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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THEOPHYLLINE AND ITS MAJOR METABOLITES IN HUMAN URINE

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SUMMARY

A highly selective automated high-performance liquid chromatographic (HPLC) method has been developed for the assay of theophylline and its major metabolites in urine. The method involves direct injection of urine on to a reversed-phase column, followed by gradient elution and ultraviolet detection. Quantitation is achieved by the peak-height ratio method with reference to an internal standard, 8-chlorotheophylline. The assay is accurate and reproducible, with a sensitivity of 1 μg/ml in urine. In order to confirm its accuracy, theophylline and its major metabolites in urine were quantified after HPLC separation by both peak-height ratio of ultraviolet absorbance and liquid scintillation spectrometry after oral administration of [^{14}C]theophylline to a human volunteer. The assay was also applied to the analysis of theophylline and its major metabolites in urine following the oral administration of theophylline to three healthy volunteers.

INTRODUCTION

Theophylline (1,3-dimethylxanthine; 1,3-DMX) is a naturally occurring xanthine alkaloid found in the leaves of the plant *Thea sinensis* from which the beverage tea is prepared. In 1937, theophylline, given as aminophylline (theophylline ethylenediamine), was found to be effective in relieving bronchial asthma and rapidly became the drug of choice for the treatment of this condition. Today the main therapeutic use of theophylline is as a bronchodilator in the treatment of asthma and other obstructive airways diseases. The compound, together with the structurally related methylxanthines caffeine (1,3,7-trimethylxanthine; 1,3,7-TMX), present in coffee, and theobromine (3,7-dimethylxanthine; 3,7-DMX), found in cocoa, is also ingested in pharmacologically effective doses in the diet.

In man, theophylline is extensively metabolised by oxidation at C-8 (yielding uric acid derivatives) and/or N-demethylation yielding 1,3-dimethyluric acid (1,3-DMU; 32–55% of dose), 3-methylxanthine (3-MX; 9–36%) and 1-methyluric acid (1-MU; 14–26%) [1–7]. The formation of 3-MX has been reported to be

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saturable within the therapeutic range [3]. These metabolites, together with 8–17% unchanged theophylline, are excreted in the urine, with some 90% of the dose being excreted in 48 h. In addition, 7-N-methylation of theophylline, giving rise to caffeine, occurs in neonates [8–11].

There is great inter-individual variation in both the pharmacokinetic profile of and clinical response to theophylline in human populations [12–14]. The pharmacokinetic variation arises from inter-individual differences in metabolism and it is thus important to establish which factors are responsible for the variations observed. For such studies accurate, sensitive and reproducible assay methods for theophylline and its metabolites are vital.

In recent years a great number of assay methods have been developed for the determination of theophylline plasma levels, with most standard procedures involving high-performance liquid chromatographic (HPLC) techniques [7,13,15–20]. However, there are far fewer methods reported for the assay of theophylline metabolites, and no concensus as to appropriate methodology has yet emerged (see below). This paper describes an HPLC method for the determination of theophylline and its metabolites in urine which has advantages over previous methods in terms of speed, ease and sensitivity. The application of this assay to the study of the pharmacokinetics of theophylline and its metabolites following oral administration is described.

EXPERIMENTAL

Compounds

[8-^{14}C]Theophylline (specific activity 38 mCi/mmol, radiochemical purity > 99% by HPLC) was purchased from Amersham International (Aylesbury, U.K.).

Sources of xanthine and uric acid derivatives were as follows: theophylline (1,3-dimethylxanthine, 1,3-DMX), 8-chlorotheophylline (internal standard), 3-methylxanthine (3-MX) and 1,3-dimethyluric acid (1,3-DMU) from Sigma (Poole, U.K.); 1,7-dimethylxanthine (1,7-DMX), 3,7-dimethylxanthine (3,7-DMX), 1-methylxanthine (1-MX), 7-methylxanthine (7-MX), 1-methyluric acid (1-MU), 3-methyluric acid (3-MU), 7-methyluric acid (7-MU), 1,7-dimethyluric acid (1,7-DMU), 3,7-dimethyluric acid (3,7-DMU) and 1,3,7-trimethyluric acid (1,3,7-TMU) from Fluka (via Fluorochem, Glossop, U.K.).

