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Separation of theophylline and its analogues by micellar electrokinetic chromatography: application to the determination of theophylline in human plasma

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ABSTRACT

Micellar electrokinetic chromatographic separation of theophylline and its analogues was investigated using sodium dodecyl sulphate (SDS) as a micellar phase. The effects of pH, micelle concentration, applied voltage and temperature on the separation and preliminary quantitative analysis were studied for the determination of theophylline in human plasma. The data indicate that this technique could be used as the reference or routine method of theophylline measurement in therapeutic drug monitoring.

INTRODUCTION

Capillary electrophoresis has been developed as a very promising technique for the analysis of charged molecules, including proteins, peptides, DNA, etc. The application of this method was extended to neutral molecules by the introduction of micellar electrokinetic chromatography (MEKC), which was first developed by Terabe *et al.* [1]. The instrumental set-up of MEKC is identical with that of capillary zone electrophoresis, except that the micellar solution is used as the electrophoretic medium.

In MEKC, analytes can be separated on the basis of either the hydrophobicity or the ionic character of solutes. Selective partitioning of the analytes into the micellar phase as a pseudo-stationary phase causes them to migrate at different rates from the electrophoretic mobility. Early work [2,3] demonstrated the use of MEKC for polar neutral aromatic compounds. Applications of MEKC were expanded to neutral and charged organic molecules, including derivatized amino acids [4], water-soluble vitamins [5], pharmaceuticals [6] and nucleic acid-related compounds [7].

Therapeutic drug monitoring (TDM) uses analytical methods such as high-performance liquid chromatography (HPLC) and immunoassay to quantify drug levels in blood (often plasma/serum) specimens. There remains controversy over the clinical utility and cost effectiveness of TDM in medicine. TDM represents the practical interface between laboratory drug analysis and the often urgent medical application of the results for optimal patient treatment; consequently, clinical analyses must be simple and fast. HPLC offers infinite flexibility for simple drug analysis for TDM, but still requires relatively high analytical

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skill and is slow and expensive. As an alternative new technique, capillary electrophoresis could be a possible analytical method for TDM, being robust, accurate, precise, sensitive, specific, cheap, fast and capable of automation.

Theophylline is a bronchodilator for chronic asthma. TDM of theophylline has been done by immunoassay using an antibody against theophylline, and by HPLC [8,9]. In this paper, factors affecting the separation of theophylline and its analogues with MEKC have been studied, and the MEKC procedures for the quantitative determinations of theophylline, caffeine and paraxanthine spiked in human serum and theophylline in patient plasma are described.

EXPERIMENTAL

Apparatus

An Applied Biosystems (Foster City, CA, USA) Model 270A capillary electrophoresis system with a 72-cm (50.5 cm to detector, 50 μm I.D.) fused-silica capillary (ABI 0602-0041) with D-502A integrator (Young-In Scientific, Seoul, South Korea) was used for the data analysis. An on-column UV detector was set at 274 nm, and the temperature was kept constant at 26.5°C unless specified otherwise.

Prior to each run, the capillary was rinsed with 0.1 *M* NaOH and running buffer by the built-in vacuum system at 0.67 bar for 3–5 min. The capillary was filled with running buffer, usually 50–80 m*M* sodium dodecyl sulphate (SDS) in 25 m*M* phosphate buffer (pH 8.0) (see figure legends). The samples were introduced by the same vacuum system at 0.17 bar for 2 s (*ca.* 8.75 nl using Poiseuille's equation).

Reagents

We used HPLC-grade solvents and doubly distilled water. Theophylline, theobromine, caffeine, paraxanthine, uric acid and SDS were purchased from Sigma (St. Louis, MO, USA). Sodium tetraborate, sodium phosphate dibasic and sodium phosphate monobasic were from DukSan Pharmaceutical (Kyungkido, South Korea). Human sera were obtained as lyophilized form SRM 909 from National Institute of Science and Technology (NIST, Gaithersberg, MD, USA).

Sample preparation

The standard solution and sudan III were prepared in doubly distilled water at a concentration for each of the species. Drug concentrations in human serum (NIST SRM 909) spiked with theophylline, paraxanthine and caffeine and in patient plasma treated with theophylline were determined.

