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Separation of free and glucuronidated opioids by capillary electrophoresis in aqueous, binary and micellar media

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Abstract

The separation behavior of free and glucuronidated opioids, including codeine, dihydrocodeine, morphine, norcodeine, normorphine, codeine-6-glucuronide, morphine-3-glucuronide, dihydromorphine, nordihydrocodeine, nordihydromorphine and dihydrocodeine-6-glucuronide, by capillary zone electrophoresis (CZE) and electrokinetic chromatography (EKC) in aqueous and binary media with ethylene glycol is reported. The opioids' charges and thus their separations are shown to be strongly dependent on buffer pH, particularly in the pH range 6–12. Unconjugated codeinoids are shown to be able to form cations whereas unconjugated morphinoids and the glucuronides are of amphoteric nature with p*I* values in the 9–10 range and around 5, respectively. Due to the similarity of most pK_a values and chemical structures, all 12 opioids cannot be fully separated by aqueous and binary CZE. However, they can be resolved completely by aqueous EKC using 80 m*M* sodium dodecyl sulfate at pH 10.6. In EKC at pH 2.2 the cationic opioids strongly interact with the negatively charged dodecyl sulfate, migrate poorly resolved toward the positive electrode, are detected in the order of decreasing capacity factors and their elution times decrease with increasing surfactant concentration. With addition of ethylene glycol (up to 60%, v/v), the interaction between solutes and dodecyl sulfate is weakened and resolution is attained. An approach to measuring the viscosity of running buffers using a capillary electrophoresis apparatus is discussed.

Keywords: Buffer composition; Ethylene glycol; Opioids; Opiates

1. Introduction

Opioids, including codeine (COD), dihydrocodeine (DHC), morphine (MOR) and heroin, have been used therapeutically and/or consumed illicitly for many years. Interest in elucidating the pharmacokinetic, pharmacogenetic and pharmacodynamic properties of these opioids and their metabolites, as well as the control of drug abuse and assessment of intoxications, have prompted analysis of these substances in body fluids, tissue extracts, post mortem specimens and seizure samples. For the monitoring of opioids, innumerable analytical techniques based on chromatographic and immunological principles have been developed and are widely used. Due to appealing advantages of employing capillary electrophoresis (CE) instead of a chromatographic approach, quite a number of papers discussing the CE analysis of morphine-like compounds have been published in the past 5 years [1–15]. In these reports sodium dodecyl sulfate (SDS) containing micellar buffers (25–100 m*M* SDS) at pH 8.5–9.2 were mostly employed, either with 5–15% (v/v) of an organic solvent [1–5,13] or without an organic

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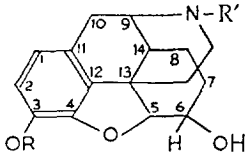
solvent [9–12,14,15] as buffer modifying agent. Furthermore, the use of a cationic surfactant, cetyltrimethylammonium bromide, instead of SDS was also reported [5,6]. Without micelles, some opioids were analyzed by capillary zone electrophoresis (CZE) at pH 6 [8] and by cyclodextrin modified CZE at pH 4 [7]. Applications included the determination of opium products in seizure samples [1–7], COD, MOR and selected metabolites in urine [8–11,13] and MOR in hair [12]. Two papers focus on the monitoring of urinary DHC and its metabolites [14,15]. Although the various separation conditions were proper for the individual issues addressed, the separation behavior of opioids was not studied systematically.

Opioids are mainly metabolized via N-demethylation, *O*-demethylation and conjugation at the hydroxyl groups [14], this providing compounds both of very similar structure and properties (after demethylation) and glucuronides (after conjugation) which have quite different chemical properties compared to those encountered with the free opioids (for structures refer to Table 1). For example, in man COD is mainly metabolized to codeine-6-glucuronide (COD6G), norcodeine (NCOD), morphine (MOR) and normorphine (NMOR). Similarly the main metabolites of DHC include dihydrocodeine-6-glucuronide (DHC6G), nordihydrocodeine (NDHC), dihydromorphine (DHM) and nordihydromorphine (NDHM). Both similarity and diversity of the compounds involved provide a challenge for the analytical procedure, particularly if free and glucuronidated substances should be analyzed simultaneously. First results obtained in our laboratory indicate that CE has the potential to fulfil this difficult task [13,14].

In this paper a systematic study of the separation behavior of the opioids mentioned above (Table 1) and ethylmorphine (EMOR, a potential internal standard for assessment of the metabolism of COD, MOR and DHC) by CE in aqueous, binary and micellar media is presented. Buffer properties varied include pH, SDS concentration and content of ethylene glycol, providing CE data under very diverse

Table 1
Chemical structures of the solutes investigated



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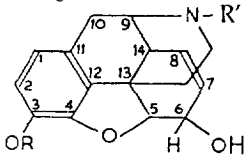

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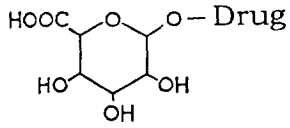

III

Compound	Abbreviation	Number	Structure	R	R′
Nordihydrocodeine	NDHC	1	I	CH_3	H
Norcodeine	NCOD	2	II	CH_3	H
Dihydrocodeine	DHC	3	I	CH_3	CH_3
Codeine	COD	4	II	CH_3	CH_3
Ethylmorphine	EMOR	5	II	C_2H_5	CH_3
Nordihydromorphine	NDHM	6	I	H	H
Normorphine	NMOR	7	II	H	H
Dihydromorphine	DHM	8	I	H	CH_3
Morphine	MOR	9	II	H	CH_3
Dihydrocodeine-6-glucuronide[a]	DHC6G	10	III		
Codeine-6-glucuronide[a]	COD6G	11	III		
Morphine-3-glucuronide[a]	MOR3G	12	III		

[a] Conjugation of the hydroxyl group at positions 3 or 6 with glucuronic acid.

separation conditions and thus leading to the elucidation of some general rules for analysis of opioids by CE.

