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Migration behaviour of benzodiazepines in micellar electrokinetic chromatography

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Abstract

The migration behaviour of a series of benzodiazepines in micellar electrokinetic chromatography was studied using three kinds of surfactants, i.e. sodium dodecyl sulphate (SDS), dodecyl trimethylammonium bromide (DoTAB) and bile salts. With SDS and DoTAB, retention factors were found to be too high to obtain adequate resolution. Both bile salts used in this study, sodium cholate and sodium deoxycholate, provided a satisfactory range of retention factors. Moreover, selectivity of the separation could be manipulated by combining these two bile salts in varying ratios.

Keywords: Benzodiazepines; Sodium cholate; Sodium deoxycholate; Surfactants

1. Introduction

The analysis of benzodiazepines (BDZs) has been extensively studied. Originally, BDZs were analysed by UV spectrophotometry but now the analysis of BDZs in pharmaceutical formulations is routinely carried out by liquid chromatography (LC) [1]. The determination of BDZs and their metabolites in body fluids has been studied by different separation techniques including thin-layer chromatography (TLC), gas chromatography (GC) and LC [2–4]. However, few reports have been specifically dedicated to BDZ analysis by capillary electrophoresis (CE) techniques [5–8]. These contributions deal with the use of sodium dodecyl sulphate (SDS) as a micelle forming surfactant, in combination with small amounts of organic modifiers in the separation buffer. In this study, the migration behaviour of a series of BDZs (Fig. 1) in micellar electrokinetic chromatography (MEKC) is evaluated with different kinds of surfactants, i.e. SDS, dodecyl trimethylammonium bromide (DoTAB) and the bile salts, sodium cholate (SC) and sodium deoxycholate (SDC). Using a mixture of the bile salts the separation of the BDZs is achieved without the need to add an organic modifier.

2. Experimental

Electrokinetic chromatography was performed on a P/ACE 2100 (Beckman, Palo Alto, CA, USA). Fused-silica capillaries (Beckman) were 0.075 mm I.D., 0.375 mm O.D., and, unless otherwise stated, 57 cm long, with the detection window at 7 cm from the capillary outlet. The temperature of the capillaries was controlled by a liquid coolant.

Fourteen benzodiazepine derivatives (bromazepam, flunitrazepam, clobazam, nitrazepam,

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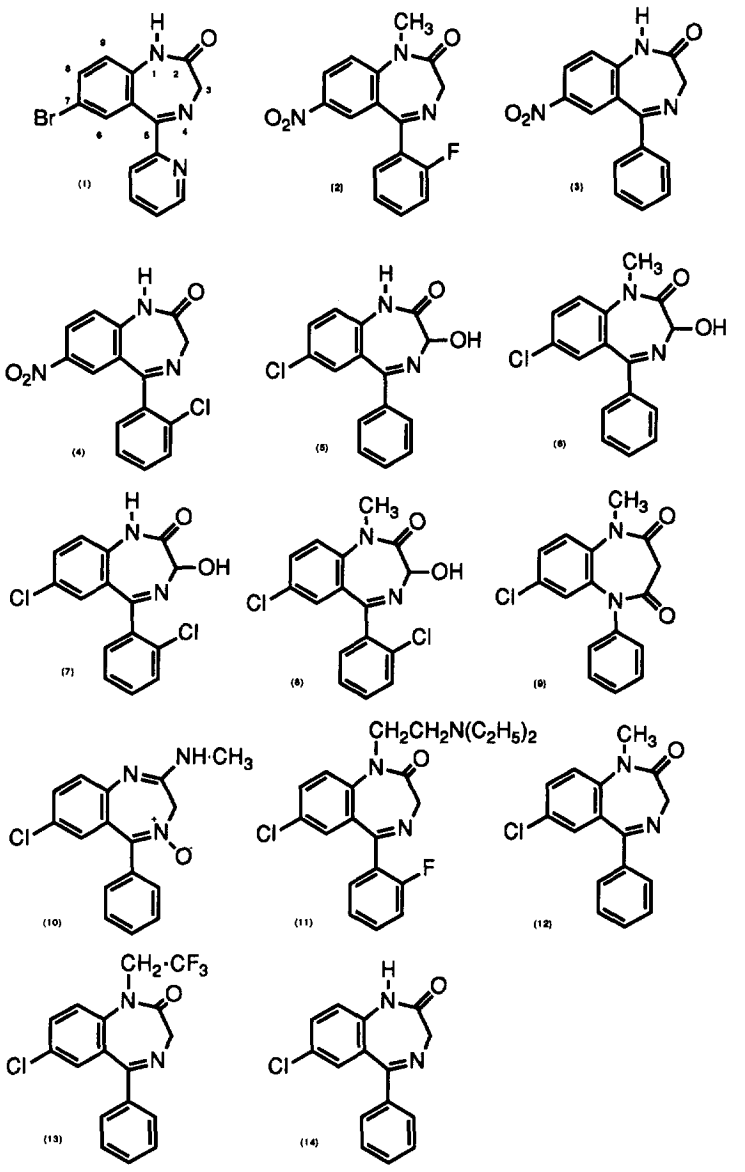

