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Determination of theophylline in plasma using different capillary electrophoretic systems

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ABSTRACT

Theophylline was determined in human plasma by capillary electrophoresis (CE). The drug was used as a model substance to study a simple sample pretreatment often used in HPLC: to 200 μl of plasma were added 400 μl of acetonitrile to precipitate the plasma proteins and the supernatant was injected into the capillary after centrifugation. Three capillary electrophoretic systems were compared with respect to migration time and electrophoretic migration reproducibility. UV detection at 280 nm was applied. The separation was preferably made in an uncoated fused-silica capillary (57 cm × 75 μm I.D.) with 10 m*M* phosphate–borate buffer (pH 9.0) as the electrophoretic buffer. A linear calibration graph was obtained in the concentration range studied, 1.8–36 μg/ml (10–200 μM). The method permits the determination of theophylline in plasma at therapeutic concentrations of 4.5–20 μg/ml (25–110 μM) with acceptable precision.

INTRODUCTION

Capillary electrophoresis (CE) has in recent years become an important separation technique owing to improvements in sensitivity, reliability and speed [1]. Various methods for the determination of drugs in body fluids has been reported recently [2–15]. Detection has been effected by UV absorbance measurements [2–10,12–14], by laser-induced fluorescence [11,14,15] and in one application by a conventional LC fluorescence detector with a laboratory made micro-LC cell [13].

To obtain the required separation for relatively small molecules, micellar phases are often added to the electrophoretic buffer. The fundamental principles of micellar electrokinetic chromatography (MEKC) have been described by Terabe and co-workers [16,17]. Micellar systems have been demonstrated to increase the separation capability for neutral and positively charged compounds. The presence of surfactants such as sodium dodecyl sulphate (SDS) has also been useful to prevent adsorption of plasma proteins on the capillary walls and rinsing with buffer between injections led to reproducibility of migration times when plasma samples were injected directly into the capillary [4,5]. It is more common, however, to rinse the capillary with both sodium hydroxide and buffer between sample injections. Micellar systems with direct injection of plasma or serum samples have been used for the determination of cefiramide [3,4], aspoxicillin [5], barbiturates [6], β-adrenoceptor blocking agents [7], thiopental [8], cicletanine enantiomers [9] and cimetidine [10]. Other sample pretreatments have included liquid–liquid extraction [8,9] and solid-phase extraction [10,11] to concentrate the sample before injection into the capillary.

Several methods for determination of drugs have also been developed without micelles in the electrophoretic buffer. Ferulic acid was determined in dog plasma after liquid–liquid extraction using 25 m*M* phosphate buffer (pH 9.2) [2]. Cytosine-β-D-arabinoside was determined in

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plasma after solid-phase extraction using citrate buffers [12]. Thiols were determined in human whole blood after pretreatment and derivatization using 0.1 *M* phosphate buffer (pH 2.5) [13]. Naproxen was determined in serum after extraction with hexane–diethyl ether. The residue was dissolved in water–ethanol (1:1) and about 10 nl were injected hydrodynamically into the capillary [14]. Anthracyclines in human plasma were determined after liquid–liquid extraction with chloroform and back-extraction into phosphoric acid. Analyte interaction with the capillary wall was prevented by using 70% acetonitrile in phosphate buffer as the electrophoretic buffer [15].

Methanol and acetonitrile have been added to the electrophoretic buffer to improve the separation [9,11] or to increase the detection response in atmospheric pressure ionization mass spectrometry [18]. Addition of acetonitrile, methanol and other organic solvents to the electrophoretic buffers reduces the electroosmotic flow in the capillary [19,20].

A simple plasma sample pretreatment in HPLC is to add methanol, acetonitrile or a strong acid to remove proteins from the plasma sample before injection on to the LC column [21]. The technique is used for compounds that are present in high concentrations where no preconcentration of the samples is needed and for hydrophilic compounds that have low recoveries in liquid–liquid extraction procedures. We were interested in exploring the technique for CE and to investigate whether it is possible to inject plasma extracts on to the capillary that contained high concentrations of acetonitrile. Theophylline was chosen as a model substance because the drug is often present in high concentrations in human plasma after therapeutic doses.

