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Separation of charged and neutral isotopic molecules by micellar electrokinetic chromatography in coated capillaries

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Abstract

Some unique separations are reported of pairs of deuterated and non-deuterated compounds by capillary zone electrophoresis (CZE) in coated capillaries in the absence and presence of surfactant micelles. Pyridine (pyridine-h_5) and [$^2H_5$]pyridine (pyridine-d_5) could be separated in plain buffer ($R = 1.1$) and in 2% Nonidet P-40 ($R = 1.5$). Owing to the good separation obtained, it was possible to assess the degree of cross-contamination when "pure" isotopes were analysed. A 1:1 mixture of benzoic-h_5 and -d_5 acid was poorly separated in the absence of detergent ($R = 0.39$) but well separated in 50 m*M* sodium dodecyl sulphate (SDS) ($R = 1.14$). Benzyl-h_5 and -d_5 alcohol were reasonably well separated in a micellar system containing 50 m*M* SDS ($R = 1.02$) (a separation previously unreported in the literature). Benzene-h_6 and -d_6 were well separated again in presence of 50 m*M* SDS ($R = 1.59$). It appears that CZE offers a unique environment for the separation of positively and negatively charged and neutral isotope mixtures, previously reported only using RP-HPLC and GC.

1. Introduction

Perhaps one of the most striking developments in capillary zone electrophoresis (CZE) has been the introduction of electrokinetic chromatography (EKC), largely developed through the efforts of Terabe [1], of which micellar EKC (MEKC), using ionic micelles as the separation carrier, is the most popular variant [2,3]. A number of useful variations have been described: (a) cyclodextrin (CD) EKC, which is especially helpful for the separation of enantiomers and aromatic isomers [4]; (b) ion-exchange EKC, which employs polyelectrolytes as the separation carriers, is utilized for the separation of isomeric ions with close or nearly identical mobilities [5]; and (c) microemulsion EKC, similar to MEKC, but closer to pure liquid–liquid partitioning [6].

A number of manipulations have also been described for enhancing selectivity, including the following. (a) The use of different micelle types: although sodium dodecyl sulphate is generally used, other types of sulphated surfactants have been reported, such as tetradecyl sulphate and dodecane sulphonate. Positively-charged micelles, such as those formed by cetyltrimethylammonium bromide, are also employed [7]. (b) Mixed micelles, among ionic and non-ionic surfactants [8]. (c) pH variations in the background electrolyte: the distribution coefficient, migration time and selectivity can be

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substantially altered by changing the ionization of analytes [9]. (d) Temperature changes: the dependence of distribution coefficients on temperature is different among analytes; therefore, temperature will also affect the selectivity [10]. (e) Addition of ion-pair reagents: *e.g.*, addition of a tetralkylammonium salt to a solution of SDS micelles for the separation of anionic and cationic analytes [11]. (f) Addition of urea: urea is an excellent protein solubilizer and, although *per se* it should not quench hydrophobic interactions, in reality it does to some extent owing to lowering of the dielectric constant of water. Its main use is in hydrogen bond breaking [12]. (g) Organic solvents: these are extensively used in RP-HPLCx to adjust the capacity factor close to the optimum value. Reports on the use of methanol, 2-propanol and acetonitrile have appeared [13,14]. It must be emphasized that there is a limit to the concentration of organic solvent, because at high concentrations it might disrupt the micelle.

Under standard MEKC conditions, both the micelle and the surrounding aqueous phase migrate in the capillary; therefore, every analyte, if it is electrically neutral, must reach the detector during the migration time window t_0 and t_{mc} (where t_0 is the transit time of the aqueous phase and t_{mc} the migration time of the micelle). It has been hinted by Terabe that, if electroosmosis is completely suppressed (in a well coated capillary), the aqueous phase will remain in the capillary and the micelle will migrate through it. In other words, the only carrier of the analyte will be solely the micelle and not the concomitant electroosmotic flow. Under these conditions, only analytes that effectively interact with the micelle will be carried past the detector (placed at the anodic end when using SDS). Positively charged species or neutral species, if unable to partition in the SDS micelle, will never appear at the detector port. To our knowledge, this separation mechanism has not been implemented so far. We show here some unique separations of both charged and neutral isotopes.