Fig. 1. The structure of benzodiazepines used in this study; the identification numbers are given in Table 1.

clonazepam, chlordiazepoxide, temazepam, lorazepam, flurazepam, diazepam, halazepam, oxazepam, lormetazepam and nordazepam) were donated by different companies marketing these drugs in Belgium.

Buffers were prepared in deionized water (Seralpur Pro 90 CN, Seral, Germany). The required pH was obtained by mixing an appropriate volume of 20 m*M* sodium borate solution and either 20 m*M* sodium dihydrogen phosphate solution or 20 m*M* sodium hydroxide solution. Varying amounts of the surfactants SDS, SC, SDC or DoTAB (all from Sigma, USA) were added to the buffer solutions at the concentration specified. Before use, these solutions were filtered through a 0.2-μm membrane. Stock solutions of each BDZ were prepared in a mixture of acetonitrile and water (1:1, v/v) at a concentration of 1 mg/ml and diluted in the sepa-

ration buffer to a concentration of 5 μg/ml before injection. Formamide (at 214 nm) or acetone (at 254 nm) was added as an EOF-marker, and dodecanophenone as the t_{mc}-marker.

Samples were introduced by pressure during 2 s, analysed with an applied voltage of 20 kV at 33°C and detected at either 214 nm (with SDS, SC and SDC), or 254 nm (with DoTAB). In between runs, the capillary was rinsed for 2 min with buffer and left equilibrating for 5 min.

3. Results and discussion

Most of the benzodiazepines studied are 1,4-benzodiazepines, except for clobazam which has a 1,5-benzodiazepine structure. Although hydrophobic, 1,4-benzodiazepines are ionogenic. They possess weak basic properties due to the nitrogen atom at position 4 that can be protonated, except for chlordiazepoxide which is a 4-N-oxide-derivative [9,10]. The pK_a-values of the protonation of N4 of 1,4-benzodiazepines are between 1.5–3.5 [9]. In this study, only neutral to alkaline pH conditions are used, and therefore most benzodiazepines are effectively non-ionic. However, some have additional acid–base features which must be considered. The presence of a nitro-group at position 7 of some 1,4-benzodiazepines (nitrazepam and clonazepam) causes an acidification of the N1 and therefore, unless substituted as in flunitrazepam, they have a second pK_a around 10.5 [9,11]. Oxazepam, temazepam, lorazepam and lormetazepam are 3-hydroxy-derivatives, but only oxazepam and lorazepam exhibit weak acid properties due to the non-substitution at N1, and can be deprotonated at higher pH values. The second pK_a value is around 11.5 [11]. Flurazepam contains a tertiary amino side chain group which exhibits strong basic property providing the second pK_a of 8.2. The available pK_a and log P (octanol/buffer, pH 7.4) of some BDZs are listed in Table 1.

