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DETERMINATION OF THEOPHYLLINE AND ITS METABOLITES IN PLASMA AND URINE BY REVERSED-PHASE LIQUID CHROMATOGRAPHY USING AN AMINE MODIFIER

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SUMMARY

A high-performance liquid chromatographic method for the determination of the concentrations of theophylline and its metabolites in plasma and urine samples is presented. The method uses the decylammonium ion, an N-alkylammonium modifier, and makes it possible to separate theophylline and its metabolites from other uric acid or xanthine derivatives, especially 1,7-dimethylxanthine, a metabolite of caffeine. The plasma and urine purification procedure is convenient, rapid and reproducible, and it can be used to determine low plasma and urine concentrations.

INTRODUCTION

In order to assess the metabolic and pharmacokinetic variations of theophylline, which influence its bronchodilator activity in various pathological situations, we have developed an analytical procedure for the determination of the drug itself and of its uric acid and xanthine metabolites.

The methods most commonly used to assay theophylline and its metabolites

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involve reversed-phase high-performance liquid chromatography (HPLC) on C_{18} alkylsiloxane columns with UV detection. Mobile phases are usually buffered solutions, at pH values of ca. 4–5. Under isocratic conditions [1,2] reducing interferences takes a very long time, and caffeine metabolites are not resolved.

The procedure can be improved by using a gradient elution programme, with ion-pair formation, according to Muir et al. [3]; xanthine and uric acids in the urine are first extracted with tetrabutylammonium (TBA) as the counter-ion. Because of its advantages this method has been extensively used, and modifications of the mobile phase (pH, ionic strength) and of the elution gradient have increased its selectivity and reproducibility [4–6]. For plasma assay, Muir et al. [7] advocate isocratic elution at pH 4.75 with TBA, giving a very fast separation (retention time, $t_R = 2$–5.5 min), but an endogenous compound (X) interferes with 1,7-dimethylxanthine (1,7-DMX).

Farish and Wargin [8], using TBA (pH 4), obtained a resolution between 1,7-DMX and theophylline, but did not describe separation of theophylline metabolites.

Voelter et al. [9] achieved ion-pair separation of anionic forms of purine and pyrimidine derivatives at pH 7.5 using TBA as cationic reagent. This procedure results in an extremely short column life.

Other ion-pair methods, using heptanesulphonic acid at pH 4 [10], trichloroacetic acid [11] or sulphonic exchange resins [12], have been suggested, but these methods fail to separate theophylline metabolites. More recently, Teunissen et al. [13] have reported the separation of urinary metabolites by ion-pair reversed-phase HPLC according to Muir et al. [3] and of plasma metabolites by reversed-phase HPLC according to Tse and Szeto [11].

For urinary determination, the most selective and sensitive method available at present is that of Tang-Liu and Riegelman [6]. However, it involves ion-pair reversed-phase chromatography with TBA at pH 4.9 with one elution gradient, which is time-consuming.

This paper presents a method for the separation of theophylline metabolites that permits plasma and urinary determinations by isocratic elution with hitherto unused modifiers. This method also separates other uric acid or xanthine derivatives, especially 1,7-DMX. The different theophylline and caffeine metabolites can be determined simultaneously by minor modification of the chromatographic parameters.

EXPERIMENTAL

Chemicals

Theophylline (1,3-dimethylxanthine, 1,3-DMX), dyphylline and caffeine (1,3,7-trimethylxanthine, 1,3,7-TMX) were obtained from Sigma (St. Louis, MO, U.S.A.); 3-methylxanthine (3-MX), 7-methylxanthine (7-MX), 1-methylxanthine (1-MX), 1,7-dimethylxanthine (1,7-DMX), 1-methyluric acid (1-MU), 3-methyluric acid (3-MU), 1,3-dimethyluric acid (1,3-DMU), 1,9-dimethyluric acid (1,9-DMU), tryptophan and decylamine (DCA) were obtained from Fluka

(Buchs, Switzerland). Methanol and acetonitrile were obtained from Merck (Darmstadt, F.R.G.).

