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HPLC Analysis of Methylxanthines and Selected Drugs in Urine Samples

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A HPLC system for separation and determination of methylxanthines and selected drugs has been developed. Teophylline, 1-methylxanthine, 3-methylxanthine, 1,3-dimethyluric acid, caffeine, paracetamol, furosemide, dexamethasone, prednisolone, cefazolin and imipenem have been determined. A RP–18e column with a RP–18 pre-column and a DAD detector were used. Gradient elution with 0.05% TFA aqueous solution with acetononitrile at the flow rate of 0.8 mL min^{-1} was applied. The developed system was used to determine the examined compounds in urine samples.

Opracowano układ chromatograficzny do rozdzielania i oznaczania metyloksantyn oraz wybranych leków. Oznaczono teofilinę, 1-metyloksantynę, 3-metyloksantynę, kwas 1,3-dimetylomoczowy, kofeinę oraz paracetamol, furosemid, deksametazon, prednizolon, cefazolinę i imipenem. Zastosowano kolumnę RP–18e z pre-kolumną RP–18 oraz detektor DAD. Fazę ruchomą stanowił układ gradientowy 0.05% wodny roztwór TFA z acetonitrylem, prędkość przepływu wynosiła 0.8 mL min^{-1}. Opracowany układ chromatograficzny zastosowano do oznaczenia badanych związków w próbkach moczu.

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Purines are ones of basic components of the building material of animal and plant cells. Exemplary derivatives of purines are *e.g.* alkaloids, like xanthines and methylxanthines. Basic components of alkaloids are xanthine and its direct metabolites: hypoxathine and uric acid. Monitoring of these compounds is very useful in medical diagnostics. For this purpose, mainly HPLC methods [1–3] are utilized. Methylxanthines are pure alkaloids which contain nitrogen atoms in a heterocyclic configuration. A group of methylxanthines include, among others, teophylline (1,3-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine). They both have practical application in medicine. Theophylline is one of the main components of bronchi relaxing drugs. Caffeine, as a stimulating system for central nervous system, is added to analgesics to intensify their activity. Due to often side effects of theophylline treatment it is important to monitor its concentration in either serum or urine of the patients. Monitoring should also include metabolites of theophylline (like 1-methylxantine, 3-methylxanthine or 1,3-dimethyluric acid). C_4 [4], C_{18} [5–10] and C_{18e} [11] -packed columns were used for determination of methylxanthines in urine samples. Gradient elution with acetic acid–methanol [4, 6–7, 11] and 0.05% aq. solution of trifluoroacetic acid–acetonitrile [7], as well as isocratic elution with acetic buffer–methanol [2] was applied. DAD [4–8, 10–11] and MS [9] detectors were most often used. The administrated drugs disturbed HPLC determination of methylxanthines. HPLC methods for determination of drugs investigated in this study in urine samples are described elsewhere. In the previous reports, a HPLC system with C_8 [13] and C_{18} [14–20] columns was used. However, these papers usually refer to the determination of a single drug, or 2–3 drugs from one group, *e.g.* only analgesics, or only antibiotics. The mobile phases used included isocratic systems: acetonitrile–phosphate buffer [13–15], methanol–water [16], methanol–TFA [17], methanol–acetic buffer [18], borate buffer [19], and gradient systems: methanol–water [20] as well. Detection was performed with a DAD detector. A method for simultaneous determination of methylxanthines with cardiac drugs, anticonvulsants, and antibiotics (sulfapyridine, sulfamethoxazole, septra) was described in the earlier paper [12]. However, there have been no reports of chromatographic systems in which teophylline and its metabolites along with drugs from different groups (analgesics, diuretics, anti-inflammatory drugs and antibiotics) were determined simultaneously. In this work, we have attempted to develop a HPLC system for simultaneous determination of five methylxanthines (teophylline, 1-methylxanthine, 3-methylxanthine, 1,3-dimethyluric acid and caffeine) and other drugs, which were administrated to patients under theophylline treatment. For separation and determination of methylxanthines (without drugs) a LiChrosorb RP–18 (7 μm) stationary phase, a 100–4.6 column, and gradient elution have been applied [10]. In this study, a 125–3 column packed with LiChroCARD Purospher RP–18e (5 μm) and accompanied with 4–4 (5 μm) pre-column packed with LiChroCARD LiChrospher 100 RP–18 stationary phase have been used. Some