The migration behaviour of the benzodiazepines is compared among buffers of different pH, ionic strengths and electroosmotic velocities. Therefore, so-called apparent capacity factors were used to standardize the retention of these compounds in each system.

Table 1
The pK_a and log P values of benzodiazepines used in this study according to [13]

Benzodiazepine	pK_a	Log P (octanol/buffer, pH 7.4)
1 Bromazepam	2.9, 11.0	1.6
2 Flunitrazepam	1.8	–
3 Nitrazepam	3.2, 10.8	2.1
4 Clonazepam	1.5, 10.5	2.4
5 Oxazepam	1.7, 11.6	2.2
6 Temazepam	1.6	–
7 Lorazepam	1.3, 11.5	2.4
8 Lormetazepam	–	–
9 Clobazam	–	–
10 Chlordiazepoxide	4.6	2.5
11 Flurazepam	1.9, 8.2	2.3
12 Diazepam	3.3	2.7
13 Halazepam	–	–
14 Nordazepam	3.5, 12.0	–

Apparent capacity factors (k'_{app}) were calculated using Eq. (1), which was originally derived by Terabe [12] for neutral compounds:

$$k'_{app} = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} \quad (1)$$

Capacity factors (k') were calculated by Eq. (2) as Khaledi et al. [13] described for anionic compounds:

$$k' = \frac{t_r - t_{ion}}{t_{ion}(1 - t_r/t_{mc})} \quad (2)$$

where t_r and t_{ion} are the migration times of the solute in the presence and absence of the micelle, respectively, and t_0 and t_{mc} are the migration times of the EOF-marker and the micelle-marker, respectively.

Unlike real capacity factors, apparent capacity factors of ionized species are affected by the ion mobility in the aqueous phase, which contributes as a pseudo-retention. Although apparent capacity factors are for that reason less fundamental in nature, their main advantage is that, for the sake of comparison, the electroosmotic flow variation in different systems can be eliminated without necessitating the measurement of t_{ion} for each ionizable compound in each system.

3.1. Elution of benzodiazepines with SDS

The migration profile of the set of BDZs was

examined with a buffer containing 50 m*M* SDS in the pH range 7–11. They eluted over a very wide range of k'_{app} values varying from 8 to ∞, proving their important solubilization by SDS micelles, and hence the considerable hydrophobicity of this class of drugs. All values remain rather unaffected by the pH. The lowest k'_{app} values (<25) were observed for bromazepam and the three 7-nitro-derivatives. All substances eluting after this group are characterized by a 7-Cl substitution. No elution was observed for the cationic flurazepam. Such high capacity factor values can not be considered appropriate according to Foley [14] and Ghowsi [15] who found that k' values between 2 and 5 are optimal in terms of resolution. Although organic modifiers can be added to the buffer to reduce capacity factors, this leads to prolonged analysis times [6]. Therefore, no further attempt was made to manipulate the selectivity of the separation with SDS.

3.2. Elution of benzodiazepines with DoTAB

Cationic surfactants have been reported to yield lower capacity factors with moderately large and hydrophobic molecules [16,17]. Selectivity changes can be expected for ionic compounds when their charge is opposite to that of the micelles.

Apparent capacity factors of neutral BDZs obtained with DoTAB (Fig. 2) were indeed lower than those found with SDS at the same concentration, but still considered too high. In this case, however, more pronounced pH effects are observed for the anionogenic species like nitrazepam, clonazepam, oxazepam and lorazepam, which are strongly retarded at higher pH values.

