Journal of Chromatography, 615 (1993) 327–333
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6788

Pharmacokinetic studies using micellar electrokinetic capillary chromatography with *in vivo* capillary ultrafiltration probes

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(First received December 15th, 1992; revised manuscript received February 12th, 1993)

ABSTRACT

Micellar electrokinetic capillary chromatography (MECC) is shown to be a quantitative method for the determination of theophylline in capillary ultrafiltrates of biological systems. MECC exhibits reproducibility in migration times of 1.3% relative standard deviation (R.S.D.) (n = 31) and peak heights of 3.0% R.S.D. (n = 28). MECC and reversed-phase liquid chromatography (LC) are shown to be complementary techniques for the determination of theophylline in ultrafiltrate samples. *In vivo* sampling of awake, freely moving rats is achieved using capillary ultrafiltration probes implanted in subcutaneous tissue. The ability of MECC coupled with *in vivo* capillary ultrafiltration to determine theophylline pharmacokinetics is demonstrated. The half-life of elimination for a 15 mg/kg intraperitoneal dose of theophylline was determined to be 3.1 ± 0.4 h for MECC and 3.2 ± 0.4 h for LC (n = 4, mean ± standard error of the mean). Concurrent results for derived pharmacokinetic parameters (area under the curve, volume of distribution, concentration at time zero and clearance) were obtained for MECC and LC.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC) was introduced by Terabe *et al.* in 1984 [1] and extended the application range of capillary zone electrophoresis (CZE) to include neutral molecules. MECC has a unique separation mechanism, combining both the charge and partition of the molecule into the micellar mobile phase. MECC has been shown to be a high-resolution separation method that requires only submicroliter samples [2,3]. MECC has been used for the determination of a number of therapeutic drugs including penicillins [4,5], antipyretic analgesic preparations [6], cephalosporins [4,7] and theophylline [8,9]. The determination of cicletanine by MECC was compared to liquid chromatography (LC) and showed comparable accuracy and precision [10].

Capillary ultrafiltration probes have been shown to be effective for *in vivo* sampling of small molecules in awake animals [11,12]. Capillary ultrafiltration probes implanted in subcutaneous tissue can provide samples at a rate of 1–3 μl/min. The ultrafiltrates obtained exhibit quantitative recovery of free drug concentration and do not contain protein or cell matter. The pharmacokinetics of theophylline in rats has been determined *in vivo* using capillary ultrafiltration [13] and microdialysis probes coupled to LC [14].

This study describes the application of MECC coupled with capillary ultrafiltration probes for the determination of pharmacokinetic parameters of theophylline. CZE has been shown to be useful for the determination of pharmacokinetic param-

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0378-4347/93/$06.00

eters when coupled with microdialysis probes for *in vivo* measurements [15]. The ability to obtain reproducible migration times and peak heights over multiple injections was investigated. Base washes and equilibrium with electrophoresis buffer were found to be necessary to achieve acceptable reproducibility. The importance of the diluent used for calibration standards is discussed. The determination of theophylline pharmacokinetics in awake, freely moving rats is demonstrated. Capillary ultrafiltration probes implanted in subcutaneous tissue provide a continuous quantitative sampling method that can be used for extended periods of time. The small sample requirement and injection method of MECC permit each sample to be analyzed multiple times. MECC and reversed-phase LC show consistent results for derived pharmacokinetic parameters.