parameters of gradient elution were changed compared to reference [10]. This modification allowed one to simultaneously separate and determine methylxanthines and selected drugs: paracetamol (4-acetaminephenol), furosemide (5-(aminosulfonyl)-4--chloro-2-([2-furanylmethyl]amino) benzoic acid), dexamethasone (9 α-fluoric-16--α-methylprednisolone), prednisolone (1-dehydrocortisone), cefazolin, and imipenem.

EXPERIMENTAL

1 mg mL^{-1} aqueous standard solutions of teophylline (THPH), 1,3-dimethyluric acid (13DMU) (Sigma) and 1-methylxanthine (1MX), 3-methylxanthine (3MX) and caffeine (CAFF) (all from Aldrich), furosemide, dexamethasone, prednisolone and cefazolin (from Sigma) and paracetamol and imipenem (from Fluka) were prepared.

Acetonitrile, water, and trifluoroacetic acid (TFA) of HPLC grade used in this work were purchased from Merck, Germany.

HPLC analyses were performed using a Merck–Hitachi chromatograph equipped with a L6200A pump and a L4500A diode-array detector (DAD). Chromatographic separations were carried out on a LiChroCARD Purospher column RP–18e, 125–3 mm, particle size 5 μm accompanied with a LiChroCARD 4–4 mm precolumn packed with LiChrospher 100 RP–18, particle size 5 μm (Merck, Germany) at a room temperature.

Gradient elution with 0.05% TFA aqueous solution (A) and acetonitrile (B) at a flow rate of 0.8 mL min^{-1} was applied. The following gradient program was applied: 0 min 95% A and 5% B, 3 min 95% A and 5% B, 10 min 90% A and 10% B, 19 min 90% A and 10% B, 30 min 80% A and 20% B, 40 min 100% A.

Urine samples from patients treated with teophylline and selected drugs were examined. Urine samples were collected within 24 h and subsequently they were frozen at the temperature of –20°C. After defrosting, 1 mL of a urine sample was acidified with 1 mL of phosphate buffer (pH 6.8; 0.067 mol L^{-1}). Then, the samples were precipitated with acetonitrile (1 mL) and centrifuged for 10 min (2500 rpm). The examined material was separated applying solid-phase extraction (SPE) using a Bakerbond System with C_{18} columns (500 mg, 3 mL). The columns were conditioned with methanol (2 × 2 mL) and water (1 × 2 mL). The prepared samples were passed through the columns. Then, the columns were dried and the analytes were eluted with methanol (1 × 1 mL) [13]. The eluates were analyzed applying HPLC. The volume of the solution in every single injection was 20 μL.

Detection was performed at different wavelengths, depending on the type of compound. For all methylxanthines the wavelength was set to $\lambda = 275$ nm, for paracetamol and cefazolin it was $\lambda = 254$ nm, for furosemide, dexamethasone and prednisolone $\lambda = 240$ nm, and for imipnem $\lambda = 300$ nm.

Calibration plots for the examined drugs were obtained using the following series of standard solutions: paracetamol 30–500 μg mL^{-1}, furosemide 10–300 μg mL^{-1}, dexamethasone 100–400 μg mL^{-1}, prednisolone 80–400 μg mL^{-1}, cefazolin 20–500 μg mL^{-1}, imipenem 20–100 μg mL^{-1}. Calibration lines for teophylline and other methylxanthines were obtained for series of solutions of the concentrations ranging from 1 to 100 μg mL^{-1}, and from 1 to 50 μg mL^{-1}, respectively. The number of experimental points taken for regression was n = 6. Every analyte was injected thrice. LOD, LOQ, and inter- and intra- day precision and accuracy of determination of methylxanthines and drugs were determined in blank urine samples (n = 3).

