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SIMULTANEOUS DETERMINATION OF THEOPHYLLINE AND ITS MAJOR METABOLITES IN URINE BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new, highly selective high-performance liquid-chromatographic (HPLC) assay for theophylline and its major metabolites in urine is described. The method utilizes an ion-pair extraction followed by separation and quantitation by reversed-phase ion-pair gradient-elution HPLC. Comparison with several other methods showed that interferences were present in too many blank urine samples to allow for the accurate quantitation of the metabolites of theophylline by direct injection—isocratic HPLC assays. Sample processing involving ion-pair complexing and extraction together with gradient-elution systems is recommended for accurate pharmacokinetic studies.

INTRODUCTION

Theophylline and its related salts comprise one of the more extensively used groups of bronchodilating agents in the treatment of chronic obstructive pulmonary diseases. The development of analytical techniques that permit the reliable determination of theophylline in plasma have contributed immensely to its therapeutic success as the clinical effects of the drug are highly correlated with the concentration in plasma. However, it is exceedingly difficult to predict a priori patient dosage requirements because of the large inter-individual variability in the clearance of the drug [1—4]. Moreover, it has been suggested that the clearance of the drug may be capacity limited since a disproportionate increase in steady-state blood concentration occurs when the dose is increased [5]. Also when concentrations above 20 mg/l are reached, the traditional log—linear decay curve is not observed; rather, the curve

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follows expected capacity-limited kinetics [6, 7]. Recently Monks et al. [8] have reported capacity-limited formation of 3-methyl xanthine in adults administered a 100-mg dose of theophylline labelled with carbon-14.

Diseased states, diet, smoking, genetic and environmental factors have been shown to markedly influence the clearance of theophylline [9—12]. Since the drug is eliminated primarily by hepatic metabolism [2], these factors presumably cause an increase or a decrease in the rate of one or more of these metabolic pathways. Studies on the metabolism of theophylline have shown that approximately 10% of the drug is eliminated by renal excretion, while the remainder is metabolized to 3-methyl xanthine (13—35%), 1-methyl uric acid (15—19%) and 1,3-dimethyl uric acid (35—40%) [13—16]. However, the inter-individual variability in these studies was very high and the urinary recovery of the drug highly variable. These and more recent studies used assays for theophylline and its metabolites which proved tedious, difficult to reproduce or gave inadequate resolution between the compounds of interest and endogenous interfering compounds in urine [13—18].

For these reasons, a new assay for urinary theophylline and its metabolites has been developed which is based on a combination of normal and ion-pair liquid—liquid extraction, with subsequent quantitation by reversed-phase ion-pair gradient elution high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Instruments and instrumental conditions

The assay was performed on an HPLC system consisting of two Altex Model 100A pumps, an Altex Model 420 solvent gradient programmer and an Hitachi Model 100-30 variable wavelength ultraviolet (UV) detector set at 280 nm (Altex, Berkeley, CA, U.S.A.).

The column was a reversed-phase 5-μm Ultrasphere ODS, 25 cm × 4.6 mm I.D. (Altex); a slurry-packed precolumn (4.0 cm × 2.5 mm I.D.) of Lichrosorb RP-2 (10 μm) was attached to the system between the injector and analytical column.

Injections were made by means of a Waters Intelligent Sample Processor, Model 710A (Waters Assoc., Milford, MA, U.S.A.).

Data analysis was performed with a Spectra-Physics SP 4100 computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

Reagents

Sources of the xanthine derivatives were: theophylline and β-hydroxyethyl theophylline from Sigma, St. Louis, MO, U.S.A.; caffeine from Eastman Kodak, Rochester, NY, U.S.A.; 3-methyl xanthine, 1-methyl xanthine, 1,7-dimethyl xanthine, 1-methyl uric acid, and 1,3-dimethyl uric acid from Adams Chemical, Round Lake, IL, U.S.A.

Sodium acetate (anhydrous), sodium bicarbonate and ammonium sulphate were of analytical grade. Tetrabutyl ammonium hydrogen sulphate (TBA) was obtained from Aldrich, Milwaukee, WI, U.S.A. Chloroform and ethyl acetate were of UV grade, the methanol was HPLC grade and they were all purchased from Burdick & Jackson Labs., Muskegon, MI, U.S.A. Isopropanol, reagent grade, was supplied by Mallinckrodt, St. Louis, MO, U.S.A.

