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Separation, pre-concentration, and HPLC analysis of methylxanthines in urine samples

An SPE method, using RP18 phases, for the simultaneous extraction of caffeine, theobromine, theophylline, paraxanthine, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1-methyluric acid, 1,3-dimethyluric acid, 1,7-dimethyluric acid, and 1,3,7-trimethyluric acid from urine has been developed. Besides a gradient HPLC system for the analysis of the compounds of interest on a LiChrosorb RP-18 (7 μm) column with mobile phase containing 0.05% aq. solution of trifluoroacetic acid and acetonitrile has been elaborated. The procedure has been successfully applied to the analysis of methylxanthines and methyluric acids in urine of patients with chronic asthma treated with theophylline and in urine of healthy subjects.

Key Words: Methylxanthines; Methyluric acids; SPE; HPLC; Urine

Received: May 8, 2004; revised: July 17, 2004; accepted: August 5, 2004

DOI 10.1002/jssc.200401841

1 Introduction

Methylxanthines belong to the group of purine alkaloids. They are basic compounds present in tea and mate leaves, coffee grains, guarana fruit, cocoa and cola nuts [1]. Caffeine and theophylline are used to alleviate neonatal apnoea, to relieve bronchial spasm, and in the therapy of asthma [2]. Besides they are components of over-the-counter drug mixtures for pain, cold, and cough [3]. The analysis of these compounds in body fluids is important not only for monitoring of asthma, neonatal apnoea, or bronchial spasm, but also for non-invasive assessment of various isoforms of cytochrome P450 (CYP) activity [4, 5] or in doping analysis [6, 7].

Liquid chromatography with UV absorbance detection is the method most frequently used for the analysis of methylxanthines and their metabolites. Usually non-polar chemically bonded stationary phases and water or acetate/phosphate buffers with acetonitrile, methanol, and tetrahydrofuran as the mobile phases are used [4–12]. Before chromatographic analysis methylxanthines present both in plant samples and in body fluids are usually separated form the matrix and pre-concentrated. The most common extraction procedures for methylxanthines and methyluric acids include liquid-liquid extraction with chloroform : 2-propanol mixture (80 : 20) [8], (85 : 15) [5], or (90 : 10) [7], dichloromethane : methanol (90 : 10) [6], dichloromethane:2-propanol (90 : 10) [9], ethyl acetate : 2-propanol (93 : 7) [10], dichloromethane in acidic pH [11], chloroform [12], protein precipitation with perchloric acid or with acetonitrile [13, 14]. Although it is widely known that solid-phase extraction is rapid, simple, safer and less solvent consuming than liquid-liquid extraction and offers a wide sorbent selection, extraction of caffeine together with its metabolites with the use of SPE technique was investigated in rather few publications [15–17].

In this work new HPLC systems and an SPE procedure for sample preparation and determination of methylxanthines and their metabolites have been elaborated and applied to the analysis of urine of patients treated with theophylline.

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2 Experimental

2.1 Materials and reagents

Caffeine (CAFF), theobromine (THBR), theophylline (THPH), paraxanthine (PX), 1-methylxanthine (1MX), 3-methylxanthine (3MX), 7-methylxanthine (7MX), 1-methyluric acid (1MU), 1,3-dimethyluric acid (1,3-DMU), 1,7-dimethyluric acid (17DMU), 1,3,7-trimethyluric acid (137TMU) and β-OH-ethyltheophylline (I.S.) were purchased from Sigma-Aldrich (Steinheim, Germany). The structures of the analysed compounds are presented in **Figure 1.**

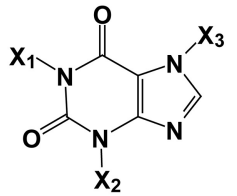
HPLC grade water, acetonitrile, and trifluoroacetic acid (TFA) were purchased from Merck, Darmstadt, Germany.

Analytical grade chloroform, methanol, 2-propanol, ethyl acetate were purchased from PoCh, Gliwice, Poland.

