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Simultaneous analysis of theophylline, caffeine and eight of their metabolic products in human plasma by gradient high-performance liquid chromatography

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SUMMARY

A method has been developed for the simultaneous determination of methylxanthines and many of their metabolites in plasma. Specially developed extraction columns (Celute-MX) were used and the extracts were separated on a 25-cm ODS column (particle size, 3 μm) at 50°C with a mobile phase gradient. The compounds were detected with a diode array detector at two analytical wavelengths. The value of drug to metabolite ratios has been explored and elevated ratios have been found when metabolic clearance was impaired by disease or co-administered drugs. Large concentrations of pharmacologically active metabolites have been found in renal failure. The assay has proved to be reliable and valuable in the elucidation of therapeutic and metabolic problems involving the methylxanthines.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) and its demethylated metabolites paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) are ubiquitous in human plasma due to the almost universal dietary intake of coffee, tea and many soft drinks[1,2]. In addition, theobromine may be present as a consequence of chocolate consumption[2]. The presence of these pharmacologically active dietary methylxanthines may influence patient compliance with theophylline therapy which is frequently used in the treatment of asthma and chronic obstructive pulmonary disease; they may also complicate the evaluation and interpretation of theophylline therapeutic drug monitoring (TDM).

The methylxanthines are extensively metabolised by the hepatic microsomal mixed function oxidase (cytochrome P_{450}) system so that, for example, less than 10% of administered theophylline and less than 2% caffeine are excreted unchanged in the urine[3–6]. Many of these metabolites are pharmacologically active and the importance of considering their contribution to the total biological effect in TDM has been suggested, especially in cases of renal insufficiency or failure[7–9].

The hepatic clearance by the premature new-born of theophylline and caffeine,

administered for the prevention of apnoea and bradycardia, is poor in comparison with children and adults[10]. A significant proportion of administered theophylline is N_7-methylated to caffeine which accumulates in plasma so that it may account for about a third of the total plasma methylxanthines in this group of patients[11].

Other co-administered drugs impair hepatic theophylline clearance and result in toxicity[12–14]; reduced clearance of drugs is a common complication of some forms of liver disease[15–18]. Fasting plasma caffeine concentrations and the determination of caffeine clearance may provide clinically valuable information for assessing the severity of hepatic dysfunction[19–21] because the routine biochemical liver function tests do not directly indicate cytochrome P_{450} status[16,22], which may provide clinically important indications of potentially abnormal drug metabolism.

The interrelationships of administered methylxanthines and their metabolic products that can be expected to occur in plasma are indicated in Fig. 1. This is very similar to the scheme which was proposed by Cornish and Christman[4] on the findings of their urine studies. 1,3-Dimethyluric acid is now recognized as the principal normal metabolite of theophylline in plasma[23] as well as in urine[4], and does not undergo any further metabolic conversion to 1-methyluric acid[24].

The present method had been developed with the aim of establishing the normal disposition of the administered methylxanthines and their metabolites in human plasma. The determination of the relationships between the drug and its metabolites should permit the identification of abnormal metabolism with more precision, and provide an explanation for toxic symptoms not resolved by results from less comprehensive, routine methylxanthine TDM assays.

EXPERIMENTAL

Equipment for high-performance liquid chromatography (HPLC)

A Perkin-Elmer series 2/2 pump unit (Perkin-Elmer, Beaconsfield, U.K.) was run in the gradient mode. The settings were optimized for each column with 250 × 4.5 mm I.D. 3-μm ODS Apex I columns [100 000 to 120 000 plates per meter (Jones Chromatography, Hengoed, CF8 1QA, Wales, U.K.)] were typically: flow-rate, 0.8 ml/min; gradient starting conditions, 0% A/(A + B); gradient rate, 2.1% A/min. The
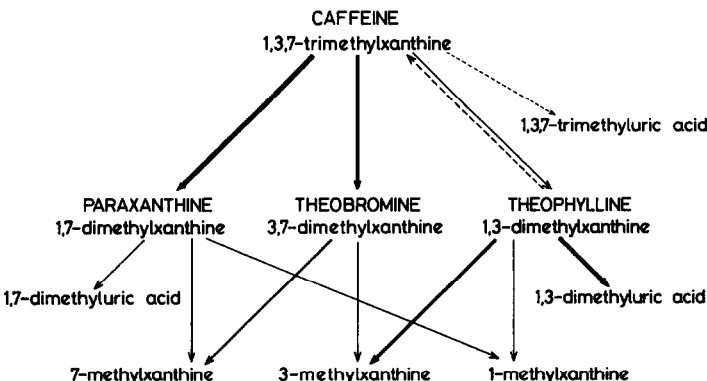

Fig. 1. Interrelationships of the methylxanthines and methyluric acids determined in plasma.

analytical column was maintained at 50°C with an HPLC column block heater (Jones Chromatography). To minimise the isocratic element at the start of the chromatograms the reconstituted extracts were injected 2.5 min after the gradient started. An ISS-100 autosampler (Perkin-Elmer) was used in the slave mode and controlled by the chromatography workstation (see below).

The methylxanthines and methyluric acids were determined with a UV absorbance detector. A Perkin-Elmer LC-75 set at 273 nm (with the absorbance range set at × 0.04) was used throughout the development of the method. Recently it was replaced with an LC-135 diode array UV detector (Perkin-Elmer). This has been used as a dual-wavelength detector so that the methyluric acids could be monitored closer to their λ_{max} (285 nm) without any loss of detector sensitivity for the methylxanthines (λ_{max} 273 nm). Channel 1 was set to 270 nm (band-width 15 nm) and channel 2 set to 285 nm with the same band-width.

The timed events for HPLC (gradient start, reset and autosampler start) were controlled by a Maxima™ chromatography workstation [software version 2.1, Dynamic Solutions Co. Ventura, CA, U.S.A. (purchased from Jones Chromatography] run on an IBM PC-AT (IBM, Basingstoke, U.K.) which was used for storing raw chromatographic data and all subsequent processing. The detector auto-zero was activated by the autosampler 2.4 min after the injection of the sample. Chromatographic data acquisition was started 2 min after sample injection and terminated after the last peak of interest (caffeine) at 25 min, to conserve space on the hard disk. A Hitachi 672-XD plotter (Jones Chromatography) was used to produce the chromatograms from data stored in Maxima™ that illustrate this work.

All the pipetting steps in the sample preparation were performed with a Hamilton Microlab-M equipped with a 250-μl syringe (1725 TLL) (V.A. Howe & Co., London, U.K.) except for reconstitution of the dried extracts, where a 1000-μl syringe (1001 TLL) was used.

All the water that was used to prepare the mobile phase and other aqueous solutions that were injected into the HPLC system was 'polished' before use with a Barnstead Water I apparatus (Gallenkamp, Loughborough, U.K.). This treatment effectively removed contaminants that accumulate in HPLC-grade water during storage. These were responsible for elevated back pressures which compromised the successful prolonged use of 25-cm 3-μm columns.

Reagents

Pure [purum grade >98% (HPLC)] 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1-methyluric acid, 3-methyluric acid, 7-methyluric acid, 1,7-dimethylxanthine, 1,7-dimethyluric acid, 1,3-dimethylxanthine, 1,3-dimethyluric acid, 3,7-dimethylxanthine, 3,7-dimethyluric acid, caffeine and 1,3,7-trimethyluric acid were purchased from Fluka (Fluorochem, Glossop, U.K.). β-Hydroxyethyltheophylline from Sigma (Poole, U.K.). 3-Ethylxanthine was a gift from Napp Laboratories. (Cambridge, U.K.). HPLC-grade acetonitrile, methanol, water, tetrahydrofuran stabilised with butylated hydroxytoluene (BHT), dichloromethane and isopropanol from Rathburn (Walkerburn, U.K.). Sodium acetate (Aristar), acetic acid (Aristar), orthophosphoric acid (Analar) and triethanolamine (Analar) were supplied by BDH (Poole, U.K.).