Solvent A was a 0.01 *M* solution of sodium acetate and 0.005 *M* tetrabutyl ammonium hydrogen sulphate in distilled water, with the pH adjusted to 4.75 by 10 *M* NaOH. Solvent B contained the same amounts of salts, but included 50% (v/v) of methanol.

Solvent A was filtered through a Millipore filter type HA 0.45 μm and solvent B through a Millipore filter type BD 0.60 μm (Millipore, Bedford, MA, U.S.A.).

The internal standard solution was prepared by dissolving β-hydroxyethyltheophylline in methanol (50 mg/l). The extraction solution consisted of ethyl acetate—chloroform—isopropanol (45:45:10, v/v). A buffer solution (pH 11) used in the extraction procedure was prepared from 90 ml of 0.1 *M* anhydrous sodium carbonate and 10 ml of 0.1 *M* sodium bicarbonate.

Procedure

A 1.0-ml volume of the internal standard solution was evaporated to dryness in a 15-ml centrifuge tube at 35°C under a stream of nitrogen. An aliquot (0.5 ml) of the urine to be assayed was transferred to the tube and mixed with 0.5 ml of a 0.1 *M* TBA solution and 1.0 g of ammonium sulphate was added, followed by vortexing for 60 sec. A 250-μl volume of buffer (pH 11) was added to adjust the pH to approximately 6.0—6.5 using pH indicator paper.

The mixture was extracted with 10 ml of ethyl acetate—chloroform—isopropanol (45:45:10, v/v) by vortexing for at least 2 min. After centrifugation (5 min at 2000—3000 rpm), 5 ml of the organic layer were transferred and evaporated to dryness at 35°C under a stream of nitrogen.

The residue was vortexed for 60 sec to dissolve it in 0.5 ml of a 0.01 *M* solution of sodium acetate containing 10% (v/v) methanol adjusted to pH 4.75 by 10 *M* NaOH. Then a second 0.5-ml volume of a solution containing 0.01 *M* sodium acetate and 0.05 *M* TBA adjusted to pH 4.75 was added and the vortexing procedure was repeated for 60 sec. The two-step reconstitution procedure was adopted to ensure adequate dissolution of several of the poorly soluble methyl uric acid ion pairs. The concentration of TBA was necessary to avoid dissociation of the ion pairs which caused several split peaks in the resultant chromatograms.

The analysis was performed by solvent gradient elution controlled by the Altex solvent gradient programmer in which the concentration of methanol in the elution was varied from 4.5% to 23%.

The program was started with 9% solvent B and increased by a five-step* gradient program to a final concentration of 46% B. This was accomplished over a 31-min period. The solvent gradient was reduced back to 9% in 2 min and allowed to equilibrate for 15 min between automatic injections.

Quantitation of theophylline and metabolite peaks is achieved by the internal standard peak area ratio method. A standard curve for each compound was prepared by spiking blank urine obtained from a volunteer who had abstained for at least 48 h from caffeine-containing food and beverages (chocolate, tea, coffee, cola, etc.). The standard samples are prepared by evaporat-

*9—12% in 10 min, 12—30% in 15 min, 30—40% in 5 min, 40—46% in 1 min, 46—99% in 2 min.

ing, together with the internal standard solution, appropriate volumes of methanolic stock solutions containing theophylline and metabolites. The residue was reconstituted in 0.5 ml of blank urine (60 sec) followed by assay as described above.

RESULTS

The results of the analysis of a solution containing the test compounds,

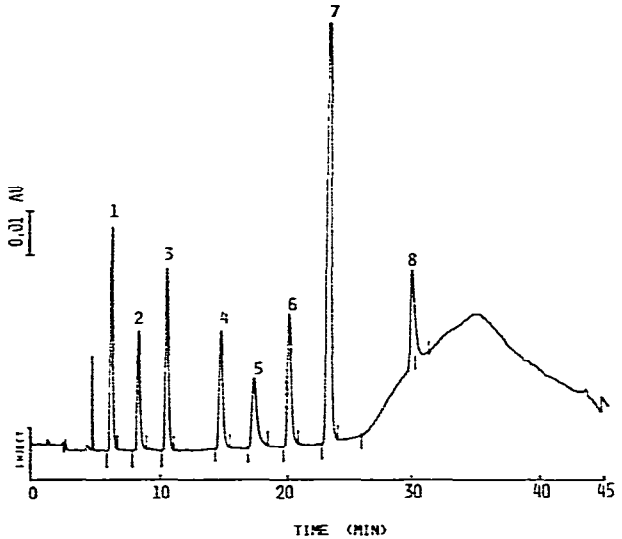

Fig. 1. Urine spiked with standard mixtures: (1) 3-MX, (2) 1-MX, (3) 1-MU, (4) 1,3-MU, (5) 1,7-MX, (6) 1,3-MX (theophylline), (8) caffeine, each at 20 μg/ml; and (7) internal standard (β-hydroxyethyltheophylline) at 50 μg/ml.