2.2 Instrumental conditions

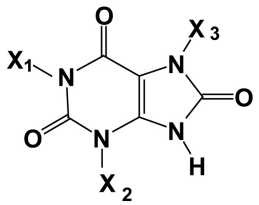
Chromatographic analysis was carried out with a Merck-Hitachi L4500A liquid chromatograph with a DAD detector (Merck-Hitachi, Darmstadt, Germany) and LiChrosorb RP-18 (7 μm), 100–4.6 column, in a gradient elution

METHYLXANTHINES



$X_1=CH_3$,	$X_2=H$,	$X_3=H$	1-METHYLXANTHINE (1MX)
$X_1=H$,	$X_2=CH_3$	$X_3=H$	3-METHYLXANTHINE (3MX)
$X_1=H$,	$X_2=H$	$X_3=CH_3$	7-METHYLXANTHINE (7MX)
$X_1=CH_3$,	$X_2=CH_3$	$X_3=H$	1,3-DIMETHYLXANTHINE, THEOPHYLLINE (THPH)
$X_1=CH_3$,	$X_2=H$	$X_3=CH_3$	1,7-DIMETHYLXANTHINE, PARAXANTHINE (PX)
$X_1=H$,	$X_2=CH_3$,	$X_3=CH_3$	3,7-DIMETHYLXANTHINE, THEOBROMINE (THBR)
$X_1=CH_3$,	$X_2=CH_3$,	$X_3=CH_3$	1,3,7-TRIMETHYLXANTHINE, CAFFEINE (CAFF)

METHYLURIC ACIDS



$X_1=CH_3$,	$X_2=H$	$X_3=H$	1-METHYLURIC ACID (1MU)
$X_1=CH_3$,	$X_2=CH_3$	$X_3=H$	1,3-DIMETHYLURIC ACID (13DMU)
$X_1=CH_3$,	$X_2=H$,	$X_3=CH_3$	1,7-DIMETHYLURIC ACID (17DMU)
$X_1=CH_3$,	$X_2=CH_3$	$X_3=CH_3$	1,3,7-TRIMETHYLURIC ACID (137TMU)

Figure 1. Structures of methylxanthines and methyluric acids.

Original Paper

mode. The programme of the optimum elution profile is presented in **Table 1.** The temperature of the analysis was set at 40°C.

The best monitoring wavelength for methylxanthines is 274 nm and methyluric acids are better detected at 289 nm.

The samples were injected by means of a Rheodyne valve with 0.02 mL loop. The injection valve loop was flushed 2× with 0.05 mL of methanol and 3× with 0.05 mL of the sample before injecting the sample.

Three injections were performed for standards and samples.

2.3 Sample collection

Urine samples were analysed immediately or they were stored at −20°C until analysis.

Table 1. Optimum gradient elution profile for the separation of methylxanthines and methyluric acids on LiChrosorb RP-18 (7 μm) column.

t_R [min]	A [%]	B [%]	Flow rate [mL/min]
0	95	5	0.80
3	95	5	0.80
10	90	10	0.80
19	90	10	0.80
30	80	20	1.60
40	100	0	0.80

Fresh urine samples were taken from patients treated for chronic asthma. The samples were collected 3, 6, and 18 h after dosing 100, 200, and 300 mg of theophylline. For each therapeutic dose the urine of 6 patients was analysed. The total volume of urine generated by the volunteers was collected.

The urine of 6 healthy controls was analysed for comparison purposes.

Blank urine sample was obtained from a healthy control after a 5-day methylxanthine diet.

2.4 Sample extraction methods

The compounds of interest were extracted from the samples with the use of BAKERBOND SPE-12G system (SPE BAKERBOND spe & trade; J.T. Baker Inc., Phillipsburg, USA) and RP-18 cartridges (J.T. Baker Inc., Phillipsburg, USA).

The SPE methods proposed in Merck Application Guide [18] and Baker Application Notes [19] have been investigated.

The influence of various parameters, namely volume (0.6–2.0 mL) and pH adjustment of the sample (pH = 2, pH = 4, or pH = 7), pH of conditioning solutions (pH = 2, pH = 4, or pH = 7), different elution solvents (various volumes of methanol and chloroform) and different sizes of RP18 SPE cartridges (3 mL/500 mg and 1 mL/100 mg) on the extraction efficiencies of methylxanthines and methyluric acids have been investigated (data not shown).

Mixtures of solvents used in liquid-liquid extraction methods have also been used for elution of methylxanthines and methyluric acids from RP-18 cartridges.

The final SPE procedure was as follows: conditioning the 1 mL RP18 phase with 2×1 mL of methanol followed by 2×1 mL of water, processing 2 mL of urine sample mixed with 0.6 mL buffer of pH = 4, and after washing the cartridge with 2×1 mL of water, elution of methylxanthines and their metabolites with 5×0.5 mL of chloroform : 2-propanol (80 : 20) mixture, evaporation under a stream of nitrogen and dissolution in 0.2 mL of 0.05% aq. trifluoroacetic acid (a component of the mobile phase).

The samples were diluted as necessary.

3 Method validation procedures

3.1 Precision, accuracy, and recovery

Within-run precision and accuracy was calculated from repeated analysis ($n = 4$) of blank urine spiked with standards during one working day.