The degassed mobile phase was prepared with 0.1 *M* sodium acetate buffer (pH 4.0) containing either 0.75 m*M* decylamine and 20 ml/l acetonitrile, (2%, v/v) for plasma assay, or 0.75 m*M* decylamine and 10 ml/l acetonitrile (1%, v/v) for urinary assay. A Sep-Pak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) was used for urine purification.

Chromatography

Chromatography was performed on a Varian 5000 instrument equipped with a stainless-steel column [Ultrasphere IP (5 μm), 15 cm×0.4 cm I.D.; Beckman Instruments, Berkeley, CA, U.S.A.] and a guard column (4 cm×0.2 cm I.D.) filled with RP-18 LiChroprep (particle size 25–40 μm). UV detection was performed with a Spectroflow 773 (Kratos Analytical Instruments, Westwood, NJ, U.S.A.) set at 280 nm with a sensitivity of 0.02 (plasma) or 0.10 (urine) a.u.f.s. A peak integrator CR-3A (Shimadzu, Kyoto, Japan) was used, and automatic injections of 20 μl were performed with a WISP injector (Waters Assoc.). All separations were carried out at room temperature (24°C) and a flow-rate of 2 ml/min.

Standards

For plasma studies, standard solutions of theophylline and its metabolites (1-MU, 3-MX, 1,3-DMU) were prepared. The calibration curve was based on xanthine- and oxopurine-free plasma loaded with theophylline (2–25 mg/l), theophylline metabolites (0.05–5 mg/l) and internal standard dyphylline (20 mg/l), using stock solutions at concentrations of 100 mg/l prepared in a 0.002 *M* aqueous solution of sodium hydroxide. The loaded plasma samples were divided and stored at −20°C until analysis.

For urinary studies, a xanthine- and oxopurine-free urine sample was loaded with theophylline metabolites (up to 100 mg/l), theophylline (up to 50 mg/l) and 1,9-DMU as internal standard (20 mg/l).

Analytical procedure

Stock dyphylline solution (100 μl) and acetonitrile (200 μl) for protein precipitation were added to plasma (100 μl). After vortex-mixing for 1 min and centrifugation at 3000 *g* for 5 min, the supernatant was transferred and evaporated to dryness under nitrogen at 45°C. The residue was reconstituted with 100 μl of mobile phase and, after mixing, 20 μl were injected into the loop. The separation took ca. 15 min.

For urine assay, 300 μl of 1,9-DMU stock solution were added to a 1-ml urine sample, and a 400-μl aliquot of the mixture was purified on a C_{18} Sep-Pak column. After washing with 2 ml of 0.1 *M* sodium acetate buffer (pH 4.4), elution was performed with 1 ml of a mixture of 0.1 *M* sodium acetate buffer (pH 4.4)–acetonitrile (80:20, v/v). A 20-μl volume of eluate was then injected into the chromatograph, and the Sep-Pak column was washed with methanol (5 ml) and distilled water (5 ml) before re-use.

