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Review

Pharmaceutical applications of micelles in chromatography and electrophoresis

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Abstract

This review surveys the use of micelles as separation media in chromatography and electrophoresis. Applications to pharmaceuticals whose molecular masses are relatively small are focused on in this review. In high-performance liquid chromatography (HPLC), chromatography using micelles and reversed-phase stationary phases such as octadecylsilylized silica gel (ODS) columns is known as micellar liquid chromatography (MLC). The main application of MLC to pharmaceutical analysis is the same as in ion-pair chromatography using alkylsulfonate or tetraalkylammonium. In most cases, selectivity is much improved compared with other short alkyl chain ion-pairing agents such as pentanesulfonate or octanesulfonate. Direct plasma/serum injection can be successful in MLC. Separation of small ions is also successful by using gel filtration columns and micellar solutions. In electrophoresis, especially capillary electrophoresis (CE), micelles are used as pseudo-stationary phases in capillary zone electrophoresis (CZE). This mode is called micellar electrokinetic chromatography (MEKC). Most of the drug analysis can be performed by using the MEKC mode because of its wide applicability. Enantiomer separation, separation of amino acids and closely related peptides, separation of very complex mixtures, determination of drugs in biological samples etc. as well as separation of electrically neutral drugs can be successfully achieved by MEKC. Microemulsion electrokinetic chromatography (MEEKC), in which surfactants are also used in forming the microemulsion, is successful for the separation of electrically neutral drugs as in MEKC. This review mainly describes the typical applications of MLC and MEKC for the analysis of pharmaceuticals. © 1997 Elsevier Science B.V.

Keywords: Reviews; Pharmaceutical analysis

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1. Introduction

Micelles, which are formed by the surfactant above the critical micelle concentration (CMC), are successfully used in separation analysis, especially in liquid chromatography. In most cases, long alkyl chain surfactants such as sodium dodecyl sulfate (SDS) are added to the mobile phase in the reversed-phase mode high-performance liquid chromatography (HPLC). This technique using an aqueous micellar solution is called micellar liquid chromatography (MLC) [1–3]. The enhancement of selectivity and the adjustment of retention of ionic solutes can be easily achieved by MLC compared with the typical ion-pair liquid chromatography. Therefore MLC is applicable for the simultaneous separation of a wide variety of active ingredients in pharmaceutical formulations such as cold medicines or ointments. Other than the selectivity manipulation, through the protein solubilization capability of the micelles, direct plasma/serum injection can be performed by MLC [4,5]. The determination of drugs, whose blood concentration level is relatively high, is clinically performed by using MLC mode. Separation of small ions also can be successful by employing gel filtration columns and micelles [6]. The micellar solutions have been used in thin-layer chromatography (TLC) [7–9] to improve the selectivity as in HPLC.

In capillary electrophoresis (CE), the technique which uses a micellar solution as a running buffer solution in capillary zone electrophoresis (CZE) is very popular as micellar electrokinetic chromatography (MEKC) [10–15]. MEKC enables the separation of electrically neutral analytes by the electrophoretic technique, because the separation principle is based on that of chromatography. Nowadays various surfactants other than SDS have been employed for MEKC [15]. MEKC is powerful for the separation of complex mixtures such as natural products and Chinese crude drugs [16–21]. Direct separation of enantiomers of drugs has been successful by MEKC using chiral surfactants or chiral additives such as cyclodextrins (CDs) [22–25]. Separation of highly hydrophobic drugs and compounds has been performed by MEKC using bile salts [26–29] or adding organic solvents [30–32] or CDs [33,34]. Determination of drugs in plasma is also successfully achieved in MEKC by a direct sample injection method [35–40], similar to MLC. Microemulsion electrokinetic chromatography (MEEKC), in which microemulsions are used as pseudo-stationary phases, has been used for the separation of the electrically neutral or hydrophobic analytes. Surfactants are used as microemulsion-forming agents. On the other hand, high molecular surfactants such as methacrylate block copolymers are found to be useful as pseudo-stationary phases of EKC.

In the present paper, separation analysis of pharmaceuticals by HPLC and CE utilizing micelles is described. MLC and MEKC are especially focused on. In MLC, determination of several active ingredients in cold medicines and ointments is described.

The applications of MLC to the separation of Chinese crude drugs and narcotics are also mentioned. In MEKC, recent applications to pharmaceuticals are summarized.

2. Micellar liquid chromatography

Selectivity and peak shapes in MLC are much improved compared with the usual ion-pair chromatography using short alkyl chain ion-pairing agents. Direct injection of the plasma/serum samples is successful by MLC. Enantiomer separation can be also achieved when chiral surfactants are employed for the micellar mobile phase. However, through the recent advances in new packing materials such as restricted access materials (Pinkerton columns etc.), chiral stationary phases, ODS columns using high purity (metal free) silica gel etc., MLC mode is usually used for selectivity enhancement in the pharmaceutical analyses.

2.1. Ion-pairing mimicry

In most cases, MLC is used for the enhancement of selectivity in the separation analysis of drugs. Compared with typical ion-pairing agents such as pentanesulfonate or octanesulfonate, selectivity is much improved, especially for the separation of active ingredients in pharmaceutical preparations. For the basic drugs such as alkaloids etc., the MLC mode is effective for the decrease of peak tailing. For hydrophilic drugs, which elute almost at the solvent front in the reversed-phase mode using a typical ODS column with no organic solvents, solute retention times can be increased with the addition of surfactants to the mobile phase (i.e., MLC mode). Some examples of these applications are described below.

2.1.1. Determination of active ingredients in cold medicines and ointments

The general drugs (so-called over-the-counter; OTC drugs) such as cold medicines or ointments contain many active ingredients. To separate these active ingredients in a single HPLC run, the gradient mode must be employed because of their quite different hydrophobicities. The gradient elution, however, is not suited for routine analysis as a quality control method because of relatively poor reproducibility and time-consuming analysis. Usually, several HPLC conditions in the isocratic mode are used for the determination of these active ingredients. In most cases, ion-pair HPLC using short alkyl chain ion-pairing agents such as pentanesulfonate or tetrabutylammonium is employed because of the existence of the basic or acidic active ingredients. In the method development of isocratic modes for these active ingredients, it is important to avoid a long retention time, which makes the continuous analysis time-consuming. SDS is effective and often used for the selectivity manipulation of these basic drugs. As an example, application to an OTC cold medicine containing acetaminophen, L-ascorbic acid, phenylpropanolamine hydrochloride, tipepidine hibenzate and chlorpheniramine maleate is shown in Fig. 1 [41], where three (phenylpropanolamine, tipepidine, chlorpheniramine) active ingredients are separated by MLC. For the other two drugs (L-ascorbic acid, acetaminophen), pentanesulfonate was used as the ion-pairing agent. Another example is shown in Fig. 2. Five active ingredients in an ointment were separated within 20 min with almost the same intervals under the conditions [42]. Among the five active ingredients, three (prednisolone, crotamiton, glycyrrhetinic acid) are electrically neutral under the conditions. Therefore, in the reversed-phase mode, their retention times are determined by the concentration of an organic solvent (%) used in the mobile phase. The retention times of the other two active ingredients (dibucaine, chlorhexidine), which are both basic, can be manipulated through the change of the concentration of SDS. The effect of SDS concentration on the retention of five active ingredients is shown in Fig. 3. In the method development for this ointment, first, the content of 2-propanol was fixed at 40%. Then the SDS concentration was adjusted to elute each active ingredient with sufficient resolution. Finally an internal substance (I.S.), phenanthrene, was selected so that it elutes between two active ingredients. It was impossible to separate five active ingredients within 30 min in the other reversed-phase conditions. In this ointment, 2-propanol was selected to elute crotamiton as a single peak. There are two isomers in crotamiton. The ratio of *cis*-isomer to *trans*-isomer

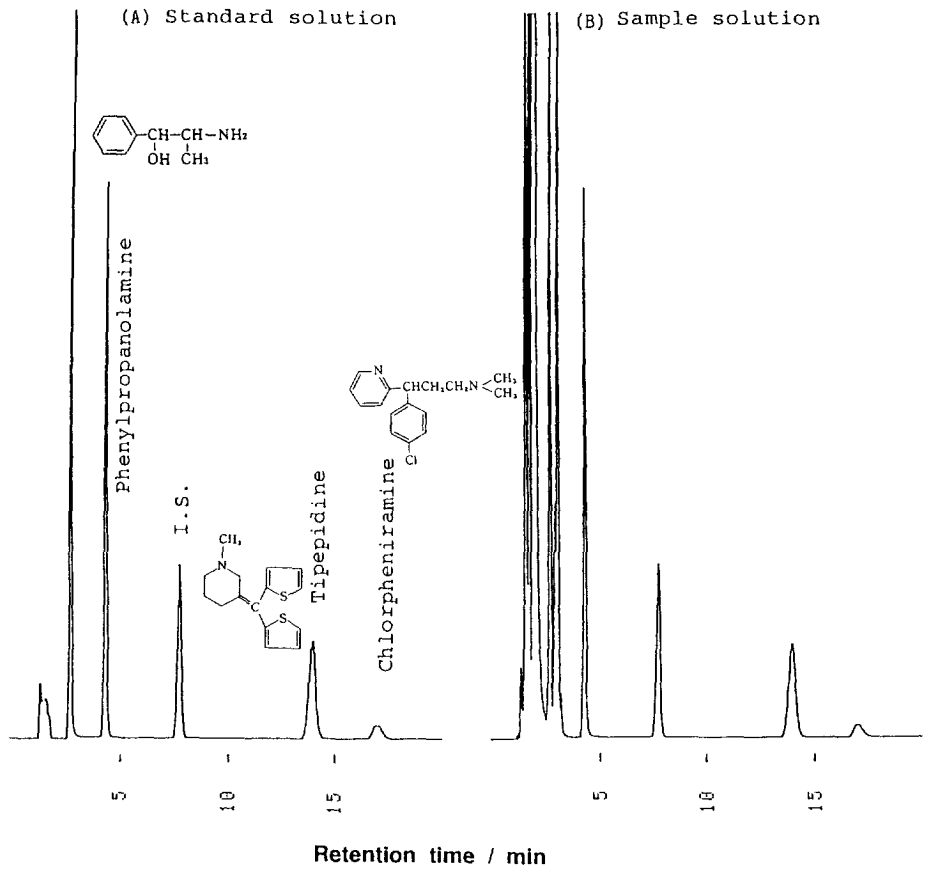

Fig. 1. MLC determination of three active ingredients in the cold medicine. (A) Standard solution, (B) sample solution. Conditions: column, Nucleosil $5C_{18}$ (15 cm×4.6 mm, 40°C); mobile phase, 0.2% SDS in 0.1% phosphoric acid–acetonitrile (55:45, v/v). I.S. = butyl parahydroxybenzoate. Flow-rate, 1.0 ml min^{-1}. Detection, 210 nm.

