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Separation and determination of lipophilic corticosteroids and benzothiazepin analogues by micellar electrokinetic chromatography using bile salts

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ABSTRACT

The separation of corticosteroids and benzothiazepin analogues by micellar electrokinetic chromatography (micellar EKC) was studied in comparison with capillary zone electrophoresis. The separation of these substances was not succesful under neutral and alkaline conditions because they migrated with the same velocity as that of the electroosmotic flow. Micellar EKC with sodium dodecyl sulphate (SDS) solutions was also not successful because these substances migrated with almost the same velocity as that of the SDS micelle, owing to their high lipophilicity. The use of bile salts, which have a similar skeleton to corticosteroids, as the micellar phase permitted the separation of these substances with high theoretical plate numbers (150 000–350 000) within a short time (*ca.* 15 min). Sodium cholate was particularly useful. The effects of bile salt concentration, pH and the addition of methanol were investigated. Micellar EKC was also applied to the determination of the drug substances in tablets and cream using the internal standard method and to purity testing of drug substances and tablets.

INTRODUCTION

Micellar electrokinetic chromatography (micellar EKC) is a type of liquid chromatography based on micellar solubilization and the instrumental technique of capillary zone electrophoresis (CZE)[1,2]. CZE has been shown to be a very powerful separation technique for ionic and biological substances[3,4]. Although CZE separation can be applied only to ionic species, owing to the separation mechanism involved, micellar EKC permits the separation of neutral substances[5–7] because its separation principle is the same as that of chromatography.

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The greatest advantage of this technique is its capability to separate neutral substances. In addition, selectivity and peak shapes can be much improved with this technique for some ionic substances[8–13] in comparison with CZE because of the presence of the micellar phase.

The determination of antibiotics in plasma by micellar EKC was reported by Nakagawa and co-workers[14,15] using a direct sample injection method. According to their method, pretreatment of the plasma sample was not necessary because the plasma proteins, which can interfere with drug analysis, were solubilized by the micelles and hence eluted later than the drugs. The use of surfactants in high-performance liquid chromatography (HPLC) was established by Armstrong and Terrill[16] as micellar chromatography, in which serum or plasma is injected directly without any pretreatment[17].

Chiral separation by micellar EKC was also successful, using chiral surfactants such as bile salts[18,19] or mixed micelles[20], in which chiral additives are solubilized into the micelle. In the former method, the chiral separation was achieved with chiral micelles alone without any metals that form chelate complexes.

Recently, Bushey and Jorgenson[21] reported the separation of dansylated methylamine and dansylated trideuteromethylamine by micellar EKC. The closely related isotopic compounds were also successfully separated under the optimum conditions by CZE[22]. These results show that ultra-high resolution can be obtained by micellar EKC or CZE.

Application of CZE and micellar EKC to the separation of water-soluble substances has been well studied recently[8–13,23–26]. However, few papers have considered the separation of lipophilic substances. These non-ionic solutes cannot be separated by CZE owing to its separation principle, or by micellar EKC using sodium dodecyl sulphate (SDS) because of the strong solubilization effect of the micelle, that is, such solutes migrate with almost the same migration time as that of the micelle. The utility of micellar EKC for the separation of hydrophobic substances has only recently begun to be explored. The use of organic modifiers in the operating buffer solution has been reported to be effective for the separation of such compounds[27,28].

This paper concerns the separation of corticosteroids and benzothiazepin analogues, which are relatively lipophilic, by micellar EKC with bile salts. Bile salts seem to have a relatively small solubilization effect compared with SDS micelles from the smaller micelle–water partition coefficients for 1-pentanol and 1-heptanol than those of SDS[29]. We would expect some characteristic solubilization effect from the structures of bile salts similar to those of corticosteroids. The effects of the pH of the buffer, organic modifier content and surfactant concentrations were studied and the separation mechanism is discussed with respect to the structures of four bile salts. The application of this technique to the determination of drug substances in commercial preparations by the internal standard method and the purity testing of drug substances and tablets are also described. The results obtained are compared with those obtained by HPLC.

EXPERIMENTAL

Apparatus and procedure for micellar EKC

Micellar EKC was performed with the same apparatus as described previous-

ly[12]. A fused-silica capillary tube (650 mm × 50 μm I.D.) (Scientific Glass Engineering, Ringwood, Victoria, Australia) was used as a separation tube. A d.c. voltage was applied between the two ends of the tube through platinum electrodes dipped into the buffer solution in the reservoirs with a Model HJLL-25PO high-voltage d.c. power supply (Matsusada Precision Devices, Kusatsu, Shiga, Japan), which delivered up to +25 kV. The electric current was monitored between the negative electrode and the negative terminal of the power supply with an ammeter throughout the operation. Migrating solute bands were detected by the on-column measurement of UV absorption (210 or 220 nm) across the axis of the tube at a position 150 mm from the negative end with a Uvidec-100-VI (Jasco, Tokyo, Japan). A Chromatopac C-R2AX (Shimadzu, Kyoto, Japan) was used for data processing. Sample solution was injected by siphoning from the positive end into the fused-silica tube filled with the buffer solution in advance. Micellar EKC was carried out at ambient temperature (*ca.* 25°C). Regarding the reproducibility of the system, the coefficients of variation of the migration times of the solutes with repeated injections (n = 5–7) were within 1% from run to run and within 3% from day to day in micellar EKC.

Reagents

Hydrocortisone, triamcinolone, betamethasone, hydrocortisone acetate, dexamethasone acetate, triamcinolone acetonide, fluocinolone acetonide and fluocinonide were obtained from Sigma (St. Louis, MO, U.S.A.). Fourteen benzothiazepin analogues (diltiazem and its derivatives and metabolites) were obtained from our laboratory (Tanabe Seiyaku, Osaka, Japan). These test samples are listed with their structures in Tables I and II. Sample 1 in Table II is diltiazem, which is a calcium antagonist with coronary vasodilatory activity[30]. All samples were used as received and dissolved in methanol at a concentration of *ca.* 0.5–1 mg/ml to give adequate peak heights in the study of the separation. Several kinds of diltiazem hydrochloride and its tablets from different companies were used as samples for the purity testing. Diltiazem tablets (Herbessor) and fluocinonide cream (Topsym) (Tanabe Seiyaku) were used for the analysis of commercial preparations.

