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Journal of Chromatography A, 792 (1997) 475–482

JOURNAL OF CHROMATOGRAPHY A

Separation of neutral substances by non-aqueous capillary electrophoresis through interactions with cationic additives

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Abstract

The electrophoretic behaviour of neutral substances in non-aqueous capillary electrophoresis systems with tetraalkylammonium ions or long-chain trimethylammonium ions accompanied by different counter ions added have been investigated. Separation of neutral substances was only achieved in the highly dipolar aprotic solvents acetonitrile or propylene carbonate. The long-chain trimethylammonium ions were, however, not sufficiently soluble in acetonitrile. Thus, the studies comparing various types of ammonium ions were performed using propylene carbonate. The separation of the test solutes was improved with increasing concentrations of additives in the solvent. The type of ammonium ion as well as the counter ion were found to be important for the size of the separation window obtained. The separation principle was used for the determination of the water insoluble vitamin K_1 as well as the preservatives methylparaben and propylparaben in a pharmaceutical product. © 1997 Elsevier Science B.V.

Keywords: Pharmaceutical analysis; Buffer composition; Vitamins; Parabens; Hydroxybenzoates; Quaternary ammonium compounds; Non-aqueous capillary electrophoresis

1. Introduction

The first separations of uncharged organic molecules in open tubular capillaries were accomplished in micellar aqueous solutions by Terabe et al. [1,2]. Since then a number of approaches to separate uncharged species have appeared [3–10]. The principle of micellar electrokinetic chromatography (MEKC) is, however, still the most widely used technique for the separation of neutral substances. In MEKC, the separation of highly hydrophobic analytes may be difficult due to an extreme partition towards the micelle and addition of organic modifiers in significant amounts in order to reduce the affinity of the hydrophobic analyte may cause disintegration of the micelles [11]. Non-aqueous electrophoresis media may be particular useful for the separation of compounds insoluble in water considering the solubility, however, a suitable separation mechanism for neutral substances has to be developed. Until now only two reports on the separation of uncharged compounds in non-aqueous media has appeared in the literature [12,13]. Wahlbroehl and Jorgenson demonstrated in 1985 that tetraalkylammonium perchlorate added to acetonitrile (MeCN) or mixtures of MeCN and water resulted in the separation of the neutral solutes benzo[*ghi*]perylene, perylene, pyrene, 9-methylantracene, naphthalene and mesityl oxide [12]. The mobility of the solutes increased with increasing hydrophobicity. The mechanism of these

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0021-9673/97/$17.00
PII S0021-9673(97)01011-X

separations was proposed to be solvophobic association of the neutral organic molecules with the tetraalkylammonium ions and thereby forming a positive charged species that migrates in an electrical field. Furthermore, a dynamic equilibrium between an associated species with a positive charge and the dissociated species with no charge rather than a saturable binding between the neutral solute and the tetraalkylammonium ion was suggested. Thus, characteristics of the used solvent as well as of the additive may be very important as this type of interaction may be very dependent on the solvation of the additives as well as of the analytes to be separated. Very recently, Miller et al. published a paper on the separation of polycyclic aromatic hydrocarbons (PAHs) in non-aqueous buffer systems based on MeCN with planar organic cations, e.g. tropylium tetrafluoroborate or 2,4,6-triphenylpyrylium tetrafluoroborate or a mixture of these compounds added [13]. The separation of the PAHs was improved in these systems compared to what was achieved by Wahlbroel and Jorgenson [12]. They found that charge-transfer interactions, electrostatic as well as dispersive forces play an important role for the separation of the PAHs. The present paper explores the use of various classes of solvents as well as different types of quaternary ammonium ions and counter ions for the separation of hydrophobic solutes in non-aqueous CE systems. The structures of the test substances are given in Fig. 1.

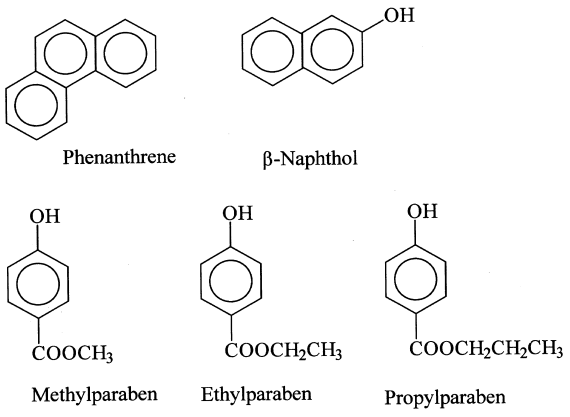

Fig. 1. The chemical structures of the test substances used.