RESULTS AND DISCUSSION

Chromatographic separation and determination of five methylxanthines (caffeine, 1-methylxantine, 3-methylxantine, 1,3-dimethyluric acid, teophylline) and drugs belonging to four groups (paracetamol, furosemide, dexamethasone, prednisolone, cefazolin, imipenem) has been performed. Drugs were determined in three combinations used in treatment (Tab. 1). The obtained chromatogram is shown in Figure 1 and the corresponding retention times are listed in Table 2.

Table 1. Determined methylxanthines and other drug mixtures

No.	Methylxanthines					Drugs					
						Analgesics	Diuretics	Demulcents		Antibiotics	
	THPH	1MX	3MX	13DMU	CAFF	Paracetamol	Furosemide	Dexamethasone	Prednisolone	Cefazolin	Imipenem
1	+	+	+	+	+	+	+		+		+
2	+	+	+	+	+	+	+		+	+	
3	+	+	+	+	+	+	+	+		+	

Table 2. Retention times and recoveries of methylxanthines and drugs

	Compound	t_R, min	Added, µg mL^{-1}	Found, µg mL^{-1}	Recoveries, %
Methylxanthines	1MX	0.90	10	10.2	102
	3MX	1.28	10	9.5	95
	13DMU	2.93	10	9.6	96
	THPH	3.55	10	9.7	97
	CAFF	14.53	10	9.8	98
Drugs	Paracetamol	2.03	200	202	101
	Furosemide	34.95	200	158	79
	Dexamethasone	28.99	200	199	99.5
	Prednisolone	29.09	200	167	83.5
	Cefazolin	7.60	200	201	100.5
	Imipenem	8.88	200	150	75

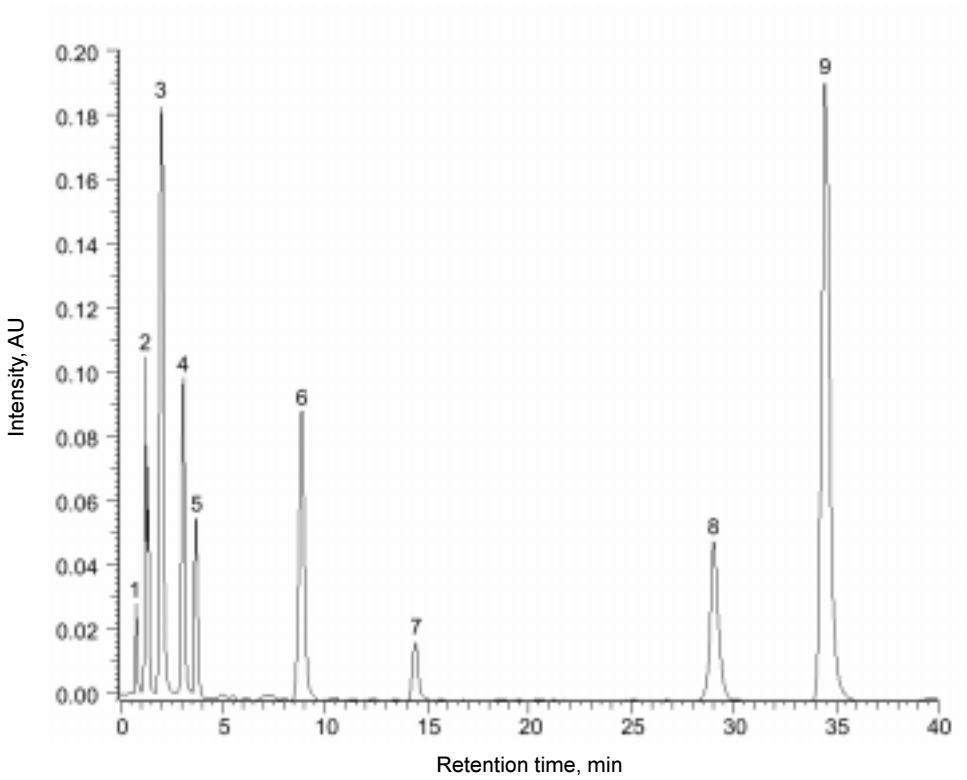

Figure 1. Chromatogram of methylxanthines and drugs (1 – 3MX, 2 – 1MX, 3 – paracetamol, 4 – 13DMU, 5 – THPH, 6 – imipenem, 7 – CAFF, 8 – prednisolone, 9 – furosemide)

