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DETERMINATION OF THE URINARY METABOLITES OF CAFFEINE AND THEOPHYLLINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A COMPARATIVE STUDY OF A DIRECT INJECTION AND AN ION-PAIR EXTRACTION PROCEDURE

N R SCOTT

Biochemistry Department, Bromley Hospital, Bromley, Kent (U K)

and

J CHAKRABORTY* and V MARKS

Clinical Biochemistry Division, Biochemistry Department, University of Surrey, Guildford, Surrey (U K)

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SUMMARY

Chromatographic separation of methylxanthine metabolites was achieved using a Hypersil octadecylsilane column with a simple concave gradient elution programme of 0—12 75% acetonitrile in 1% tetrahydrofuran, pH 4 8, and the eluted components were detected by monitoring their absorption at 280 nm An extraction procedure involving the formation of an ion-pair complex was developed which gave significant improvements over previously described methods including a shorter chromatographic run of 20 min A thorough comparison of this procedure with a more convenient alternative involving direct injection of diluted urine specimens showed that the latter analysis was adequate for the quantitation of the major urinary metabolites of caffeine and theophylline

INTRODUCTION

Theophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) are potent bronchodilators and widely used in the treatment of asthma and neonatal apnoea However, large amounts of these xanthines may also be

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absorbed by the consumption of tea, coffee, cocoa, chocolate, cola-flavoured drinks and caffeine-containing analgesics and antacids [1]

Despite their widespread usage, the metabolism of these compounds has until recently received little attention. In 1952, Brodie et al [2] established that the major urinary metabolite of theophylline in man was 1,3-dimethyluric acid. This was later confirmed by Cornish and Christman [3] who reported that the main excretory products after theophylline ingestion were 1,3-dimethyluric acid, 1-methyluric acid, 3-methylxanthine and unchanged theophylline (10%). They also investigated the metabolism of caffeine and theobromine and found that for caffeine, 1-methylxanthine and 1-methyluric acid were the major urinary metabolites. To a lesser degree 1,7-dimethylxanthine, 7-methylxanthine, 1,3-dimethyluric acid and unchanged caffeine were also excreted Following the ingestion of theobromine, the major urinary metabolites were reported to be 7-methylxanthine, unchanged theobromine and 7-methyluric acid However, the separation procedures used in these early studies were very cumbersome involving anion-exchange and paper chromatography.

In recent years, high-performance liquid chromatography (HPLC) has improved the analysis of xanthine metabolites. In 1974, Thompson et al. [4] separated theophylline metabolites using an Aminex A-5 cation-exchange resin but the procedure required an initial pre-fractionation of urine samples using a Dowex 2-X8 anion-exchange resin (Cl^-) Aldridge et al [5] determined caffeine metabolites using a simple gradient elution system on a reversed-phase column following organic extraction of urine samples by means of chloroform—isopropanol (85 15) However, the HPLC procedure used in studies of caffeine metabolism in man [6, 7] failed to resolve 7-methylxanthine from 1-methyluric acid, theophylline from 1,7-dimethylxanthine or 3,7-dimethyluric acid from 1-methylxanthine. In addition, extraction recoveries were considerably low for 1-methyluric acid (36.3%), 7-methyluric acid (55.6%) and 3,7-dimethyluric acid (58 5%). Recently, it has been shown that the recovery of methylxanthines from urine may be significantly improved by the formation of an ion-pair complex prior to extraction [8]

We describe in this paper a gradient elution system which offers improved selectivity and facilitates the separation and quantitation of thirteen metabolites within a 20-min period In order to optimize sample preparation a comparison is made between results obtained from the direct injection of diluted urine samples and an ion-pair extraction procedure similar to that previously described by Tang-Liu and Riegelman [8]

EXPERIMENTAL

Materials

Caffeine, tetrahydrofuran (AR), ethyl acetate (AR), chloroform (AR) and isopropanol (AR) were purchased from BDH (Poole, U K), theophylline, theobromine, proxyphylline (7B-hydroxypropyltheophylline), 1-methyluric acid, 1,7-dimethylxanthine and tetrabutylammonium hydrogen sulphate from Sigma (Poole, U K.); 7-methyluric acid, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, 1,7-dimethyluric acid and 1,3,7-trimethyluric acid from Adams Chemical Co. (Round Lake, IL, U.S.A.)

