separation efficiency of primary and secondary glycosides was comparable to that of the γ-CD system.

Sodium cholate (NaC)-borate buffers have been successfully employed for the separation and determination of lipophilic corticosteroids and benzothiazepine analogues by MECC [7]. Test runs with 50 m*M* NaC-30 m*M* borate and 100 m*M* NaC-30 m*M* borate buffers yielded only partial separation of the cardenolide mixtures (data not shown). With an NaC–SDS–borate buffer, however, a good separation of the cardenolides was obtained (Tables II and III). Increasing the NaC concentration resulted in a decrease in the pseudo-effective mobilities, $m_{eff,S}$, of the cardiac glycosides (Fig. 6). This effect seemed to be qualitatively similar to that of the concentration of γ-CD on the migration behaviour of the sample compounds (Fig. 4). The concentration of NaC had virtually no influence on the resolution.

Gdf and Gdgm are two cardiac glycosides that are anomeric at C4′ and they cannot be distinguished by conventional HPLC. A sample isolated from *Digitalis lanata* showed one main peak in the HPLC trace that was diminished by *ca.* 30% after treatment with glucosidase. It was difficult to explain this phenomenon using HPLC. MECC analysis with the three different buffers revealed that the sample contained two main components and that one of them was degraded completely by glucosidase (Table IV). By admixture of a Gdgm standard it could be

TABLE II

SEPARATION OF PRIMARY GLYCOSIDES

Pseudo-effective mobilities, $m_{eff,S}$ ($\times 10^8$ $m^2/V \cdot s$), and electroosmotic flow, m_{EOF} ($\times 10^8$ $m^2/V \cdot s$) (first he), elution number (second line) and number of plates (third line).

Buffer	Conditions	m_{EOF}	DC	LC	Gev	Ggr	PA	LA
30 m*M* borate-50 m*M* SDS–10 m*M* β-CD (pH 9.3)	25 000 kV/m	5.58	-2.37 1 26500	-3.62 3 –	-2.91 2 38 400	-2.37 1 –	-4.43 4 33 100	-4.56 5 38000
30 m*M* borate-50 m*M* SDS–10 m*M* y-CD (pH 9.3)	25 000 kV/m	5.76	-3.12 3 88400	-3.88 4 68 250	-2.24 2 134 650	-2.19 1 160000	-4.09 5 68600	-4.28 6 97 350
22.5 m*M* borate-37.5 m*M* SDS–7 *M* urea (pH 9.3)	32 050 kV/m	5.41	-4.09 1 119 230	-4.4 4 147 550	-4.38 3 192420	-4.13 2 85 950	-4.53 5 157 500	-4.56 6 150 900
30 m*M* borate-25 m*M* SDS–25 m*M* NaC (pH 9.3)	21430 kV/m	5.65	-3.15 1 85 500	-3.54 2 99200	-4.2 4 129 000	-3.85 3 114 000	-4.39 5 25 700	-4.44 6 35300

TABLE III

SEPARATION OF SECONDARY GLYCOSIDES

Pseudo-effective mobilities, $m_{eff,S}$ ($\times 10^8$ $m^2/V \cdot s$), and electroosmotic flow, m_{EOF} ($\times 10^8$ $m^2/V \cdot s$) (first line), elution number (second line) and number of plates (third line).

Buffer	Conditions	m_{EOF}	Dg	β-Ddn	α-AcDg	E v	Dt	a-MDt
30 m*M* borate-50 m*M* SDS–10 m*M* β-CD (pH 9.3)	28 570 kV/m	4.9	-2.64 1 5450	-3.11 3 5600	-3.45 4 9200	-3.06 2 15900	-3.5 5 13400	-3.57 6 14900
30 m*M* borate-50 m*M* SDS–10 m*M* y-CD (pH 9.3)	25 000 kV/m	5.49	-2.74 2 10000	-3.62 3 31200	-3.99 5 25800	-2.03 1 23300	-3.69 4 11000	-4.12 6 19 100
22.5 m*M* borate-37.5 m*M* SDS–7 *M* urea (pH 9.3)	32 050 kV/m	5.44	-3.78 1 3450	-4.17 2 10500	-4.44 4 6000	-5.22 3 7500	-4.46 5 14300	-4.52 6 17 800
30 m*M* borate-25 m*M* SDS–25 m*M* NaC (pH 9.3)	21430 kV/m	5.88	-2.25 1 15 800	-2.87 2 10250	-3.51 3 20 700	-3.83 4 31900	-4.15 5 9300	-4.31 6 8000

