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Determination of substituted purines in body fluids by micellar electrokinetic capillary chromatography with direct sample injection

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ABSTRACT

Many substituted purines (theobromine, caffeine, paraxanthine, theophylline and uric acid, as well as other methylated xanthines and uric acids) can easily be separated and analysed in one run using micellar electrokinetic capillary chromatography with a borate–phosphate buffer containing 75 m*M* sodium dodecyl sulphate (pH ≈9). Serum, saliva and urine samples collected after the self-administration of caffeine and serum samples from patients receiving theophylline or caffeine pharmacotherapy were screened for substituted purines. The data presented show the ease of using on-column multi-wavelength detection for investigating the feasibility of direct sample application, the characterization of sample pretreatment procedures and peak confirmation by comparing absorption spectra. It is shown that the determination of purines in serum and saliva samples, including therapeutic concentrations of caffeine and theophylline, can be accomplished without any sample pretreatment, whereas sample extraction is required for the determination of purines in urine. Quantitative data for the determination of micromolar amounts of theophylline (samples from adult patients) and caffeine (samples from infants born prematurely) in serum samples compare well with data obtained by non-isotopic immunoassays. Micellar electrokinetic capillary chromatography with the direct injection of serum or saliva samples requires only microlitre volumes of sample and several different compounds can be determined within a few minutes.

INTRODUCTION

Theophylline is a potent bronchodilator and respiratory stimulant and is widely used in the treatment of asthma [1]. Both theophylline and caffeine are used for the treatment of apnoea in infants born prematurely [2,3]. As a consequence of the variation of pharmacokinetics between patients, it is widely recognized that it is necessary to monitor concentrations of drugs in individual patients to ensure the maximum clinical response and to avoid undesirable side-effects associated with overdoses of these compounds. Caffeine clearance is also used as a liver function test, which requires the determination of caffeine concentrations in serum or saliva samples [4]. Non-isotopic immunoassays [3,5] and many chromatographic methods [3,5–7] have been developed as instrumental approaches for the determination of theophylline and caffeine in body fluids.

High-performance capillary electrophoresis (HPCE) and micellar electrokinetic capillary chromatography (MECC, an interface between electrophoresis and chromatography) are attractive approaches for the determination of pharmaceuticals in body fluids [8–16]. In MECC two distinct phases are used, an aqueous and a micellar or pseudo-stationary phase. These two phases are established by buffers containing surfactants [*e.g.* sodium dodecyl sulphate (SDS)] above their critical micellar concentration. An MECC analysis is performed in equip-

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ment designed for HPCE, *i.e.* in an open-tubular capillary of very small internal diameter. A high-voltage d.c. electric field is applied along the column, causing a movement of the entire liquid (the so-called electro-osmotic flow) and migration of the charged micelles. Non-ionic solutes partition between the two phases and elute with zone velocities between those of the two phases. Their elution order is essentially based on the degree of partitioning [14]. In such a system several substituted purines of very similar structure can be well separated with a buffer of about pH 8 containing 50 m*M* SDS [17].

In a previous investigation [15] the advantages of using fast-scanning, multi-wavelength detection for the HPCE–MECC determination of barbiturates in serum samples and urine was shown. This approach allowed peak confirmation and peak purity to be evaluated by comparing the absorption spectra. The objectives of the work described in this paper were to investigate (i) the suitability of direct sample introduction and various extraction procedures for the determination of caffeine, theophylline, paraxanthine, uric acid and related compounds in human serum, saliva and urine samples by MECC using on-column, fast-scanning polychrome detection, and (ii) the determination of theophylline and caffeine in serum samples by MECC compared with non-isotopic immunoassays.

EXPERIMENTAL

Chemicals and origin of samples

All chemicals used were of analytical-reagent or research grade. The purines were obtained from Fluka (Buchs, Switzerland), except uric acid, which was purchased from Merck (Darmstadt, Germany). Bovine plasma was prepared by centrifugation of bovine blood (from the local slaughterhouse) and blank human serum samples were obtained by centrifugation of the authors' blood (1500 *g* for 10 min). Blank saliva samples were obtained from a subject who does not drink any beverages containing caffeine or other methylxanthines. Serum samples were collected in the routine drug assay laboratory where they were received for therapeutic drug monitoring of patients receiving theophylline pharmacotherapy and of infants born prematurely and receiving treatment with caffeine or theophylline. After centrifugation, all samples were assayed for theophylline or caffeine by an automated enzyme immunoassay and stored at −20°C until required.

Self-administration of caffeine

Coffee was prepared from disposable bags of instant coffee packaged for the study of the overnight clearance of caffeine from saliva [4]. A bag containing 2 g of instant decaffeinated coffee powder spiked with 140 mg of caffeine was used to prepare a cup of coffee. One or two cups were administered.

Immunoassays

Caffeine concentrations were determined in serum and saliva samples by an automated enzyme-multiplied immunoassay technique (EMIT; Syva, Palo Alto, CA, USA) using a Cobas Bio centrifugal analyser (F. Hoffmann-La Roche, Diagnostica, Basle, Switzerland) as previously described by Zysset *et al.* [3]. Theophylline concentrations were measured using an EMIT (Syva) procedure similar to that for caffeine and by an automated fluorescence polarization immunoassay (FPIA) on a TDx analyzer (Abbott Laboratories, Irving, TX, USA). The FPIA assays were performed according to the manufacturer's instructions. The EMIT assays for caffeine and theophylline are designed to determine caffeine and theophylline concentrations from 5.2 to 155 μM (1–30 μg/ml) and 13.9 to 222 μM (2.5–40 μg/ml), respectively. The FPIA assay operates between 2.8 and 222 μM (0.51–40 μg/ml).

Electrophoretic instrumentation and running conditions

The instrument with multi-wavelength detection used in this work has been described previously [15]. Briefly, it features a 75 μm I.D. fused-silica capillary of about 90 cm length (Product TSP/075/375, Polymicro Technologies, Phoenix, AZ, USA) and a fast-scanning multi-wavelength detector (Model UVIS 206 PHD) with an on-column capillary detector cell (No. 9550-0155; both from Linear Instruments, Reno, NV, USA) towards the capillary end. The effective separation distance was 70 cm. A constant voltage of 20 kV was applied. The cathode was on the detector side. Sample application occurred manually through dipping the anodic capillary end into the sample vial and lifting it 34 cm for a specified time interval (typically 5 s). Multi-wavelength data were

read, evaluated and stored by a Mandax AT 286 computer system using the 206 detector software package version 2.0 (Linear Instruments), with windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Conditioning for each experiment was performed by rinsing the capillary with 0.1 *M* sodium hydroxide solution for 3 min and with buffer for 5 min. Throughout this work the 206 detector was used in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals (26 wavelengths). With these settings the sampling rate was 3.69 data points per second and wavelength unit.