3.3. Elution of benzodiazepines with bile salts

Bile salts exhibit both hydrophilic and hydrophobic faces [18,19] which lower the polarity difference between the aqueous and the micellar phase. This characteristic was exploited to improve the separation of hydrophobic molecules [19–21]. The disadvantages of bile salts are that they contain some impurities and exhibit rather high absorbance at low UV wavelength ($\lambda \leq 210$ nm). The unconjugated carboxylates, like SC and SDC, can only be used in neutral to alkaline solution [22]. Bile salts are chiral

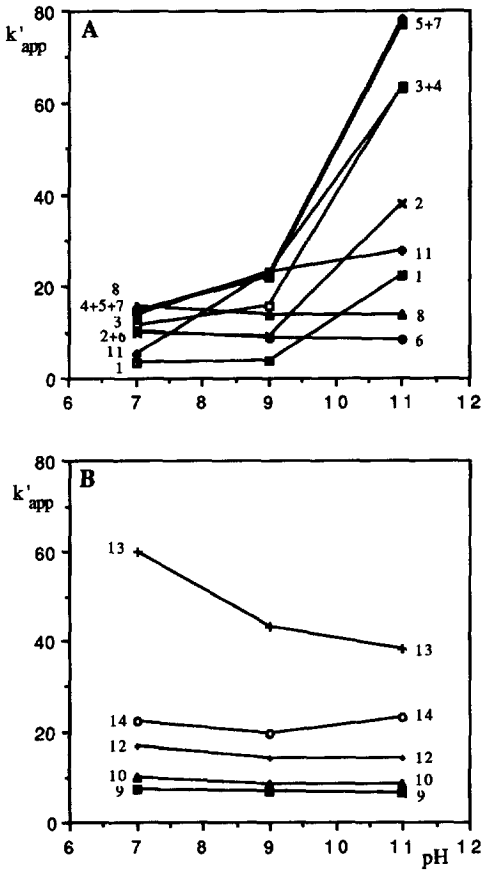

Fig. 2. Apparent capacity factors of BDZs as a function of pH with DoTAB (50 m*M* in 20 m*M* borate or borate–phosphate, other conditions see Section 2).

and can interact differently with racemates. Among the BDZs studied, the 3-hydroxy-derivatives (oxazepam, lorazepam, temazepam and lormetazepam) are racemic. Peak broadening and even peak splitting have been observed for these compounds when SC or SDC was used at low temperature [23]. Since a temperature of 33°C was found to suppress the chiral recognition [23], this temperature was applied throughout all experiments, unless otherwise stated.

SDC, the deoxy-form of SC, is more hydrophobic than SC [20]. This is in accordance with our observations: the apparent capacity factors of the BDZs are consistently higher (approximately a factor of 2) with SDC than with SC, at equivalent molar concentration (Fig. 3 and Fig. 4). In the pH range 7–9, only small variations in the apparent retention

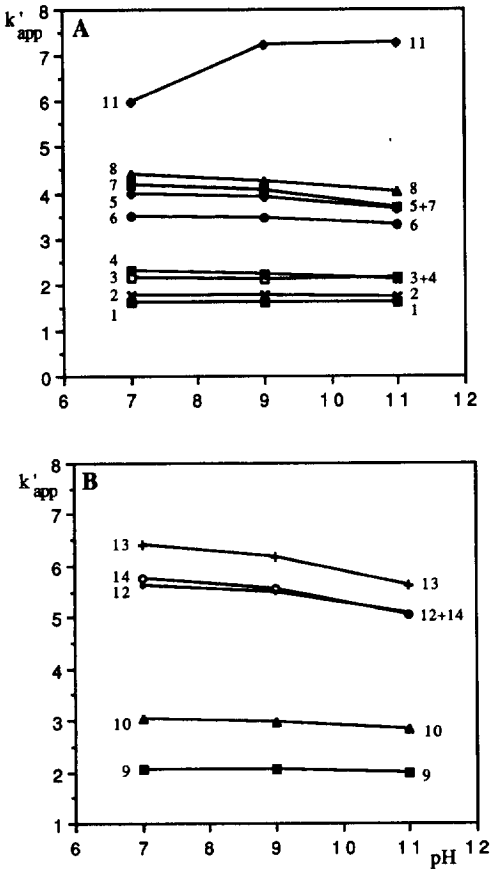

Fig. 3. Apparent capacity factors of BDZs as a function of pH with SDC (50 m*M* in 20 m*M* borate or borate–phosphate, other conditions see Section 2).