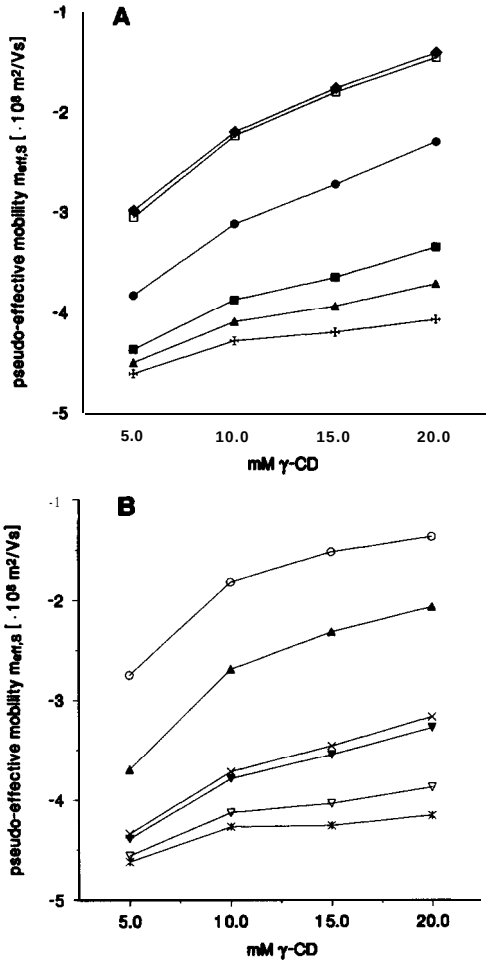

Fig. 4. Influence of γ-CD concentration on $m_{eff,S}$ of (A) primary and (B) secondary cardiac glycosides. (A) ◆ = Ggr; ❑ =Gev; ● =DC; ■ =LC; ▲ = PA; +=LA. (B) ○ = Ev; A = Dg; x = β-mDn; ▼ = Dt; V = α-AcDg; * = a-MDt. Conditions as in Tables II and III.

shown that the Gdgm is deglucosylated to digitoxigeninglucomethyloside (Dtgm) whereas Gdf is resistant to the glucosidase treatment (Fig. 7).

The capability of analysing complex mixtures such as plant-leaf extracts with the MECC systems would be of great interest. In order to decide whether a main constituent of ***Digitalis lanata*** leaf extracts was Gdf, Gdgm or a mixture of the two, 50% methanolic extracts were analysed .

With γ-CD the two cardenolides of interest migrated too close to the electroosmotic flow

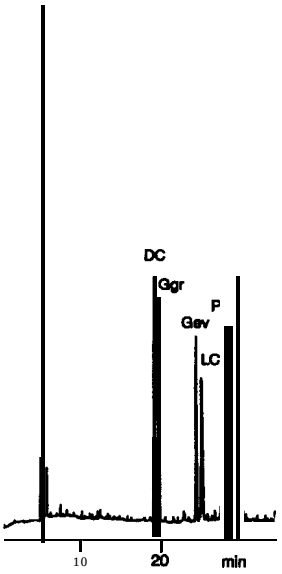

Fig. 5. Electropherogram of six primary cardiac glycosides. Buffer, 22.5 m*M* $Na_2B_4O_7$–37.5 m*M* SDS-7 *M* urea (pH 9.3); capillary length, 0.8 m (0.5 m to the detector); detection at 225 nm, range 0.005 (attenuation 3); voltage, 25 kV; current, 26 μA; 60-s hydrostatic injection (0.1 m high).

(EOF) whereas other cardiac glycosides migrate very close to the micellar front. For this reason electropherograms of crude cell extracts could hardly be evaluated. With NaC-SDS-borate and 7 *M* urea-SDS-borate buffers, cardiac glyco-

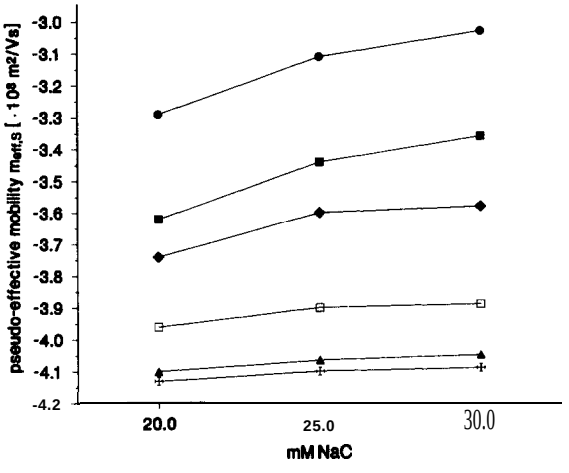

Fig. 6. Influence of NaC concentration on $m_{eff,S}$ of primary cardiac glycosides. ● =DC; ■ = LC; ◆ =Ggr; Cl= Gev; A = PA; + = LA. Conditions as in Table II.