Sodium dodecyl sulphate (SDS) from Nacalai Tesque (Kyoto, Japan) and sodium cholate (SC), sodium taurocholate (STC), sodium deoxycholate (SDC) and sodium dehydrocholate (SDHC) from Tokyo Kasei Kogyo (Tokyo, Japan) were used as anionic surfactants. Sudan III from Nacalai Tesque was used as a tracer of the micelle[2]. These were dissolved in a buffer solution prepared by mixing 0.02 *M* sodium dihydrogenphosphate solution with 0.02 *M* sodium tetraborate solution to give appropriate pH values. These solutions were filtered through a 0.45-μm membrane filter prior to use. All other chemicals and solvents were of analytical-reagent grade from Katayama Kagaku Kogyo (Osaka, Japan).

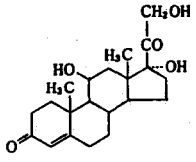
Procedure for purity testing

About 0.03 g of diltiazem hydrochloride was weighed and dissolved in 20 ml of methanol. The solution was used as the sample solution. Diltiazem tablets were ground and the resulting powder was weighed in an amount approximately equivalent to 0.03 g of diltiazem according to the label claim and placed in a test-tube, then 20 ml of methanol were added for extraction. The tube was warmed in a water-bath at *ca.* 40°C

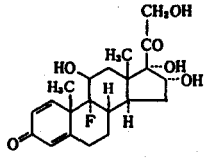
TABLE I

CORTICOSTEROIDS

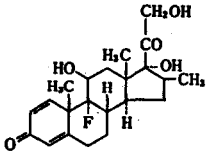
Hydrocortisone



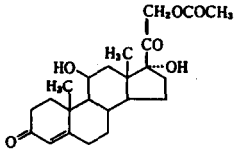
Triamcinolone



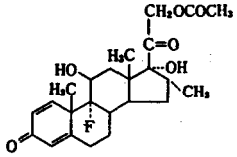
Betamethasone



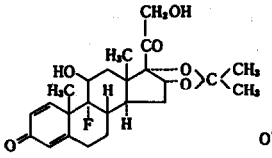
Hydrocortisone acetate



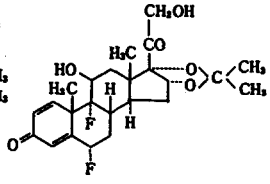
Dexamethasone acetate



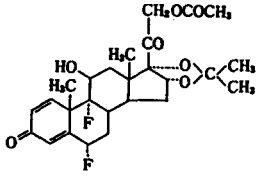
Triamcinolone acetonide



Fluocinolone acetonide



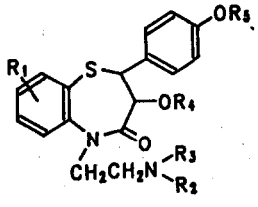
Fluocinonide



Symbol	*Name*	*Symbol*	*Name*
a	Hydrocortisone	e	Dexamethasone acetate
b	Triamcinolone	f	Triamcinolone acetonide
c	Betamethasone	g	Fluocinolone acetonide
d	Hydrocortisone acetate	h	Fluocinonide

TABLE II

DILTIAZEM AND ITS RELATED COMPOUNDS



No.	R_1	R_2	R_3	R_4	R_5
1	H	CH_3	CH_3	$COCH_3$	CH_3
2	H	CH_3	CH_3	H	CH_3
3	H	H	CH_3	H	CH_3
4	H	CH_3	CH_3	H	H
5	H	H	CH_3	H	H
6	H	(NH[a]	−)	$COCH_3$	CH_3
7	Cl	CH_3	CH_3	$COCH_3$	CH_3
8	Cl	H	CH_3	H	CH_3
9	Cl	H	H	$COCH_3$	CH_3
10	Cl	CH_3	CH_3	H	H
11	Cl	(NH[a]	−)	$COCH_3$	CH_3
12	H	CH_3	CH_3	Aromatic[b]	CH_3

[a] Des-$CH_2CH_2NR_2R_3$ form (thiazepin form).

[b] Substituted benzoyl.

for 10 min with occasional shaking, then cooled. The solution was filtered through a membrane filter (0.45 μm) and used as the sample solution. The peak areas of the substances obtained from each solution were measured with the data processor and the content of impurities was determined by the area percentage method.

Procedure for determination of drug substances in products

Ten diltiazem tablets (Herbessor, 30-mg tablets) were weighed and ground. One tenth of the powder was weighed accurately into a 50-ml volumetric flask and 30 ml of methanol were added for extraction. The flask was warmed in a water-bath at *ca.* 40°C for 10 min with occasional shaking, then cooled. Internal standard solution (exactly 10 ml), which was prepared by dissolving 0.3 g of diltiazem derivative (sample 7 in Table II) in 100 ml of acetonitrile, was added and then sufficient water was added to the flask to make 50 ml. This solution was filtered and used as the sample solution. Authentic diltiazem (*ca.* 0.03 g) was weighed accurately into a 50-ml volumetric flask and exactly 10 ml of internal standard solution were added and then sufficient water to make 50 ml. This solution was used as the standard solution.

About 1 g of fluocinonide cream was weighed accurately into a test-tube and 5 ml of methanol and 5 ml of internal standard solution, which was prepared by dissolving 0.02 g of diltiazem derivative (sample 11 in Table II) in 100 ml of methanol, were added. The tube was then warmed at *ca.* 40°C for 5 min. After cooling, this methanol solution was filtered with a membrane filter (0.45 μm) and 0.1 ml of water was added to 1.0 ml of this filtrate. This solution was filtered again with a membrane filter (0.45 μm) and used as the sample solution. Authentic fluocinonide (about 5 mg) was weighed accurately and dissolved in exact 50 ml of methanol. Exactly 5 ml of this solution were combined with exactly 5 ml of internal standard solution and 1 ml of water. This solution was used as the standard solution.