For quantitation the model 270A capillary electrophoresis system (Applied Biosystems, San Jose, CA, USA) was used. This apparatus features automated capillary rinsing, sampling and execution of the electrokinetic run. For these experiments it was equipped with a 50 μm I.D. fused-silica capillary with an effective separation length of 35 to 45 cm. A Model D-2000 chromato-integrator (Merck-Hitachi, Darmstadt, Germany) was used for recording the pherograms and for quantitation by peak-area measurements. The integrator sampling period was set to one data point per 200 ms throughout this work. Unless otherwise stated, before each run the capillary was rinsed with 0.1 *M* sodium hydroxide solution (1 min) and with buffer (2 min). The injection of the sample was achieved by vacuum suction for 1–2 s. A constant voltage of 15—30 kV was applied and the temperature was set at 40°C.

Electrophoresis buffers and standard solutions

For monitoring the purines a buffer of 75 m*M* SDS, 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4 (pH $\approx$ 9) was used. All standard solutions of purines were prepared in buffer or methanol at concentrations of 100–360 μg/ml. The blank and patient samples were spiked by adding known aliquots of these standard solutions to the body fluids prior to sample injection or extraction. For quantitation, aliquots of the methanolic standard solutions were added to a glass test-tube with a conical bottom, evaporated to dryness under a stream of nitrogen (40°C) and reconstituted with either 0.5 ml of bovine plasma, blank human serum or patient serum.

Direct injection of body fluids

Serum, saliva and urine samples were either injected as received or, prior to injection, filtered using 0.2-μm Nalgene (25 mm diameter) disposable syringe filters (Nalge Company, Rochester, NY, USA).

Extraction of purines

The rapid extraction of substituted purines from serum (or bovine plasma) and saliva samples was achieved using Sep-Pak C_{18} cartridges (Waters, Division of Millipore, Milford, MA, USA), which are reversed-phase octadecylsilane-bonded silica columns. These were conditioned immediately before use by drawing 2 ml of methanol followed by an equal volume of water through the column with a plastic syringe. The columns were loaded by the application of a mixture of 0.5 ml of serum (bovine plasma) or saliva sample and 0.5 ml of 20 m*M* phosphate buffer (pH 7), then rinsed with 2 ml of water and dried with an equal volume of air. The columns were eluted into a test-tube with 0.6 ml of methanol before evaporation to dryness under a gentle stream of nitrogen at 30–40°C. The residue was dissolved in 200 μl of running buffer.

Liquid–liquid extraction for the determination of substituted purines in serum (bovine plasma) and saliva samples by MECC was performed using a modification of the method of Zysset *et al.* [3]. Into an 11-ml screw-capped Sovirel test-tube, 0.5 ml of serum (bovine plasma) or saliva sample, 0.2 g of ammonium sulphate and 8 ml of chloroform–isopropanol (1:1, v/v) were added. After vigorous shaking for 30 min and centrifugation at 1500 *g* for 10 min the organic layer was transferred into a test-tube with a conical bottom and evaporated to dryness under a gentle stream of nitrogen at 70°C. The residue was dissolved in 200 μl of running buffer, shaken for 30 s, taken up by a 2-ml plastic syringe and filtered through a Nalgene syringe filter (0.2 μm, 25 mm diameter).

The purines were extracted from urine using Bond Elut Certify cartridges and the Vac Elut setup (both from Analytichem International, Harbor City, CA, USA). With minor alterations, the manufacturer's instructions for acidic and neutral drugs were used, as described previously for the determination of barbiturates [15]. After extraction the dried residue was dissolved in 100–200 μl of running buffer.

RESULTS AND DISCUSSION

MECC of substituted purines in serum, saliva and urine samples

The three-dimensional electropherogram shown in Fig. 1A represents the absorbance *versus* retention time *versus* wavelength relationship for a model mixture consisting of ten substituted purines. These compounds are found in the metabolic pathways of caffeine and theophylline in humans [18]. Each component is characterized by its retention and migration behaviour, with theobromine (compound 1) being the fastest (the least interaction with the micelles) and uric acid (compound 10) the slowest. Fig. 1B shows the data at three wavelengths only, indicating that these purines are readily detected at 280 nm and that there is baseline resolution except between 1-methyluric acid (compound 8) and 7-methyluric acid (compound 9). Under the investigated experimental conditions, several other purines were found to co-elute with one of these compounds. 3,7-Dimethyluric acid formed a mixed zone with 7-methylxanthine, 1-methylxanthine with 1-methyluric acid, and 1,3- and 1,7-dimethyluric acid with 3-methylxanthine. The absorbance spectrum of each compound can be extracted from the data points as so-called time slices. When the background absorption was subtracted, the spectra compared well with those measured on a standard spectrophotometer. Background-subtracted, normalized spectra are used to aid comparisons (see below).

Two extraction methods were investigated to monitor caffeine and theophylline in serum samples. Fig. 2 shows three-dimensional pherograms of theobromine, caffeine and theophylline extracted from spiked bovine plasma using the liquid–liquid extraction (panel A) and the Sep-Pak C_{18} (panel B)

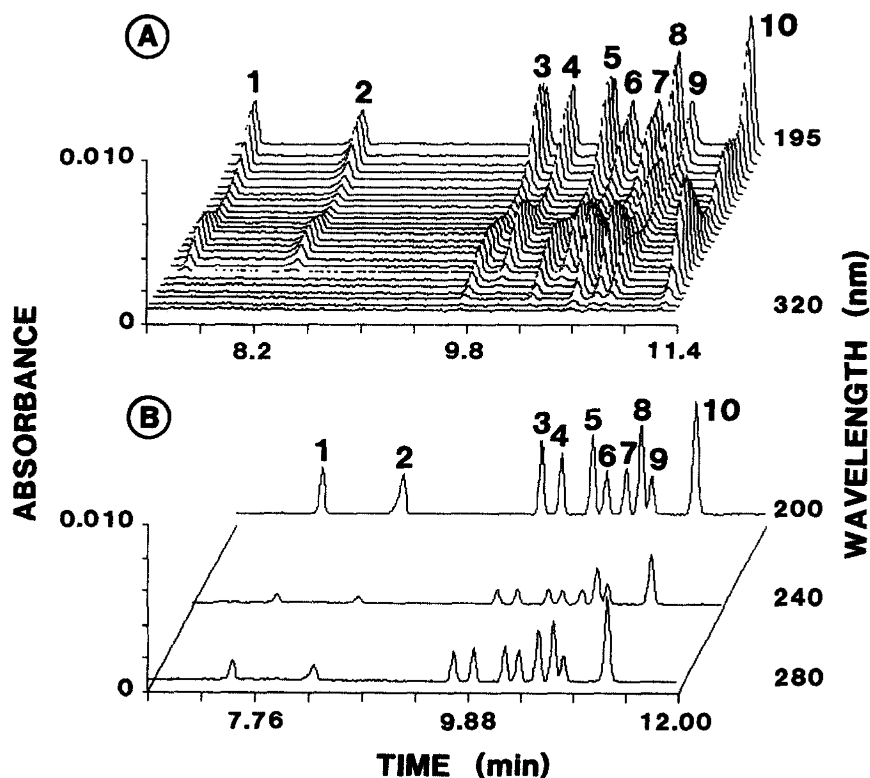

Fig. 1. Three-dimensional electropherograms of a model mixture of ten substituted purines (A) and pherograms at three wavelengths of the same run (B). The applied voltage was a constant 20 kV and the current was 75 μA. Peaks: 1 = theobromine (3,7-dimethylxanthine); 2 = caffeine (1,3,7-trimethylxanthine); 3 = paraxanthine (1,7-dimethylxanthine); 4 = theophylline (1,3-dimethylxanthine); 5 = 7-methylxanthine; 6 = 3-methylxanthine; 7 = 3-methyl uric acid; 8 = 1-methyl uric acid; 9 = 7-methyl uric acid; 10 = uric acid.