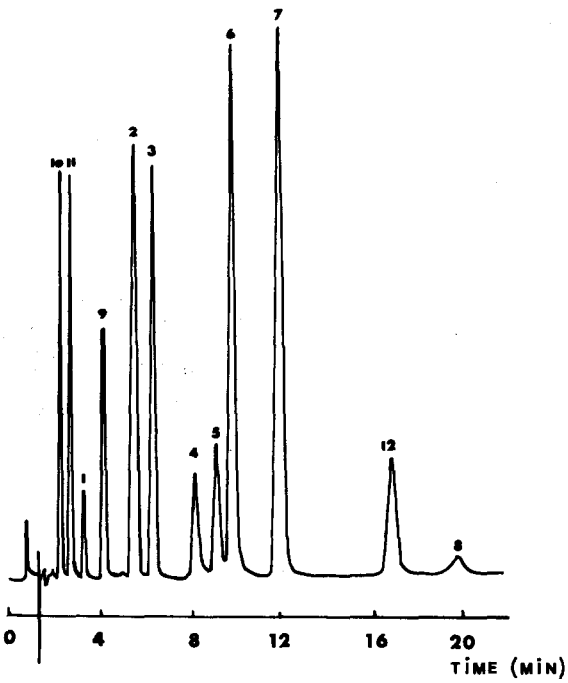

Fig. 1. HPLC of a mixture of the following compounds: 1=3-MX (0.5 mg/l); 2=1-MU (2 mg/l); 3=1,3-DMU (2.2 mg/l); 4=tryptophan (2.2 mg/l); 5=1,7-DMX (2.5 mg/l); 6=theophylline (6 mg/l); 7=dyphylline (8 mg/l); 8=caffeine (2 mg/l); 9=1-MX (1.5 mg/l); 10=3-MU (1.5 mg/l); 11=7-MX (1.5 mg/l); 12=1,9-DMX (4 mg/l). Mobile phase, 0.75 m*M* DCA and 20 ml/l acetonitrile in buffer (pH 4.0).

RESULTS

Fig. 1 shows the resolution of a mixture of theophylline, its metabolites (1-MU, 3-MX, 1,3-DMU), 1,7-DMX and tryptophan, usually found in plasma, and Fig. 2 shows the results with loaded plasma. The linearity of standard curves was checked at the concentrations studied (0.05–5 mg/l for metabolites and 2–25 mg/l for theophylline), with correlation coefficients up to 0.999.

The limit of quantitation, corresponding to a 2:1 signal-to-noise ratio, was 0.01 mg/l for 3-MX and 1-MU, 0.02 mg/l for 1,3-DMU and 0.1 mg/l for theophylline.

A precision study on loaded plasma included reproducibility (day-to-day) over fifteen days (Table I) and repeatability (within-run) on thirty samples (Table II).

A chromatogram from loaded urine is shown on Fig. 3; the linearity of standard curves was checked at the concentrations studied (5–50 mg/l for 3-MX, 1-MU and theophylline, 10–100 mg/l for 1,3-DMU) with correlation coefficients up to 0.997. The limit of quantitation for each compound, after extraction, was 0.5 mg/l for 3-MX, 1-MU, and 1,3-DMU, and 1 mg/l for theophylline. The precision study is presented in Tables III (day-to-day) and IV (within-day). Because of the purification, coefficients of variation were higher for urine than for plasma assays.

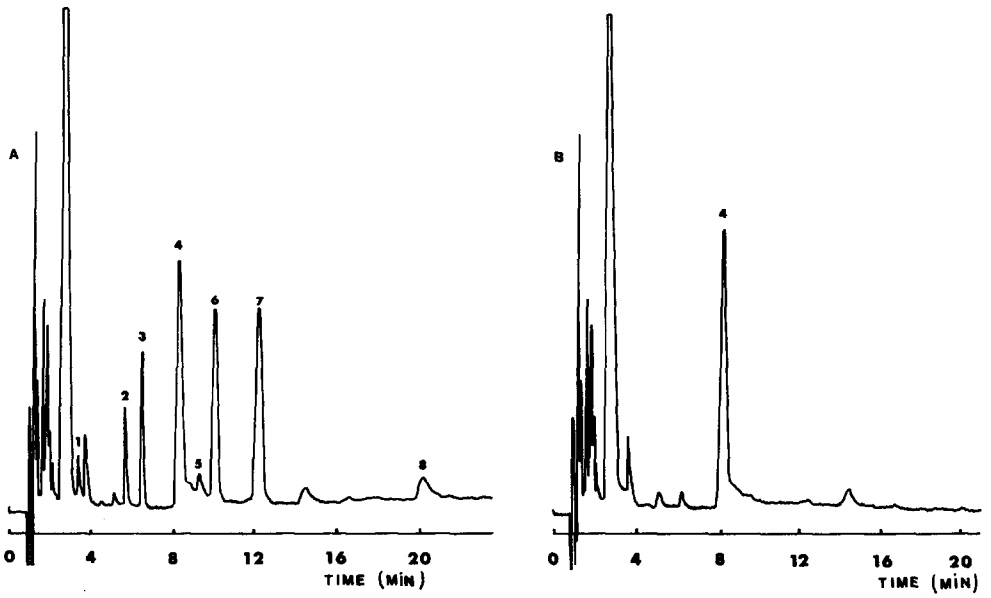

Fig. 2. HPLC of plasma spiked with theophylline and metabolites. (A) 1=3-MX (0.2 mg/l); 2=1-MU (0.5 mg/l); 3=1,3-DMU (1 mg/l); 4=tryptophan (8 mg/l); 5=1,7-DMX (1 mg/l); 6=theophylline (8 mg/l); 7=dyphylline (10 mg/l); 8=caffeine (4 mg/l). (B) Blank plasma. Mobile phase, 0.75 m*M* DCA and 20 ml/l acetonitrile in buffer (pH 4.0).