Between-day precision and accuracy was calculated from repeated analysis ($n = 4$) of blank urine spiked with standards on five consecutive working days.

The efficiency of the SPE procedures was evaluated by comparing peak area ratios to internal standard, with and without extraction.

3.2 Calibration curves

Calibration curves as peak area ratios (compound/internal standard) vs. standard concentrations were obtained with the use of a least-squares linear regression method.

3.3 Limit of detection and quantification

The limit of detection (LOD) was based on a signal-to-noise ratio of 3 : 1 at the baseline.

The limit of quantification (LOQ) was based on a signal-to-noise ratio of 10 : 1 at the baseline.

4 Results and conclusions

4.1 Chromatography

A gradient system for the separation of methylxanthines and methyluric acids was developed. The optimum gradient profile for the compounds of interest is presented in Table 1. Retention times and relative retention times [min] of the compounds in this system are presented in **Table 2**.

Retention times of these compounds in urine after solid-phase extraction are also presented in Table 2. As can be seen from the Table, urine matrix influences the retention times of the compounds of interest. Methylxanthines and methyluric acids were identified in these samples by the standard addition method. Absorption spectra of the compounds of interest were also compared.

A chromatogram of urine sample is presented in **Figure 2.** Interference from endogenous compounds in the sample matrix was not observed.

Resolution factors for the compounds of interest were higher than 1.1, providing satisfactory resolution of the analytes [20].

Table 2. Retention times of methylxanthines and methyluric acids in standard solution and urine samples [min].

	Retention times of standards [min]	Relative retention times	Retention times in the urine sample [min]	Relative retention times
1 MU	8.32	0.32	8.43	0.31
7 MX	8.80	0.34	8.91	0.33
3 MX	10.37	0.40	10.13	0.38
1 MX	11.20	0.43	11.25	0.42
13 DMU	13.07	0.50	13.36	0.50
THBR	15.31	0.59	15.49	0.58
17 DMU	16.05	0.62	16.19	0.61
PX	18.00	0.69	18.37	0.69
THPH	18.35	0.71	18.75	0.70
137 TMU	19.44	0.75	20.05	0.75
I.S.	25.89	1.00	26.77	1.00
CAFF	26.37	1.01	27.20	1.02

Table 3. SPE recoveries obtained on octadecylsilane sorbents using chloroform, chloroform:2-propanol (80:20), and ethyl acetate:2-propanol (93:3) as the elution solvents.

Analyte	Chloroform	Chloroform : 2-propanol (80 : 20)	Ethyl acetate : 2-propanol (93 : 7)
1 MU	53.8	86.0	0.0
7 MX	59.5	92.2	79.3
3 MX	68.0	96.3	87.8
1 MX	48.0	92.2	74.0
13 DMU	1.9	85.5	0.0
THBR	100.1	92.1	89.6
17 DMU	2.0	90.7	0.0
PX	98.0	98.3	92.4
THPH	99.1	97.2	85.7
137 TMU	101.2	86.3	0.0
CAFF	103.5	93.2	73.3

4.2 Solid-phase extraction

For sample preparation the SPE methods proposed in the Merck Application Guide [18] and in Baker Application Notes [19] have been examined. As neither chloroform nor methanol, used as the elution solvents for SPE on RP18 cartridges, gave satisfactory recoveries for the compounds of interest (even after optimisation of their volumes), we have introduced some modifications into the extraction methods. For the elution of methylxanthines and methyluric acids we have applied mixtures of solvents used in liquid-liquid extraction methods.

Recoveries for the compounds of interest from blank urine samples spiked with 5 ng of standards obtained using chloroform, chloroform : 2-propanol (80 : 20), and ethyl acetate : 2-propanol (93 : 7) mixtures as the elution solvents are presented in **Table 3.**

As can be seen from the Table, the best results were obtained with the use of a chloroform : 2-propanol (80 : 20) mixture. This procedure has been applied to urine analysis.

4.3 Method validation procedures

Precision, accuracy, and recovery studies were conducted by spiking blank urine samples with known amounts of standards of methylxanthines and methyluric acids. The obtained data were compared with the results calculated from regression equations of SPE processed samples (**Table 4**).

Within-day and between-day precision and accuracy for three different additions of methylxanthines and methyluric acids in blank urine samples ($n = 4$) in this method are presented in **Table 5.**

Table 4. Data on regression equations for determination of methylxanthines and methyluric acids after SPE in urine.

