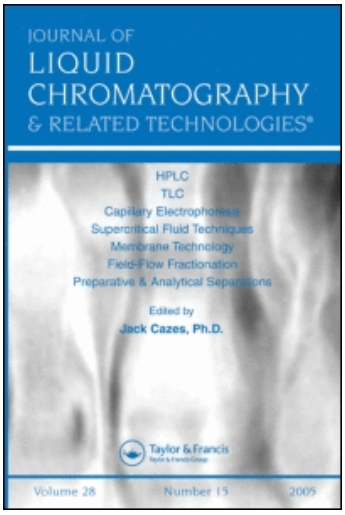

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An HPLC Method for the Determination of Theophylline and Its Metabolites in Serum and Urine

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AN HPLC METHOD FOR THE DETERMINATION OF THEOPHYLLINE AND ITS METABOLITES IN SERUM AND URINE

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ABSTRACT

A urine and a serum assay have been developed to quantitate theophylline and its major metabolites:1,3-dimethyluric acid, 3-methylxanthine and 1-methyluric acid. Reverse phase chromatography follows a serum acetone extraction procedure and a urine anion exchange clean-up procedure. Lower limits of sensitivity are 0.04 µg/ml for serum metabolites and 1 µg/ml for urine metabolites. Both assays are free of interference from endogenous substances. These assays have been tested successfully in pharmacokinetic and metabolic studies of theophylline.

INTRODUCTION

Measurement of the bronchodilator theophylline (1,3-dimethylxanthine) and its metabolites in various biological fluids has been the subject of much interest, as investigators study the pharmacokinetic characteristics and metabolic pathways of

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the drug (1-5). The major metabolic products of theophylline have been identified as 3-methylxanthine (3-mx), 1,3-dimethyluric acid (1,3-mu) and 1-methyluric acid (1-mu) (1,4). These metabolites arise from the 8-oxidation (1,3-mu), 1-demethylation (3-mx) and consecutive 3-demethylation and oxidation (1-mu) of theophylline (6). Methylation of theophylline occurs in infants (7) and is also said to occur in adults (2), with detectable serum levels of caffeine (1,3,7-trimethylxanthine) having been observed after multiple dose administration of theophylline.

To date, various assay methods have been developed for the determination of theophylline and its metabolites in plasma or serum and urine (1,4,5,8-15), but have not generally proven to be reproducible in our laboratory. Two investigators (5,8) describe a serum assay that resolved only 1 metabolite in addition to theophylline. Others (9,11,14) were able to resolve metabolites and parent drug in aqueous solution, but failed to show subsequent resolution in human sera. Tang-Liu et al (4) have developed an assay suitable for their analytical and pharmacokinetic work, which has been previously described.

Publication of urine analyses have been more abundant. However, we concur with Muir et al (10) who, in a recent comparative discussion, stated that previously reported procedures have not demonstrated sufficient selectivity and sensitivity. Published work by Tang-Lui and Riegelman (15) describes the most selective and sensitive assay to date but the procedure is time-

consuming and requires access to a pump that can automatically vary its rate of delivery of an individual solvent. In view of this, new methods have been developed in our laboratory that simply and reliably quantitate theophylline and its known metabolites in both serum and urine.

MATERIALS AND METHODS

Reagents

Reagents used were anhydrous crystalline theophylline, caffeine, ß-hydroxyethyl theophylline, tetrabutyl ammonium hydrogen sulfate and an anion exchange resin (DOWEX-1) chloride form, 200 to 400 mesh, all obtained from Sigma Chemical, St. Louis, Missouri. 3-Methylxanthine, 1,3-dimethyluric acid and 1-methyluric acid were purchased from Adams Chemical, Roundlake, Illinois. Mobile phase constituents used were methanol (HPLC grade) and water (HPLC grade) from Burdick and Jackson, Muskegon, Michigan and sodium phosphate monobasic from Fisher Scientific, Fair Lawn, New Jersey.