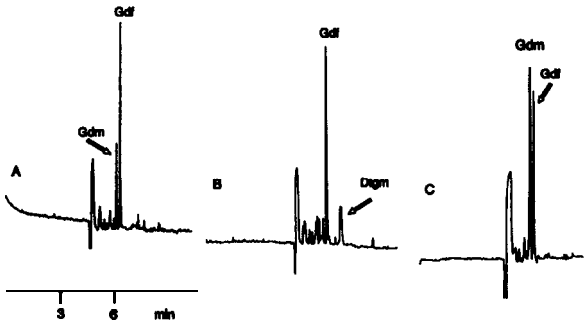

Fig. 7. Electropherograms of cardiac glycosides from *Digitalis lanata.* Buffer, 30 m*M* $Na_2B_4O_7$–50 m*M* SDS-28 m*M* γ-CD (pH 9.3); capillary length, 0.75 m (0.45 m to the detector); detection at 225 nm, range 0.082 (attenuation 4); voltage, 20 kV; current, 38 μA; 30-s hydrostatic injection (0.1 m high). (A) Before glycosidase treatment; (B) after glycosidase treatment; (C) with Gdm standard.

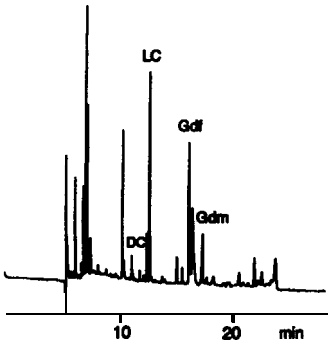

Fig. 8. Electropherogram of a crude cell extract from *Digitalis lanata.* Buffer, 30 m*M* $Na_2B_4O_7$–25 m*M* SDS-25 m*M* NaC (pH 9.3); capillary length, 0.7 m (0.4 m to the detector); voltage, 20 kV; current, 40 μA; methanolic cell extract diluted with electrophoresis buffer; other conditions as in Fig. 2.

sides are sufficiently separated from both the EOF and the micellar front (Table IV). We obtained good and reproducible electropherograms with these systems. However, the migration times differed considerably from those of the standard samples. This difficulty could be overcome by diluting the samples two-fold with the electrophoresis buffer prior to the analysis (Fig. 8). Nevertheless, it will remain necessary to add internal standards for an exact identification until more experience with this method is gained.

An alternative to the analysis of crude extracts would be to carry out a preseparation by HPLC and to separate further the fractions known to contain two or more substances by MECC. This would make identification of single compounds easier and more reliable. We injected a DC-containing fraction from an HPLC separation of a ***Digitalis lanata*** leaf extract. In order to allow on-line capillary detection in MECC, the 200-μl fraction had to be concentrated twofold by evaporation. Subsequently we were able to show that the fractions contained no Ggr. This compound is barely separated from DC by HPLC

TABLE IV

SEPARATION OF TWO ANOMEIUC GLYCOSIDES (Gdf AND Gdm)

Pseudo-effective mobiities, $m_{eff,S}$ ($\times 10^8$ $m^2/V \cdot s$), and electroosmotic flow m_{EOF} ($\times 10^8$ $m^2/V \cdot s$) (first line), elution number (second line) and number of plates (third line).

Buffer	Conditions	m_{EOF}	Gdf	Gdm	Dtgm
30 m*M* borate-50 m*M* SDS-20 m*M* γ-CD (pH 9.3)	26 666 kV/m	5.6	-1.31 2 193 800	-1.18 1 248780	-2.12 3 61600
22.5 m*M* borate-37.5 m*M* SDS-7 *M* urea (pH 9.3)	32 050 kV/m	5.4	-4.23 1 135 100	-4.27 2 120 400	-4.98 3 57 300
30 m*M* borate-25 m*M* SDS-25 m*M* NaC (pH 9.3)	28 570 kV/m	5.15	-3.5 1 23008	-3.68 2 38600	-3.81 3 83 700

but has clearly different migration times in y-CD and in NaC-modified MECC (data not shown).