EXPERIMENTAL

Micellar electrokinetic capillary chromatography

Electrophoresis was performed using an Applied Biosystems 270-HT electropherograph (Foster City, CA, USA). Fused-silica capillaries of 70 cm (50 cm to the detector) with dimensions of 50 μm I.D. and 375 μm O.D. (Polymicro Technologies, Tucson, AZ, USA) were used. An on-column UV detector, set at 274 nm, was used. The capillary was temperature-controlled at 30°C. The samples were cooled using an Isotemp cooling bath (Fisher, Pittsburg, PA, USA) set at −4°C. Prior to the start of a set of samples (for pharmacokinetic experiments) and every nine samples, the capillary was rinsed for 5 min with deionized water, 5 min with 0.25 *M* NaOH and 5 min with electrophoresis buffer at 5080 mmHg. The capillary was then equilibrated for 5 min. Before each sample was introduced, the capillary was rinsed with 0.25 *M* NaOH for 1 min and with electrophoresis buffer for 4 min. The column was equilibrated at 21 kV for 5 min. The electrophoresis buffer was 25 m*M* sodium phosphate at pH 8.0 with 80 m*M* sodium dodecyl sulphate (SDS). The sample was introduced using the vacuum system for 5 s using 1270 mmHg (15 nl injected). Separations were conducted at 300 V/cm.

Liquid chromatography

The determination of theophylline was carried out with a BAS 201A chromatograph (West Lafayette, IN, USA) using a Biophase II C_{18}, 3 μm particle size, 100 mm × 3.2 mm I.D. column at 35°C and a flow-rate of 1 ml/min. A 5-μl injection loop was used. A mobile phase of 100 m*M* sodium phosphate buffer (pH 6.1) with 5% acetonitrile was used. The metabolites and theophylline were quantitated using UV detection at 274 nm.

Materials

Theophylline, 3-methylxanthene, 1-methylxanthene and 1,3-dimethyluric acid were purchased from Sigma (St. Louis, MO, USA) and used as received. All buffers were made from analytical-grade materials purchased from Aldrich (Milwaukee, WI, USA). HPLC-grade acetonitrile and sterile Ringer's solution (Baxter, McGraw Park, IL, USA) were used as received. All solutions were made with double-distilled, deionized water and filtered through a 0.45-μm Nylon filter.

Subjects and surgery

Hooded Long Evans rats, 370–412 g (Harlan–Sprague Dawley, Indianapolis, IN, USA) were used. The animals were housed in a temperature-controlled room under a 12-h light–dark cycle. Food and water were provided *ad libitum*. Subjects were anesthetized with a 10:1 (100 mg/ml) mixture of ketamine–xylazine (1 ml/kg). Subjects were weighed to the nearest gram while anesthetized. A 5-mm incision was made in the back between the shoulders. A second incision was made in the back of the animal 5.1–7.6 cm posterior to the shoulders. A thin-walled 13-gauge needle was inserted through the two incisions. A UF-3-16 capillary ultrafiltration probe, with three loops of polyacrylonitrile–methallylsulfone membrane, 16 cm × 310 μm O.D. × 220 μm I.D. (BAS) was inserted into the needle. The needle was then carefully removed, leaving the probe in the subcutaneous tissue. The probe was sutured to the skin to secure it. The two incisions were then sutured closed. The animal was then transferred to an awake animal sampling system

and connected to the swivel. The animals were allowed to recover from surgery for between 4 and 12 h before experiments were conducted.

In vivo pharmacokinetic experiments

The awake animal system used has been previously described [11,13]. Briefly, the animal was attached to a counter balanced arm through a wire tether. The arm supended a single channel swivel. The combination of the swivel and swinging arm provided the animal with complete mobility about the cage. The capillary ultrafiltration probe was connected to the swivel with 110 μm I.D. TPTFE tubing (BAS). The swivel was likewise connected to a Minipulse 2 (Gilson, Villier-le-Bel, France) peristaltic pump. The peristaltic pump was equipped with 0.007 in. tubing with a total internal volume of 6.2 μl. The peristaltic pump was always set to produce a flow-rate of 3 μl/min. Samples were collected using a CMA 140 fraction collector (BAS/CMA, West Lafayette, IN, USA). Ultrafiltrate samples were collected for at least 1 h prior to all experiments. Theophylline was administered intraperitonealy in a 15 mg/kg dose in Ringer's solution. Samples were collected at timed intervals of 15 min.