Aqueous stock solutions of parent drug (theophylline), metabolites (1-mu, 3-mx, 1,3-mu) and internal standard (ß-hydroxyethyl theophylline) were prepared on a daily basis. For serum assays, both theophylline and internal standard were dissolved to give concentrations of 200 µg/ml while metabolites were combined as a 20 µg/ml solution. For urine assays, theophylline and metabolites were prepared as a 50 µg/ml solution and

the internal standard as a 25 µg/ml solution. The mobile phase in both serum and urine assays consisted of methanol (solvent A) and 10 mM sodium phosphate (solvent B) adjusted to pH 4.5. The solvent system was degassed through 0.5 micron Durapore filter (Millipore, Bedford, Massachusetts).

Instrumental Conditions

The HPLC system consisted of a Perkin Elmer Series 2 liquid chromatograph pump, an LC-85 variable UV spectrophotometric detector and an LC autocontrol. The detection wavelength was set at 275 nm in order to optimize the absorption of all compounds of interest. The methylated xanthines absorb maximally at 273 nm while the methylated uric acids absorb at 290 nm. Samples were injected by means of a Perkin Elmer ISS-100 automatic injector. A Perkin Elmer Sigma 10 Data Processing System initiated the solvent program. Detector response was set at 0.02 absorbance units full scale for serum assays and 0.04 for urine assays. All determinations were performed at a solvent flow rate of 2.0 ml/minute.

Urine assays were performed using a µBondapak C18 10 micron column (3.9 mm I.D. X 30 cm) (Waters Scientific, Mississauga, Ontario). For serum analysis, the column was changed to an Ultrasphere ODS 5 micron (4.6 mm I.D. x 250 mm) (Beckman Instruments, Toronto, Ontario). This improved resolution of compounds of interest from endogenous material. Recently, this

Ultrasphere ODS 5 micron column was tried in the urine assay and was found to provide similar resolution.

Procedure

Serum assay: Three ml of acetone containing the internal standard (8-hydroxyethyl theophylline) (65 ng/ml) were added to 200 µl of serum. Samples were acidified with 20 µl of glacial acetic acid to increase the recovery of 1 methyluric acid, vortexed for 2 minutes and placed in a centrifuge for 10 minutes. The organic layer were separated and evaporated to dryness by means of a Buchler Vortex Evaporation set at a temperature of 40°C. The residue was reconstituted with 125 µl of distilled water and 100 µl were injected onto the column. The methanol phosphate buffer solvent system was delivered in a stepwise linear gradient fashion, consisting of methanol at 6% for 8 minutes, increased from 6 to 21% in 5 minutes, retained at 21% for 7 minutes, then reset to 6% (Figure 1). The column was equilibrated for 10 minutes before the next injection. Pressure registered by the column remained between 3800 and 4200 psi.

Urine Assay: Pasteur capillary pipettes (145 mm length) (Maple Leaf) with distal ends plugged with glass wool were filled with 8 ml (0.42 g) of Dowex-1 suspended in distilled water with gentle stirring. Filled columns were washed with 6 ml deionized water. A sample volume of 200 µl was introduced onto the resin followed by 200 µl of distilled water containing the internal