The specially developed sample preparation columns (Celute-MX tubes) were

supplied by Jones Chromatography and Nucleopore 0.4-μm polyester filter membranes were from Sterilin (Hounslow, U.K.).

Glass test tubes (50 × 6 mm and 75 × 10 mm) and other glassware were purchased from Philip Harris Scientific (London, U.K.). Micro sample vials were supplied by Chromacol (London, U.K.).

Preparation of mobile phases

Stock acetate buffer was prepared by adjusting 20 mM sodium acetate to pH 4.0 with concentrated acetic acid[25]. Before storage, it was vacuum filtered through a 0.4-μm polyester membrane. For use it was diluted 1:1 with water.

The mobile phase for pump A was composed of 25% acetonitrile and 2.0% tetrahydrofuran (THF) in 10 mM acetate buffer, while that for pump B was 0.01% THF in 10 mM acetate buffer (all v/v). This latter solution was also used for the reconstitution of the plasma extracts and in the rinse reservoir of the ISS-100 autosampler. The ready mixed mobile phases were vacuum filtered through a 0.4-μm polyester membrane filter before use and degassed by maintaining under reduced pressure for a few minutes.

Preparation of internal standard solution

The primary internal standard, 3-ethylxanthine (approximately 2.2 mg) and a second reference compound, β-hydroxyethyltheophylline (approximately 3.4 mg) were dissolved with a few drops (<0.5 ml) triethanolamine in <5 ml water before being made up to 100 ml with stock (20 mM, pH 4.0) acetate buffer. Both compounds were designated as reference compounds in the Maxima component list. In addition, β-hydroxyethyltheophylline could be used in post-chromatographic analysis as an alternative internal standard in the event of there being a chromatographic interference with 3-ethylxanthine.

Sample preparation for HPLC analysis

Volumes of 100 μl of plasma or serum were added to 35 μl of internal standard in 50 × 6 mm glass test tubes. After mixing, they were transferred to Celute-MX sample preparation tubes. In each batch of up to 30 samples, the standard calibration plasma was included in quadruplicate. Ten minutes after the last transfer of sample to the Celute tubes the methylxanthines and methyluric acids were eluted with 2 × 1.5 ml isopropanol–dichloromethane (10:90, v/v) into 10 × 75 mm glass tubes. These extracts were evaporated to dryness in a block heater set to <37°C under a gentle stream of oxygen-free nitrogen. When dry, the extracts were reconstituted in 100 μl of mobile phase B, transferred to the injection vials and capped. Before they were loaded into the autosampler they were spun for 5 min in a refrigerated centrifuge (4400 rpm, MSE™ benchtop Chilspin, Fisons plc, Loughborough, U.K.) as a precaution against any small particulate material blocking the top frit of the analytical column. Normally, 20μl of the prepared sample (standard calibration plasma or unknowns) were injected.

Preparation of standard calibration serum

The raw material for the standard calibration serum was pooled human serum. It was spun in a refrigerated centrifuge after which the fatty surface layer was re-

moved and the remaining fluid decanted from the pellet of debris. An aliquot of this pool was set aside for analysis.

Stock high concentration (approximately 100 mg/100 ml) aqueous solutions of all the analytes were prepared. These were used to spike the pooled serum to the intended approximate concentrations and the volume of the spiked serum made up to 1.0 l with more of the pooled blank serum. Since there were detectable concentrations of some of the methylxanthines in the blank, the precise concentrations in the calibration standard were determined as follows.

Of the blank serum, 20 aliquots were prepared for HPLC analysis according to the sample preparation method already described. Since the expected peak areas were small, 30 μl of the reconstituted sample were injected. The mean and standard deviation (S.D.) of the peak areas were determined. A further batch of 20 aliquots of the spiked serum were similarly prepared and analysed except that 20 μl were injected.

Using these data the concentration of the methylxanthines and methyluric acids in the spiked serum were determined.

Example for caffeine. The mean peak area for caffeine in the blank serum (determined from 20 × 30 μl injections) is 2.58 area counts (S.D. = 0.094). The volume of spiked serum injected was 20 μl, therefore, the corrected area for the blank is (2.58·20)/30 = 1.72 area counts.

The total volume of aqueous methylxanthines and methyluric acids added was 50 ml, therefore, the peak area for the blank in spiked serum is (1.72·950)/1000 = 1.63 area counts.

The mean peak area for the spiked serum determined from 20 × 20 μl injections was 11.99 area counts (S.D. = 0.54).

The peak area counts in the spiked serum which are due to the added caffeine = (peak area counts in the spiked serum) minus (volume-corrected peak area counts in the blank) = 11.99 − 1.64 = 10.35 area counts; 10 ml aqueous caffeine were added containing 53.03 μM.

Thus 53.03 μM caffeine gives 10.35 area counts. Therefore the total caffeine in the spiked serum is (added caffeine μM·area counts total caffeine)/area counts added caffeine = (53.03·11.99)/10.35 = 61.43 μM.

RESULTS AND DISCUSSION

Sample preparation

During the development of the sample preparation packing, the performance of batches of the Celute-MX tubes was assessed by determining the recoveries for all analytes together with the internal standards. An aqueous solution of the analytes was prepared from the concentrated stock solutions that were used for the preparation of the standard calibration plasma, and diluted to approximately the same concentration. Plasma was avoided because injection of unextracted plasma samples would severely reduce the life-expectancy of the analytical column. Provided that the conditioned Celite had sufficient buffering capacity to adjust the pH of plasma to the optimal value for the extraction, this test gave a valuable indication of how well it would perform with plasma samples.

Internal standard solution (35 μl) was added to each of fifteen 100-μl aliquots of aqueous standard solution. Five of these were transferred directly to injection vials

(the non-extracted standards), capped and were subsequently used to calibrate the analytical method. The remaining ten aliquots were carried through the sample preparation procedure described in the method, but the residues were reconstituted to 135 μl rather than 100 μl so that the dilution was the same as that of the non-extracted calibration standards. Of each of the fifteen samples 25 μl were injected into the HPLC system.

The recoveries of all the compounds including the two internal standards were assessed by external standard analysis calibrated to the mean of the five non-extracted aqueous injections. Internal standard analysis of the same data was also performed, and the results of a typical Celute-MX batch test are shown in Table I. Comparison of the recoveries assessed by internal and external standard analysis provided an indication of the magnitude of the liquid handling losses. The internal standard analysis results of > 100% indicated that the recovery of the internal standard was not as good as that of the dimethylxanthines and caffeine, which was confirmed by the external standard analysis figures.

Initial studies using acetonitrile precipitation[26] or isopropanol–chloroform (10:90)[27] showed that extraction of the monomethylxanthines was poor or non-existent from these neutral conditions. Subsequent studies including the methyluric acids and during evaluation of a bonded phase sample preparation method[28] confirmed these findings. The extraction of the monomethylxanthines and dimethyluric acids was significantly improved when the sample was acidified with a small quantity of orthophosphoric acid prior to extraction with dichloromethane–isopropanol. The bonded phase method could be similarly modified to include all the analytes by the use of acidic water (pH 4.0 with orthophosphoric acid) for the wash steps. Unfortunately, this was accompanied by a serious increase in the number and frequency

TABLE I

CELUTE-MX BATCH TEST

Devised to assess the performance of individual batches of the conditioned celite, Celute-MX. Details in discussion of sample preparation.