Other materials were purchased: sodium acetate trihydrate (Analar grade) and Scintran Cocktail T from BDH (Poole, U.K.); HPLC-grade acetonitrile from Rathburn (Walkerburn, U.K.); creatinine reagent kit (No. 555) from Sigma.

Theophylline tablets (125 mg; Nuelin tablets, Batch No. 3EO1A; Riker Labs., Loughborough, U.K.) were supplied by the Pharmacy of St. Mary's Hospital.

Methylxanthine-free urine was obtained from a healthy male volunteer who had abstained from methylxanthine-containing foods and beverages for four days.

High-performance liquid chromatography

The HPLC system consisted of two M6000A solvent delivery systems, an M720 system controller, a WISP 710B autoinjector and an M440 UV detector equipped

with a 280-nm filter (all from Waters Assoc., Harrow, U.K.). The stainless-steel analytical column (250×5 mm I.D.) was packed with 5-μm Hypersil-ODS (C_{18} reversed-phase; Shandon Southern Products, Runcorn, U.K.). Mobile phase A consisted of 1.28 g/l sodium acetate trihydrate containing 0.4% (v/v) glacial acetic acid, pH 4, and mobile phase B consisted of 1.28 g/l sodium acetate trihydrate containing 20% (w/v) acetonitrile and 0.5% (v/v) glacial acetic acid. Both solutions were filtered and degassed with a Millipore HA (0.45 μm) solvent filtering system prior to use.

Compounds were eluted from the column using a linear gradient from 0 to 80% solvent B over 22 min with a hold of 5 min at the final conditions. The flow-rate was 1 ml/min throughout, and a 10-min equilibration period was allowed between each injection.

Assay method

Urine was diluted to a creatinine concentration of 20 mg/dl, and duplicate 1.5-ml aliquots were spiked with 12.5 μg 8-chlorotheophylline as internal standard (250 μl of a 50 μg/ml solution in diluted methylxanthine-free urine). After centrifugation at 1500 *g* for 10 min, the supernatant was placed in a WISP vial, and 6–20 μl were injected on to the HPLC column via the WISP autoinjector.

The method was established using methylxanthine-free urine spiked with theophylline and its metabolites in the range 0–20 μg/ml, treated as above. Quantitation was achieved by measuring the ratio of the heights of the peaks of the compound of interest and that of the internal standard, and relating this to previously constructed standard curves. Calibration curves were established freshly each time the assay was used.

Human volunteer studies

[^{14}C] Theophylline study. A single healthy male volunteer, age 37 years, weight 95 kg, who gave his informed consent, participated in the investigation, which was approved by the Ethical Committee of St. Mary's Hospital and Medical School. He abstained from all foods and beverages containing methylxanthines (tea, coffee, cocoa, chocolate and cola drinks) for seven days prior to and during the study. Control urine (0–24 h) was collected on the day prior to the study. At 9.00 a.m. on the study day the subject took a solution of theophylline (200 mg containing 30 μCi in 200 ml water) orally after an overnight fast. Food was withheld for a further 2 h. Serial urine samples were collected up to 52 h after dosing, their volumes and pH recorded, and aliquots stored at −20°C prior to analysis.

Conventional theophylline tablet study. Three healthy volunteers, two males and one female, aged 25, 27 and 25 years, weight 65, 70 and 55 kg, respectively, participated in the study. After four days abstention from all dietary methylxanthines, the three volunteers each received three 125-mg tablets of theophylline orally with 100 ml water after an overnight fast. Food was withheld for a further 2 h and serial urine samples were collected, their volumes and pH recorded, and aliquots frozen at −20°C until analysis.