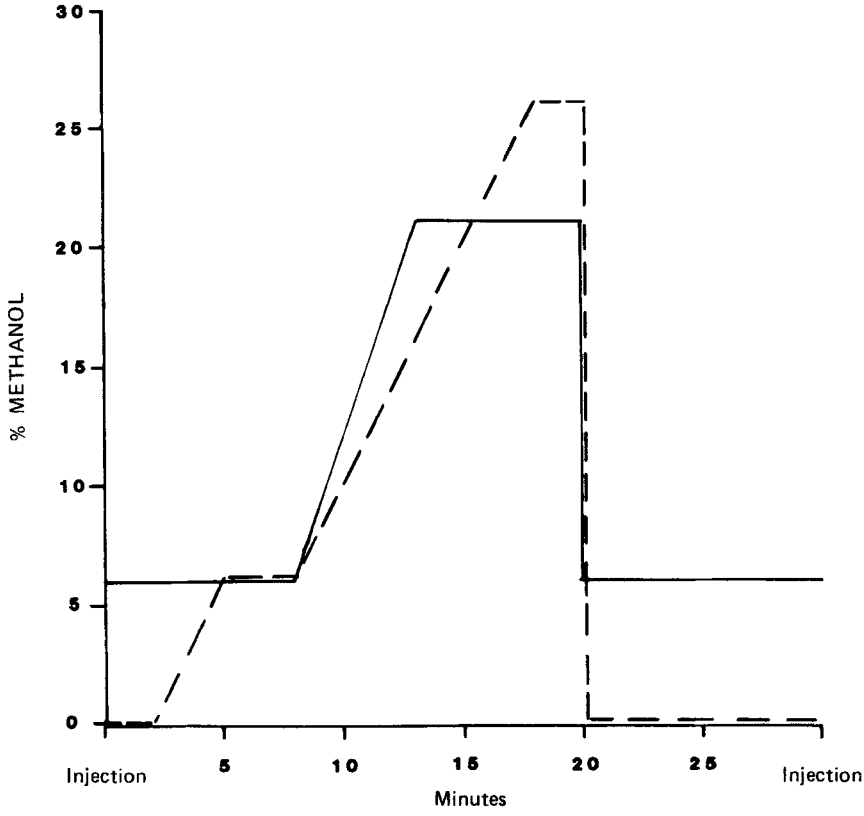

FIGURE 1: Solvent gradient program for serum (——), and urine assay (---). The percentage of methanol is shown as a function of elution time. Injections of sample occur every 30 minutes.

standard (25 µg/ml). Two ml of tetrabutylammonium hydrogen sulfate (TBA) 0.05 M were added to elute the desired compounds. Fifty µl of the eluant were injected onto the column. New columns were prepared for each sample analyzed. The mobile phase consisted of phosphate buffer with methanol at 0% for the first 2 minutes, increased at 2% per minute from 2 to 5 minutes and again from 8 to 18 minutes, (Figure 1). A 10-minute lagtime between

injections was used to equilibrate the column. The operating pressure was approximately 4000 psi.

RESULTS

Figure 2 shows the separation of an aqueous mixture of theophylline and its metabolites obtained with the described procedure (serum gradient program). Linearity of detector response was demonstrated by injecting onto the column, known amounts of theophylline and metabolites in aqueous solution. The resulting calibration curves were obtained by plotting the amount injected against the measured peak height. Curves were linear over a 20 fold variation in concentration, encompassing the range of serum concentration values expected from metabolites and theophylline after therapeutic administration of the latter (Figure 3). The correlation coefficient calculated for each compound was greater than 0.998.

Determinations of theophylline and metabolite concentrations in serum and urine were performed using an internal standard technique, where the peak height of compound of interest is compared to that of the internal standard. The appropriate equation for quantitation is:

$$\text{Conc} = \text{resp factor X conc int std} \quad X \quad \frac{\text{peak height unknown}}{\text{peak height int std}}$$

The relative response factor for each compound is previously determined by analyzing a sample in which the concentration of