Samples were extracted with ethyl acetate as described previously [10]: 100 μl of standard solution, spiked serum or patient plasma were added to 900 μl of ethyl acetate in a glass tube, vortex-mixed vigorously for 30 s, and centrifuged for phase separation. A 500-μl volume of the organic phase was transferred to a clean glass tube. This procedure was repeated three times. The collected 1.5 ml of organic phase were evaporated with nitrogen gas and reconstituted with 50 μl of distilled water. This reconstituted sample was injected for capillary electrophoresis.

RESULTS AND DISCUSSION

Prior to the application of MEKC to theophylline monitoring for TDM, the factors affecting the separation of theophylline and its analogues (see Fig. 1 and Table I) by MEKC were studied.

In electropherograms of the similar six components in 25 m*M* phosphate buffer solution (pH

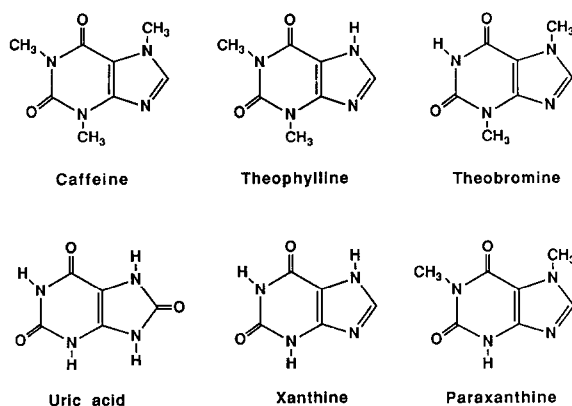

Fig. 1. Structures of theophylline and its analogues.

TABLE I

pK_a VALUES OF THEOPHYLLINE AND ITS ANALOGUES [11]

Compound	pK_a
Caffeine	12.3
Theobromine	9.9
Theophylline	8.9
Xanthine	0.8, 7.4, 11.1
Uric acid	5.4, 11.3
Paraxanthine	–[a]

[a] Not given.

8.0) without micelles, xanthine and uric acid were difficult to separate, as were theophylline and paraxanthine, and caffeine comigrated with the electroosmotic flow. A typical electropherogram with SDS is shown in Fig. 2. Methanol was used as a marker of the electroosmotic flow, and sudan III comigrated with the micelle fraction. In order to obtain optimum separation conditions for theophylline in human plasma, the effects of the SDS concentration and the pH in the running buffer, the applied voltage and the ambient temperature were investigated [12]. Because the separation of these solutes with MEKC is based on their hydrophobicity and ionic characteristics, the micelle concentration and the pH of the running buffer are the chief factors affecting the resolution. The migration time increases with increasing SDS concentration, and the degree of increase is dependent on the hydrophobicity of the molecules: the most hydrophobic molecule, caffeine, shows the largest increase, followed by paraxanthine, theophylline, theobromine, uric acid and xanthine. This trend is consistent with previous results published by Terabe and co-workers [13,14]. Theophylline, which has a similar hydrophobicity to paraxanthine, can be well resolved from paraxanthine by increasing the SDS concentration to above 60 mM.

The pH effect on migration time was examined with 25 mM phosphate buffer containing 80 mM SDS over the pH range 5.8–8.0. The migration times of caffeine, paraxanthine, theophylline and theobromine remained almost constant and the electroosmotic velocity also remained steady over this pH range. This implies that the micellar solubilization of these four solutes does not vary over this pH range. The migration time of xan-