2. Experimental

2.1. Drugs and chemicals

COD, COD6G and MOR were a gift of the Institute of Pharmacy, University of Bern (Bern, Switzerland). DHC and its synthesized metabolites DHC6G, NDHC, DHM and NDHM were received from Mundipharma (Basel, Switzerland). Morphine-3-glucuronide (MOR3G) was purchased from Sigma (St. Louis, MO, USA). NMOR, NCOD and EMOR were purchased as methanolic solutions (1.0 mg/ml base) from Alltech (State College, PA, USA). SDS was from Bioprobe (Basel, Switzerland) and ethylene glycol was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Preparation of sample solutions and running buffers

The stock sample solutions (1 mg/ml) of MOR, NMOR, NCOD and EMOR were prepared with methanol, whereas all other compounds were dissolved in 2 m*M* H_3PO_4 aqueous solution. Sample solutions were prepared by adding appropriate volumes of the stock solutions to water. With the exception of COD6G (10 μg/ml), the final concentration of the opioids was 20 μg/ml each and the sample solution further contained 8% (v/v) methanol and 0.3 m*M* H_3PO_4. The phosphate running buffers were prepared by adding appropriate volumes of 0.2 *M* H_3PO_4, 0.2 *M* NaH_2PO_4, 0.2 *M* Na_2HPO_4 and 1 *M* NaOH into water or into mixtures of water and ethylene glycol, providing the desired pH value and keeping the total phosphate concentration at 0.04 *M*. The pH of aqueous buffers was determined by a Ross combination pH electrode (Orion, Boston, MA, USA). Measuring the pH of a binary solution with an electrode standardized with aqueous buffers is of no thermodynamic value [16]. Thus, the pH value given for a binary system refers to that of an aqueous buffer with the same phosphate composition. The running buffers with SDS were prepared by dissolving specified amounts of SDS in the phosphate buffer.

2.3. Capillary electrophoresis

CE was performed using a 270A-HT CE system (Applied Biosystems, San Jose, CA, USA) equipped with a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 41 cm (22 cm) total (effective) length. The capillary oven was thermostated by heated forced air and the temperature was set at 35°C. Capillary conditioning was effected by rinsing with running buffer for 10 min applying a vacuum of 20 in.Hg (67.7 kPa) at the outlet end. Sample was introduced by applying a vacuum of 5 in.Hg (16.9 kPa) for 1 s with aqueous running buffers or for 2 s with binary systems. UV absorbance detection was effected at 210 nm. Positive voltages (positive electrode on sampling side) or negative voltages (negative electrode on sampling side) applied and currents are given in the figure legends.

2.4. Measurement of viscosity with the CE apparatus

After injection of a short sample plug (<0.4 mm) into the buffer-filled capillary, the capillary inlet end was returned to the buffer vial. Then a constant vacuum (16.9 kPa) was applied and the electropherogram recording was initiated. According to the Poiseuille equation [17], the viscosity (η) of the buffer is directly proportional to the time (t) elapsed for the sample plug to reach the detector:

$$\eta = \frac{r^2 P t}{8 L_t L_e} \quad (1)$$

where P is the pressure difference between the capillary ends, r is the capillary radius, and L_e and L_t are the effective and total length of the capillary, respectively. The viscosity of water was determined to be 0.000756 Ns/m^2. Literature values are 0.000719 Ns/m^2 at 35°C and 0.000765 Ns/m^2 at 32°C [17]. Parts of the capillary (19 and 5 cm on outlet and inlet ends, respectively) were not thermo-

stated. Thus, the average temperature of the whole capillary is expected to be somewhat lower than 35°C, this accounting for the difference in viscosity values. The precision is further hampered by an uncertainty of the value of P. Using a standard liquid of well known viscosity (water, for example) for calibration, however, permits the determination of precise viscosity values of buffers according to $\eta = \eta_0 t/t_0$, where η_0 and t_0 refer to the tabulated viscosity and the measured time interval, respectively, of a standard liquid (e.g., water). This latter approach was employed to determine the viscosities of the CE buffers used in this work.

3. Results and discussion

3.1. Capillary zone electrophoresis in aqueous medium

As is shown in Fig. 1A, the effective electrophoretic mobility (μ_{eff}), calculated from the migration times of solutes (t) and sample solvent (t_{EO}) according to $\mu_{eff} = L_e/E^*(t^{-1} - t_{EO}^{-1})$ where L_e is the effective capillary length and E is the electric field strength [18], was determined to be strongly dependent on buffer pH. A positive (negative) electrophoretic mobility means that the solute is positively (negatively) charged and migrates electrophoretically toward the negative (positive) electrode. At pH<7 the amine groups of the free morphinoids (MOR, NMOR, DHM and NDHM) and free codeinoids (COD, NCOD, DHC and NDHC) are fully protonated and the difference in mobility is only based on molecular size and/or shape. As a result, the component with largest molecular weight (EMOR) moved more slowly than the rest. The steady increase in μ_{eff} with decreasing pH (pH<7 in Fig. 1A) is caused by a steady decrease of the ionic strength (I) between 66.1 and 22.4 mM at pH 6.89 and 2.23, respectively.