It was possible to detect theophylline in therapeutic concentrations of 4.5–20 μg/ml (25–110 μM) with acceptable precision. Three electrophoretic systems were compared with respect to migration time and electrophoretic mobility for theophylline when repeated injections of theophylline dissolved in dilute electrophoretic buffers and in plasma extract were made.

EXPERIMENTAL

Materials

Theophylline was kindly obtained from Kabi Biopharma (Stockholm, Sweden). Acetonitrile was of LiChrosolv quality (E. Merck, Darmstadt, Germany) and water was purified with a Milli-Q reagent water system (Millipore, Bedford, MA, USA). All other chemicals were of analytical-reagent grade and were used as received.

Capillary electrophoresis instrumentation and methods

A Beckman (Palo Alto, CA, USA) P/ACE System 2100 capillary electrophoresis system was used. The electrophoretic separation was performed either in a fused-silica capillary, eCAP (Beckman), or in a coated capillary, CElect H175 (Supelco, Bellefonte, PA, USA). The dimensions were 57 cm × 75 μm I.D. × 375 μm O.D. The electrophoretic buffers were prepared from an aqueous solution of sodium dihydrogenphosphate to which disodium tetraborate decahydrate solution was added to give pH 9.0. Sodium dodecyl sulphate (SDS), 25 m*M*, was added to the final solution in some of the experiments. The buffers were filtered through a 0.45-μm Miller-HV filter (Millipore) prior to use. Hydrodynamic injections were made by applying a 0.5 p.s.i. (3.45 kPa) pressure for 1–5 s using the "low-pressure" device in the instrument at the inlet end of the capillary. Detection was performed by on-column measurement of UV absorbance at 280 nm at a position 50 cm from the capillary inlet. All separations were performed at 12 kV. The current was continuously monitored during analysis using the second data collection channel in the P/ACE.

Reproducibility of migration times. Variations in migration times and electrophoretic mobilities after repeated injections of plasma samples were first studied using uncoated capillaries. Study 1 was performed with an electrophoretic buffer consisting of 25 m*M* SDS in 10 m*M* sodium phosphate–borate buffer (pH 9.0) and study 2 with 10 m*M* sodium phosphate–borate buffer (pH 9.0) as the electrophoretic buffer. To each

experiment a new uncoated fused-silica capillary (eCAP) was used. The new uncoated capillary was rinsed for 5 min with 1 *M* sodium hydroxide solution, 2 min with water, 2 min with 0.1 *M* hydrochloric acid and 2 min with water by pressurizing the inlet of the capillary at 138 kPa ("high pressure") using the standard pressure device of the CE instrument followed by 30 min with electrophoretic buffer using the "low-pressure" device. Thereafter nine determinations of acetone (10% solution in water) were made to monitor the electroosmotic flow in the capillary. Acetone was injected for 1 s (5 nl injected).

A 10 μg/ml standard solution of theophylline in the electrophoretic buffer diluted 1:10 with water and plasma extracts with theophylline (see *Sample preparation*) was then injected. One injection of theophylline standard solution was followed by five injections of plasma extract from the same vial. The cycle was repeated 7–9 times. The theophylline sample was injected for 5 s (26 nl injected). Acetone was also injected (1 s) with each theophylline sample to monitor the electroosmotic flow.

A standardized washing procedure, 1 min with 0.1 *M* sodium hydroxide solution and 3 min with electrophoretic buffer using "high pressure", was applied before each injection. Study 1 was repeated once and in the second experiment the capillary was rinsed for 4 min with electrophoretic buffer only before injections. The separation was made at 12 kV for 10 min.

Study 3 was performed with a coated capillary. The coated capillary (CElect) was treated according to the manufacturer's instructions, *i.e.*, no initial washing procedure was performed. The electrophoretic buffer was 20 m*M* sodium phosphate–borate (pH 9.0). The equilibration of the capillary with buffer was monitored by the injection of acetone as described above, performing a separation at 12 kV for 10 min. Then the same cycle, one injection of theophylline standard solution followed by five injections of plasma extracts with theophylline (3 s), was performed. With each injection acetone was also injected (1 s) to monitor the electroosmotic flow. The separation was made at 12 kV for 26 min. Before each injection, the capillary was rinsed for 1 min with 0.1 *M* sodium hydroxide solution and 3 min with electrophoretic buffer using "high pressure".