	Calibration curves		r	Concentration range [μg/mL]	LOD [μg/mL]
	Slope	Intercept			
1 MU	0.0260 ± 0.0006	−0.0004 ± 0.0015	0.999	0.100–50.000	0.050
7 MX	0.0269 ± 0.0006	0.0013 ± 0.0022	0.999	0.100–50.000	0.050
3 MX	0.0247 ± 0.0002	−0.0033 ± 0.0015	0.999	0.100–30.000	0.050
1 MX	0.0240 ± 0.0006	0.0050 ± 0.0017	0.998	0.100–30.000	0.050
13 DMU	0.0116 ± 0.0002	0.0110 ± 0.0020	0.999	0.100–50.000	0.060
THBR	0.0263 ± 0.0008	0.0076 ± 0.0029	0.998	0.100–30.000	0.050
17 DMU	0.0195 ± 0.0002	0.0060 ± 0.0005	0.999	0.100–50.000	0.050
PX	0.0156 ± 0.0005	0.0175 ± 0.0046	0.996	0.100–20.000	0.060
THPH	0.0253 ± 0.0003	0.0093 ± 0.0013	0.999	0.100–30.000	0.050
137 TMU	0.0083 ± 0.0002	0.0029 ± 0.0007	0.998	0.100–20.000	0.070

Table 5. Recovery and within-day and between-day precision ($n = 3$).

		Within-day				Between-day			
		Measured [ng]	SD [ng]	RSD [%]	Recovery [%]	Measured [ng]	SD [ng]	RSD [%]	Recovery [%]
1MU	5	4.30	0.11	2.6	86.0	4.42	0.19	4.3	88.3
	10	8.83	0.12	1.4	88.3	8.83	0.32	3.6	88.3
	20	17.84	0.23	1.3	89.2	17.90	0.51	2.8	89.5
7MX	5	4.61	0.09	2.0	92.2	4.68	0.20	4.3	93.5
	10	9.33	0.13	1.4	93.3	9.35	0.36	3.9	93.5
	20	18.82	0.24	1.3	94.1	18.80	0.48	2.6	94.0
3MX	5	4.82	0.09	1.9	96.3	4.82	0.18	3.7	96.3
	10	9.68	0.14	1.4	96.8	9.63	0.41	4.3	96.3
	20	19.30	0.25	1.3	96.5	19.44	0.52	2.7	97.2
1MX	5	4.61	0.08	1.7	92.2	4.71	0.21	4.5	94.2
	10	9.31	0.11	1.2	93.1	9.42	0.33	3.5	94.2
	20	18.86	0.21	1.1	94.3	19.12	0.41	2.1	95.6
13DMU	5	4.28	0.09	2.1	85.5	4.46	0.22	4.9	89.1
	10	8.61	0.12	1.4	86.1	8.91	0.35	3.9	89.1
	20	17.64	0.25	1.4	88.2	18.04	0.45	2.5	90.2
THBR	5	4.61	0.11	2.4	92.1	4.56	0.19	4.2	91.1
	10	9.33	0.14	1.5	93.3	9.11	0.36	4.0	91.1
	20	18.82	0.23	1.2	94.1	18.42	0.46	2.5	92.1
17DMU	5	4.54	0.1	2.2	90.7	4.54	0.22	4.8	90.8
	10	9.21	0.16	1.7	92.1	9.08	0.38	4.2	90.8
	20	18.44	0.25	1.4	92.2	18.20	0.36	2.0	91.0
PX	5	4.92	0.11	2.2	98.3	4.97	0.24	4.8	99.3
	10	9.82	0.11	1.1	98.2	9.93	0.46	4.6	99.3
	20	19.66	0.26	1.3	98.3	19.90	0.51	2.6	99.5
THPH	5	4.86	0.09	1.9	97.2	4.87	0.23	4.7	97.3
	10	9.73	0.12	1.2	97.3	9.73	0.31	3.2	97.3
	20	19.66	0.25	1.3	98.3	19.60	0.56	2.9	98.0
137TMU	5	4.32	0.11	2.5	86.3	4.41	0.21	4.8	88.2
	10	8.73	0.13	1.5	87.3	8.82	0.31	3.5	88.2
	20	17.62	0.23	1.3	88.1	17.86	0.61	3.4	89.3
CAFF	5	4.66	0.12	2.6	93.2	4.66	0.19	4.1	93.1
	10	9.31	0.12	1.3	93.1	9.31	0.32	3.4	93.1
	20	18.84	0.24	1.3	94.2	19.22	0.55	2.9	96.1

