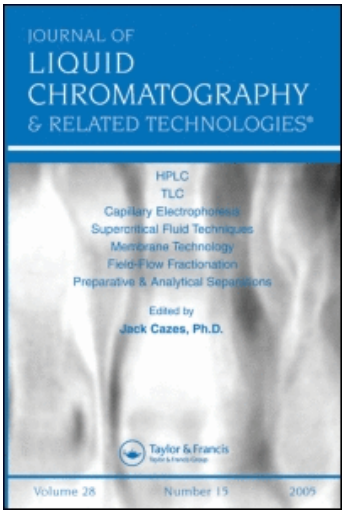

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I. N. Papadoyannis[a]; V. F. Samanidou[a]; K. A. Georga[a]
[a] Laboratory of Analytical Chemistry Chemistry Department Aristotle, University of Thessaloniki GR, Thessaloniki, Greece

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SOLID-PHASE EXTRACTION STUDY AND PHOTODIODE ARRAY RP-HPLC ANALYSIS OF XANTHINE DERIVATIVES IN HUMAN BIOLOGICAL FLUIDS

I. N. Papadoyannis,* V. F. Samanidou, K. A. Georga

Laboratory of Analytical Chemistry
Chemistry Department
Aristotle University of Thessaloniki
GR - 54006 Thessaloniki Greece

ABSTRACT

An automated reverse phase high performance liquid chromatography (HPLC) - photodiode array method using a multi linear gradient elution is described for the simultaneous analysis of nine xanthines: xanthine, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, isocaffeine, theobromine, paraxanthine, theophylline and caffeine. The separation method development was based on mobile-phase optimisation and off-line solid-phase extraction (SPE) from human biological fluids: blood serum and urine. Eluent consisted of 0.05 M CH_3COONH_4 and methanol (90:10 v/v) changing to (70:30 v/v) over a period of 20 min. Identification of xanthines was achieved by photodiode - array detector and quantitation was performed at 270 nm. Isocaffeine was used as internal standard at a concentration of 3.06 ng/μL. High extraction recoveries were achieved from Merck RP-18 cartridges using 1% hydrochloric acid as eluent, requiring small volumes, 40 μL of blood serum and 100 μL of urine.

The separation of xanthines was achieved on octylsilica, using a Silasorb C_8, 10μm, 250x4.6 mm i.d. analytical column thermostated at 32 ^{0}C and proved to be highly selective, sensitive, reproducible, accurate and rapid regarding the nine compounds. Detection limits ranged from 2 to 3 ng for 20 μL injected volume while linearity holds up to 20 ng/μL for each compound.

INTRODUCTION

The term xanthine derivatives (methylxanthines), in the case of the present study, refers to mono - di and tri-methylated derivatives of xanthine.

Caffeine (1,3,7-trimethylxanthine, 1,3,7 TMX) and its dimethylated metabolites: theophylline (1,3-dimethylxanthine, 1,3 DMX) and theobromine (3,7-dimethylxanthine, 3,7 DMX) three of the most well known compounds that belong to the group of methylxanthines, are ubiquitous in human biological fluids, due to the dietary intake of coffee, tea and cola drinks. Additionally, theobromine is present due to chocolate consumption.[1]

Theophylline is a mild diuretic agent, a moderate myocardial and central nervous stimulant and a powerful bronchodilator. Caffeine is utilised in the therapy of neonatal apnoea.[2,3]

The methylxanthines are extensively metabolised by the hepatic microsomal mixed function oxidase (cytochrome P_{450}) system so that less than 10% of theophylline and less than 2% of caffeine are excreted unchanged in the urine.

The presence of the pharmacologically active dietary methylxanthines may influence patient compliance with theophylline therapy used in the treatment of asthma and chronic obstructive pulmonary disease. They may also alterate the evaluation and interpretation results of theophylline therapeutic drug monitoring.[1]

Patients on theophylline medication for obstructive airway diseases and consuming caffeine containing beverages excrete the whole spectrum of metabolites in the urine.

It is advantageous to be able to measure the individual metabolites and assess their contribution to the total xanthine level.

Figure 1 presents the interrelationships of methylxanthines and their metabolic products expected to occur in serum.[1]

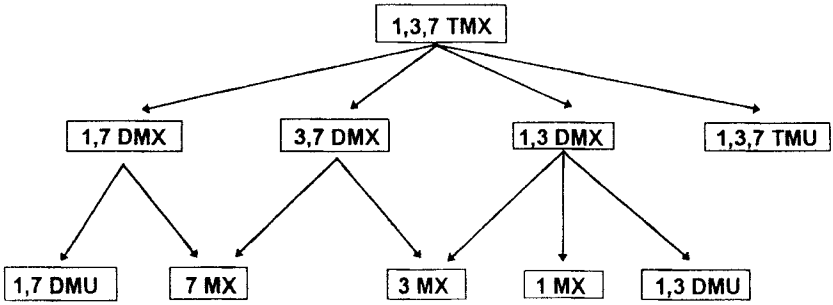

Figure 1. The Interrelationships of the xanthine derivatives and their metabolic products expected to occur in plasma. (U=uric acid).

