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NEW SIMPLIFIED MICROASSAY FOR THE QUANTITATION OF THEOPHYLLINE AND ITS MAJOR METABOLITES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic technique is presented for simultaneous determination of theophylline, 3-methylxanthine, 1-methyluric acid, 1,3-dimethyluric acid and caffeine in serum using β-hydroxyethyltheophylline (BHET) as an internal standard. An aliquot of a serum sample (100 μl) was added to 40 μl of an internal standard solution (BHET; 100 μg/ml) and vortexed. A 20% trichloroacetic acid solution (60 μl) was then added; the mixture was vortexed, centrifuged and 100 μl were injected onto a C_8 column maintained at 45°C. Inter- and intra-day variability of the assay for all compounds was less than 4.3%. Sensitivity ranged from 10 ng/ml for 1-methyluric acid to 25 ng/ml for theophylline. Drugs commonly coadministered with theophylline did not interfere with the assay.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is a bronchial smooth muscle relaxant

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used clinically in the treatment of asthma [1]. Theophylline is metabolized extensively in vivo to three major metabolites: 3-methylxanthine (3-MX), 1-methyluric acid (1-MU) and 1,3-dimethyluric acid (1,3-DMU) [2, 3]. Although less potent than theophylline, 3-MX may contribute to some extent to the therapeutic effects of theophylline by relaxing the smooth muscle of the bronchi in man [4, 5]. It has also been shown that 1,3-DMU relaxes the smooth muscle of the guinea pig trachea [4, 5]. In addition, both 3-MX and 1,3-DMU increase contractility of the heart in the guinea pig [4, 5]. Owing to the pharmacological activity of these metabolites, it is important to measure the concentrations of theophylline as well as its metabolites when examining the relationship of the pharmacokinetics of this drug to its pharmacological effects.

Most of the assays developed to measure the concentrations of theophylline and its three major metabolites in serum have required a large volume of sample, tedious sample preparation and long chromatogram run times. Furthermore, many of these assays exhibited poor resolution of the compounds of interest [6—15]. This prompted us to develop a method which would require small sample volumes (making it a valuable tool for clinical pediatric research as well as animal experimentation), a relatively short and simple sample preparation and a shorter chromatogram run time as well as lower limits of detection.

EXPERIMENTAL

Apparatus

A Series 2 liquid chromatograph dual-pumping system was used in conjunction with an LC-100 column oven and an LC-75 UV spectrophotometric detector set at 273 nm (Perkin-Elmer, Norwalk, CT, U.S.A.). A packed pre-column containing μBondapak C_{18}/Corasil reversed-phase liquid chromatography packing material (Waters Assoc., Milford, MA, U.S.A.) was used in conjunction with a high-speed C_8 column (5-μm particles, 125 mm × 4.6 mm I.D., Perkin-Elmer, Norwalk, CT, U.S.A.) for the chromatographic separation.

Reagents and standards

The sources of the xanthines and uric acids used to make the standards and quality controls were as follows: 1-MU and 1,3-DMU from Adams Chemical (Round Lake, IL, U.S.A.); 3-MX from Aldrich (Milwaukee, WI, U.S.A.); caffeine from Fisher Scientific (King of Prussia, PA, U.S.A.); theophylline from Eastman-Kodak (Rochester, NY, U.S.A.); and β-hydroxyethyltheophylline (BHET) from Pierce (Rockford, IL, U.S.A.). Tetrabutylammonium phosphate (Pic A Reagent) was obtained from Waters Assoc. Sodium acetate and methanol (Fisher Scientific) were of HPLC grade. Glacial acetic acid and 20% trichloroacetic acid solution were also obtained from Fisher Scientific.

The internal standard stock solution was prepared by diluting 100 mg of BHET in 100 ml of distilled water. This stock solution was further diluted 1:10 with distilled water to yield the desired concentration of 100 μg/ml. Aliquots of the internal standard were stored at −20°C and remained stable for approximately six months.

