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Short communication

Determination of theophylline and its metabolites in human urine and plasma by high-performance liquid chromatography

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Abstract

A method for the quantitation of theophylline (13DMX) and the three metabolites, 1-methyluric acid (1MU), 3-methylxanthine (3MX) and 1,3-dimethyluric acid (13DMU) in human plasma and urine has been developed. The method is based on a simple one-step liquid–liquid extraction with ethylacetate–2-propanol followed by isocratic, reversed-phase high-performance liquid chromatography with UV detection (detection wavelength: 273 nm). The overall mean recoveries ranged from 86 to 95% for the four compounds. The detection limit was 1 μM for 1MU, 3MX and 13DMU and 2 μM for 13DMX in urine, and 0.1 μM for 1MU, 3MX and 13DMU and 0.2 μM for 13DMX in plasma. The intra-day and inter-day coefficient of variation was <6% and <9%, respectively, and the accuracy was within ±10% in both urine and plasma.

The simple but sensitive method is highly suitable for the development of theophylline as a probe drug for assessing CYP1A2 activity in man.

Keywords: Theophylline; 1-Methyluric acid; 3-Methylxanthine; 1,3-Dimethyluric acid

1. Introduction

Theophylline (1,3-dimethylxanthine, 13DMX) is a bronchodilating agent used in the management of asthma. In humans, theophylline is eliminated almost exclusively by the cytochrome P-450 mediated hepatic oxidation, predominantly by 8-hydroxylation to 1,3-dimethyluric acid (13DMU), and this pathway accounts for about half of the total theophylline clearance [1]. In addition theophylline is N-demethylated to 1-methylxanthine (1MX) and 3-methylxanthine (3MX). The former is further oxidized by xanthine oxidase [2] to 1-methyluric acid (1MU), which is the only theophylline 1-demethylation product seen in human plasma and urine. The cytochrome P-450 enzyme CYP1A2 has been shown to catalyse 80–90% of the N-demethylations and about 50% of the 8-hydroxylation of theophylline in vitro [3,4], and the remainder of the 8-hydroxylation is believed to involve CYP2E1 [4,5]. Caffeine is commonly used as a model drug for assessing CYP1A2 activity in man [6–8], but the proposed caffeine metabolic ratios all seem to have their shortcomings due to the very complex metabolism of caffeine and the involvement of other P-450s [9,10]. Although theophylline is chemically closely related to caffeine, it has a much simpler metabolism pattern compared to caffeine. Hence, assessment of theo-

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phylline metabolism and especially the two N-demethylation pathways could give a more precise measurement of the CYP1A2 activity in humans. Thus, theophylline has the potential for being developed as a model drug for the assessment of CYP1A2 in man.

An HPLC method was developed for simultaneous quantitation of theophylline and metabolites in urine and plasma. Methods for the analysis of theophylline and metabolites have been published previously, but commonly highly toxic solvents such as chloroform [11] and acetonitrile [12–14] are used for the extraction of theophylline and metabolites from urine and plasma. In order to improve the working safety in the laboratory the aim was to use a less hazardous solvent for the extraction of theophylline and metabolites from urine and plasma. Here we describe an isocratic and sensitive HPLC method based on a simple one-step extraction procedure with ethylacetate–2-propanol as extraction solvent.

2. Experimental

2.1. Chemicals and reagents

Theophylline anhydrous (M_r=180.2, purity>98%) and β-hydroxyethyltheophylline (M_r=244.2, purity>98%) were purchased from Sigma (st. Louis, MO, USA), and 1-methyluric acid (M_r=182.1, purity>98%), 3-methylxanthine (M_r=166.4, purity>98%) and 1,3-dimethyluric acid (M_r=196.2, purity>99%) were purchased from Fluka (Buchs, Switzerland). Ethylacetate, 2-propanol, ethanol and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from either Merck or Sigma. Water was purified by osmosis and distillation.

2.2. Preparation of standard solutions

Stock solutions of 5 m*M* 1MU, 3MX, 13DMU, 13DMX and the internal standard, β-hydroxyethyltheophylline (β-OH-ET), were prepared in 30% v/v ethanol in distilled water. The mixtures were used to prepare the appropriate standards in plasma and urine collected from a subject after four days of xanthine-free diet.

