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Optimized micellar electrokinetic chromatographic separation of benzodiazepines

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Abstract

Although capillary zone electrophoresis is an inadequate method for the separation of electrically neutral benzodiazepines, their separation can be achieved by micellar electrokinetic chromatography (MEKC). The cyclodextrin (CD)-modified MEKC separation of nine benzodiazepines was obtained within 24 min at 15 kV on a 470 mm × 75 μm I.D. silica capillary at 30°C using 50 m*M* borate–50 m*M* sodium dodecyl sulfate–20 m*M* γ-CD–2 *M* urea (pH 9.2) containing 1% of tetrahydrofuran as the running buffer. The effects of γ-CD concentration, urea concentration, percentage of organic solvent and applied voltage on the resolution were studied.

Keywords: Micellar electrokinetic chromatography; Buffer composition; Optimization; Benzodiazepines; Cyclodextrins

1. Introduction

Benzodiazepines (BZDs) are for the most part psychotropic substances used in the management of many psychiatric disorders [1]. The determination of benzodiazepines in biological fluids requires specific and sensitive techniques that can detect not only the parent drug but also its metabolites, which are often pharmacologically active [2].

A number of methods, particularly immunochemistry, spectrophotometry, fluorimetry, gas chromatography (GC), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), have been reported for the analysis of benzodiazepines [1–3]. Immunochemical techniques allows benzodiazepine-like materials to be detected but they do not discriminate between the parent drug and its metabolites. UV spectrophotometric and fluorimetric methods lack specificity and sensitivity. Although fairly sensitive, GC is time consuming and often requires prederivatization or prehydrolysis of the sample. Among these techniques, HPLC in the reversed-phase (RP) mode seems to be the most suitable for the analysis of thermally labile compounds [3].

It is well established that capillary electrokinetic techniques such as capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) allow the highly efficient separation of minute amounts of many classes of compounds such as pharmaceutical drugs [4]. However, like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [5], benzodiazepines are mostly neutral and of

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similar hydrophobicity. Hence a good separation of these substances by conventional CZE cannot be expected. It is then necessary to extend the concept of using a mobile phase and a pseudo-stationary phase to the utilization of buffer modifiers such as cyclodextrins (CDs), organic solvents and urea to improve the selectivity of the separation [6].

This paper describes the CZE and CD–MEKC separations of nine benzodiazepines in aqueous–organic solution. In addition, the effects of sodium dodecyl sulfate (SDS), β-CD, urea, organic solvents and applied voltage on the migration time window, the selectivity and the resolution were elucidated from the electrophoretic separation of some common standards. The usefulness of CD-modified MEKC as an analytical tool for the qualitative separation of benzodiazepines is demonstrated.

2. Experimental

2.1. Apparatus

All experiments were carried out on an HPCE P/ACE System 2000 instrument (Beckman, Palo Alto, CA, USA). Separations were performed on a 47 cm long (40 cm to the detector cell) × 75 μm I.D. fused-silica capillary tube (Beckman) with a built-in detection window. The UV absorption detector was set at 254 nm for CZE and 214 nm for CD–MEKC experiments. Samples were introduced pneumatically by application of 300 kPa pressure for 5 s. In all experiments a constant voltage was applied with the anode at the inlet and the cathode at the outlet side. The column temperature was kept constant at 30°C. Electrophoretic mobilities and peak areas were monitored using Beckman GOLD 6.01 software.

2.2. Chemicals

Alprazolam, bromazepam, chlordiazepoxide, clobazam, clonazepam, clorazepate, oxazepam, prazepam, triazolam and anthracene were purchased from Sigma (St. Louis, MO, USA). Sodium phosphate (NaH_2PO_4/Na_2HPO_4), boric acid and 1*M* sodium hydroxide (Prolabo, Rhône Poulenc, Manchester, UK) were of analytical-reagent grade. HPLC-grade distilled water (Baker, Deventer, Netherlands) was used to prepare buffers and standard solutions.

Sodium dodecyl sulfate (SDS) was obtained from Sigma, urea from Prolabo and γ-CD from Merck (Darmstadt, Germany). Acetonitrile, ethanol, methanol and tetrahydrofuran (Baker), were of high-purity grade.

2.3. Stock standard solutions

Drug stock standard solutions (1 g/l) were prepared in methanol, stored at 4°C and found to be stable for several months [3]. Anthracene solutions (1 g/l) were prepared in tetrahydrofuran.

2.4. Running buffers

For CZE, a buffer solution was prepared by mixing 0.04 *M* sodium dihydrogenphosphate–0.04 *M* sodium monohydrogenphosphate solution (pH 6.9) with 20% (v/v) acetonitrile. Methanol was used as an electroosmotic flow marker because it is poorly incorporated into the micelles and is successfully UV detected [7].

For CD–MEKC, a borate buffer (0.05 *M*) was prepared from boric acid by adding sodium hydroxide to reach pH 9.2. Running buffers consisted of 0.05 *M* borate buffer containing SDS (0.05 *M*), urea (2–4 *M*) and γ-CD (0–50 m*M*). Some buffers were modified with 1% (v/v) tetrahydrofuran. Prazepam ($C_{19}H_{17}ClN_2O$), the most lipophilic compound, was used as a micellar marker.

2.5. Choice of sample electrolyte

Hydrophobic compounds such as benzodiazepines do not dissolve in water and standard solutions are often prepared in methanol. When a sample is poorly soluble in methanol, 1% tetrahydrofuran (THF) or dimethyl sulfoxide (DMSO) is added to methanol.