Blank serum was obtained from normal volunteers who had abstained for

TABLE I

CONCENTRATION OF STANDARDS AND QUALITY CONTROLS

	Concentration (μg/ml)				
	3-MX	1-MU	1,3-DMU	Theophylline	Caffeine
Standard 1	30.00	15.00	30.00	40.00	20.00
Standard 2	15.00	7.50	15.00	20.00	10.00
Standard 3	7.50	3.75	7.50	10.00	5.00
Standard 4	4.70	2.35	4.70	6.25	3.12
Standard 5	2.35	1.18	2.35	3.12	1.56
Standard 6	1.18	0.59	1.18	1.56	0.78
Standard 7	0.59	0.30	0.59	0.78	0.39
Standard 8	0.30	0.15	0.30	0.39	0.20
Standard 9	0.15	0.075	0.15	0.195	0.10
Standard 10	0.075	0.038	0.075	0.098	0.05
Standard 11	0.038	0.019	0.038	0.049	0.025
Standard 12	0.019	0.010	0.019	0.025	0.013
Quality control A	22.50	11.25	22.50	30.00	15.00
Quality control B	6.10	3.05	6.10	8.12	4.05

48 h from all beverages and foods containing caffeine and other xanthines. The standards were made by dissolving 80 mg of theophylline, 60 mg of 3-MX, 30 mg of 1-MU, 60 mg of 1,3-DMU and 40 mg of caffeine in 1000 ml of blank serum. This stock solution was further diluted with blank serum to obtain twelve serum standards and quality-control samples A and B in the concentrations shown in Table I. Standards and quality controls as well as all patient samples were stored at −70°C due to evidence of degradation of the metabolites at −20°C.

Extraction procedure

Blood samples were centrifuged for 5 min at 1380 *g* to separate the serum from the blood clots. The serum was harvested with a Pasteur pipet and transferred to a 12 × 75 mm labeled test tube. Into a microcentrifuge tube were placed 40 μl of the internal standard solution and 100 μl of the serum sample. Proteins were precipitated by the addition of 60 μl of 20% trichloroacetic acid solution to the serum and internal standard. The mixture was vortexed for 30 s.

After centrifugation of the sample in a Beckman Microfuge at 15 600 *g* for 4 min, the supernatant was transferred to a second labeled microcentrifuge tube and centrifuged for 1 min. A 100-μl quantity of this sample was injected directly onto the chromatographic system.

Chromatographic conditions

The mobile phase consisted of 10 m*M* sodium acetate and 0.005 *M* tetrabutylammonium phosphate with 4% methanol. After lowering the pH to 4.5 with glacial acetic acid, the mobile phase was magnetically stirred and aspirated under a vacuum until degassed.

A flow-rate of 2.0 ml/min was used which generated a pressure of 10 MPa. The column was maintained at a constant temperature of 45°C through the use

of a column oven. Detector sensitivity was set at 0.04 absorbance units. The chart speed was set at 4 mm/min. The total run time selected was 14 min. The concentrations of theophylline and its metabolites in patient samples were calculated using the peak-area ratios of the compound of interest and the use of the time of best fit describing the relationship between peak-area ratios and concentration for the serum standards.

Extraction recovery

The assay recovery of theophylline and its metabolites was assessed at the concentrations present in quality-control samples A and B (see Table I). Five replicates of each quality-control sample were extracted and injected. Five injections of each quality-control sample prepared in aqueous solutions were