TABLE I

DAY-TO-DAY REPRODUCIBILITY OF THE HPLC ASSAY IN PLASMA SAMPLES

Compound	Standards 1		Standards 2	
	Concentration (mean ± S.D., $n=15$) (mg/l)	Coefficient of variation (%)	Concentration (mean ± S.D., $n=20$) (mg/l)	Coefficient of variation (%)
3-MX	0.7 ± 0.041	5.7	1.5 ± 0.041	2.8
1-MU	2.1 ± 0.097	4.7	1.0 ± 0.057	5.5
1,3-DMU	2.2 ± 0.075	3.4	0.98 ± 0.034	3.4
Theophylline	11.7 ± 0.25	2.1	20.6 ± 0.44	2.1

TABLE II

WITHIN-DAY REPRODUCIBILITY OF THE HPLC ASSAY IN PLASMA SAMPLES

Compound	Concentration (mean ± S.D., $n=30$) (mg/l)	Coefficient of variation (%)
3-MX	0.37 ± 0.013	3.6
1-MU	0.30 ± 0.022	7.3
1,3-DMU	0.73 ± 0.017	2.3
Theophylline	10.21 ± 0.37	3.6

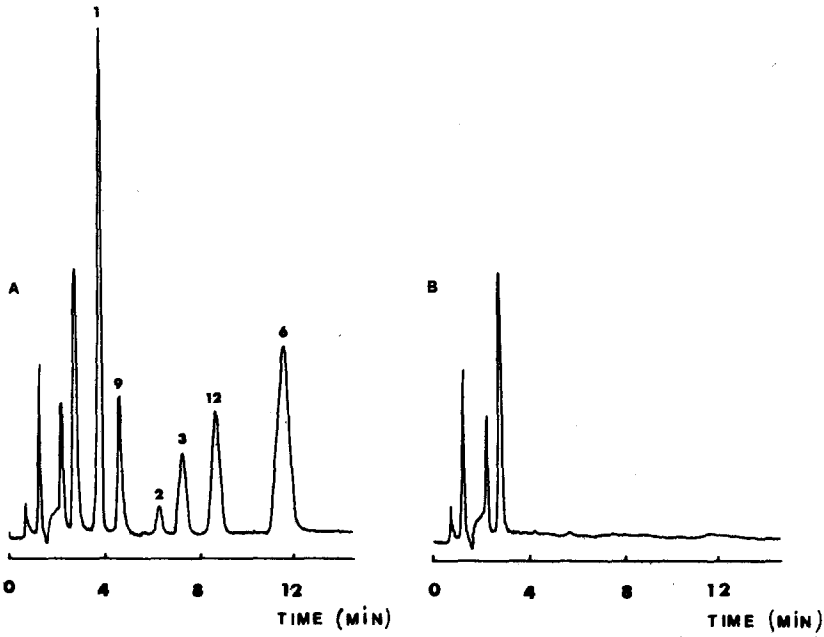

Fig. 3. HPLC of theophylline and metabolites from extracted urine. (A) 1=3-MX (50 mg/l); 2=1-MU (5 mg/l); 3=1,3-DMU (8 mg/l); 6=theophylline (50 mg/l); 9=1-MX (20 mg/l); 12=1,9-DMU (23 mg/l). (B) Blank urine. Mobile phase, 0.75 m*M* DCA and 10 ml/l acetonitrile in buffer (pH 4.0).