Compound	Internal standard analysis		External standard analysis	
	Mean (μM)	Yield (%)	Mean (μM)	Yield (%)
7-Methylxanthine	14.62	89.7	13.63	83.6
3-Methylxanthine	15.27	96.0	14.25	89.5
1-Methylxanthine	17.02	99.7	15.88	93.0
1,3-Dimethyluric acid	20.34	95.4	18.97	89.0
3,7-Dimethylxanthine	27.87	101.5	26.00	94.7
3-Ethylxanthine (int. std)	(50.00)	(100.0)	46.65	93.3
1,7-Dimethyluric acid	9.78	96.2	9.13	89.7
1,7-Dimethylxanthine	24.82	101.4	23.15	94.6
1,3-Dimethylxanthine	58.40	101.2	54.48	94.4
1,3,7-Trimethyluric acid	9.39	101.0	8.76	94.2
β-Hydroxyethyltheophylline	50.45	100.9	47.07	94.1
1,3,7-Trimethylxanthine	60.28	101.4	58.11	94.6

of chromatographic interferences which seriously reduced the reliability of the meth-od. Other alternatives using bonded phases, suggested by Dr. P. A. Harris (Analyt-ichem)[29] and Professor M. Burk (University of Arizona)[30], were found to be unable to retain the full range of related compounds that are extracted by Celute-MX from plasma.

The efficiency and convenience of the extraction was improved by the use of Chem-Elut[TM] tubes (formally Clin-Elut, from Analytichem). Celite in these tubes provides a solid support for the liquid–liquid extraction but was found to produce variable recoveries for the metabolites. The pH of damp native Celite was found to be alkaline[31], and the extraction of the metabolites was found to be unreliable if the bed was not acidified throughout the full length. This was improved if the Celite was preconditioned and buffered so that the tubes were homogeneous from top to bot-tom.

The Celite packing for the Celute-MX tubes has been developed empirically and conditioned to give maximal recoveries of the 10 related compounds. In practice, the Celute Batch Test has provided valuable data for the assessment of extraction performance, and has been found to give a good indication of expected efficiency when used to extract methylxanthines and methyluric acids from plasma, confirming the observations for theophylline, theobromine and caffeine that there is no difference between extraction from water or plasma when the sample is acidified[32]. In addition, each batch was tested with the standard plasma in order to establish that the buffering capacity of the Celute-MX was sufficient to adjust the plasma samples to the opti-mum pH for the extraction. If this was inadequate, the recovery of the metabolites was inferior, the dimethyluric acids being the most vulnerable.

The recovery of monomethyluric acids was poor using the Celute-MX columns, and attempts to improve their yields were abandoned when it was realised that they were accompanied by a reduction in selectivity; the increased occurrence of chroma-tographic interferences made the method unreliable. 3,7-Dimethyluric acid was not included as C_8-oxidation of theobromine is not thought to occur in humans[33] and with the present instruments it was not possible to reliably separate it from 3-methyl-xanthine.

Gradient vs. isocratic elution

The monomethylxanthines were not retained or separated in the isocratic meth-od[25] that was initially employed, and when the concentration of acetonitrile in the mobile phase was reduced to overcome this problem, there was an unacceptable increase in the retention times for theophylline and caffeine. In a detailed study of the retention behaviour of the methylxanthines[2], the capacity factors $[k' = (t_R - t_0)/T_0,$ where t_R is the retention time of the compound and t_0 the retention time of an unretained compound] for the analytes included in this assay were shown to range from 1.83 to 29.00 (Table II). A chromatogram of the standard calibration plasma is given in Fig. 2, and shows how the use of a mobile phase gradient enabled adequate retention and separation of the early eluting compounds without unacceptably wide caffeine and theophylline peaks. The k' in this gradient system (k'_{grad}) at 50°C are given in Table II and range from 0.87 to 3.43.   ,

Maintaining the analytical column at 50°C ensures reproducible separations and requires lower concentrations of organic solvent[34]. Separation of paraxanthine

TABLE II

CONCENTRATION AND RETENTION DATA FOR CHROMATOGRAM OF THE STANDARD CALIBRATION PLASMA (FIG. 2).

Capacity factors for each compound separated at 50°C by gradient elution (k'_{grad}) were calculated from the equation ($t_R - t_0/t_0$). The capacity factors determined in isocratic conditions at room temperature[2] are included for comparison.

Peak label	Compound	Concentration (μM)	t_R (min)	k'_{grad} (50°C)	k' (ref. 2)
7-mx	7-Methylxanthine	16.3	8.52	0.87	1.83
3-mx	3-Methylxanthine	15.9	9.42	1.07	2.46
1-mx	1-Methylxanthine	17.1	10.13	1.23	3.00
1,3-di m-ua	1,3-Dimethyluric acid	21.3	11.87	1.61	2.95
Theobromine	3,7-Dimethylxanthine	27.5	12.48	1.74	5.21
3-ex (int.std.)	3-Ethylxanthine	–	13.64	2.00	–
1,7-di m-ua	1,7-Dimethyluric acid	10.2	14.19	2.12	3.39
Paraxanthine	1,7-Dimethylxanthine	24.5	15.20	2.34	8.00
Theophylline	1,3-Dimethylxanthine	57.7	15.60	2.43	9.16
1,3,7-tri m-ua	1,3,7-Trimethyluric acid	9.3	16.95	2.73	7.78
β-OH-et (ref.)[a]	β-Hydroxyethyltheophylline	–	17.61	2.87	–
Caffeine	1,3,7-Trimethylxanthine	61.4	20.17	3.43	29.00

[a] ref. = Reference compound in Maxima component list. This and the int. std (also designated ref.) are used to determine the relative retention times of the component peaks and ensures correct peak indentification.

from theophylline was achieved by the addition of the polar modifier THF to mobile phase A, and this also helped the separation of 1,3-dimethyluric acid from theobromine.

The main reason for continuation of the gradient so long after caffeine has eluted is to ensure the elution of trimethoprim and sulphamethoxazole (the constituents of Septrin and Bactrim). If these late eluting compounds remain on the column at the end of the gradient they come off in the following chromatograms as very wide peaks. The ability to use a purge step (of mobile phase A or stronger solution of acetonitrile) would permit a considerable reduction in the full cycle time, thereby increasing the throughput of samples.

Method validation

Within-run ($n = 20$) and between-run ($n = 26$) precision studies were conducted on pools of low (P0), medium (P3), high (P2) and very high (P1) spiked human serum and the results using the LC-75 UV detector are given in Tables III and V. The between run data were acquired over a period of six months; every time a batch of standards and samples was set up for analysis the set of four precision test samples was included and the results set aside until a sufficient number had been acquired. Four different batches of Celute-MX packing were used during this period. The precision tests have been repeated with the recently acquired diode array detector, and the results are given in Tables IV and VI.

The within-run and between-run precision tests using the LC-75 UV detector were mediocre for the methylxanthines, the lower concentrations of acceptable preci-
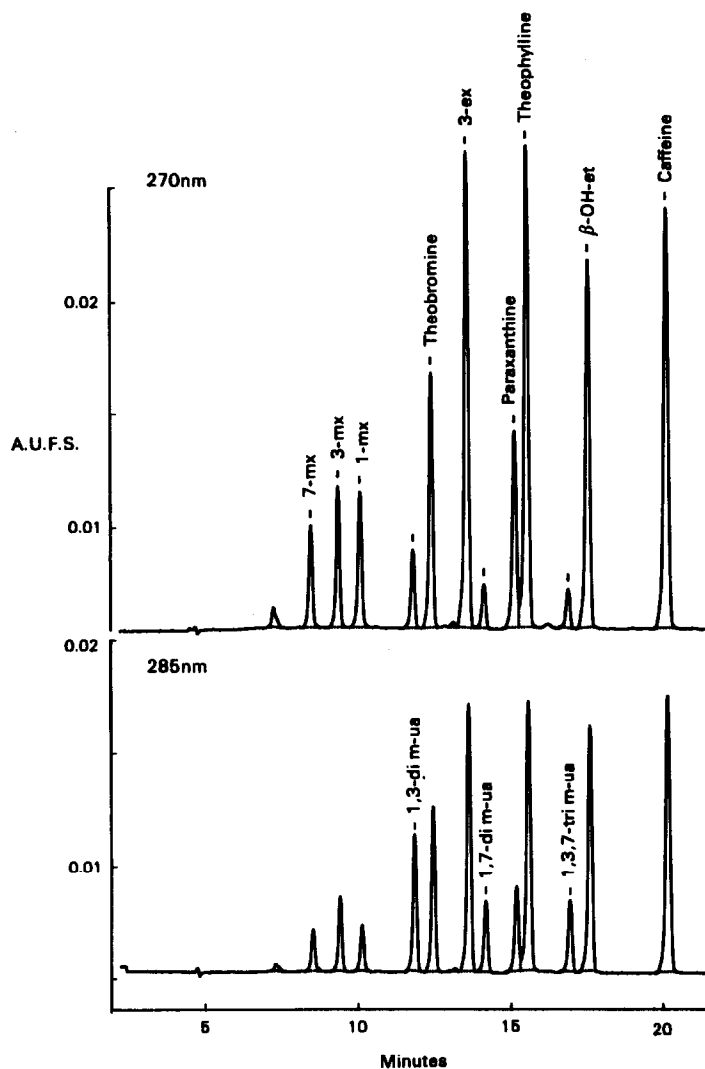
Fig. 2. Chromatogram of the current standard calibration plasma. Concentrations, abbreviations and retention data are given in Table I.