The electroosmotic mobility is regarded to be proportional to $\sigma\epsilon^{1/2}/\eta I^{1/2}$ [18], where σ is the surface charge density, η the viscosity and ϵ the dielectric constant. For qualitative purposes, it is assumed that electrophoretic mobilities are equally dependent on the ionic strength, this yielding a simple relationship for a corrected electrophoretic mobility (μ_{cor})

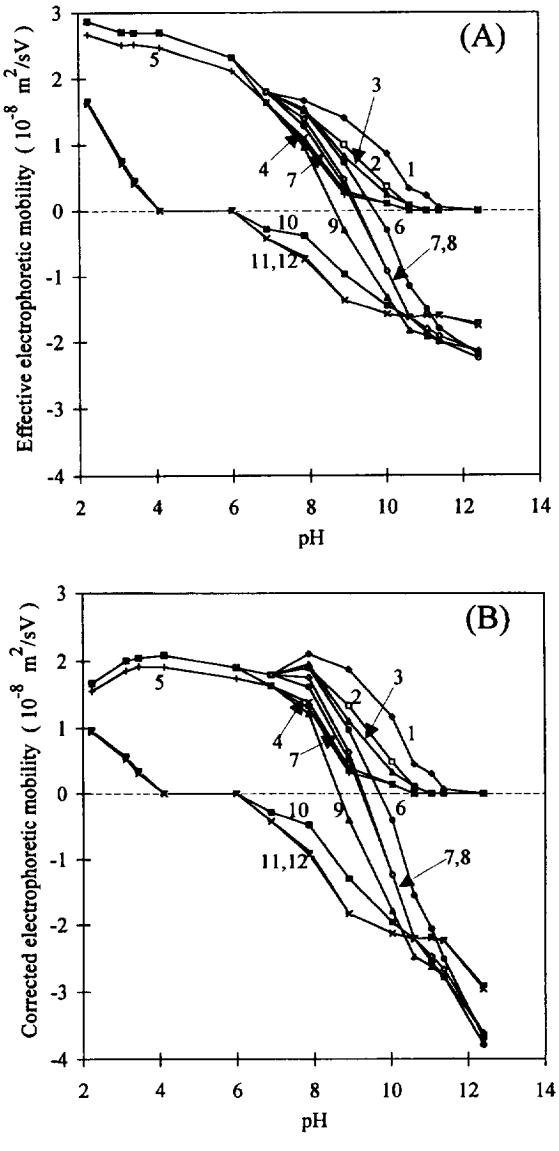

Fig. 1. The pH dependence of (A) effective electrophoretic mobilities and (B) corrected electrophoretic mobilities of opioids in 0.04 M phosphate buffers. The standard buffer used for the calculation of μ_{cor} was 0.04 M phosphate at pH 6.9 ($I_0 = 0.0661$ M). For CZE at pH 12.4–5.0 the applied constant voltage was 10 kV and the currents varied between 77 and 17 μA. For the experiments at pH 4.1–2.2 the voltage was 20 kV and the currents were 43–51 μA. The numbers correspond to the solute numbers listed in Table 1.

$$\mu_{cor} = \mu_{eff}(\frac{\epsilon_0}{\epsilon})^{1/2}(\frac{\eta}{\eta_0})(\frac{I}{I_0})^{1/2} \quad (2)$$

where the subscript 0 refers to the properties of a standard buffer. A change in μ_{cor} can be regarded purely as a change in charge if the temperature variation due to Joule-heating is neglected. The variation of viscosity with pH within the pH 2–12 range was determined to be <2% and thus was neglected for the calculation of μ_{cor} (Fig. 1B). Changes of ϵ were also disregarded. The ionic strength was calculated according to [19] and by taking the OH^- concentration into consideration as well. As expected, for pH<7, the corrected mobilities of the unconjugated solutes changed very little (Fig. 1B). At pH>7, μ_{cor} decreased due to the gradual loss of a proton. For pH>10 the free morphinoids became negatively charged due to the dissociation of the phenolic hydroxyl group. No negative mobility was observed for the free codeinoids in the investigated pH range (2.2–12.4). These substances have only an aliphatic hydroxyl group which cannot dissociate at pH≤12.4.

Most pK_a values of the test solutes are not available from the literature but can be simply estimated from the mobility data measured at two pH values at which a solute is partly ionized [20,21]. The difference in the calculated pK_a values using effective and corrected electrophoretic mobilities was found to be small (~0.1). Thus, changes in ionic strength for buffers with $\Delta pH \sim 1$ appear to be negligible [20]. Data obtained are summarized in Table 2. The estimated pK_{a_1} values for MOR and COD are close to those found in the literature (8.1 and 8.2 for MOR and COD, respectively, [22]). The same is true for pK_{a_2} of MOR (9.85 [23]). The pK_{a_1} values of the free codeinoids in Table 2 refer to the protonation of the amine group. The pK_{a_1} and pK_{a_2} of the glucuronides refer to the dissociation of the carboxyl group and the protonation of the amine group, respectively. Not unlike the glucuronides, free morphinoids can also form zwitterions. The pK_{a_1} and pK_{a_2} of the morphinoids are ascribed to the dissociation of the phenolic hydroxyl group and the protonated amine group, respectively. The data presented in Table 2 further show that the basic power of the secondary amines (NDHC, NCOD, NDHM and NMOR) are stronger (larger pK_a values) than their tertiary amine derivatives (DHC, COD, DHM and MOR, respectively). This is consistent with the literature where the pK_a value of desethylamiodarone was determined to be larger than that of amiodarone [20]. NCOD, COD, NMOR and MOR have smaller pK_a values than their saturated derivatives NDHC, DHC, NDHM and DHM, respectively.