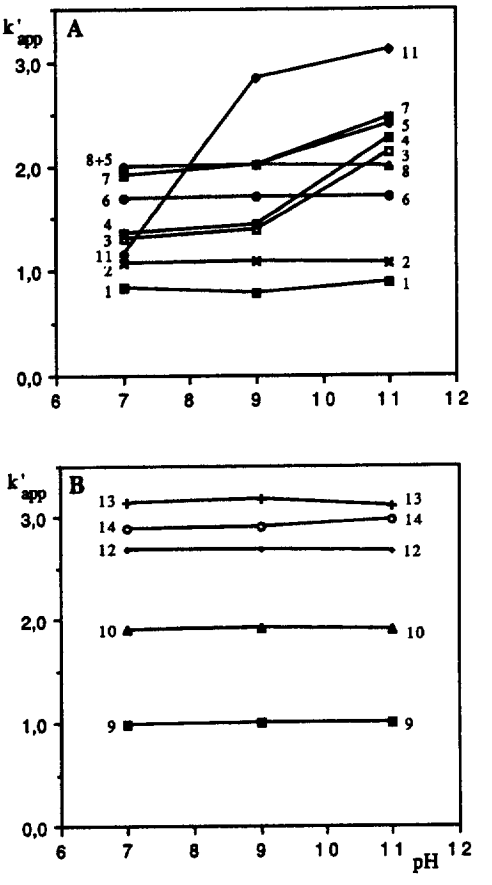

Fig. 4. Apparent capacity factors of BDZs as a function of pH with SC (50 m*M* in 20 m*M* borate or borate–phosphate, other conditions see Section 2).

factors are observed, except for flurazepam. However, with SC at pH 11, the ionization of oxazepam, lorazepam, nitrazepam and clonazepam becomes apparent. Although for these compounds a similar retardation effect was observed with DoTAB, the reasons for this behaviour with bile salts is different. With DoTAB, ion-pair association between the cationic micellar surface and the increased fraction of anions obviously plays a role, but this cannot be the case with the anionic bile salts.

For ionic compounds, the net mobility (corrected for the electroosmotic flow) can be expressed in the following way:

$$\mu_{ep} = (f_{HA,mc} + f_{A^-,mc})\mu_{mc} + f_{A^-,aq}\mu_{A^-}$$

where f represents the fraction of the species HA or A^- in the micellar (mc) or aqueous (aq) phase. When a larger fraction is ionized, $f_{HA,mc}$ is reduced and this is not compensated by an increase in $f_{A^-,mc}$ (charge repulsion). However, for low capacity factors, the contribution of μ_{mc} is already small. On the other hand, the increased contribution from $f_{A^-,aq}$ (at the expense of $f_{HA,aq}$ which does not contribute to the mobility) is strongly felt, as is seen with SC. In the case of SDC, the capacity factors are higher and some compensation occurs between the changes in the contributions from μ_{A^-} and μ_{mc} and therefore the pH effect is less well observed.

Strong pH effects are also observed with flurazepam which is positively charged at pH 7. Clearly, this compound is less tightly associated with the bile salt micelles than it was with SDS. The

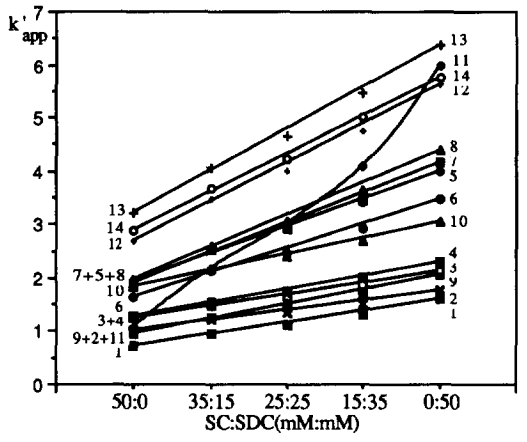

Fig. 5. Apparent capacity factors of BDZs in a 20 m*M* borate–phosphate buffer (pH 7) containing different proportions of SC and SDC, other conditions see Section 2.

effects of the cathodic migration in the aqueous phase can be observed in the lower apparent capacity factors.