2. Experimental

2.1. Chemicals

Octadecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, tetrapropylammonium bromide, tetramethylammonium hydrogensulfate monohydrate, tetraethylammonium hydrogensulfate, tetrapropylammonium hydrogensulfate, tetrabutylammonium hydrogensulfate, tetrahexylammonium hydrogensulfate, tetramethylammonium perchlorate, tetraethylammonium perchlorate, tetrapropylammonium perchlorate and thiourea were all of analytical grade and obtained from Fluka (Buchs, Switzerland). Hexadecyltrimethylammonium hydrogensulfate, hexadecyltrimethylammonium bromide, phenanthrene, methyl 4-hydroxybenzoate (methylparaben), ethyl 4-hydroxybenzoate (ethylparaben), propyl 4-hydroxybenzoate (propylparaben) and vitamin K_1 (phytomenadion) of USP quality were supplied by E. Merck (Darmstadt, Germany). Dodecyltrimethylammonium bromide, tetrahexylammonium bromide, tetraethylammonium chloride, tetrapropylammonium chloride, tetrabutylammonium chloride, tetrabutylammonium perchlorate and β-naphthol were obtained from Sigma (St. Louis, MO, USA). Formamide, N-methylformamide (NMF) and mesityl oxide 98% were purchased from Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO), dimethylformamide (DMF), propylene carbonate (4-methyldioxolone-2) and acetic acid were purchased from Riedel-de Häen (Seelze, Germany). Methanol (MeOH) and acetonitrile (MeCN) were of HPLC-grade and obtained from Labscan (Dublin, Ireland). All chemicals were used as received from the supplier.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed using a Beckman P/ACE 5010 instrument (Fullerton, CA, USA) equipped with a UV detector (operated at 254 nm). The samples were loaded at the anode by applying a pressure (0.5 p.s.i.g.; ca. 3.5 kPa) for 3 s and the separations were carried out at 25°C by applying a voltage gradient of 25 kV. The air circulation over the samples in the autosampler

carrousel was reduced in order to minimise evaporation of the sample as well as of the electrophoresis medium during the experiments. Furthermore, the part of the lever arms entering the vials was replaced with one made of PTFE in order to make it more resistant to the organic solvents used. Data acquisition was accomplished with a NEM personal computer 486 with System Gold software.

The capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA) and used with the dimensions 47 cm (40 cm to the detector)×50 μm unless otherwise stated. Prior to use, new capillaries were rinsed with 1 *M* sodium hydroxide for 1 h, 0.1 *M* sodium hydroxide for 20 min, distilled water for 20 min and subsequently with methanol for 20 min followed by 15 min flushing with the electrophoresis medium. The capillary was flushed with the electrophoresis medium for 15 min when changing the composition of the electrophoresis medium. The capillary was flushed with the electrophoresis medium for 3 min prior to each separation. The electrophoresis medium in the inlet as well as outlet vials were replaced after each run when using a bromide salt and after three runs when using other salts.

Electrophoretic parameters were determined using mixtures containing ca. 0.2 mg/ml of phenanthrene, β-naphthol, propylparaben, ethylparaben, methylparaben and thiourea [electroosmotic flow (EOF marker] dissolved in the solvent, being the major constituent of the electrophoresis medium.

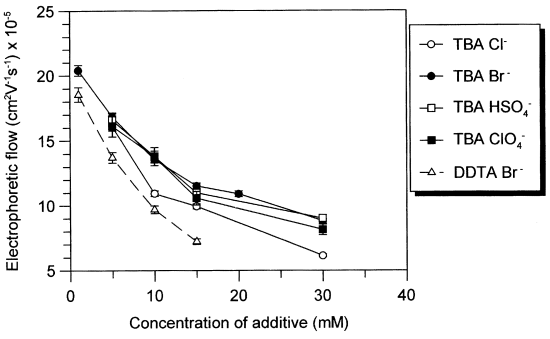

Fig. 2. The electroosmotic flow (thiourea) as a function of the concentration of various tetrabutylammonium (TBA) salts or dodecyltrimethyl ammonium bromide (DDTA Br^-) in propylene carbonate.

