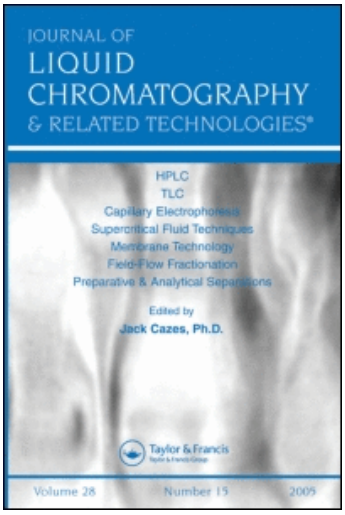

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A Bidimensional HPLC System for Direct Determination of Theophylline in Serum

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A BIDIMENSIONAL HPLC SYSTEM FOR DIRECT DETERMINATION OF THEOPHYLLINE IN SERUM

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ABSTRACT

A bidimensional HPLC system combining steric gel exclusion and reverse phase ODS columns for determination of theophylline in serum is reported. Factors involved in the development of the method and its performance are discussed. This technique is a practical alternative for the determination of theophylline levels in serum without any clean up procedure before chromatography.

INTRODUCTION

Theophylline, commonly used in the treatment of asthma, and in the apnea of the premature infants, is characterized by a large interindividual variation of its clearance. For monitoring of this drug, numerous assays have been reported; a review of these techniques has been done by Berthou et al (1). At present, enzyme immuno assays and HPLC are the prevailing methods. Some advantages for the latter technique would be the possible simultaneous measurement of theophylline and its metabolites. Most of them resemble each other and differ only in the composition of the mobile phase, pH, proportion of the organic solvent,

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presence of tetrabutylammonium sulfate as ion pair, on choice of the internal standard. These procedures require a deproteinization step by acetonitrile (2,3), trichloracetic acid (4) or ammonium sulfate (5,6) followed by the injection of the supernatant onto the chromatograph or by extraction of the drug by organic solvents (5,6,7,8,9,10,11). Additional steps such as centrifugation and solvent evaporation are necessary after the drug extraction.

We propose a bidimensional chromatographic system combining a steric exclusion and a reverse phase ODS columns for quantitation of the theophylline by direct injection of serum samples without requiring any clean up procedure.

MATERIAL AND METHODS

Solvents and Chemicals

Acetonitrile HPLC grade is purchased from Fisher Scientific (Montreal, Canada). All other reagent grade chemicals are obtained from Baker (Canlab, Montreal, Canada). Theophylline is purchased from Sigma (St-Louis, Mo, USA)., 1-methylxanthine and 3-methylxanthine from ICN pharmaceuticals (NY, USA), 3-methyluric acid and 1,3-dimethyluric acid from Adams chemicals (Round Lake, Il, USA). Stock solutions of theophylline of 1 mg/ml are prepared in methanol; theophylline metabolites, 5 mg/ml, are prepared in 0.05M phosphate buffer pH 7.4.

Chromatographic Instrumentation and Conditions

The bidimensional HPLC system consists of 2 parts: (Fig 1) the steric gel exclusion part consists of a protein column (I-60

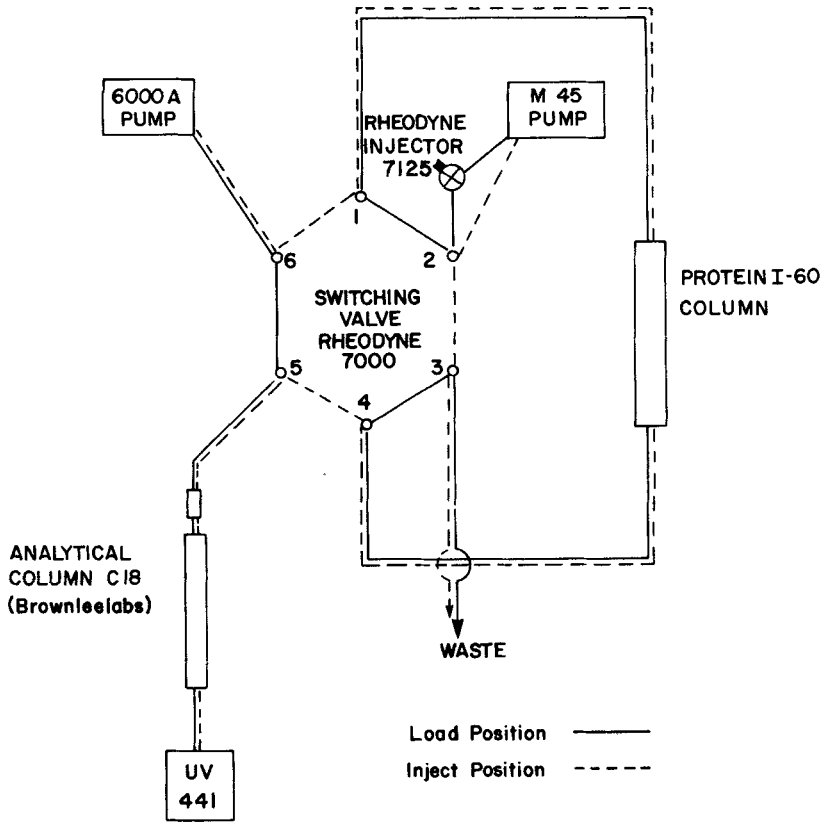

Fig 1 = Diagram of the bidimensional chromatographic system.