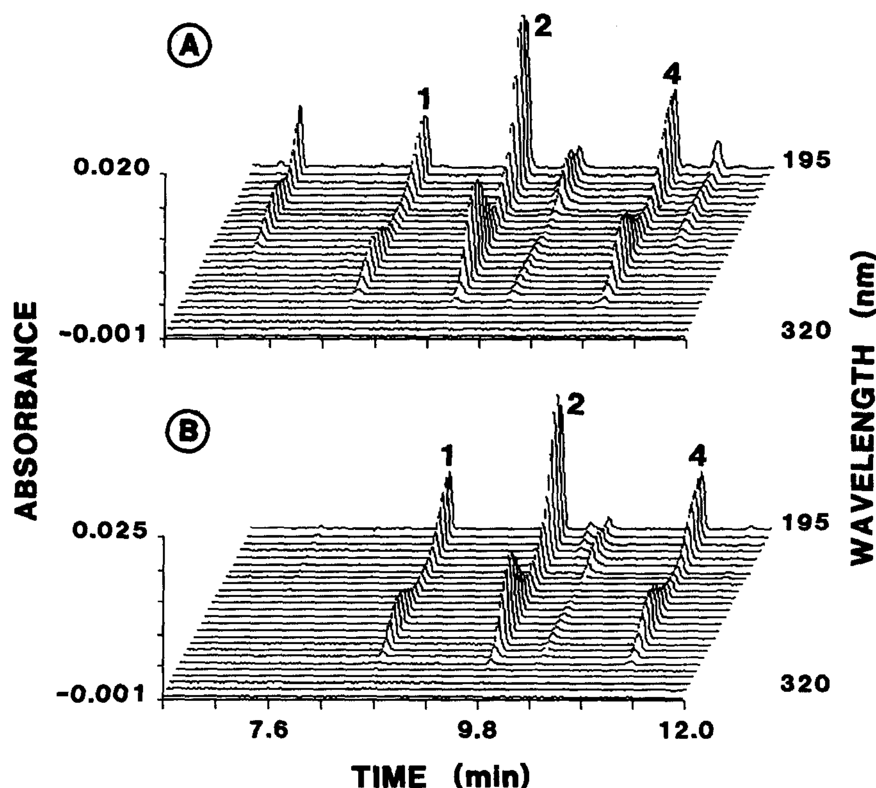

Fig. 2. Three-dimensional data plot obtained after liquid–liquid (A) and Sep-Pak (B) extraction of bovine plasma spiked with theobromine (*ca.* 140 μM), caffeine (*ca.* 250 μM) and theophylline (*ca.* 140 μM). The applied voltage was a constant 20 kV and the current was 74 μA.

pretreatment procedures. The three purines could easily be identified by comparing their spectra with those obtained from Fig. 1 (data not shown), as well as by their retention behaviour, *i.e.* time of detection relative to a known compound such as theobromine. With both procedures, the three compounds extracted well and provided well resolved pherograms when detected at or close to 280 nm. With the solid-phase method, however, a lower number of components was extracted from the serum matrix.

The data presented in Fig. 3A and B were obtained with a human serum sample which was only passed through a 0.2-μm syringe filter before sample injection. This serum sample was prepared 1 h after the self-administration of 140 mg of caffeine. It represents the same sample previously investigated as a blank probe for the determination of barbiturates in another buffer system [15]. As in this previous investigation, the data shown in panel B show that components eluting between caffeine (peak 2) and uric acid (peak 10) can be analysed with direct sample injection, *i.e.* without extraction. Analysis by an EMIT gave a caffeine concentration of 22.8 μM. Serum proteins are dissolved by SDS and elute (as a very braod zone) after uric acid. For comparison, the three-dimensional pherogram of a serum sample collected 2.5 h after caffeine self-administration (EMIT 21.3 μM caffeine) and extracted using the liquid–liquid extraction procedure is presented in Fig. 3C. In both instances, the spectral information allowed the positive identification of caffeine (peak 2), its metabolite paraxanthine (peak 3) and uric acid (peak 10), as is illustrated by the normalized spectra of panels D, E and F, respectively.

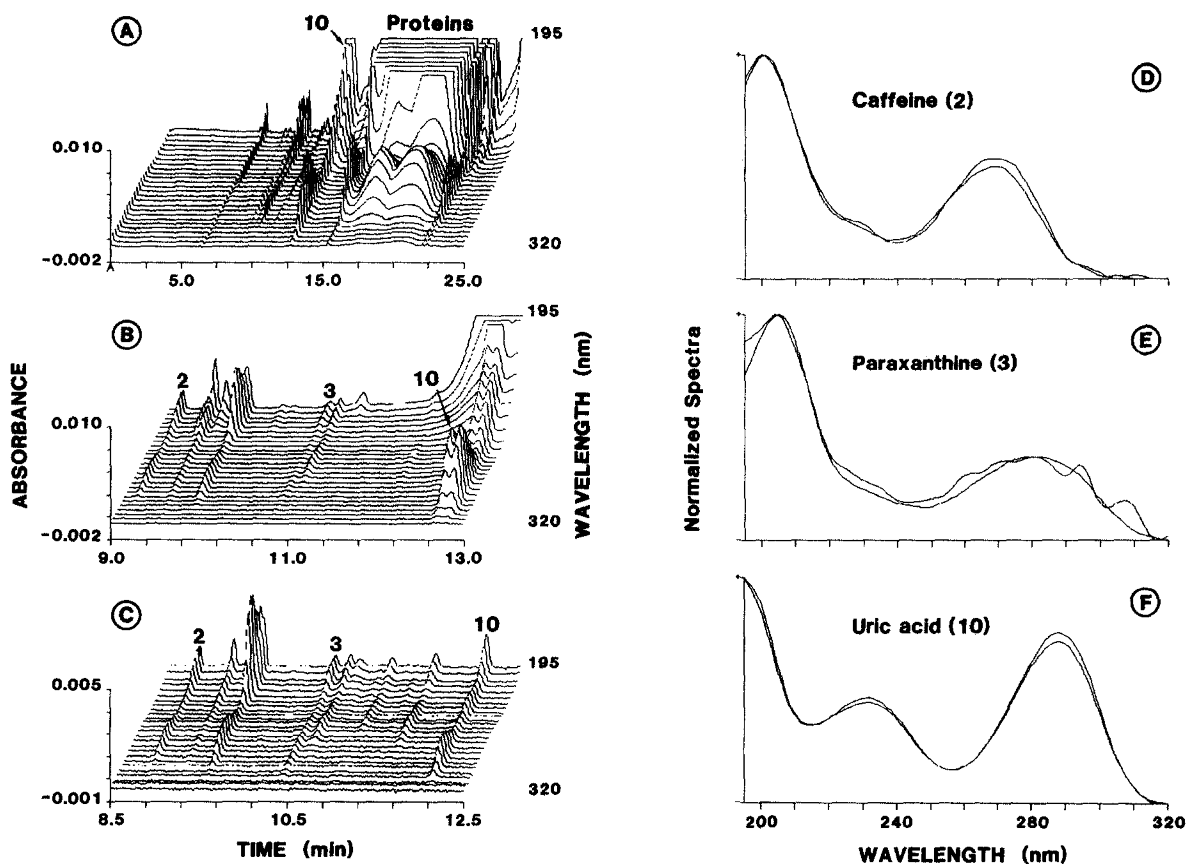

Fig. 3. Three dimensional data plots of a directly injected serum sample (complete pherogram in A and expanded section in B) and after liquid–liquid extraction (C). Power conditions as in Fig. 1. Spectral identity proof of eluting caffeine (D), paraxanthine (E) and uric acid (F) zones are presented as a comparison of the background corrected time slices with computer-stored, normalized spectra.