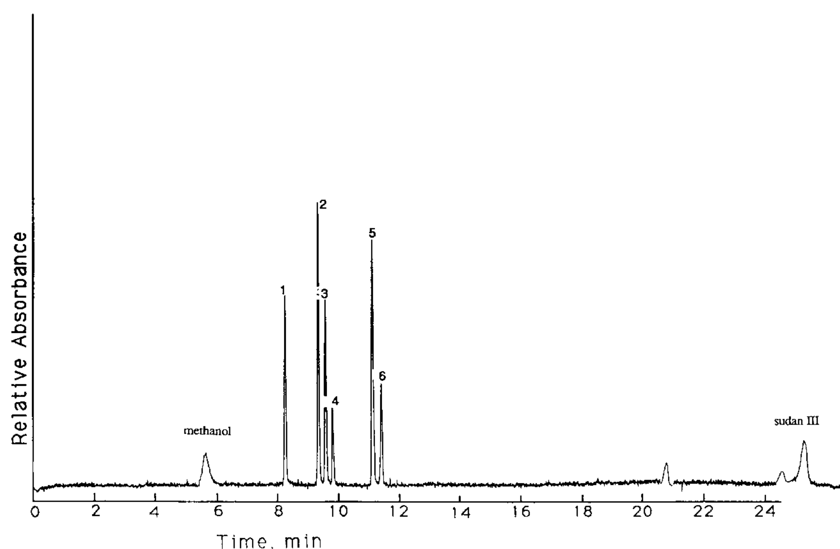

Fig. 2. Typical electropherogram of theophylline and its analogues in MEKC. Peaks: 1 = theobromine; 2 = theophylline; 3 = paraxanthine; 4 = xanthine; 5 = caffeine; 6 = uric acid. Buffer, 25 mM phosphate buffer (pH 8.0) containing 80 mM SDS; applied voltage, 21 kV; separation capillary, 720 mm × 0.05 mm I.D. fused silica; oven temperature, 26.5°C; detection wavelength, 274 nm.

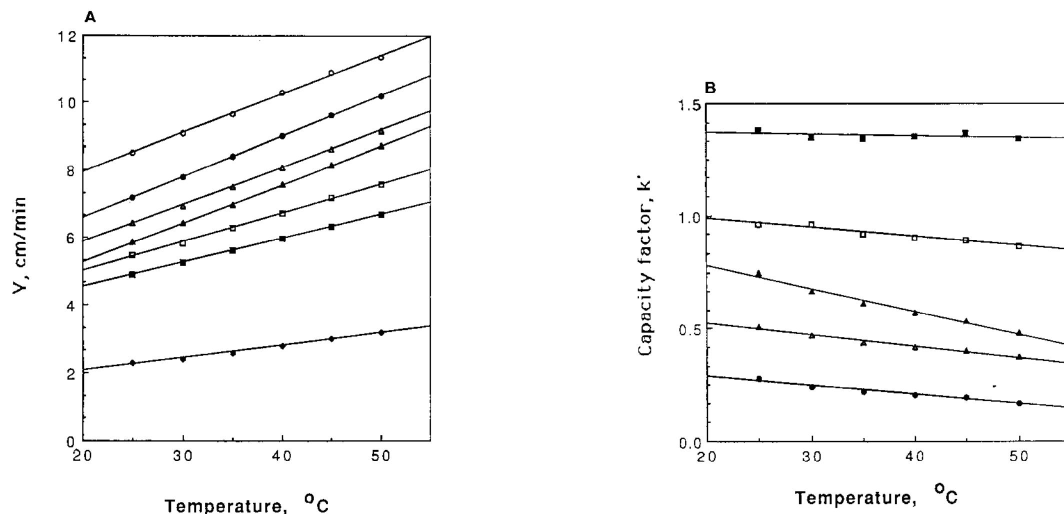

Fig. 3. Temperature effect on migration rate (A) and capacity factor (B). Separation conditions as in Fig. 2, except SDS concentration (50 m*M*), applied voltage (20 kV) and oven temperature (25°C). Symbols: ○ = methanol; ● = theobromine; △ = theophylline; ▲ = caffeine; □ = xanthine; ■ = uric acid; ◆ = Sudan III.

thine (pK_a = 7.4) increased markedly with increasing pH, and that of uric acid (pK_a = 5.4) showed a small increase between pH 5.8 and 6.6, and stayed constant above pH 6.6. These results suggest that micellar partitioning of solute around the pK_a is extremely sensitive to the pH, and that the optimization of MEKC for ionic solutes is easily manipulated by changing the pH around the pK_a of the solute.