In CZE, it has been demonstrated that best sepa-

Table 2
Estimated pK_a values in different media[a]

Compound	Media			
	Water		Water–ethylene glycol (1:1, v/v)	
	pK_{a_1}	pK_{a_2}	pK_{a_1}	pK_{a_2}
Nordihydrocodeine	10.1		9.1	
Norcodeine	9.7		8.3	
Dihydrocodeine	9.5		8.0	
Codeine	8.4		7.1	
Ethylmorphine	8.3		7.1	
Nordihydromorphine	8.8	10.4	8.5	
Normorphine	8.4	10.1	7.9	
Dihydromorphine	8.5	10.1	7.9	
Morphine	8.3	9.8	7.0	
Dihydrocodeine-6-glucuronide	3.0	8.2		7.5
Codeine-6-glucuronide	2.9	8.0		7.2
Morphine-3-glucuronide	2.9	7.9		7.1

[a] The pK_a values in water and the binary medium were calculated according to the corrected electrophoretic mobility data given in Fig. 1B and Fig. 3B, respectively.

rations are achieved with a buffer pH close to the pK_a or the isoelectric point of solutes [20,24,25]. This was also found to be true for the opioids. Best separations could be obtained between pH 8–9, conditions under which the elution order of the partly ionized solutes was almost purely determined by pK_a, i.e., the solutes were essentially detected in the order of decreasing pK_a values (Figs. 1 and 2). Due to the rather small differences in pK_a values and chemical structures of most test solutes, however, complete separation of the 12 compounds was found to be impossible using aqueous CZE (Fig. 2A). Variation of the phosphate concentration between 40 and 100 m*M* and addition of β-cyclodextrin (20 m*M*) did not improve the separation significantly (data not shown).

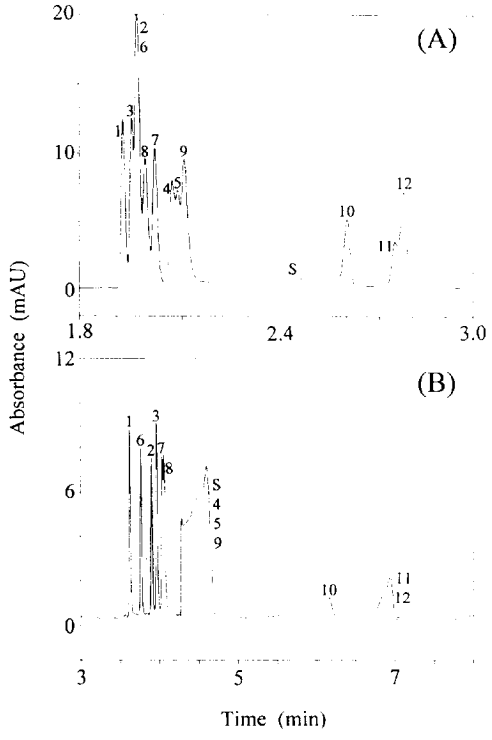

Fig. 2. Typical electropherograms obtained by (A) aqueous CZE and (B) binary CZE. The running buffers were 0.04 *M* phosphate (pH 7.9) containing (A) 0% and (B) 50% (v/v) ethylene glycol. The applied voltages (currents) were 10 (37) and 25 kV (35 μA), respectively. The viscosity of the binary medium was determined to be 3.8 fold higher than that of the aqueous buffer. S denotes sample solvent. Peak identification as in Table 1.

3.2. Capillary zone electrophoresis in binary medium

The use of binary media instead of pure aqueous solutions may lead to different separation patterns [20]. In this work, ethylene glycol–water mixtures have been investigated. Ethylene glycol was chosen due to its rather high boiling point (198.9°C [17]), which prevents problems associated with evaporation of liquid, and due to its viscosity (19.9 cP = 0.0199 Ns/m^2 at 20°C [17]), which is much higher than that of pure water (1.002 cP at 20°C [17]). In contrast to aqueous solutions, binary systems can exhibit drastic changes in both viscosity and dielectric constant [26]. Therefore, for the sake of data comparison, corrected electrophoretic mobilities were calculated according to Eq. (2) using viscosity values determined with the CE instrument (cf., Section 2.4) and ϵ values obtained by $\epsilon = 78.5\ (1-x) + 37.7x$ [26], where x is the volume fraction of ethylene glycol and 78.5 and 37.7 are the dielectric constants of pure water and ethylene glycol, respectively [17]. For a buffer pH of 6.9, corrected electrophoretic mobility data as function of the volume fraction of ethylene glycol are presented in Fig. 3A. The data reveal that the corrected electrophoretic mobility (the amount of charge) of each solute decreases as the volume fraction of ethylene glycol is increased. This was found to be true for anions and cations. It was interesting to learn that the glucuronides could not be analyzed in presence of 70 or 80% ethylene glycol (peaks became very broad). Furthermore, the pH dependence of the corrected mobility in 50% (v/v) ethylene glycol was explored (Fig. 3B) and pK_a values were determined as described above (Table 2). It should be noted that the pH values (cf., Section 2) and thus also the pK_a values given for binary systems are not thermodynamic properties. This, however, does not prevent us in using these values for comparison of migration behavior in aqueous and binary media. As was stated previously [20], pK_a values in a binary system of more basic power than water should be lower than those observed in aqueous solution. This was also found to be true for opioids in a binary medium formed with ethylene glycol ($pK_a = 14.22$ [17]; see data presented in Table 2). It is interesting to note that although the effective electrophoretic mobility in the binary systems could

