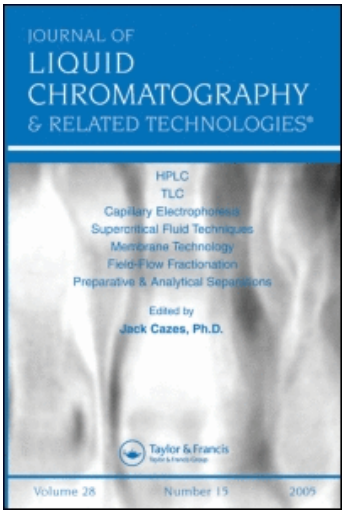

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A New Simplified Microassay for the Quantitation of Theophylline in Serum by High-Performance Liquid Chromatography

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A NEW SIMPLIFIED MICROASSAY FOR THE QUANTITATION OF THEOPHYLLINE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An improved, rapid and simplified HPLC method is presented for the quantitation of theophylline in serum. The internal standard, beta-hydroxypropyl theophylline (20 ul of a 100 ug/ml solution) is added to 50 ul of serum. Serum proteins are precipitated by the addition of 30 ul of 20% trichloroacetic acid solution. The mobile phase consists of a sodium acetate buffer with 8% acetonitrile. Chromatogram run time is 8 minutes. The sensitivity limit is 0.10 ug/ml. This method is interference free from the major metabolites of theophylline and other drugs commonly coadministered with theophylline.

INTRODUCTION

Theophylline , a bronchodilating agent, is used clinically in the treatment of asthma, as well as other pulmonary diseases characterized by bronchospasm.(1,2) The therapeutic serum theophylline concentration for bronchodilation has been shown to be 10-20 ug/ml.(3) Toxic symptoms appear with increasing frequency when the serum theophylline concentration exceeds 20 ug/ml.(4) Due to large variation in clearance rates of theophylline, serum levels of the drug must be monitored to enable the clinician to optimize the therapeutic benefits, while decreasing the probability of toxicity.(5)

There have been many theophylline assays described to date. However, many of these require large sample volumes, complicated sample preparation, long chromatogram run times, exhibit interferences with other compounds and are not sensitive enough for accurate pharmacokinetic evaluations.(2,6-14)

This prompted us to develop an HPLC method for the quantitation of theophylline in serum which utilizes a small sample volume, a short and simple sample preparation, rapid chromatogram run time, has increased sensitivity and is free of interferences from metabolites of theophylline, as well as many drugs commonly coadministered with theophylline.

MATERIALS AND METHODS

Apparatus

A U6K Injector and an M45 Pump System were used in conjunction with an M440 Absorbance Detector set at 280 nm (Waters Associates, Milford, MA, USA). A uBondapak reversed-phase C18 precolumn cartridge (Waters Associates, Milford, MA, USA) was used in conjunction with a uBondapak reversed-phase C18 Radial-Pak cartridge (100 mm long, 8 mm I.D., Waters Associates, Milford, MA, USA) for the chromatographic separation.

Reagents and Standards

The sources of the compounds used to make the standards and internal standard are as follows:

theophylline (Fisher Scientific, King of Prussia, PA, USA) and beta-hydroxypropyl theophylline (BHPT) (Sigma Chemical Co., St. Louis, MO, USA). Sodium acetate and acetonitrile (Fisher Scientific, King of Prussia, PA, USA) were of HPLC grade. Glacial acetic acid and 20% trichloroacetic acid solution were also obtained from Fisher Scientific (King of Prussia, PA, USA).

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

Xanthine-free serum was obtained from normal volunteers who had abstained from all foods and beverages containing caffeine and other xanthines for 48 hours. The standards were made by dissolving 100 mg of theophylline in 100 ml of xanthine-free serum. This stock solution was further diluted with

TABLE 1
CONCENTRATIONS OF STANDARDS

Standard	Theophylline Concentration(ug/ml)
1	50.00
2	25.00
3	10.00
4	5.00
5	2.50
6	1.00
7	0.75
8	0.50
9	0.25
10	0.10
11	0.00

xanthine-free serum to obtain 10 serum standards in the concentrations shown in Table 1. A xanthine-free serum sample was used as 0.0 ug/ml. Standards and patient samples were also stored at -20°C.

Extraction Procedure

Blood samples were centrifuged for 5 minutes at 1,380 x G to separate the serum from the whole blood. The serum was harvested with a Pasteur pipet and transferred to a 12 x 75 mm labeled test tube. Into a microcentrifuge tube was placed 20 microliters of the internal standard solution and 50 microliters of the serum sample. Serum proteins

were precipitated by the addition of 30 microliters of 20% trichloroacetic acid solution added to the serum and internal standard. The mixture was vortexed for 30 seconds.