According to Ackermans et al. [8], the addition of methanol to sample solutions does not

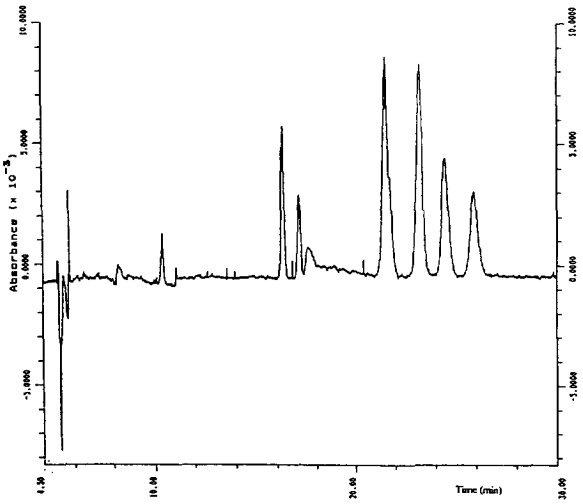


Fig. 1. CD–MEKC of a benzodiazepine mixture. Electrolyte, 50 m*M* SDS–50 m*M* γ-CD–2 *M* urea in 50 m*M* borate buffer (pH 9.2); sample solution, methanol–tetrahydrofuran–30 m*M* γ-CD solution (9:1:90, v/v/v); column, 470 mm × 75 μm I.D. fused silica; applied voltage, 15 kV; detection wavelength, 214 nm.

disturb the electroosmotic flow but can induce other consequences, e.g., a decrease in the migration time of solutes, distortion of micelles and loss of current. These effects become critical when the proportion of methanol reaches 30% and above. Further, injections of samples dissolved in pure organic solvent must be avoided in order to prevent electric field discontinuity and loss of current [9]. The electropherogram presented in Fig. 1 shows a good peak efficiency because the ionic strength of the sample buffer is lower than that of the running buffer. Samples of benzodiazepines were prepared in 30 m*M* γ-CD solutions from the stock standard solution at a concentration of 10 mg/ml.

All samples and solutions were filtered through a 0.22-mm Nalgene filter (Sybron, Rochester, NY, USA) before use in the capillary electrophoresis equipment.

The capillary column was conditioned by a pneumatic rinse with the separation buffer for 1 min immediately prior to injection. The column was washed with 0.1 *M* NaOH (1 min), then rinsed with water (1 min) between runs.

Table 1
pK_a and α values of benzodiazepines

Compound	pK_1	pK_2	α
Alprazolam	2.4		0.99
Bromazepam	2.9	11	0.99
Chlordiazepoxide	4.6		0.99
Clobazam	–	–	–
Clonazepam	1.5	10.5	0.99
Clorazepate	3.5	12.5	0.99
Diazepam	3.4	–	0.99
Nitrazepam	3.2	10.8	0.99
Oxazepam	–	–	–
Prazepam	2.7		0.99
Triazolam	–	–	–

3. Results and discussion

A 40 m*M* phosphate buffer (pH 6.9) containing 20% (v/v) acetonitrile was used as the running electrolyte to separate the benzodiazepine mixture by CZE.

In Table 1 are listed the p*K* values of benzodiazepines. Dissociation coefficients were calculated from the equation

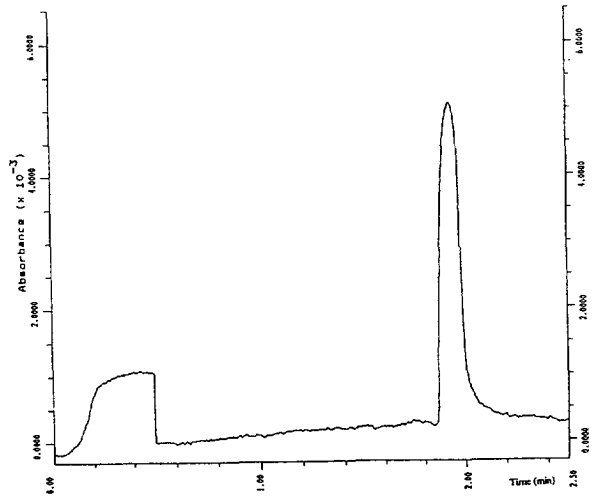


Fig. 2. Electropherogram of benzodiazepines. Column, 470 mm × 75 μm I.D. fused silica; applied voltage, 10 kV; detection wavelength, 254 nm; electrolyte, 40 m*M* phosphate buffer (pH 6.9)–20% (v/v) acetonitrile; current, 85 mA.

$$\alpha = \frac{1}{10^{(pK_1 - pH)} + 10^{(pH - pK_2)} + 1} \quad (1)$$

At pH around 7, benzodiazepines are dissociated and seem to be electrically balanced or neutral, like alprazolam, chlordiazepoxide and prazepam. As shown in Fig. 2, they electromigrate at the same velocity determined by the electroosmotic flow and no separation can be achieved.

In CD–MEKC, a neutral CD was added to the micellar solution. CDs will not interact with the micelles because of the hydrophilic nature of the outside surface and will migrate with the same velocity than that of the bulk solution. The migration velocity and hence the migration time of a hydrophobic compound depends on the partitioning of the solute between the CD and the micelle. This technique is limited to solutes which can fit into the CD cavity.

According to Terabe et al. [5], the capacity factor $\tilde{k}'$ of a highly hydrophobic solute in DC-MEKC is given by

$$\tilde{k}' = \frac{n_{mc}}{n_{CD}} = K \cdot \frac{V_{mc}}{V_{CD}} \quad (2)$$

where n_{CD} and n_{mc} are the total amount of solute included in the CD and the amount of the solute incorporated in the micelle, respectively, V_{CD} and V_{mc} are the volumes of CD and of the micelle, respectively, and K is the distribution coefficient.

The capacity factor is also calculated from migration time data by the following equation, similar to that derived for electrokinetic chromatography with micellar solutions [10]:

$$\tilde{k}' = \frac{t_R - t_0}{\left(1 - \frac{t_R}{t_{mc}}\right) t_0} \quad (3)$$

where t_R, t_0 and t_{mc} are the migration times of the sample solute, a solute entirely excluded from the micelle and the micelle, respectively.

A peak capacity can be defined similarly to that suggested by Giddings [11]:

$$n = 1 + \frac{N^{1/2}}{4} \cdot \ln\left(\frac{t_{mc}}{t_0}\right) \quad (4)$$

According to Eq. 4, the peak capacity depends on the migration time window.