of crotamiton obtained from commercial sources is about 5:95 [42]. The ratio of isomers has been determined by gas chromatography (GC) [43]. However, to determine the total content of crotamiton in an ointment, it is better to elute crotamiton as one peak. 2-Propanol was selected for this purpose.

2.1.2. Separation of narcotics such as codeine and morphine

The narcotics such as opium alkaloids and Chinese crude drugs contain many active ingredients. Most of them are basic compounds and typically reversed-phase mode with SDS is employed for the separation analyses, especially those in pharmaceutical preparations. Examples for the determination of codeine phosphate and dihydrocodeine phosphate in pharmaceutical preparations (10% powder, 1% powder, tablet) by HPLC with SDS are shown in Fig. 4A [44]. Tetrahydrofuran was used for the separation of codeine and dihydrocodeine. By using the same conditions as in Fig. 4A, determination of morphine in pharmaceutical preparations (morphine hydrochloride, opium alkaloids hydrochloride; tablets, powder, injection) was also successful. A typical chromatogram is shown in Fig. 4B [45]. The MLC with SDS is also used for the determination of scopolamine and *l*-hyoscyamine in extract of belladonna root or scopolia rhizome (both are Chinese crude drugs). Belladonna extract is used for the cold medicine for nose disease and scopolia extract is used as a stomach medicine. Separation of scopolamine and *l*-hyoscyamine in pharmaceutical preparations is

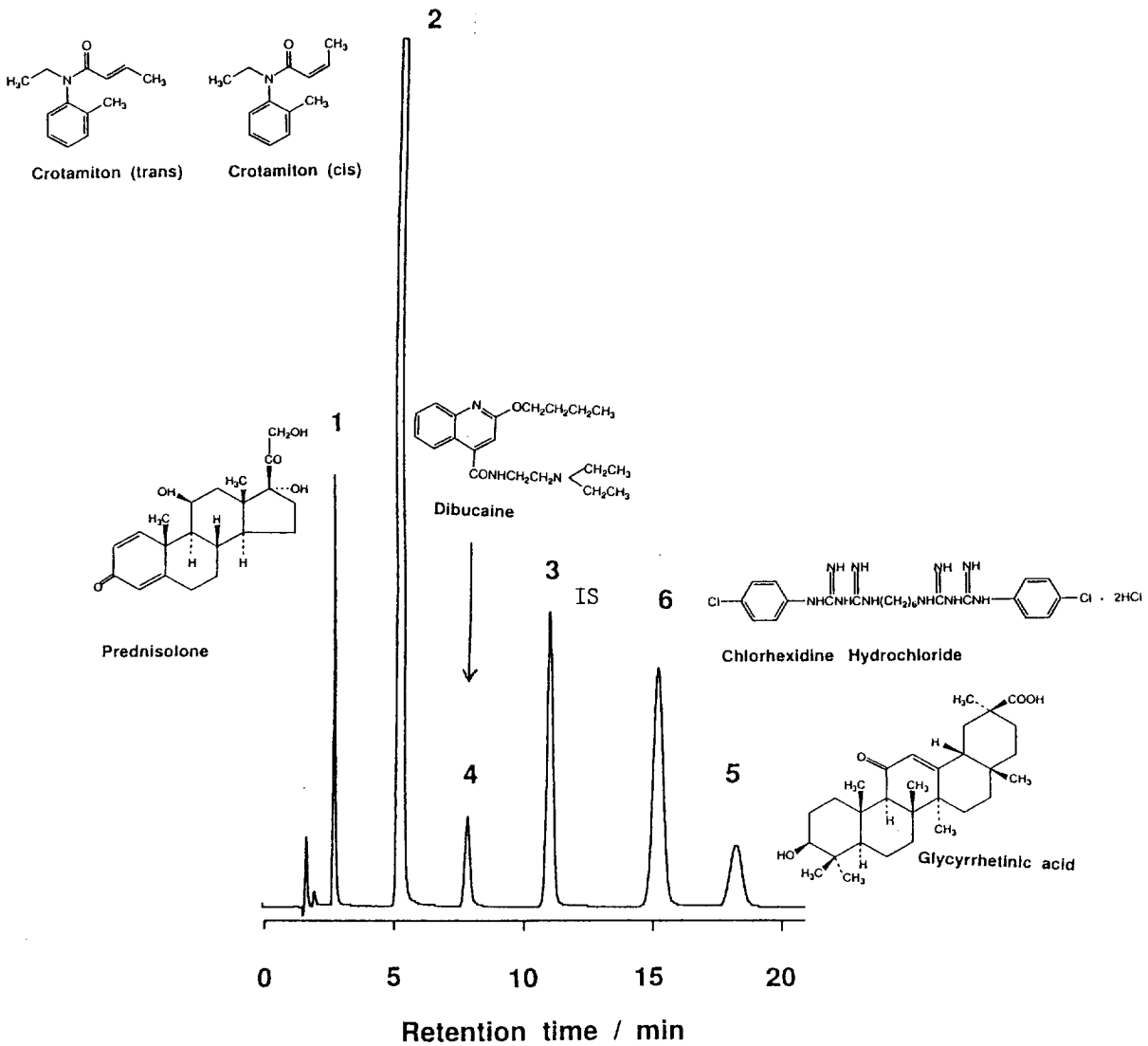

Fig. 2. MLC determination of five active ingredients in the ointment. Solutes: 1 = prednisolone, 2 = crotamiton, 3 = phenanthrene (I.S.), 4 = dibucaine, 5 = glycyrrhetinic acid, 6 = chlorhexidine. Conditions: column, Zorbax RX-C_8 (15 cm × 4.6 mm, 40°C); mobile phase, 0.05 *M* sodium dihydrogenphosphate (pH 3.0 adjusted by phosphoric acid) containing 0.3% SDS–2-propanol (60:40, v/v). I.S. = phenanthrene. Flow-rate, 0.8 ml min^{-1}. Detection, 270 nm [42].

shown in Fig. 5. The MLC mode was effective for the separation of those active ingredients and the other compounds in the pharmaceutical preparations, although these two alkaloids can be separated by the usual reversed-phase mode. Determination of alkaloids in Ephedra herb is also successful by the MLC with SDS as shown in Fig. 6 [46]. Phenylpropanolamine, pseudoephedrine, ephedrine and methylephedrine were baseline resolved within 15 min. Total alkaloid content in Ephedra herb determined by the method was found to be around 1% [46].

2.1.3. Determination of hydrophilic drugs by the reversed-phase mode

Determination of active ingredients in pharma-

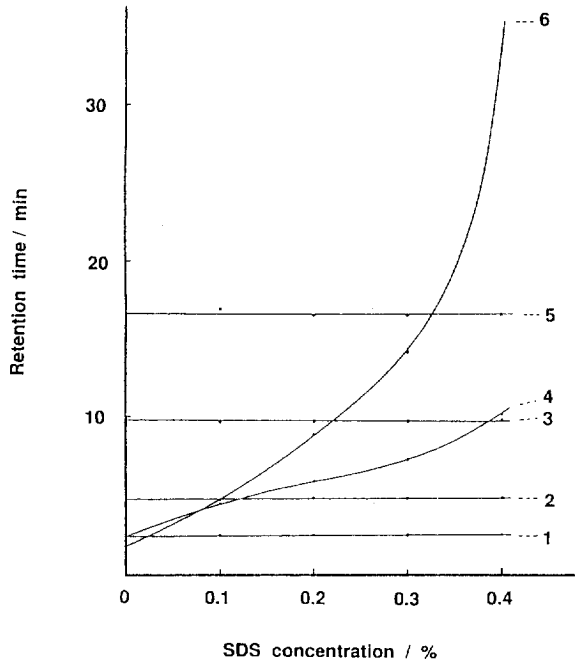

Fig. 3. Effect of SDS concentration on the retention of five active ingredients. Solute numbers are the same as in Fig. 2. Mobile phase, 0.05 *M* sodium dihydrogenphosphate (pH 3.0 adjusted by phosphoric acid) containing SDS–2-propanol (60:40, v/v). Flow-rate, 1.0 ml min^{-1}. Other conditions are the same as in Fig. 2 [42].

ceutical preparations is usually performed by using HPLC, especially in reversed-phase mode with an ODS column. However, sometimes it is difficult to separate very hydrophilic compounds in the reversed-phase mode with an ODS column. These elute with the solvent front in most cases even with no organic solvent mobile phase. In such cases, the addition of the surfactants to the mobile phase is effective. One example is the separation of carnitine hydrochloride, which is used as gastrointestinal medicines [47]. The Japanese pharmaceutical Codex 1993 (Japan) has adopted ion-pair HPLC using pentanesulfonate [48]. The concentration of an organic modifier is 0% according to the codex 1993. The crotonic betain, which is a main impurity of carnitine, elutes just before the main peak under the conditions. By employing the MLC mode, i.e., an SDS mobile phase, the resolution of the two compounds was markedly improved as shown in Fig. 7A. The organic modifier (methanol) 30% was necessary for the elution of carnitine in the MLC mode. This means that selectivity manipulation through changing the organic modifier (concentration and species) can be easily performed in the MLC mode because of the relatively high concentration of the organic modifier. One application by this mode is shown in Fig. 7B. The small content of carnitine in food supplements, in which many other amino acids, proteins, etc. are contained, was successfully determined by HPLC with an ODS column and micellar mobile phases [49].