sion being in excess of 3.0 μM and rather higher for the methyluric acids where these latter were being detected well away from their maximum absorption wavelength (λ_{max} = 285 nm). The use of a second detector set to the λ_{max} of the methyluric acids improved the precision for low concentrations of these compounds.

The minimum concentration for acceptable precision [coefficient of variation (C.V.) = 5%] with the present instruments and an injection volume of 20 μl is 1.5–2.0 μM and is linear to 500 μM. At 285 nm the internal standard (3-ethylxanthine) is not being detected at its λ_{max}, but as this is a consistently large peak, the errors are less significant than those resulting from determination of low concentrations of methyl-uric acids at 273 nm. The performance is critically dependent upon the sample prep-

TABLE III

WITHIN-RUN PRECISION STUDY WITH LC-75 DETECTOR ($n = 20$)

Compound	P0	P3	P2	P1
7-Methylxanthine				
Mean (μM)	0.20	6.43	28.83	55.13
S.D.	0.11	0.28	1.56	1.45
C.V. (%)	55.1	4.4	5.4	2.6
3-Methylxanthine				
Mean (μM)	2.51	8.36	28.06	53.66
S.D.	0.16	0.21	0.56	1.10
C.V. (%)	6.5	2.5	2.0	2.0
1-Methylxanthine				
Mean (μM)	0.31	5.50	23.67	48.80
S.D.	0.14	0.17	0.38	0.81
C.V. (%)	44.5	3.0	1.6	1.6
1.3-Dimethyluric acid				
Mean (μM)	4.47	20.84	45.39	64.21
S.D.	0.40	0.30	0.80	1.75
C.V. (%)	9.0	1.4	1.8	2.7
Theobromine				
Mean (μM)	3.17	28.45	77.53	216.62
S.D.	0.22	0.15	0.79	6.86
C.V. (%)	6.9	0.5	1.0	3.1
1,7-Dimethyluric acid				
Mean (μM)	4.25	7.89	11.37	12.25
S.D.	0.39	0.23	0.34	0.65
C.V. (%)	9.2	3.0	3.0	5.3
Paraxanthine				
Mean (μM)	2.59	13.05	33.68	92.26
S.D.	0.33	0.18	0.32	3.22
C.V. (%)	12.6	1.4	0.9	3.5
Theophylline				
Mean (μM)	39.21	67.38	123.46	283.06
S.D.	0.43	0.43	0.96	10.60
C.V. (%)	1.1	0.6	0.8	3.7
1,3,7-Trimethyluric acid				
Mean (μM)	3.86	7.87	13.02	22.10
S.D.	0.46	0.21	0.40	0.57
C.V. (%)	11.9	2.7	3.1	2.5
Caffeine				
Mean (μM)	6.47	32.12	80.59	223.61
S.D.	0.43	0.39	0.80	11.45
C.V. (%)	6.4	1.2	1.0	5.1

aration and a better understanding of the mechanism might lead to more uniform yields and improved precision. However, this group of compounds exhibits a wide range of polarity and other chromatographically important parameters, so the necessity for some compromise should be anticipated. In the latter case, priority should be

TABLE IV

WITHIN-RUN PRECISION STUDY WITH LC-135 DETECTOR (n = 20)

Compound	P0	P3	P2	P1
7-Methylxanthine				
Mean (μM)	0.55	6.75	30.98	56.54
S.D.	0.77	0.33	1.32	1.21
C.V. (%)	11.9	4.9	4.3	2.1
3-Methylxanthine				
Mean (μM)	2.61	8.52	25.83	55.36
S.D.	0.11	0.18	0.56	0.85
S.V. (%)	4.1	2.1	2.2	1.5
1-Methylxanthine				
Mean (μM)	0.81	5.64	22.98	49.04
S.D.	0.05	0.09	0.18	0.60
C.V. (%)	6.8	1.5	0.8	1.2
1.3-Dimethyluric acid				
Mean (μM)	4.85	21.44	47.17	66.10
S.D.	0.16	0.47	1.46	1.23
C.V. (%)	3.2	2.2	3.1	1.9
Theobromine				
Mean (μM)	3.66	29.06	83.85	209.79
S.D.	0.09	0.39	1.03	3.10
C.V. (%)	2.5	1.4	1.2	1.5
1,7-Dimethyluric acid				
Mean (μM)	3.85	8.25	11.84	12.87
S.D.	0.26	0.23	0.34	0.26
C.V. (%)	6.8	2.8	2.9	2.0
Paraxanthine				
Mean (μM)	3.01	13.31	33.90	87.43
S.D.	0.11	0.19	0.33	1.22
C.V. (%)	3.7	1.4	1.0	1.4
Theophylline				
Mean (μM)	40.67	68.12	123.34	270.27
S.D.	0.59	0.92	1.13	2.66
C.V. (%)	1.4	1.4	0.9	1.0
1,3,7-Trimethyluric acid				
Mean (μM)	4.47	7.94	13.22	21.22
S.D.	0.22	0.13	0.16	0.21
C.V. (%)	4.9	1.6	1.2	1.0
Caffeine				
Mean (μM)	7.72	32.54	80.48	212.33
S.D.	0.20	0.58	1.06	3.33
C.V. (%)	2.6	1.8	1.3	1.6

given to the major metabolites of caffeine and theophylline, namely paraxanthine and 1,3-dimethyluric acid, since these have been found to be the most valuable in the investigation of abnormal cases.