The data shown in Fig. 4 were obtained with a serum sample from a patient receiving theophylline pharmacotherapy (EMIT 95 μM). The suitability of direct sample introduction for drug monitoring is shown with the example of panel A. A clear theophylline zone is produced within the analytical window. Fig. 4B shows the three-dimensional pherogram after Sep-Pak extraction and Fig. 4C the spectral proof of identity for the theophylline zone. The corresponding single-wavelength data for 200 and 280 nm are presented in Fig. 5. These data clearly show that well resolved theophylline peaks are obtained with either method when monitored at 280 nm. The serum concentration of theophylline was determined as 87 μM using Sep-Pak clean-up and detection at 280 nm (see below) and 96 μM using FPIA. Thus, MECC has the potential to determine serum theophylline at concentrations of pharmacological interest (the therapeutic range of this compound is 55–110 μM) and without elaborate sample pretreatment.

The direct injection of serum samples in MECC allows rapid analysis which can be performed on very small sample volumes (a few microlitres), such as those from infants born prematurely, samples which are typically too small to be pretreated. The three-dimensional pherograms presented in Fig. 6 were obtained with two unfiltered serum samples from infants. All the assigned peaks could be verified by comparing normalized time slices, and well

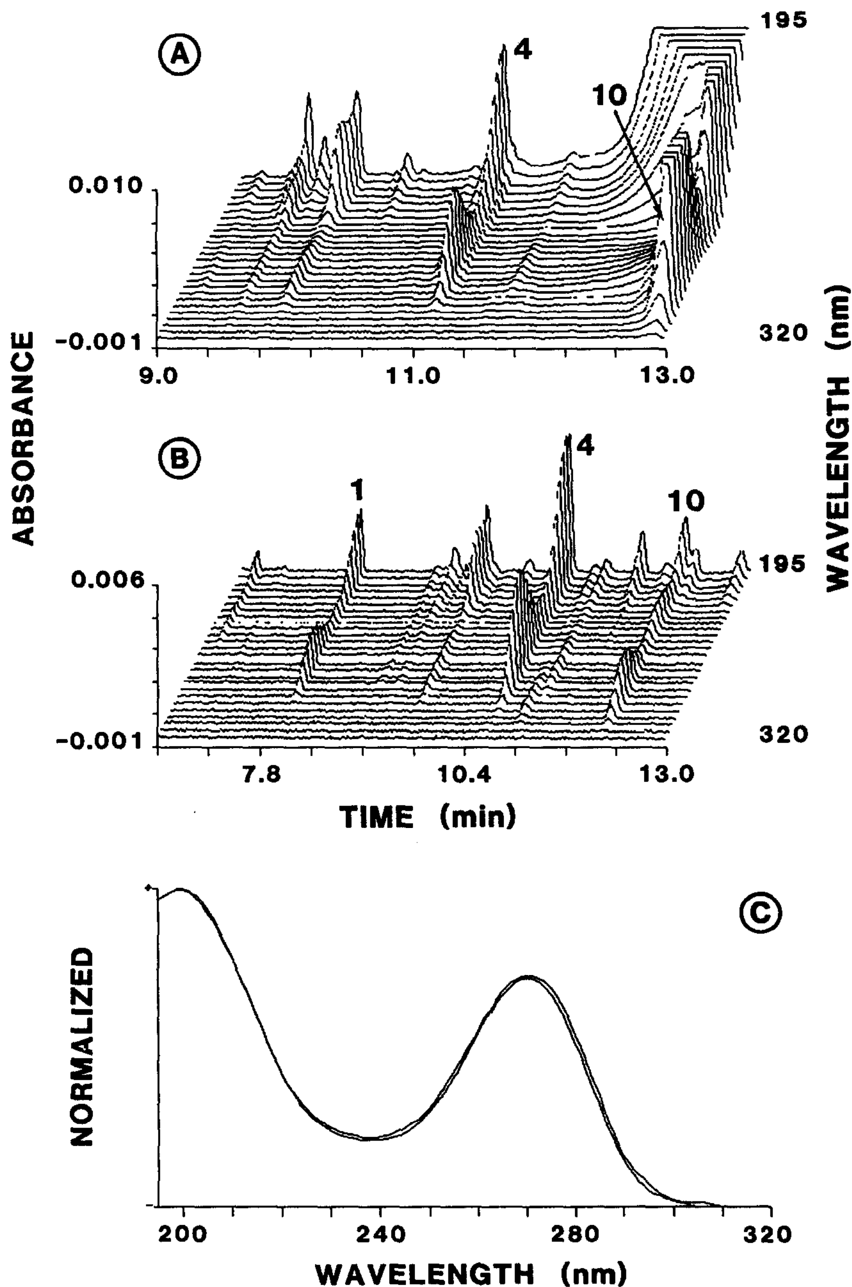

Fig. 4. Data for a directly injected (A) and a Sep-Pak-extracted (B) serum sample of a patient receiving theophylline pharmacotherapy. For the extraction, theobromine (40 μM; peak 1 in panel B) was added as an internal standard. Power conditions as in Fig. 1. Panel C shows a background corrected time slice of the theophylline zone of panel B compared with that of a computer-stored reference spectrum.

resolved pherograms were obtained at 280 nm (data not shown), indicating that the direct injection of serum samples from infants could be used for the determination of purines. Serum concentrations of caffeine, determined by EMIT, were 14 (Fig. 6A) and 107 (Fig. 6B) μM. The theophylline concentra-