2.2. Direct plasma/serum injection

Direct injection of biological fluids is successful in MLC mode [4,5]. Precipitation and column plugging can be eliminated by the use of micellar mobile phases. Micellar mobile phases are able to solubilize proteins that are present in the sample matrix and thus prevent their precipitation. The major advantage of using micellar mobile phases for the analysis of biological fluids is the complete elimination of all sample preparation, which results in a significant labor and time saving. When a serum sample is injected, the surfactant interacts with the serum proteins and displaces the bound drugs. The displaced drug then can freely partition into the stationary phase. The solubilized proteins generally elute near or at the solvent front, while the drug elutes later depending on the hyrophobicity. Separation of serum bilirubin [50], determination of caffeine, theophilline and theobromine in urine [51], determination of nicotine and cotinine in human urine [52], determination of theophiline in human serum [53] etc. were successfully performed by the MLC mode.

Selectivity can be manipulated mainly through the concentration of the surfactant and the organic solvent and buffer pH [54,55]. Typically SDS and polyoxyethylene(23)dodecanol (Brij 35) have been widely used as surfactants for the assay of drugs in biological fluids because of their effective serum protein solubilizing ability. As for the organic modifiers, the types and proportions are limited in the MLC. The addition of organic modifiers to the micellar mobile phases decreases the micelle formation. High concentration of organic modifiers causes the destruction of the micelles, leading to the protein precipitation. Propanol and acetonitrile up to ~10% have been successfully used. Surfactant concentra-

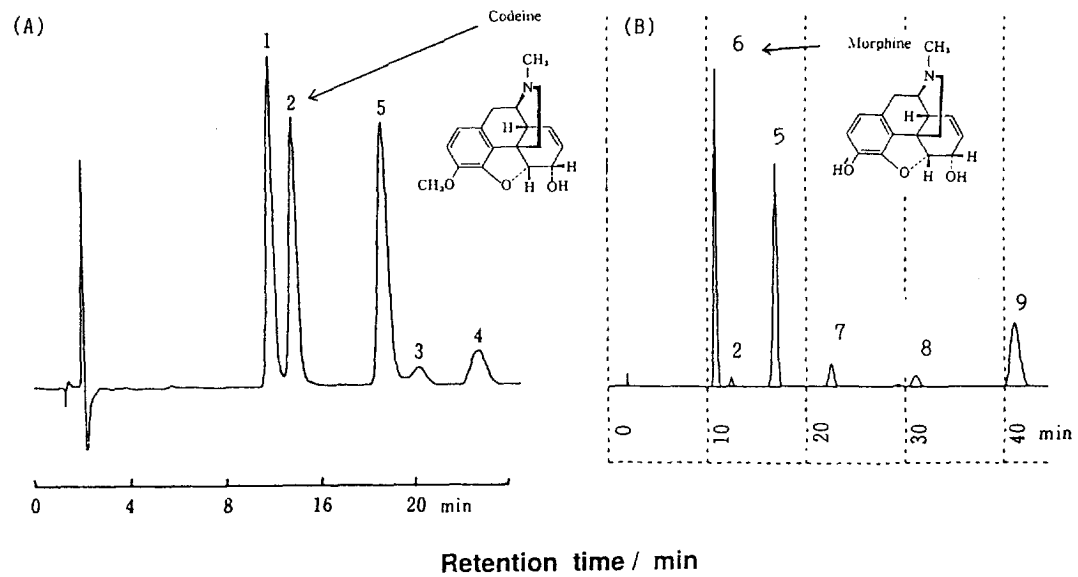

Fig. 4. (A) MLC determination of codeine, dihydrocodeine and related compounds. (B) MLC separation of opium alkaloids and atropine injection. Solutes: 1=dihydrocodeine, 2=codeine, 3=hydrocodon, 4=methylcodeine and dihydromethylcodeine, 5=etilefrine (I.S.), 6=morphine, 7=papaverine, 8=thebaine, 9=noscapin. Conditions: column, Nucleosil $5C_{18}$ (15 cm×4.6 mm, 40°C); mobile phase, 0.2% SDS in 0.1% phosphoric acid (pH 3.0 adjusted by NaOH)–tetrahydrofuran (240:70, v/v). I.S.=etilefrine. Flow-rate, 1.0 ml min^{-1}. Detection, (A) 280 nm, (B) 285 nm [44,45].

tion can have a profound effect on the retention of drugs. That is, increasing the micelle concentration decreases the retention time of the neutral solutes. For ionic drugs, buffer pH is another important factor for their retention. For most analytes, the concentration of SDS is generally 20~100 m*M* and the buffer pH is maintained between 3 and 7. The pH and SDS concentration are determined according to the hydrophobicity and pK_a of the analytes. In addition to separation, the detection is one of important problems in the MLC because sample concentration by the extraction etc. is not usually performed. For typical aromatic compounds, the detection limit ranges between 0.2~5 μg ml^{-1} using a UV detector. Recently so-called restricted access materials such as internal-surface reversed phases, shielded hydrophobic phases, and semi-permeable surfaces [56–59] have been successfully used for the direct injection of plasma/serum without extensive sample clean-up processes.

2.3. Enantiomer separation

Enantiomer separation is successful by the MLC mode through the addition of chiral surfactants. This method can be regarded as chiral mobile phase method in direct HPLC enantiomer separation. Bile salt micelles have been successfully applied to the separation of enantiomers by Hinze et al. [60,61] and Hu et al. [62–64]. All bile salts investigated showed enantiorecognition for 2,2′-dihydroxy-1,1′-dinaphthyl and 1,1′-dinaphthyl-2,2′-diyl hydrogenphosphate. An example is shown in Fig. 8, where 70% acetonitrile in 0.15 *M* sodium cholate buffer solution was used as the mobile phase [62]. In this case, the authors employed microcolumn HPLC to decrease the consumption of the buffer solution containing bile salts.

3. Micellar exclusion chromatography

Size exclusion chromatography of small molecules such as metal cations [65], inorganic anions [66], organic anions [67] has been performed by using size exclusion columns with micellar solutions. The retention of the solutes is controlled mainly by the two equilibria. One is the partition between the

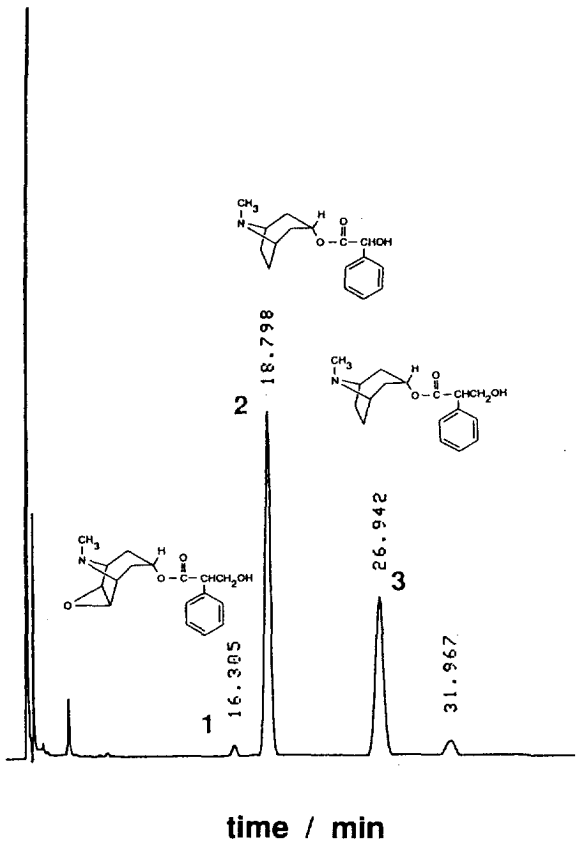

Fig. 5. MLC determination of scopolamine and *l*-hyoscyamine in scopolia extract. Solutes: 1 = scopolamine, 2 = homatropine (I.S.), 3 = *l*-hyoscyamine. Conditions: column, Nucleosil $5C_{18}$ (15 cm × 4.6 mm, 40°C); mobile phase, 0.28% SDS in 0.05 *M* sodium dihydrogenphosphate containing 1% triethylamine (pH 2.5 adjusted by phosphoric acid)–acetonitrile (76:24, v/v). Flow-rate, 1.0 ml min^{-1}. Detection, 210 nm.

micelles and the bulk solvent in the mobile phase and the other is the partition between the stationary phases and the mobile phase. Partition to the micelles dominantly controls the retention at surfactant concentrations higher than CMC. By introducing micelles, different solutes' partition to the micelles enables the different retentions, leading to the separation. This mode is termed as micellar exclusion chromatography.