The occurrence of co-chromatographic interferences with the analytes and in-

TABLE V

BETWEEN-RUN PRECISION STUDY WITH LC-75 DETECTOR ($n = 26$)

Compound	P0	P3	P2	P1
7-Methylxanthine				
Mean (μM)	0.65	7.52	29.13	56.28
S.D.	0.81	0.66	1.58	3.42
C.V. (%)	124.0	8.7	5.4	6.0
3-Methylxanthine				
Mean (μM)	2.52	8.39	28.31	53.87
S.D.	0.29	0.37	0.75	1.39
S.V. (%)	11.6	4.4	2.7	2.6
1-Methylxanthine				
Mean (μM)	0.49	5.87	23.75	47.91
S.D.	0.35	0.77	1.01	1.76
C.V. (%)	71.8	13.0	4.3	3.7
1.3-Dimethyluric acid				
Mean (μM)	4.78	21.17	47.38	64.46
S.D.	0.74	1.24	1.01	2.92
C.V. (%)	15.4	5.8	2.1	4.5
Theobromine				
Mean (μM)	3.60	28.95	79.30	206.63
S.D.	0.52	0.65	1.22	3.31
C.V. (%)	14.5	2.3	1.5	1.6
1,7-Dimethyluric acid				
Mean (μM)	3.67	7.50	11.63	12.66
S.D.	0.54	0.58	0.70	0.96
C.V. (%)	14.8	7.8	6.0	7.6
Paraxanthine				
Mean (μM)	2.71	13.40	34.50	88.02
S.D.	0.47	0.59	0.55	1.65
C.V. (%)	17.2	4.4	1.6	1.9
Theophylline				
Mean (μM)	39.86	68.62	125.30	268.33
S.D.	0.97	1.35	1.78	3.52
C.V. (%)	2.4	2.0	1.4	1.3
1,3,7-Trimethyluric acid				
Mean (μM)	4.17	7.94	13.57	22.93
S.D.	0.77	0.81	1.05	1.46
C.V. (%)	18.5	10.2	7.7	6.4
Caffeine				
Mean (μM)	7.20	32.43	82.44	208.20
S.D.	0.66	1.09	1.45	5.52
C.V. (%)	9.2	3.4	1.8	2.7

ternal standards have been found to be rare, but may occasionally be suspected from the peak shape or from unusual drug to metabolite ratios. An example of an interference with the internal standard is given in Fig. 5. In this case β-hydroxyethyltheophylline was used as the internal standard to overcome the problems arising from

TABLE VI

BETWEEN-RUN PRECISION STUDY WITH LC-135 DETECTOR (n = 14)

Compound	P0	P3	P2	P1
7-Methylxanthine				
Mean (μM)	0.66	7.11	29.35	57.80
S.D.	0.58	0.89	1.50	2.83
C.V. (%)	86.9	12.5	5.1	4.9
3-Methylxanthine				
Mean (μM)	2.77	8.57	29.07	56.22
S.D.	0.19	0.36	1.47	1.80
C.V. (%)	7.0	4.2	5.1	3.2
1-Methylxanthine				
Mean (μM)	0.69	5.67	23.91	49.51
S.D.	0.32	0.25	0.51	1.71
C.V. (%)	45.6	4.5	2.1	3.5
1.3-Dimethyluric acid				
Mean (μM)	5.00	21.38	47.57	66.85
S.D.	0.44	1.40	1.77	2.60
C.V. (%)	8.9	6.6	3.7	3.9
Theobromine				
Mean (μM)	4.05	30.03	81.23	214.07
S.D.	0.29	0.84	2.87	8.27
C.V. (%)	7.3	2.8	3.5	3.9
1,7-Dimethyluric acid				
Mean (μM)	3.97	7.94	11.49	12.21
S.D.	0.27	0.55	0.88	1.07
C.V. (%)	6.9	6.9	7.7	8.7
Paraxanthine				
Mean (μM)	3.17	14.02	34.88	89.71
S.D.	0.35	0.49	1.35	3.33
C.V. (%)	10.9	3.5	3.9	3.7
Theophylline				
Mean (μM)	42.13	70.41	127.76	275.03
S.D.	1.62	2.36	4.75	11.32
C.V. (%)	3.8	3.4	3.7	4.1
1,3,7-Trimethyluric acid				
Mean (μM)	4.48	8.04	12.78	21.16
S.D.	0.55	0.50	1.05	1.99
C.V. (%)	12.4	6.2	8.2	9.4
Caffeine				
Mean (μM)	8.40	34.18	84.83	217.78
S.D.	0.65	1.24	3.34	9.14
C.V. (%)	7.8	3.6	3.9	4.2

the inadvertent installation of a column requiring a different composition of mobile phase in order to achieve acceptable separation. Examination of the maximum absorption wavelength, peak purity indices (both available from the LC-135 print-out) and the peak area absorption ratios (Table VII) may be used to confirm or refute

these suspicions. Metronidazole, paracetamol (Fig. 6) and some of the cephalosporin antibiotics are frequently observed in the chromatograms. The major reason for the use of an alternative internal standard was the increasing occurrence of the cephalosporins since many of these antibiotics had similar retention times to β-hydroxyethyltheophylline which was the only internal standard used at that time.

There has been good correlation between plasma theophylline levels determined by this method and other commercial assays including Abbot's TDX® (Abbot Laboratories, Maidenhead, U.K.) and Syva's EMIT™ (Syva U.K., Maidenhead, U.K.). A small number of samples that produced higher theophylline levels by the Ames Seralyzer® (Miles Laboratories, Slough, U.K.) than by TDX, EMIT and HPLC[35], were found by this method to contain exceptionally high levels of theophylline metabolites, principally 1,3-dimethyluric acid ($> 100~\mu M$) and the chromatograms were typical of renal failure patients being treated with theophylline (Fig. 4)[36].

Early in the development of this method the recovery of caffeine was extremely unreliable. The temperature ($> 60°C$) used for evaporation of the organic extracts was found to be responsible and reduction of the temperature of the hot block to $< 37°C$ improved the precision to values similar for other compounds in the assay. The explanation is thought to be that caffeine, unlike theophylline, sublimes ($178°C$)[37], and care should therefore be taken with the evaporation step. Examination of the precision tests with the LC-75 indicate that this might be a contributory factor in the inferior reproducibility for caffeine in comparison with dimethylxanthines.

Clinical applications

For patients with normal hepatic cytochrome P_{450} and renal function the concentration of plasma methylxanthine metabolites has been found to depend upon the concentration of the parent drug. A numerical expression of the relationship was required to establish the normal ranges that had been observed and to accommodate

TABLE VII

MAXIMUM ABSORPTION WAVELENGTHS AND PEAK AREA ABSORBANCE RATIOS

Compound	Maximum absorption wavelength (nm)[a]	Peak area ratio (270 nm to 285 nm)[b]
7-Methylxanthine	269	2.27
3-Methylxanthine	271	1.81
1-Methylxanthine	268	2.94
1,3-Dimethyluric acid	287	0.56
3,7-Dimethylxanthine	274	1.53
3-Ethylxanthine (int std.)	271	1.77
1,7-Dimethyluric acid	285	0.62
1,7-Dimethylxanthine	270	2.28
1,3-Dimethylxanthine	271	1.78
1,3,7-Trimethyluric acid	289	0.58
β-Hydroxyethyltheophylline	274	1.48
1,3,7-Trimethylxanthine	274	1.51

[a] Determined with a Perkin-Elmer LC-135 diode array detector.
[b] Determined from the peak of the within-run test plasma P2 (Table V), band-width for both wavelengths = 15 nm (*i.e.*, 262.5–277.5 nm for the 270 nm channel, and 277.5–292.5 nm for the 285 nm channel).

the considerable range of drug concentrations that were encountered. Molar ratios were the most obvious and the convention 'drug to metabolite' was chosen because the whole numbers normally obtained were considered to be more convenient to assess than the reciprocal values.

Some metabolites may be formed from two compounds (Fig. 1). For example, 3-methylxanthine is a demethylated product of both theobromine and theophylline and therefore a ratio for theophylline to 3-methylxanthine in the presence of significant concentrations of (dietary) theobromine would not accurately reflect either the demethylation of theophylline or the clearance of 3-methylxanthine. This difficulty is overcome to some extent by inclusion of both possible sources of the metabolite; in this case (theobromine + theophylline) to 3-methylxanthine. However, this is not a complete solution because the demethylation of both compounds is uneven[4,33].

The drug to metabolite ratios have been determined on a large number of samples, and values for the normal ranges of the main groups of patients that are served by this laboratory are given in Table VIII. These should be regarded as preliminary since the majority of the samples used to obtain these data was apparently taken at random with respect to dose and time. Other studies have shown that these ratios vary depending upon the dose-to-sample interval[38] and better documentation is necessary to establish the normal ranges with more precision. In practice, the most valuable ratios have proved to be caffeine to paraxanthine and theophylline to 1,3-dimethyluric acid. That is, ratios including metabolites which have no other source in plasma than the cytochrome P_{450} mediated conversion of the parent compound.