The sample solution and the standard solution were introduced into the capillary tube by siphoning (about 10 cm height, 10 s) for micellar EKC analysis. The injection volume in the system was of the order of 1 nl[12]. The ratios of the peak area of each ingredient to that of the internal standard were measured with the data processor and the content of each ingredient in a tablet or cream was calculated.

Procedure for capillary washing

To maintain good peak shapes and reproducible retention data, the capillary tube was flushed with the working buffer solution from a manually operated syringe for 1 min each time after a sample solution had been injected. The tube was washed according to the following procedure each time when the solution was replaced: the capillary was flushed with water for *ca.* 1 min, then swept with 0.1 *M* potassium hydroxide solution and allowed to stand for *ca.* 30 min, flushed with water again until the eluted solution showed neutral using a pH test paper and finally filled with the working solution and allowed to stand for at least 30 min before operation.

RESULTS AND DISCUSSION

CZE separation and micellar EKC with SDS solutions

Corticosteroids are very lipophilic and are not soluble in water, hence the octanol–water partition coefficients are infinity. Benzothiazepin analogues (diltiazem

and related compounds) are soluble in water in their salt forms, however. Their octanol–water partition coefficients change in accordance with the pH values of aqueous solutions and are also infinity under neutral and alkaline conditions because their pK_a values are around 7. Corticosteroids are electrically neutral in the range pH 7–9, and consequently they migrated with almost the same velocity as the electroosmotic velocity, which was evaluated from the migration time of methanol[2], with very broad and tailing peaks in the CZE mode using pH 7–9 buffer solutions. Diltiazem analogues also eluted with around the migration time of the electroosmotic flow with very broad and tailing peaks in the CZE mode using pH 7–9 buffer solutions.

As micellar EKC has been applied successfully to the separation of electrically neutral or non-ionic compounds[5–7], we tried to separate these corticosteroids and diltiazem-related compounds by micellar EKC with SDS solutions. The solutes migrated with almost the same migration time as that of Sudan III with 0.05–0.1 *M* SDS at pH 7–9. This result shows that these solutes are almost totally solubilized by the SDS micelle, because Sudan III is considered to be a marker of the micelle[2]. The separation of these solutes was slightly improved by decreasing the SDS concentration to 0.01–0.02 *M*, but the peaks were very broad and showed tailing as in CZE separation. These results may indicate that adsorption of these solutes on the capillary wall occurs in the absence of SDS or at low SDS concentrations.

The use of organic modifiers in the operating solutions has been reported to be effective for the separation of such compounds by reducing the capacity factors[28]. However, addition of organic modifiers results in long migration times owing to a decrease in electroosmotic flow and selectivity is not much improved.

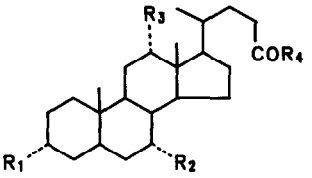
Micellar EKC with bile salts

Bile salts, which have a skeleton similar to that of corticosteroids, are biological surfactants synthesized in the liver. They form small aggregates because of the bulky structure of the monomer. The structures of the bile salts used are shown in Table III with critical micellar concentrations (CMC)[31]. Three (or two) hydroxyl groups at the 3α-, (7α-) and 11α-positions of bile salts are all oriented in the same direction, nearly perpendicular to the steroidal frame, and consequently the bile salts have both a hydrophilic and a hydrophobic face in the molecule. Therefore, the bile salt molecules tend to combine with each other at the hydrophobic face in an aqueous phase, as shown diagrammatically in Fig. 1[32]. Hence bile salts are considered to form a primary micelle with up to ten monomers.

Micellar EKC was performed with buffer solutions of pH 9.0 containing bile salts. First, a mixture of seven alkyl *p*-hydroxybenzoates (see Fig. 2) was injected to examine the power of micellar solubilization. These solutes migrated with almost identical velocities and coeluted at *ca.* 7 min in CZE at pH 9.0. However, micellar EKC using 0.05 *M* SDS or N-lauroyl-N-methyltaurate permitted a successful separation[12]. Micellar EKC using bile salts was also successful except for sodium dehydrocholate (SDHC). A typical electropherogram of seven alkyl *p*-hydroxybenzoates in CZE and chromatograms obtained by employing sodium cholate (SC) and sodium deoxycholate (SDC) are shown in Fig. 2. The elution pattern of these solutes in SDHC was almost the same as that of the electropherogram in CZE, indicating that SDHC has no effect on micellar solubilization or does not form micelles, although it has been reported that SDHC has a capability for inclusion complex formation with various organic solvents[33].

ABLE III

TRUCTURE OF BILE SALTS



Bile salt	*Abbreviation*	R_1	R_2	R_3	R_4	*CMC (mM)*	N^a
Sodium cholate	SC	OH	OH	OH	ONa	12.5	3
Sodium taurocholate	STC	OH	OH	OH	$NHCH_2CH_2SO_3Na$	4.0	3.5
Sodium deoxycholate	SDC	OH	H	OH	ONa	6.4	14
Sodium dehydrocholate	SDHC	=O	=O	=O	ONa	–	–

[a] Aggregation number.

In general, the migration time varies in a regular and predictable fashion with repeated substitution of a group in a sample molecule. Often the capacity factor will depend linearly on the number of repeating groups. This relationship is well known as Martin's rule in chromatography. In micellar EKC, the capacity factor is given by[1]

$$\tilde{k}' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})} \tag{1}$$

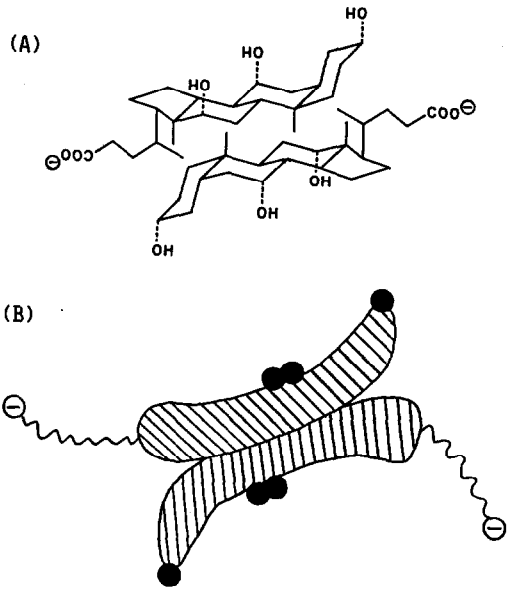


Fig. 1. (A) A possible structure and (B) its diagrammatic representation of a two-molecular aggregate of sodium cholate in aqueous solution. The hydroxy groups are representated by the filled circles and the carboxylate groups by the open circles.