All examined compounds were well separated. The measured retention times indicate that the proposed method can not be used to simultaneous determination of dexamethasone and prednisolone. However, these drugs are not used simultaneously in the treatment so such determination is unnecessary. In order to estimate accuracy of determination, recovery studies were performed. For this purpose 200 μg of drugs and 10 μg of methylxanthines were added to 1 mL-in-volumes urine samples of healthy people, thus not accepting either teophylline or other drugs. Chromatogram of the sample obtained after SPE procedure (see Experimental section) is shown in Figure 2. It spans only over 10 min, because after that time only baseline was recorded. Urine sample of a healthy human (blank sample) was submitted to SPE procedure and chromatogram of the eluat (after elution with methanol) from C_{18} columns on Bakerbond system was recorded at four wavelength (240, 254, 275 and 300 nm). While carrying out chromatographic separation of urine samples, time change of the wavelength was suggested. It was possible only in case when the wavelength was self-evident and the determined methylxanthines and drugs exhibited maximum of absorption and retention times. Due to the application of the time change of the wave-

length, chromatograms of urine sample exhibiting the peaks of particular analytes were free from the matrix effect.

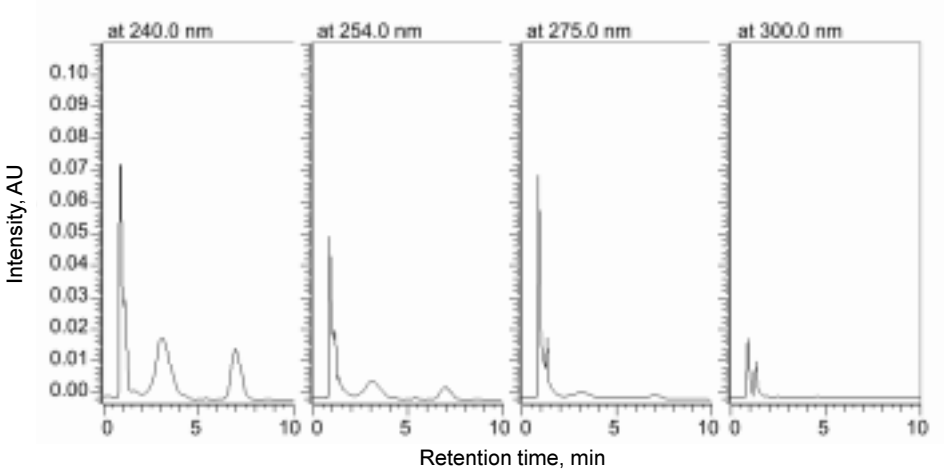

Figure 2. Chromatograms of blank sample (eluat) of urine from healthy patient after SPE procedure

Recoveries of particular compounds are given in Table 2. The applied sample preparation method allowed one to achieve recoveries of 75–101% for the examined drugs and 95–102% for the examined methylxanthines. Regression parameters of the calibration plots are listed in Table 3.

Table 3. Regressions parameters of calibration plots of methylxanthines and drugs

	Compound	Slope	Intercept	R^2	LOD, ng	LOQ, ng
Methylxanthines	1MX	115690	–8834.4	0.999	1	3.3
	3MX	40952	–33960	0.998	1	3.3
	13DMU	85515	–77913	0.997	1	3.3
	THPH	38370	112375	0.996	1.2	4
	CAFF	33294	366812	0.994	1.2	4
Drugs	Paracetamol	46151	376561	0.999	1.6	5.2
	Furosemide	27913	473779	0.971	2	6.6
	Dexamethasone	64309	-6×10^6	0.997	2	6.6
	Prednisolone	11372	2×10^6	0.966	2	6.6
	Cefazolin	18147	-3×10^6	0.995	1	3.3
	Imipenem	14323	296673	0.999	2	6.6

Intra- and inter- day precision and accuracy of the applied method for three different additions of methylxanthines and drugs are presented in Table 4.