type 7.8 mm x 300 mm) with a precolumn filled with the guard column support (Cat. No. 85290), connected to an M-45 pump (all from Waters Assoc., Milford, MA, USA) and a injection valve (Model 7125, Rheodyne, Berkeley, CA, USA) with a 100 µl loop. The mobile phase used for the steric gel exclusion is a 0.02 M triethylamine acetate buffer pH 7.4 at a flow rate of 1 ml/min. The analytical part consists of an ODS reversed stationary phase column (Spherisorb 5 µm 250 x 3 mm, Brownlee, Santa Clara, CA, USA)

protected by an MPLC guard column (Brownlee). The mobile phase used is a 0.01 M acetate buffer (pH 4) - acetonitrile (90:10 v/v) pumped by an 6000A pump (Waters Assoc., Milford MA, USA) at a flow rate of 1 ml/min. The two parts are linked by a switching valve (Model 7000, Rheodyne, Berkeley, CA, USA).

The detection system consists of a fixed wavelength detector (model 441, Waters Assoc., Milford, MA, USA) operated at 280 nm and a recorder (Model 561, Hitachi, Japan).

Procedure

The standard curve is prepared by adding theophylline 1, 2, 4, 8, 16, 32 µg/ml to drug free serum samples. Three aliquots are used at each concentration. These are assayed as described below. For analysis of serum samples, a 100 µl aliquot is loaded in the injection valve. The switching valve, Rheodyne 7000, is set at load position (fig 1); nine minutes after the injection of the sample in the steric gel exclusion part, the swithching valve is then set to inject position for 2 minutes permitting the transfer of theophylline to the analytical column. The switching valve is reset to load position for the reequilibration of the protein column by the triethylamine buffer for 10 minutes before the injection of the next serum sample, while chromatographic separation is carried out on the analytical column.

Quantitation

All measurements are done by peak heights. Recovery is estimated by comparing the peak heights of the standard curve in

serum with that obtained on chromatographing the stock theophylline solution on the ODS column. The slope and intercept of the standard curve are obtained by linear regression of peak height on concentration ($Y = Ax + B$).

Method Validation

A kinetic profile of theophylline is established on a rabbit (new Zealand, 15 kg) following an IV dose of 10 mg/kg. Blood samples (2 ml) are collected from the ear vein at 0, 6, 15, 60, 240 and 360 minutes after the dose. Serum is then separated and transferred. Aliquots are used for theophylline determination following the Soldin and Hill method (12) and by the bidimensional on line HPLC proposed.

RESULTS AND DISCUSSION

Chromatography

The elution diagram of serum proteins, theophylline and its metabolites from I-60 protein column is monitored by collecting 1 ml fractions and reading the absorbance at 280 mn (Fig 2). The elution volume of serum proteins is 5.5 ml which corresponds to the void volume of this column. The exclusion of the protein column used is 20 000 allowing the elimination of the proteins from the sample injected.

Despite the fact that steric exclusion chromatography separates on the basis of molecular size, we observe different elution volumes for methyluric acid derivatives, theophylline and its