2.3. Extraction procedure

2.3.1. Urine

To 40 μl of urine (acidified to ca. pH 4 by the addition of 300 μl 1 *M* HCl to 10 ml of urine), 50 μl of a 300 μ*M* internal standard solution were added in a 10-ml glass test tube, and the total volume was adjusted to 400 μl with acetate buffer (0.01 *M*, pH 4.0).

Subsequently, 5 ml ethylacetate–2-propanol (93:7, w/w) were added. The mixture was shaken for 10 min, and after centrifugation for 10 min at 1000 *g* the tube was maintained at −30°C until the aqueous phase was frozen. The organic phase was transferred to a conical glass test tube and evaporated to dryness at 55°C under a steam of nitrogen. The residue was reconstituted in 300 μl mobile phase and vortex-mixed for 10 s. A 40-μl aliquot was injected onto the column.

2.3.2. Plasma

To 500 μl of plasma, 25 μl of a 300-μl internal standard solution and 75 μl 1 *M* HCl were added in a 10-ml glass test tube (acidification of sample to ca. pH 4). Subsequently, 5 ml ethylacetate–2-propanol (90:10, w/w) were added. The mixture was shaken for 10 min, and after centrifugation for 10 min at 1000 *g* the tube was maintained at −30°C until the aqueous phase was frozen. The organic phase was transferred to a conical glass test tube and evaporated to dryness at 55°C under a steam of nitrogen. The residue was reconstituted in 300 μl mobile phase by maintaining the tubes in an ultrasonic bath until the residue was dissolved. After centrifugation for 15 min at 13 000 *g*, a 40-μl aliquot of the supernatant was injected onto the column.

2.4. Apparatus and chromatographic conditions

Chromatography was performed using Hitachi instruments (Hitachi, Tokyo, Japan): an AS-2000 autosampler with a 100-μl injector loop, a T-6300

column thermostat, a L-6200 intelligent pump and an L-4250 UV–VIS detector with variable wavelength. The system was controlled through a D-6000 HPLC interface module and a personal computer (IBM). Data acquisition was performed by Hitachi HPLC-manager, version 2, 1992. Separations were achieved using a Beckman ultrasphere ODS column, 5 μm, 250×4.6 mm I.D. (Beckman, Fullerton, CA, USA). The mobile phase consisted of acetate buffer (0.01 *M*, pH 4.0) and methanol (93:7, w/w for urine and 91:9, w/w for plasma). The aqueous phase was filtered through a Millipore filter (0.45 μm) and the mobile phase was degassed prior to use. The column temperature was maintain at 30°C by a column thermostat and the flow-rate was kept at 1 ml/min from 0 to 11 min, at 1.5 ml/min from 11 to 17 min, and at 2.5 ml/min from 17 to 30 min. The column effluent was quantified at a wavelength of 273 nm.

3. Results

3.1. Selectivity

Baseline separation was obtained between theophylline, the three metabolites and the internal standard under the applied conditions (Fig. 1b and 1e). About 30 min were required for the analysis. The retention times in minutes were in urine 8.2, 9.3, 14.2, 20.9 and 28.4 for 1MU, 3MX, 13DMU, 13DMX and β-OH-ET, respectively, and in plasma 7.0, 7.9, 12.3, 20.2 and 24.2 for 1MU, 3MX, 13DMU, 13DMX and β-OH-ET, respectively. Some impurities were seen in the blank chromatograms (Fig. 1a and 1d), but none were coeluting with any of the analytes. Typical chromatograms after extraction from urine and plasma from a volunteer who had taken theophylline is seen in Fig. 1c and 1f.