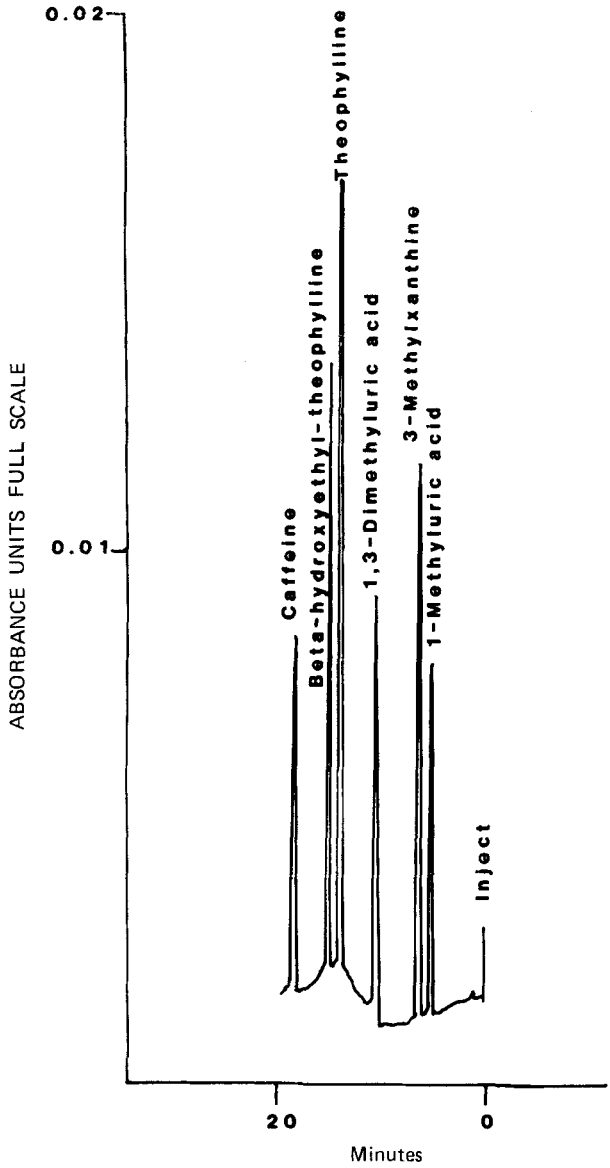

FIGURE 2: The HPLC separation of theophylline and its metabolites in aqueous mixture. Beta-hydroxyethyl theophylline serves as the internal standard.

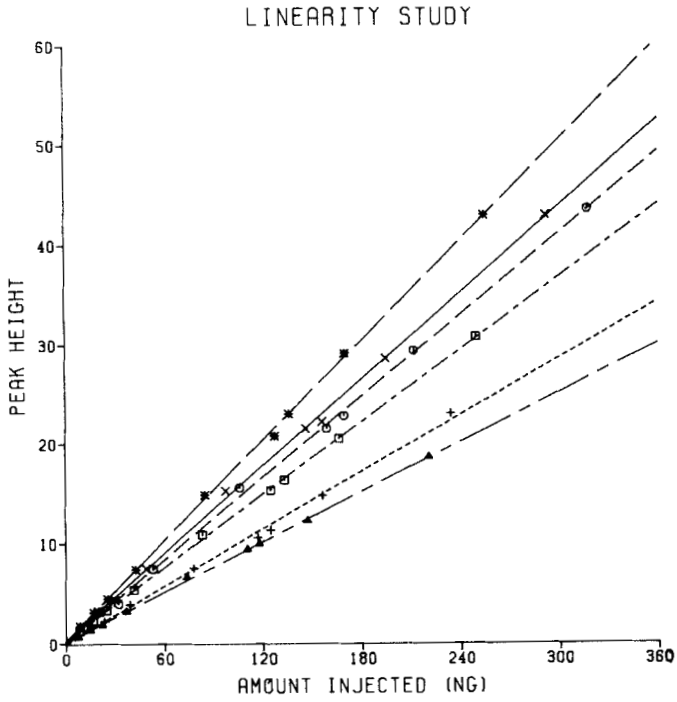

FIGURE 3: Theophylline and metabolite standard curves. Peak heights are proportional to amount injected: 1-methyluric acid (x), 3-methylxanthine (0), 1,3-dimethyluric acid (+), theophylline (*), 8-hydroxyethyl theophylline (□), caffeine (Δ).

both the component of interest and internal standard is known:

$$\text{resp factor} = \frac{\text{concentration of compound}}{\text{concentration of standard}} \times \frac{\text{peak height of standard}}{\text{peak height of compound}}$$

Table 1 presents the recovery and precision data obtained from the serum assay. The absolute recovery from serum was measured following the addition of a known concentration of compound to drug free serum and comparing the peak height obtained after extraction to that obtained after direct injection of the substance in aqueous solution. Six determinations

TABLE 1

Absolute Recovery and Retention Time of Theophylline and Metabolites in Serum (n=12)

Compound	Retention Time	Absolute Recovery	CV (%)
ß-hydroxyethyl theophylline	19 min	102%	1.6%
theophylline	17 min	98%	5.6%
1,3-mu	14 min	96%	6.1%
3-mx	10.5 min	88%	8.1%
1-mu	8.5 min	66%	7.3%

were performed at two concentrations of metabolites (0.5 µg/ml, 1 µg/ml) and two concentrations of theophylline (10 µg/ml, 20 µg/ml). Similar data are presented for the urine assay (Table 2). Six determinations were performed at 20 µg/ml and 40 µg/ml.