TABLE III

DAY-TO-DAY REPRODUCIBILITY OF THE HPLC ASSAY IN URINE SAMPLES

Compound	Standards 1		Standards 2	
	Concentration (mean ± S.D., $n=15$) (mg/l)	Coefficient of variation (%)	Concentration (mean ± S.D., $n=15$) (mg/l)	Coefficient of variation (%)
3-MX	11.3 ± 1.3	11.4	51.2 ± 1.9	3.8
1-MU	16.3 ± 1.2	7.3	Not determined	Not determined
1,3-DMU	36.5 ± 2.4	6.6	7.9 ± 0.8	10.6
Theophylline	10.7 ± 1.2	11	49 ± 4.3	8.8

TABLE IV

WITHIN-DAY REPRODUCIBILITY OF THE HPLC ASSAY IN URINE SAMPLES

Compound	Concentration (mean ± S.D., $n=20$) (mg/l)	Coefficient of variation (%)
3-MX	12.3 ± 0.6	4.8
1-MU	1.7 ± 0.2	10.3
1,3-DMU	7.3 ± 0.6	8.2
Theophylline	8.0 ± 0.8	10.8

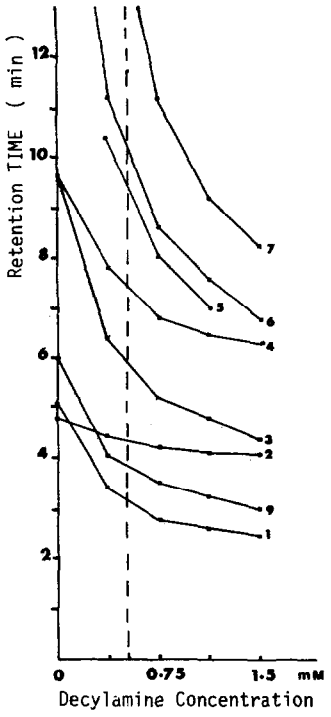

Fig. 4. Variation in retention time with DCA concentration in mobile phase containing 2% acetonitrile and 0.1 *M* acetate buffer (pH 4.0). Curves: 1 = 3-MX; 2 = 1-MU; 3 = 1,3-DMU; 4 = tryptophan; 5 = 1,7-DMX; 6 = theophylline; 7 = dyphylline; 9 = 1-MX.

DISCUSSION

Decylamine is a hydrophobic long alkylated cationic ammonium which, when fixed on ODS gel, modifies the polarity of these reversed phases by mechanisms described elsewhere [14–18] with ion-pair reversed-phase chromatography, using modifiers. Column loading with decylammonium cation is demonstrated by the long time required to reach a steady state in the column and the simultaneous DCA decrease in the mobile phase.

The fixation of DCA on the stationary phase produces a strongly positive charge density, which reduces the retention time of the non-polar dimethylxanthine derivative (1,3-DMX) and of the weakly polar dimethyluric acid derivative (1,3-DMU). This "column effect" is enhanced by increasing the DCA concentration from 0 to 1.5 m*M* (close to the limit of solubility) in the mobile phase (Fig. 4). The shorter and approximately constant retention time of the 1-methyluric acid derivative is independent of the DCA concentration. This behaviour may be attributed to the solvent–solute interaction, especially in the presence of acetonitrile. It is called here the "solvent effect". With the method we propose, an ion-pair mechanism is excluded for the different uric acid derivatives, since it would lead to an increase in retention times as reported by Tang-Liu and Riegelman [6] who performed the separation at pH 4.9 in the presence of 5 m*M* TBA.

TABLE V

pK_a VALUES OF XANTHINE AND URIC ACID DERIVATIVES

Compound	pK_1	pK_3	pK_7	pK_9	Ref.
1-MX		7.9	12.2		19
		7.7	12.05		20
7-MX	~13	8.33			20
	>13	8.42			19
3-MX	11.92		8.45		19
	11.90		8.32		20
1,3-DMX			8.68		19
			8.77-8.80		20
1-MU		10.6		5.75	20
		11.69±0.1		5.48±0.1	21
				5.87±0.03	–*
3-MU	>12			5.75	20
				6.2	19
				6.02±0.1	21
7-MU		10.90±0.1		5.45±0.05	21
1,3-DMU				5.75	20
				6.22±0.05	21
9-MX	>13	6.12			19
1,7-DMX		8.81			20
		8.65			19
1,9-DMU		5.11	11.98		21
		5.99			19
Caffeine	14				20

*UV spectrophotomeric data determinated by the authors.