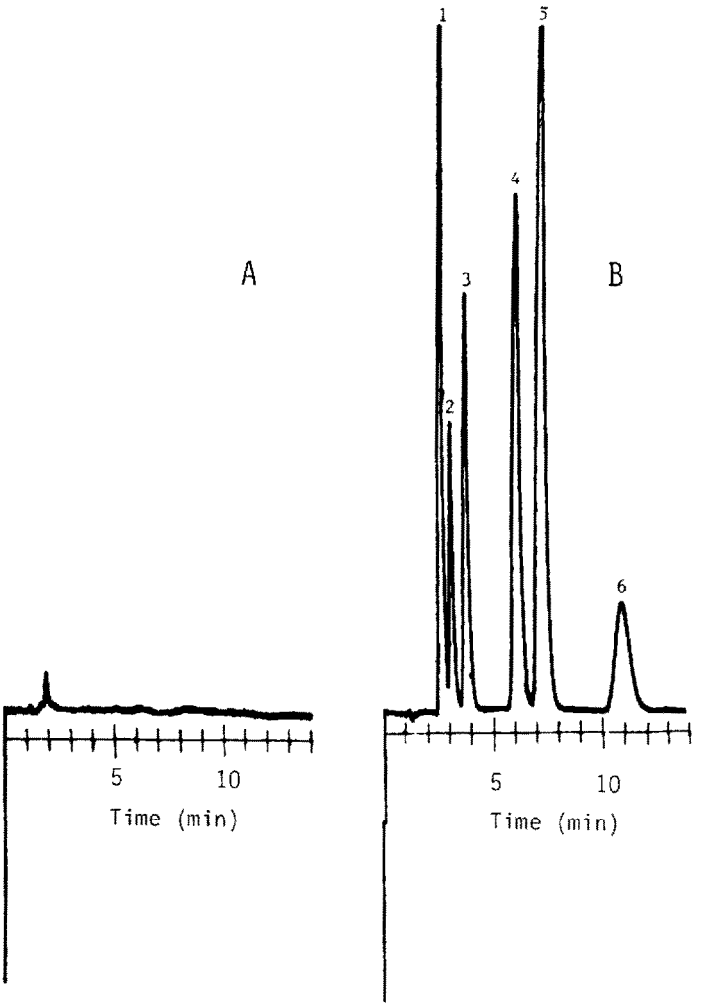

Fig. 1. (A) Chromatogram of a blank serum sample from a subject who had abstained from all caffeine-containing foods and beverages for 48 h. (B) Chromatogram resulting from injection of standard 2. Peaks: 1 = 3-MX; 2 = 1-MU; 3 = 1,3-DMU; 4 = theophylline; 5 = BHET; 6 = caffeine.

directly injected. The assay recovery for each compound was computed using the following equation:

$$\text{Percentage recovery} = \frac{\text{peak area extract}}{\text{mean peak area direct injections}} \times 100.$$

RESULTS

The chromatogram resulting from the injection of an extract from a blank serum sample obtained from a volunteer who had abstained from all caffeine-containing foods and beverages for 48 h is shown in Fig. 1A. Fig. 1B illustrates the response to injection of standard 2 (Table I). The retention times of 3-MX, 1-MU, 1,3-DMU, theophylline, BHET and caffeine were 2.73, 3.22, 3.90, 6.28, 7.42 and 11.19 min, respectively. Comparison of Fig. 1A and B illustrates the lack of interference of endogenous components of serum with this assay.

Typical standard curves relating the peak-area ratios of each compound to the concentration of that compound in the standard (Table I) are presented in Fig. 2. Each standard curve showed good linearity over the range of concentrations examined. The mean least-squares regression equations as well as the mean coefficients of determination for the standard curves of theophylline and its metabolites for four consecutive days are presented in Table II.