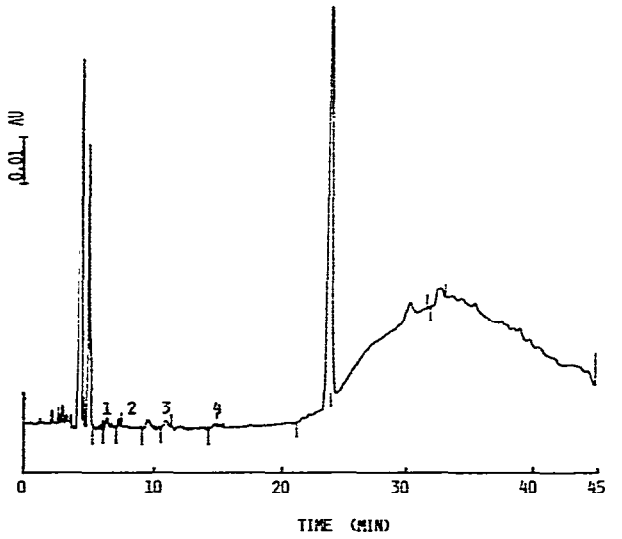

Fig. 2. Chromatogram of a patient's urine collected before theophylline administration, spiked with internal standard (50 μg/ml).

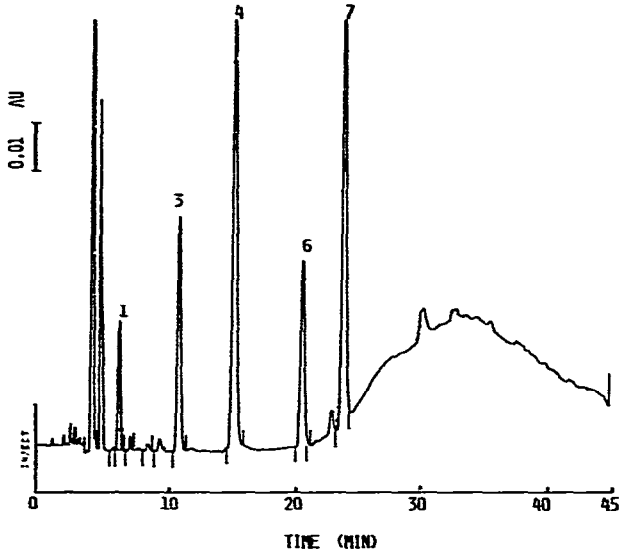

Fig. 3. Chromatogram of a patient's urine collected 3 h after theophylline administration. (1) 3-MX, (3) 1-MU, (4) 1,3-MU, (6) theophylline, and (7) internal standard.

of a blank urine sample and of a sample of urine taken from a volunteer 12 h after a 320 mg dose of theophylline, are shown in Figs. 1, 2 and 3, respectively. Recovery, assay precision and accuracy studies were performed six times for each compound at seven concentrations, varying from 2 to 150 μg/ml, and the results are shown in Table I.

DISCUSSION

Extraction procedure

In order to minimize sample workup, direct injection of samples was attempted under various circumstances, but was unsuccessful as interfering peaks did not allow the adequate resolution of the compounds of interest. Attempts to improve selectivity by pre-extraction at several pH values and with various extraction solvents were also unsuccessful.

Due to the large difference in physico-chemical properties of the methyl xanthines and the methylated uric acids, selection of a simple organic extraction solvent providing a high extraction coefficient for both groups was not possible. Thus, even at low pH, where both the methyl xanthines ($pK_a \approx 8.5$) and the methyl uric acids ($pK_a \approx 5.5$) are predominantly in the unionized form, extraction of the methyl uric acids from aqueous to organic (chloroform—isopropanol, 95:5, v/v) solvent was very inefficient (less than 15%) by virtue of their high polarity. Increasing the polarity of the extraction solvent (chloroform—isopropanol, 50:50, v/v) increased the extraction efficiency, but also resulted in an unacceptable degree of extraction of other, interfering, compounds.