DISCUSSION

CDs have been successfully applied for the separation of several hydrophobic compounds. They have been especially useful for chiral separations in capillary electrophoresis. Nishi et al. [14] suggested that with the addition of cyclodextrins to the electrophoresis buffer a new equilibrium is established between the micellar phase, the aqueous phase and the hydrophobic cavity of the CDs.

Whereas the hydrophobic cavity of α-CD appears to be too small for an interaction with the cardiac glycosides, their migration behaviour is clearly changed by β-CD and γ-CD. We could not find an explanation for the observation that two structurally related compounds (DC and LC) migrated with very broad peaks, an effect that was completely abolished with γ-CD. The separation efficiency with y-CD is higher than that with β-CD. Separation in these systems is based on a complicated dynamic equilibrium between the borate complex in the aqueous phase, inclusion in the CDs and solubilization by the SDS micelles (Fig. 9).

Urea increases the solubility of hydrophobic compounds in aqueous systems and can therefore also be used as a modifier of very lipophilic molecules in MECC. Terabe *et al.* [6] investigated the separation mechanism of urea-modified MECC and came to the conclusion that it is mainly based on the shift of the partition equilibrium between aqueous and micellar phases. They stressed that urea broadens the migration time window and is therefore very suitable for high resolution MECC.

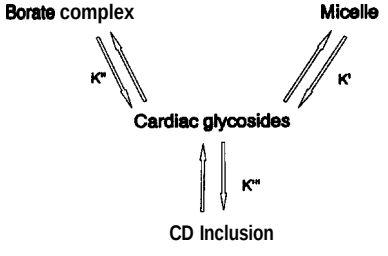

Fig. 9. Separation mechanism of CD-modified MECC.

Bile salts seem to have a smaller solubilizing effect than SDS, which makes them also suitable for analyses of very lipophilic compounds. The similar structure to the aglycones of the cardenolides could make specific solubilization effects possible. Although a good separation with NaC-borate buffers was not possible, the results with the mixed NaC-SDS-borate buffer were very good. It is possible that mixed micelles are formed, the solubilization properties of which are especially suitable for the separation of these compounds [15]. An investigation of the solubilization mechanisms of the different systems would require more comparative analyses. Additional structural analysis of the cardenolides may yield further information on their conformation in different solvents.

All buffer systems investigated show a higher separation efficiency for primary than for secondary cardiac glycosides. A number of explanations for this observation are possible. Borate, which is a constituent of all the buffer systems, can interact better with the hydroxyl groups on the terminal glucosyl residue on primary cardiac glycosides. Additionally, this group of cardenolides shows a greater polarity than secondary cardiac glycosides, which could lead to a better incorporation into the micelles.

We have shown in this paper that MECC can be applied to the separation of Gdf and Gdgm, two cardenolides of identical polarity that comigrate in HPLC. The resolution of these very similar compounds could be readily achieved without prior derivatization or hydrolysis. It is of considerable advantage to have three different MECC methods at hand for the analysis of cardenolides. As there are obvious differences in the solubilization behaviour of the buffers, a sample could be run with different buffers to ensure a reliable identification of a particular compound.

Owing to wall adsorption and perhaps to changes in the formation of micelles, crude tissue extracts have proved to be difficult to analyse by MECC. This is why so far only a few electropherograms of complex mixtures have been published. We were able to achieve a very good resolution with the NaC-SDS-borate and 7 *M* urea-SDS-borate buffers, especially when the

extracts were diluted with the electrophoresis buffer.

CONCLUSIONS

MECC using the three different buffer systems established allows a reliable separation and identification of cardiac glycosides even if they are inseparable by HPLC. It can be used for the analysis of crude cell extracts and for the investigation of HPLC fractions. The possibility of injecting very small sample volumes broadens the range of application of MECC when only limited amounts of sample are available. MECC can be recommended as a method for the routine analysis of cardenolide-containing samples. It is both more economical and less threatening to the environment than HPLC, where large amounts of acetonitrile are used. We conclude that MECC has all the potential to become a valuable tool for the investigation of the biosynthesis, transport and degradation of cardiac glycosides.

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