Acetonitrile, HPLC-grade, was obtained from Rathburn Chemicals (Walkerburn, U.K.).

A stock standard solution (100 mg/l) was prepared by dissolving 10 mg of each xanthine metabolite in approximately 35 ml of 0.08 *M* disodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) adjusting the pH to 7 with 1 *M* phosphoric acid and then diluting to 100 ml with distilled water.

Equipment

A gradient elution HPLC system was used consisting of an Applied Chromatography Systems (ACS) 300/02 dual-channel reciprocating pump and an ACS 750/36 decilinear programmer. Chromatography was performed using a 250 × 4.5 mm I.D. stainless-steel column containing 5-μm Hypersil octadecylsilane (Shandon, London, U.K.). Eluted peaks were detected at 280 nm by an ACS 750/11E UV monitor fitted with a 10-μl flow cell. Samples were injected via a Rheodyne loop injector fitted with a 50-μl loop.

Procedure

Direct injection. Centrifuged urine samples and the stock standard were diluted 1:10 with an aqueous solution of the internal standard, proxyphylline (10 mg/l). An aliquot (50 μl) of the diluted sample was injected directly into the column without further preparation.

Ion-pair extraction. Centrifuged urine (200 μl), 50 μl of internal standard solution (200 mg/l proxyphylline), 200 μl of 0.1 *M* tetrabutylammonium hydrogen sulphate and 100 μl of pH 11 buffer solution (0.1 *M* sodium carbonate–0.1 *M* sodium bicarbonate, 90:10) were vortex-mixed for 30 s in a stoppered tube, before and after the addition of approx. 0.5 g of ammonium sulphate. The mixture was then extracted by vortex-mixing with 5 ml of a solution containing ethylacetate–chloroform–isopropanol (45:45:10) for at least 1 min. After centrifugation for 5 min at 2000 rpm (1500 *g*), a 4-ml aliquot of the organic layer was transferred to a clean tube and evaporated to dryness at 45°C under a stream of air. The residue was dissolved in 500 μl of 1% tetrahydrofuran, pH 4.8, 50 μl of which were injected into the column.

Caffeine metabolites were separated using a gradient elution system, the eluent for pump A being 1% tetrahydrofuran in 10 m*M* acetate buffer, pH 4.8 and for pump B, 15% acetonitrile and 1% tetrahydrofuran in 10 m*M* acetate buffer, pH 4.8. Solvent A was pumped for 5 min before introducing a stepwise gradient of 5, 10 and 2% increase in B per min, each step being pumped for 5 min. A flow-rate of 1.5 ml/min was maintained throughout.

For quantitation, the peak-height ratios of eluted metabolites to the internal standard were compared with those obtained from the injection of corresponding standard mixtures processed in a similar manner.

Urine samples were obtained from inpatients receiving theophylline therapy and normal volunteers receiving a controlled dietary intake (300, 450 or 600 mg) of caffeine in the form of pre-weighed aliquots of instant coffee. Following a 24-h period of equilibration, the volunteers collected two consecutive 24-h urine samples for determination of xanthine metabolites. For comparison, xanthine metabolites were also measured in random samples of urine obtained from a group of Mormon volunteers on a strict caffeine-free diet. All

participants were instructed to exclude other dietary sources of theobromine, theophylline or caffeine throughout the study

RESULTS

The resolution of a standard mixture (50 mg/l) of xanthine metabolites achieved by the described chromatographic procedure is shown in Fig. 1. The retention times ranged from 3.6 min for uric acid to 18.0 min for the internal standard proxyphylline (Table I)

The recovery and linearity of the extraction procedure were investigated by assaying a series of aqueous standard solutions containing methylxanthines over a concentration range of 0– 200 mg/l (Table II). The recovery from urine was determined by measurement of methylxanthine levels in a sample before and after the addition of a known amount of methylxanthine standard (50 mg/l) A linear plot of ratios of concentrations against the ratios of peak heights was obtained for each metabolite relative to the internal standard (mean coefficient of correlation = 0.998) The calculated recovery values in aqueous and urine samples were consistent with the exception of 1,7-dimethylxanthine which