2. Experimental

2.1. Materials

Benzene (benzene-h_6), [$^2H_6$]benzene (benzene-d_6), benzoic (benzoic-h_5) acid, [$^2H_5$]-benzoic (benzoic-d_5) acid, pyridine (pyridine-h_5), [$^2H_5$]pyridine (pyridine-d_5), benzyl (benzyl-h_5) alcohol, [$^2H_5$]benzyl (benzyl-d_5) alcohol, aniline (aniline-h_5), [$^2H_5$]aniline (aniline-d_5), 3-(morpholino)propanesulphonic acid (MOPS), SDS, Tris and Nonidet P-40 were purchased from Sigma (St. Louis, MO, USA). N-Acryloylaminoethoxyethanol was synthesized as described by Chiari *et al.* [15]. Ammonium peroxodisulphate and N,N,N′,N′-tetramethylenediamine (TEMED) were obtained from Bio-Rad Labs. (Hercules, CA, USA).

2.2. Methods

CZE was performed in a Waters Quanta 4000 capillary electrophoresis system (Millipore, Milford, MA, USA). For the experiments, 75-μm coated (modified Hjertèn procedure) fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA), 40 cm long (31 cm to the detector) were used. All the separations were carried out in coated capillaries using 50 m*M* acetate–Tris (pH 5.0, 4.7 and 4.0) buffer in the presence of Nonidet P-40 or SDS and 50 m*M* MOPS–NaOH (pH 7.2). The samples were loaded by hydrostatic pressure and the separations were carried out at room temperature. The detector was set at 254 nm.

2.3. Separation conditions

For the separation of pyridine from pyridine-d_5, the running buffer was 50 m*M* acetate–Tris (pH 5.0), injection for 5 s by hydrostatic pressure and run at 12 kV and 26.3 μA toward the cathode (Fig. 1A). The sample concentration was 1.45 m*M* for the hydrogenated form and 1.44 m*M* for the deuterated form. The sepa-

ration in Fig. 1B was obtained using 50 m*M* acetate–Tris (pH 5.0) and 2% Nonidet P-40 as running buffer, at 12 kV and 28.8 μA.

The separation of aniline from aniline-d_5 (Fig. 2) was performed in 50 m*M* acetate–Tris (pH 4.75) at 20 kV and 50 μA; the sample, 1.3 m*M*, was injected for 5 s by hydrostatic pressure.

For the separation of benzoic acid from benzoic-d_5 acid, the running buffer was 50 m*M* acetate–Tris (pH 4.0) with injection for 5 s by hydrostatic pressure and run at 25 KV and 26.3 μA towards the anode (Fig. 3A). The separation in Fig. 3B was carried out in 50 m*M* acetate–Tris (pH 5.0) in the presence of 50 m*M* SDS at 15 kV and 47 μA. The sample concentrations were 40 and 39 m*M*, respectively.

Benzene was separated from benzene-d_6 (Fig. 4) using 50 m*M* MOPS–NaOH (pH 7.2) with 50 m*M* SDS as running buffer, run at 13 kV and 48 μA towards the anode. The sample (3 m*M*) was injected for 10 s by hydrostatic pressure. The same conditions were used in the separation of benzyl alcohol from benzyl-d_5 alcohol (Fig. 5); the sample concentration was 0.15 m*M*.

2.4. Coating the capillary wall

The following procedure gave the best results. The capillary was first treated with 100 μl of 1 *M* NaOH for 5 h, then rinsed and flushed with 100 μl of 0.1 *M* HCl followed by 100 μl of 0.1 *M* NaOH. After 1 h it was rinsed with water and acetone, filled with a 1:1 solution of Bind Silane [3-(trimethoxysilyl)propyl methacrylate] in acetone and then incubated overnight. After this treatment, the capillary was flushed with air for 5 min and then washed with 20 m*M* phosphate buffer (pH 7.0). The capillary was filled with 6% N-acryloylaminoethoxyethanol solution in the same degassed buffer containing the appropriate amount of catalyst (0.5 μl of TEMED and 0.5 μl of 40% ammonium peroxodisulphate per millilitre of gelling solution). Polymerization was allowed to proceed overnight at room temperature and then the capillary was emptied by means of a syringe.

3. Results

Fig. 1A shows the separation of an artificial mixture of pyridine-h_5 and -d_5 (1:1) in an electroosmosis-free, coated capillary. The separation was conducted in acetate buffer, titrated to pH 5 (close to the p*K* values of the two components) so as to maximize charge differences, with migra-