4. Micellar electrokinetic chromatography

Drugs can be analyzed by the CE techniques.

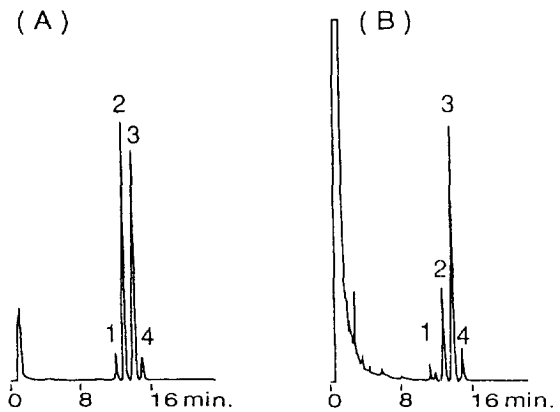

Fig. 6. MLC determination of alkaloids in Ephedra herb. (A) standards, (B) extract of Ephedra herb. Solutes: 1 = phenylpropanolamine, 2 = pseudoephedrine, 3 = ephedrine, 4 = N-methylephedrine. Conditions: column, L-column ODS (15 cm × 4.6 mm, 45°C); mobile phase, 0.78% SDS solution–acetonitrile–phosphoric acid (640:360:1, v/v). Detection, 210 nm [46].

Usually only filtration is performed on sample solution, prior to use. However, drugs having no UV absorbance must be derivatized as in HPLC analysis because typical commercial CE instruments are equipped with a UV detector only. Carbohydrates and oligosaccharides have been analyzed as pyridylaminated (PA) [68–70] or 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives [71,72]. Amino acids have been separated as dansyl (DNS) derivatives, o-phthaldialdehyde (OPA) derivatives, 3,5-dinitrobenzoyl derivatives, phenylthiohydantoin (PTH) derivatives etc. Diastereomeric derivatizations have also been employed for MEKC enantiomer separation [73–75]. Chiral derivatization reagents employed for HPLC have been utilized in the CE techniques. The concept for sample preparation or treatment in CE is the same as in HPLC. However, preparation of sample solutions of relatively high concentrations is needed in CE purity testing, to detect less than 0.1% related substances, because of low concentration sensitivity of CE instruments. According to the guidance for registration applications on the content and qualification of impurities in new drugs, impurities above apparent levels of 0.1% must be identified [76]. These levels are not so difficult for typical pharmaceuticals in CE. If sample concentration is too low, it must be concentrated prior to CE analysis. Isotachophoretic preconcentration technique [77,78] or field-amplified sample

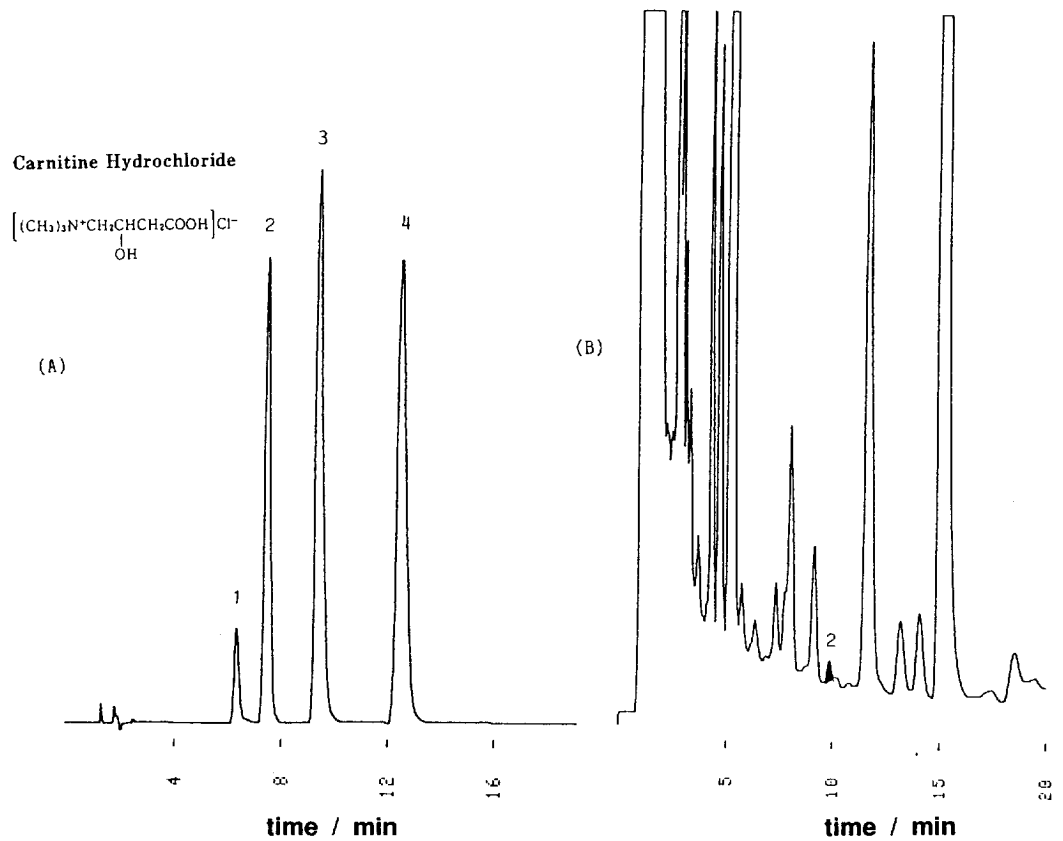

Fig. 7. (A) MLC separation of carnitine and related compounds. (B) MLC determination of carnitine in food supplements. Solutes: 1 = crotonic betaine, 2 = carnitine, 3 = acetylcarnitine, 4 = ethyl parahydroxybenzoate (I.S.). Conditions: column, Inertsil ODS-2 (15 cm × 4.6 mm, 40°C); mobile phase, 0.1% SDS in 0.05 *M* sodium dihydrogenphosphate (pH 3.0 adjusted by phosphoric acid)–methanol (70:30, v/v). Flow-rate, 1.0 ml min^{-1}. Detection, 210 nm.

stacking technique [79], should be employed. The sample stacking in MEKC has been accomplished by dissolving the neutral solutes in a low concentration micellar solution. Other than sample stacking, sample matrix usually influences the separation, especially, peak height and peak shape in CE including MEKC [80–82].

Ratio of peak areas, i.e., area percentage method is generally used for MEKC purity testing of drugs as in HPLC. In CE including MEKC, usually normalisation of peak areas with migration times is performed because peak area increases with an increase in migration times [83]. It is recommended to employ an I.S. method for assay of drugs to compensate the variance in sample injection. MEKC assay of active ingredients in pharmaceutical preparations have all been performed using an I.S. method. The results obtained corresponded well to HPLC results within R.S.D. of 3%. Altria et al. reported CE (including MEKC) application to the analysis of pharmaceuticals and drug related impurities with a view to quantitative aspects and validation exercises [84–88].

Selectivity can be easily manipulated through the changing the micellar solution, or employing additives to the micellar solution. In particular, change of the surfactant in MEKC, this corresponds to the change of the column in HPLC, is effective [89,90]. Different pseudo-stationary phases give different selectivity for the separation of drugs. A variety of surfactants have been employed for MEKC. Typically long alkyl-chain surfactants such as SDS have been employed. Non-ionic long alkyl-chain surfactants such as Brij 35 and Tween 20 have also been

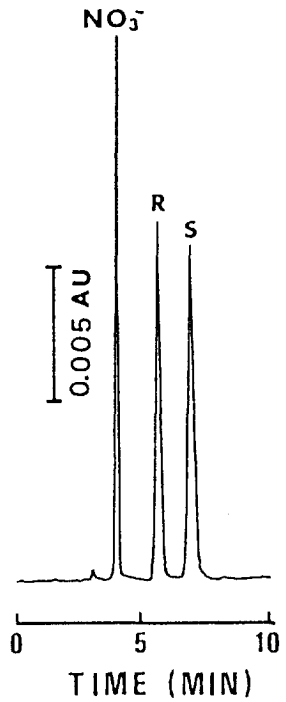

Fig. 8. Enantiomer separation of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate (BNDHP) by MLC using sodium cholate. R=(*R*)-(−)-BNDHP, S=(*S*)-(+)-BNDHP. Conditions: column, LiChrosorb RP-18 (15 cm×0.35 mm, 20°C); mobile phase, 70% acetonitrile solution containing 0.15 *M* sodium cholate. Flow-rate, 2.8 μl min^{-1}. Detection, 237 nm [62].

successfully used with other ionic surfactants for MEKC separation. Different selectivity has been obtained by using a mixed micelle. When ionic interaction is strong in MEKC with SDS alone, i.e., the analytes have a cationic nature and migrate with the micelle, the addition of non-ionic surfactants will be successful through the reduction of the surface charge in the mixed micelle. Cationic surfactants are effective for the separation of basic drugs such as catecholamines [91], β-blockers [92] and imipramine analogues [93]. Alkylsaccharides surfactants such as octyl-β-D-glucopyranoside have been utilized in MEKC under the alkaline borate conditions by El Rassi et al. [94–96]. In situ charged micelles are formed and the charge density of the micelle can be adjustable by pH and/or the borate concentration. Bile salts have been also used for MEKC. These are anionic surfactants from biological sources. They have steroidal structures and probably form helical micelles having reversed micelle conformation. Non-conjugated types of bile salts (sodium cholate, sodium deoxycholate etc.) must be used at a pH higher than 5, although taurine conjugated bile salts can be applicable even at pH 3. Compared with long alkyl chain surfactants, bile salts have relatively weak solubilization power. Corticosteroids and benzothiazepin analogues, which were almost totally solubilized in SDS micelle because of its high hydrophobicity and were not separated by MEKC with SDS, have been successfully separated with sodium cholate, sodium taurocholate etc. [26,97,98]. Apart from the separation power for hydrophobic compounds, enantiomer separation has been successful with bile salts [99–105].