The extraction recoveries of theophylline and its metabolites as well as the

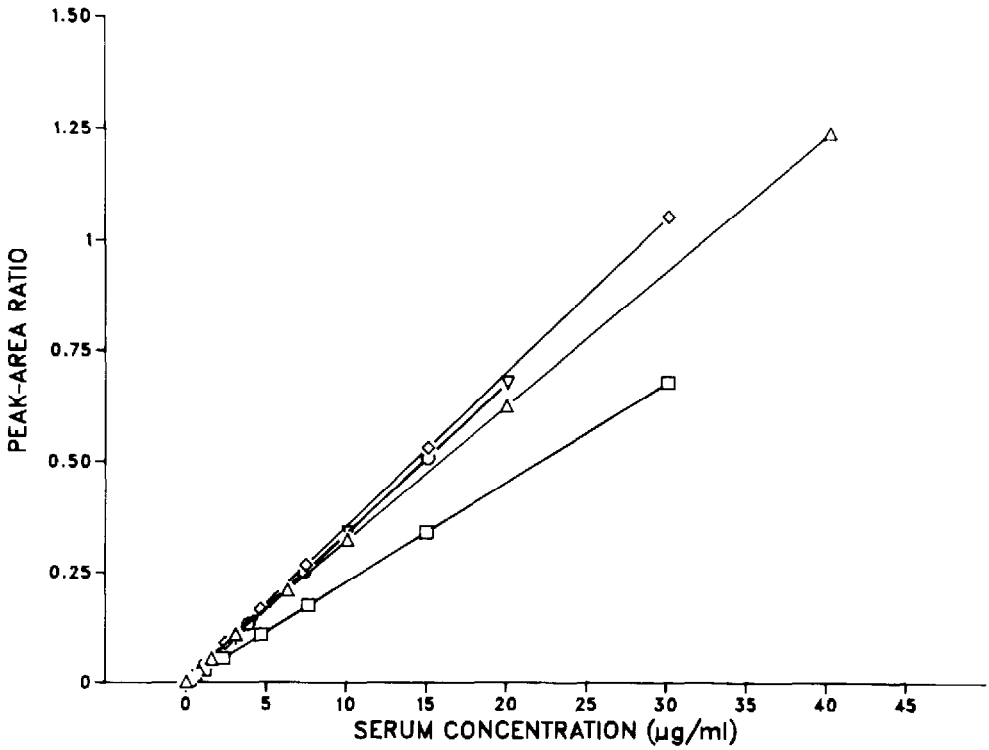

Fig. 2. Representative correlation curves illustrating the relationship between the peak-area ratio to concentration. (△) Theophylline; (◇) 3-MX; (○) 1-MU; (□) 1,3-DMU; (▽) caffeine.

TABLE II

MEAN REGRESSION EQUATIONS AND r^2 VALUES FOR STANDARD CURVES

Compound	Regression equation	r^2
3-MX	$0.040x + 0.001$	0.9997
1-MU	$0.025x - 0.001$	0.9993
1,3-DMU	$0.028x - 0.001$	0.9988
Theophylline	$0.030x - 0.001$	0.9988
Caffeine	$0.031x + 0.001$	0.9993

TABLE III

EXTRACTION RECOVERY AND SENSITIVITY LIMITS OF THE ASSAY FOR THEOPHYLLINE AND ITS METABOLITES IN SERUM

Compound	Recovery (%)*		Sensitivity limit** (ng/ml)
	A	B	
Theophylline	92.9 ± 1.9	76.0 ± 3.3	30
3-MX	92.4 ± 2.8	72.9 ± 0.5	20
1-MU	94.0 ± 6.5	77.9 ± 0.4	10
1,3-DMU	96.2 ± 1.4	74.2 ± 1.4	20
Caffeine	81.1 ± 0.7	66.6 ± 2.4	10

*Performed at concentrations present in quality control samples A and B (see Table I); values reported are the mean ± S.D. of five measurements.
**Based on a signal-to-noise ratio of 2.5.

sensitivity limits for each of these compounds are presented in Table III. The assay recoveries appeared to be concentration-dependent with extraction recovery ranges at low concentrations ranging from 66.6 to 77.9% while those at high concentrations varied from 81.1 to 96.2%. Recovery of the internal standard was calculated to be 98.5%.

The intra-day variability of the assay for each of four consecutive days as well as the inter-day variability of the assay over the same four days are presented for quality-control samples A and B in Tables IV and V. All intra-day coefficients of variation were less than 4.3% while inter-day values were less than 3.8%.