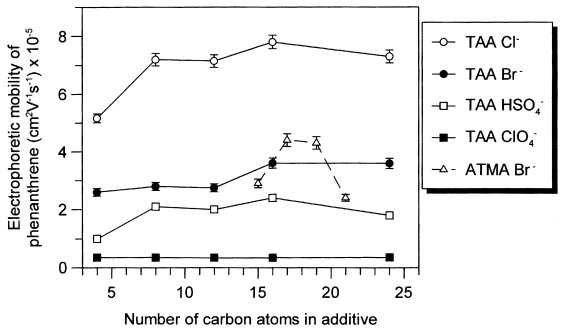

Fig. 3. The electrophoretic mobility of phenanthrene as a function of the number of carbon atoms of the tetraalkylammonium ion (TAA) or the alkyltrimethylammonium ion (ATMA) added. All ammonium salts were added in concentrations of 10 m*M* to propylene carbonate.

In Figs. 1–3 data points represent the average of at least three experiments. The error bars indicate the standard deviation.

2.3. Sample preparation of vitamin K_1 product

One volume of Konakion drops 20 mg vitamin K_1/ml (Hoffman-La Roche, Basel, Switzerland) was evaporated by vacuum centrifugation using a Maxi Dry Lyo-system (Heto, Allerød, Denmark). The remains were reconstituted in 4 volumes of propylene carbonate with 0.2 mg/ml of ethylparaben added as an internal standard. Calibration standards were prepared in propylene carbonate. The prepared sample was injected into the capillary for 3 s (3.5 kPa). The electrophoresis medium consisted of 20 m*M* tetradecylammonium bromide in propylene carbonate. The separation was performed in 27 cm (20 cm to the detector)×50 μm capillary by applying a voltage of 25 kV at 25°C.

3. Results and discussion

3.1. Choice of EOF marker

Walbroel and Jorgenson demonstrated the separation of mesityl oxide and formamide in a mixture of MeCN and water containing tetrahexylammonium

perchlorate [12]. Thus it may not be a trivial task to find a suitable substance to be used as the EOF marker. A number of flow markers were investigated and it was found that mesityl oxide was difficult to detect in some systems. Formamide has been used in the previous studies in this field [12,13], but small amounts (0.1%) of formamide or DMSO in the sample injected was found to influence the separation selectivity dramatically. Thiourea was easily detected and did not influence the separation selectivity. Whether or not thiourea serves as a true marker of EOF or a migrator under the conditions investigated is, however, not known, but out of the EOF marker candidates investigated, thiourea was found to be the best choice.

3.2. Choice of organic solvent

Representative solvents with different physical/chemical properties according to the Kolfhoff classification [14] (Table 1) were chosen in order to study the electrophoretic behaviour of the neutral test compounds in various solvents with quartenary ammonium compounds added. Kolfhoff divides solvents into two main groups. Solvents which have a stable lysate ion are called amphiprotic, all other solvents aprotic [14]. Separation was only successful when using MeCN and propylene carbonate. Even small amounts (ca. 0.5%) of methanol or acetic acid, added to MeCN or propylene carbonate resulted in loss of the separation. It has earlier been demonstrated that water added in concentrations up to 50% to MeCN improve the separation of hydrophobic solutes [12]. This was explained by an increase of the interaction between hydrophobic species in the presence of water. The opposite may be the case when introducing methanol or acetic acid into the systems. MeCN was, however, found to be a poor solvent for the long-chain trimethylammonium salts. Thus propylene carbonate was chosen for further studies into the effect of the type of ammonium ion and MeCN was only used for the comparison of various salts of tetrabutylammonium ions.