Surfactants mentioned above form molecular aggregates of surfactants. There is a CMC and aggregation number for each surfactant. On the other hand, high molecular mass surfactant such as block copolymers [106–109] can form micelles with one molecule. This has some advantages over normal low molecular mass surfactant, e.g., stability, rigidity, controllable size etc. CMC is essentially zero, therefore, micelle concentration is independent of temperature, buffer concentration, and additives such as organic solvents. High background in MEKC–MS with normal surfactant micelle may be reduced with high molecular mass surfactants [109]. Copolymers of methacrylate types and polymerized vinyl group-terminated surfactant such as sodium 10-undecylenate have been applied for MEKC separation [110–112]. Recently starburst dendrimers, which is not the micelle, were also employed for the separation of neutral analytes [113–115]. Perspectives and recent applications of MEKC in drug analysis were reviewed previously [13–15].

4.1. Separation analysis

4.1.1. Amino acids and peptides

MEKC is effective for the separation of complex mixtures. Typical examples are the separation of amino acids and peptides. Amino acids are used as injections or tablets for nutritional purposes. Most amino acids have been employed for MEKC analysis as DNS, PTH and OPA-derivatives to enable detection. Chiral derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)

[73] and L-Marfey's reagent [116] has been used for enantiomer separation of amino acids. A great number of papers have been published on amino acid separation by MEKC [117–140] and the optimization of the separation of amino acid derivatives (around 20) has been achieved through the changing the surfactant concentration, adding organic modifiers, adding another surfactant etc. More than 18 amino acids derivatives (DNS, PTH etc.) have been separated within 30 min. One example is shown in Fig. 9, where 26 analytes except di-DNS-Lys, di-DNS-His and di-DNS-Tyr were baseline resolved [140]. Sandra et al. reported the separation of 17 fluorescein isothiocyanate (FITC) derivatized amino acids within 15 min by using a combination of mixed micelle (SDS/Brij 35) and the addition of 2-propanol [136].

Peptides are ionic analytes and can be separated by CZE mode. The advantage of MEKC over CZE in the separation of peptides is the wide choice of selectivity manipulation. Many peptides are known as biologically active compounds and pharmaceuticals will be produced by derivatization or imitation of these compounds. For synthetic peptides, related peptides having similar electrophoretic mobilities are sometimes contaminated. The MEKC separation will be useful for these samples. A typical example is the separation of [Leu^{13}]motilin and [Met^{13}]motilin, in which only one neutral amino acid residue is different among 22 amino acid residues. The successful separation was achieved by MEKC through the addition of organic solvents [141] or MEKC with non-ionic surfactant Tween 20 [142]. Enkephalins (5 amino acid residues) and angiotensins (7–10 amino acid residues) were successfully separated by MEKC with SDS [143] or Tween 20 [142]. Insulins from

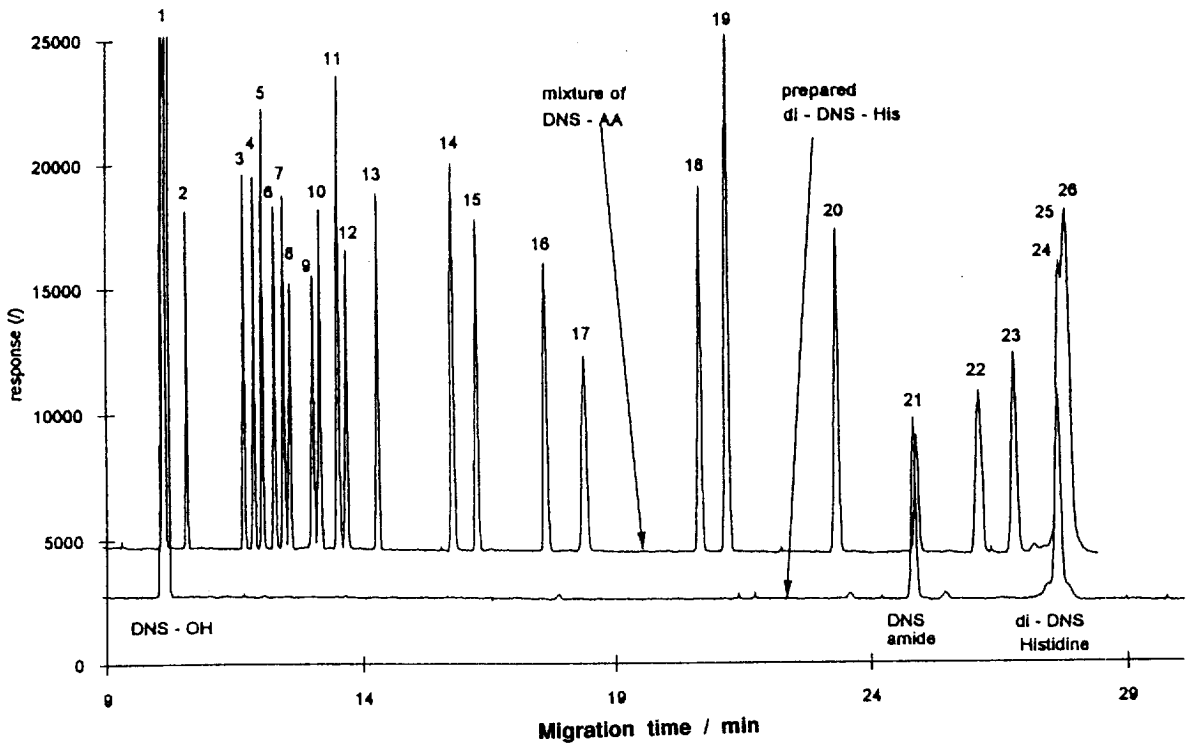

Fig. 9. Separation of DNS-amino acids by MEKC using SDS. Conditions: capillary, 57.5 cm (effective length 50 cm)×0.05 mm, 10°C; buffer, 125 m*M* SDS in 20 mM borax; applied voltage, 25 kV; detection, 214 nm. Solutes: 1=DNS-OH, 2=DNS-HyPro, 3=DNS-Thr, 4=DNS-Ser, 5=DNS-Asn, 6=DNS-Gln, 7=DNS-Ala, 8=DNS-Glu, 9=DNS-α-Aba, 10=DNS-Asp, 11=DNS-Gly, 12=DNS-Val, 13=DNS-Cys acid, 14=DNS-Pro, 15=DNS-Met, 16=DNS-Ile, 17=DNS-Leu, 18=DNS-Phe, 19=DNS-Trp, 20=di-DNS-Cys, 21=DNS-amide, 22=DNS-Lys, 23=DNS-Arg, 24=di-DNS-Lys, 25=di-DNS-His, 26= di-DNS-Tyr [140].

different origins were also successfully separated by MEKC with organic solvents [141].

4.1.2. Vitamins and antibiotics

Water soluble vitamins have been successfully separated by the CZE [144–146]. However, selectivity is much improved by employing MEKC mode [147–151]. Therefore, most of simultaneous separations have been performed by MEKC. For fat-soluble vitamins such as retinoic acid, CDs [152] or organic solvents [153,154] were added to the micelle solution.

Separation of antibiotics are successful by CZE because most of them are ionic species [143]. For a simultaneous separation of mixtures, however, MEKC mode is much suited [155,156] because of the similar electrophoretic mobilities of the analytes in CZE. One example is the determination of cephradine and its related impurities as shown in Fig. 10 [157]. By using 20 m*M* phosphate–borate buffer (pH 9.0) containing 0.05 *M* SDS and 0.1% Brij 35, cephradine, cephalexin and all related impurities were baseline resolved. In this case, Brij 35 was added to avoid bandbroading of cepharadine after repeated injections of the preparations (sodium carbonate injection). The R.S.D. for recovery testing was 0.88%, detection limit of cepharadine was 0.53 $\mu g\ ml^{-1}$. The R.S.D. value for repeatability was 0.4% for both migration times and peak areas of cepharadine. There were not significant differences between the results obtained with the MEKC method and those by the validated HPLC method [157].

4.1.3. Determination of active ingredients in cold medicines

The simultaneous determination of several active ingredients in pharmaceutical preparations has been successfully achieved by MEKC. A typical example is a cold medicine [158–161]. Except for chlorpheniramine, whose label claim is smallest among active ingredients used in cold medicines, R.S.D. values of 1–3% and assay results of almost 100% were obtained [160,161]. A typical example of the separation of eleven active ingredients which are used for a cold medicine is shown in Fig. 11 [161], where acetonitrile was added to separate noscapin and chlorpheniramine. In general, it is difficult to determine these drugs simultaneously by conventional reversed-phase HPLC in the isocratic mode as mentioned in MLC. However, these drugs, including cationic, anionic and neutral, could be separated by MEKC with a relatively short analysis time. The assay results obtained by MEKC and validation exercises compared with HPLC are good enough for assay of pharmaceuticals [158–161].