Drugs commonly administered with theophylline were tested for interference. The following compounds in the concentrations indicated did not interfere with the assay: 1,7-dimethylxanthine (40 μg/ml), prednisone (5 μg/ml), prednisolone (5 μg/ml), metaproterenol (30 μg/ml) and terbutaline (50 ng/ml).

DISCUSSION

It has been observed that the pharmacological activity of theophylline is highly correlated with its concentration in serum [16]. The optimal therapeutic range of theophylline has been shown to be 10—20 μg/ml [17].

TABLE IV

INTRA-DAY VARIABILITY OF THE ASSAY

Compound	Concentration added (μg/ml)	Mean concentration found (μg/ml)	Coefficient of variation (%)
Day 1			
3-MX	22.50	22.19	1.26
	6.10	6.22	1.29
1-MU	11.25	11.05	3.62
	3.05	2.95	3.73
1,3-DMU	22.50	22.34	0.67
	6.10	6.39	3.29
Theophylline	30.00	29.21	2.67
	8.12	8.07	4.34
Caffeine	15.00	15.00	0.67
	4.06	4.09	1.47
Day 2			
3-MX	22.50	22.55	0.62
	6.10	6.07	1.81
1-MU	11.25	11.16	1.43
	3.05	3.02	3.64
1,3-DMU	22.50	22.75	0.40
	6.10	6.09	1.31
Theophylline	30.00	30.09	0.33
	8.12	8.16	0.86
Caffeine	15.00	15.13	0.73
	4.06	4.07	1.97
Day 3			
3-MX	22.50	22.83	0.88
	6.10	6.25	1.28
1-MU	11.25	11.49	1.65
	3.05	2.93	2.73
1,3-DMU	22.50	23.05	0.69
	6.10	6.32	1.27
Theophylline	30.00	30.42	0.53
	8.12	8.63	2.67
Caffeine	15.00	15.43	0.26
	4.06	4.04	2.73
Day 4			
3-MX	22.50	22.23	1.89
	6.10	6.24	1.44
1-MU	11.25	11.18	0.98
	3.05	3.06	1.96
1,3-DMU	22.50	22.42	0.58
	6.10	6.09	1.48
Theophylline	30.00	29.74	0.53
	8.12	8.12	0.99
Caffeine	15.00	15.04	0.33
	4.06	4.00	1.50

TABLE V

INTER-DAY VARIABILITY OF THE ASSAY

Compound	Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
3-MX	22.50	22.45 ± 0.38	1.69
	6.10	6.19 ± 0.12	1.94
1-MU	11.25	11.22 ± 0.29	2.58
	3.05	2.99 ± 0.11	3.68
1,3-DMU	22.50	22.64 ± 0.31	1.37
	6.10	6.22 ± 0.19	3.05
Theophylline	30.00	29.86 ± 0.61	2.04
	8.12	8.24 ± 0.31	3.76
Caffeine	15.00	15.15 ± 0.19	1.25
	4.06	4.05 ± 0.09	2.22

However, owing to differences in metabolism, there may be wide inter-individual variation within this range. For this reason, dosages must be individualized in order to optimize therapy [18].

It becomes important to measure the concentration of the metabolites of theophylline concurrent with the parent drug in serum owing to the bronchodilating and myocardial stimulating activity they each possess. An individual with a subtherapeutic serum theophylline concentration may be deriving some benefit from the additive bronchodilating effects of 3-MX and 1,3-DMU, while cardiotoxicity may be observed at subtherapeutic serum theophylline concentrations owing to the effects of 3-MX and 1,3-DMU.