After centrifugation of the sample in a microcentrifuge at 15,000 x G for 4 minutes, the supernatant was transferred to a second labeled microcentrifuge tube and centrifuged for 1 minute. A 20 microliter quantity of this sample was injected directly onto the chromatographic system.

Chromatographic Conditions

The mobile phase consisted of 10 mmol/L sodium acetate buffer with 8% acetonitrile. After lowering the pH to 4.0 with glacial acetic acid, the mobile phase was magnetically stirred and aspirated under a vacuum until degassed.

A flow rate of 4.8 ml/min was used at a pressure of 8 MPa. The system was at ambient temperature. Detector sensitivity was set at 0.2 absorbance units. The chart speed was 4 mm/min. The total run time selected was 8 minutes. The

concentration of theophylline in patient samples was calculated using the peak area ratio of theophylline and the use of the line of best fit describing the relationship between peak area ratio and concentration for the serum standards.

Extraction Recovery

The assay recovery of theophylline and the internal standard was assessed at the concentrations present in standards 1,3 and 5 (see Table 1). Five replicates of each of the three standards were extracted and injected. Five injections of each of the three standards prepared in aqueous solutions were directly injected. The assay recovery at each concentration was computed using the following equation:

$$\% \text{ Recovery} = \frac{\text{Peak Area Extract}}{\text{Mean Peak Area Direct Injection}} \times 100$$

RESULTS

The chromatogram resulting from the injection of an extract from a xanthine-free serum sample obtained from a volunteer who had abstained from all

xanthine containing foods and beverages for 48 hours is shown in Figure 1A. Figure 1B illustrates the response to injection of Standard 3 (Table 1). The retention times of theophylline (THEO) and BHPT were 2.93 and 6.27 minutes, respectively. Comparison of Figures 1A and 1B illustrates the lack of interference from endogenous components of serum with this assay. A chromatogram resulting from the injection of an aqueous solution containing the major metabolites of theophylline is shown in Figure 2. The retention times of these compounds are listed in Table 2.

A typical standard curve relating the peak area ratio to the concentration of theophylline in the

TABLE 2
RETENTION TIMES FOR THEOPHYLLINE AND ITS MAJOR METABOLITES

Compound	Retention Time
1-Methyluric acid	1.27 minutes
3-Methylxanthine	1.39 minutes
1,3-Dimethyluric acid	1.76 minutes
1,7-Dimethylxanthine	1.82 minutes
Theophylline	2.93 minutes
Caffeine	5.58 minutes
BHPT	6.27 minutes

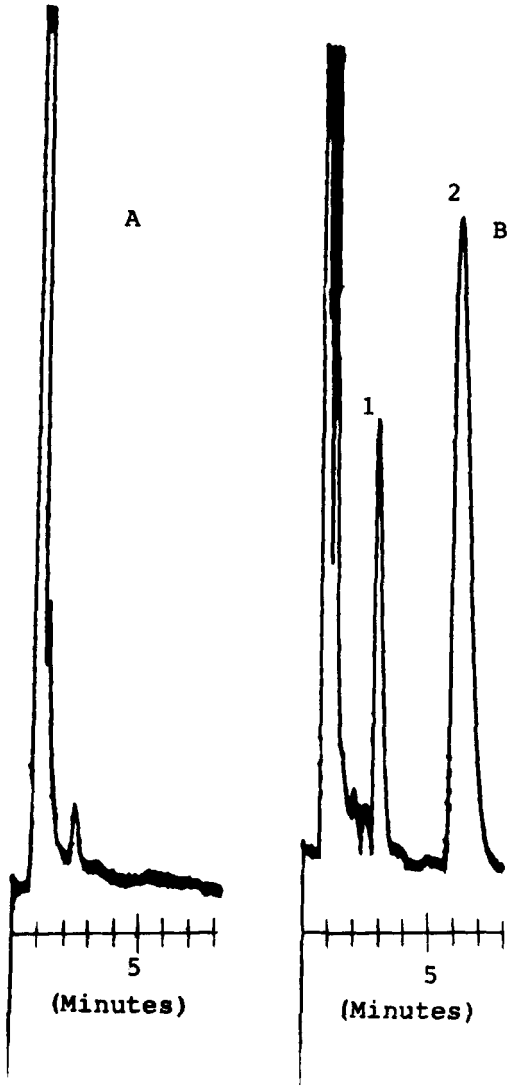

Figure 1A: Chromatograph resulting from the injection of a xanthine-free serum sample obtained from a volunteer who had abstained from all xanthine containing foods and beverages for 48 hours.