3.1. Optimization of the resolution in MEKC

The resolution of a pair of neutral solutes is expressed through the relationship [11,12]

$$R_s = \frac{N^{1/2}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{\tilde{k}'_2}{1 + \tilde{k}'_2} \cdot \frac{1 - \frac{t_0}{t_{mc}}}{1 + \left(\frac{t_0}{t_{mc}}\right)\tilde{k}'_1} \quad (5)$$

with

$$f(\tilde{k}') = \frac{\tilde{k}'_2}{1 + \tilde{k}'_2} \cdot \frac{1 - \frac{t_0}{t_{mc}}}{1 + \left(\frac{t_0}{t_{mc}}\right) \cdot \tilde{k}'_1} \quad (6)$$

N is the number of theoretical plates, α is the selectivity, given by $\tilde{k}'_2/\tilde{k}'_1$, $\tilde{k}'_1$ and $\tilde{k}'_2$ are the capacity factors of the first and second solutes, t_0 is the migration time of an unretained solute moving at the electroosmotic flow-rate and t_{mc} is the migration time of a neutral solute completely retained in the micelle.

Eq. (5) predicts the incidence of efficiency, selectivity and $f(\tilde{k}')$ on resolution. As t_{mc} approaches infinity, the last term in $f(\tilde{k}')$ becomes equal to unity and the resultant equation for resolution becomes identical with that in conventional HPLC.

For conventional chromatography, R_s increases with increasing k'. However in MEKC, the last two factors in Eq. 6 decrease at large $\tilde{k}'$ values. The optimum value of $\tilde{k}'$ is dependent on the elution window width and on the ratio t_0/t_{mc}.

The effects of several modifiers on experimental parameters such as migration time window and selectivity are discussed below.

3.2. Optimization of the surfactant type and concentration

Terabe et al. [5] suggested the use of buffer additives such as an anionic surfactant (SDS), γ-CD or urea for the separation of electrically neutral and highly hydrophobic compounds. In

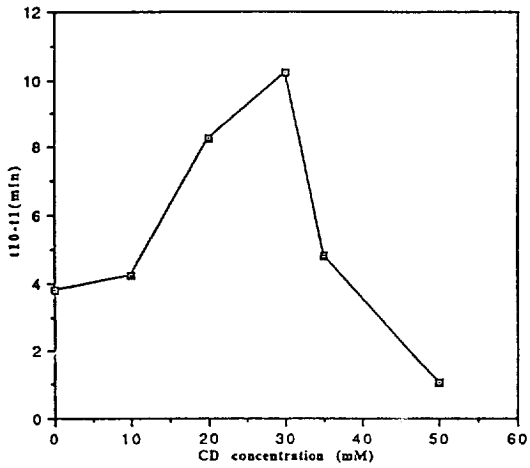


Fig. 3. Variation of the migration time window with the γ-CD concentration. Column, 470 mm × 75 mm I.D. fused silica; electrolyte, 50 m*M* SDS–γ-CD–2 *M* urea in 50 m*M* borate buffer (pH 9.2); applied voltage, 15 kV.

MEKC, SDS provides a selectivity similar to that in RPLC. It is easy to use and allows the separation of low to moderately hydrophobic compounds [1]. Concentrations of SDS between 25 and 100 m*M* are commonly reported. Higher concentrations would result in the generation of large electrophoretic currents and could cause band broadening due to Joule heating. In this work, the SDS concentration was set at 50 m*M*.

3.3. Modification with γ-cyclodextrin

The migration time window is defined as $t_{10} - t_0$, the difference between the migration time of a solute which has no interaction with the micelle and the migration time of a solute which totally dissolves in the micelles [13]. Anthracene was used as the micellar marker, although it is more hydrophobic that benzodiazepines. Prazepam was assumed to be the most hydrophobic and the largest molecule among the benzodiazepines investigated because it hardly penetrates the small cavity of γ-CD. This assumption is confirmed by the fact that the migration time of prazepam is not affected by the γ-CD concentration.

The effect of varying the γ-CD concentration on the width of the migration time window is illustrated in Fig. 3. The plot exhibits a maximum at 30 m*M* γ-CD. However, at this concentration, no separation between clonazepam and chlordiazepoxide was achieved, as shown in

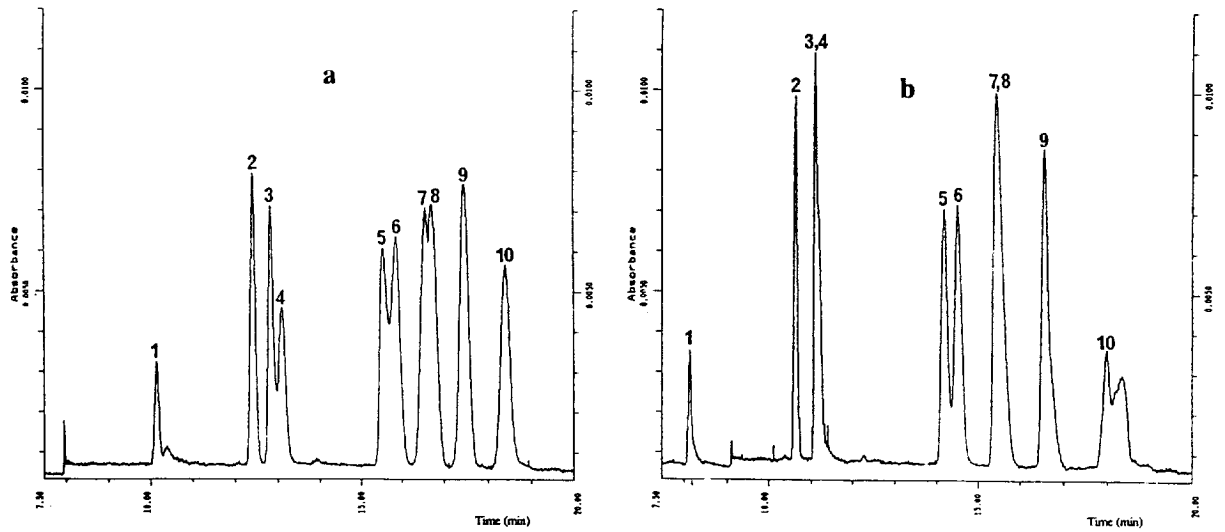


Fig. 4. Effect of γ-CD concentration on CD–MEKC separation of benzodiazepines. Conditions as in Fig. 2 except for the γ-CD concentration: (a) 20 and (b) 30 m*M*. Analytes: 1 = anthracene; 2 = bromazepam; 3 = clonazepam; 4 = chlordiazepoxide; 5 = clobazam; 6 = oxazepam; 7 = alprazolam; 8 = clorazepate; 9 = triazolam; 10 = prazepam.