Pharmacokinetic parameters

Theophylline pharmacokinetics follows an open two-compartment model with first-order absorption [16]. The disposition of theophylline can be described by the following equation:

$$C_t = A_1 e^{-\alpha t} + A_2 e^{-\beta t} - (A_1 + A_2) e^{-k_a t}$$

where α is the fast distribution constant, β is the elimination constant and k_a is the absorption constant. The absorption and fast distribution process are both occurring simultaneously. Therefore, it is difficult to evaluate the rate at which distribution occurs [17]. Thus, only the slow disposition (elimination) half-life was determined for the β term. The half-life of elimination was determined by plotting the log of the concentration against time [13,18]. Pharmacokinetic parameters were determined using standard methods [13] for individual subjects, and then the mean and standard error of the mean (S.E.M.) were computed for the group.

RESULTS AND DISCUSSION

Quantitative aspects of MECC

When compared to LC, the reproducibility of peak areas and migration times of capillary electrophoresis has been inferior [10,19–21]. These problems have been associated with the unique electroosmotic pumping system used. Variations in electroosmotic flow are most commonly a result of adsorption of protein and other positively charged ions to the negatively charged capillary surface. In MECC this can still be a problem even with the high concentration of SDS (80 m*M*) present in the buffer [22]. The use of capillary ultrafiltration probes as a sampling method matches capillary electrophoresis systems quite well because protein and cell matter are excluded and small volumes are obtained.

The reproducibility of several washing methods was investigated. Theophylline and three of its metabolites were used to provide a group of charged and neutral molecules. Multiple injections, without NaOH rinses between injections, resulted in a gradual decrease in electroosmotic flow, increasing migration time. Multiple injections with NaOH washes and complete system flushes at least every ten injections provided reproducibility of migration times of 1.3% relative standard deviation (R.S.D.) ($n = 31$) and 3.0% R.S.D. ($n = 28$) for peak height. Fig. 1 illustrates the reproducibility of the MECC method described. The reproducibility is similar to that of previously reported work using automated instrumentation with hydrodynamic injections [23]. In comparison with LC, MECC tended to have a higher probability of an error in peak height. The average R.S.D. for MECC was 20.9%, whereas that of LC was 15.6%. These data also reflect the variations between animals. Due to the small volume injected, 15 nl, each sample was electrophoresed three times to insure statistically significant results. The separation of theophylline requires approximately 6–10 min by MECC, whereas the analysis can be done by LC in 4 min.

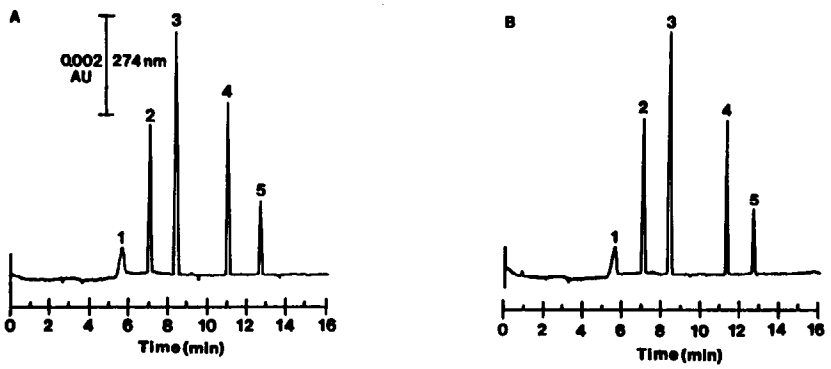

Fig. 1. Effect of eighteen continuous injections of theophylline and its metabolites with NaOH and buffer rinses after each determination, with additional system flushes every ten samples. (A) First injection; (B) eighteenth injection. Peaks: 1 = methanol; 2 = 3-methylxanthene; 3 = theophylline; 4 = 3-methyluric acid; 5 = 1,3-dimethyluric acid. Separation conditions are given in the text.

The total analysis time for MECC is much greater though, as long as 25 min, due to the need for rinsing and equilibrating the capillary between each sample. Due to the small volume injected in CZE, 10–15 nl, the concentration of sample becomes a limiting factor. LC is less limited in this manner, for the sample size required can range from a few nanoliters to over 20 μl.