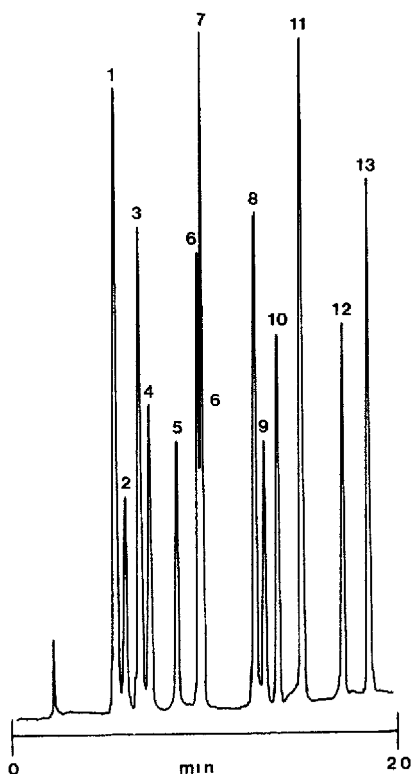


Fig 1 Chromatogram of a methylxanthine standard solution (50 mg/l) Chromatographic conditions as described in text, 0 2 a u f s Peaks 1 = 7-methyluric acid, 2 = 7-methylxanthine, 3 = 1-methyluric acid, 4 = 3-methylxanthine, 5 = 1-methylxanthine, 6 = 1,3-dimethyluric acid, 7 = theobromine, 8 = 1,7-dimethyluric acid, 9 = 1,7-dimethylxanthine, 10 = theophylline, 11 = 1,3,7-trimethyluric acid, 12 = caffeine, 13 = proxyphylline (internal standard)

TABLE I

CHROMATOGRAPHY RETENTION DATA OF METHYLXANTHINE METABOLITES

Compound	Retention time (min)	Capacity factor (*k*)
Uric acid	3 6	0 6
7-Methyluric acid (7-MU)	5 3	1 4
7-Methylxanthine (7-MX)	6 0	1 8
1-Methyluric acid (1-MU)	6 8	2 1
3-Methylxanthine (3-MX)	7 2	2 3
1-Methylxanthine (1-MX)	8 6	2 9
1,3-Dimethyluric acid (1,3-MU)	9 5	3 3
Theobromine (Theobr)	9 7	3 4
1,7-Dimethyluric acid (1,7-DMU)	12 0	4 5
1,7-Dimethylxanthine (1,7-DMX)	12 7	4 8
Theophylline (Theoph)	13 5	5 1
1,3,7-Trimethyluric acid (1,3,7-TMU)	14 6	5 6
Caffeine	16 7	6 6
Proxyphylline (internal standard)	18 0	7 2

TABLE II

METHYLXANTHINE RECOVERIES AND WITHIN BATCH PRECISION OF ANALYSIS

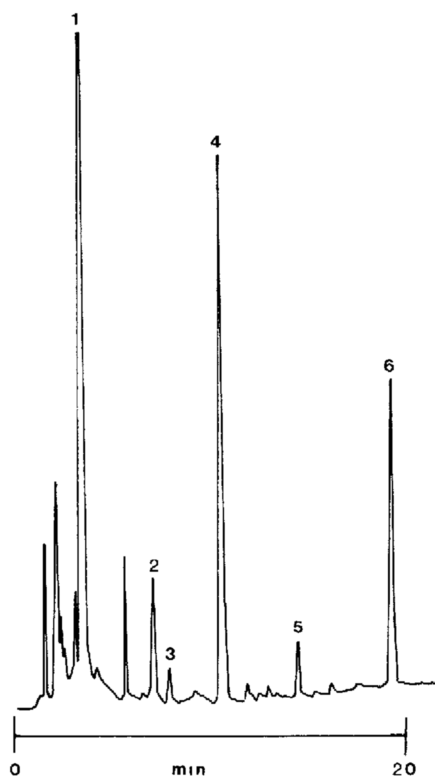
Urine A 24 h sample obtained from a volunteer after a caffeine intake of 600 mg per 24 h Urines B and C separate urine specimens collected from Mormon subjects and spiked with methylxanthines to give in each case a final concen tration of 27 78 mg/g of creatinine Results are given as mean of four determinations $\bar{x}$ = Mean concentration found in urine (mg/g of creatinine), C V = coefficient of variation (%)