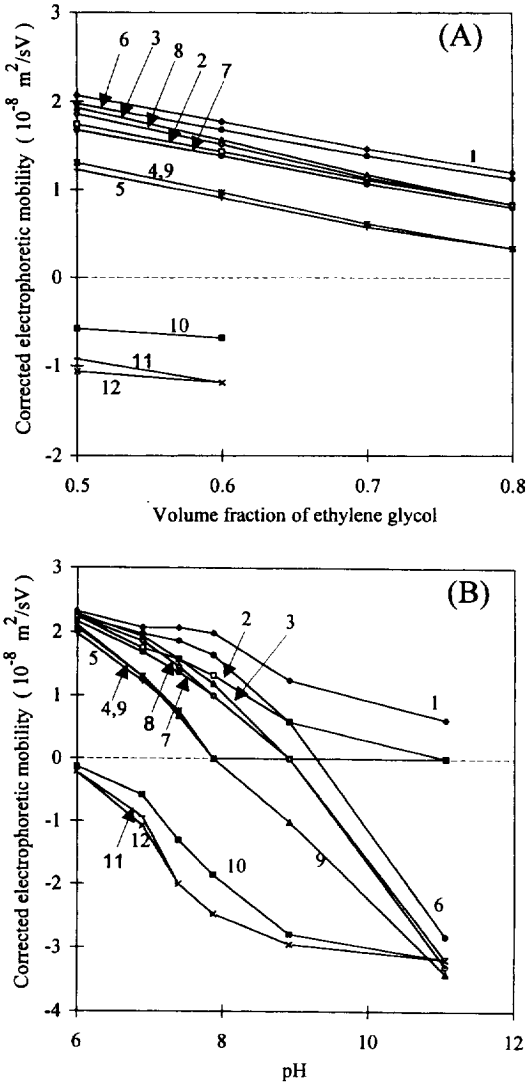

Fig. 3. Opioid separation as function of (A) volume fraction of ethylene glycol in the 0.04 *M* phosphate buffers (pH 6.9) and (B) pH of buffers containing 50% (v/v) ethylene glycol and 0.04 *M* phosphate. The standard buffer used for the calculation of μ_{eof} was the same as that employed for the data presented in Fig. 1. The applied voltage was 25 kV and the currents were (A) 25–8 μA for the CZE with 50–80% ethylene glycol and (B) 18–43 μA for the CZE at pH 6.0–11.1. Solute numbers as in Table 1.

be several times smaller than that in aqueous medium, the corrected mobility data are comparable (Fig. 1B and Fig. 3B). This indicates the validity and illustrates the usefulness of the relationship given in Eq. (2). Although the elution order in binary CZE was somewhat different from that in aqueous CZE, full separation of the 12 test solutes was still unachievable (Fig. 2B).

3.3. Micellar electrokinetic chromatography in aqueous medium

In micellar electrokinetic chromatography (MEKC), an effective electrophoretic mobility for charged and uncharged solutes can be calculated using the same relationship as for CZE (see above; [27]). The data presented in Fig. 4 show that the effective electrophoretic mobility is dependent on SDS concentration and buffer pH. As is stated in the literature [20,27–29], SDS was determined to drastically influence the migration of cations and neutral solutes (free codeinoids in Fig. 4A). Furthermore, due to the electrostatic repulsion dodecyl sulfate was found to have little effect on anions (unconjugated morphinoids and glucuronides in Fig. 4A). In addition to hydrophobic interaction, electrostatic interaction is an important factor determining retention in MEKC. Thus, within the pH range 8–11, separation and elution order are very sensitive to a change in pH (Fig. 4B). In contrast to the impact of the pH variation, an SDS concentration change between 20 and 100 m*M* appears to hardly influence the elution order of the tested opioids.

For the understanding of the retention mechanism, capacity factors (k') were calculated according to [27,30]

$$k' = \frac{\mu_s - \mu_0}{\mu_{mc} - \mu_s} = K\nu(C_{SDS} - CMC) \qquad (3)$$

where μ_s and μ_{mc} are the electrophoretic mobilities of a solute and the micelles, respectively, μ_0 is the mobility of the solute without micelles, K the distribution coefficient, C_{SDS} is the total SDS concentration, *CMC* is the critical micellar concentration and ν is the partial specific volume of micelles (0.2515 l/mol for SDS [30]). In our experiments μ_{mc} was not measured but can be calculated from the μ_s values at two SDS concentrations using Eq. (3). With *CMC* = 4 m*M* for phosphate buffers [27], the average calculated μ_{mc} was determined to be $-5.14 \cdot 10^{-8}$ m^2/s V, a value which is close to that reported in the

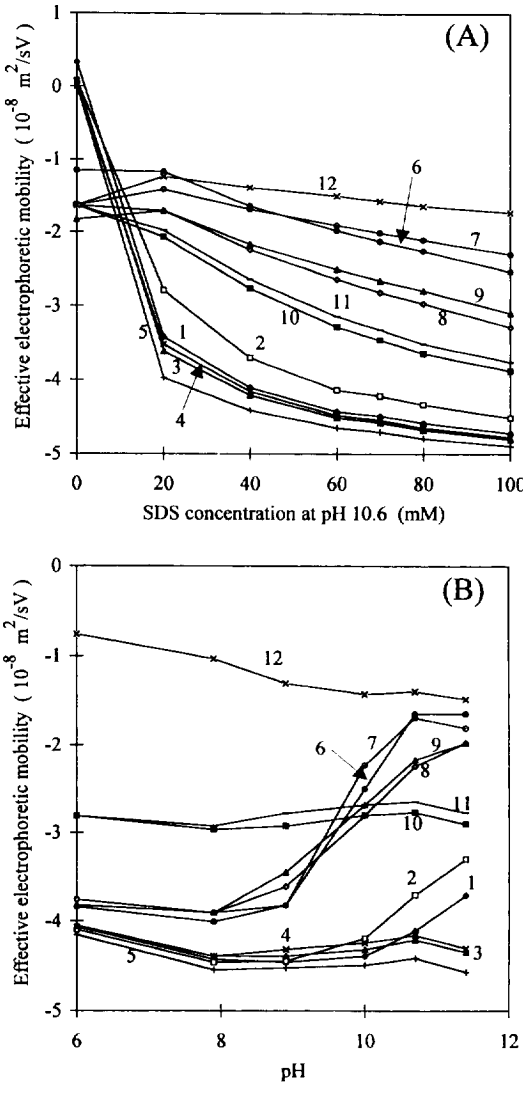

Fig. 4. Effects of (A) SDS concentrations and (B) buffer pH on the effective electrophoretic mobility in MEKC. The running buffers were (A) 0.04 *M* phosphate (pH 10.6) with various amounts of SDS and (B) 0.04 *M* phosphate containing 40 m*M* SDS with various pH values. The voltage was 10 kV and the currents were (A) 46–67 μA for 20–100 m*M* SDS and (B) 27–56 μA at pH 6.0–11.4. Solute numbers as in Table 1.