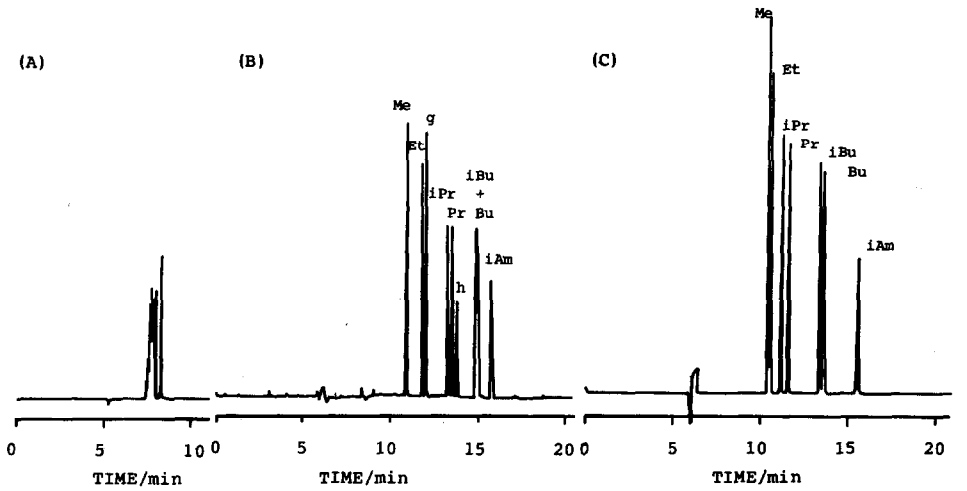


Fig. 2. (A) Separation of seven alkyl *p*-hydroxybenzoates by CZE with a 0.02 *M* phosphate–borate buffer solution (pH 9.0), and separation of those with fluocinolone acetonide (g) and fluocinonide (h) by micellar EKC with (B) 0.1 *M* sodium cholate (pH 9.0) and with (C) 0.05 *M* sodium deoxycholate (pH 9.0). Me, methyl; Et, ethyl; iPr, isopropyl; Pr, *n*-propyl; iBu, isobutyl; Bu, *n*-butyl; iAm, isoamyl *p*-hydroxybenzoate. Applied voltage, 20 kV.

where t_0, t_R and t_{mc} are the migration times of an unincorporated solute, the solute and the micelle, respectively.

The $\tilde{k}'$ values of seven alkyl *p*-hydroxybenzoates were calculated according to this equation from the data shown in Fig. 2, where these esters and samples g and h are separated by micellar EKC with 0.1 *M* SC solution of pH 9.0. The plots of log $\tilde{k}'$ versus *n* (carbon number of the alcohol part in alkyl *p*-hydroxybenzoates) are approximately

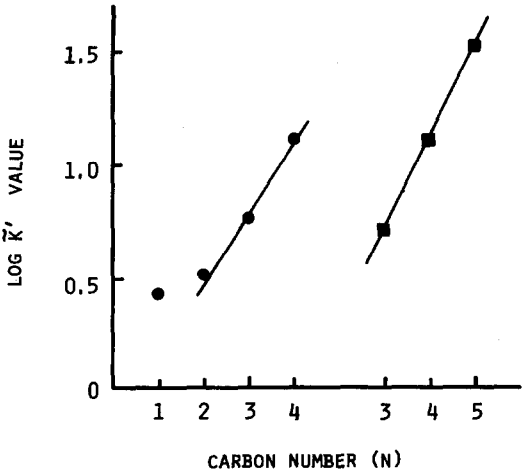


Fig. 3. Dependence of log $\tilde{k}'$ on the carbon number (*n*) of the alcohol part of alkyl *p*-hydroxybenzoates in micellar EKC with sodium cholate. Conditions as given in Fig. 2B. ●, Me, Et, Pr and Bu; ■, iPr, iBu and iAm.

linear, as shown in Fig. 3, except for $n = 1$. This indicates that partition or interaction between the solutes and the micelle in micellar EKC employing bile salts follows a retention mechanism similar to that in reversed-phase HPLC.

From the results, corticosteroids and diltiazem-related compounds were analysed by micellar EKC with SC, sodium taurocholate (STC) and SDC solutions. Typical chromatograms are shown in Fig. 4. The solutes were successfully separated within *ca.* 15 min by use of bile salts in comparison with CZE or micellar EKC with SDS solutions. The theoretical plates numbers calculated from the equation[34]

$$N = 2\pi(t_R h/A)^2 \quad (2)$$

where t_R, h and A are migration time, peak height and peak area, respectively, were 200 000–350 000.