For these reasons, extraction conditions were selected such that the methyl xanthines could be extracted in the unionized form by normal liquid—liquid

TABLE I

RECOVERY AND PRECISION STUDIES

For percentage recovery $n = 6$; C.V. = coefficient of variation.

Concentration (mg/l)	1,3-MX		3-MX		1-MU		1,3-MU		1-MX	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
2	95.5	12.0	84.3	5.8	100.3	17.6	105.3	11.1	86.3	13.4
5	103.9	5.4	95.5	6.2	87.8	16.3	96.1	3.9	97.1	6.6
10	101.9	5.5	94.6	3.3	95.4	14.2	97.9	4.5	100.8	4.9
25	101.0	1.8	94.6	2.2	92.0	11.2	95.1	1.9	98.12	2.3
50	104.5	4.7	97.0	2.6	94.0	10.7	98.2	2.8	99.5	5.3
100	94.9	2.0	92.6	3.9	91.3	2.3	94.4	3.1	94.5	1.3
150	103.6	4.4	94.4	3.5	98.6	18.2	96.5	4.4	97.5	4.2
r^2	0.9970		0.9973		0.9592*		0.9975		0.9976	

*For individual calibration curves $r^2 > 0.998$ (see text).

extraction and the more polar methylated uric acids by means of an ion-pair liquid—liquid extraction. The latter type of extraction has been shown to be particularly efficient for polar and ionized compounds [19, 20]. A pH of 6.0—6.5 was determined to be optimal in accomplishing the extraction; at this pH range, the methyl xanthines are unionized and can be extracted with slightly polar organic solvents or mixtures (e.g., chloroform or ethyl acetate with a small percentage of isopropanol). On the other hand, the methyl uric acids are ionized at this pH, and form an ion pair with the tetrabutyl ammonium counter ion, which can be efficiently extracted in the solvents mentioned above. Extraction was enhanced by adding high concentrations of ammonium sulphate to the system to produce a salting-out effect. The optimum counterion concentration was found to be 0.1 *M*. Lower concentrations produced low and variable extraction efficiencies for 1-methyl uric acid (1-MU) and 1,3-dimethyl uric acid (1,3-MU), while higher concentrations permitted an unacceptable degree of co-extraction of interfering polar compounds. Similar observations were made on the effect of varying isopropanol and ammonium sulphate concentrations. Thus, with low concentrations of isopropanol or ammonium sulphate, poor extraction of both the methyl xanthines and the methyl uric acid ion pairs was noted, while high concentrations, again, produced an unacceptable degree of co-extraction of interfering polar compounds, resulting in much-reduced selectivity.

Chromatographic procedure

Similar considerations prompted the use of an ion-pairing chromatographic system. Thus, in the absence of the counter ion, the retention of the methyl-uric acids was inadequate for acceptable resolution. Addition of the tetrabutylammonium counterion to the solvent system markedly increased the retention of these compounds despite the fact that the pH of the analytical mobile phase was lower than their pK_a values. Small changes in pH (± 0.25 pH units) produced marked changes in the retention time of 1-MU and 1,3-MU. Thus, lowering the pH to 4.5 resulted in a marked reduction in the retention time and resolution of these compounds by virtue of reducing the fraction of these molecules in the ionic state, capable of ion pairing. Increasing the pH to 5.0 resulted in the opposite effect: the retention time of 1-MU and 1,3-MU increased by virtue of the higher equilibrium concentration of the paired, neutral species. The pH of 4.75 was optimal for resolution and was used in all analyses. The concentration of tetrabutyl ammonium ion was also varied at constant pH. As would be expected, reduced concentration of the ion caused a small reduction in the retention time of the methyl uric acids, and increased concentration produced an increased retention, although this effect was not as marked as that of pH. A tetrabutyl ammonium concentration of 0.005 *M* was determined to be optimum.

All chromatographic conditions were optimized by maximizing resolution and selectivity between small endogenous peaks and by optimized chromatography of the standard mixture shown in Fig. 2, which contained a mixture of 3-methyl xanthine (3-MX), 1-methyl xanthine (1-MX), 1,3-dimethyl xanthine (1,3-MX, theophylline), 1,7-dimethyl xanthine (1,7-MX, paraxanthine), 1,3,7-trimethyl xanthine (caffeine), 1-methyl uric acid (1-

MU), 1,3-dimethyl uric acid (1,3-MU) and β-hydroxyethyltheophylline (internal standard). This system allows the selective determination of theophylline and its metabolites including 1,7-MX; these compounds are not resolved in many other HPLC procedures [16—18].