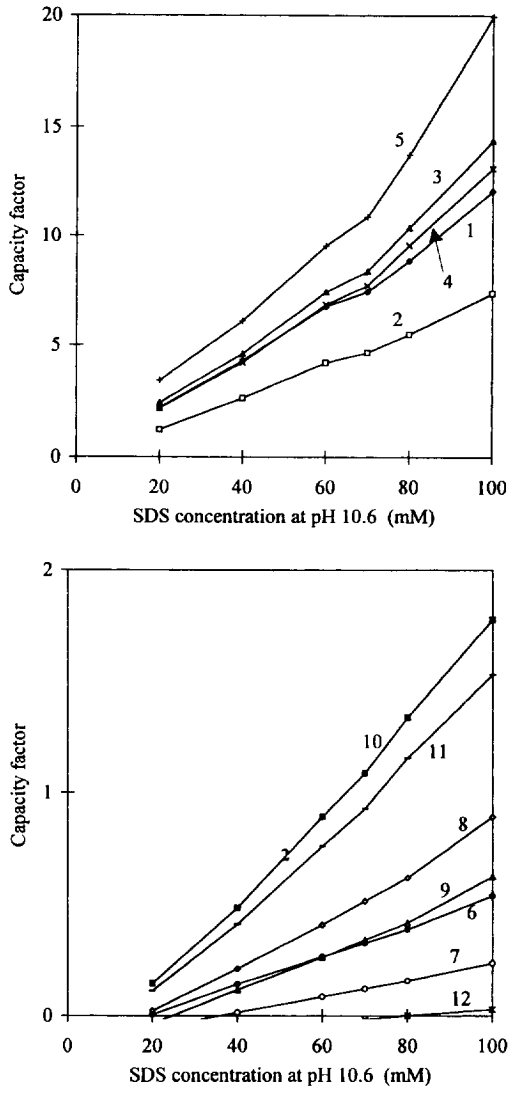

Fig. 5. Plots of capacity factors against SDS concentration at pH 10.6. The capacity factor was calculated according to Eq. (3) using the mobility data given in Fig. 4A. Solute numbers as in Table 1.

literature ($-5.38 \cdot 10^{-8}$ m^2/s V [27]). The data presented in Fig. 5 reveal that several anions (solutes 7, 9, 12) can have a negative k'. Negative k' values of anions have been reported previously [29]. They occur because μ_0 and μ_s were measured in media of different ionic strengths and different viscosities (η increased ~1% per 10 m*M* SDS; μ_s decreased as ionic strength and viscosity increased). If the interaction between the solute and micelles becomes too weak, the absolute value of μ_s may be smaller than that of μ_0, this resulting in a negative k'. Thus, for precise calculation of k', the ionic strength should be taken into account. Unfortunately, no theoretical model describing ionic strength correction for micellar solutions is available.

The distribution coefficient K for each opioid at pH 10.6 can be estimated by linear regression analysis of the data presented in Fig. 5 (cf., Eq. (3)). These data and those obtained at other pH values are presented in Fig. 6. All correlation coefficients were within 0.9795–0.9997. The magnitude of K is directly related to the elution order and can change drastically with pH. K values of the free morphinoids increased about two orders of magnitude upon conversion from the anionic form (pH 11.4) to the cationic form (pH 6.0). Due to the blocking of the phenolic group, a smaller change (half to one order of magnitude) was observed for the free codeinoids which are neutral at pH 11.4 and become gradually positively charged as the pH is lowered to pH 6.0. A similar increase in K has been reported for other solutes changing from neutral substances to cations [27] or from anions to neutrals [27,29]. K values of the glucuronides were found to change very little (2–3 times) over the pH range (6.0–11.4), a range in which these solutes are negatively charged (Fig. 1). Codeinoids are shown to be characterized by larger K values than the morphinoids. The higher pK_a values and larger molecular sizes of the codeinoids

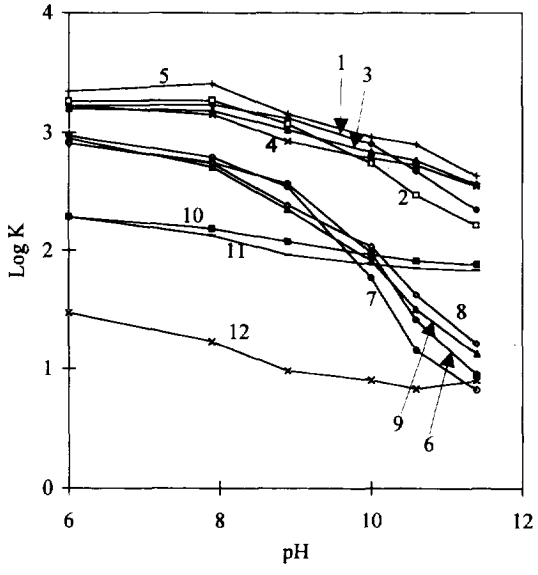

Fig. 6. Distribution coefficient as function of pH. K values represent the slope obtained by linear regression analysis of k' vs. C_{SDS} data at specified pH values (cf., Eq. (3)). The applied voltage for all the experiments was 10 kV and the currents were 19–70 μA, depending on pH and C_{SDS}. Solute numbers as in Table 1.

are attributed for that behavior. Furthermore, the elution order (related to K) among the free codeinoids or the free morphinoids changes as function of pH (Fig. 4B and Fig. 6). At pH>10, DHC, COD, DHM and MOR were determined to be more strongly incorporated into the dodecyl sulfate micelle compared to their corresponding N-demethylated compounds NDHC, NCOD, NDHM and NMOR, respectively. The opposite is true for pH< 10. When pH>8, NMOR, MOR, NCOD and COD had smaller values of K than their saturated counterparts NDHM, DHM, NDHC and DHC, respectively. The opposite order was observed for pH<8.