Table 4. Inter- and intra-day precision of determination of methylxanthines and drugs

		Within-day				Between-day			
Compound	Input μg mL^{-1}	Measured, μg mL^{-1}	SD μg mL^{-1}	CV, %	Recovery, %	Measured, μg mL^{-1}	SD, μg mL^{-1}	CV, %	Recovery, %
1MX	1	1.01	0.03	2.7	101	0.99	0.01	1.5	99
	5	5.00	0.02	1.6	100	5.00	0.02	1.6	100
	10	10.20	0.02	2.2	102	10.15	0.01	1.3	101
3MX	1	0.95	0.03	3.4	95	0.98	0.02	2.2	98
	5	4.97	0.08	8.2	99	4.95	0.06	6.1	99
	10	9.50	0.05	4.8	95	9.81	0.05	4.8	98
13DMU	1	0.97	0.02	1.94	97	0.96	0.02	1.79	96
	5	4.92	0.02	1.94	98	4.92	0.03	2.00	98
	10	9.71	0.03	2.38	97	9.67	0.04	2.13	97
THPH	1	0.97	0.02	2.14	97	0.99	0.01	0.97	99
	5	4.97	0.01	0.53	99	4.98	0.01	0.59	99
	10	9.82	0.02	1.53	98	9.79	0.03	1.27	97
CAFF	1	0.95	0.01	1.05	95	0.95	0.02	1.81	95
	5	4.92	0.02	1.78	98	4.91	0.02	1.81	98
	10	9.85	0.01	1.31	98	9.82	0.02	1.74	98
Paracetamol	100	101.0	0.01	1.70	101	100.0	0.01	1.70	100
	150	149.5	0.01	1.71	99	149.0	0.01	1.95	99
	200	202.0	0.02	1.35	101	202.0	0.02	1.35	101
Furosemide	100	79.0	0.02	1.79	79	79.5	0.03	2.67	79.5
	150	119.0	0.11	3.70	79	120.0	0.12	3.79	80
	200	162.0	0.02	2.18	81	163.0	0.02	1.74	81

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Table 4. (Continuation)

		Within-day				Between-day			
Compound	Input μg mL^{-1}	Measured, μg mL^{-1}	SD μg mL^{-1}	CV, %	Recovery, %	Measured, μg mL^{-1}	SD, μg mL^{-1}	CV, %	Recovery, %
Dexamethasone	100	99.5	0.01	0.71	99.5	100.5	0.06	3.33	100.5
	150	147.5	0.02	2.40	98	146.5	0.03	2.41	98
	200	200.0	0.04	3.54	100	199.0	0.05	2.84	99.5
Prednisolone	100	83.0	0.02	1.70	83	84.0	0.01	0.84	84
	150	124.5	0.01	0.57	83	124.0	0.01	1.14	83
	200	166.0	0.01	0.85	83	167.0	0.01	0.42	83.5
Cefazolin	100	100.5	0.01	0.70	100.5	101.5	0.01	0.70	101.5
	150	149.7	0.00	0.24	99.8	150.5	0.01	0.47	100
	200	200.5	0.01	0.35	100	200.0	0.01	0.71	100
Imipenem	25	18.5	0.04	3.82	74	18.7	0.02	1.89	75
	50	37.8	0.01	0.56	76	37.3	0.01	1.33	75
	100	75.2	0.00	0.47	75	75.7	0.01	1.40	76

The proposed chromatographic system was subsequently used to examine real samples. Urine samples were taken from patients, who were treated with teophylline and analgesics, diuretics, and antibiotics. The contents of drugs and methylxanthines in urine samples were determined from the calibration lines. The obtained results are presented in Table 5.

Table 5. The contents of methylxanthines and other drugs in urine samples of treated patients

	Compound	Marked, mg 24 h^{-1}				
		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Methylxanthines	3MX	55.00 ± 0.38	10.24 ± 0.10	28.98 ± 0.12	7.68 ± 0.20	10.00 ± 0.24
	1MX	13.60 ± 0.12	10.12 ± 0.14	29.42 ± 0.16	43.50 ± 0.20	15.82 ± 0.02
	13DMU	22.40 ± 0.20	16.62 ± 0.06	68.06 ± 0.08	85.38 ± 0.78	37.14 ± 0.14
	THPH	107.96 ± 0.20	15.52 ± 0.08	124.06 ± 0.08	13.34 ± 0.04	158.22 ± 0.72
	CAFF	16.22 ± 0.12	–	20.36 ± 0.16	–	–