The dependence of the velocity on the applied voltage was investigated, and a linear increase with increasing voltage was found. However, a positive deviation from the linearity was observed above 25–30 kV, which was magnified by raising the SDS concentration. In this case, a temperature rise caused by Joule heating at high applied voltage reduces the viscosity of solvent.

During the quantitative analysis of solutes by MEKC, it is important to maintain the reproducibility of the migration time and the peak area [15]. Temperature is a crucial factor in the reproducibility. The variation of migration rate with the air-circulated ambient temperature is shown in Fig. 3A. When this temperature increases, the velocities of electroosmotic flow, micelles and solutes are linearly increased, because of several factors: buffer viscosity, chemical equilibria, pH, etc.

The electroosmotic velocity can be described by the classical formula [16]

$$v_{eo} = \frac{\varepsilon \zeta}{\eta} E \qquad (1)$$

where ε is the permittivity of the liquid, ζ is the zeta potential, η is the viscosity of the liquid, and E is the electrical field strength. The value of v_{eo} is linearly correlated with $1/\eta$ in eqn. 1, and $1/\eta$ is linearly dependent on the temperature. Therefore, the increase of v_{eo} with temperature is linear, as shown in Fig. 3A. The increase of micelle ve-

TABLE II

DETECTION LIMITS UNDER THE SAME CONDITIONS IN FIG. 3

Compound	Detection limit (mol)
Theobromine	$1.1 \cdot 10^{-13}$
Theophylline	$2.8 \cdot 10^{-14}$
Caffeine	$1.0 \cdot 10^{-13}$
Xanthine	$6.6 \cdot 10^{-14}$
Uric acid	$1.6 \cdot 10^{-13}$

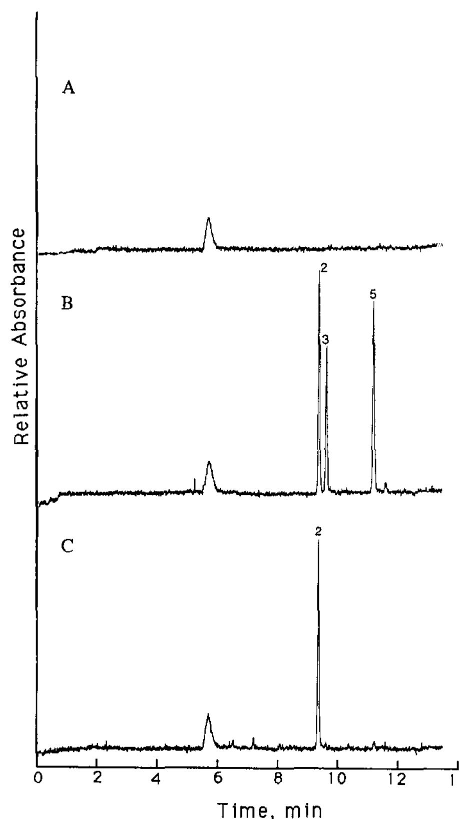

Fig. 4. Electropherogram of (A) blank human serum, (B) human serum spiked with theophylline (2), paraxanthine (3) and caffeine (5) and (C) plasma from a patient treated with theophylline. Serum and plasma samples were pretreated as described in Experimental, and the separation conditions were as in Fig. 2.

locity with temperature is less, presumably because the electrophoretic velocity, which is the negative direction, also increases as the temperature rises. The variation of the solute flow could be explained by a change in the distribution of solute between micelles and buffer, and the change in the electrophoretic mobility.

The temperature-dependent alteration of distribution of solutes between the micellar and aqueous phases is measured by calculating the capacity factor, k'. This is defined by the ratio n_{mc}/n_{aq}, as described previously [1], and can be calculated from retention times as follows:

$$k' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})} \quad (2)$$

where t_R is the retention time of a neutral solute that comigrates with the electroosmotic front. We used the capacity factors as a measure of the relative distribution of solutes between the micellar and aqueous phases at various temperatures. Fig. 3B shows that xanthine and uric acid have high k' values, caused by the acidity rather than the hydrophobicity. Caffeine, which is the most hydrophobic solute, shows the most marked variation of capacity factor with changing temperature. The k' value of uric acid, which is the most hydrophilic solute, is nearly constant over the range 25–50°C. These results indicate that temperature changes affect the chemical equilibrium of solutes between the micellar and aqueous phase.