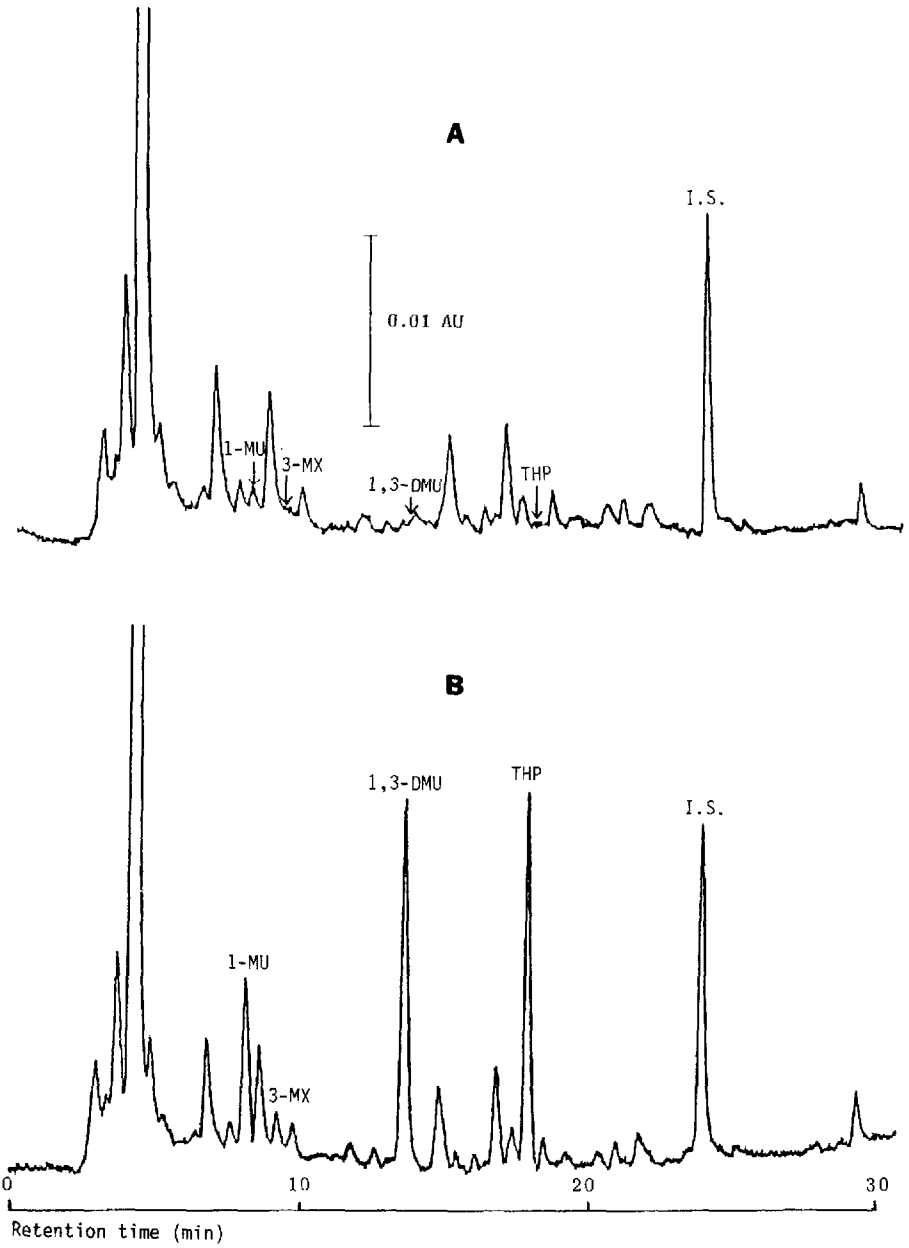

Fig. 1. HPLC traces from the analysis of theophylline and its major metabolites in urine. (A) Urine of a volunteer after seven days abstention from all dietary methylxanthines, spiked with 12.5 μg/ml 8-chlorotheophylline as internal standard (I.S.). (B) Urine of the same volunteer collected 0-2 h following a 200-mg oral dose of theophylline, spiked with 12.5 μg/ml 8-chlorotheophylline (I.S.). Peaks: 1-MU = 1-methyluric acid; 3-MX = 3-methylxanthine; 1,3-DMU = 1,3-dimethyluric acid; THP = theophylline. Detector sensitivity, 0.05 a.u.f.s., other details as in text.