Compound*	Direct injection				Ion pair extraction					
	Urine A		Urine B		Urine A		Urine C		Recovery (mean ± S D) (%)	
	$\bar{x}$	C V	$\bar{x}$	C V	$\bar{x}$	C V	$\bar{x}$	C V	Aqueous	Urinary
7 MU	7 84	22 5	29 2	16 8	4 59	19 6	24 04	6 33	71 5 ± 2 43	74 5 ± 4 9
7 MX	19 55	10 5	35 18	7 1	17 65	6 2	29 34	4 25	73 8 ± 2 3	81 3 ± 6 0
1-MU	99 92	0 84	25 42	8 0	80 94	3 6	26 99	1 90	64 7 ± 8 4	65 9 ± 8 8
3 MX	10 23	9 7	30 78	6 1	12 1	10 3	29 75	3 12	84 2 + 2 7	89 6 ± 4 4
1 MX	56 47	7 9	28 15	4 4	54 7	2 1	33 31	3 71	93 4 ± 8 4	85 4 ± 15 1
1 3 DMU	8 3	5 2	30 55	8 9	7 51	24 2	26 01	7 41	81 4 ± 6 8	73 7 ± 1 3
Theobromine	8 96	8 2	30 73	8 3	4 37	21 2	31 85	5 37	95 1 ± 2 7	96 3 ± 2 9
1,7 DMU	36 31	1 27	29 35	7 4	36 74	8 3	29 06	4 04	85 2 ± 7 5	83 6 ± 3 8
1,7 DMX	19 92	8 14	31 52	9 4	14 74	9 8	29 03	1 86	101 4 ± 5 2	79 3 ± 5 3
Theophylline	4 67	5 57	32 28	5 9	ND**	ND**	31 32	5 19	92 4 ± 8 4	85 4 ± 11 0
1,3,7 TMU	4 38	19 6	30 7	5 5	5 51	15 9	27 34	2 95	85 0 ± 2 7	91 9 ± 4 1
Caffeine	7 25	13 8	31 3	6 4	5 04	16 0	29 18	3 57	97 5 ± 4 2	97 8 ± 5 4

*For meaning abbreviations, see Table I
**ND = Not detected

showed a slightly lower recovery from urine. For the direct-injection procedure, detector linearity was studied and found to be acceptable over the range required (mean coefficient of correlation = 1.0)

The analysis of 24-h urine samples from patients receiving theophylline therapy produced peaks which corresponded to theophylline, uric acid, 1-methyluric acid, 3-methylxanthine and 1,3-dimethyluric acid (Fig 2) Urinary excretion products identified from individuals receiving a controlled intake of caffeine included the compounds named above plus caffeine, 7-methyluric acid, 7-methylxanthine, 1-methylxanthine, theobromine, 1,7-dimethyluric acid, 1,7-dimethylxanthine and 1,3,7-trimethyluric acid (Fig 3) A summary of urine



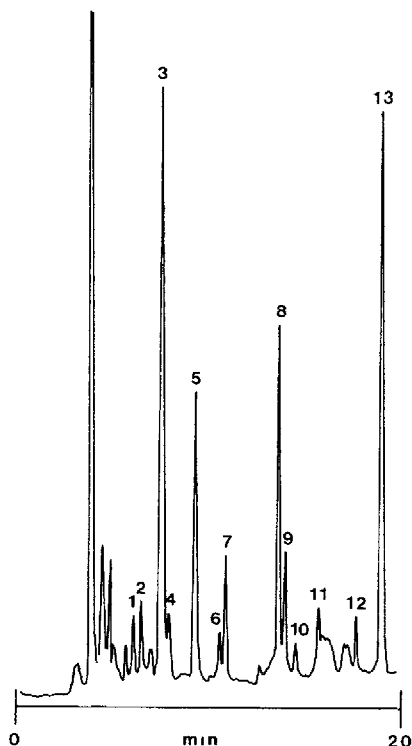


Fig. 2. Chromatogram of a urine sample received from a subject receiving theophylline therapy (direct injection technique). Chromatographic conditions as described in text, 0.1 a.u.f.s. Peaks: 1 = uric acid, 2 = 1-methyluric acid, 3 = 3-methylxanthine, 4 = 1,3-dimethyluric acid, 5 = theophylline, 6 = proxyphylline (internal standard).