With the present method the control of the different factors (pH, ionic strength, DCA and acetonitrile concentrations) leads to a suitable resolution of xanthine derivatives (dioxopurines) and uric acid derivatives (trioxopurines).

Influence of pH, DCA and acetonitrile

The separations we observed were due to differences in the polarity of the solutes in the buffered aqueous solution (pH 4.0) and to the DCA modifier, which modulate the retention of less polar solutes in the stationary phase. DCA ensures substantial reduction of the retention time for all solutes, except the monomethyluric acid derivatives, and a suitable separation of all derivatives, with a reduced acetonitrile concentration in the mobile phase.

The pK_a values of the different solutes studied are listed in Table V. The xanthine derivatives with a weak acid character (pK_a ca. 8) were clearly distinguished from the uric acid derivatives (pK_a ca. 6), called "acidic". Thus, at pH 4.0, xanthine derivatives are in a non-ionic form and uric acid derivatives are slightly ionized (1–10% depending on substitution), and the chromatographic separations were almost entirely due to the polarity of each solute under fixed conditions.

Monomethylxanthine derivatives with many tautomeric conformations or strong hydrogen-bonding character are relatively polar, so that the elution order 7-MX, 3-MX, 1-MX was justified. 1-MX is more acidic (pK_a 7.9) than the other two and has limited tautomeric forms

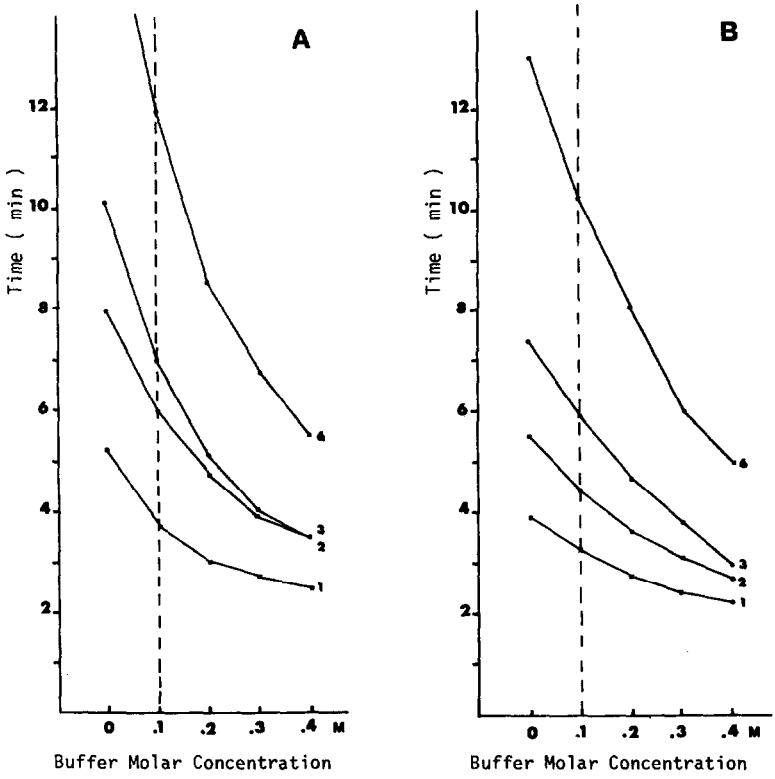

Fig. 5. Variation of retention time with buffer molar concentration of mobile phase at pH 4.0: (A) acetonitrile = 0%, decylamine = 0.5 m*M*; (B) acetonitrile = 2%, decylamine = 0.5 m*M*. Curves: 1 = 3-MX; 2 = 1-MU; 3 = 1,3-DMU; 6 = theophylline.

The dimethylxanthine derivatives with weaker acidic character are essentially non-polar, having two N-methyl groups. For the same reasons of tautomeric conformations or hydrogen-bonding interactions, 3,7-DMX and 1,7-DMX have shorter retention times than 1,3-DMX (theophylline). The latter has neither tautomeric forms nor hydrogen-bond donor activity. In the presence of DCA, the retention of all derivatives is reduced, as their approach to the non-polar sites in the stationary phase is restricted.