DISCUSSION

The serum extraction procedure resulted in the immediate precipitation of serum proteins. Figure 4-A shows the chromatogram of theophylline-free serum from a volunteer who had abstained from xanthine containing food and beverages for the previous 48 hours, and again after ingestion of 10 mg/kg of oral theophylline (Figure 4-B). No endogenous substances were found to interfere with the analysis. In this assay, caffeine elutes at 20 minutes. Since all volunteers received only 1 therapeutic dose of theophylline, no quantifiable peaks of caffeine were observed at its elution time (2). The range of 24 hour serum concentrations of

TABLE 2

Absolute Recovery and Retention Time of Theophylline and Metabolites in Urine (n=12)

Compound	Retention Time	Absolute Recovery	CV (%)
ß-hydroxyethyl theophylline	18 min	89%	10.2%
theophylline	17 min	70%	8.6%
1,3-mu	14 min	73%	10.3%
3-mx	11 min	78%	11.1%
1-mu	9.8 min	72%	7.3%

the major metabolites seen after one dose (6-10 mg/kg) of theophylline was from 0.04 µg/ml to 0.6 µg/ml for 1-methyluric acid and 3-methylxanthine and from 0.05 µg/ml to 1.5 µg/ml for 1,3-dimethyluric acid. The limit of sensitivity of this assay for all compounds is 0.04 µg/ml.

The urine assay involves a preliminary clean up procedure, adapted from the work of Thompson et al (8). It entails anion exchange separation where Dowex, a strongly basic anion exchange resin, binds the polar ionized compounds of interest as well as the endogenous interfering substances. The methylated xanthines, being weak bases, are not preferentially retained by the column and are eluted with the water. The polar methylated uric acids are retained by the column and will elute following the introduction of an ion with higher affinity for the resin. In the present example, the sulfate ion contributed by the TBA has a fairly high selectivity for the resin. Figure 5-A shows the efficiency of this procedure in removing endogenous interfering compounds

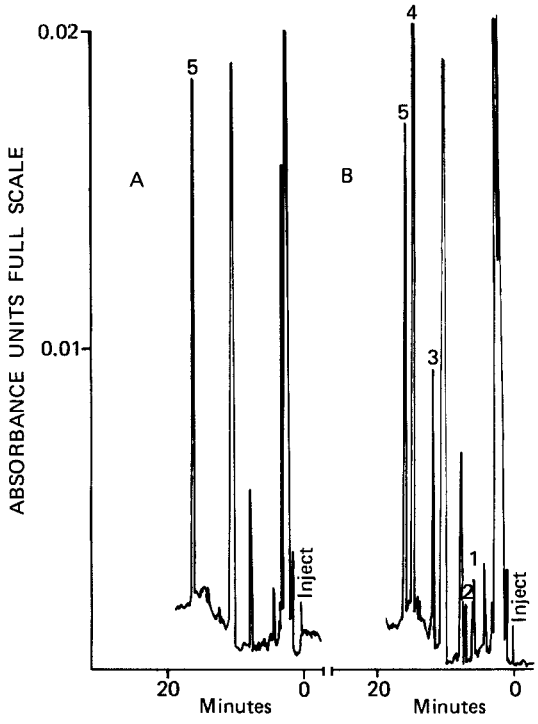

FIGURE 4: A. Chromatogram of a volunteer's theophylline-free serum after a 48 hour abstinence from dietary xanthines: 5) internal standard (ß-hydroxyethyl theophylline). B. Volunteer's serum after ingestion of 10 mg/kg theophylline: 1) 1-methyluric acid, 2) 3-methylxanthine, 3) 1,3-dimethyluric acid, 4) theophylline, 5) internal standard.

from a volunteer's drug free urine. Figure 5-B is a urine chromatogram of the same subject after the administration of 10 mg/kg of theophylline. The assay sensitivity for all compounds is 1 µg/ml.