The retention times observed were: 3-MX, 6 min; 1-MX, 8 min; 1-MU, 11 min; 1,3-MU, 15 min; 1,7-MX, 17 min; theophylline, 20 min; β-hydroxyethyltheophylline, 23 min; and caffeine, 30 min. As the retention times of endogenous uric acid and xanthine are substantially shorter than the first peak of interest (3-MX) these compounds do not interfere with the present procedure.

The use of an exhaustively silylated column together with a replaceable precolumn resulted in maintained column performance after the injection of several thousand samples over a period of approximately one year. The high-efficiency column (number of theoretical plates was approximately 1000 per cm), together with a fast time constant detector, allowed for the resolution of rapidly eluting peaks when the system was run isocratically with 10% methanol. However, in order to maximize quantitative estimation of the peaks and to elute endogenous peaks with long retention times, the gradient elution system was ultimately used for routine assays. The choice of 280 nm as the detection wavelength was to optimize the absorbance of both the methyl xanthines (λ_{max} = 270 nm) and methyl uric acids (λ_{max} = 290 nm). Although both of these groups of compounds have significant absorbance at 254 nm, more interference and spurious peaks were observed at this wavelength.

Quantitation, reproducibility and accuracy

Peak area ratios appeared to give more reproducible results than peak height ratios. The values for recovery show that theophylline, 3-MX and 1,3-MU can be extracted reproducibly at concentrations of up to 400 μg/ml. Above 150 μg/ml the standard curve for 1-MU became non-linear. The individual calibration curves for 1-MU (between 2 and 150 μg/ml) showed excellent linearity ($r^2 > 0.998$) but relatively large differences in slopes between assays were seen (see Table I). This was probably due to very minor variations in extraction conditions; e.g. pH, organic extractant composition between assays. For the other compounds the standard curve was linear over a wider range and showed good reproducibility. The limit of sensitivity (defined as three times the baseline noise) for the compounds measured as a pure substance in aqueous solutions was approximately 100 ng/ml. However, even though some subjects abstained from caffeine intake for longer than 48 h, their blank urine samples contained small residual peaks at retention times corresponding to 3-MX, 1-MU and 1,3-MU, which represents approximately 1—2 μg/ml of these compounds. Thus the limit of sensitivity of the assay is dependent upon these blank values, which possibly originate from unavoidable dietary intake of various xanthine derivaties.

These results indicate that the procedure has sufficient selectivity, sensitivity, precision and accuracy to be suitable for pharmacokinetic studies.

Comparison with other HPLC assays of urinary theophylline and metabolites

Desiraju et al. [17] proposed a direct assay of theophylline and metabolites

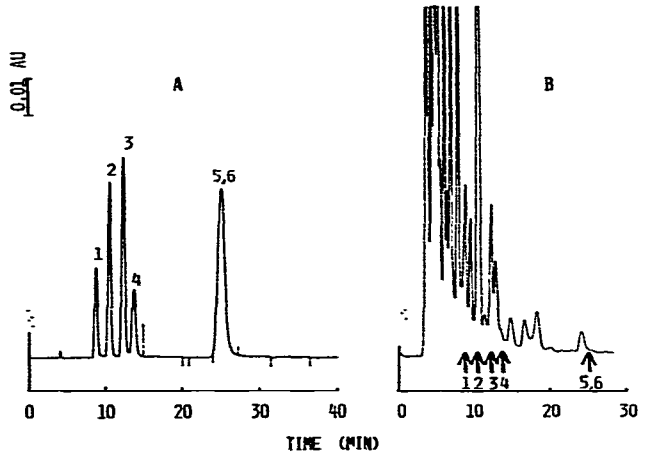

Fig. 4. Chromatograms of a standard mixture (A) and blank urine (B) run using the method of Desiraju et al. [17]. Arrows indicate elution times for compounds in standard test mixture. See Fig. 1 for identification code.