With either SC or SDC, some BDZs remained unseparated. Manipulation of the selectivity by using a mixture of the two bile salts was already investigated [24]. It was found that at pH 7, and with the exception of flurazepam, there was an almost linear relationship between the apparent capacity factor of BDZs and the proportion of the two bile salts (Fig. 5). In this way, by selecting a bile salt ratio, the selectivity for some BDZs can be manipulated without extending the analysis time. From these data, an optimal surfactant system for a particular mixture can be predicted. Almost all BDZs could be separated from each other by one of those conditions, except for oxazepam and lorazepam which eluted close to each other in all of the conditions examined.

Fig. 6 shows the complete separation of 13 out of the 14 BDZs investigated. The separation was obtained with a 20 m*M* borate–phosphate buffer (pH 7), containing 35 m*M* SDC and 15 m*M* SC, operated at an applied voltage of 20 kV and a temperature of 35°C. A capillary of 87 cm total length was used to reduce the effect of the injection plug on efficiency, and thus resolution. Accordingly, the temperature was raised to 35°C to suppress the chiral recognition of the 3-hydroxy-BZDs, [23]. Nevertheless, the members of this group (compounds **5–8** in Fig. 1) still exhibit broader peaks than the other compounds. Under these conditions only oxazepam and lorazepam are not separated. The repeatability was evaluated by performing six successive injections of a sample mixture of 11 BDZs at a concentration of 10 μg/ml each. The excluded compounds were oxazepam which co-eluted with lorazepam, and lormetazepam and nordazepam that showed the smallest resolution with lorazepam and diazepam, respectively. High repeatability of the migration times was observed (R.S.D.<0.2%). For the short injection time used (2 s) the variation of peak areas is significantly reduced by using an internal standard (clonazepam). The relative standard deviation of the

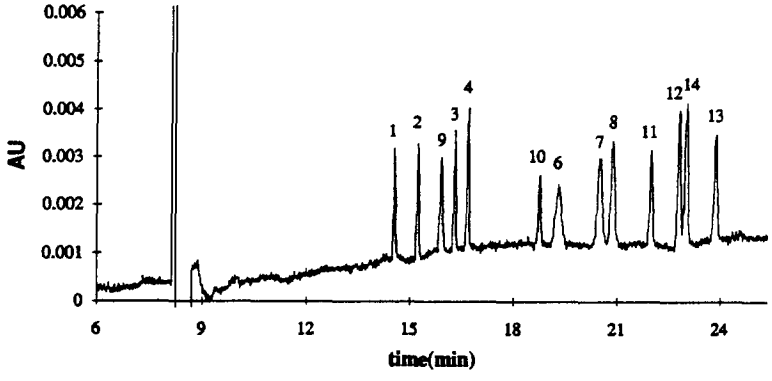

Fig. 6. MEKC separation of 13 BDZs buffer, 20 m*M* borate–phosphate (pH 7) containing 15 m*M* SC+35 m*M* SDC; fused-silica capillary, 87 cm total length, temperature 35°C, other conditions see Section 2.

peak area ratios were better than 5%. Calibration of the 11 baseline resolved BDZs in the concentration range 5–50 μg/ml showed good linearity of the peak area ratios as a function of concentration ($r>0.999$) with an intercept of the regression line not significantly different from zero.