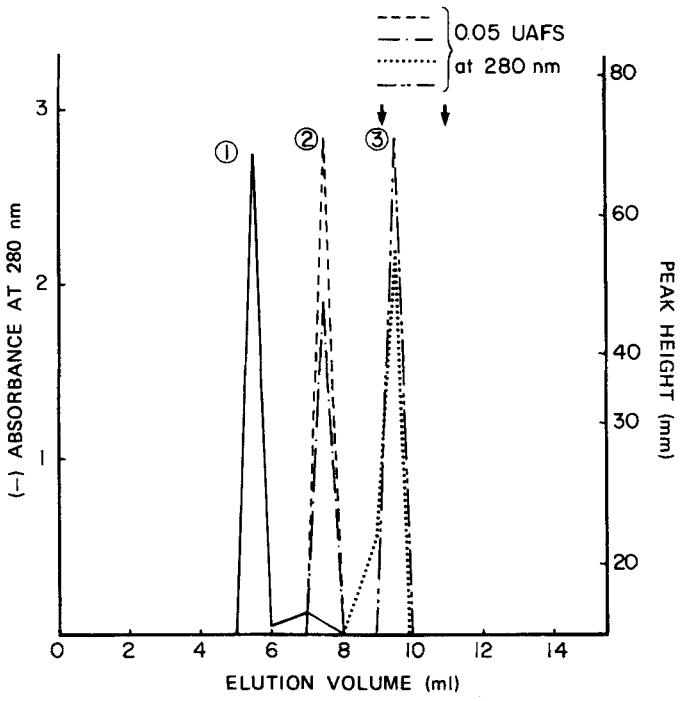

Fig 2 = Elution profile of (1) serum proteins (100 µℓ) (——) (2) 1-methyluric acid (1 µg) (---), 1,3-dimethyluric acid (1 µg) (—·—·) (3) 3-methylxanthine (1 µg) (....) and theophylline (2 µg) (—··—··) from the I-60 protein column. (↓↓) Elution volume transferred to the analytical column.

basic metabolites. The calculated Kav is 0.224 for 1-methyluric acid and 1,3-dimethyluric acid and 0.454 for theophylline and 3-methylxanthine metabolite; (Kav = $\frac{Ve - Vo}{Vt - Vo}$, Ve = elution volume of the compound, Vo and Vt are the void volume and the total bed volume respectively). Thus some interaction may occur between the column packing material and the compounds tested which could explain the difference in the Kav obtained for theophylline and its metabolites. This interaction affects also the recovery.

TABLE 1

Percentage of Theophylline Recovery Following the Gel Exclusion Chromatography

Mobile Phase Tested	Percentage of Recovery (Mean ± Standard Error) (n, Number of Assays)
1. 0.2 M phosphate buffer pH 7.4	76.33% ± 0.27 (n = 9)
2. 0.5 M phosphate buffer pH 7.4	82.60% ± 1.45 (n = 8)
3. 0.02 M triethylamine acetate buffer pH 7.4	95.15% ± 0.42 (n = 18)

Several mobile phases for the gel exclusion system have been tested and the results reported in Table 1.

As shown in Table 1, the concentrations tested are from 1 to 32 µg/ml of theophylline. Higher recovery is obtained by increasing the ionic strenght of the mobile phase as observed with the 0.5 M phosphate buffer. But high variations in the recovery are noted. The triethylamine acetate buffer allows the best and constant recoveries for all the concentrations tested. The use of serum samples for theophylline measurement is required for the protein column long life. The fibrinogen in the plasma hamper rapidly the resolution of I-60 column; furthermore it prevents the injection of plasma volumes exceeding 20 µl. The elution profile of the I-60 column is controlled routinely after the analysis of a hundred samples; any modification could be corrected by the replacement of the precolumn support.

Chromatograms from the dual column system are shown in Fig 3. Good resolution is obtained due to the reconcentration on the

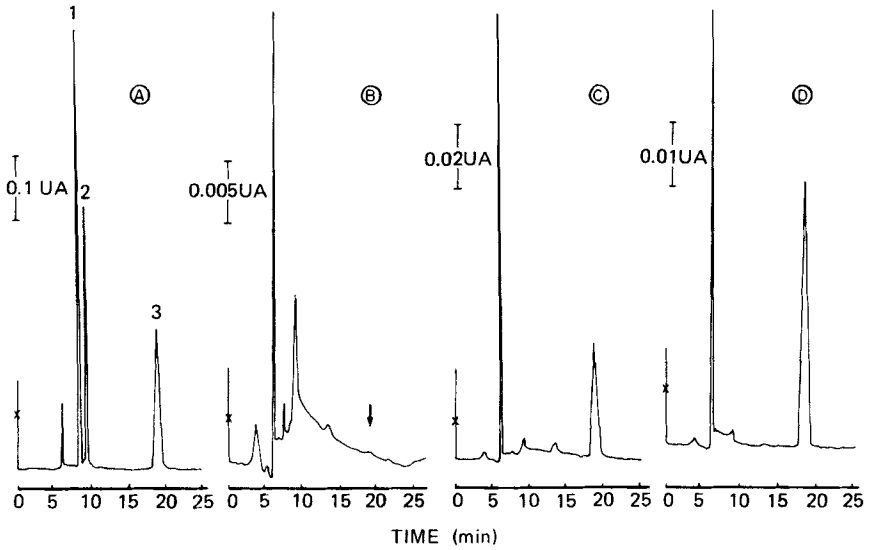

Fig 3 = Chromatography of theophylline and its metabolites. (A) chromatograms of aqueous standards. Peaks: 1, 3-methylxanthine (5 µg); 2, 1-methylxanthine (5 µg); 3, theophylline (5 µg). (B) serum blank. (C) blank serum spiked with 8 µg/ml theophylline. (D) rabbit 1-h serum sample following an IV dose of 10 mg/kg theophylline (estimated concentration 9.8 µg/ml).

analytical column of the 2 ml eluate from the I-60 column. Basic metabolites which coelute with theophylline in the gel exclusion system are well separated on the analytical ODS column (Fig 3 A). No interference peak is observed at the retention time of 18.6 minutes corresponding to that of theophylline.