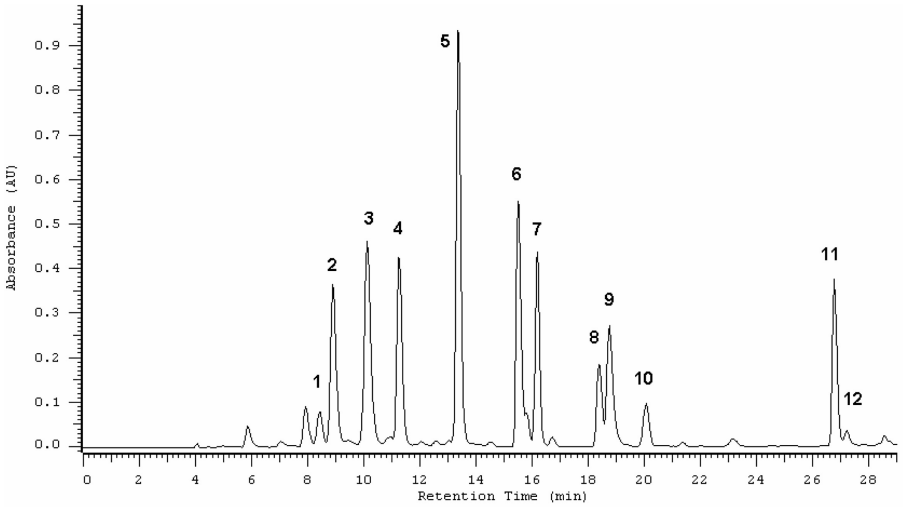


Figure 2. Chromatogram of urine sample in the gradient system described in the text. Retention times of the determined compounds [min]: 1MU(**1**) = 8.43, 7MX(**2**) = 8.91, 3MX(**3**) = 10.13, 1MX(**4**) = 11.25, 13DMU(**5**) = 13.36, THBR(**6**) = 15.49, 17DMU(**7**) = 16.19, PX(**8**) = 18.37, THPH(**9**) = 18.75, 137TMU(**10**) = 20.05 I.S.(**11**) = 26.77 and CAFF(**12**) = 27.20.

Table 6. Urinary concentrations of methylxanthines and methyluric acids

	Urine of healthy controls ($n = 6$) [μg/mL]	Urine of patients treated with theophylline ($n = 18$) [μg/mL]
1U	30–60	20–80
7MX	17–37	7–45
3MX	6–16	13–36
1MX	10–23	10–25
13U	4–7	65–155
THBR	1–2	3–20
17U	7–20	17–96
PX	9–11	5–15
THPH	<2	9–30
137U	2–3	<16
CAFF	<3	<4

4.4 Analysis of urine samples

In the clinical phase of the study, theophylline metabolite concentrations were determined in urine samples of patients with chronic asthma treated with theophylline and of healthy controls. Oral theophylline formulations are used in chronic asthma to reduce the incidence and severity of respiratory symptoms. Rates of inactivation of theophylline vary extensively among patients and doses must be adjusted to each person's response. Cardiac, hepatic, or pulmonary disease, fever, cigarette smoking, age, and the presence of other drugs will influence the hepatic metabolism of theophylline. Concomitant food ingestion has variable effects on absorption of oral preparations. Adverse effects include restlessness, nausea, and insomnia. The therapeutic index of theophylline is low, and overdose can result in seizures and fatal cardiac arrythmias. When patients are treated with high dose preparations, plasma concentrations of drug should be monitored. Optimal levels range from 5 to 20 μg/mL.

The concentrations of methylxanthines and methyluric acids in the urine of patients with chronic asthma treated with theophylline and in the urine of healthy subjects are presented in **Table 6.**

Significant differences in concentrations of THBR, THPH, 3MX, 17DMU, and 13DMU between the two groups of subjects were observed. Good correlations between taken dose and observed concentrations of theophylline in urine could be noted. High concentrations in urine were observed starting from the third hour after taking the drug orally, with a maximum after six hours and slow tempering during the next twelve hours after which approximately one third of the maximal concentration was present.

5 Concluding remarks

A novel SPE method, using octadecylsilane phases, for the simultaneous extraction of methylxanthines and methyluric acids from body fluids has been developed. Application of mixtures of solvents used earlier for liquid-liquid extraction provided high recovery rates for the analytes. The described method is simple, rapid, accurate, and less solvent consuming than liquid-liquid extraction. The SPE procedure together with a gradient HPLC system, elaborated by our team, has been applied to the analysis of methylxanthines and methyluric acids in the urine of patients with chronic asthma treated with theophylline and in the urine of healthy subjects. This analysis proved the usefulness of this method in urine analysis. The procedure is also considered to be applicable for the evaluation of individual variations of CYP1A2 activity.

Acknowledgements

This work was supported by the State Committee for Scientific Research (Poland), Project No. 4#T09A# 08922.

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