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Table 5. (Continuation)

	Compound	Marked, mg 24 h^{-1}				
		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Drugs	Paracetamol	393.92 ± 0.14	134.96 ± 0.08	446.90 ± 0.08	–	–
	Furosemid	86.04 ± 0.04	77.26 ± 0.10	93.40 ± 0.02	–	–
	Cefazolin	–	–	–	436.20 ± 0.16	433.42 ± 0.12

The following compounds were determined in the examined urine samples: teophylline (t_R= 4.25 min), 1-methylxanthine (t_R = 1.23 min), 3-methylxanthine (t_R = 0.91 min), 1,3-dimetyluric acid (t_R = 3.07 min), caffeine (t_R = 15.25 min), paracetamol (t_R = 2.10 min), furosemide (t_R = 36.35 min) and cefazolin (t_R = 8.05 min). Exemplary chromatogram of a urine sample of a patient treated with teophylline and other drugs is shown in Figure 3. The differences between retention times of the analytes in model samples (Fig. 1) and in real urine sample are a result of a matrix effect. The presence of particular analytes was confirmed by standard addition method and from UV spectra recorded within the range 200–400 nm.

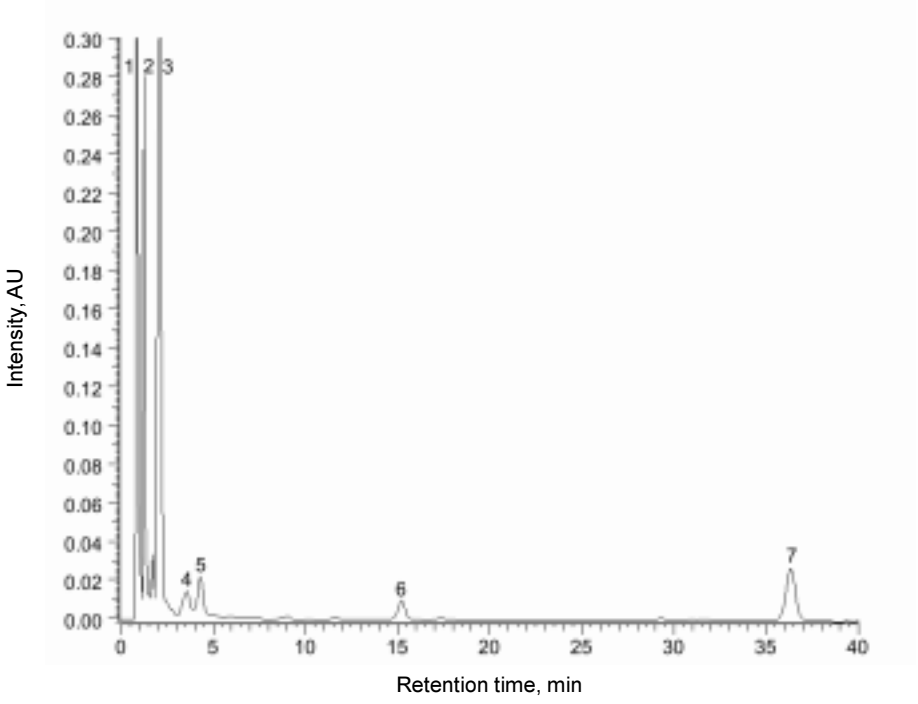

Figure 3. Chromatogram of urine samples (1 – 3MX, 2 – 1MX, 3 – paracetamol, 4 – 13DMU, 5 – THPH, 6 – CAFF, 7 – furosemide) of the treated patients

The chromatographic system described in this paper allows one to determine teophylline, its metabolites, and also other drugs, which are administrated to patients. It should be emphasized that until now there has been no published report on a chromatographic system suitable for simultaneous determination of these types of drugs. Small amount of urine (1 mL) needed to perform the analysis, fast and easy sample preparation, and convenient analysis time make the developed method applicable to the estimation of the content of particular drugs, methylxanthines, and their metabolites in urine samples.

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