A capillary equilibrated with 25 m*M* SDS in 10 m*M* sodium phosphate–borate buffer (pH 9.0) was filled with buffer diluted 1:10 with water (study 4) and plasma extract (study 5) to simulate the injection situations. Acetone and standard theophylline solution were then injected and the migration times of the compounds were monitored when 12 kV was applied.

Calculations. The calculation of column efficiency was based on $N = 5.54\ (t_m/w_{0.5})^2$, where N is the number of theoretical plates, t_m is the migration time of the compound and $w_{0.5}$ is the peak width at half-height.

The electroosmotic flow, u_{eo}, was calculated by $u_{eo} = L_d L_t / V t_0$, where L_d is the capillary length to the detector, L_t the total length of the capillary, V the applied voltage and t_0 the time for a neutral marker (acetone) to reach the detector [1]. The observed electrophoretic mobility, $u_{ep,obs}$, was calculated in a similar way using t_m, the migration time for the substance. The mobility of the substance, u_{ep}, was then obtained from the relationship $u_{ep} = u_{ep,obs} - u_{eo}$.

The injection volume was calculated from the flow obtained in the capillary during injection. The flow was measured from the time, t_n, needed for a neutral compound to reach the detector applying the "low-pressure" device in the instrument and the volume of the capillary to the detector, $(\pi r^2 L_d)/t_n$.

Sample preparation

Stock solutions of theophyline were prepared in methanol and further dilutions were made with 0.05 *M* phosphate buffer (pH 7.4).

A calibration graph for theophylline in plasma was prepared from plasma samples containing 0.45, 0.9, 1.8, 2.7, 4.5, 9.0, 18, 27 and 36 μg/ml. Quality control plasma samples of theophylline contained 2.7, 9.0 and 18 μg/ml. The plasma samples were stored at −20°C until analysed.

In the work-up procedure, plasma samples were deproteinized with acetonitrile. To 200 μl plasma were added 400 μl of acetonitrile in an

Eppendorff tube. The tubes were capped, shaken for 2–3 min and centrifuged at 6890 *g* (6000 rpm) for 5 min. The supernatant was transferred into a 400-μl vial inserter and injected into the CE. The plasma extract for the reproducibility studies was prepared in the same way in a large batch and stored at −70°C until used. The concentration of theophylline in plasma was 10 μg/ml.

When plasma samples from patients were analysed the CE conditions used in study 2 described above were used. Daily the capillary was rinsed for 5 min with 1 *M* sodium hydroxide solution, 2 min with water, 2 min with 0.1 *M* hydrochloric acid and 2 min with water using "high pressure" and then equilibrated with electrophoretic buffer for 30 min using "low pressure".

RESULTS AND DISCUSSION

The described CE instrumentation offers the possibility of obtaining highly reproducible results owing to the standardized washing procedure that can be programmed and automation of injection. Three different CE systems were compared to obtain the best CE system for the determination of theophylline in plasma with little sample pretreatment.

Reproducibility of migration times

The results from study 1 are given in Fig. 1, where the obtained run-to-run variability for acetone, standard theophylline dissolved in buffer diluted 1:10 with water, theophylline in plasma extract and an endogenous plasma peak are shown. Fig. 1A gives the migration times found. The relative standard deviation (R.S.D.) of the migration time for theophylline in the plasma extract was 2.1% (36 injections) and 0.54% of the standard theophylline (8 injections). After a total of 55 injections some erroneous values were obtained with unexpectedly long migration times. When the capillary had been rinsed using the same procedure as for a new capillary, the migration times were restored.

When study 1 was repeated with a 4-min rinse with buffer only between sample injections, the theophylline peak in the plasma extract disappeared after ten injections whereas the endogenous plasma peak could still be recorded. It seems that theophylline was adsorbed on the capillary walls when the capillary was rinsed only with buffer between injections. Other systems, in which plasma was injected directly, have been reported to be stable when the capillary was rinsed with buffer between injections [4,5]. These separations were performed in 50 μm I.D. capillaries with 20 m*M* buffers. Theophylline standard solution injected dissolved in dilute buffer gave the same result as in Fig. 1A, *i.e.*, R.S.D. = 0.72% for the migration time (eight injections).