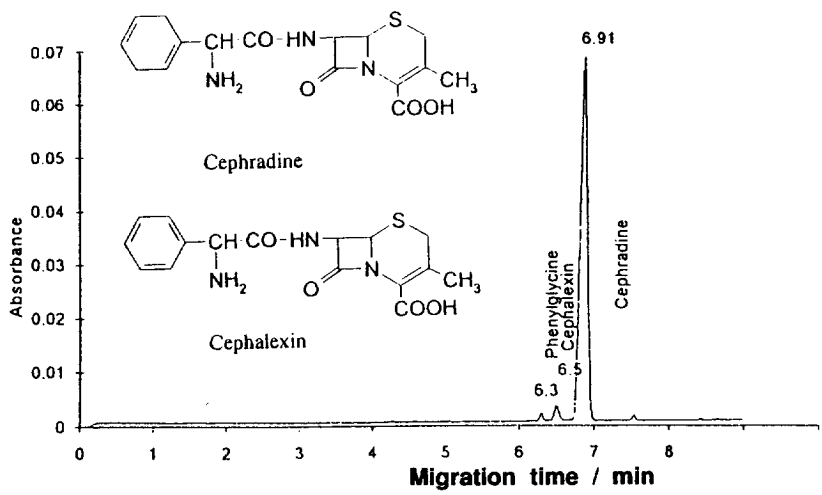

Fig. 10. Determination of cephradine and its related impurities by MEKC using SDS and Brij 35. Conditions: capillary, 57 cm (effective length, 50 cm)×0.075 mm, 25°C; buffer, 0.02 *M* borate–phosphate buffer (pH 9.0) containing 0.05 *M* SDS and 0.1% Brig 35; sample, 1 $mg\ ml^{-1}$ 0.5 p.s.i. for 5 s (1 p.s.i.=6894.76 Pa); applied voltage, 20 kV; detection, 214 nm [157].

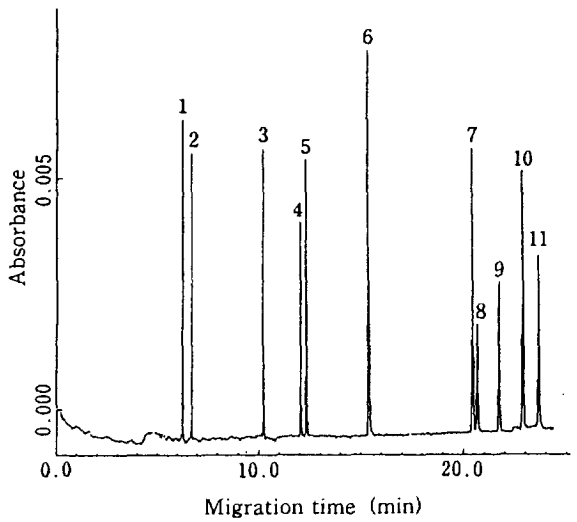

Fig. 11. MEKC separation of eleven active ingredients used for the cold medicines. Conditions: capillary, 57 cm (effective length 50 cm)×0.075 mm, 25°C; buffer, 0.015 *M* borate–phosphate buffer (pH 8.0) containing 0.1 *M* SDS and 7% acetonitrile; applied voltage, 18 kV; detection, 214 nm. Solutes: 1 = acetaminophen, 2 = caffeine, 3 = guaifenesin, 4 = bucetin, 5 = ethenzamide, 6 = phenirefrin hydrochloride, 7 = phenylpropanolamine hydrochloride, 8 = dihydrocodeine phosphate, 9 = DL-methylephedrine hydrochloride, 10 = noscapine, 11 = chlorpheniramine maleate [161].

4.1.4. Natural products and Chinese crude drugs

Chinese crude drugs and Chinese drug preparations contain many biologically active components, which are usually ionic and hydrophobic. In order to estimate the quality of these samples, a simple and high resolution method should be developed. MEKC is effective for the separation of the complex mixtures. Flavonoids [16–21], cardiac glycosides [162], lignans [163], lappaconitine [164] and limonoid glucosides [165] in plant extracts, and illicit drugs [166–174] such as codeine and cocaine have been separated by MEKC with SDS or CTAB. Separation of amphetamine and related compounds together with caffeine is shown in Fig. 12 [174], where cationic surfactant, CTAB and organic solvent (dimethylformamide) were used for the buffer solution. Determination of glycyrrhizin in Chinese drug preparations [175,176], ginsenosides in Ginseng Radix [177], and sennoside A and B [178,179] in Chinese drug preparations were successful by MEKC with SDS or bile salts. CDs or organic solvents such as acetonitrile were found to be effective for the separation of relatively hydrophobic or basic Chinese drugs.

4.1.5. Corticosteroids and estrogens

Steroids, which are electrically neutral drugs, can be separated only by using MEKC mode. However, these drugs are too hydrophobic to separate by conventional MEKC with SDS alone. Bile salt micelles have been found to be effective for the separation of corticosteroids [26,27,29] and estrogens [28]. Additives such as CDs, urea and organic solvents to the micellar solutions were also effective for the separation of these steroids [26–29,180–185]. On the other hand, low concentration of SDS [29] and cationic surfactant dodecyltrimethylammonium bromide (DTAB) [183] were also successfully used for the separation of steroids. Papers concerning the separation of steroids by MEKC are summarized in Table 1.

4.1.6. Enantiomers

Direct enantiomer separation can be achieved by MEKC using various techniques [22,24]. One approach is using a chiral micelle. Bile salt surfactants have been successfully used for enantioseparation of several drugs. Enantiomeric separations of some DNS-amino acid derivatives [100], trimetoquinol hydrochloride [99,102], β-carboline derivatives [99], binaphthyl compounds [99], diltiazem hydrochloride [99,103], verapamil and related compounds [186] etc. were successful by using bile salts as chiral selectors. Among the bile salts employed, sodium taurodeoxycholate (STDC) was the most effective for the enantiorecognition of the solutes. This may ascribed to the following two points: one is the increased solubilizing capability by the lack of the hydroxyl group at C-7 position, and the other is a low pK_a value due to the sulfonate group in comparison with other bile salts having a carboxyl group. As an example, enantiomer separation of verapamil and related drugs by MEKC with bile salts is shown in Fig. 13 [186]. Methanol and polyoxyethylene ether were added to the buffer solution containing SDC.

Acylamino acid surfactants such as sodium N-dodecanoyl-L-valinate (SDVal) [187–192] and alkoxyacylamino acid [193–195] have been also employed

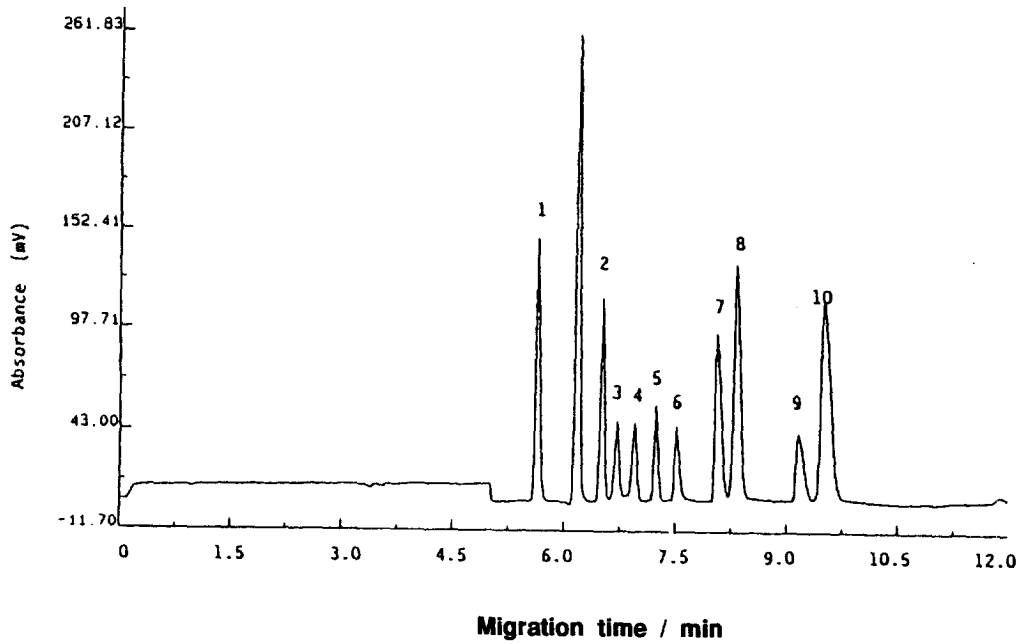

Fig. 12. MEKC separation of 10 amphetamines and related substances. Conditions: capillary, 72 cm (effective length 50 cm)×0.075 mm, 30°C; buffer, 0.01 *M* sodium tetraborate (pH 11.5) consisting of 1% ethanolamine, 11% dimethylformamide, 88% 0.025 *M* CTAB; applied voltage, −15 kV; detection, 254 nm. Solutes: 1=*p*-aminobeozoic acid (I.S.), 2=caffeine, 3=norephedrine, 4=pseudonorephedrine, 5=ephedrine, 6=pseudoephedrine, 7=amphetamine, 8=methylenedioxyamphetamine, 9=methamphetamine; 10=methylenedioxymethamphetamine [174].

for the separation of enantiomers by MEKC. Some amino acid derivatives [187,188] and PTH-amino acids [189,190] were successfully enantioseparated by using SDVal. Some saponins such as digitonin [189], glycyrrhizic acid [196], and β-escin [196] have been used for the MEKC enantiomer separation. In this case, a mixed micelle of saponin and SDS was essential. In MEKC enantiomer separation, a mixed micelle of two chiral surfactants or a combination of a chiral surfactant and a chiral additive such as CDs are effective.