Fig. 4, and peak tailing was observed for triazolam.

In spite of a loss of resolution between oxazepam and clobazam, a 20 m*M* γ-CD concentration leads to sharp and symmetrical peaks and acceptable resolution between clonazepam and chlordiazepoxide on the one hand and clorazepate and alprazolam on the other.

The dependence of the migration time on the γ-CD concentration was investigated over the concentration range 0–50 m*M*, as shown in Fig. 5. A decrease in the electroosmotic flow and a shortening of the migration time of the solute with increasing γ-CD concentration was observed. According to Yik et al. [6], at higher CD concentrations, solubilization in the cavity increases and solutes migrate with the electroosmotic flow.

The affinity [5,6,14] of several solutes towards SDS and without γ-CD was explored. Clorazepate and triazolam tend to be completely solubilized into the micelles, as confirmed by their migration times being similar to that of the micelle (Fig. 6). These compounds could not be successfully separated by MEKC using an SDS solution without modifier. On the same electropherogram, bromazepam, clonazepam and chlordiazepoxide are not totally incorporated into the SDS micelles and are detected earlier.

According to Terabe et al. [5], in CD–MEKC the elution order should agree with the tendency of the solutes to form inclusion complexes with the CD in the presence of SDS. A solute having a shorter migration time is more strongly included in the CD cavity than a solute having a longer migration time. At a 20 m*M* γ-CD concentration, the migration times of bromazepam, clonazepam and chlordiazepoxide decrease by

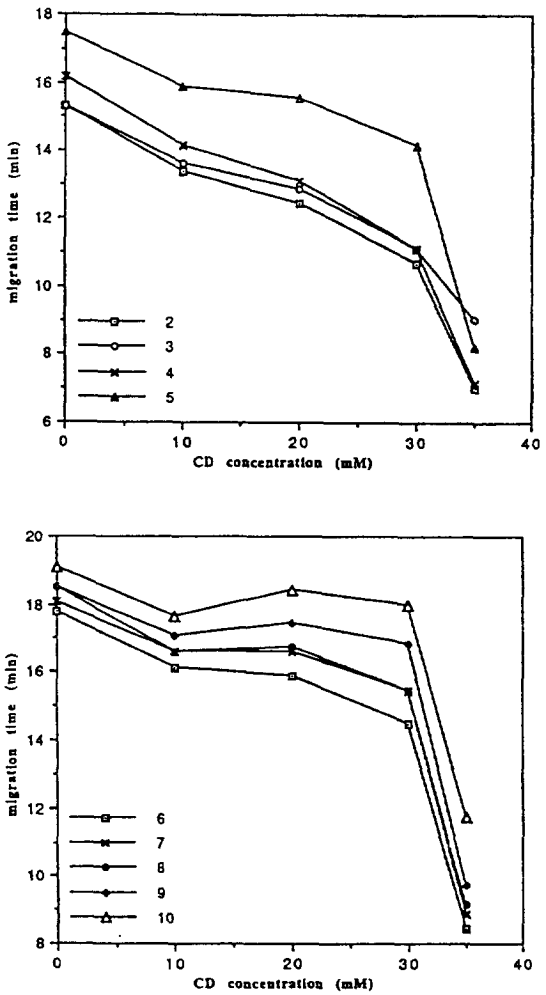


Fig. 5. Variation of migration time with γ-CD concentration. Conditions as in Fig. 3. Numbers on lines correspond to peaks in Fig. 4.

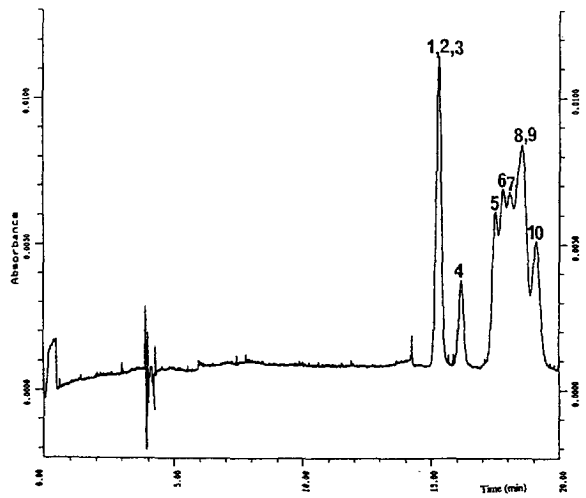


Fig. 6. CD–MEKC separation of benzodiazepines without CD. Conditions as in Fig. 2. For peak identification, see Fig. 3.

about 20%, so they form stable inclusion complexes with γ-CD and hydrophobic interactions with SDS are less effective. Prazepam can reasonably be considered to be totally incorporated into the SDS micelles as its migration time only decreases by 3%. This justifies the choice of prazepam as the micellar marker.

To explain this elution order, the hydrophobicity, size and molecular structure of benzodiazepines must be considered. According to Terabe et al. [5], the ratio of the solute incorporated into the micelle depends on its hydrophobicity but the inclusion complex formation of the solute with CD depends also on the concordance of the solute molecular size with the CD cavity diameter. Prazepam and triazolam molecules are too large to enter the hydrophobic internal cavity of γ-CD.

3.4. *Buffer modification with urea*

In CD–MEKC, urea is used to increase the solubility of β- and γ-CD in the aqueous phase [15]. Increasing the applied voltage at a constant urea concentration (4 *M*) results in a decrease in the migration times but the resolution becomes poor and the baseline unsteady as a result of Joule heating effects (Fig. 7).

At constant applied voltage (15 kV), the current decreases on increasing the urea concentration from 2 to 4 *M*. This is in agreement with Terabe et al.'s observation that the current decreases by 50% on increasing the urea concentration from 0 to 8 *M* [10]. Urea concentrations from 2 to 6 *M* are usable.