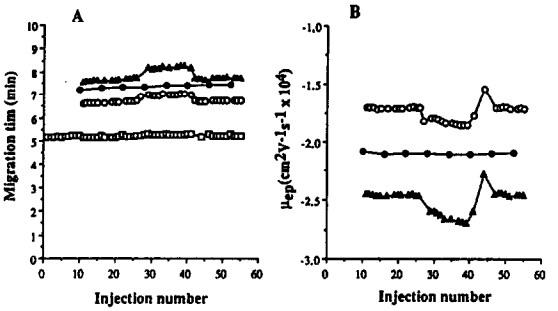

Fig. 1. Reproducibility in micellar capillary electrophoresis. Capillary, uncoated fused silica, 75 μm I.D. × 57 cm, 50 cm to the detector; temperature, 30°C; voltage, 12 kV; current, 31 μA; buffer, 25 m*M* SDS in 10 m*M* phosphate–borate buffer (pH 9.0); injection volume, 26 nl (5-s pressure injection). (A) Variation of migration times for repeated injections; (B) variation of electrophoretic mobility for repeated injections. □ = Acetone; ● = theophylline (standard); ○ = theophylline (plasma); ▲ = endogenous plasma peak.

The electrophoretic mobilities for the compounds were calculated and are given in Fig. 1B. The graph shows that fluctuations in the electroosmotic flow could not explain the larger variation in migration times for theophylline in the plasma extract compared with theophylline dissolved in dilute buffer. The variations in the electrophoretic mobility for theophylline in plasma extract and the endogenous plasma peak with time are the same. It can also be noticed that theophylline in the plasma extract has a shorter migration time than theophylline dissolved in dilute buffer. Theophylline is negatively charged to 70% at pH 9.0 and did not seem to be partitioned into the micelles as the electrophor-

etic mobility for the standard theophylline was roughly the same with and without SDS in the electrophoretic buffer (compare Figs. 1B and 2B).

For study 2, the migration times for 60 injections are shown in Fig. 2A. After 39 injection there was a break in power and the system was stopped for 10 h. The study was continued without any additional washing of the capillary. A small jump in migration times can be seen in Fig. 2A after 39 injections but they remained fairly constant. Inspection of the peak shape for the theophylline plasma peak showed, however, a tendency for fronting. The asymmetry factor was 0.62 for theophylline for the first injection of plasma extract after the stop. This fronting of the peaks increased thereafter and the last set of plasma samples had asymmetry factors of about 0.31. This can be compared with asymmetry factors in the range 0.94–1.04 before the stop. We concluded that some adsorption of plasma components on the capillary wall occurred during the run and that additional washing was needed after a certain number of injections. The R.S.D. for the migration time of theophylline in the plasma extract was 1.8% (40 injections).

The electrophoretic mobility was calculated for each run and is shown in Fig. 2B. There is also a tendency in this system for theophylline in plasma extracts to migrate faster than theophylline standard, but the differences between the electrophoretic mobility for theophylline standard and theophylline in plasma extracts are much smaller than in study 1.

In study 3, a coated capillary (CElect) was used. In this system the injection volumes had to be decreased from 26 to 16 nl (3-s injections). The design of the study was the same as for the earlier studies but the separation time was increased to 26 min owing to the longer migration times of the compounds in this system. The migration times for acetone, theophylline standard and plasma extract are given in Fig. 3A and the electrophoretic mobilities in Fig. 3B. The plasma peak was not recorded owing to the long migration time. The migration times for theophylline increase with time and the fluctuations in migration times are very pronounced for theophylline in the plasma extract, R.S.D. = 10% (fourteen injections). The electrophoretic mobility of theophylline in the plasma extract was surprisingly constant, R.S.D. = 0.65%. The observed variation in migration times seems to be due to changes in the electroosmotic flow during the study. Acetonitrile may interact with the capillary wall and the coating does not seem to be compatible with acetonitrile. Very stable migration times have been reported on this type of capillary when peptides and proteins were injected in a pure aqueous system [22].