in which urine was directly injected into a 30-cm Waters Bondapak C_{18} column using 12% methanol in 0.05 *M* KH_2PO_4 as the eluent. With this system, the authors reported that the major endogenous urinary constituents eluted in the first 7 min and that only the peaks for hypoxanthine and xanthine were subject to interference since theophylline metabolite peaks eluted at longer retention times. We have been unable to validate these results. Fig. 4A is a reproduction of the chromatogram of a standard mixture (10 μl; 50 μg/ml) in water under the above conditions. Fig. 4B is a chromatogram of a typical blank urine sample (10 μl) under the above conditions. The arrows indicate the positions where theophylline and its metabolites should elute. It can be seen that interferences of the order of 10—100 μg/ml are present in this region. Similar results were obtained with other blank urine samples. Clearly, the degree of accuracy and precision required for pharmacokinetic studies can not be met in all circumstances.

Grygiel and co-workers [18] also reported a urinary assay which was a modification of the plasma assay of Orcutt et al. [21]. These workers had to extract the acidified urine with dichloromethane to assay theophylline; however, they report being able to separate the metabolites of theophylline by direct injection of 10 μl of urine into a 5-μm reversed-phase column (15 cm) using 10 m*M* acetate buffer (pH 5) as the eluent. The retention times were reported as 1-MU = 4.5, 3-MX = 10, 1-MX = 12.5, 1,3-MU = 15.5 min. Two separate assay procedures were therefore employed to measure theophylline and its metabolites in urine. Fig. 5A represents the chromatogram of a standard mixture of theophylline and metabolites under the above conditions using a 25 cm column, and Fig. 5B is a typical blank urine. The arrows once again indicate the positions where the metabolites should elute. Elimination of methanol from the mobile phase allows better resolution of some peaks (e.g., 2 and 4) but interferences were still present. In addition, carry-over peaks from previous injections, representing compounds with long retention times, were frequently seen. Other urines gave similar results. Examination

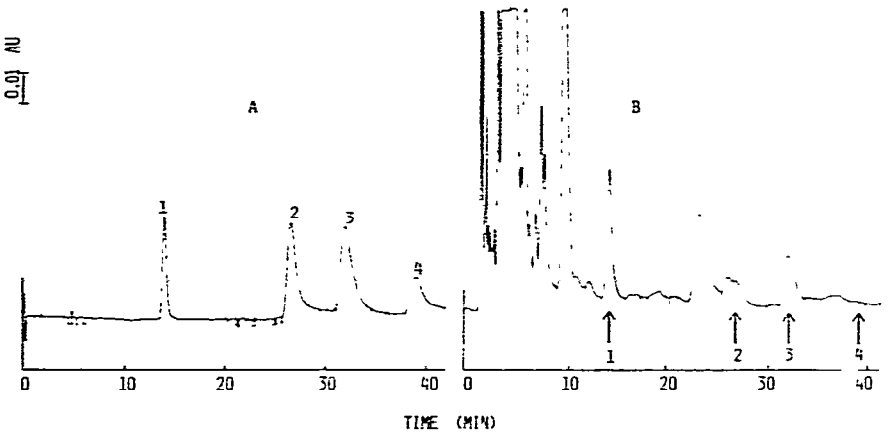

Fig. 5. Chromatograms of a standard mixture (A) and blank urine (B) run using the method of Grygiel et al. [18]. Arrows indicate elution times for compounds in standard test mixture. See Fig. 1 for identification code.

of Fig. 4A and B and Fig. 5A and B indicate the difficulties of utilizing these assays for the quantitative analysis of theophylline and its metabolites in most urine samples.

In addition to these assays, Aldridge et al. [22] investigated caffeine metabolism in the newborn using an HPLC assay to estimate the metabolites. The assay involves an extraction step from urine saturated with ammonium sulphate using chloroform—isopropanol (85:15, v/v) as the solvent. The residue of the organic layer was separated using a μBondapak C_{18} column and a solvent gradient comprising 1.5% (v/v) acetonitrile up to 7.5% in 0.5% (v/v) acetic acid over 15 min. The authors reported that the extraction step eliminated interfering peaks from endogenous compounds. With the important exception that 1,3-MX and paraxanthine were not resolved, the method appears to separate the metabolites of theophylline. However, the extraction efficiency of the methylated uric acids was low (in particular 1-MU, 36% ± 12%) by virtue of the polarity of these compounds.

The use of the ion-pair extraction procedure reported herein improves markedly the extraction efficiency of the uric acid derivatives, particularly of 1-MU with an average extraction efficiency of approximately 95% (see Table I).

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