The relative migration order observed in Fig. 4 almost follows the lipophilicity of the solutes. Among corticosteroids, the solutes which have the foundamental steroid structure, that is, having no ester structure at the hydroxy group on the C-21 position and having no acetonide structure at the hydroxy groups on the C-16 and C-17 positions (samples a, b and c in Table I) eluted first, followed by the acetate type (d and e), the acetonide type (f and g) and finally fluocinonide, which has both acetate and acetonide structures. The lipophilicity of corticosteroids also increases in the order mentioned above. For diltiazem-related compounds, elution of the solutes follows in the order diltiazem analogues (samples 1–6), their chlorinated derivatives (7–11) and

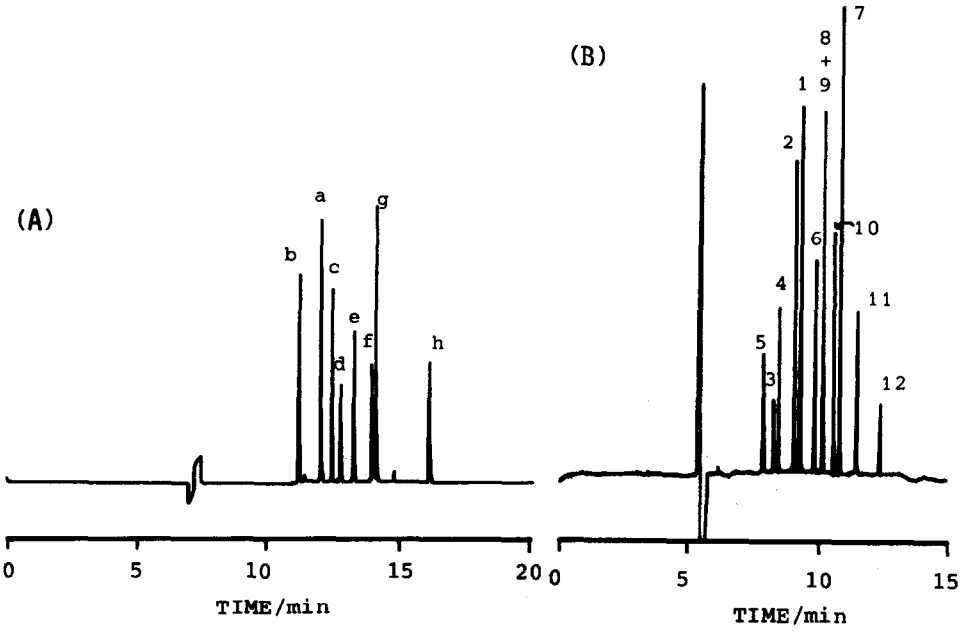


Fig. 4. Typical chromatograms of (A) eight corticosteroids and (B) twelve benzothiazepin analogues. Conditions: buffer, 0.02 *M* phosphate–borate (pH 9.0) containing (A) 0.1 *M* sodium cholate and (B) 0.05 *M* sodium taurocholate; applied voltage, 20 kV; temperature, ambient; detection wavelength, 210 nm. Solutes are given in Tables I and II.

substituted benzoyl compound (12). Samples 2–5 are metabolites of diltiazem (1) and samples 8–10 are metabolites of its chlorinated compound (7). These metabolites migrated faster than their original compounds because of an increase of hydrophilicity.

Micellar EKC with 0.05 *M* SDC solution (pH 9.0) was also performed and typical chromatograms are shown in Fig. 5. The migration pattern of corticosteroids is almost the same as that in 0.1 *M* SC shown in Fig. 4, except for sample f. For sample b, the migration time was shorter than for other samples over the all bile salts and the elution order was altered by the bile salt concentrations, as mentioned later. The separation of twelve benzothiazepin analogues by use of SDC was not successful. Six diltiazem analogues and five chlorinated analogues were eluted as groups of incompletely resolved peaks even at pH 9.0 (Fig. 5B). From these results, we selected SC or STC as the surfactant for micellar EKC in the following experiments.

Among four bile salts, SDHC had no effect on solubilization of the solutes. SDC, in contrast, had the greatest solubilization effect, although the selectivity decreased in comparison with SC and STC. No distinct difference in the migration of the solutes was observed between SC and STC, between which only the ionic groups are different. The effect of ionic interaction observed in the separation of ionic solutes by micellar EKC[12,13,35] did not contribute to the migration of the solutes, because the solutes are electrically neutral. The structural difference in the steroidal part between SDC and SC or STC is only in one hydroxyl group (see Table III); the hydroxyl group at the C-7 position in SC or STC is replaced with a hydrogen atom in SDC. The solubilization effect of SDC seems to be much increased by this substitution from the data on the effect of surfactant concentration on the migration times of the solutes (see below). The reported CMC of SDC is smaller than that of SC[31]. This supports the observation of stronger solubilization of SDC.

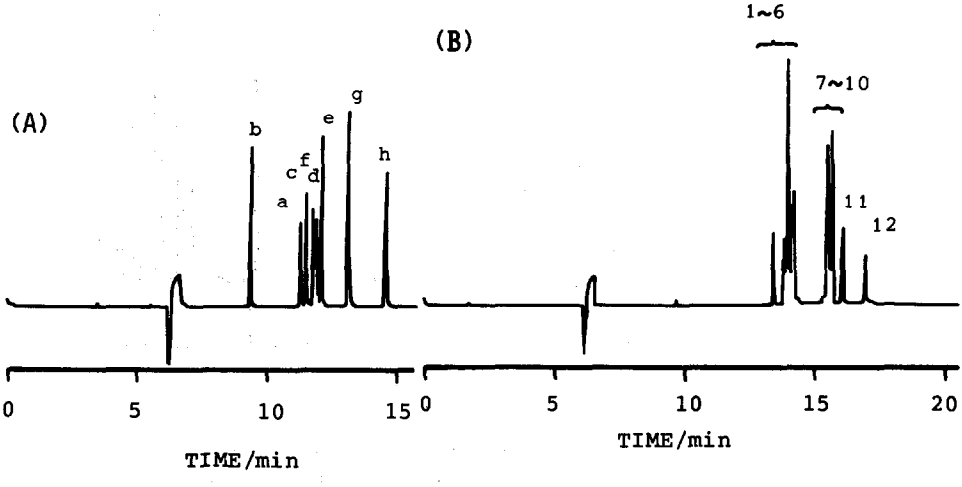


Fig. 5. Typical chromatograms of (A) eight corticosteroids and (B) twelve benzothiazepin analogues by micellar EKC with sodium deoxycholate (0.05 *M*). Other conditions as in Fig. 4.