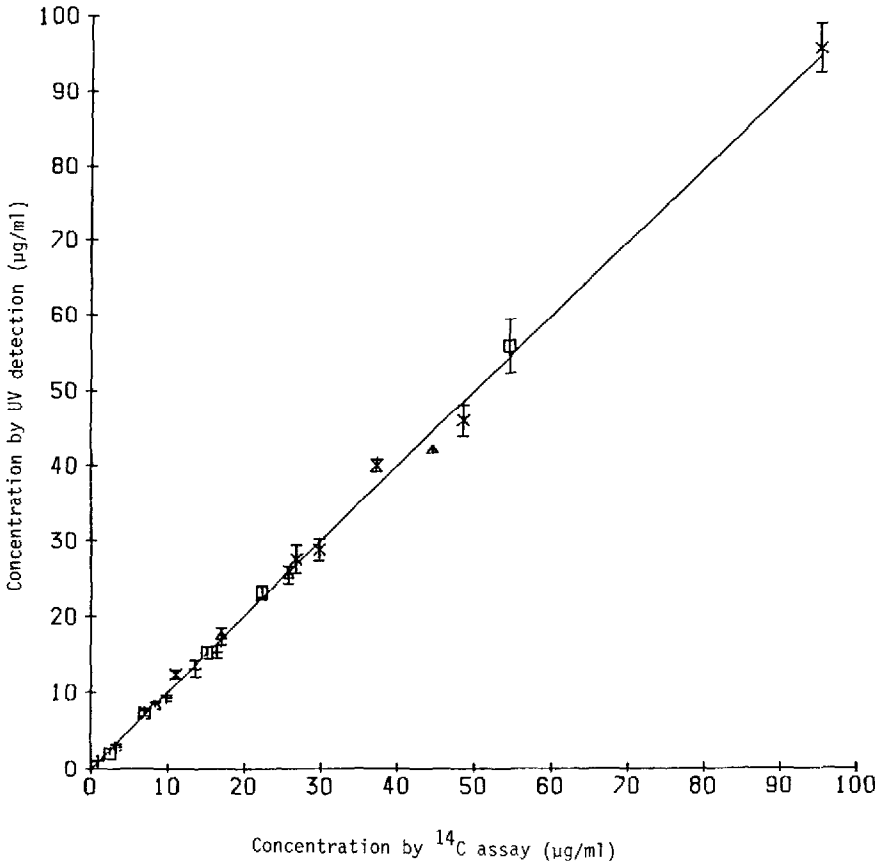

Fig. 2. Correlation curve for the analysis of theophylline and its major metabolites by HPLC using (ordinate) UV detection at 280 nm and (abscissa) radiochemical detection in the urine of a volunteer who took 200 mg [8-^{14}C]theophylline orally in solution. Details of the experiment are provided in the text. For each compound, only a single line representing the mean is shown for the sake of clarity. Parameters for the individual compounds are as follows:

Compound	r	Slope	Intercept
Theophylline (+)	0.986	0.904	0.587
1,3-Dimethyluric acid (×)	0.997	0.996	0.576
3-Methylxanthine (△)	0.995	0.963	−0.231
1-Methyluric acid (□)	0.997	1.027	−0.217

Intra-assay variation

Blank urine was spiked with theophylline and its major metabolites at concentrations of 1 and 10 µg/ml. Six samples were prepared at each concentration, and each sample was injected on to the HPLC column twice. The coefficient of variation (%) of the peak-height ratio was determined as the standard error/mean.

Inter-assay variation

Calibration curves. Fresh calibration curves covering the concentration range 0–20 µg/ml were constructed on six separate occasions, and day-to-day variation in the slope of the peak-height ratio versus concentration line was determined.

TABLE I

QUANTITATION OF THEOPHYLLINE AND ITS METABOLITES IN 0–48 h URINE OF THREE VOLUNTEERS GIVEN 375 mg THEOPHYLLINE ORALLY

Subjects 1 and 2 were males aged 25 and 27 years respectively. Subject 3 was female aged 25 years.