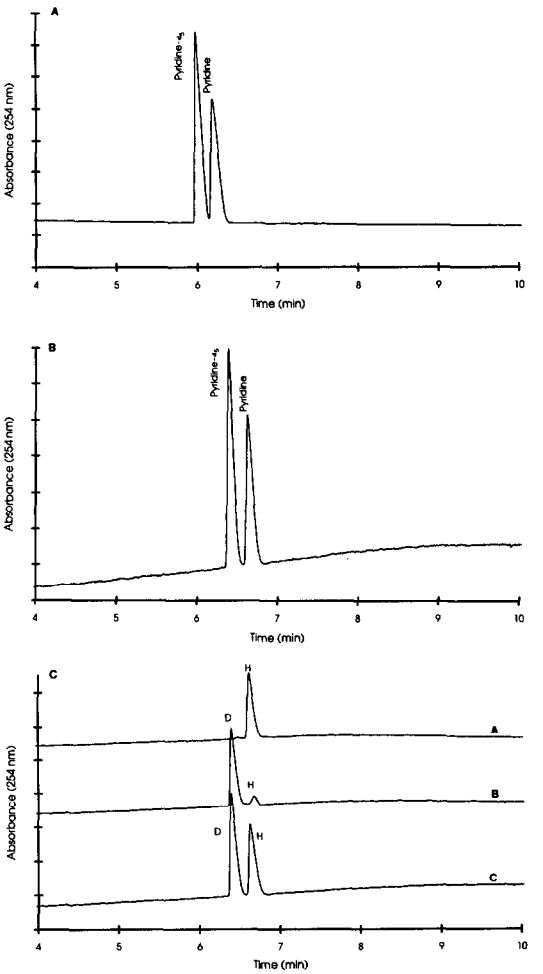

Fig. 1. Separation of pyridine-h_5 and -d_5 by CZE. Conditions: coated capillary; background electrolyte, 50 m*M* acetate–Tris (pH 5.0); run, 12 kV at 26.3 μA towards the cathode; sample injection, 5 s by hydrostatic pressure. (A) Separation of the 1:1 mixture; (B) same as A, but in the presence of 2% Nonidet P-40 in the running buffer; (C) overlap of three different runs: (trace A) pure pyridine-h_5, (trace B) pure pyridine-d_5 and (trace C) 1:1 mixture.

tion towards the cathode. Under these conditions there is essentially baseline separation between the two species, with pyridine-d_5 carrying the highest fractional charge, as it is eluted first.

An even more striking separation is obtained in presence of neutral micelles of Nonidet P-40 (Fig. 1B) under otherwise identical experimental conditions as in Fig. 1A. The elution order is still the same and the transit times are only 10% longer, possibly owing to the increment of viscosity caused by the detergent. However, the peak resolution (R) has now increased from 1.1 to 1.5. Fig. 1C shows an overlap of three different runs (in 2% Nonidet P-40), consisting of the 1:1 mixture, pure pyridine-h_5 and the corresponding pyridine-d_5. One can thus appreciate the relative contamination of the single components: pyridine-d_5 is contaminated by *ca.* 1% of pyridine-h_5.

Fig. 2 depicts the separation of an artificial mixture of aniline-h_5 and -d_5 (1:1) in an electroosmosis-free coated capillary. The separation was carried out in acetate buffer, titrated at pH 4.75 in order to maximize charge differences, with migration towards the cathode.

Fig. 3A shows the separation of benzoic-h_5 and -d_5 acid in a coated capillary, in acetate buffer (pH 4.0) in the absence of micelles. The migration is now towards the anode (reverse polarity). There is a clear hint of peak splitting, indicating that the presence of deuterium (in the aromatic ring) has some effect on the pK value. Given the elution order, it appears that benzoic-h_5 acid carriers the highest fractional charge, as it is eluted first. When the same run was repeated under MEKC conditions (50 mM SDS), a striking effect was seen on the resolution of the two peaks, which however maintained the same order of elution times, while doubling the transit times (Fig. 3B) ($R = 0.38$ in the absence and 1.14 in the presence of SDS).

Fig. 4 shows the separation of neutral species, benzyl-h_5 and -d_5 alcohol, in the presence of SDS micelles (50 mM). Under these conditions, both compounds would be lost at the cathodic reservoir if they did not interact with the SDS micelle, which now acts as the sole carrier. The

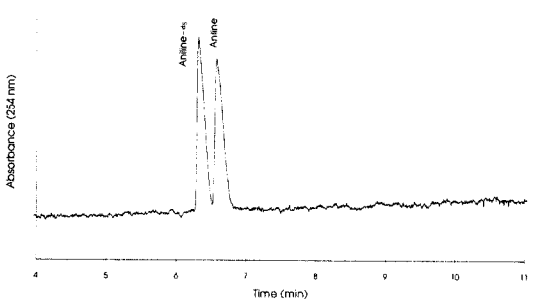

Fig. 2. Separation of aniline-h_5 and -d_5 by CZE. Conditions: coated capillary; background electrolyte, 50 mM acetate–Tris (pH 4.75); run, 20 kV 50 μA towards the cathode; sample injection, 5 s by hydrostatic pressure.