Separation of the 3 major metabolites of theophylline from endogenous urinary xanthines and uric acids eluting from the column at similar retention times has plagued investigators in

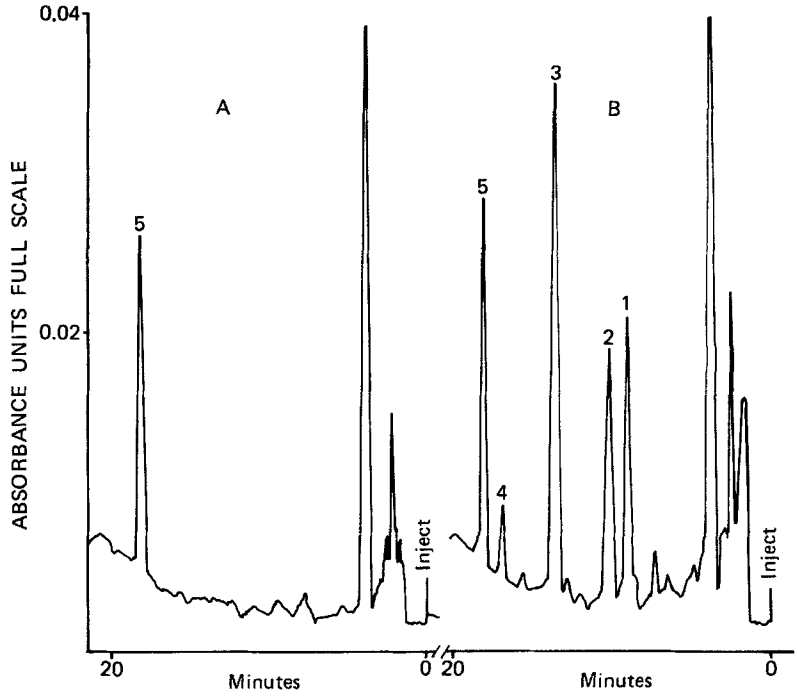

Figure 5: A. Chromatogram of volunteer's theophylline-free urine maintained on a xanthine-free diet for 48 hours: 5) internal standard (ß-hydroxyethyl theophylline). B. Volunteer's urine after ingestion of 10 mg/kg theophylline: 1) 1 methyluric acid, 2) 3-methylxanthine, 3) 1, 3-dimethyluric acid, 4) theophylline, 5) internal standard.

this area of chromatography (3). While urine composition will vary among individuals (15), usually a xanthine-free diet was found to abolish interfering peaks.

Our procedure has sufficient selectivity and precision for practical use, as demonstrated by the results obtained from the following pharmacokinetic study. A single intravenous dose of theophylline as aminophylline (363 mg) was administered to a

TABLE 3

Recovery of Theophylline and its Metabolites from the Urine of A Human Volunteer

Compound	% Recovered (Dose 1)	% Recovered (Dose 2)
theophylline	14.3%	16.8%
1,3-dimethyluric acid	39.2%	40%
1-methyluric acid	17.7%	16.33%
3-methylxanthine	11%	13.2%
Total	82.3%	86.3%
Collection period	48 hours	48 hours

normal male subject on 2 separate occasions. Total (mole per cent) urinary recovery of theophylline and its metabolites after a 48 hour urine collection was found to be 82.3% and 86.3% (Table 3).

This study was repeated in 7 additional subjects who received a dose of 6 mg/kg intravenously on 2 separate occasions. Assuming theophylline is 80% of the aminophylline dose administered, the molar percent urinary recovery of theophylline varied from as low as 73% to as high as 123% (mean 95%). Two explanations for greater than 100% recovery can be put forward: 1) even though subjects consented to xanthine-free diets for 48 hours prior to dosing, some dietary intake of xanthine containing foods might have occurred (10); 2) aminophylline USP standards have allowable limits of $\pm$ 7%. Some subjects may have received more than the labelled dose of aminophylline.