3.3. Choice of quartenary ammonium ion

The electrophoretic behaviour of the hydrophobic test solutes by adding increasing amounts of symmetrical or asymmetrical quartenary ammonium compounds to non-aqueous electrophoresis media were investigated. Generally, the separation window was enlarged with increasing concentrations of the

Table 1
Classification of organic solvents according to their Brønsted acid–base behaviour modified after Kolthoff [14]

Solvent designation		Relative acidity	Relative basicity	Examples
Amphiprotic	Neutral	+	+	MeOH, glycerol, phenol, *tert.*-butylalcohol
	Protogenic	+	−	Sulphonic acid, formic acid, acetic acid
	Protophillic	−	+	Liquid ammonia, formamide, N-methylacetamide, NMF
Aprotic	Dipolar protophillic	−	+	DMSO, DMF, tetrahydrofurane, 1,4 dioxane, pyridine
Aprotic	Dipolar protophobic	−	−	MeCN, acetone, nitrobenzene, sulfolane, propylene carbonate
	Inert	−	−	Aliphatic hydrocarbons, benzene, 1,2-dichloroethane, tetrachloromethane

− indicates weaker and + indicates stronger acid or base than water.

additive in propylene carbonate concurrently with a decrease in mobility of the test solutes. Furthermore, the mobility of an analyte was increased with increasing hydrophobic characteristics of the solute. Thus the general migration order of the test solutes were phenanthrene followed by β-naphthol, propylparaben, ethylparaben, methylparaben and thiourea.

The positive charge is more evenly distributed over the large bulky symmetrical ammonium ions compared to the asymmetrical long-chain ammonium ions. From studies on adsorption of quartenary ammonium ions to silica surfaces, it is known that the long-chain asymmetrical quartenary ammonium ions interact more strongly with the silanol groups than do symmetrical quarternary ammonium ions in an aqueous environment [15,16]. A similar difference in the strength of the interactions with the capillary wall is also seen in propylene carbonate leading to a more pronounced decrease of the electroosmotic flow when using asymmetrical ammonium compared to the use of symmetrical ammonium ions (Fig. 2). The effect of the number of carbon atoms in the additive on the difference in mobility of a very hydrophobic solute (phenanthrene) and the flow marker (thiourea) is displayed in Fig. 3. The size of the separation window exhibits an optimum around 17–19 carbon atoms in the case of the long-chain trimethylammonium ions whereas the separation window is fairly independent of the number of carbon atoms in the tetraalkylammonium compounds tested. Cetylpyridinium bromide ions was also found to enable a good selectivity towards the test solutes as the size of the separation window was in the order of what was obtained by the long-chain trimethylammonium ions. Background UV absorbance is, however, a drawback compared to the aliphatic ammonium compounds.

3.4. Choice of counter ion

The solubility of the hydrogensulfate, bromide and chloride salts of tetraalkylammonium ions are relatively high in MeCN and propylene carbonate compared to the solubility of the salts of the long-chain alkyltrimethylammonium ions in these solvents. The bromide salts of the long-chain alkyltrimethylammonium were found to be sufficiently soluble in propylene carbonate but not in MeCN. All counter ions tested except the perchlorates provided stable, reproducible electrophoretic conditions.

Another very important aspect in the choice of counter ion is the impact on the size of the separation window as seen from Table 2. The chloride salts of the tetraalkylammonium ions provides a larger separation window than does the bromides, hydrogensulfates or the perchlorates in propylene carbonate as well as in MeCN. This may reflect the differences in the solvation of the anions that accompany the ammonium ions in the solvents. Tetraalkylammonium ions are in general better solvated in propylene carbonate as well as in MeCN than in water. The opposite is the case regarding the anions used as seen from Table 3 [17]. The solvation decreases in the order $ClO_4^- > Br^- > Cl^-$ in propylene carbonate as well as in MeCN. From the data shown in Tables 2 and 3, there seems to be some sort of correlation between the solvation and the size of the

Table 2
Comparison of the electrophoretic mobility of phenanthrene in MeCN or propylene carbonate with 15 m*M* of various tetrabutylammonium salt added

Additive	Electrophoretic mobility of phenanthrene ($cm^2 V^{-1} s^{-1}$)×10^{-5} (R.S.D.%)	
	MeCN	Propylene carbonate
Tetrabutylammonium chloride	32.3 (4.1)	7.8 (4.5)
Tetrabutylammonium bromide	16.4 (3.2)	3.2 (3.1)
Tetrabutylammonium hydrogensulfate	10.1 (2.3)	2.3 (4.6)
Tetrabutylammonium perchlorate	10.5 (32.4)	0.1 (43.7)

Conditions: Capillary: 47 cm (40 cm to the detector)×50 μm, 25°C, 25 kV. Detection: UV-absorbance at 254 nm. For other conditions please refer to the Section 2.