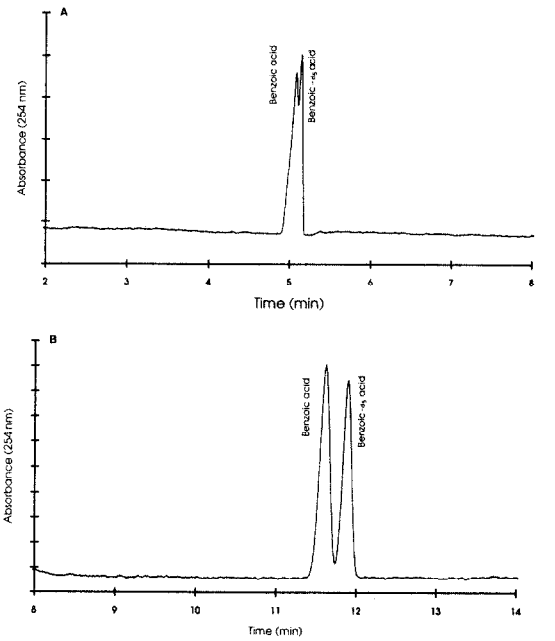

Fig. 3. Separation of benzoic-h_5 and -d_5 acid by CZE. Conditions: coated capillary; background electrolyte, 50 mM acetate–Tris (pH 4.0); run, 25 kV at 26.3 μA towards the anode; sample injection, 5 s by hydrostatic pressure. (A) Separation of the 1:1 mixture; (B) same as A, but in the presence of 50 mM acetate–Tris (pH 5.0) with 50 mM SDS in the running buffer, run at 15 kV and 47 μA.

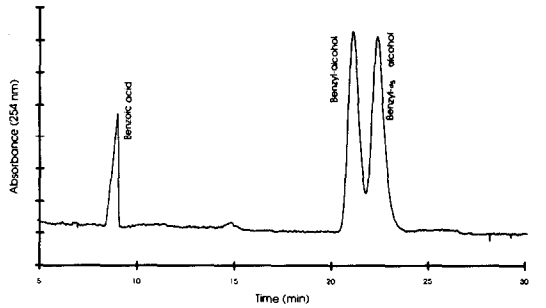

Fig. 4. Separation of benzyl-h_5 and -d_5 alcohol by MEKC. Conditions: coated capillary; background electrolyte, 50 m*M* MOPS (pH 7.2) containing 50 m*M* SDS; run, 13 kV at 48 μA towards the anode; sample injection, 5 s by hydrostatic pressure.

fact that benzyl-h_5 alcohol has a shorter transit time than the deuterated compound means that, in principle, the former has a higher affinity for the SDS micelle. This is truly a unique separation, previously unreported, to our knowledge, in the literature (note also the very good *R* value of 1.02).

In Fig. 5 shows the separation of another pair of neutral compounds, benzene-h_6 and -d_6, again in a micellar system containing 50 m*M* SDS in 50 m*M* MOPS buffer (pH 7.2). The separation of the 1:1 mixture is truly remarkable, and it must occur via a mechanism similar to that for the

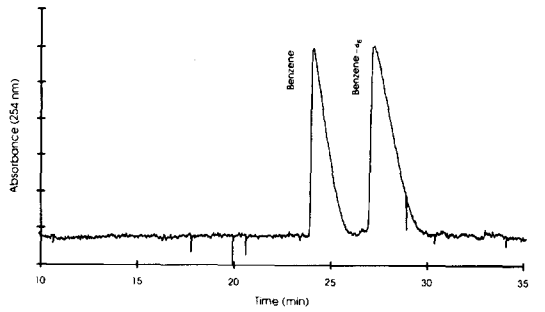

Fig. 5. Separation of benzene-h_6 and -d_6 by MEKC. Conditions: coated capillary; background electrolyte, 50 m*M* MOPS (pH 7.2) containing 50 m*M* SDS; run, 13 kV at 48 μA towards the anode; sample injection, 10 s by hydrostatic pressure.

separation of benzyl alcohols, as benzene-h_6 runs in front of the deuterated form (the *R* value here is also fairly high, 1.59).

4. Discussion

Some unique features of these separations are worth discussing.