Fig. 3. Chromatogram of a urine sample collected 0—24 h following oral ingestion of 500 mg caffeine (ion-pair extraction technique). Chromatographic conditions as described in text, 0.2 a.u.f.s. Peak identification as in Fig. 1.

methylxanthine levels found in this group of normal volunteers and those obtained from the Mormon control group is shown in Table III. Statistical analysis of results obtained for each procedure using a Student t-test and linear regression analysis demonstrates a favourable correlation for the major metabolites, 1-methyluric acid (r = 0.867, $P < 0.1$), 1-methylxanthine (r = 0.930, $P < 0.05$) and 1,7-dimethyluric acid (r = 0.783, $P < 0.1$). However, the direct-injection results show a slight positive bias and for the remaining metabolites, the correlation between the two procedures decreases in proportion to concentration although a significant difference is only observed for unchanged caffeine.

Analysis of the results obtained from the Mormon control group show that for extracted urines the blank levels are acceptable whilst significant values are observed for certain metabolites using the direct-injection procedure. In the case of 1-methylxanthine, 3-methylxanthine and theobromine the mean blank values using direct injection were affected by the detection of an interfering peak on a single occasion.

Within-batch precision of both procedures was studied by replicate analysis

TABLE III

URINARY EXCRETION OF METHYLXANTHINE METABOLITES IN HEALTHY SUBJECTS

For the Mormon group, one random specimen was collected from each subject With the rest of the subjects, two 24-h urine samples were collected and analysed separately and the average value used for evaluation of the mean value for the respective subject group (n = 2, 4 or 5)

Compound*	Concentration found in urine (mean ± S D) (mg/g of creatinine)			
	Caffeine intake per 24 h			
	300 mg (n = 4)	450 mg (n = 5)	600 mg (n = 2)	Caffeine-free diet (Mormon group) (n = 13)
Direct injection				
7-MU	4 73 ± 1 30	4 4 ± 2 09	11 65 ± 1 53	0 66 ± 0 72
7-MX	10 9 ± 1 27	14 64 ± 2 49	18 8 ± 6 0	3 15 ± 3 35
1-MU	51 9 ± 6 85	80 24 ± 27 65	120 6 ± 26 6	2 68 ± 1 89
3-MX	3 13 ± 4 93	13 7 ± 5 04	10 47 ± 5 33	3 61 ± 2 82
1-MX	30 2 ± 7 01	48 66 ± 9 50	62 87 ± 4 76	5 16 ± 4 64
1,3-DMU	2 98 ± 3 61	7 14 ± 1 49	9 1 ± 2 31	3 06 ± 2 36
Theobromine	2 13 ± 2 66	8 96 ± 2 82	13 83 ± 6 48	3 39 ± 5 87
1,7-DMU	27 1 ± 9 12	24 98 ± 6 49	35 2 ± 1 48	0 59 ± 0 75
1,7-DMX	14 63 ± 1 03	16 08 ± 5 22	20 65 ± 1 65	0 22 ± 0 78
Theophylline	NM**	1 66 ± 1 16	4 9 ± 0 51	1 48 ± 1 66
1,3,7-TMU	NM**	3 48 ± 2 91	3 33 ± 0 81	0 47 ± 0 70
Caffeine	17 93 ± 6 45	12 16 ± 7 34	4 75 ± 1 01	0 32 ± 0 80
Ion-pair extraction				
7-MU	5 43 ± 1 29	4 28 ± 1 31	5 1 ± 1 17	0 75 ± 0 92
7-MX	10 21 ± 2 52	17 34 ± 2 61	17 58 ± 1 86	0 17 ± 0 43
1-MU	50 32 ± 14 75	75 14 ± 39 8	98 2 ± 11 3	0 79 ± 0 66
3-MX	7 93 ± 1 56	12 6 ± 2 07	11 48 ± 1 40	0 62 ± 0 72
1-MX	28 68 ± 7 63	47 52 ± 9 49	54 9 ± 5 06	1 08 ± 1 09
1,3-DMU	5 13 ± 2 53	5 82 ± 0 92	6 83 ± 0 74	0 2 ± 0 33
Theobromine	5 35 ± 1 21	6 06 ± 3 90	6 7 ± 1 53	0 37 ± 0 73
1,7-DMU	24 38 ± 7 97	24 76 ± 7 88	30 93 ± 6 67	0 35 ± 0 45
1,7-DMX	15 75 ± 2 08	15 94 ± 1 88	19 95 ± 1 01	0 78 ± 1 58
Theophylline	2 43 ± 1 72	3 16 ± 2 23	2 63 ± 1 18	1 42 ± 1 60
1,3,7-TMU	2 05 ± 0 74	1 92 ± 0 64	2 7 ± 0 48	0 35 ± 0 36
Caffeine	7 4 ± 3 56	5 73 ± 2 36	7 03 ± 1 15	0 30 ± 0 58