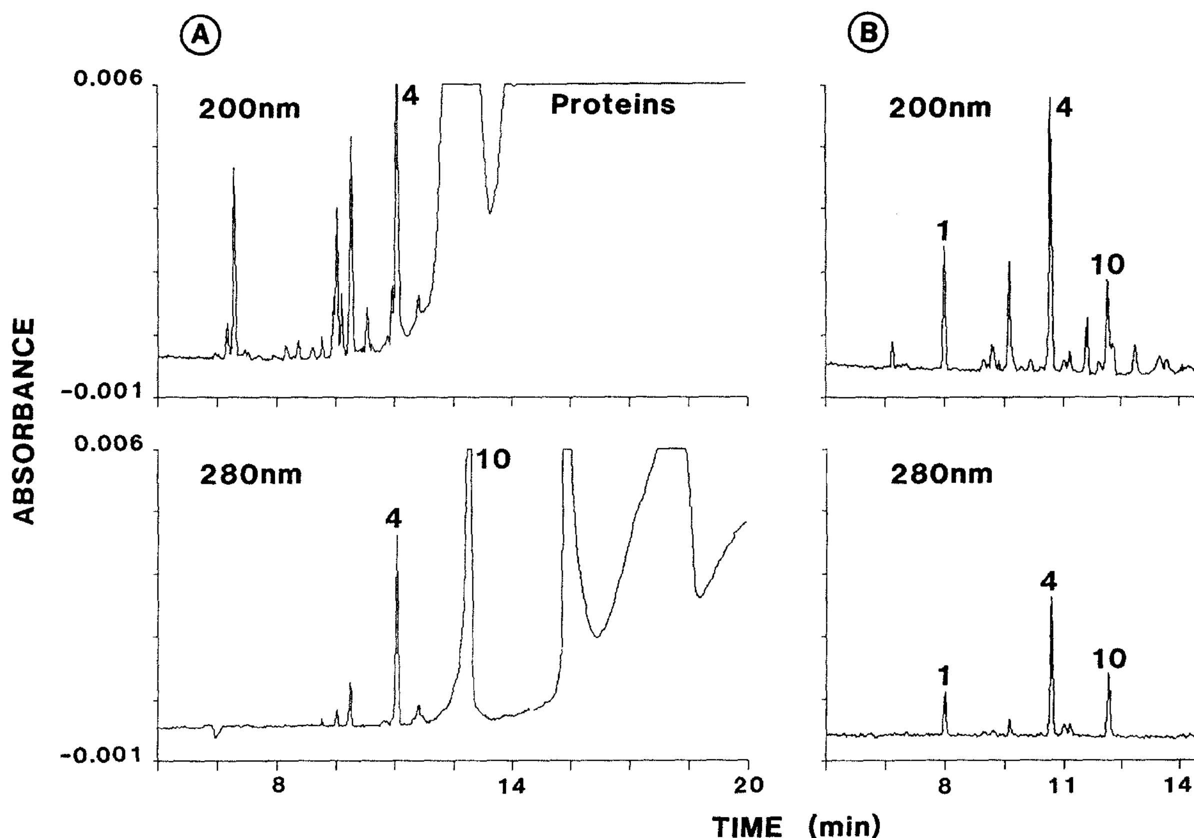

Fig. 5. Single-wavelength pherograms (200 and 280 nm) of the data presented in Fig. 4A and B.

tions were 35.7 and 3.5 μM, respectively, using FPIA. An analysis of the sample shown in Fig. 6A, but filtered prior to injection, gave no change in the caffeine and theophylline peaks. The two serum samples are interesting because they show metabolic differences in the drugs given. Theophylline was given in the sample of Fig. 6A and caffeine in the sample of Fig. 6B. Not surprisingly, caffeine is clearly detected as a metabolite of theophylline in infants born prematurely [18] using MECC (Fig. 6A), whereas no substantial amount of theophylline is found during caffeine pharmacotherapy (Fig. 6B).

The determination of caffeine [4] and theophylline [19] in saliva has been reported as being a useful alternative to the monitoring of these compounds in serum samples. This non-invasive method has great potential, particularly for children, as it avoids the potential trauma associated with venipuncture. As with the serum samples, it was of interest to elucidate the possibilities of direct injection of saliva compared with different sample extraction procedures. Fig. 7A and B shows multi-dimensional pherograms obtained after the direct injection of a filtered saliva sample which was collected about 1 h after the self-administration of 280 mg of caffeine. For that sample, concentrations of caffeine and theophylline in saliva were determined to be 29.8 (EMIT) and <2 μM (FPIA), respectively. Fig. 7A shows the pattern measured between 0 and 25 min, showing a much reduced protein content of saliva compared with that of serum samples. The expanded caffeine–uric acid window of the same data is presented in Fig. 7B and the corresponding data from a blank saliva sample from a subject who did not take any caffeine-containing beverages nor food is given in Fig. 7C. Fig. 7D shows the pherogram obtained with the saliva sample of Fig. 7A and B

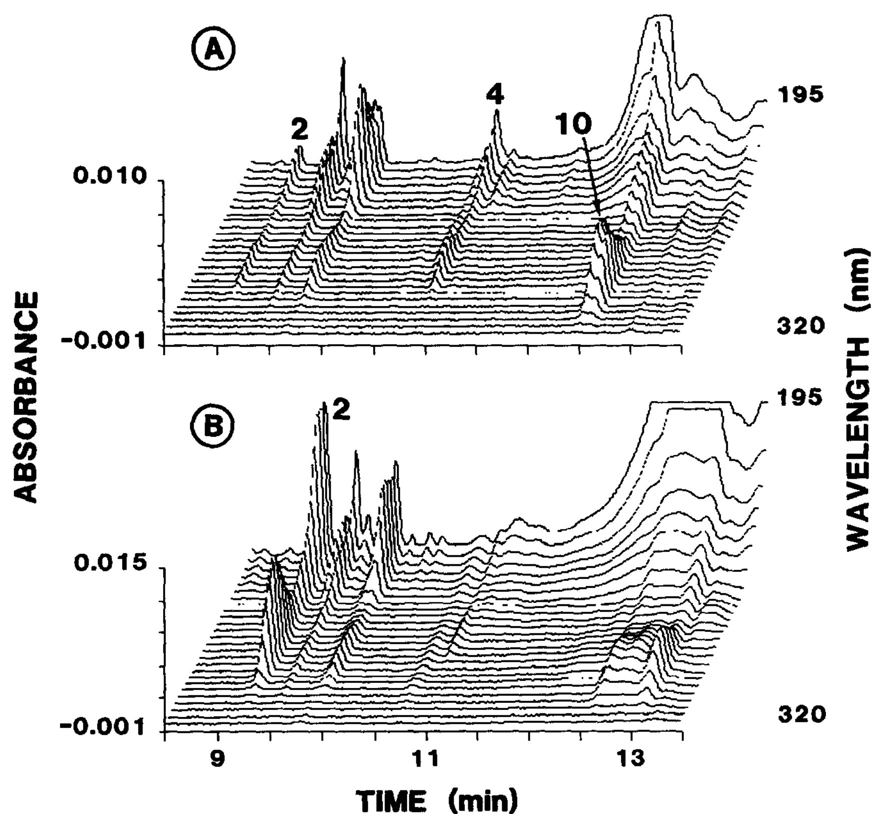

Fig. 6. Multi-wavelength data obtained by the direct injection of serum samples from two infants born prematurely. (A) Infant with theophylline treatment; (B) with caffeine pharmacotherapy. Power conditions as in Fig. 1.

but after liquid–liquid extraction as a clean-up procedure. The assigned peaks of caffeine, paraxanthine and uric acid could easily be verified by comparing normalized, background corrected time slices (data not shown).