4.1. Uniqueness of isotope separations by CZE or MEKC

To our knowledge, the only report that has appeared so far on the use of CZE for isotope separations is that by Terabe *et al.* [16], who achieved a remarkable separation between oxygen isotopic benzoic acids (of three types, $C_6H_5C^{16}O_2H$, $C_6H_5C^{16}O^{18}OH$ and $C_6H_5C^{18}O_2H$). They calculated that the difference in p*K* values, due to the oxygen replacement, was about 1% and they could obtain such a separation by adjusting the pH of the background electrolyte close to the p*K* values (pH 3.89). However, when separating all three isotopes, under different electroosmotic flow velocities (which they tried to modulate by addition of 0.1% hydroxypropylcellulose), they ended up with long separation times (up to 100 min) and poor plate counts. Even our separations of charged compounds (pyridine-h_5 and -d_5, aniline-h_5 and -d_5 and benzoic-h_5 and -d_5 acid) are different from the separations obtained by Terabe *et al.* [16], because they reported a direct effect on the p*K* values due to the presence of ^{18}O in the protolytic group. Conversely, in our case, the deuteration is distal, *i.e.*, it is in the rings, and not in the dissociating groups. Nevertheless, it has been suggested that even substitutions with deuterium atoms in neighbouring groups could alter the ionization constant by as much as 10%, owing to the slightly greater electron density near the ^2H-bonded carbon atom compared with that carrying only ^1H. This means that deuterium should have an electron-donating inductive effect as compared with hy-

drogen, and in the case of deutero-substituted acids this effect is revealed by their lower acidity. This mechanism would seem to be operative in, *e.g.*, Fig. 3A and B: the fact that benzoic-d_5 acid is the later eluting species suggests that it has a lower fractional charge than benzoic-h_5 acid.

4.2. Other examples in the literature

Whereas isotope separations in the CZE literature are scanty, many such separations exist in liquid and gas chromatography. An interesting theoretical study has been proposed by Poshkus [17]. He suggested three factors which drive such separations in adsorption chromatography: (a) the difference in the potential functions of the interaction between the isotopic molecules and the adsorbent surface (a quantum mechanical effect); (b) the differences of the quantum effects in adsorption for the external (translational and rotational) degrees of freedom of isotopic molecules, due to the difference in their masses and moments of inertia; and (c) the different changes of intramolecular vibrational energy of the isotopic molecules on adsorption, due to differences in the masses of the vibrating atoms. Tanaka and Thornton [18] reported the separation of deuterated and protiated palmitic acid and were able to measure secondary isotope effects on hydrophobic binding of these molecules to μBondapak C_{18} beads. Baweja [19] applied reversed-phase HPLC to the separation of deuterium and hydrogen analogues of a series of aromatic hydrocarbons. From the fact that the deuterated forms are eluted before their protiated analogues, he postulated that Van der Waals forces are operational. The C–^{1}H bonds induces greater forces of attraction between itself and the stationary phase than those generated between the C–^{2}H bond and the stationary phase, so that the deuterated-compound moves ahead of the protiated species. Cartoni and Ferretti [20] also separated pairs of isotopic molecules (*e.g.*, benzene-h_6 and -d_6) on the same principle of HPLC, hinting at the possibility of isolating pure isotopic molecules on a preparative scale. In a similar approach, Possanzini *et al.* [21] separated mixtures of isotopic molecules by GC on porous polymer beads. They hypothesized that the enhancement of resolution could be connected with the mass transfer in the gas phase and in the mobile phase and in the adsorption–desorption rates.

5. Conclusions and practical hints

Although in its infancy, the technique of MEKC under fully suppressed electroomosis, where the micelle acts as the sole carrier, could open up a new area in the vast field of micellar separations, especially for neutral analytes. There are some practical aspects that can be pointed out here. First, especially with neutral, aromatic analytes, one should try to avoid solubilization in plain organic solvents. These solvents will disrupt the SDS micelle at the injection port, leaving a plug of pure organic solvent and suppressing any chance of the analyte interacting with SDS micelles and being transported at the anode past the detector. Thus, such analytes should preferably be dissolved in aqueous–organic solvents, already containing SDS micelles. On the positive side, there is a unique feature of our coating (which is both extremely resistant to hydrolysis and highly hydrophilic), namely the slow adsorption of SDS. It is known that, in polyacrylamide-coated capillaries, electroosmosis is quickly restored, in SDS–MEKC, because a layer of SDS molecules is adsorbed (by hydrophobic interaction, via the alkyl chain) on the coating, thus regenerating a layer of negative charges on the wall. This process is greatly quenched with our coating with the novel monomer, owing to its extreme hydrophilicity. In addition, if, on prolonged use, some electroosmotic flux is generated, the surface can be simply restored by a conditioning step (even under drastic alkaline conditions, if necessary), removing the loosely bound SDS. This is a unique advantage that no conventional polyacrylamide coating will ever offer.

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