The electroosmotic flow velocity, V_{eo}, and the electrophoretic velocity of the micelle, V_{ep}, are given by the equations [16]

$$V_{eo} = -\frac{\varepsilon \zeta_c E}{\eta} \tag{7}$$

$$V_{ep} = \frac{2\zeta_m f(\kappa_a) E}{3\eta} \tag{8}$$

where ε is the dielectric constant of the mobile phase, ζ_c and ζ_m are the zeta potentials for the column wall and the micelles, respectively, η is the viscosity of the mobile phase and $f(\kappa_a)$ is the electric field strength, which depends on the micelle shape and size.

Taking into account the sign of the migration direction, the apparent migration velocity of the SDS micelle is given by the sum of V_{eo} and V_{ep}:

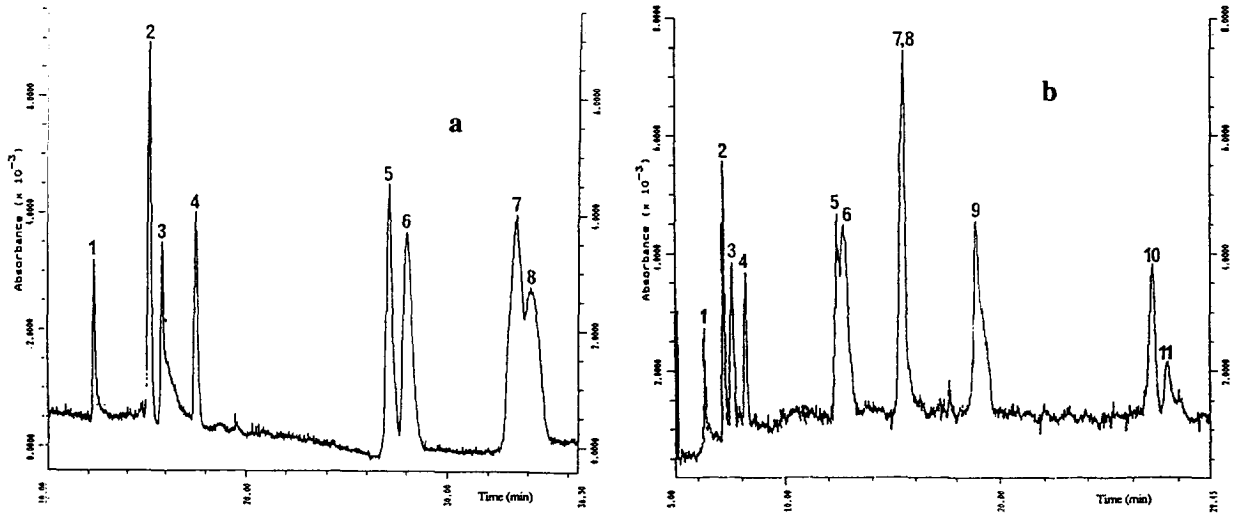


Fig. 7. Effect of applied voltage on CD–MEKC separation of benzodiazepines. Electrolyte, 50 m*M* SDS–30 m*M* γ-CD–4 *M* urea in 50 m*M* borate buffer (pH 9.2); applied voltage, (a) 15 and (b) 25 kV. Peak identification as in Fig. 3, 11 = impurity.

$$V_{mc} = V_{ep} + V_{eo} = -\frac{\varepsilon\left(\zeta_c - \frac{2}{3}\zeta_m f(\kappa_a)\right)}{\eta} \quad (9)$$

The t_0/t_{mc} ratio is expressed by:

$$\frac{t_0}{t_{mc}} = \frac{V_{mc}}{V_{eo}} = 1 - \left(\frac{2|\zeta_m| f(\kappa_a)}{3|\zeta_c|}\right) \quad (10)$$

The t_0/t_{mc} decreased from 0.2554 to 0.1182 when the urea concentration increased from 2 to 4 *M*. The migration time of the electroosmotic marker (MeOH) increases by 30% with increasing the urea concentration, which indicates a decrease in the zeta potential ζ_c. The t_0/t_{mc} ratio is considerably reduced, and the migration time window $t_{mc} - t_0$ is expanded, as t_{mc} is increasing faster.

Further, on increasing the urea concentration, the resolution between oxazepam and clobazam was improved, but the analysis time was doubled from 19 to 40 min. By increasing the applied voltage at constant urea concentration of 4 *M*, the analysis time was shortened but the resolution and baseline again deteriorated as a result of Joule heating effects. Hence, the op-