Effect of bile salt concentration and pH on the separation

The effect of the bile salt concentration on the migration times of the solutes was investigated with SC and STC solutions (pH 9.0). The results are shown in Fig. 6 for corticosteroids and Fig. 7 for benzothiazepin analogues. The migration times increased with increase in bile salt concentration, although the electroosmotic flow did not change significantly over the whole concentration range. The migration time of Sudan III was almost the same as for sample 12 in Fig. 7. This can be ascribed to a larger micelle phase ratio at high than at low SC concentrations. However, the increase in migration times was very slight when the SC concentration was increased from 0.1 to 0.15 *M* in comparison with the increase from 0.05 to 0.1 *M*, as shown in Figs. 6 and 7.

In micellar EKC, the total range of migration of electrically neutral solutes is limited between the migration time of water (t_0), which is measured with methanol, and that of the micelle (t_{mc}), which is measured with Sudan III. The value of t_{mc}/t_0 can be regarded as a parameter indicating the total width of the migration time window. The value of t_{mc}/t_0 calculated from Fig. 2, in which Sudan III migrated just after isoamyl *p*-hydroxybenzoate, is 2.7. The value of t_{mc}/t_0 observed in micellar EKC with SDS solutions usually lies between 4 and 5[7]. The slight change in migration times between 0.1 and 0.15 *M* SC and the narrow migration time window at 0.15 *M* SC can be ascribed to the small t_{mc}/t_0 value in micellar EKC with bile salts. It is interesting that a typical solute concentration would be 3 m*M* (calculated for hydrocortisone), and the greatest change in migration behaviour in Figs. 6 and 7 occurred as the concentration of micelles changed from about 5 to about 10 m*M*, assuming an aggregation number of 10. Optimum separation was obtained at *ca.* 0.1 *M* SC.

The use of STC instead of SC did not bring about any significant change in selectivity in comparison with the results shown in Figs. 6 and 7. When concentrations of STC higher than 0.1 *M* were employed, the baselines became noisy. The purity of the STC used might be responsible.

The pH dependence of the migration time was examined with 0.1 *M* SC solutions

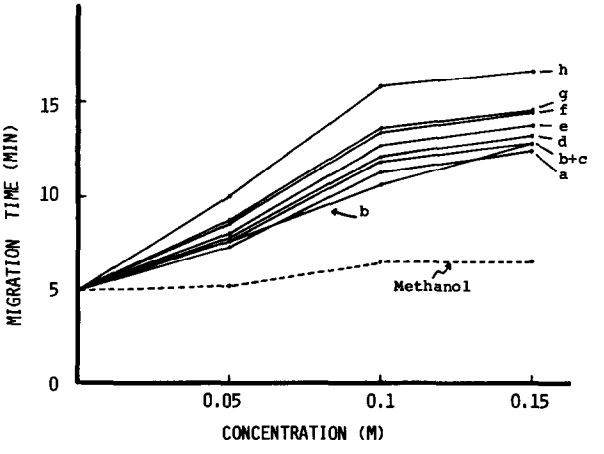


Fig. 6. Effect of bile salt concentration on the migration time of corticosteroids. Buffer, 0.02 *M* phosphate–borate (pH 9.0) containing sodium cholate. Other conditions as in Fig. 4.

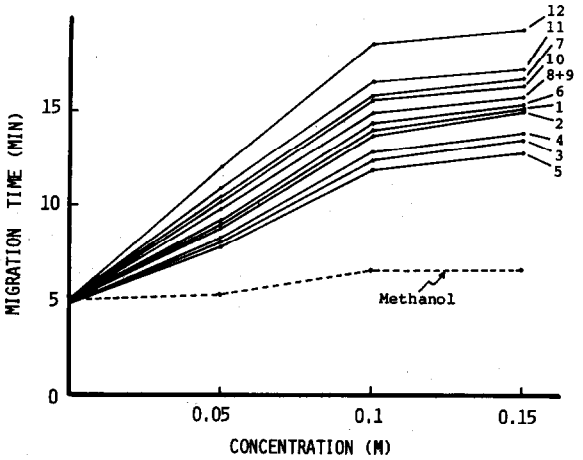


Fig. 7. Effect of bile salt concentration on the migration time of benzothiazepin analogues. Conditions as in Fig. 6.

in the pH range 7–9 and the results are shown in Fig. 8. The migration behaviour of corticosteroids did not alter over the whole pH range, because they are electrically neutral. However, the selctivity between diltiazem (sample 1) and its metabolites or chlorinated diltiazem (sample 7) and its metabolites deteriorated at low pH. It was useful to use high pH solutions to improve the separation of benzothiazepin analogues. The ionic effect due to an ionizable phenol will probably contribute to migration at high pH (9.0), because metabolic solutes have phenolic hydroxy groups. That is, electrostatic repulsion between the solutes and the anionic micelle will decrease the incorporation of these solutes by the micelle. Unincorporated anionic solutes,

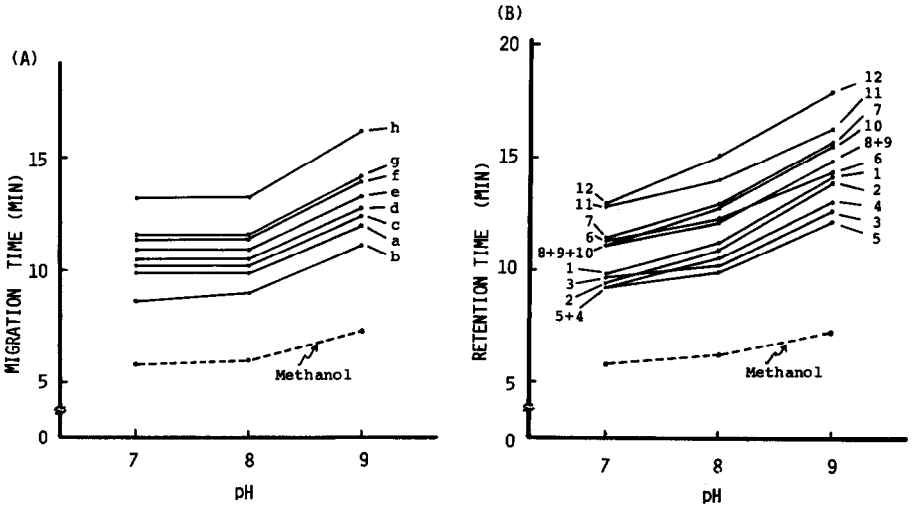


Fig. 8. pH dependence of the migration time of (A) cortocosteroids and (B) benzothiazepin analogues. Buffer, 0.02 *M* phosphate–borate containing 0.1 *M* sodium cholate. Applied voltage, 20 kV.

however, will migrate in the opposite direction to the electroosmotic flow by electrophoresis of solutes themselves. Therefore, it is difficult to predict how the migration time of ionizable solutes will be altered by changes in pH.