Table 3
Solvation of selected anions and cations in MeCN or propylene carbonate relative to water expressed as standard molar Gibbs energies (kJ/mol) at 25°C of transfer of single ions from water to the solvent

Single ion	Solvent	
	MeCN	Propylene carbonate
H^+	46.4	50
Li^+	25	23.8
Na^+	15.1	14.6
K^+	8.1	5.3
$(CH_3)_4N^+$	3	−11
$(CH_3CH_2)_4N^+$	−7	−13
$(CH_3CH_2CH_2)_4N^+$	−13	−22
$(CH_3CH_2CH_2CH_2)_4N^+$	−31	−31
F^-	71	56
Cl^-	42.1	39.8
Br^-	31.3	30
I^-	16.8	13.7
CN^-	35	36
ClO_4^-	2	−3

The data are taken from the compilation of Marcus [17]. A positive value means that the ion is better solvated by water than by the solvent; a negative value means that the ion is more strongly solvated after transfer to the organic solvent.

separations window. However, other factors like the ability of the additive to form ion pairs in the solvent may also play a role. No data on the solvation of HSO_4^- ions in propylene carbonate or MeCN was available.

The type of counter ion does also influence the selectivity. The migration order of β-naphthol and propylparaben was reversed in propylene carbonate when using tetraalkylammonium chloride compounds in concentrations above 20 m*M*. This indicates that the relative mass-to-charge ratio of the analyte–additive complexes may change and that other factors other than a simple interaction between the analyte and the ammonium ion may play a role.

4. Application

The separation of water-insoluble neutral solutes in pharmaceutical products is demonstrated using Konakion drops 20 mg/ml. Vitamin K_1 is quantitated together with the preservatives propylparaben and methylparaben in a product that also contains Cremofor EL and water. As electrophoresis medium, tetradecylammonium bromide was used as the additive to propylene carbonate. The electrophoresis medium in the inlet vial became yellow–brown coloured during the electrophoretic run when using the bromide salts. This is probably caused by electrode processes leading to the formation of bromine. It was found that it was necessary to replace the electrophoresis medium after each run in order to obtain reliable quantitative determinations. The sample preparation procedure was found to be critical as the presence of water in the sample deteriorated the peak shapes especially of the preservatives. Thus, the water in the sample was evaporated using vacuum centrifugation and the remains were reconstituted in propylene carbonate with ethylparaben added as an internal standard. The selectivity of the method towards vitamin K_1 and some of its degradation products are demonstrated by the electropherogram of Konakion drops exposed to daylight (Fig. 4). The correlation coefficients (r) were determined to 0.9993, 0.9981, 0.9996 for vitamin K_1 (range 3.005–6.001 mg/ml), propylparaben (range 0.020–0.739 mg/ml) and methylparaben (range 0.081–0.964 mg/ml), respectively. The ranges given correspond to the concentration in the final sample solution. The repeatability of the method was tested at three concentration levels (n=6 at each level) in the ranges given above. The relative standard deviation was found be around 3% for all three compounds tested at all concentration levels tested. Konakion drops were analysed in six replicates. A concentration corresponding to 98.% (R.S.D.=4.95%), 96% (R.S.D.=4.45%) and 82% (R.S.D.=2.26%) of the expected concentration of vitamin K_1, propylparaben and methylparaben, respectively, were found in the pharmaceutical product.

5. Conclusions

The separations of neutral substances obtained in non-aqueous capillary electrophoresis systems by the addition of tetraalkylammonium ions to the electrophoresis medium have been investigated. The properties of the organic solvent in the electrophoresis medium was found to be very important in order to achieve separation. The type of the counter ion accompanying the ammonium ions was found to be