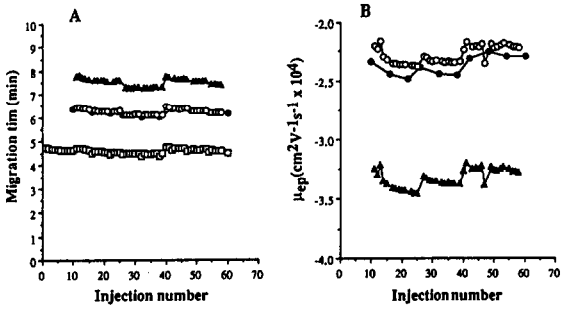

Fig. 2. Reproducibility in capillary electrophoresis. Capillary, uncoated fused silica, 75 μm I.D. × 57 cm, 50 cm to the detector; temperature, 30°C; voltage, 12 kV; current, 20 μA; buffer, 10 m*M* phosphate–borate buffer (pH 9.0); injection volume, 26 nl (5-s pressure injection). (A) Variation of migration times for repeated injections; (B) variation of electrophoretic mobility for repeated injections. Symbols as in Fig. 1.

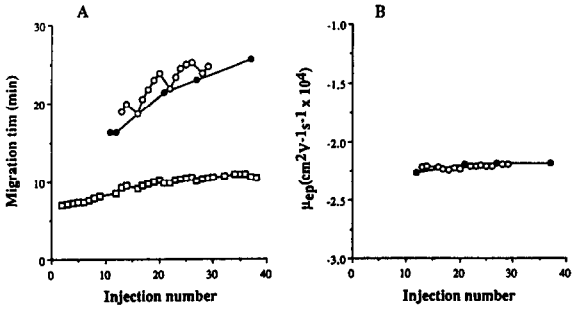

Fig. 3. Reproducibility in capillary electrophoresis. Capillary, coated, CElect, 75 μm I.D. × 57 cm, 50 cm to the detector; temperature, 30°C; voltage, 12 kV; current, 37 μA; buffer, 20 m*M* phosphate–borate buffer (pH 9.0); injection volume, 16 nl (3-s pressure injection). (A) Variation of migration times for repeated injections; (B) variation of electrophoretic mobility for repeated injections. Symbols as in Fig. 1.

Sample solvent and electroosmotic flow

It is preferable to inject the sample in a solvent where the conductivity of the sample is lower than in the surrounding buffer. The sample will then move very fast to the boundary between the injection plug and the buffer solution when the voltage is applied. A concentration of the sample at the boundary occurs and highly efficient peaks are obtained. A threefold improvement in detectability has been reported for peptides injected dissolved in water compared to the electrophoretic buffer [23].

In our studies, theophylline was negatively charged and was swept toward the cathode and the detector by the electroosmotic flow in the capillary. To obtain an idea of the velocities in the injection plug, studies 4 and 5 were performed. For a tenfold dilution of the electrophoretic buffer with water, $u_{ep,obs} = 8.0 \cdot 10^{-4}$ was calculated for theophylline. In the plasma extract, however, a much lower value, $u_{ep,obs} = 1.5 \cdot 10^{-4}$, was obtained. This was the lowest value of $u_{ep,obs}$ obtained in all the electrophoretic buffers used in studies 1–3 (Table I).

Addition of 50% of acetonitrile to the electrophoretic buffer has been shown to reduce the electroosmotic flow by *ca.* 40% [19,20]. We observed a small decease in u_{eo} in studies 2 and 3 when plasma extracts were injected. In contrast, in study 1, with SDS present a small increase in u_{eo} was observed for the plasma extracts.

A strong electroosmotic flow in the capillary seems desirable for our separation. The same high efficiency, $N = 210\,000$, was obtained for the theophylline peaks, injected dissolved in dilute buffer and in the plasma extract, in study 2 with the highest electroosmotic flow. Highly efficient peaks were obtained despite the long injection plug of 6 mm for a 26-nl injection.

The discontinuity of the solvent in the capillary caused a decrease in current over the capillary until the injection plug had migrated to the capillary end. This was most pronounced for the plasma extracts with SDS in the system. Electropherograms of theophylline are given in Fig. 4 together with the recorded current. Fig. 4A shows theophylline dissolved in dilute buffer

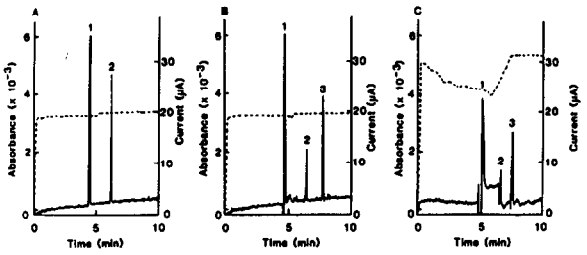

Fig. 4. Electropherograms of theophylline. (A) Standard theophylline, 10 μg/ml, dissolved in electrophoretic buffer diluted 1:10 with water; (B) plasma extract with theophylline, corresponding to 10 μg/ml plasma. Conditions in (A) and (B) as in Fig. 2. (C) Plasma extract with theophylline, corresponding to 10 μg/ml plasma. conditions as in Fig. 1. Peaks: 1 = acetone (neutral marker for the electroosmotic flow); 2 = theophylline; 3 = endogenous plasma peak.