Besides employing chiral surfactants, enantiomer separation has been achieved by using some chiral additives such as CDs (CD-MEKC) [197–200] to the SDS micelle. By CD-MEKC with SDS or bile salts, some DNS-amino acids [197], barbiturates [198], naphthalene-2,3-dicarboxaldehyde-labeled (CBI) amino acids [199], cicletanine [200], etc. were enantioseparated.

When direct enantiomer separation of an analyte by MEKC is not successful and the analyte has some reactive groups such as amino or carboxyl groups in a molecule, diastereomeric derivatization methods should be applied to the MEKC, similarly to HPLC. Separation of D- and L-amino acids, which were derivatized with a chiral reagent such as GITC or Marfey's reagent as diastereomers, was successfully achieved by MEKC with an SDS solution alone [73,116]. Enantiomer separation by MEKC has been reviewed elsewhere [15,22,24].

4.2. Clinical analysis

Determination of drugs and their metabolites in biological fluids such as urine and serum has been successfully performed by MEKC [201–215]. In clinical analysis, usually drugs at low concentration in complex matrixes must be analyzed. Therefore extraction procedures with disposable cartridges are often employed. Proteins in serum samples are precipitated by adding an organic solvent. These pretreatment procedures are similar to those in HPLC except for direct injection method in MEKC as mentioned below. Thormann et al. have extensively

Table 1
Application of MEKC to the separation of steroids

Analytes	Buffer solution	Ref.
Corticosteroids (8 mixtures) Triamcinolone, hydrocortisone Betamethasone, fluocinonide	50–150 m*M* (pH 7.0–9.0) SC, STC, SDC, STDC	[26]
Hydrocortisone acetate Dexamethasone acetate	50 m*M* SDS, 6 *M* urea (pH 9.0)	[181]
Triamcinolone acetonide Fluocinolone acetonide	50 m*M* SDS, 4 *M* urea, 15 m*M* γ-CD (pH 9.0)	[34]
Corticosteroids (17 mixtures) Triamcinolone, fludrocortisone Prednisone, hydrocortisone Cortisone, prednisolone	50–100 m*M* (pH 9.0) SGDC, SGDC + STDC, STDC SDS + SGDC, SDS + SGDC + STDC	[27]
Corticosterone, progesterone Fludrocortisone acetate Cortisone acetate Prednisolone acetate Hydrocortisone-21-acetate Triamcinolone acetonide Fluocinolone acetonide 6α-Methyl prednisolone Deoxycorticosterone Prednisone acetate	50–100 m*M* (pH 9.0) SGDC, SGDC + STDC, STDC 75 m*M* SDS + ACN, urea, β-CD	[182]
Corticosteroids (10 mixtures) Triamcinolone, aldosterone Cortisone, cortisol, corticosterone Dexamethasone, deoxycorticosterone 11-Dehydrocorticosterone Deoxycorticosterone acetate	25 m*M* SDS (pH 9.5) 50 m*M* SGDC (pH 6.5)	[29]
Estrogens (10 mixtures) 17 β-Estradiol, estriol 16-Keto-17 β-estradiol 2-Methoxyestradiol, 2-hydroxyestradiol 4-Hydroxyestradiol, 4-hydroxyestrone Estrone, 2-methoxyestrone 16α-Hydroxyestrone	50 m*M* SDS + 20% MeOH (pH 7.0)	[180]
Estrogens (10 mixtures) 17 β-Estradiol, 17 α-estradiol Equilenin, estrone, equilin, estriol 17 β-Dihydroequilin 17 β-Dihydroequilenin 17 α-Dihydroequilin 17 α-Dihydroequilenin	50 m*M* SDS + 15 m*M* β-CD (pH 8.0) 75 m*M* SC (pH 8.0)	[28]
Other mixtures Testosterone, 17-deoxycorticosterone Hydrocortisone, dimethyltestosterone Testosterone propionate, cortisone	50 m*M* DTAB + trioctylphosphine (pH 7.4)	[183]
Progesterone, testosterone	100 m*M* SDS + 10% MeOH (pH 7.0)	[184]
Free and conjugated steroids (13 mixtures)	50 m*M* SDS + 16% ACN (pH 8.0)	[185]

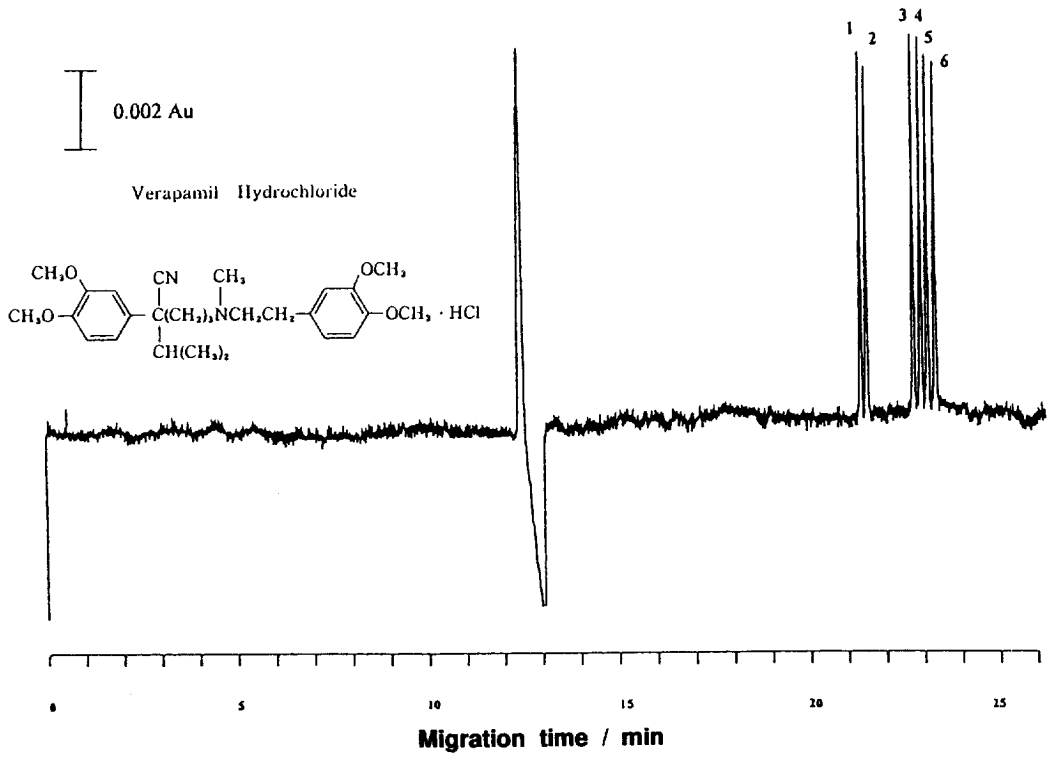

Fig. 13. Enantiomer separation of verapamil, norverapamil and gallopamil by MEKC using SDC and polyoxyethylene-4-dodecyl ether. Conditions: capillary, 75 cm (effective length 65 cm)×0.050 mm, room temperature; buffer, polyoxyethylene-4-dodecyl ether mole fraction of 0.3, 25% methanol, 0.05 *M* SDC; applied voltage, 20 kV; detection, 210 nm. Solutes: 1=(−)-gallopamil, 2=(+)-gallopamil, 3=(−)-verapamil, 4=(+)-verapamil, 5=(−)-norverapamil, 6=(+)norverapamil. Norverapamil (N-desmethyl form) is a major metabolite of verapamil and gallopamil is a methoxy derivative of verapamil [186].

investigated for MEKC determination of drugs in body fluids, employing photodiode array defector [207,208,213]. Lukkari and Siren investigated the determination of β-blockers in human biological fluids by using MLC and MEKC with cetyltrimethylammonium bromide (CTAB) [215]. Separation of ten β-blockers in human serum by MEKC with CTAB is shown in Fig. 14 [215]. They say that MEKC sample preparation was less time-consuming although detection limit in the MEKC was low. MEKC monitoring of drugs and its metabolites in body fluids have been recently reviewed [216–218].