Effect of organic modifiers

Optimization of resolution has been also attained through adding an aqueous organic modifier[27,28,36] in addition to the parameters mentioned above. The effect of an organic solvent on the separation of the solutes was investigated by use of methanol at concentrations up to 20%. The result for corticosteriods is shown in Fig. 9. The migration times of the solutes increased with increase in the methanol concentration. This is ascribed to the decrease in the electroosmotic flow. The addition of 20% of methanol decreased the electroosmotic flow by about half (from 1.24 to 0.64 mm/s). The migration order and selectivity were not influenced except for sample b, probably because these solutes have nearly the same structure and they are electrically neutral. For the benzothiazepin analogues the same tendency (increase in migration time) was observed with increase in the methanol concentration without a change in elution order. However, the selectivety was much improved in the separation of a mixture of fourteen cold medicines, in which ionic compounds were mixed with neutral compounds[37].

Qualitative analysis of diltiazem hydrochloride and its tablets

On the basis of the results mentioned above, a buffer solution of pH 8.0 containing 0.1 *M* SC was selected for qualitative and quantitative analysis of diltiazem hydrochloride and its tablets from the viewpoint of successful separation and short analysis times. A chromatogram of diltiazem containing 0.3–0.5% of its related substances (samples 2–5) is shown in Fig. 10B, indicating a satisfactory separation. A typical chromatogram obtained from diltiazem hydrochloride (supplied by FE)

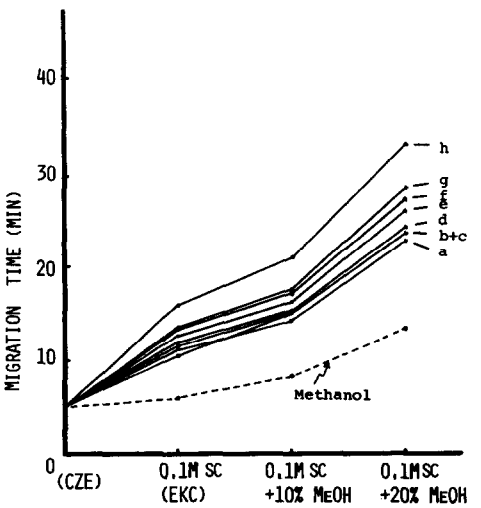


Fig. 9. Effect of organic modifier (methanol) in micellar EKC of corticosteroids in comparison with 0.02 *M* phosphate–borate buffer solution without bile salts (CZE) and with 0.1 *M* sodium cholate (EKC).

according to the procedure described under Experimental is shown in Fig. 10A. About 0.2% of an impurity (sample 2) was detected. The detection limit was *ca.* 0.1% at a signal-to-noise ratio of 3. Other results with samples from commercial sources and our laboratory are summarized in Table IV. These values agreed well with the values obtained by HPLC. Qualitative analysis was thus successfully performed by micellar EKC with the same precision as in an HPLC analysis.

Quantitative analysis of diltiazem tablets and fluocinonide cream

Diltiazem tablets were analysed by the internal standard (I.S.) method using sample 7 as an I.S.. The conditions are the same as in Fig. 10. The reproducibilities (coefficients of variation, C.V.) of the migration times and peak area ratios obtained with the standard solution by repeated injections ($n = 5$) were 0.7% and 2.2% respectively, were comparable to those obtained in previous studies[37–39]. The calibration graph for diltiazem obtained by the peak-area ratio method gave excellent linearity over the range 40–120% of the assay concentration with a correlation coefficient $r = 0.998$ and passed through the origin. The recovery was almost 100%.

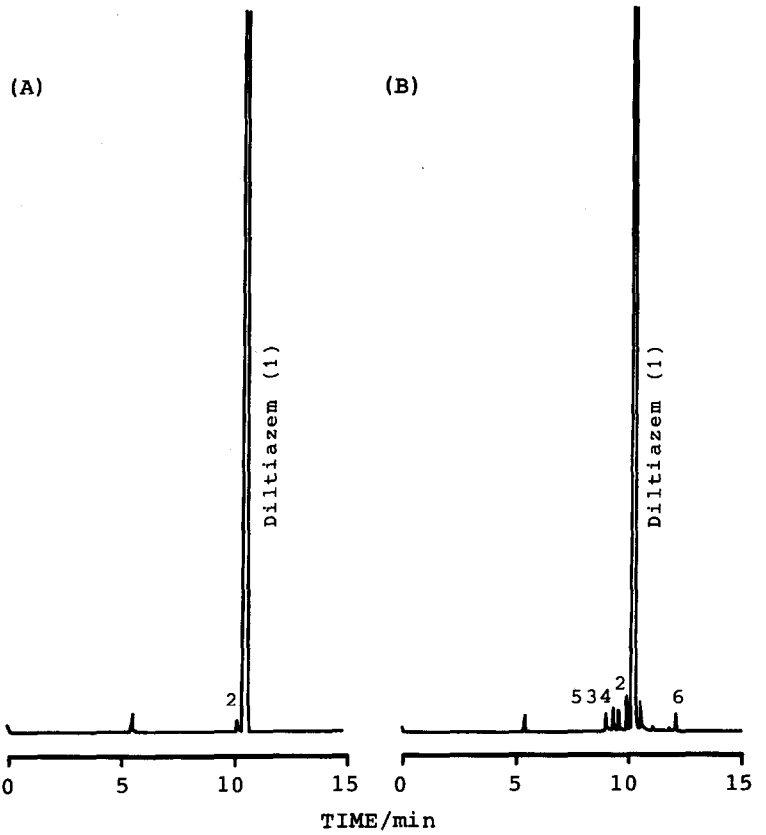


Fig. 10. Typical chromatograms obtained in the purity testing of diltiazem. (A) Diltiazem hydrochloride supplied by FE and (B) authentic diltiazem with 0.3–0.5% of related substances added. Buffer, 0.02 *M* phosphate–borate (pH 8.0) containing 0.1 *M* sodium cholate. Other conditions as in Fig. 4.