The analysis of urine from a subject with regular coffee consumption by direct injection reveals the presence of many highly concentrated compounds (Fig. 8A and B). With this approach, only uric acid could be reliably assigned. Thus, for the determination of caffeine and other purines, sample clean-up is necessary, as is shown by the pherograms presented in Fig. 8C–E. With the liquid–liquid extraction method developed for serum and saliva samples (Fig. 8C), many compounds became even more concentrated than in urine alone, whereas with the Sep-Pak procedure (Fig. 8D) the urine matrix could be simplified, but the determination of caffeine was not possible. However, the Bond Elut Certify method allowed the unambiguous determination of caffeine and paraxanthine in this urine sample (Fig. 8E).

Theophylline and caffeine concentrations in serum samples from patients

The determination of theophylline in human serum samples by MECC was performed by the external and internal standard methods using theobromine as the reference compound. All measurements were made on an ABI instrument. Peak areas of single injections were used as the basis for data evaluation. Two approaches were investigated, the direct injection of serum samples and simplification of the sample matrix by Sep-Pak extraction prior to sample analysis. With direct sample injection calibration graphs were constructed with spiked human serum samples in the concentration range 10–120 μM (five data points) and with 60 μM theobromine as an internal standard. Detection was at 280 nm. A typical pherogram is shown in Fig. 9B. The cali-

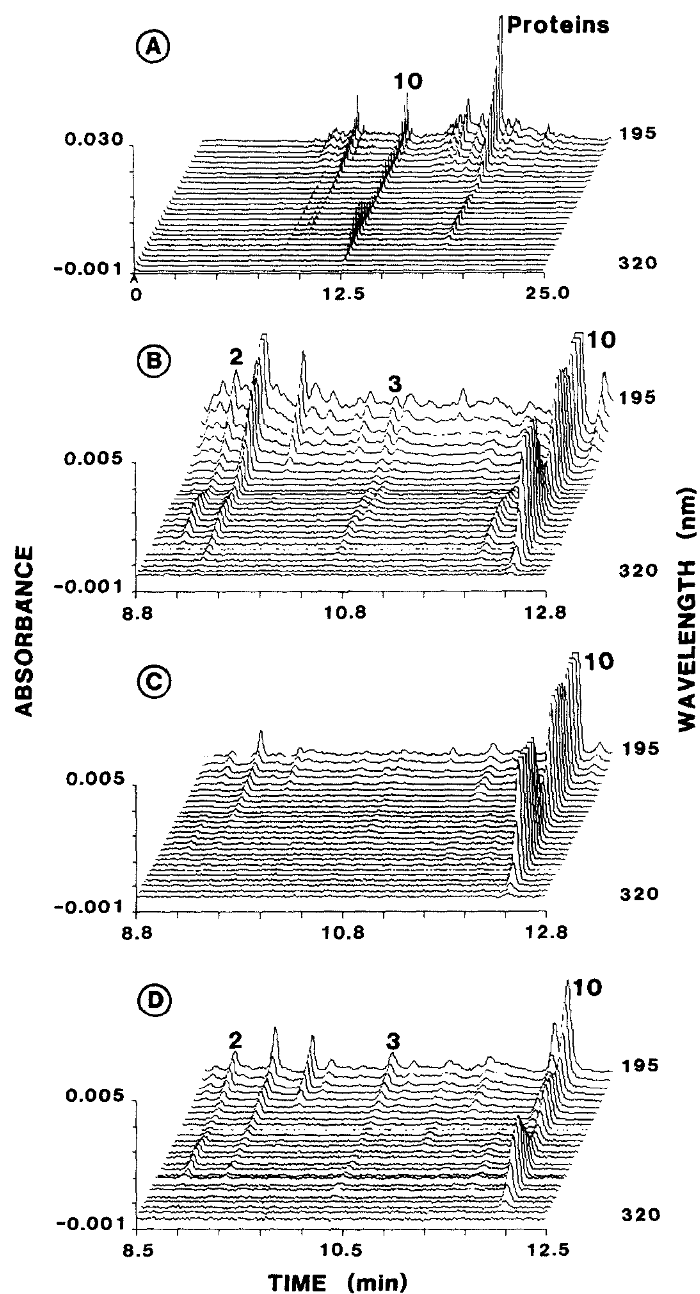

Fig. 7. Pherograms of saliva samples. (A) Complete data plot between 0 and 25 min and (B) expanded section of the same run with direct injection of a saliva sample which was collected 1 h after the self-administration of 280 mg of caffeine. (C) Data obtained after the direct injection of a saliva sample from a subject who does not consume any caffeine. (D) Data plot of the saliva sample of panels A and B but after liquid–liquid extraction. Power conditions as in Fig. 1.

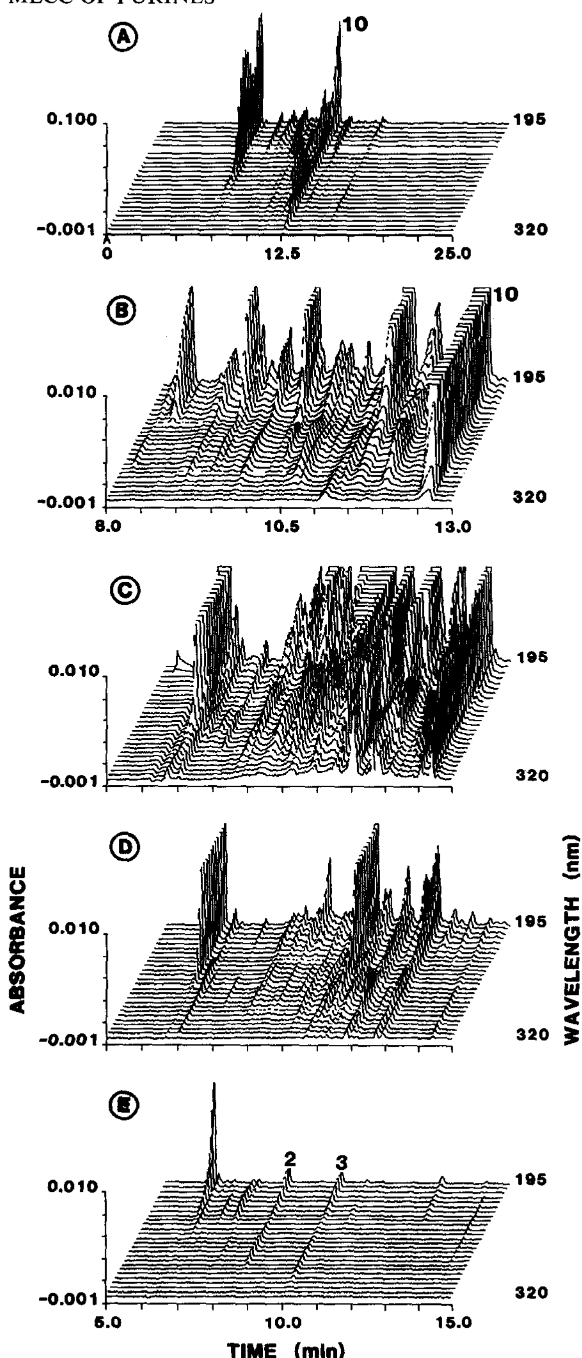

Fig. 8. Three-dimensional pherograms of a urine sample. The complete data plot of a directly injected urine sample is presented in panel A and part of the data on a ten-fold more sensitive absorbance scale in panel B. Three-dimensional data plots of the same urine sample obtained after (C) liquid–liquid, (D) Sep-Pak and (E) Bond Elut Certify extraction are also shown. Power conditions as in Fig. 1.