Fig. 7A shows that all the 12 solutes could be fully separated having 80 mM SDS at pH 10.6. Due to strong electrostatic attraction full separation could not be achieved at pH 6.0 (Fig. 7B). Furthermore, at pH 2.2 all the solutes were completely positively charged and the separation became even worse (Fig. 7C). At this low pH, electroosmosis is too weak and incapable of transporting the micelles towards the cathode. Thus, for registration of the data presented in Fig. 7C, the polarity of the electric field had to be changed. It should be noted that this configuration corresponds to case VIII according to the general classification of MEKC modes [31]. In case VIII the solutes elute in the order of decreasing capacity factors. In Fig. 7C EMOR is first detected and MOR3G is the last peak, which is opposite to that seen Fig. 7A and Fig. 7B (normal mode of MEKC, case IV [31]). Case VIII can be useful to shorten the detection time for those solutes that are strongly incorporated into the micelles. This can be achieved either at low pH (<5) [20,32–34] (Fig. 7C) or by increasing surfactant concentration when micelles migrate opposite to electroosmosis [34].

3.4. Electrokinetic chromatography in binary medium

The configuration shown in Fig. 7C was further explored via addition of ethylene glycol. Large amounts of organic solvents may result in disruption of micelles [35]. In this case single surfactant ions instead of micelles act as the carrier and the separation mode is ionic electrokinetic chromatography [36]. Whether there be micelles or monomers, however, the interaction of solutes with surfactants can

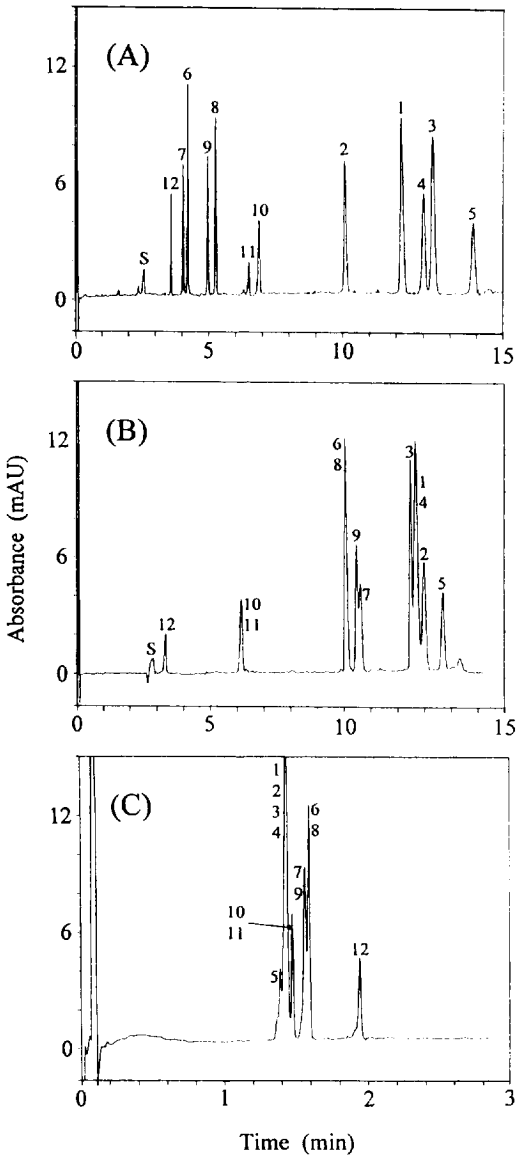

Fig. 7. MEKC electropherograms obtained with (A) 80 m*M* SDS at pH 10.6, (B) 40 m*M* SDS at pH 6.0 and (C) 40 m*M* SDS at pH 2.2. The phosphate concentration in the running buffers was 0.04 *M*. The applied voltages were (A,B) 10 kV and (C) −20 kV and the currents were (A) 61 μA, (B) 27 μA and (C) 71 μA. Peak identification as for Fig. 2.

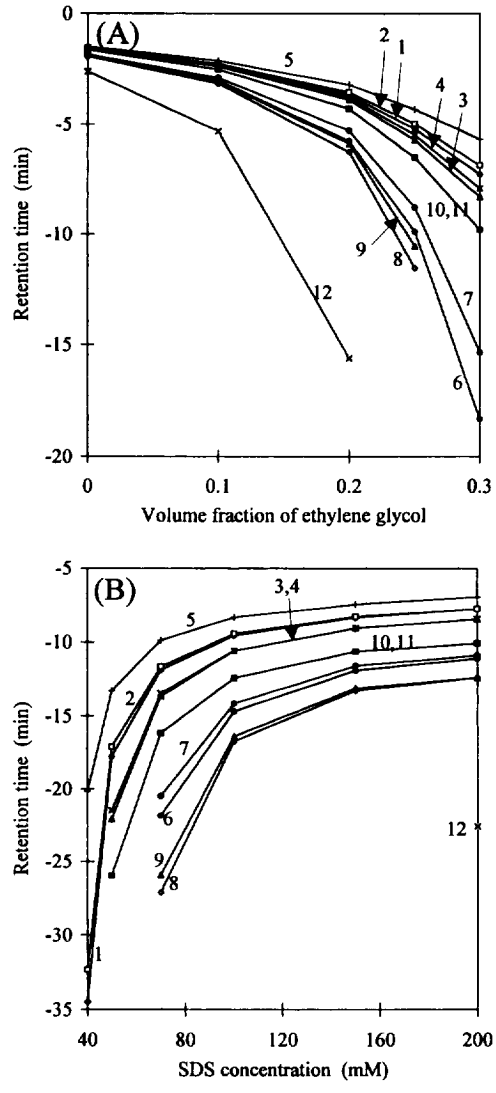

Fig. 8. Effects of (A) volume fraction of ethylene glycol and (B) SDS concentration on the retention time in binary EKC at pH 2.2. The SDS concentration for (A) was 20 m*M* and the volume fraction of ethylene glycol for (B) was 0.5. The running buffers always contained 0.04 *M* phosphate (pH 2.2). The applied voltage was −20 kV and the currents were (A) 64–25 μA for 0–30% (v/v) ethylene glycol and (B) 19–60 μA for 50–200 m*M* SDS. Solute numbers as in Table 1.