Figure 1B: Chromatograph resulting from the injection of standard 3 using the following peak identifications: (1) THEO and (2) BHPT.

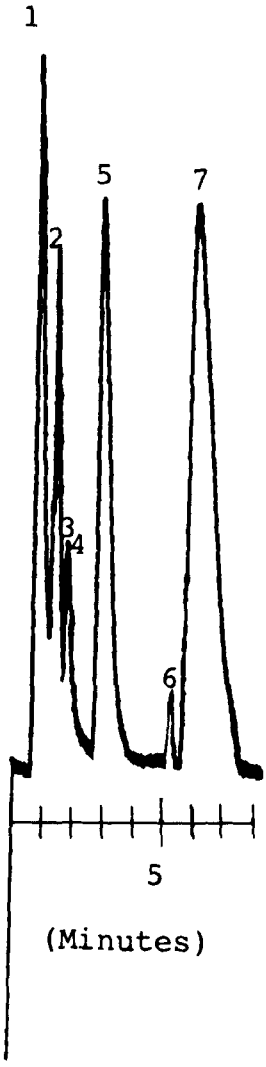

Figure 2: Chromatograph resulting from the injection of an aqueous solution containing theophylline, its major metabolites and BHPT using the following peak identifications: (1) 1-methyluric acid, (2) 3-methylxanthine, (3) 1,3-dimethyluric acid, (4) 1,7-dimethylxanthine, (5) theophylline, (6) caffeine, and (7) beta-hydroxypropyl theophylline (internal standard).

standards is presented in Figure 3. Each standard curve showed good linearity over the range of concentrations examined. The least squares regression equations as well as the coefficients of variation for the standard curves of theophylline for five consecutive days are presented in Table 3.

The extraction recoveries of theophylline are presented in Table 4. The sensitivity limit for theophylline was found to be 0.1 ug/ml based on a signal to noise ratio of 2.5. The assay recovery appeared to be slightly concentration dependent with extraction recovery ranges from 95.4% to 100%. Recovery of the internal standard was calculated to be 98%.

TABLE 3

MEAN REGRESSION EQUATIONS AND r^2 VALUES FOR STANDARD CURVES

	Regression Equation	r^2 Value
Day 1	0.032x+0.007	0.9998
Day 2	0.032x+0.008	0.9998
Day 3	0.032x+0.006	0.9998
Day 4	0.028x+0.004	0.9995
Day 5	0.027x+0.005	0.9994

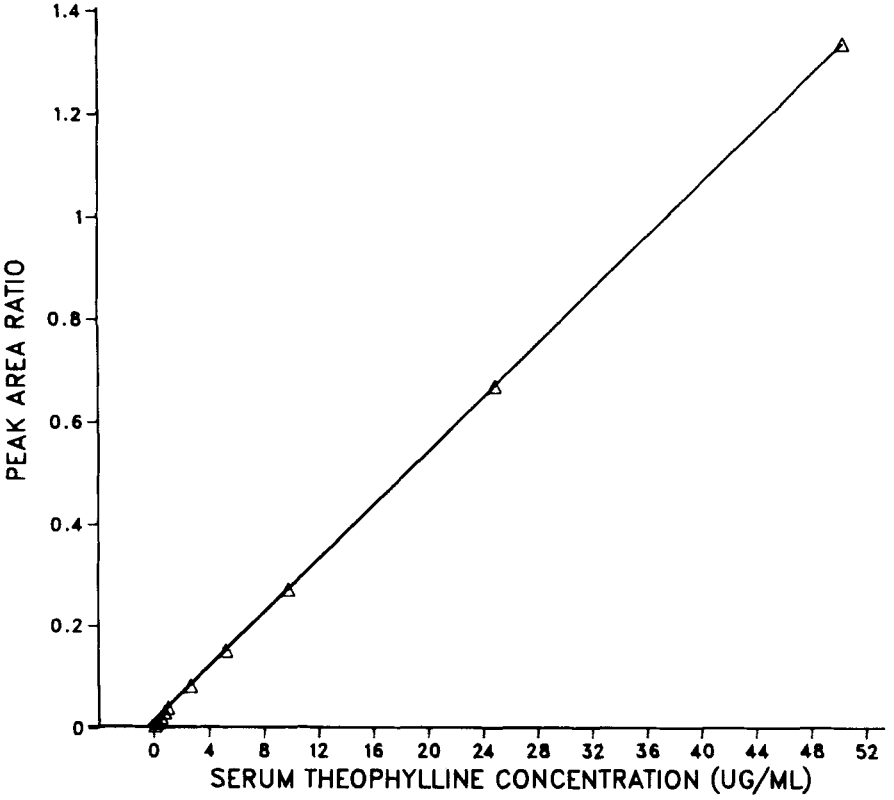

Figure 3: Typical standard curve relating the peak area ratio to the concentration of theophylline.