*For meaning abbreviations, see Table I
**NM = Present but not measurable

(n = 4) of a 24-h urine sample collected from a volunteer receiving a controlled daily caffeine intake of 600 mg (Table II, urine A) and also from the analysis of Mormon control urines spiked with methylxanthine metabolites equivalent to a concentration of 25 mg/l (Table II, urines B and C) Quantitation of the metabolites (Table II) showed overall mean coefficient of variation (C.V.) values of 8 64% for direct injection and 8 12% for the extraction procedure with corresponding ranges of 4.4% (1-methyluric acid) to 19 7% (7-methyluric acid) and 2 75% (1-methyluric acid) to 15 81% (1,3-dimethyluric acid)

Between-batch analyses gave mean C.V. values of 8 58% for direct injection (ranging from 1.5% for caffeine to 15.5% for 7-methyluric acid) and 8 04% for the extraction procedure (covering C.V of 3% for 1-methylxanthine up to 12 2% for 1,7-dimethylxanthine)

DISCUSSION

Most studies of urinary metabolites [5–7] have utilised the procedure described by Aldridge et al [5] This involves extraction of urine with a chloroform–isopropanol mixture (85.15) before separation of the metabolites on a C_{18} μBondapak column (Waters Assoc) using a concave gradient of 1 5—7 5% acetonitrile in 0.5% acetic acid. This method is, however, incapable of resolving 7-methylxanthine from 1-methyluric acid, theophylline from 1,7-dimethylxanthine or 3,7-dimethyluric acid from 1-methylxanthine in contrast to the procedure described herein which eliminates most of these problems of non-selectivity

In order to optimize sample preparations in terms of selectivity, recovery, precision and preparation time, we have assessed in this study the use of direct injection of diluted urine and a procedure involving the formation and extraction of an ion-pair complex. The extraction procedure described shows excellent selectivity and much improved recovery from previous techniques based on chloroform—isopropanol extraction Variations in recovery are relatively insignificant using the direct-injection technique and, with a much reduced preparation time, it has obvious advantages over extraction techniques. Also, in our experience, we have found the requirement of peak-height correction using the internal standard to be minimum. However, it is apparent that the technique has limitations due to high blank values and the occasional presence of an interfering peak. These problems were not resolved by the use of ultrafiltration or C_{18} Sep-Pak (Waters Assoc) purification prior to injection

The chromatography procedure described achieves separation of the xanthine metabolites within 20 min, and a re-equilibration period of 5 min permits the analysis of approximately eighteen samples in one working day or more if automatic sampling is used Using the extraction procedure a batch of twenty samples may be processed for chromatography in approx 1 h Experience has shown that the resolution of certain metabolites may vary slightly with different batches of Hypersil ODS necessitating a slight adjustment to the pH of the eluting solvents to achieve optimum separation. Whilst the resolution of 1,3-dimethyluric acid and theobromine is relatively poor, these represent only minor metabolites of caffeine metabolism and for theophylline metabolite studies, theobromine interference in the measurement of 1,3-dimethyluric acid levels is negligible

Both procedures have proved robust in use with no problems of late eluting peaks and minimal deterioration of column performance. When required, peak resolution may be rapidly restored by repacking the top few millimetres of the columns and the use of a guard column is considered unnecessary The use of direct injection offers a slight improvement in the within-batch precision when compared with the extraction procedure but for the major metabolites both procedures show excellent reproducibility.

In summary, we feel that both the procedures described in this study have some value in investigating the pharmacokinetics and metabolism of methylxanthines In studies where a precise estimation of metabolite values is required the extraction procedure described is the method of choice However, experience has shown that, providing suitable cut-off values are applied to compensate for the potentially high blank values, the convenience of direct injection of diluted urine is of value where quantitation is only required for the major metabolites Applications include screening for toxicity or detecting alterations in the pattern of metabolite excretion resulting from diseased states such as alcoholic liver disease

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