be reduced with organic solvents and the retention behavior can also be explained with the concept of capacity factor. As is shown with the data presented in Fig. 8A, increasing amounts of ethylene glycol lead to improvement of separation via weakening of the solute–surfactant interactions. Like mobility, the signs of retention time (t_R) also indicate the migration directions of solutes [31,32]. The negative t_R means that the solute migrates toward the positive electrode. As for a case VIII configuration, solutes

elute in the order of decreasing capacity factors [31]. With 30% (v/v) ethylene glycol the capacity factor became so small that MOR3G was not detected within 40 min of power application (Fig. 8A). Increasing the SDS concentration can result in larger capacity factors and thereby shorter detection times (absolute value of t_R), as is shown with the data depicted in Fig. 8B. With 50% ethylene glycol and 40 m*M* SDS only EMOR, NCOD and NDHC were detectable within 60 min. Other solutes migrated too slowly or toward the negative electrode. When the SDS concentration was increased to 200 m*M*, all solutes could be observed within 23 min. The detection time decrease observed in conjunction with an SDS concentration increase is opposite to that encountered in the normal MEKC mode (case IV [31]). Typical electropherograms obtained in binary medium at pH 2.2 are shown in Fig. 9. Generally, a larger amount of ethylene glycol and a lower SDS concentration led to better separation but made the detection time longer.

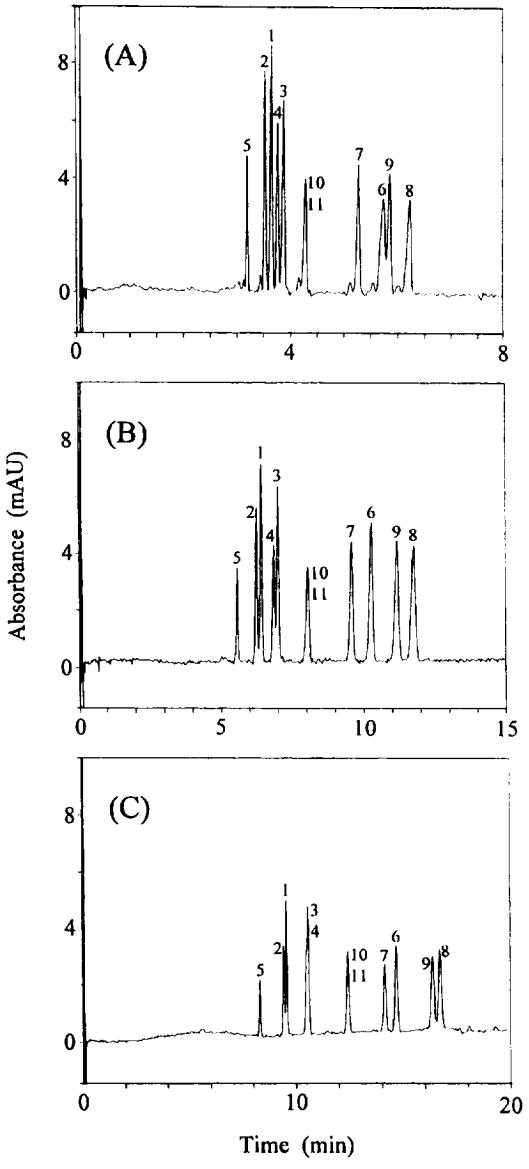

Fig. 9. EKC chromatograms obtained in binary buffers containing 0.04 *M* phosphate (pH 2.2). The volume fraction of ethylene glycol and SDS concentrations were (A) 0.2 and 20 m*M*, (B) 0.35 and 40 m*M* and (C) 0.5 and 100 m*M*, respectively. The voltage was −20 kV and the currents were (A) 33 μA, (B) 26 μA and (C) 30 μA. Peak identification as for Fig. 2.

4. Conclusions

Free codeinoids are shown to be positively charged below about pH 10 and are neutral at higher pH values, whereas free morphinoids are amphoteric compounds with p*I* values between about 9 and 10. Furthermore, glucuronides are ampholytes with p*I* values around 5. Thus, buffer pH is the most important factor for the separation of opioids by both CZE and EKC. Separability in CZE is best at a pH around the pK_a or p*I*. Furthermore, in EKC with dodecyl sulfate and at low pH, ethylene glycol is shown to be an efficient agent for improvement of the separation of positively charged compounds which otherwise interact (electrostatic attraction) too strongly with the negatively charged surfactant. All 12 test solutes can be fully separated by aqueous MEKC at pH 10.6 and having 70–100 m*M* SDS. Compared to the buffer used previously (pH 9.2; 75 m*M* SDS [14]), this system provides quicker separations (and therefore faster runs). For complete resolution of the investigated opioids without SDS, no aqueous or binary medium with ethylene glycol was found. However, for metabolic investigations after administration of DHC, DHC and its metabolites NDHC, DHM, NDHM and DHC6G can also be separated by (A) binary CZE at pH 7.9 with 40–60% ethylene glycol, (B) binary EKC at pH 2.2 with 50% ethylene glycol and 70–200 m*M* SDS, (C) binary EKC at pH 2.2 with 30–40% ethylene glycol and 40

m*M* SDS and (D) binary EKC at pH 2.2 with 20% ethylene glycol and 20 m*M* SDS. The latter three systems (B–D) also apply to the separation of COD and its metabolites NCOD, MOR, NMOR, COD6G and MOR3G and might also be applicable to metabolic investigations with other opioid drugs, including ethylmorphine, hydrocodone and hydromorphone. Further work dealing with the determination of opioids and their metabolites in body fluids is currently under way.

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