The drug to metabolite ratios have been extremely useful for the interpretation of results on exceptionally toxic samples from patients who have deliberately taken an overdose of theophylline. For example, a theophylline to 1,3-dimethyluric acid ratio

TABLE VIII

PRINCIPAL DRUG TO METABOLITE MOLAR RATIOS DETERMINED IN PLASMA OF FOUR GROUPS OF PATIENTS

Ratio	Mean	S.D.	Suggested normal range (mean ± S.D.)
Group I: neonates treated with caffeine (n = 186)			
Caffeine to paraxanthine	57.4	21.6	35.8–79.0
Caffeine to theobromine	52.4	21.5	30.9–73.9
Caffeine to theophylline	39.6	13.7	25.9–53.3
Group II: neonates treated with theophylline (n = 221)			
Theophylline to caffeine	3.7	2.7	1.0–6.4
Theophylline to 1,3-dimethyluric acid	22.2	9.0	13.2–31.2
Group III: children treated with theophylline (n = 159)			
Theophylline to 1,3-dimethyluric acid	15.2	6.8	8.4–22.0
(Theophylline + theobromine) to 3-methylxanthine	31.0	17.7	13.3–48.7
Caffeine to paraxanthine	2.1	1.8	0.3–3.9
Group IV: adults treated with theophylline (n = 50) (small group investigated as part of a correlation study)			
Theophylline to 1,3-dimethyluric acid	11.3	3.5	7.8–14.8
Caffeine to paraxanthine	2.0	0.9	0.3–3.9

within the normal range has been demonstrated for a plasma theophylline concentration of 736 μM (132 mg/l), indicating that the ratios are to a great extent independent of concentration. In other situations they may be used to estimate the quantity of metabolite that would be expected for a given concentration of parent drug and hence gauge the magnitude of metabolic impairment.
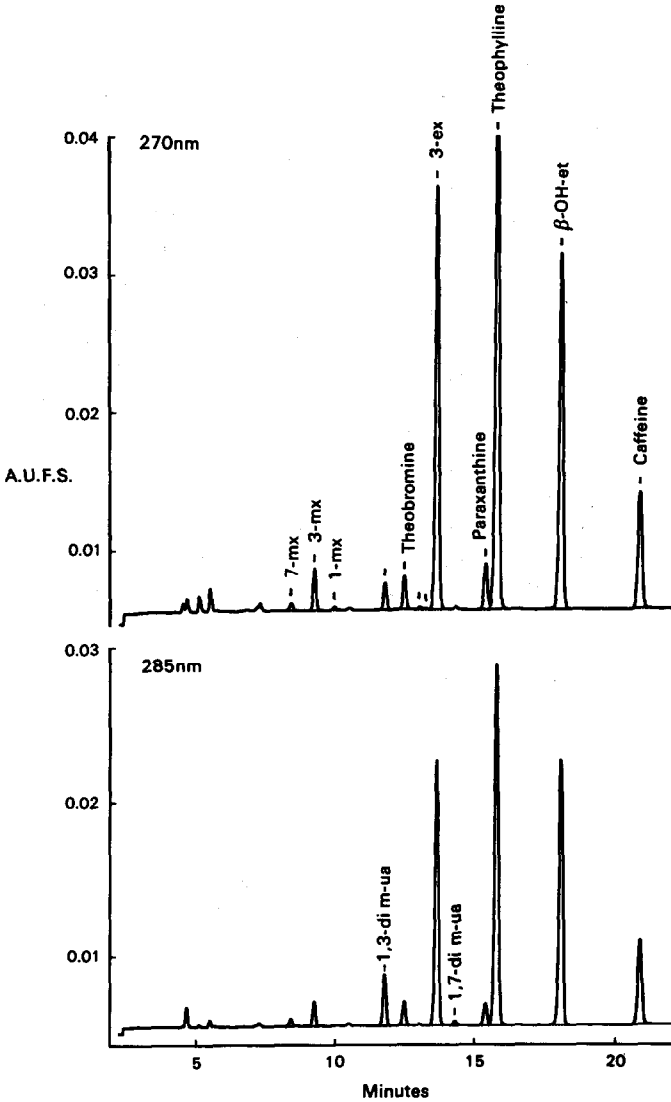

Fig. 3. Theophylline within the recommended therapeutic range, moderate caffeine intake and a normal plasma methylxanthine profile. For abbreviations, see Table II. Concentrations: 7-methylxanthine, 1.0 μM; 3-methylxanthine, 4.8 μM; 1-methylxanthine, 0.4 μM; 1,3-dimethyluric acid, 6.7 μM; theobromine, 3.9 μM; 1,7-dimethyluric acid, 0.6 μM; paraxanthine, 6.6 μM; theophylline, 78.0 μM; 1,3,7-trimethyluric acid, 0.0 μM; caffeine, 19.0 μM. Drug to metabolite ratios (suggested normal reagents in parenthesis): theophylline to 1,3-dimethyluric acid, 11.7 (7.8–14.8); (theophylline + theobromine) to 3-methylxanthine, 17.2 (15.0–50); caffeine to paraxanthine, 2.9 (0.3–3.9); paraxanthine to 1,7-dimethyluric acid, 11.0 (8.0–20).

EXAMPLES

The normal adult plasma methylxanthine profile has been included for comparison with the abnormal examples which demonstrate situations when this assay has been of value and should result in a revision of the therapeutic management.

Theophylline within the recommended therapeutic range, moderate caffeine intake and a normal plasma methylxanthine profile (Fig. 3)

This shows a typical plasma methylxanthine profile for an adult with a moderate dietary caffeine intake and who is receiving theophylline therapy for asthma. The plasma concentration of theophylline, 78.0 μM (15 mg/l), is within the recommended therapeutic range of 55–110 μM (10–20 mg/l) and 1,3-dimethyluric acid is present at the expected concentration for normal theophylline C_8-oxidation and renal clearance. The majority of the 3-methylxanthine is the product of N_1-demethylation of theophylline. The paraxanthine and theobromine concentrations are consistent with normal hepatic clearance of caffeine and no dietary theobromine. The presence of 1,7-dimethyluric acid in samples from caffeine-consuming adults is normal. A value for the paraxanthine to 1,7-dimethyluric acid ratio similar to that of the other C_8-oxidation ratio (theophylline to 1,3-dimethyluric acid) is a common finding.

Gross accumulation of theophylline metabolites in renal failure (Fig. 4)

It is shown here that the theophylline concentration of 213 μM (38 mg/l) is well above the recommended therapeutic range and would be expected to cause serious clinical symptoms of theophylline toxicity. In addition, the gross accumulation of both principal theophylline metabolites is consistent with renal failure[36]. Both compounds are pharmacologically active and contribute significantly to the toxic condition[8,9]. The accumulation of 1,3-dimethyluric acid confirms the report that it does not undergo further demethylation to form 1-methyluric acid[24]. 1,3,7-trimethyluric acid was detected and its identity confirmed by the 270 to 285 nm absorbance ratio.

The probable reason for the toxic level of theophylline in this case is clinical overdose because the hepatic clearance of theophylline is not affected by renal failure[39]. Theophylline toxic symptoms occur in renal failure cases even when the plasma concentration is below the therapeutic range[36] and therefore its use in this condition should only be considered when the metabolites can be monitored to establish that the form of renal support employed (*e.g.*, haemodialysis) is able to remove them from plasma.

Theophylline toxicity due to hepatic clearance impaired by another drug (Fig. 5)

The theophylline concentration of 262 μM (47 mg/l) (Fig. 5) confirmed the clinical diagnosis of theophylline toxicity. The mean adult theophylline to 1,3-dimethyluric acid ratio of 11.3 (Table VIII), indicates that the expected concentration for 1,3-dimethyluric acid would be 23 μM. The level of 1.8 μM is <10% of the expected concentration and suggests that C_8-oxidation is seriously impaired causing theophylline accumulation through diminished hepatic clearance. The possibility of an adverse drug reaction was suspected and subsequently it was confirmed that the patient had recently completed a ten-day course of erythromycin.