It was also observed that standards made in different diluents, Ringer's solution and basal ultrafiltrate, provided different results when examined by MECC but no difference when studied by LC. A 30% difference in slope was obtained for standards made in Ringer's solution and basal ultrafiltrate and analyzed by MECC. The standards made in Ringer's solution resulted in sharper, taller peaks with greater peak area, underestimating concentrations of ultrafiltrate samples. This was attributed to the conductivity in the sample plug and the magnitude of focusing that took place during electrophoresis [24,25]. The ultrafiltrate samples have a higher ionic strength than the micellar buffer resulting in defocusing of the zones. All concentrations were calculated for MECC using standards made in basal ultrafiltrate for this reason.

In vivo pharmacokinetics of theophylline

Capillary ultrafiltrate samples were collected every 15 min and analyzed immediately by LC, then 25 μl were stored at 0°C until analyzed by MECC. The rate of sample collection was 3.0 ±

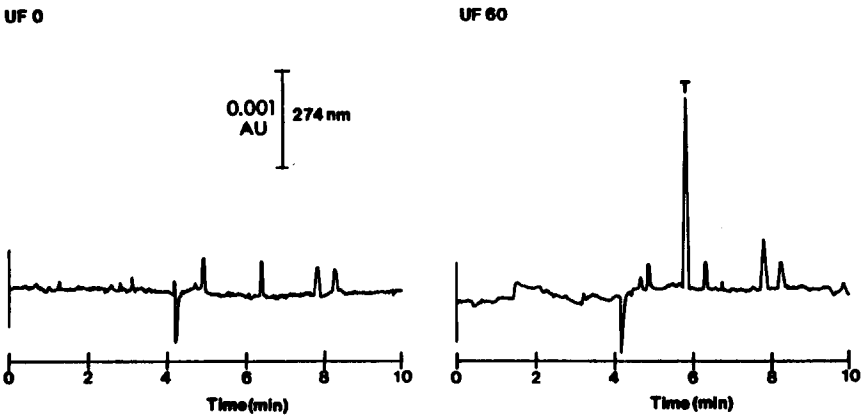

Fig. 2. Comparison of the MECC separation of *in vivo* capillary ultrafiltrates before (UF 0) and 60 min after (UF 60) a 15 mg/kg theophylline dose. The peak for theophylline (10 μg/ml) is marked with a T. Separation conditions are given in the text.

TABLE I

COMPARISON OF DERIVED PHARMACOKINETIC PARAMETERS FOR THEOPHYLLINE DOSED INTRAPERITONEALLY AT 15 mg/kg BY MECC AND HPLC

For all parameters mean ± S.E.M. are given ($n = 4$); $t_{1/2}$ elimination = elimination half-life; AUC = area under the curve; V_d = volume of distribution; Cl = clearance; C_0 = concentration at time zero.

Parameter	MECC	HPLC	% Difference
$t_{1/2}$ elimination (h)	3.1 ± 0.4	3.2 ± 0.4	3.2
AUC (μg min/ml)	3845 ± 503	4024 ± 403	4.5
V_d (l)	0.41 ± 0.01	0.40 ± 0.02	2.5
Cl (ml/min)	1.5 ± 0.2	1.5 ± 0.1	0.0
C_0 (mg/l)	16.1 ± 1.7	18.0 ± 1.1	11.1

0.2 μl/min, and samples were obtained every 15 min. An example of theophylline determination by MECC in capillary ultrafiltrates, before and 60 min after dosing, is illustrated in Fig. 2. The derived pharmacokinetic parameters for MECC and LC for a 15 mg/kg intraperitoneal dose are presented in Table I. The results correspond very well, with a very small percentage difference between the two methods. Using the Student's t-test, no statistically significant difference was obtained. The variation observed in C_0 (concentration at time zero) was most likely due to the storage of samples. The correlation between the two methods for the determination of theophylline in rat ultrafiltrates is illustrated in Fig. 3. A slope of 0.954 was obtained for the average of four subjects. Fig. 4 illustrates concurrent theophylline disposition (mean ± S.E.M.) by MECC and LC ($n = 4$).