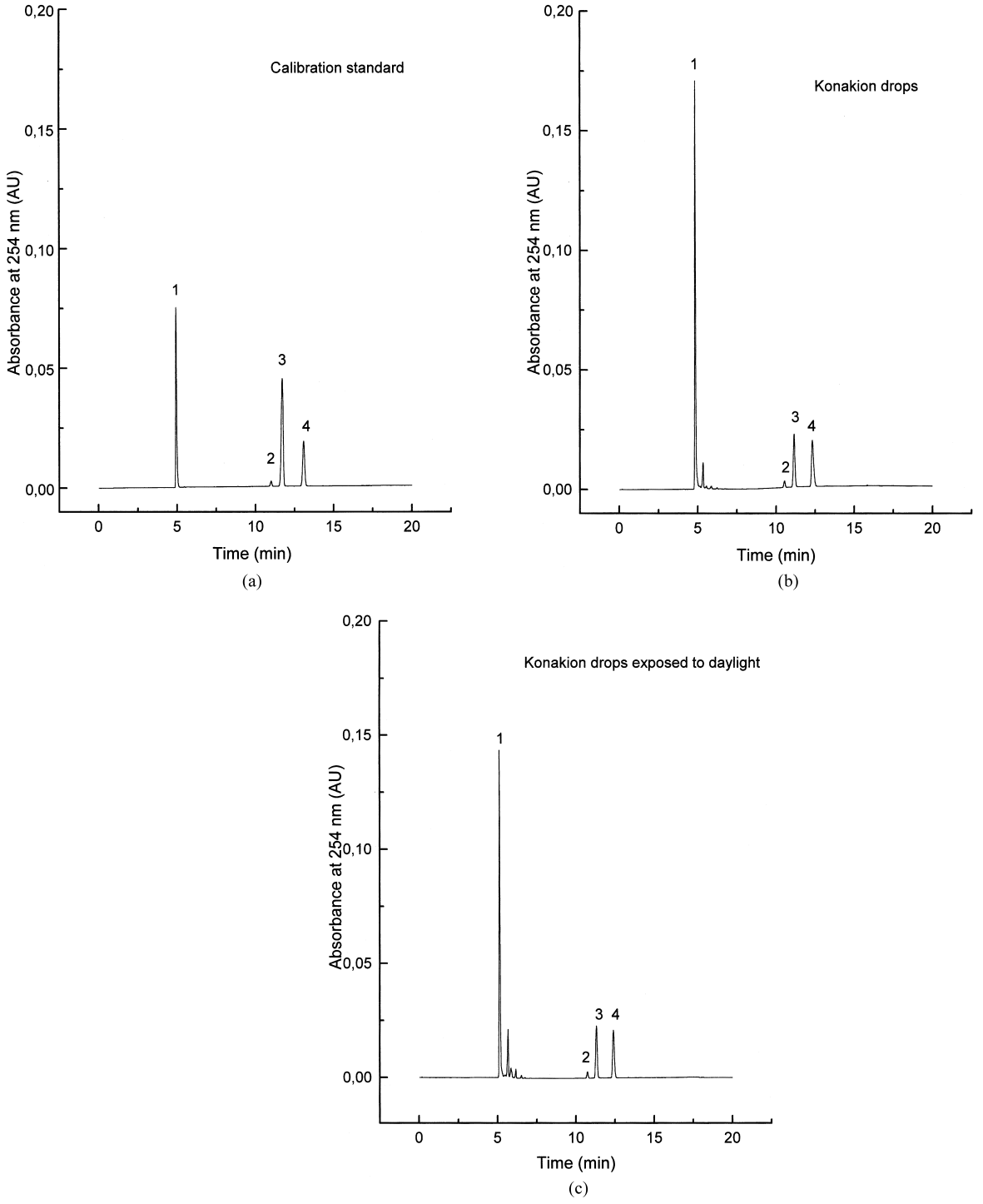

Fig. 4. Separation of vitamin K_1(1), propylparaben (2) and methylparaben (4) in Konakion drops. Ethylparaben (3) is added as an internal standard. Capillary: 27 cm (20 cm to the detector)×50 μm. Electrophoresis medium: 20 m*M* tetradecylammonium bromide in propylene carbonate. Voltage: 25 kV. Current produced: ca. 7 μA. See the Section 2 for other conditions.

very important for the size of the separation window available. In the case of the tetraalkylammonium ions, chloride salts should be preferred over bromide, hydrogensulfate or perchlorate in order to provide a large separation window. The bromide salts of long-chain trimethylammonium ions provided a larger separation window than tetraalkylammonium bromides. The applicability of the technique was demonstrated by a quantitative determination of the water insoluble vitamin K_1 as well as preservatives in a pharmaceutical product. Thus, non-aqueous CE may be an alternative to MEKC for the separation of hydrophobic solutes having a low solubility in aqueous media.

Acknowledgements

This work was supported by the Danish Technical Research Council grant No. 9501141.

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