The most acidic uric acid derivatives are 1-MU and 7-MU. At pH 4.0 they also are in non-ionic form, and in the absence of DCA their elution order follows the same sequence as the xanthine series. However, each uric acid derivative is more rapidly eluted than the corresponding xanthine derivative. Thus, for a mobile phase of 0.1 *M* sodium acetate (pH 4.0) – acetonitrile (2%), without DCA, 3-MU is more rapidly eluted than 3-MX, and 7-MU, 1-MU and 1,3-DMU are more rapidly eluted than 7-MX, 1-MX and 1,3-DMX, respectively (Fig. 5).

1,3-DMU is the least polar metabolite, due to its two N-methyl groups, and it has the longest retention time on the C_{18} reversed-phase column. Even when acetonitrile was added in the mobile phase, its retention time remained high (t_R = 9.5–10 min) (Fig. 4). Conversely, in the presence of DCA and the absence of acetonitrile, the retention time decreases owing to the column effect (t_R = 7

min). In the presence of DCA and acetonitrile, therefore, the marked decrease of t_R results from the solvent and column effects (Fig. 4).

The retention time for 1-MU remains approximately constant (4.4–4.8 min) (Fig. 4) for a mobile phase (pH 4.0) with fixed acetonitrile concentration (2%), whether DCA is present or not. This is unrelated to a column effect and only due to the solvent effect (t_R=4.5 min) (Fig. 5B). Acetonitrile, with a high dipole moment (μ=3.84 D) [22] is a better solvent for uric acid derivatives, and the retention times are longer in its absence (t_R=6 min) (Fig. 5A).

Purification procedure

Plasma assays were performed after minimal sample treatment, i.e. without extraction, but by acetonitrile protein precipitation, which permits determination of xanthine or uric acid derivatives without interferences.

Urinary determinations needed prior purification to remove more or less polar endogenous compounds. Thus, 400 μl of urine loaded with internal standard were filtered on a C_{18} Sep-Pak column. The more polar endogenous compounds were discarded by a first elution with acetate buffer, and a second elution with acetonitrile (20%) was performed to extract the purine derivatives. An optimum acetonitrile concentration helps to differentiate between polar and non-polar compounds. Concentrations lower than 10% give incomplete recovery of metabolites, whereas with a higher concentration (50%) non-polar contaminants are eluted and the duration of chromatographic analysis for optimum resolution is increased (2 h). With the method presented, recoveries were 80% for 1,3-DMU and 1,9-DMU, 75% for theophylline, 70% for 3-MX and 66% for 1-MU.

Comparison with other methods

After comparative studies on theophylline assays [gas chromatography (GC), HPLC, enzyme immunoassay (EIA), gas chromatography–mass spectrometry (GC–MS)] [23], the French Society for Clinical Biology recommended HPLC as reference method [23,24].

For validation, 144 plasma samples from theophylline-treated subjects were assayed by three methods: the method presented here (X), the normal-phase HPLC method ($Y1$) described by Aulagner et al. [24] and the enzyme-multiplied immunoassay technique (EMIT) EIA method ($Y2$) (Fig. 6).

Linear regression equations and correlation coefficients were $Y1=0.989X-0.018$ ($r=0.987$); $Y2=1.029X+0.107$ ($r=0.964$) and $Y2=1.035Y1+0.216$ ($r=0.972$).

The method presented here was in good agreement with the normal-phase HPLC method with no bias, whereas a lesser correlation with the EMIT-EIA method was observed. Similarly there was no close correlation between the normal-phase HPLC method and the EMIT-EIA method. Finally, Student's t test showed non-significant differences between the HPLC methods ($t=1.166$), whereas significant differences were observed between our method and the EMIT-EIA method ($t=2.153$, $p<0.05$) and between the latter method and normal-phase HPLC ($t=3.165$, $p<0.01$).