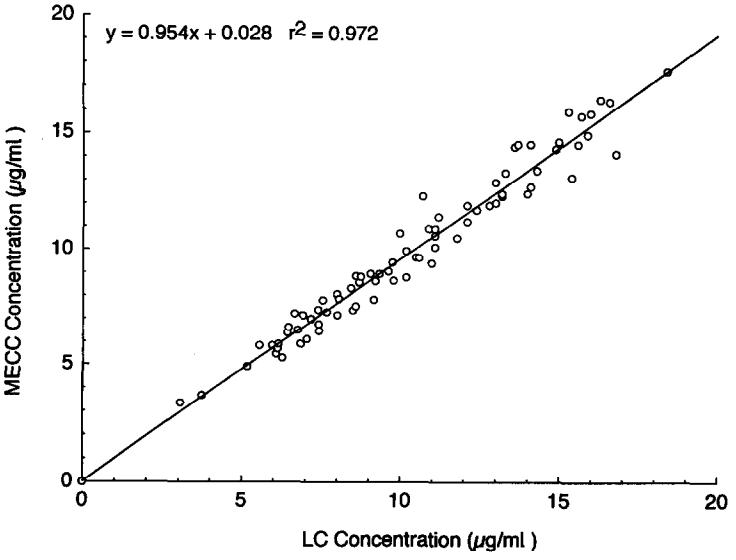

Fig. 3. Correlation of theophylline in rat ultrafiltrates determined by MECC and LC. The slope of the line is 0.954. For both LC and MECC four subjects were used. The average of triplicate determinations during each experiment for MECC are reported.

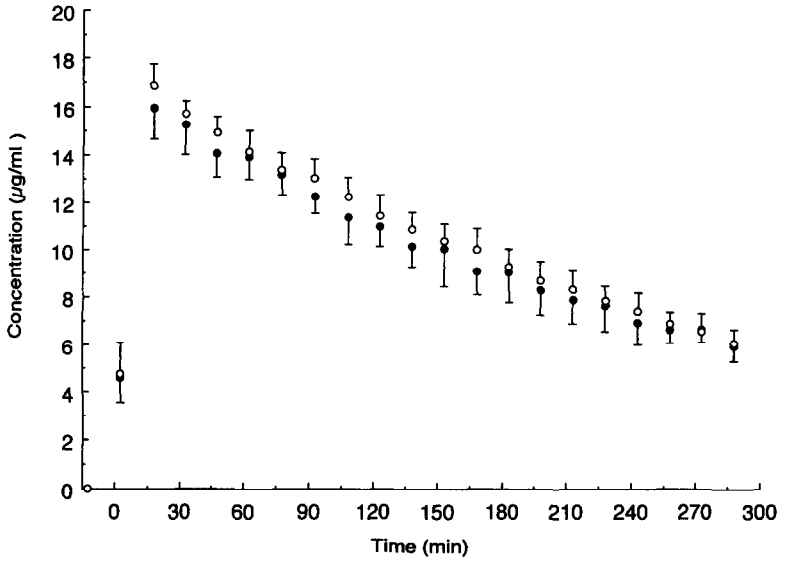

Fig. 4. Comparison of the disposition of theophylline determined by MECC (●) and LC (○). For LC, each point represents the mean ± S.E.M. ($n = 4$). The MECC data represent the mean ± S.E.M. of triplicate determinations during each experiment, and a total of four experiments.

CONCLUSIONS

MECC has been shown to be an effective method for determination of theophylline pharmacokinetics in capillary ultrafiltrates. Reproducible migration times and peak heights can be obtained using short base rinses and equilibration of the column. MECC is advantageous for use with *in vivo* sampling probes due to the small volume required for analysis. Capillary ultrafiltration probes provide samples with quantitative recovery of free theophylline concentration from awake, freely moving animals. Samples can be acquired at a rate of 1–3 μl/min. Comparison of MECC and LC for the analysis of theophylline pharmacokinetics show consistent results.

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