Subject	Percentage of dose administered				
	1-MU	3-MX	1,3-DMU	Theophylline	Total
1	18.1	11.8	45.5	13.4	88.8
2	17.4	11.3	43.5	11.5	83.7
3	14.7	8.2	42.1	14.9	79.9
Mean ± S.D.	16.7 ± 1.8	10.4 ± 1.9	43.7 ± 1.7	13.3 ± 1.7	84.1 ± 4.5

Standards. On six separate occasions methylxanthine-free urine was spiked with theophylline and its major metabolites at 1 and 10 μg/ml and assayed as described. Inter-assay coefficients of variation were determined.

Correlation between UV peak-height ratio method and radiochemical quantitation of theophylline and its major metabolites

Urine samples obtained from a volunteer who had received [^{14}C]theophylline orally were injected on to the HPLC column and assayed for theophylline and its metabolites using UV detection by the peak-height ratio method with reference to calibration curves. In addition, successive 0.3-ml fractions of eluent were collected, Scintran Cocktail T added, and radioactivity determined by liquid scintillation counting using a Packard TriCarb 4640 scintillation spectrometer. The correlation coefficient between the two techniques was determined.

RESULTS

HPLC separation

The system affords baseline separation of a number of methylxanthine and methyluric acid standards and the internal standard, 8-chlorotheophylline. The retention times (min) of these are: 3-MU, 5.2; 7-MU, 6.9; 1-MU, 8.2; 7-MX, 8.2; 3-MX, 9.2; 1-MX, 10.5; 1,3-DMU, 13.4; 3,7-DMX, 15.9; 1,7-DMU, 16.8; 1,3-DMX, 17.6; 1,7-DMX, 17.6; 1,3,7-TMU, 18.7; 1,3,7-TMX, 22.5; 8-chlorotheophylline, 23.6. Fig. 1A shows the chromatogram of a volunteer's urine (diluted to 20 mg/dl creatinine) after seven days abstention from dietary methylxanthines. No interfering peaks are present other than very small amounts of a compound which co-chromatographs with 1-MU. Fig. 1B shows the same volunteer's urine collected between 0 and 2 h after a 200-mg dose of theophylline.

Calibration curves

All calibration curves of peak-height ratio of the compound of interest to internal standard versus concentration for theophylline and its major metabolites

in human urine are linear in the range 0–20 μg/ml (correlation coefficients > 0.990 in each case).

Limit of detection

The absolute limit of detection (signal-to-noise ratio of 2) of theophylline and its metabolites by this assay is 0.5 μg/ml, but values below 1 μg/ml are not reported.

Reproducibility

Intra-assay variation. The intra-assay coefficients of variation for theophylline and its metabolites at a concentration of 1 μg/ml is less than 7.5% and is 1.6% at 10 μg/ml ($n=12$ in both cases).

Inter-assay variation. The inter-assay variation in the slope of the peak-height ratio versus concentration graph is up to 20%. It is therefore necessary to construct a new calibration curve on each occasion. Using freshly prepared calibration curves, the inter-assay variation is less than 9.7 and 5.3% at 1 and 10 μg/ml, respectively ($n=6$ for each compound).

Correlation between UV peak-height ratio method and radiochemical method of quantitation of theophylline and its major metabolites. Fig. 2 shows the correlation curve obtained when the above two methods for the quantitation of theophylline and its metabolites were compared. Concentrations by HPLC assay are plotted against values obtained by liquid scintillation spectrometry. The mean ($\pm$S.D.) correlation coefficient is 0.994 ± 0.005 with a slope of 0.973 ± 0.053 ($n=10$ for each compound).

Application of the assay to theophylline metabolism studies in man

Table I presents details of the excretion of theophylline and its metabolites, assayed by HPLC as described in this paper. In the 0–48 h urine of volunteers given 375 mg theophylline, the total recovery of theophylline and metabolites was $84.1 \pm 4.5\%$ of the dose, comprising $16.7 \pm 1.8\%$ 1-MU, $10.4 \pm 1.9\%$ 3-MX, $43.7 \pm 1.7\%$ 1,3-DMU and $13.3 \pm 1.7\%$ theophylline (mean $\pm$ S.D., $n=3$).