An alternate method was developed for the separation of the group of 3-hydroxy BDZs. Oxazepam and lorazepam showed partial separation only with SC at pH 11. Fig. 7A shows the apparent capacity factors of the four derivatives as a function of pH of the buffer containing 50 m*M* SC. It is illustrative how the erratic changes of the apparent capacity factors of oxazepam and lorazepam, resulting from partitioning as well as ionization effects, are removed by calculating the real capacity factors (Fig. 7B). In spite of the longer migration times at pH>9, also expressed in larger apparent capacity factors, smaller capacity factors are indeed calculated for oxazepam and lorazepam, reflecting the electrostatic repulsion between the anionic solutes and the anionic micelles.

A separation buffer of pH 10.2 was then chosen for the investigation of the effect of organic modifier. Results show that an organic solvent such as acetonitrile reduced the electrophoretic mobilities of the negatively charged compounds (i.e. oxazepam and lorazepam) to a smaller extent than the neutral ones (i.e. temazepam and lormetazepam) (Fig. 8). A possible reason is that the organic solvent causes the pH of the buffer to increase [25] and therefore oxazepam and lorazepam became more ionized and are retarded in the capillary. The complete separation of the four 3-hydroxy-benzodiazepines was already reported before [23].

It must be noted that for particular mixtures of BDZs, simple buffer systems can be used, with omission of the organic modifier. Baseline resolution of diazepam and nordazepam was obtained with an adapted buffer system of 20 m*M* borate buffer (pH 9) containing 60 m*M* SC. This system was used to determine the drug diazepam and its metabolites

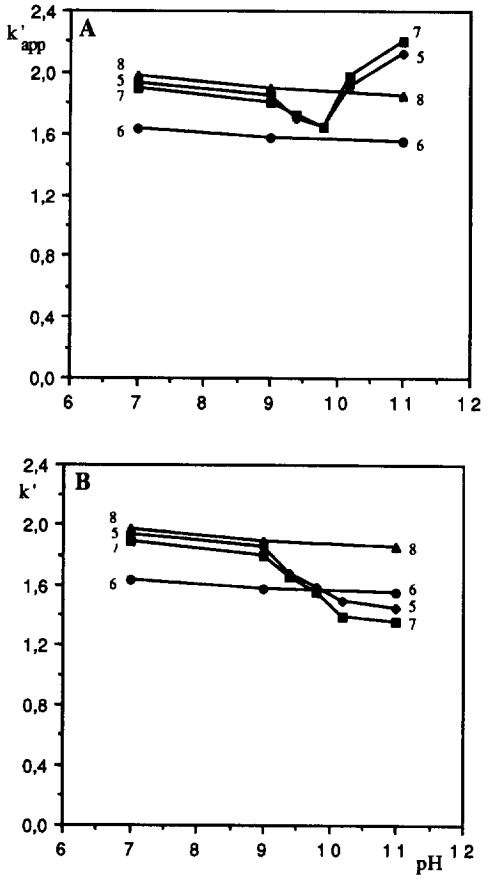

Fig. 7. Apparent capacity factors (A) and capacity factors (B) of 3-hydroxy-benzodiazepines as a function of pH of the buffer containing 50 m*M* SC; other conditions see Section 2.

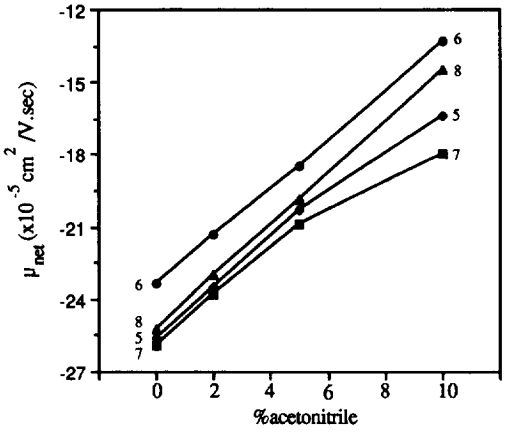

Fig. 8. Electrophoretic mobilities of 3-hydroxy-benzodiazepines as a function of % acetonitrile in the separation buffer of 20 m*M* borate (pH 10.2) containing 50 m*M* SC; other conditions see Section 2.