Experience has shown that in cases of theophylline overdose, caffeine to paraxanthine ratios in the range 5 to 9 are not unusual and are thought to reflect caffeine
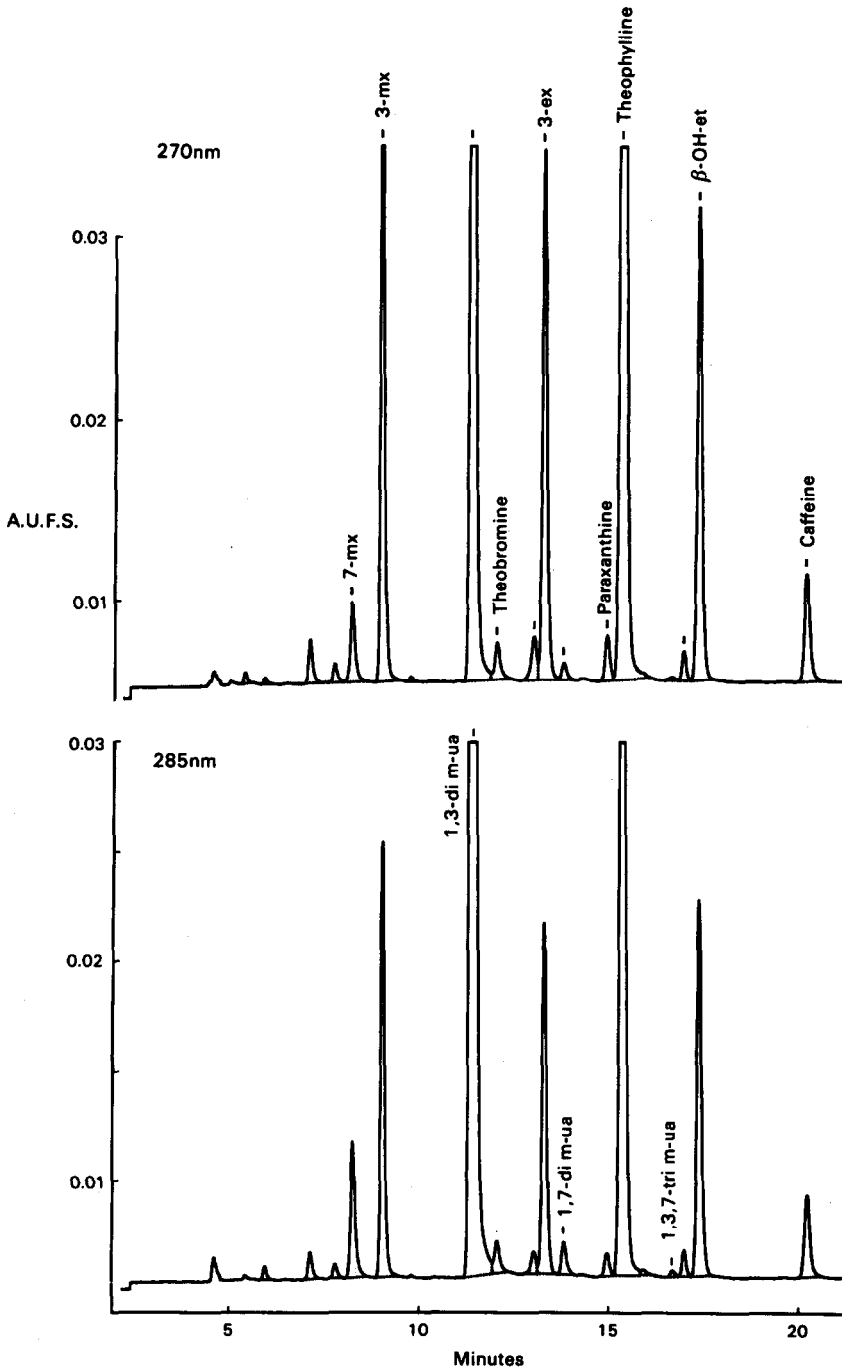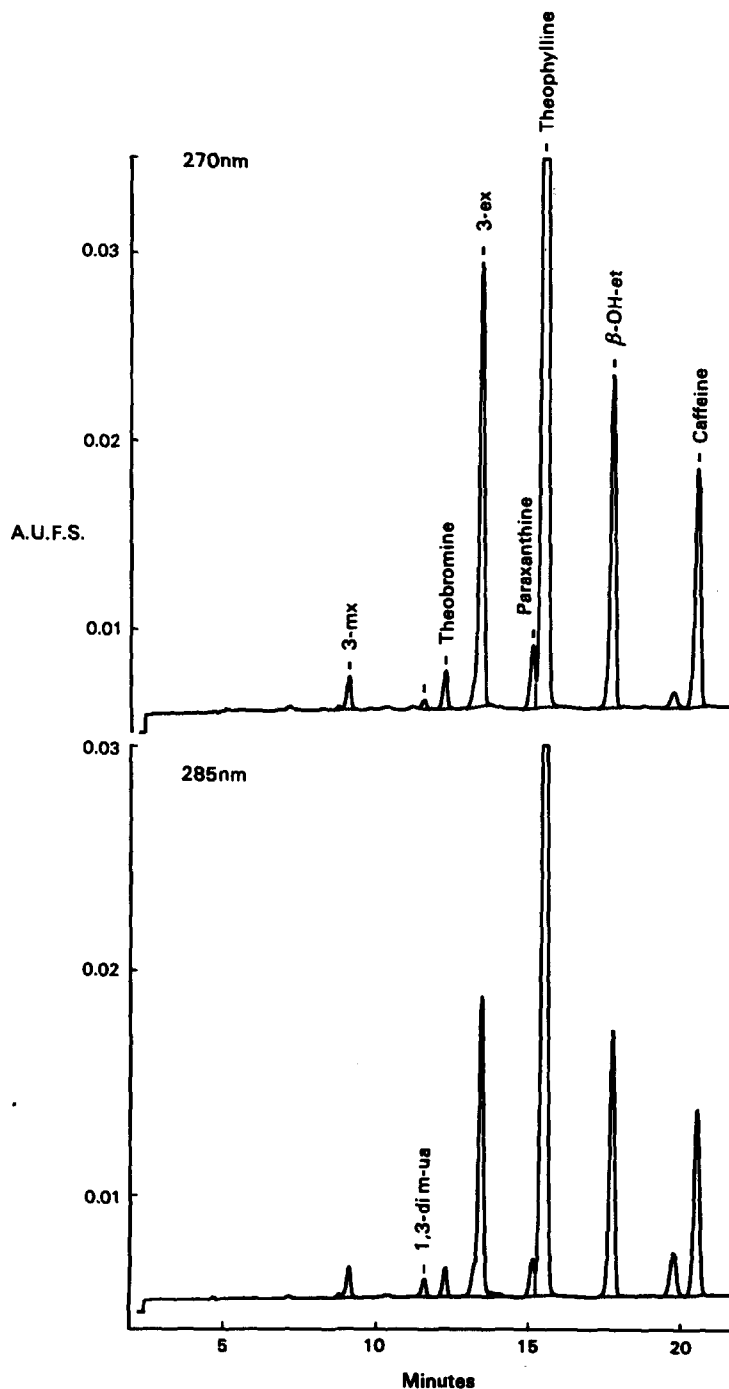
Fig. 4. Gross accumulation of theophylline metabolites in renal failure. For abbreviations, see Table II. Concentrations: 7-methylxanthine, 0.0 μM; 3-methylxanthine, 61.4 μM; 1-methylxanthine, 0.4 μM; 1,3-dimethyluric acid, 522.4 μM; theobromine, 4.5 μM; 1,7-dimethyluric acid, 4.6 μM; paraxanthine, 5.0 μM; theophylline, 213.2 μM; 1,3,7-trimethyluric acid, < 1.0 μM; caffeine, 13.7 μM. Drug to metabolite ratios (suggested normal ranges in parenthesis): theophylline to 1,3-dimethyluric acid, 0.4 (7.8–14.8); (theophylline + theobromine) to 3-methylxanthine, 3.6 (15–50); caffeine to paraxanthine, 2.7 (0.3–3.9); paraxanthine to 1,7-dimethyluric acid, 1.1 (8.0–20).