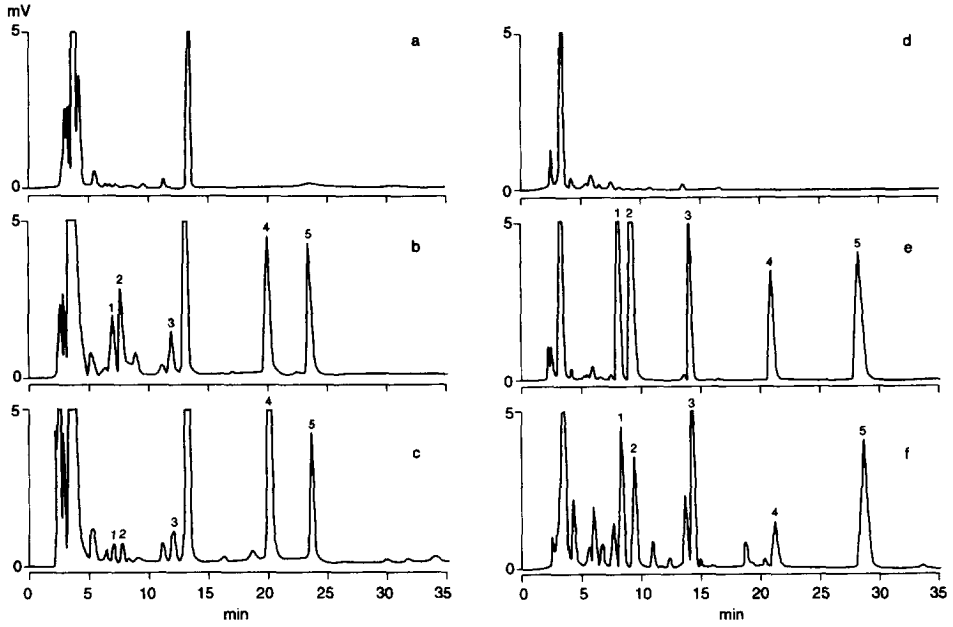

Fig. 1. (a) Blank plasma from a volunteer after four days of xanthine-free diet. (b) Extraction of standard mixture added to xanthine-free plasma in concentrations of 2.5 μM for 1, 2 and 3, and 10 μM for 4; 25 μl of 300 μM 5 was added to the sample before extraction. (c) An amount of 500-μl plasma extract from a healthy volunteer 3 h after the ingestion of a single dose of 260 mg theophylline containing: 1, 0.9 μM; 2, 0.6 μM, 3, 2.0 μM; 4, 26.6 μM; 25 μl of 300 μM 5 was added before extraction. (d) Blank urine from a volunteer after four days of xanthine-free diet. (e) Extraction of standard mixture added to xanthine-free urine in concentrations of 150 μM; 50 μl of 300 μM 5 was added to the sample before extraction. (f) An amount of 40-μl urine extract from the same volunteer as in (c) containing: 1, 95.0 μM; 2, 45.6 μM; 3, 184.9 μM; 4, 52.7 μM; 50 μl of 300 μM 5 was added to the sample before extraction. Peaks: 1=1-methyluric acid; 2=3-methylxanthine; 3=1,3-dimethyluric acid; 4=1,3-dimethylxanthine; 5=β-hydroxyethyltheophylline (internal standard).

3.2. Recovery

The absolute recoveries of the metabolites were assessed ($n=10$) at five concentration levels by comparing the peak area after extraction with the peak area obtained from direct injection of equivalent quantities of pure standard. In urine the five concentration levels were 25, 50, 100, 200 and 300 μM for all compounds. The recoveries were 89% (77–105%), 95% (86–113%), 86% (81–97%) and 93% (90–95%) for 1MU, 3MX, 13DMU and 13DMX, respectively, given as mean values and ranges for the five concentration levels. The recovery of the internal standard was 87%. In plasma the concentration levels were 0.25, 2.0, 4.0, 6.0 and 8.0 μM for 1MU, 3MX and 13DMU, and 1.0, 8.0, 16.0, 24.0 and 32.0 μM for 13DMX. The recoveries were 87% (75–124%), 91% (84–108%), 86% (79–106%), and 90% (89–92%) for 1MU, 3MX, 13DMU and 13DMX, respectively, given as mean values and ranges for the five concentration levels. The mean recovery of the internal standard was 86% in plasma.

3.3. Linearity

The linearity of detector response to different concentrations of each compound was determined at concentrations of 25, 50, 100, 200 and 300 μM in urine and in plasma the concentration levels were 0.25, 2.0, 4.0, 6.0 and 8.0 μM for 1MU, 3MX and 13DMU, and 1.0, 8.0, 16.0, 24.0 and 32.0 μM for 13DMX. The standard curves for all compounds were linear over the investigated concentration range, the r-values were 0.995, 1.000, 0.996 and 0.999 for 1MU, 3MX, 13DMU and 13DMX, respectively, in urine, and 0.998, 1.000, 1.000 and 0.999 for 1MU, 3MX, 13DMU and 13DMX, respectively, in plasma.