DISCUSSION

The HPLC assay method described above allows the accurate and reproducible separation and quantitation of theophylline and its metabolites in urine. Its main advantage over other methods in the literature is that it does not involve extraction, which is time-consuming and can result in differential losses of the compounds of interest. Because of the wide range of polarities of the various methylxanthines and uric acids, it is difficult to obtain satisfactory recoveries of all the required compounds by the same method, as conditions which allow good extraction of one compound will not necessarily afford acceptable recoveries of another.

Various HPLC assay methods have been developed for the determination of theophylline and its metabolites in urine [7,13,15,18,20,21]. These all suffer from one or more of the following problems: they have not proven reproducible in our

laboratory, are time-consuming or are not specific or sensitive enough for our purposes. Most of the reported methods involve an extraction procedure whereby theophylline is easily recovered but the polar metabolites are not, due to their poor solubility in most organic solvents. The method of Thompson et al. [13] is time-consuming as methylxanthine and methyluric acid fractions must be separated before injection onto the HPLC column. Similarly, Grygiel et al. [16] described an assay which requires two separate HPLC runs for theophylline and its metabolites. Furthermore, Muir et al. [21] reported that this method was unsatisfactory due to co-elution and carry-over from compounds with long retention times. Aldridge et al. [22] extracted metabolites from urine into chloroform–isopropanol (85:15, v/v) prior to injection on to the HPLC column, and although they reported elimination of interfering peaks, the extraction efficiency of methyluric acids was low, especially that of 1-MU, which was only 36%. The method described by St Pierre et al. [20] necessitated a preliminary anion-exchange extraction. Muir et al. [21] developed an assay which appeared to be more sensitive and specific than any previously described, but it involves an ion-pair liquid–liquid extraction followed by ion-pair gradient-elution HPLC, and is therefore both lengthy and complicated. Experimental conditions including pH, ionic strength and temperature affect ionic equilibria and therefore these parameters must be strictly controlled if reproducible results are to be obtained. Rapid degeneration of column packing due to the ion-pairing agent is also a problem.

This brief survey shows that the literature contains no description of an HPLC assay for theophylline and its metabolites in urine which would be suitable for routine pharmacokinetic studies intended to explore certain of the origins of human inter-individual variations in theophylline metabolism. We have found that our assay is simpler and quicker than others reported to date, whilst remaining highly sensitive and specific. Although urine is injected directly on to the HPLC column, the life of a column is not unacceptably short as small injection volumes (usually 6 μl) are used. Additionally, the use of a protective precolumn increases column life considerably (see also Muir et al. [17]).

Although the run-time for one sample is 38 min (including equilibrium time) by this method, each run generates data for theophylline and three other compounds. The present assay method is also suitable for the measurement of theophylline and its metabolites in other body fluids, providing proteins are first precipitated using 10% (w/v) trichloroacetic acid.

There occurred inter-day variation in the slope of the graph of peak-height ratio versus concentration of up to 20%, which probably arises from minor variations in pH, temperature or composition of the mobile phase. As a result, it is essential to establish new calibration curves each time the assay is used, reducing the inter-day variations to the levels quoted above.

280 nm was chosen as the wavelength of detection, as a compromise between absorbances of the methylxanthines (λ_{max} ca. 270 nm) and the methyluric acids (λ_{max} ca. 290 nm). Although both classes of compound absorb well at 254 nm, so too do a greater number of interfering compounds.

Methylxanthine-free diets were imposed on volunteers taking part in the study in order to prevent analytical interference from dietary theophylline, the caffeine

metabolite paraxanthine which co-chromatographs with theophylline, or those metabolites common to both caffeine and theophylline (1-MX and especially 1,3-DMU and 1-MU) which would confuse the results if derived from dietary caffeine. Although subjects abstained from methylxanthine-containing foods and beverages for at least four days prior to the experiment, blank urine sometimes contained small residual peaks (up to 2 μg/ml equivalent) at retention times corresponding to 1-MU and 3-MX. These may have been due to unintentional consumption of methylxanthines, as theophylline and caffeine are frequently used as food additives at levels up to 2.8% by weight, in a variety of foods. However, as the diet was strictly controlled, they more likely arise from other endogenous compounds which co-elute with theophylline metabolites. The sensitivity of the assay is limited by these background levels.