4.3. Direct plasma/serum injection

Direct injection method has been successfully employed in MEKC [35–40], similarly to MLC. Without a surfactant (CZE mode), plasma protein peaks interfered with the peaks of the solute and protein adsorption occurred, causing a change of the velocity of the EOF. Rinsing of the capillary with an alkaline solution or an SDS solution is required to recover the capillary surface or reproducible migration times when plasma samples are injected in CZE mode. With a surfactant, the migration times of the protein peaks shift longer, and the useful analytical window, which is defined between the solvent peak and the plasma protein peaks, appears. The migration time of the analyte of interest must be manipulated to migrate before the protein peaks. The surfactant also breaks the drug–protein complexes, therefore, the total amount of bound and unbound drug could be determined by this method. The use of a micellar solution also prevents protein adsorption on the capillary wall, due to the solubilization of the protein hence the electrostatic repulsion between the solubil-

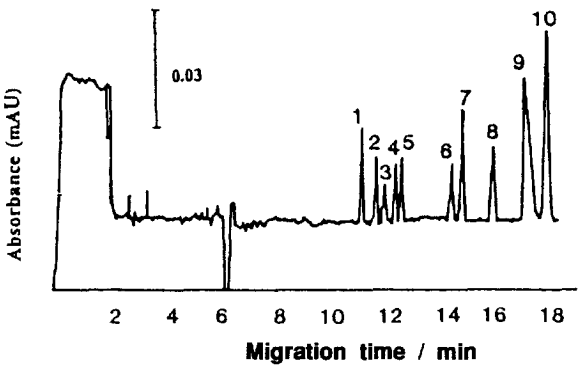

Fig. 14. Separation of 10 β-blockers spiked to human serum by MEKC with CTAB. Conditions: capillary, 56 cm×0.05 mm (~25°C); buffer, 0.08 *M* sodium dihydrogenphosphate and 0.08 *M* disodium hydrogenphosphate (pH 7.0) containing 0.01 *M* CTAB; applied voltage, −26 kV; detection, 210 nm. Solutes: 1 = acebutolol, 2 = nadolol, 3 = timolol, 4 = atenolol, 5 = metoprolol, 6 = oxprenolol, 7 = pindolol, 8 = alprenolol, 9 = labetalol, 10 = propranolol [215].

ized proteins and the capillary wall. The detection limit in the direct plasma/serum injection method of aromatic drugs usually lies around 1 μg ml^{-1} in UV detection.

4.4. Determination of some physicochemical properties of drugs

The capacity factor k' can be related to the distribution coefficient K of an analyte between the micellar and aqueous phases through

$$k' = K\frac{V_{mc}}{V_{aq}} \quad (1)$$

where V_{mc} and V_{aq} are volumes of micellar and aqueous phases. Here V_{mc} can be determined from a micellar concentration c_{mc}, which is equal to the concentration of a surfactant c_{sf} minus CMC, and partial specific volume of micelle *v*. *v* can be determined easily (e.g., 0.87 ml g^{-1} for SDS) [67]. V_{aq} is equal to the rest of solution. Thus, V_{mc}/V_{aq} can be described as

$$\frac{V_{mc}}{V_{aq}} = \frac{v(c_{sf} - CMC)}{1 - v(c_{sf} - CMC)} \quad (2)$$

When micellar concentrations are low, the denominator at the right side of Eq. (2) may be approximated to be equal to unity, i.e.,

$$k' = Kv(c_{sf} - CMC) \quad (3)$$

The plots of the capacity factor vs. surfactant concentration have shown linear relationship for both neutral and charged analytes and distribution coefficient has been obtained from slopes of these plots [11,219,220].

Hydrophobicity of drugs is one of the important physicochemical properties to evaluate the biological effects because a drug has to pass across various biomembranes, which have a lipoid nature. In a development of a new drug, logarithmic partition coefficients between *n*-octanol and water (P_{oct}) have been usually determined. Traditionally, log P_{oct} values are measured using the 'shake-flask' method combined with UV or HPLC assay. Recently MEKC was used to determine the log P_{oct} values as in HPLC [221–224]. Linear log–log relationships were found between both the micelle–water partition coefficient (log K) in MEKC and *n*-octanol–water partition coefficient (log P_{oct}) [225]. The capacity factor k' and the micelle–water partition coefficient K is proportional as shown in Eq. (3). Therefore, these linear relationships can be expressed as

$$\log k' = a \log P_{oct} + b \quad (4)$$

where a and b are constants that can be determined from the observed data. Thus, the technique is performed by analyzing a set of standards of known log P_{oct} under the given MEKC conditions. The logarithmic capacity factor of each standard obtained is plotted against its log P_{oct} to form a linear calibration curve. Then log P_{oct} of an unknown analyte is calculated from the obtained capacity factor and the calibration curve. Over 100 analytes with widely varying functionality were investigated and good correlation was observed [222].

5. Microemulsion electrokinetic chromatography

Microemulsion electrokinetic chromatography (MEEKC) is also one branch of electrokinetic chromatography (EKC) and has a capability of separating electrically neutral analytes as in MEKC. Micro-

emulsions (o/w) are prepared by mixing oil, water, a surfactant and a cosurfactant such as a medium alkyl chain alcohol. Therefore microemulsion can be used as a micelle-like pseudo-stationary phase in EKC. The MEEKC has been successfully employed for the separation of electrically neutral compounds [226–228] and the selectivity difference was investigated. Recently, separations of water soluble and lipid soluble vitamins [229] and steroids [29] were reported by using MEEKC with SDS or tetramethyltetradecylammonium bromide (TTAB). As an example, separation of the test mixture of ten steroids is shown in Fig. 15 [29]. The microemulsion consisted of *n*-hexane (0.81%), SDS (3.31%) and *n*-butanol (6.61%) was employed in the separation. Compared with MEKC, different selectivity was obtained by MEEKC. MEEKC was also used for the determination of the hydrophobicity of analytes. The results obtained by MEEKC were compared with those obtained by MEKC with SDS [230] and a good relationship was found.

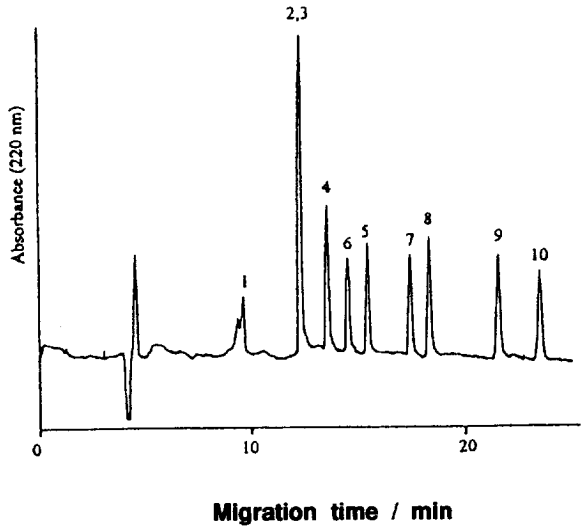

Fig. 15. Separation of ten steroids by MEEKC. Conditions: capillary, 60 cm (effective length 50 cm)×0.050 mm, room temperature; buffer, *n*-hexane (0.81%), SDS (3.31%), *n*-butanol (6.61%) with 0.02 *M* phosphate buffer (pH 10.0) (89.28%, w/w); applied voltage, 15 kV; detection, 220 nm. Solutes: 1 = triamcinolone, 2 = aldosterone, 3 = cortisone, 4 = cortisol, 5 = dexamethasone, 6 = 11-dehydrocorticoster one, 7 = corticosterone, 8 = cortexolone, 9 = deoxycorticosterone, 10 = deoxycorticosterone acetate [29].

6. Conclusions

Micelles or surfactants have been successfully used in the separation analysis, especially in LC and CE. HPLC using surfactants, i.e., micellar liquid chromatography (MLC) has the capability of enhancing selectivity and injecting directly plasma/serum samples. Separation of basic pharmaceuticals and the simultaneous determination of active ingredients in the preparations have been successful by MLC. However, through the development of special columns, such as restricted access columns, chiral columns, ODS using high purity (metal free) silica gels, MLC mode will be mainly used for the selectivity manipulation in pharmaceutical analysis. On the other hand, in electrophoresis, through the introduction of micelles to the running buffer solution of CZE, i.e., micellar electrokinetic chromatography (MEKC), electrically neutral or non-ionic analytes have been separated as well as ionic analytes. MEKC has many attractive advantages over CZE. Separation of every kind of drugs, including cationic, neutral and anionic, is possible in a single run within a relatively short time. MEKC is powerful for the separation of complex mixtures such as natural products and Chinese crude drugs because of its high resolution. Direct enantiomer separation also can be successful using chiral selectors such as chiral surfactants or chiral additives. The enhancement of selectivity can be easily achieved through various techniques (changing surfactant species and its concentration etc.). The direct injection method of biological samples such as serum can be successful in MEKC. Other than separation, physicochemical properties also can be determined by MEKC. In conclusion, the great merits of MEKC (or CE), seem to be the easy method development and the small scale analytical method (small reagents and sample requirement, the fact that there is no need for organic solvents, high cost columns etc.). Therefore, MEKC will be much used for the enantiomer separation and physicochemical properties determination in the pharmaceutical analysis.

7. Abbreviations

ACN	Acetonitrile

CBI	Naphthalene-2,3-dicarboxaldehyde
CD-MEKC	Cyclodextrin modified MEKC
CDs	Cyclodextrins
CE	Capillary electrophoresis
CMC	Critical micelle concentration
CTAB	Cetyltrimethylammonium bromide
CZE	Capillary zone electrophoresis
DNS	Dansyl
DTAB	Dodecyltrimethylammonium bromide
EKC	Electrokinetic chromatography
FITC	Fluorescein isothiocyanate
GC	Gas chromatography
GITC	2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiacyanate
HPLC	High-performance liquid chromatography
I.S.	Internal standard
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MLC	Micellar liquid chromatography
ODS	Octadecylsilylized silica gel
OPA	o-Phthalaldehyde
OTC	Over the counter
PA	Pyridylaminated
PMP	1-Phenyl-3-methyl-5-pyrazolone
PTH	Phenylthiohydantoin
R.S.D.	Relative standard deviation
STDC	Sodium taurodeoxycholate
SC	Sodium cholate
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SD Val	Sodium N-dodecanoyl-L-valinate
SGDC	Sodium glycodeoxycholate
STC	Sodium taurocholate
STDC	Sodium taurodeoxycholate
TTAB	Tetramethyltetradecylammonium bromide
TLC	Thin-layer chromatography

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