TABLE 4

EXTRACTION RECOVERY OF THE ASSAY

Concentration (ug/ml)	Recovery (%)[1]
50.00	100.0 (3.0)
10.00	98.0 (6.0)
2.50	95.4 (7.0)

[1] Values reported are the mean (±SD) of 5 measurements.

The inter-day variability of the assay over 5 consecutive days is presented for the standards in Table 5. All inter-day coefficients of variation were less than 6.41%.

Drugs commonly administered with theophylline, as well as the major metabolites of theophylline were tested for interference. These compounds included: acetaminophen, aspirin, Bactrim, caffeine, 8-chlorotheophylline, 7-(2,3-dihydroxypropyl)theophylline, 1,3-dimethyluric acid, 1,7-dimethylxanthine, Erythromycin, 3-methylxanthine, 1-methyluric acid,

TABLE 5
INTER-DAY VARIABILITY OF THE ASSAY

Concentration (ug/ml)	Mean (ug/ml)	S.D.	C.V.
50.00	49.82	0.158	0.32
25.00	25.34	0.311	1.23
10.00	10.10	0.214	2.12
5.00	4.96	0.178	3.59
2.50	2.34	0.150	6.41
1.00	1.00	0.006	0.60
0.75	0.75	0.006	0.80
0.50	0.49	0.010	2.01
0.25	0.25	0.007	2.80
0.10	0.11	0.006	5.45
0.00	0.00	0.000	0.00

prednisolone, prednisone, metaproteronol, terbutaline and chlorpheniramine maleate.

DISCUSSION

Optimal bronchodilating effects have been shown to occur at serum concentrations of 10-20 ug/ml.(3) Wide inter- and intra-individual variations in clearance rates is exhibited and may be caused by many factors, including: age, weight, diet, smoking, absorption, concurrent drug therapy and other disease states.(3) For this reason, it becomes important to quantitate the serum concentration of theophylline in order to maximize the therapeutic benefits while decreasing the risk of toxicity.

Many assays have been developed to quantitate theophylline in serum(2,6-14), however, some are very tedious to perform and do not yield acceptable results for pharmacokinetic studies.
Some assays described exhibit interference between theophylline and a metabolite of caffeine,

1,7-dimethylxanthine.(6,8,9) A method proposed by Peng(10) is less sensitive than the method proposed herein. This method does not employ the use of an internal standard. The lack of an internal standard as a reference could mask problems in peak size due to inadvertant injection of an air bubble or sample leak within the system. Theobromine and dyphylline were shown to interfere with theophylline in this method. Evenson proposed a method which gives incomplete separation of theophylline from an endogenous plasma peak when the supernatant of trichloroacetic acid deproteinized plasma is chromatographed on an ion-exchange column.(9,15) Although a method proposed by Kabra(12) has a run time of only 70 seconds, the sensitivity limits are not as great, sample preparation is tedious and, in some cases, dyphylline was shown to interfere with theophylline analysis. A method described by Park(13), while exhibiting increased sensitivity, requires a long and complicated sample preparation. Starkey(2) proposed a method that requires a large

sample volume and has a long chromatogram run time. A method proposed by Muir(6), requires a large sample volume, tedious sample preparation, has a long chromatogram run time and is less sensitive than the method proposed herein. Although a method proposed by Bock(7) requires only a 40 second run time, poor separation of theophylline from 1,7-dimethylxanthine is exhibited and the analytical column has a very short lifetime which would increase the cost of the assay. A method proposed by Adams(14), requires the use of a column oven and exhibits interference between dyphylline and theophylline, as well as between the internal standard (8-chlorotheophylline) and dimenhydrinate. In this method, the pH of the mobile phase was critical to the retention time of the internal standard.

In summary, a fast and easy HPLC method for the determination of theophylline in serum is presented. This method requires a small sample volume, short sample preparation and a short chromatogram run

time. No interferences are seen with any major metabolites of theophylline or drugs commonly coadministered with theophylline. The small sample volume and sensitivity limit of 0.1 ug/ml makes this assay an excellent choice for the performance of pharmacokinetic studies and in the management of pediatric patients.

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