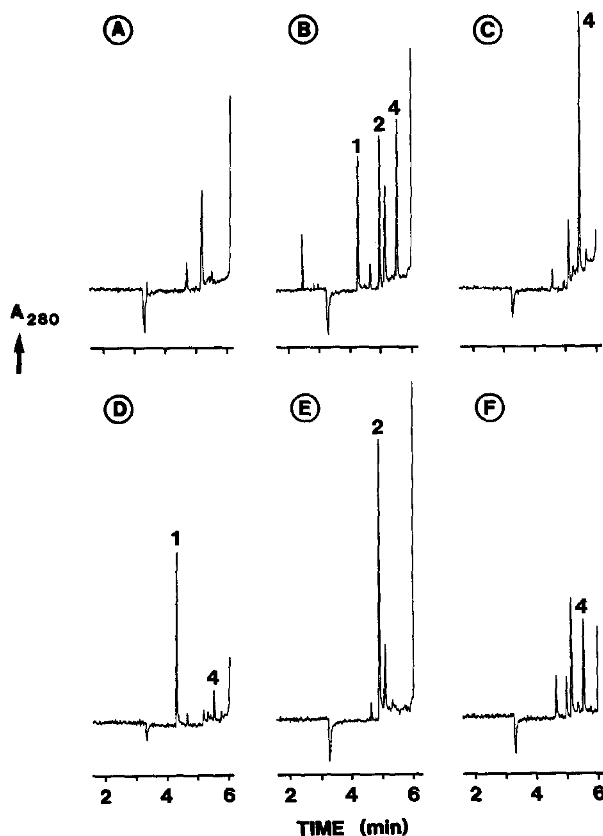

Fig. 9. Single-wavelength electropherograms (280 nm) obtained with direct injection of serum samples using the automated instrument. The injection time was 1 s, the effective capillary length was 37 cm and the applied voltage was a constant 15 kV (32 μA current) in all instances. (A) Blank serum sample; (B) blank serum sample spiked with theobromine, caffeine and theophylline (60 μM each); (C) sample from patient receiving theophylline; (D) sample from patient receiving theophylline spiked with theobromine (60 μM); (E) sample from infant (caffeine treatment); (F) sample from infant (theophylline treatment).

bration graphs were linear (typical correlation coefficient 0.995; slope 37.7; y-intercept 5.2 μM). Similar correlations were obtained with external data evaluation, *i.e.*, without the incorporation of the internal standard. It was interesting to find that bovine plasma could not be used as a calibration matrix for the direct sample injection because of interfering peaks. For Sep-Pak extraction calibration graphs were constructed with bovine plasma spiked with theophylline in the concentration range 5–160 μM (six data points) and with 40 μM theobromine as an internal standard. Detection was at 200 and 280 nm. The graphs showed good linearities with

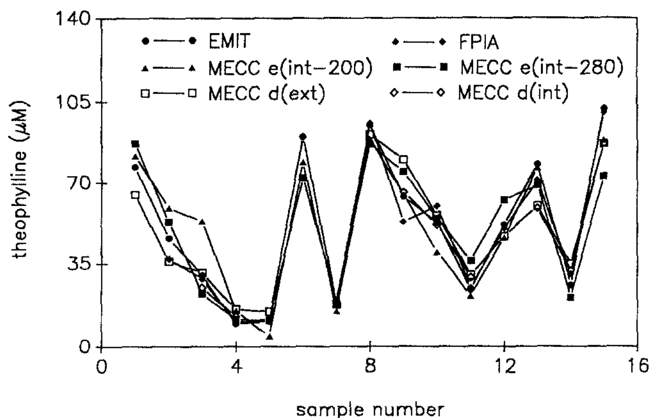

Fig. 10. Theophylline concentrations in serum samples from fifteen patients determined by MECC with direct injection (evaluated with and without internal standard and denoted by d(int) and d(ext), respectively), MECC after solid-phase extraction and with detection at 200 and 280 nm (both evaluated with internal standard and denoted by e(int-200) and e(int-280), respectively). EMIT and FPIA.

correlation coefficients of 0.998 and 0.995, slopes of 35.05 and 24.04 and y-intercepts of -10.6 and -3.21 μM for the two sets of data, respectively. Slightly lower correlations were obtained using the external standard method.

Fifteen serum samples from patients receiving theophylline treatment were analysed by MECC and their theophylline concentrations compared with those obtained by two non-isotopic immunological techniques, EMIT and FPIA. All the data correlated well (Fig. 10 and Table I). It is interesting to note that the data from the two immunological procedures correlate better (coefficient 0.988, see Table I) than the MECC data evaluated with different approaches (coefficients between 0.895 and 0.984). The MECC data were slightly lower than those obtained by EMIT (Fig. 11A), indicating the presence of cross-reactivity of other purines in the immunoassays. The data summarized in Table I show that with direct sample injection better correlations with the EMIT data were obtained compared with Sep-Pak extraction. This suggests that more reliable theophylline concentrations are determined without extraction. Furthermore, after extraction, more reliable data were obtained when monitored at 280 than when monitored at 200 nm, this difference being attributed to the difference in the number of detected peaks at the two wavelengths (Fig. 5). Several serum samples also showed distortions of the theophylline peaks, which were not seen in longer capillary columns. The deviations, however, are insignificant with respect to the relatively wide treatment range used for theophylline. With direct sample injection good reproducibility was obtained. The mean retention time (relative standard deviation; R.S.D.) for theophylline of ten consecutively injected samples was 5.47 min (0.6%). The R.S.D. of peak areas was 5–7%. The theophylline concentrations determined with and without the inclusion of the internal standard were essentially equal. This is in contrast to the results with purines (data not shown) and barbiturates [15,16] using sample extraction prior to analysis.

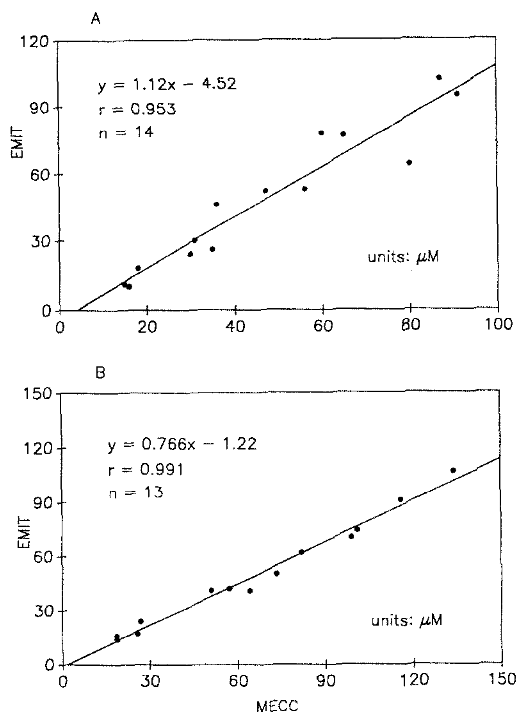

Fig. 11. Comparison of drug concentrations determined by EMIT and MECC (obtained with direct injection of serum sample and without incorporation of an internal standard). (A) Theophylline concentration in serum samples from adult patients; (B) caffeine concentrations in serum samples from infants born prematurely.