3.4. Precision

The intra-day precision of the method was evaluated by repeated analysis ($n=10$) of samples of urine and plasma. Five concentrations of theophylline and the three metabolites were investigated in urine: 25, 50, 100, 200 and 300 μM, and the coefficients of variation were 3% (1–6%), 3% (1–6%), 3% (1–5%) and 3% (1–5%) for 1MU, 3MX, 13DMU and 13DMX, respectively, given as mean values and ranges for the five concentration levels. The coefficient of variation of the internal standard was 3%. In plasma the concentration levels were 0.25, 2.0, 4.0, 6.0 and 8.0 μM for 1MU, 3MX and 13DMU, and 1.0, 8.0, 16.0, 24.0 and 32.0 μM for 13DMX. The coefficients of variation were 5% (3–11%), 3% (3–4%), 6% (2–11%) and 4% (2–9%) for 1MU, 3MX, 13DMU and 13DMX, respectively, given as mean values and ranges for the five concentration levels. The coefficient of variation of the internal standard was 3% in plasma.

3.5. Reproducibility

Inter-day reproducibility was assessed for five following days at three concentration levels: 37.5, 125, and 250 μM for the four compounds in urine, and in plasma 1.0, 3.0 and 7.5 μM for 1MU, 3MX and 13DMU and 4.0, 12.0, 30.0 μM for 13DMX. The coefficients of variation are shown in Table 1 and Table 2.

Table 1
Reproducibility and accuracy of theophylline (13DMX), 1-methyluric acid (1MU), 3-methylxanthine (3MX) and 1,3-dimethyluric acid (13DMU) in urine

Compound	37.5 μM			125 μM			250 μM		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
1MU	36.2	6	3	122.6	6	2	261.1	4	4
3MX	33.9	1	10	115.3	9	8	232.9	6	7
13DMU	33.6	5	3	124.2	6	0.6	260.6	6	4
13DMX	36.4	9	3	120.9	6	3	241.2	4	4

Samples spiked in xanthine-free urine were analysed once a day for five days.

Table 2
Reproducibility and accuracy of theophylline (13DMX), 1-methyluric acid (1MU), 3-methylxanthine (3MX) and 1,3-dimethyluric acid (13DMU) in plasma

Compound	1.0 μM			3.0 μM			7.5 μM		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
1MU	1.10	1	10	3.12	1	4	7.57	2	1
3MX	1.06	5	6	3.13	5	4	7.63	4	2
13DMU	1.04	6	4	3.02	1	0.8	7.61	2	1
	4.0 μM			12.0 μM			30.0 μM		
	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)	Mean	C.V. (%)	Accuracy (%)
13DMX	3.89	7	3	11.87	5	1	29.53	4	2

Samples spiked in xanthine-free plasma were analysed once a day for five days.

3.6. Accuracy

Samples spiked to three concentration levels were analysed once a day for five days. The concentrations were 37.5, 125, and 250 μM for the four compounds in urine, and in plasma the levels were 1.0, 3.0 and 7.5 μM for 1MU, 3MX and 13DMU and 4.0, 12.0, 30.0 μM for 13DMX. The mean estimates and deviations from the spiked values are shown in Table 1 and Table 2.

3.7. Limit of detection and quantification

The limit of detection, based on a signal-to-noise ratio of 3:1 was 1 μM for 1MU, 3MX and 13DMU and 2 μM for 13DMX in urine, and 0.1 μM for 1MU, 3MX and 13DMU and 0.2 μM for 13DMX in plasma. The limit of quantification based on a coefficient of variation less than 20% for repeated analysis ($n=10$) was 4 μM for 1MU and 3MX and 5 μM for 13DMU and 13DMX in urine, and 0.2 μM for 1MU, 3MX, 13DMU and 13DMX in plasma.

4. Discussion

This study describes an isocratic reversed-phase HPLC method developed for simultaneous quantitation of theophylline and three metabolites in plasma and urine. In order to improve the occupational safety in the laboratory we have developed a method where the less hazardous compound ethylacetate is used as an extraction solvent. Our method demonstrates good overall recovery, accuracy and precision, and low detection limits of all compounds, and thus provides a valuable tool for developing theophylline as a model drug for the assessment of CYP1A2 activity in vivo.

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