The HPLC system described is also capable of resolving a number of other related xanthine and uric acid compounds and thus may have widespread applicability in metabolic studies involving the related methylxanthines, caffeine and theobromine, and certain other analogues which are used therapeutically. Although by this method 1-MU and 7-MX are not resolved and theophylline and paraxanthine are not resolved, the former pair can be resolved by slight alterations in the gradient system. The resolution of theophylline and paraxanthine may be achieved by the inclusion of an ion-pairing agent such as tetrabutylammonium hydroxide in the solvent mixture, which increases the retention time of theophylline relative to paraxanthine [17,18].

The applicability of our assay to pharmacokinetic studies of theophylline is described. When given orally as theophylline, the 48-h urinary profile of theophylline and its major metabolites was similar to results previously reported [1,3,4,6,7,23]. The analytical method is currently being applied to further studies of theophylline metabolism and disposition in animals and man.

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REFERENCES

1 H.H. Cornish and A.A. Christman, J. Biol. Chem., 228 (1957) 315.
2 J.W. Jenne, H.T. Nagasawa and R.D. Thompson, Clin. Pharmacol. Ther., 19 (1976) 375.
3 T.J. Monks, J. Caldwell and R.L. Smith, Clin. Pharmacol. Ther., 26 (1979) 513.
4 J.J. Grygiel and D.J. Birkett, Clin. Pharmacol. Ther., 28 (1980) 456.
5 D.D.-S. Tang-Liu and S. Riegelman, Res. Commun. Chem. Pathol. Pharmacol., 34 (1981) 371.
6 M.J. Arnaud and C. Welsch, in N. Rietbrock, B.G. Woodcock and A.H. Staib (Editors), Theophylline and Other Methylxanthines, Vol. 3, Vieweg, Braunschweig, 1982, p. 135.
7 U. Gundert-Remy, R. Hildebrandt, N. Hengen and E. Weber, Eur. J. Clin. Pharmacol., 24 (1983) 71.
8 C. Bory, P. Baltassat, M. Porthault, M. Bethenod, A. Frederich and J.V. Aranda, Lancet, i (1978) 1204.
9 J.V. Aranda, A.T. Louridas, B.B. Vitullo, P. Thom, A. Aldridge and R. Haber, Science, 206 (1979) 1319.

10 M.J. Boutroy, P. Bert, P. Monin, R.J. Royer and M.J. Royer-Morrot, Lancet, i (1979) 820.
11 K.-Y. Tserng, K.C. King and F.N. Takieddin, Clin. Pharmacol. Ther., 29 (1981) 594.
12 J.W. Jenne, E. Wyze, F.S. Rood and F.M. MacDonald, Clin. Pharmacol. Ther., 13 (1972) 349.
13 R.D. Thompson, H.T. Nagasawa and J.W. Jenne, J. Lab. Clin. Med., 84 (1974) 584.
14 L. Hendeles, M. Weinberger and G. Johnson, Clin. Pharmacokin., 3 (1978) 294.
15 R.K. Desiraju and E.T. Sugita, J. Chromatogr. Sci., 15 (1977) 563.
16 J.J. Grygiel, B.S. Lindon, M.H. Wing, B.S. Farkas and D.J. Birkett, Clin. Pharmacol. Ther., 26 (1979) 660.
17 K.T. Muir, M. Kunitani and S. Riegelman, J. Chromatogr., 231 (1981) 73.
18 D.D.-S. Tang-Liu and S. Riegelman, J. Chromatogr. Sci., 20 (1982) 155.
19 R.L. Williams, R.A. Upton and A. Bostrom, J. Asthma, 20 (1983) 27.
20 M.V. St-Pierre, A. Tesoro, M. Spino and S.M. MacLeod, J. Liq. Chromatogr., 7 (1984) 1593.
21 K.T. Muir, J.H.G. Jonkman, D.-S. Tang, M. Kunitani and S. Riegelman, J. Chromatogr., 221 (1980) 85.
22 A. Aldridge, J.V. Aranda and A.H. Niems, Clin. Pharmacol. Ther., 25 (1979) 447.
23 D.D.-S. Tang-Liu, R.L. Williams and S. Riegelman, Clin. Pharmacol. Ther., 31 (1982) 358.