The determination of caffeine in serum samples from infants born prematurely was performed by the external standard method using peak areas of single injections as the basis for data evaluation.

TABLE I

LINEAR REGRESSION ANALYSIS DATA OF COMPARATIVE SERUM THEOPHYLLINE CONCENTRATIONS

The acronyms d(int) and d(ext) represent data obtained with direct injection of serum samples and data evaluation based on internal and external calibration, respectively. e(int-280) and e(int-200) refer to Sep-Pak extraction and detection at 280 and 200 nm, respectively.

Assay 1 (x-axis)	Assay 2 (y-axis)	n	Slope	y-Intercept (μM)	r
MECC d(int)	MECC d(ext)	8	1.01	3.44	0.984
MECC d(ext)	EMIT	14	1.12	−4.52	0.953
MECC d(int)	EMIT	8	1.23	−7.62	0.972
MECC d(ext)	MECC e(int-280)	14	0.976	2.36	0.913
MECC d(int)	MECC e(int-280)	8	0.969	1.37	0.936
MECC e(int-280)	EMIT	15	1.06	−1.79	0.930
MECC e(int-200)	EMIT	14	1.03	−0.213	0.947
MECC e(int-200)	MECC e(int-280)	14	0.964	1.89	0.895
FPIA	EMIT	15	1.01	0.503	0.988

Samples were directly injected and pherograms were measured at 280 nm. Typical pherograms are depicted in Fig. 9E and F. Calibration graphs were constructed with spiked human serum samples in the concentration range 10–120 μM (five data points). Linear relationships with correlation coefficients between 0.995 and 0.999, slopes of 0.0117 to 0.0135 and y-intercepts ranging from 0.9 to 3.2 μM caffeine were obtained. Fifteen samples from infants receiving either theophylline or caffeine treatment were analysed and the caffeine concentrations compared with those obtained by EMIT. The data correlated well (Fig. 11B), but the MECC data were consistently higher than the caffeine concentrations determined by EMIT. No explanation could be found for this deviation. In fact, higher EMIT concentrations were expected because of cross reactivity with other substituted purines, including paraxanthine and theophylline. To gain an insight into this problem further investigations are required. The average retention time was 4.93 min with an R.S.D. of 1.7% (n = 10). Occasionally, a sudden increase in elution time to values between 5.2 and 6.7 min was observed. If this occurred a 15-min wash with 1 M sodium hydroxide solution had to be used to restore the correct measuring conditions. Reproducibility was improved by rinsing with 0.1 M sodium hydroxide solution and buffer for 3 and 5 min, respectively, between each run.

It is interesting to add that caffeine concentrations greater than 50 μM originated from caffeine pharmacotherapy, whereas caffeine concentrations less than 40 μM were found during treatment with theophylline, when caffeine is produced as metabolite. The conclusion is reached that MECC with direct sample injection and without the inclusion of an internal standard produces pharmacologically meaningful data which can be used for monitoring caffeine in serum samples of infants during treatment with theophylline or caffeine.

CONCLUSIONS

Using body fluids as samples, fast-scanning polychrome detection is an interesting approach for the characterization and identification of eluting zones in MECC. The multi-wavelength data reveal the suitability of the direct injection of serum or saliva samples (no protein removal required) for the determination of micromolar amounts of caffeine, theophylline, paraxanthine and uric acid by MECC. The direct injection of urine is not possible for the determination of methylated xanthines at these concentrations.

The determination of theophylline and caffeine in serum samples by MECC with on-column UV adsorption detection can be used for concentrations of pharmacological interest, *i.e.*, covering the therapeutic ranges of the two drugs (55–110 and 25–100 μM, respectively). The results correlate well with those obtained by homogeneous immunoassays. No sample pretreatment of any sort, including pre-

column treatment as in high-performance liquid chromatography [20], nor internal standard is required. Measurements can be performed on serum samples as small as a few microlitres and with a detection limit in the low micromolar range. Hence MECC is an attractive method for therapeutic drug monitoring, particularly for small sample volumes such as body fluids from infants. It is a rapid assay which is easy to perform. With MECC as described here, it is assumed that total drug concentrations are determined (but not proven) as a result of the release of the protein-bound portion of the drug by SDS (direct sample injection) or the extraction procedure. Free drug concentrations would be obtained if the proteins were removed (*e.g.*, with ultrafiltration) prior to sample injection or extraction.

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REFERENCES

1 A. Herxheimer, *Drug Ther. Bull.*, 17 (1979) 91.
2 M.C. Nahata, D. A. Powell and T. G. Franko, *Ther. Drug Monit.*, 5 (1983) 269.
3 T. Zysset, A. Wahlländer and R. Preisig, *Ther. Drug Monit.*, 6 (1984) 348, and references cited therein.
4 G. Jost, A. Wahlländer, U. von Mandach and R. Preisig, *Hepatology*, 7 (1987) 338.
5 R. L. Boeckx, E. M. Frith and F. E. Simons, *Ther. Drug Monit.*, 1 (1979) 65.
6 J. L. Cohen, C. Cheng, J. P. Henry and Y. L. Chan, *J. Pharm. Sci.*, 67 (1978) 1093.
7 K. K. Midha, S. S. Sved, R. D. Hossie and I. J. McGilveray, *Biomed. Mass Spectrom.*, 4 (1977) 172.
8 M. C. Roach, P. Gozel and R. N. Zare, *J. Chromatogr.*, 426 (1988) 129.
9 Y. Tanaka and W. Thormann, *Electrophoresis*, 11 (1990) 760.
10 T. Nakagawa, Y. Oda, A. Shibukawa and H. Tanaka, *Chem. Pharm. Bull.*, 36 (1988) 1622.
11 T. Nakagawa, Y. Oda, A. Shibukawa, H. Fukuda and H. Tanaka, *Chem. Pharm. Bull.*, 37 (1989) 707.
12 D. E. Burton, M. J. Sepaniak and M. P. Maskarinec, *J. Chromatogr. Sci.*, 24 (1986) 347.
13 H. Nishi, T. Fukuyama and M. Matsuo, *J. Chromatogr.*, 515 (1990) 245.
14 H. Nishi and S. Terabe, *Electrophoresis*, 11 (1990) 691.
15 W. Thormann, P. Meier, C. Marcolli and F. Binder, *J. Chromatogr.*, 545 (1991) 445.
16 P. Meier and W. Thormann, *J. Chromatogr.*, 559 (1991) 505.
17 D. E. Burton, M. J. Sepaniak and M. P. Maskarinec, *Chromatographia*, 21 (1986) 583.
18 M. J. Arnaud, in P. B. Dews (Editor), *Caffeine*, Springer, Berlin, 1984, pp. 3–38.
19 I. A. Siegel, H. Ben-Aryeh, D. Gozal, A. A. Colin, R. Szargel and D. Laufer, *Ther. Drug Monit.*, 12 (1990) 460.
20 Y. Kouno, C. Ishikura, N. Takahashi, M. Homma and K. Oka, *J. Chromatogr.*, 515 (1990) 321.