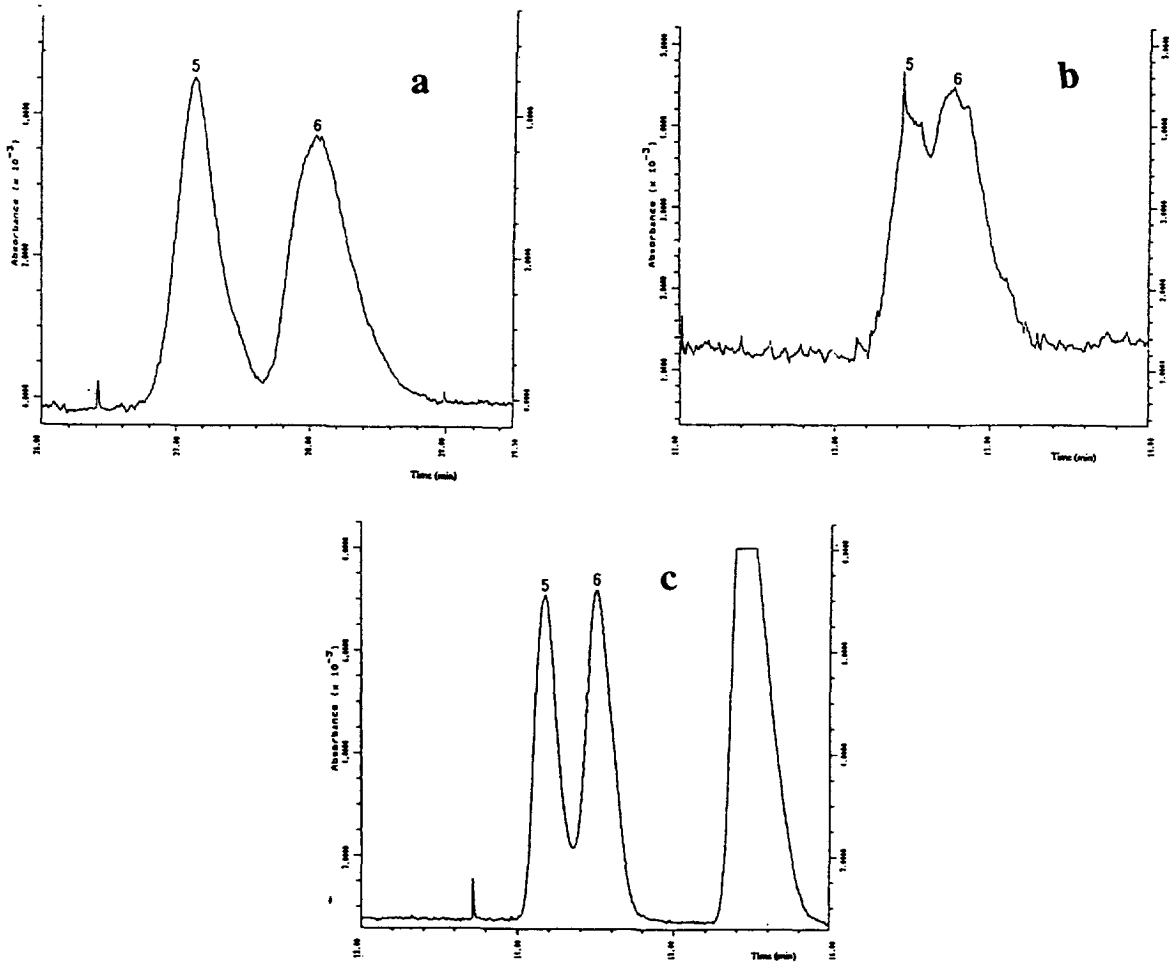


Fig. 8. Effect of urea addition on the resolution between clobazam and oxazepam. Electrolyte composition as in Fig. 6 except for the urea concentration: (a) 2 and (b) and (c) 4 *M*; applied voltage, (a) and (b) 15 kV and (c) 25 kV; current, (a) 62.2, (b) 53.8 and (c) 105.1 mA. Peak identification as in Fig. 3.

timum urea concentration in the running buffer was 2 *M* (Fig. 8).

3.5. Buffer modification with tetrahydrofuran

The effects of several organic modifiers on different parameters of the electrophoretic separation of benzodiazepines are shown in Fig. 9. By adding 1% (v/v) tetrahydrofuran to the buffer as an organic modifier, the selectivity was enhanced with an acceptable analysis time and narrow peak width.

Electropherograms were recorded in a 50 m*M* borate–50 m*M* SDS–20 m*M* γ-CD–2 *M* urea