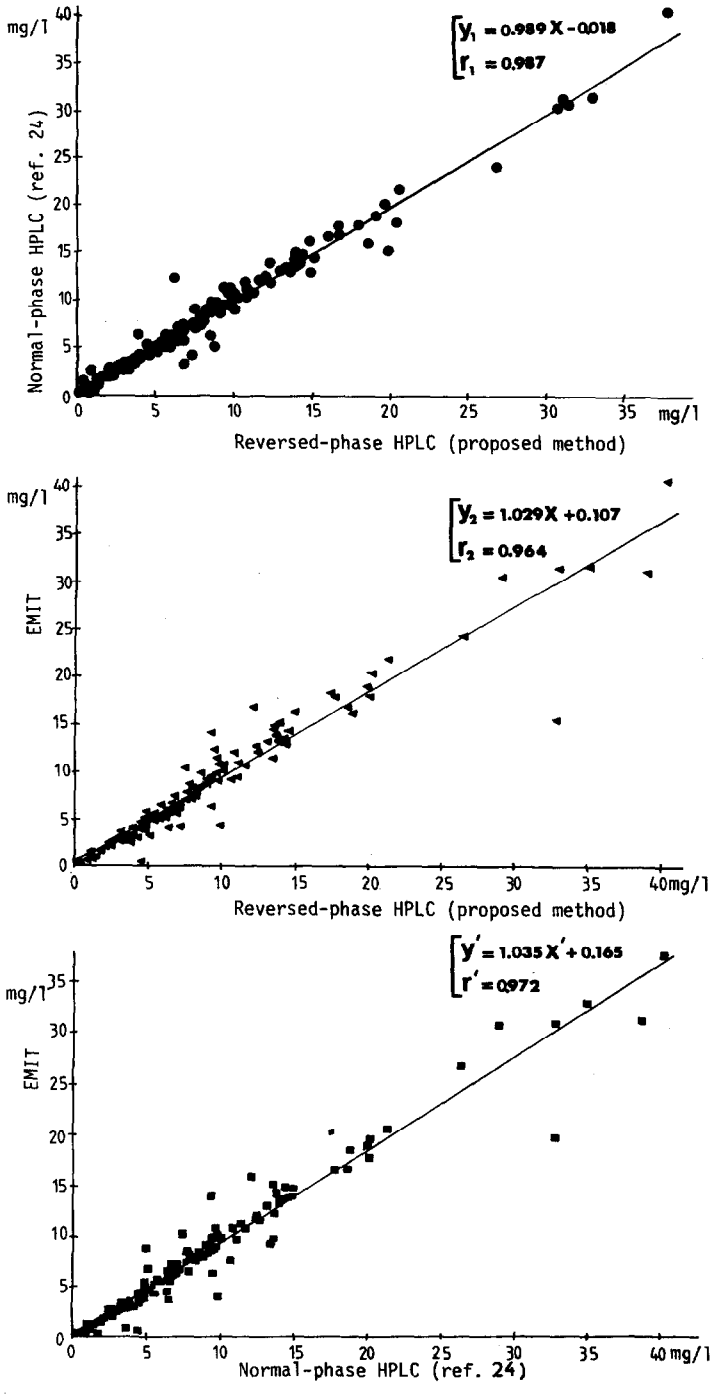

Fig. 6. Comparison of the HPLC and EMIT methods for determination of theophylline in 144 plasma samples.

TABLE VI

PLASMA LEVELS OF THEOPHYLLINE AND ITS MAJOR METABOLITES

Values are 1 h after administration of Armophylline® (300-mg oral dose).

Compound	Concentration (mean ± S.D., $n = 12$) (mg/l)
3-MX	0.06 ± 0.04
1-MU	0.17 ± 0.05
1,3-DMU	0.42 ± 0.15
Theophylline	7.74 ± 1.46

Pharmacokinetic application

Plasma levels were good agreement with those from other methods, with respect to 1,3-DMU [25,26] (C_{max} ca. 30–45 min after single oral dose), 1-MU and 3-MX [26] (C_{max} ca. 45–120 min and 6–9 h, respectively) (Table VI). Our method can be used to assess urinary levels of theophylline and its metabolites without modifying the analytical procedure.

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