TABLE IV

RESULTS OF PURITY TESTING OF DILTIAZEM HYDROCHLORIDE AND ITS TABLETS

Source	*Sample*	*Impurity (%)*
TA	Diltiazem	ND[a]
FE	Diltiazem	0.22
FA	Diltiazem	0.27
TA	Tablets	ND[a]
TO	Tablets	1.46

[a] Not detected.

The assay results are summarized in Table V and typical chromatograms are shown in Fig. 11A. The average assay value for six replicate analyses was 101.5% and the C.V. was 1.7%.

Topsym cream containing 0.5% of fluocinonide was also analysed by the I.S. method using sample 11 as an I.S. Buffer solution of pH 9.0 containing 0.1 *M* SC was used and typical chromatograms are shown in Fig. 11B. The accuracy and reproducibility of the determination are given in Table VI. The C.V. obtained by repeated injections ($n = 6$) of the same sample solution in the peak-area ratio mode was worse than that in the peak-height ratio mode. The reason is unclear but the matrix of the formulation (non-ionic surfactant) might have affected the results. The peak-height ratio mode was used in the actual assay of cream. The capillary tube was washed with the operating buffer solutions as described in the procedure for capillary washing. Tables V and VI demonstrate the capability of this technique as a quantification method.

In conclusions, it was found that corticosteroids and benzothiazepin analogues can be successfully separated by micellar EKC using bile salts with high theoretical plate numbers within *ca.* 15 min, although the separation of these solutes could not be achieved by conventional CZE or micellar EKC with SDS solutions. The successful separation indicates that it might be useful in cases of poor resolution to explore various kinds of surfactants having different molecular structures in order to improve the selectivity in micellar EKC. The results of the quantitative and the qualitative analyses suggest that micellar EKC can be a helpful complement to HPLC in the field of separation science.

TABLE V

REPRODUCIBILITY AND ASSAY RESULTS FOR DILTIAZEM TABLETS

Peak area ratio (C.V.)	*Assay values (%)*		
2.2%	99.4	102.5	99.9
	101.3	104.3	101.6

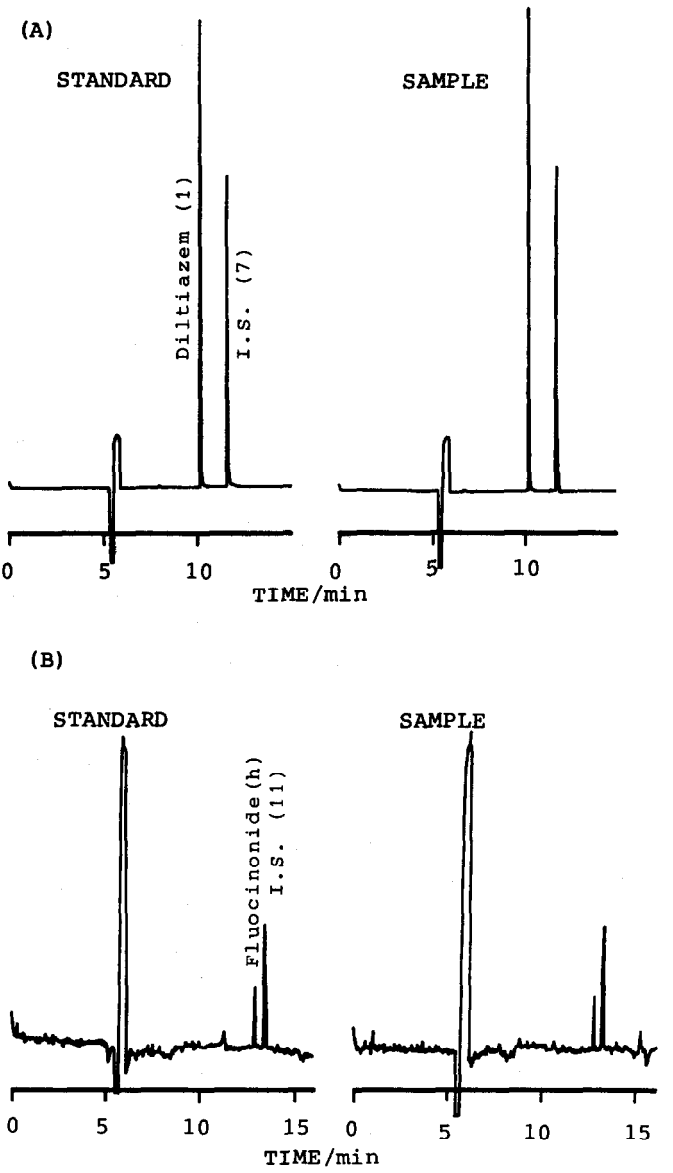


Fig. 11. Typical chromatograms obtained in the assay of (A) diltiazem tablets and (B) fluocinonide cream. Conditions: (A) as in Fig. 10; (B) as in Fig. 2B.

TABLE VI

REPRODUCIBILITY (C.V.) AND ASSAY RESULTS FOR FLUOCINONIDE CREAM

Standard solution (C.V.)		*Sample solution (C.V.)*		*Assay values (%)*		
Peak area ratio	*Peak height ratio*	*Peak area ratio*	*Peak height ratio*			
3.17%	4.59%	9.32%	2.77%	102.5	100.2	101.4
				104.4	93.0	103.6

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