Many assays have been developed to measure serum concentrations of theophylline and its metabolites [6—15]. These assays require a large sample volume, tedious sample preparation and longer chromatogram run times, and many times do not result in acceptable resolution [6—15]. Three assays developed in the late 1970s were able to resolve the metabolites and parent drug in aqueous solution but not in serum [13—15]. In a method proposed by Orcutt et al. [13] large concentrations of 1,7-dimethylxanthine interfered with theophylline determination. St.-Pierre et al. [12] developed a method using a gradient elution program. Although good separation of the parent drug and metabolites is exhibited, it requires tedious sample preparation, longer chromatogram run times and is less sensitive than the method proposed herein. Desiraju and Sugita [7] proposed an assay that requires a run time of approximately 30 min and does not completely resolve 1-methyluric acid from xanthine. This method, however, did not detect any metabolites of theophylline in patient serum. Chromatograms of serum samples from patients receiving aminophylline, included in a method proposed by Muir et al. [8], appeared to have three peaks with approximately the same retention times as 3-MX, 1-MU and 1,3-DMU, respectively, in the standard mixture. These three peaks were not identified; however, if they are endogenous components in the patient's serum, they would alter the quantitation of these three particular metabolites. A serum concentration–time profile for all compounds in one of

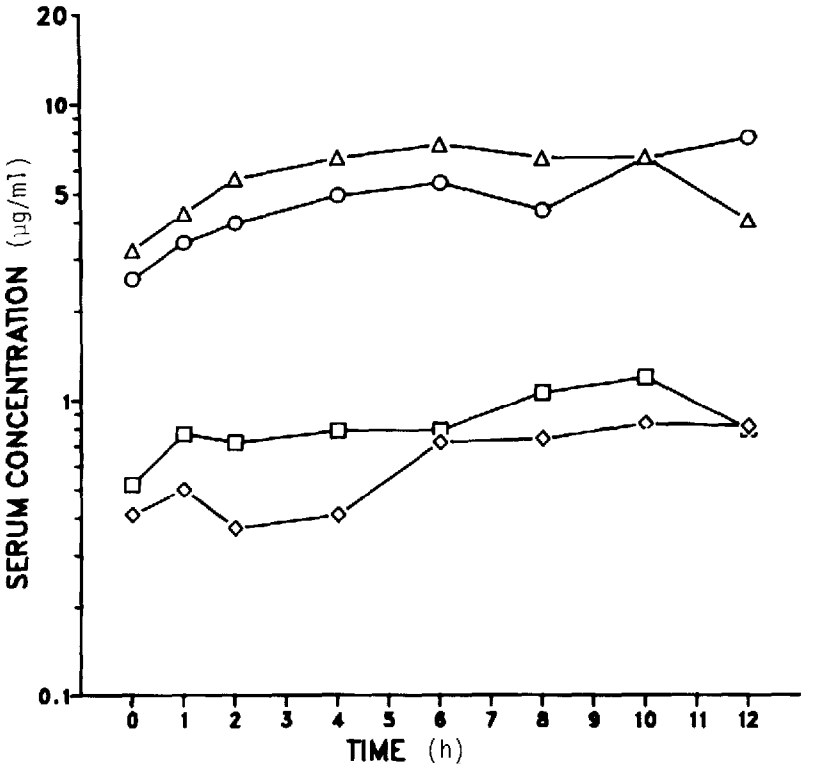

Fig. 3. Serum concentration versus time profiles for a patient following an oral theophylline dose over a 12-h dosing interval at steady state. (△) Theophylline; (◇) 3-MX; (○) 1-MU; (□) 1,3-DMU.

our patients following an oral theophylline dose over a dosing interval at steady-state is shown in Fig. 3, demonstrating the ability of our assay to detect theophylline and its metabolites under clinical conditions.

In summary, an HPLC assay for theophylline and its metabolites which is simple, requires small volumes of serum, has a short run time and increased sensitivity has been developed. Using the method outlined herein, the analysis of a standard curve and quality controls, as well as many patient samples in the course of one day is feasible, thus, providing a useful research and clinical tool which is particularly useful in situations where a limited volume of specimen exists, such as in a pediatric patient populatino and animal research.

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