Standard Curves

Using the method described above, standard curves are constructed for serum at concentrations of 1, 2, 4, 8, 16 and 32 µg/ml. The linearity of the standard curve for serum with 3 determinations at each concentration is excellent ($R^2 > 0.99$ n = 18) and a least

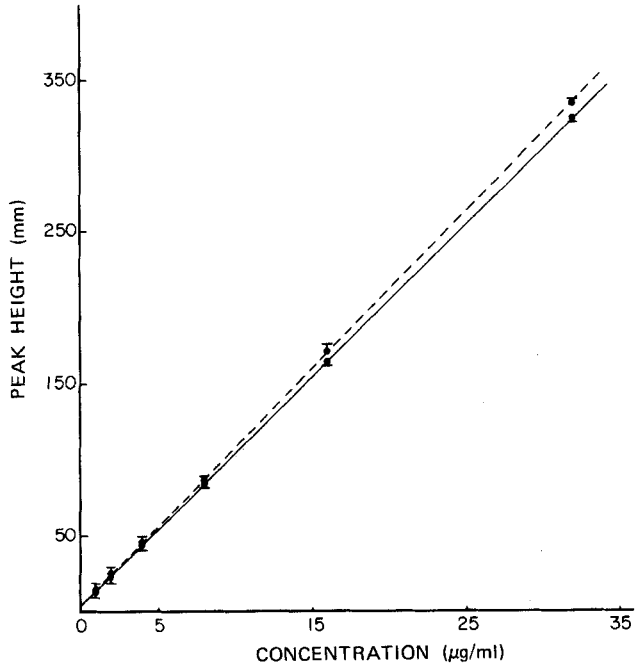

Fig 4 = Standard curves of theophylline (mean ± standard error) obtained from blank serum spiked with 1, 2, 4, 8, 16 and 32 µg/ml theophylline following the bidimensional system chromatography (----) and from standard solutions of theophylline in phosphate buffer (0.05M pH 7.4) by direct injection on the analytical column (——).

squares linear regression of peak height (mm) on concentration (µg) gives a slope of 10.12, an intercept of 0.57 and a mean coefficient of variation of 1.30%. The recoveries for the concentrations from 1 to 32 µg/ml of theophylline in serum are shown by figure 4. The mean recovery is 95.1%. It appears that the binding of theophylline to serum proteins is very weak resulting a complete dissociation in the triethylamine buffer during the gel exclusion chromatography. Because of the good recovery and the use of constant volume injection loop, the use of an *internal*

TABLE 2

Accuracy of the Method

Theoretical concentration µg/ml	n	$\bar{\Delta}$	$\bar{\Delta}$%	SE	RSD%
15	5	0.011	0.07	0.479	0.30
7.5	5	0.008	0.11	0.438	0.56

n = number of samples
$\bar{\Delta}$ = absolute error (mean)
$\bar{\Delta}$% = relative error (mean)
SE = standard error of the mean
RSD = relative standard deviation

standard is found unnecessary. The mean interday coefficient variation is 1.36%. It is calculated following the Rodbard program for the calculation of within and between assay variance (13). The accuracy of the method is tested on spiked serum samples is shown in table 2.

Method Validation

Separate aliquots of the serum of a rabbit treated with 10 mg/kg theophylline and collected at different times are used for determination of theophylline using the Soldin and Hill method and the multidimensional on line chromatography system described. The results obtained are presented in table 3. They show excellent agreement. ($Y = 1.096\ x - 0.577$; R^2: 0.989).

In conclusion, the combination of gel exclusion - reversed phase chromatography could be valuable alternative for theophylline determination in serum without any clean up procedure, advantageous compared to that with direct injection of plasma on

TABLE 3

Kinetic Profile of Theophylline After an IV Dose of 10 mg/kg

Time of blood collection after the IV dose (min)	Theophylline concentration (µg/ml)	
	Soldin and Hill Method	Bidimensional Chromatography
0	-	-
6	14.08	15.43
15	11.46	11.63
60	9.99	9.80
240	5.15	5.43
360	3.92	3.73

reversed phase column. The latter technique, proposed by some authors (14), is limited to few microliters of samples which is a factor limiting the sensitivity and presents high risk of protein precipitation on the analytical column. The bi-dimensional system developed could be fully automated, reducing the technician cost. It would be attractive for the direct simultaneous measurement of theophylline and its metabolites.

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