Few authors have been able to adequately describe an assay that consistently separates and quantitates all known metabolites

of theophylline in biological fluids. While a serum (4) and urine (15) assay have been reported that are both selective and sensitive, we have found the present assays simpler and more suitable for our laboratory purposes than previously published reports. These assays are being used in current pharmacokinetic studies of theophylline and its metabolites.

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REFERENCES

1. Jonkman, J.H.G., Tang, D., Upton, R.A. and Riegelman, S., Measurement of Excretion Characteristics of Theophylline and its Major Metabolites, Eur. J. Clin. Pharmacol., 20, 435, 1981.

2. Tang-Lui, D.D-S, and Riegelman, S., Metabolism of Theophylline to Caffeine in Adults, Res. Commun. Chem. Pathol. Pharmacol., 34, 371, 1981.

3. Melethil, S., Dutta, A., Ryan, P.B., Pingleton, S.K. and Kelly, S.J. Steady State Urinary Excretion of Theophylline and its Metabolites in the Presence of Erythromycin, Res. Commun. Chem. Pathol. Pharmacol., 35, 341, 1982.

4, Tang-Lui, D.D-S, Williams, R.L. and Riegelman, S., Nonlinear Theophylline Elimination, Clin. Pharmacol. Ther., 31, 358, 1982.

5. Gundert-Remy, U., Hildebrandt, R., Hengen, N. and Weber, E., Non-linear Elimination Processes of Theophylline, Eur. J. Clin. Pharmacol., 24, 71, 1983.

6. Birkett, D.J., Miners, J.O. and Attwood, J., Secondary Metabolism of Theophylline Biotransformation Products in Man-Route of Formation of 1-Methyluric Acid, Br. J. Clin. Pharmacol., 15, 117, 1983.

7. Bory, C., Baltassat, P., Porthault, M., Bethenod, M., Frederich, A. and Aranda, J.V., Metabolism of Theophylline to Caffeine in Premature Newborn Infants, J. Pediatr., 94, 988, 1979.

8. Thompson, R.D., Nagasawa, H.T. and Jenne, J.W., Determination of Theophylline and its Metabolites in Human Urine and Serum by High Pressure Liquid Chromatography, J. Lab. Clin. Med., 84, 584, 1974.

9. Orcutt, J.J., Kozak, P.P., Gillman, S.A. and Cummins, L.H., Micro-scale Method for Theophylline in Body Fluids by Reversed-Phase, High Pressure Liquid Chromatography, Clin. Chem., 23, 599, 1977.

10. Muir, K.T., Jonkman, J.H.G., Tang, D-S, Kunitani, M. and Riegelman, S., Simultaneous Determination of Theophylline and its Major Metabolites in Urine by Reversed-Phase Ion-Pair High-performance Liquid Chromatography, J. Chromatogr., 221, 85, 1980.

11. Naish, P.J., Chambers, R.E. and Cooke, M., Theophylline Estimation - a Comparative Evaluation of a Gas Chromatographic Method and a High Performance Liquid Chromatography Method, Ann. Clin. Biochem., 16, 254, 1979.

12. Turcant, A., Cailleux, P. and Allain, P., Separation of Xanthine Derivatives by High Pressure Liquid Chromatography and Application to Plasma Analysis, J. Liquid Chromatogr., 3, 1537, 1980.

13. Christensen, H.D. and Whitsett, T.L., Measurements of Xanthines and Their Metabolites by Means of High Pressure Liquid Chromatography, in Biological/Biomedical Applications of Liquid Chromatography, 1979, p. 507.

14. Peng, G.W., Gadalla, M.A. and Chiou, W.L., High-Performance Liquid Chromatographic Determination of Theophylline in Plasma, Clin. Chem., 24, 357, 1978.

15. Tang-Liu, D.D-S. and Riegelman, S., An Automated HPLC Assay for Simultaneous Quantitation of Methylated Xanthines and Uric Acids in Urine, J. Chromatogr. Sci., 20, 155, 1982.