The detection limits of solutes (Table II) were calculated for an injection volume of 8.75 nl, and a signal-to-noise ratio of 2. A detection limit in Table II of *ca.* 0.1 pmol means 0.1 pmol per 8.75 nl sample concentration. This sensitivity is good enough to analyse theophylline and caffeine in the clinical therapeutic range (5–20 μg/ml).

Serum samples were pretreated as described in Experimental. Blank human serum showed no peaks except the electroosmotic flow front (Fig. 4A). Human serum spiked with theophylline, caffeine and paraxanthine, which is one of possible

TABLE III

RECOVERIES OF ETHYL ACETATE EXTRACTION OF CAFFEINE AND THEOPHYLLINE SPIKED IN HUMAN SERUM

Concentration (μg/ml)	Recovery (mean ± S.D., n = 5) (%)	C.V. (%)
Caffeine		
5	94.0 ± 4.7	4.9
10	88.2 ± 3.3	3.7
20	98.0 ± 2.5	2.6
40	97.6 ± 2.5	2.3
60	94.4 ± 3.3	3.5
Theophylline		
5	97.0 ± 2.8	2.9
10	105.8 ± 1.6	1.5
20	108.5 ± 1.9	1.8
40	109.6 ± 2.3	2.1
60	96.3 ± 2.1	2.2

TABLE IV

REPRODUCIBILITY OF RETENTION TIME AND PEAK AREA OF INTRA- AND INTER-DAY RUNS

	Retention time (min)				Peak area		
	EOF[a]	Theophylline	Paraxanthine	Caffeine	Theophylline	Paraxanthine	Caffeine
Within-run (n = 14)							
Mean	5.46	8.64	8.86	10.20	13 738	9617	14 323
S.D.	0.04	0.08	0.07	0.06	661	278	445
C.V.(%)	0.78	0.96	0.84	0.54	4.81	2.89	3.11
Between-run (n = 59)							
Mean	5.57	8.77	8.89	10.33	14 113	9946	14 714
S.D.	0.16	0.33	0.35	0.44	910	575	964
C.V. (%)	2.89	3.82	3.89	4.22	6.45	5.79	6.55

[a] Electroosmotic flow.

interfering compounds in theophylline measurement, showed three peaks with the same retention times as the standard compounds in Fig. 2 (Fig. 4B). A plasma sample from patient undergoing theophylline therapy displayed a peak with the same retention time as the theophylline peak. This shows that the extraction procedure is good enough to remove the complicating components from the matrix, including proteins.

For the quantitative analysis, the correlation between the peak area and the sample concentrations (5–60 μg/ml) in human serum was studied. The respective linear regression equations for caffeine and theophylline were $y = 418.34 + 705.51x$, $r = 0.999$, and $y = 191.28 + 906.10x$, $r = 1.000$. The extraction recovery was tested by adding known amounts of theophylline and caffeine to human serum. The recovery of theophylline was 93–98% and that of caffeine 98–110% (Table III). We carried out precision tests for the retention time and peak area with human serum spiked with 10 μg/ml theophylline, paraxanthine and caffeine (Table IV). The coefficients of variation (C.V.) of the retention time were less than 1%, and those of the peak area for theophylline, paraxanthine and caffeine were 4.81, 2.98 and 3.11%, respectively. We calculated between-run C.V. with identical samples as for the within-run test, on seven days. The between-run C.V. of the retention time were 2.9–3.9%, and those of the peak area 5.8–6.5%. Large values for the between-run C.V. of the retention time indicate a change in column conditioning. Since the precision of the peak area is affected by variation in the retention time, stable and reproducible separation conditions must be established prior to the quantitative analysis.

These experiments have demonstrated the feasibility of monitoring theophylline and caffeine in human serum (plasma) by MEKC using 25 m*M* phosphate buffer (pH 8.0) containing 80 m*M* SDS, with on-column UV detection. The preliminary quantitative results suggest the suitability of MEKC as a TDM method for serum theophylline and caffeine.

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