Fig. 5. Theophylline toxicity due to hepatic clearance impaired by another drug. For abbreviations, see Table II. Concentrations: 7-methylxanthine, 0.0 μM; 3-methylxanthine, 2.4 μM; 1-methylxanthine, 0.0 μM; 1,3-dimethyluric acid, 1.8 μM; theobromine, 4.1 μM; 1,7-dimethyluric acid, 0.0 μM; paraxanthine, 8.2 μM; theophylline, 262.5 μM; 1,3,7-trimethyluric acid, 0.0 μM; caffeine, 40.9 μM. Drug to metabolite ratios (suggested normal ranges in parenthesis): theophylline to 1,3-dimethyluric acid, 145.8 (7.8–14.8); (theophylline + theobromine to 3-methylxanthine, 111.1 (15–50); caffeine to paraxanthine, 5.0 (0.3–3.9).
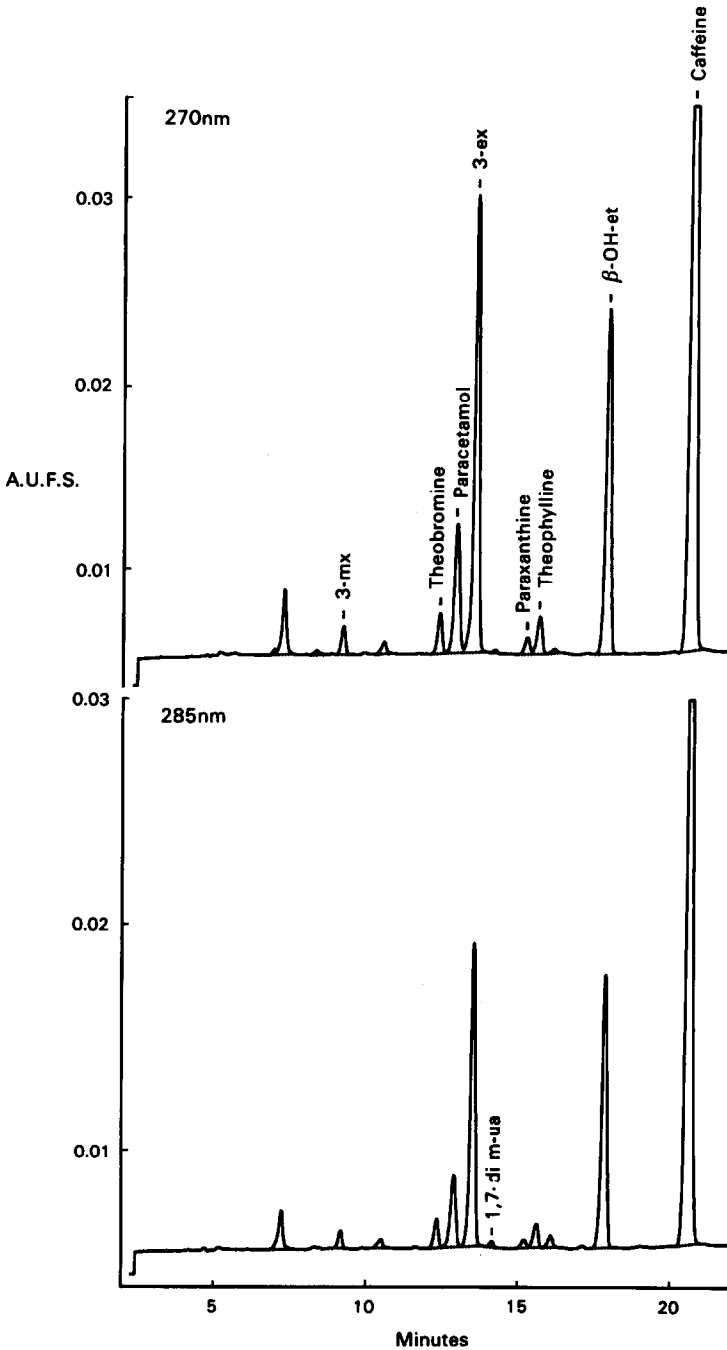
Fig. 6. Impaired demethylation in liver disease causing accumulation of dietary caffeine in plasma. For abbreviations, see Table II. Concentrations: 7-methylxanthine, 0.6 μM; 3-methylxanthine, 3.6 μM; 1-methylxanthine, 0.2 μM; 1,3-dimethyluric acid, 0.0 μM; theobromine, 4.0 μM; 1,7-dimethyluric acid, 1.7 μM; paraxanthine, 2.0 μM; theophylline, 4.1 μM; 1,3,7-trimethyluric acid, 0.0 μM; caffeine, 161.1 μM. Drug to metabolite ratios (suggested normal ranges in parenthesis): (theophylline + theobromine) to 3-methylxanthine, 2.3 (14–50); caffeine to paraxanthine, 80.6 (0.3–3.9); paraxanthine to 1,7-dimethyluric acid, 1.2 (8.0–14.0).

synthesis by N_7-methylation of theophylline[15,36,40]. While this phenomenon cannot be excluded, in this sample the high value of the (theophylline + theobromine) to 3-methylxanthine ratio of > 100 implies some impairment of demethylation.

Impaired demethylation in liver disease causing accumulation of dietary caffeine in plasma (Fig. 6)

The expected paraxanthine concentration for an adult plasma caffeine level of 161 μM (31 mg/l) with normal cytochrome P_{450} function would be approximately 80 μM (14 mg/l) (Fig. 6). The level of 2.0 μM is about 2.5% of that expected and indicates a serious impairment of demethylation causing an accumulation of dietary caffeine in the plasma.

The frequent occurrence of high plasma caffeine levels in some forms of liver disease[15,16] was the basis of the suggestion that determination of fasting caffeine concentration could provide a guide to the severity of chronic liver disease[21]. The co-determination of paraxanthine and consideration of the caffeine to paraxanthine ratio would be less dependent upon patient compliance and enable those at risk to be identified with more precision even with low caffeine levels[36,41].

The presence of theobromine and theophylline at concentrations slightly above paraxanthine may reflect a dietary component from the consumption of tea and coffee. The presence of 3-methylxanthine could reflect some renal impairment since it would not be expected for these concentrations of parent compounds, and the (theophylline + theobromine) to 3-methylxanthine ratio of 2.3 is consistent with this interpretation.

CONCLUSIONS

A reliable assay has been developed which enables the simultaneous determination of caffeine, theobromine, theophylline and most of their N-demethylated and C_8-oxidized metabolites in plasma. It has been used to identify abnormal metabolism in several cases of unexpected theophylline toxicity. Toxicity due to impairment of cytochrome P_{450} mediated methylxanthine metabolism by disease or another drug was identified by reduced concentrations of some or all of the metabolites and a corresponding increase of the molar drug to metabolite ratios. Reduced ratios due to excessive metabolite concentrations have been found in renal failure. The use of the drug to metabolite ratios compensates for large differences in concentration and aids the distinction between toxicity due to failures of metabolism and toxicity due to overdose. Therefore, this assay will elucidate impaired hepatic cytochrome P_{450} mediated clearance of methylxanthines caused by disease or other co-administered drugs.

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