TABLE I

ELECTROOSMOTIC FLOW AND OBSERVED ELECTROPHORETIC MOBILITY FOR THEOPHYLLINE IN DIFFERENT SYSTEMS

Conditions: 1 = 25 m*M* SDS in 10 m*M* phosphate–borate buffer (pH 9.0); 2 = 10 m*M* phosphate–borate buffer (pH 9.0); 3 = 20 m*M* phosphate–borate buffer (pH 9.0); 4 = buffer 1 diluted 1:10 with water; 5 = plasma extract (mainly acetonitrile); voltage, 12 kV; temperature, 30°C.
Samples: a = theophylline (10 μg/ml), dissolved in electrophoretic buffer diluted 1:10 with water; b = theophylline in plasma extract. Injection volume = 16 nl in system 3 and 26 nl in the other systems.

Parameter	1		2		3		4	5
	a	b	a	b	a	b	a	a
μ_{eo} (10^{-4} cm^2 V^{-1} s^{-1})	7.48	7.55	8.76	8.62	4.0	3.9	10	2.2
$\mu_{ep,obs}$ (10^{-4} cm^2 V^{-1} s^{-1})	5.38	5.81	6.40	6.35	1.8	1.7	8.0	1.5

from study 2. The same current profile was obtained for standard theophylline with SDS in the electrophoretic buffer. Fig. 4B shows theophylline in plasma extract from study 2 and Fig. 4C theophylline in plasma extract from study 1. To obtain reproducible results, standards and samples should be prepared in the same solvent.

Analysis of plasma samples

The electrophoretic conditions used in study 2 were chosen for the determination of theophylline in plasma from patients. The procedure was studied in the range 1.8–36 μg/ml. The correlation coefficient was 0.996 ($y = 0.024 + 0.162x$) for the calibration graph (peak-height measurements). Plasma samples from patients were analysed together with three quality control samples in each series. Two electropherograms are shown in Fig. 5A and B. The plasma sample in Fig. 5B contained theophylline (6.9 μg/ml). The reproducibility (between-series) was 8.0% at a level of 2.7 μg/ml, 4.4% at 9.0 μg/ml and 2.6% at 18 μg/ml. The limit of determination was 1.8 μg/ml (10 μM).

The reproducibility was of the same order as for our standard HPLC method [24]. The limit of determination for theophylline in plasma was lower with HPLC, however, 0.45 μg/ml (2.5 μM).

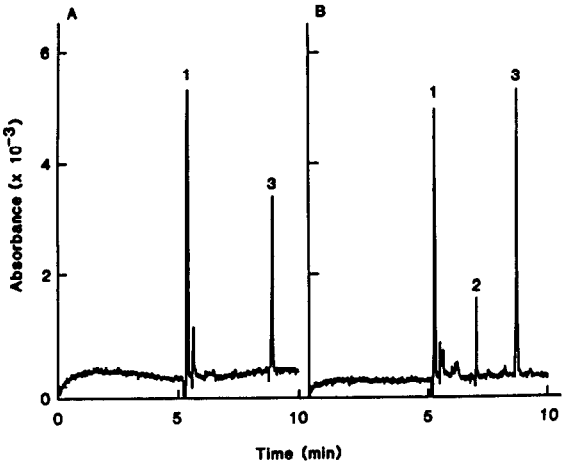

Fig. 5. Electropherograms of plasma samples. (A) Blank plasma; (B) plasma sample from a patient, containing theophylline (6.9 μg/ml). Conditions as in Fig. 2. Peaks as in Fig. 4.

This study has shown that it is possible to determine theophylline in plasma at therapeutic concentrations by CE with acceptable precision.

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