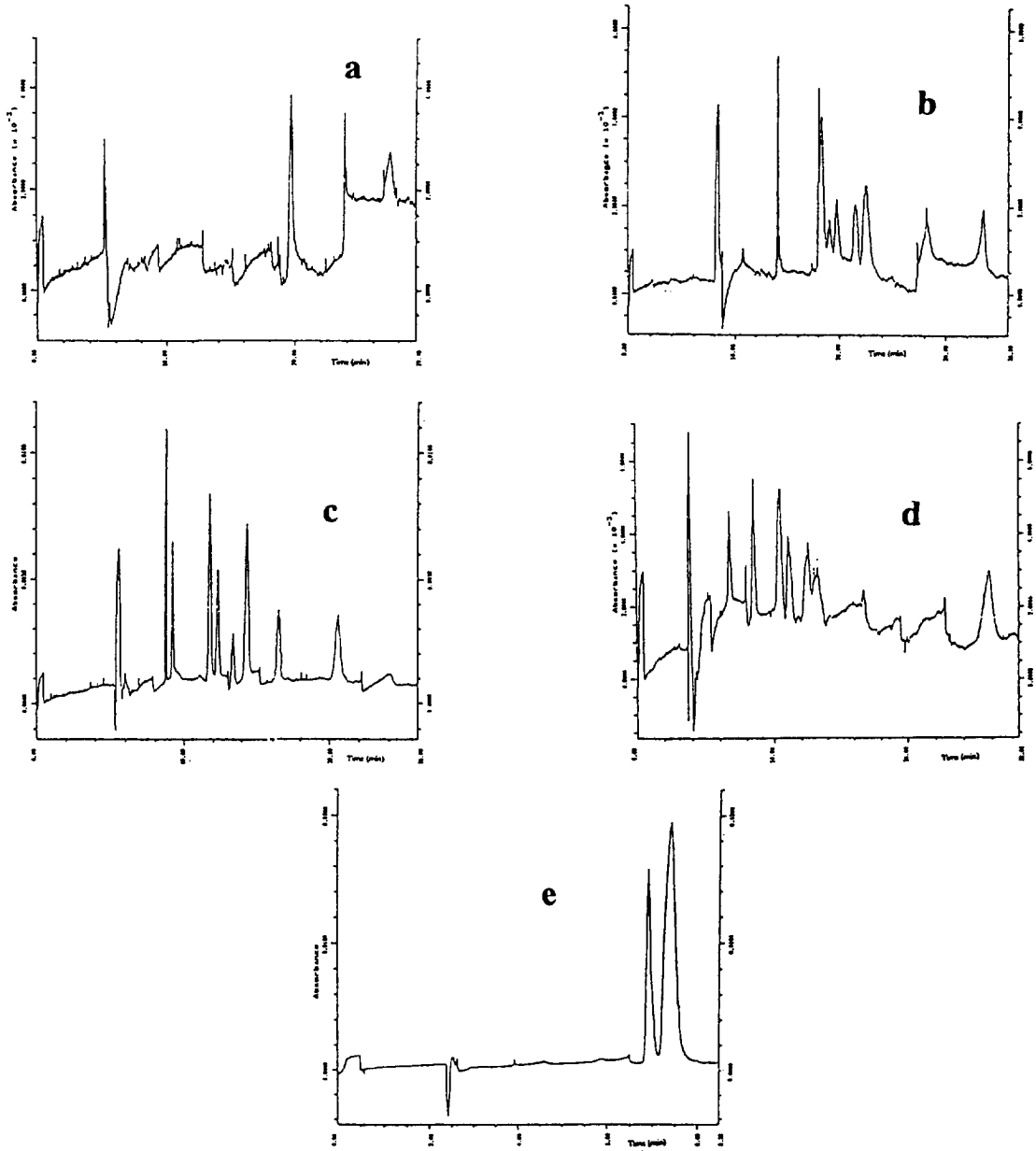


Fig. 9. Effect of the organic solvent on the peak efficiency. Electrolyte, 50 m*M* SDS in 25 m*M* borate buffer modified with 20% (v/v) organic solvent: (a) methanol; (b) ethanol; (c) tetrahydrofuran; (d) acetonitrile; and (e) water; applied voltage, 20 kV.

Table 2
Effect of addition of 1% THF to the electrolyte on the separation parameters at two applied voltages

V (kV)	THF (%)	t_{e0}		$t_{mc} = t_{10}$		t_{eo}/t_{mc}	
15	0	4.408	$\delta = 5.9\%$	18.409	$\delta = 21.3\%$	0.239	$\delta = 12.5\%$
15	1	4.668	Ratio = 1.4	22.329	Ratio = 1.4	0.209	$\delta = 3.2\%$
20	1	3.339		15.480		0.216	

Running buffer: 50 mM SDS–20 mM γ-CD–2 M urea in 50 mM borate buffer. δ = Difference (%).

buffer containing 1% (v/v) tetrahydrofuran electrolyte. As shown in Table 2, the addition of 1% tetrahydrofuran does not significantly reduce the electroosmotic flow (t_{eo} remains constant) but does increase the marker migration time (t_{mc}) from 18.41 to 22.24 min. Hence the addition of THF to the buffer contributes 12% to the decrease in t_0/t_{mc} and thus expands the migration time window.

The effect of the addition of 1% THF on resolution was estimated from the separation of three pairs of benzodiazepines poorly resolved without the THF modifier. The results are given in Table 3. For a given solute, the longer its migration time, the greater is the discrepancy between $\tilde{k}'$ measured without THF and with 1% THF. The ratio $(\alpha - 1)/\alpha$ increases faster than α; $f(\tilde{k}')$ (Eq. 6) is then much increased when

Table 3
Effect of addition of 1% THF to the electrolyte on the resolution

Parameter	Clonazepam	Chlordiazepoxide	Clobazam	Oxazepam	Alprazolam	Clorazepate
$\tilde{k}'$ (no THF)	6.34	6.89	16.3	18.94	27.83	30.65
	$\delta = 32\%$		$\delta = 37\%$		$\delta = 44\%$	
$\tilde{k}'$ (1% THF)	4.3	5.09	10.20	12.76	15.5	20.06
$\alpha = \frac{\tilde{k}'_2}{\tilde{k}'_1}$	1.0868		1.1620		1.1013	
		$\delta = 9\%$		$\delta = 8\%$		$\delta = 17\%$
	1.1837		1.2510		1.2942	
$(\alpha - 1)/\alpha$	0.07987		0.13941		0.09198	
		$\delta = 94\%$		$\delta = 44\%$		Factor: 2.47
	0.15519		0.20064		0.22732	
$f(\tilde{k}')$	0.26421		0.14765		0.09632	
		$\delta = 32\%$		$\delta = 59\%$		$\delta = 85\%$
	0.34819		0.23421		0.17772	
$\frac{\tilde{k}'_2}{1 + \tilde{k}'_2}$	0.8733		0.9498		0.9648	
		$\delta = 4\%$		$\delta = 2\%$		$\delta = 2\%$
	0.8358		0.9273		0.9525	
$\frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})\tilde{k}'_1}$	0.30255		0.15544		0.09946	
		$\delta = 38\%$		$\delta = 62\%$		$\delta = 88\%$
	0.41660		0.25257		0.18658	

Operating conditions as in Table 2. δ = difference (%).

THF is present. The addition of THF has little effect on efficiency but the resolution is improved from the selectivity change and the decrease in $\tilde{k}'_1$.

3.6. Incidence of the applied voltage and heat dissipation

The effect of voltage on the separation was investigated under the optimized operating conditions. As shown in Table 2, on increasing the applied voltage from 15 to 20 kV, the time of the analysis is decreased by a factor of 1.44 while the elution time window is not affected. The effect of the operating voltage on the resolution is discussed on the basis of the selectivity and retention measurements made at 15 and 20 kV. The results are presented in Table 4. The selectivity varies little with the applied voltage, which has a stronger effect on the $(\alpha - 1)/\alpha$ term. The applied voltage does not influence $f(\tilde{k}')$. Although the capacity factor $\tilde{k}'$ should be independent of the voltage, a significant variation of $\tilde{k}'$ is observed for the last two pairs. Finally, the voltage variation has little effect on the resolution but does modify the duration of the analysis.