(nordazepam, oxazepam and temazepam) in spiked plasma [26].

4. Conclusion

The advantages of bile salts over long hydrocarbon chain surfactants for the MEKC-separation of benzodiazepines have been described. Separation selectivity can be controlled by using mixtures of SC and SDC at pH 7.0, without any other additives. Only for the separation of oxazepam and lorazepam, the addition of 10% acetonitrile and a different pH (10.2) were required. The method for the separation of 11 benzodiazepines shows high reproducibility of the migration times and acceptable reproducibility of the peak areas when an internal standard method was applied.

References

[1] USP XXIII, United States Pharmacopoeia Conventional, 1995.
[2] J.M. Clifford and W.F. Smyth, Analyst, 99 (1974) 241.
[3] M. Chiarotti, N. De Giovanni and A. Fiori, J. Chromatogr., 358 (1986) 169.
[4] L.A. Berrueta, B. Gallo and F. Vicente, J. Pharmaceut. Biomed. Anal., 10 (1992) 109.
[5] I.M. Johansson, R. Pavelka and J.D. Henion, J. Chromatogr., 559 (1991) 515.
[6] M. Schafroth, W. Thormann and D. Allemann, Electrophoresis, 15 (1994) 72.
[7] M. Tomita, T. Okuyama, S. Sato and H. Ishizu, J. Chromatogr., 621 (1993) 249.
[8] I. Bechet, M. Fillet, P. Hubert and J. Crommen, Electrophoresis, 15 (1994) 1316.
[9] H.J. Roth., K. Eger and R. Troschutz, in M.H. Rubinstein (Editor), Pharmaceutical Chemistry, Vol. 2, Drug Analysis, Ellis Horwood, UK, 1991.
[10] J.M. Clifford, W.F. Smyth, Z. Anal. Chem., 264 (1973) 149.
[11] A.C. Moffat, J.V. Jackson, M.S. Moss and B. Widdop, Clarke's Isolation and Identification of Drugs, Pharmaceutical Press, UK, 1986.
[12] S. Terabe, K. Otsuka and T. Ando, Anal. Chem., 57 (1985) 834.
[13] M.G. Khaledi, S.C. Smith and J.K. Strasters, Anal. Chem., 63 (1991) 1820.
[14] J.P. Foley, Anal. Chem., 62 (1990) 1302.
[15] K. Ghowsi, J.P. Foley and R.J. Gale, Anal. Chem., 62 (1990) 2714.
[16] D.E. Burton, M.J. Sepaniak and M.P. Maskarinec, J. Chromatogr. Sci., 25 (1987) 514.
[17] D. Crosby and Z. El Rassi, J. Liq. Chromatogr., 16 (1993) 2161.
[18] H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, J. Pharm. Sci, 79 (1991) 519.
[19] H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, J. Chromatogr., 498 (1990) 313.
[20] H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, J. Chromatogr., 513 (1990) 279.
[21] R.O. Cole, M.J. Sepaniak, W.L. Hinze, J. Gorse and K. Oldiges, J. Chromatogr., 557 (1991) 113.
[22] H. Nishi and S. Terabe, J. Pharmaceut. Biomed. Anal., 11 (1993) 1277.
[23] S. Boonkerd, M.R. Detaevernier, Y. Michotte and J. Vindevogel, J. Chromatogr. A, 704 (1995) 238.
[24] S. Boonkerd, M. Lauwers, M.R. Detaevernier and Y. Michotte, J. Chromatogr. A, 695 (1995) 97.
[25] W. Schutzner and E. Kenndler, Anal. Chem., 64 (1992) 1991.
[26] S. Boonkerd, A. Moens, J. Vindevogel, M.R. Detaevernier and Y. Michotte, to be submitted.