To summarize, the best option is to apply as high a voltage as possible to the capillary, yielding to the highest separation efficiency in the shortest possible time. The practical limit to this approach lies in the capability of the system to dissipate the heat generated during the electrophoretic process. Indeed, many workers have found that V_{app} (or $1/t_m$) as a function of E (or V) deviates positively at high values of E, an effect attributed to the increase in the temperature of the running buffer as a consequence of Joule heating [17,18]. According to a generally accepted value of 2%/°C for the decrease in water viscosity [18], the in-column temperature deviation, ΔT, was calculated from the non-linearity of the dependence of the current on the applied voltage (Fig. 10) expressed by Gonnord and Collet [19] by analogy with the expression of Vindevogel and Sandra [18]:

Table 4
Effect of applied voltage on resolution

Parameter	Clonazepam	Chlordiazepoxide	Clobazam	Oxazepam	Alprazolam	Clorazepate
$\tilde{k}'$ (1% THF) (V = 20 kV)	4.17	4.84	9.63	11.85	14.37	18.14
	δ = 3%	δ = 5%	δ = 3.6%	δ = 7.4%	δ = 7.3%	δ = 10%
$\tilde{k}'$ (1% THF) (V = 15 kV)	4.3	5.09	10.20	12.76	15.5	20.06
$\alpha = \frac{\tilde{k}'_2}{\tilde{k}'_1}$	1.1607		1.2264		1.2624	
		δ = 1.94%		δ = 1.97%		δ = 12.46%
	1.1837		1.2510		1.2942	
$(\alpha - 1)/\alpha$	0.13845		0.18461		0.20786	
		δ = 10.8%		δ = 8%		δ = 8.56%
	0.15519		0.20064		0.22732	
$\frac{\tilde{k}'_2}{1 - \tilde{k}'_2}$	0.8228		0.9219		0.9478	
		δ = 0.84%		δ = 0.58%		δ = 0.49%
	0.8358		0.9273		0.9525	
$\frac{1 - t_0/t_{mc}}{1 - (t_0/t_{mc})\tilde{k}'_1}$	0.41248		0.25454		0.19104	
		δ = 1%		δ = 0.78%		δ = 2.4%
	0.41660		0.25257		0.18658	

Operating conditions as in Table 2. δ = difference (%).

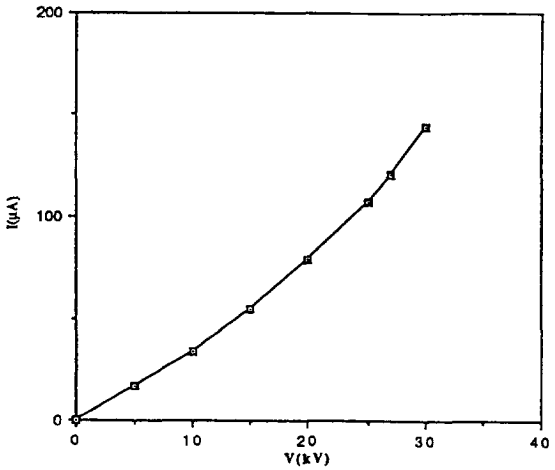


Fig. 10. Variation of current with applied voltage. Electrolyte, 50 m*M* SDS–2 *M* urea–20 m*M* γ-CD–50 m*M* borate buffer. The background electrolyte contained 1% of THF.

$$\Delta T\,(°C) = \frac{\frac{I_m}{I_{th}} - 1}{2\%} \qquad (11)$$

where I_m is the current measured at voltage V and I_{th} is the theoretical current calculated from the assumption of a linear relationship between the current intensity, I, and the applied voltage, V:

Table 5
Calculated in-column temperature increase and dissipated power as a function of applied voltage

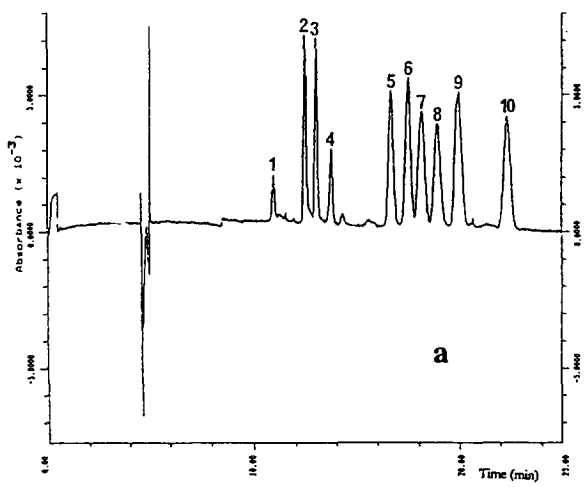
V (kV)	Buffer	
	ΔT (°C)	P (mW/cm)
5	0	1.71
10	1.66	7.30
15	4.47	17.30
20	8.51	33.10
25	14.6	57.00
27	17.7	69.70
30	22.2	91.85

Conditions as in Fig. 11.

$$I_{th} = I_{5kV} V_{kV} / 5_{kV} \qquad (12)$$

I_{5kV} is the current measured at 5 kV where Joule heating effects are smaller and 2% is the decrease in water viscosity for each 1°C increase in temperature.

The dissipated power calculated from the equation $P = VI/L$, where L is the total length of the capillary, is shown in Table 5. As the upper power limit recommended by the manufacturer is about 50 mW/cm, it can be concluded that the



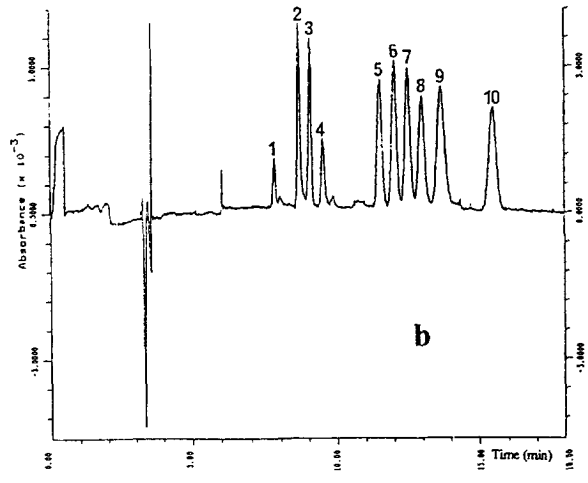


Fig. 11. CD–MEKC separation of benzodiazepines. Electrolyte, 50 m*M* SDS–2 *M* urea–20 m*M* γ-CD–50 m*M* borate buffer containing 1% of THF; applied voltage, (a) 15 and (b) 20 kV. For peak identification, see Fig. 3.

applied voltage must be kept below 20 kV. Electropherograms obtained at applied voltages of 15 and 20 kV are presented in Fig. 11.

The cooling system allows the capillary temperature to be maintained steady up to 30°C. The in-column temperature was measured to be 8.5°C at 20 kV. An increase of 17% in the electrophoretic mobilities was observed.

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