A large variety of techniques and analytical procedures for determining xanthine derivatives in biological fluids have been reported: Radioimmunoassay (RIA), fluorescence polarisation immunoassay (FPIA), enzyme immunoassay (EIA), capillary electrophoresis and several chromatographic methods with emphasis on HPLC.[4-6]

A number of HPLC methods, reverse phase and normal, have been reported for the determination of methylxanthines in biological fluids. Many of the previously reported assays failed to separate other xanthine derivatives from theophylline and, thus, have substantially overestimated theophylline levels leading to clinical problems. A few have addressed this problem by using isocratic or gradient elution, ion-exchange columns or ion-pairing reagents which improve separation but lead to rapid deterioration of column efficiency.[7-14] High performance liquid chromatographic methods that enable separation and identification of all possible metabolites are required for caffeine and theophylline analysis.

Several procedures for the preparation of biological samples have been reported. Extraction with organic solvent followed by evaporation and reconstitution of the residue and dilution of serum, with or without, eliminating proteins are generally accepted methods.[3,15]

Solid-phase extraction (SPE) recently substituted the classic liquid-liquid extraction, as being rapid, solvent consuming, which leads to reduction in pollution and offering a wide sorbent selection. Therefore, SPE is widely applied to sample preparation especially for samples of biological interest. Most of the assays developed to measure xanthine derivatives and metabolites in biological samples have required a large volume of sample, tedious sample preparation and long chromatogram run times. Furthermore, many of these assays exhibited poor resolution of the compounds of interest.[16]

This prompted us to develop a method that would require small sample volumes, a relatively short and simple sample preparation and a shorter chromatogram run time, as well as lower limits of detection.

The present method deals with the simultaneous determination of xanthine derivatives in human biological fluids, blood serum and urine, in less than 20 minutes time, after solid-phase extraction. It requires only a small sample volume, 40 μL of serum and 100 μL of urine, making it a valuable tool for clinical paediatric research.

It is sensitive and rapid providing high selectivity, satisfactory reproducibility and high accuracy.

EXPERIMENTAL

Instrumentation

A Shimadzu (Kyoto, Japan), quaternary low pressure gradient system was used for chromatographic analysis of xanthine derivatives. The solvent lines were mixed in a FCV-9AL mixer and an LC-9A pump was used to deliver the mobile phase to the analytical column which was thermostated in a CTO-6A oven.

Sample injection was performed by a SIL-9A autosampler and detection was achieved by a SPDM-6A photodiode array detector.

Chromatograms were stored on the hard disk of a Function 386 PC and printed on a SEIKOSHA SP-1900 printer.

Degassing of solvents was achieved by continuous helium sparking in the solvent flasks through a DGU-2A degassing unit.

The analytical column was a Silasorb C_8, 10μm, 250x4.6 mm i.d., purchased by Rigas Labs, Thessaloniki, Greece.

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solution, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pre-treatment of biological samples.

The SPE study was performed on a Vac-Elut vacuum manifold column processor purchased from Analytichem International, a division of Varian (Harbor City, USA).

All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rocford, IL, USA).

UV spectra for selecting the working wavelength of detection were taken using a Varian DMS 100S UV/VIS double-beam spectrophotometer.

All computations were achieved using a VIP 312 computer.

Materials and Reagents

All xanthine derivatives were purchased by Sigma (St. Louis, MO, USA).

HPLC gradient grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Ammonium acetate p.a. was also from Merck. Bis de-ionised water was used throughout analysis. Solid phase extraction cartridges C_{18} were from Merck.

The mobile phase was vacuum filtered before use through 0.2 μm membrane filters.

Standard Solutions

Stock solutions of xanthine derivatives were prepared in methanol and stored refrigerated at 4 ^{0}C. These solutions were found to be stable for at least one month.

Working methanolic solutions were prepared from stocks at concentrations 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, 10.0, 15.0 and 20.0 ng/μL. Methanolic solution of internal standard was added at a concentration of 3.06 ng/μL.

Chromatography

Peak areas of the xanthine derivatives were measured and the ratio to internal standard was compared for each compound to that of the calibration standards. Maintaining the analytical column at 32 °C ensures reproducible separations.

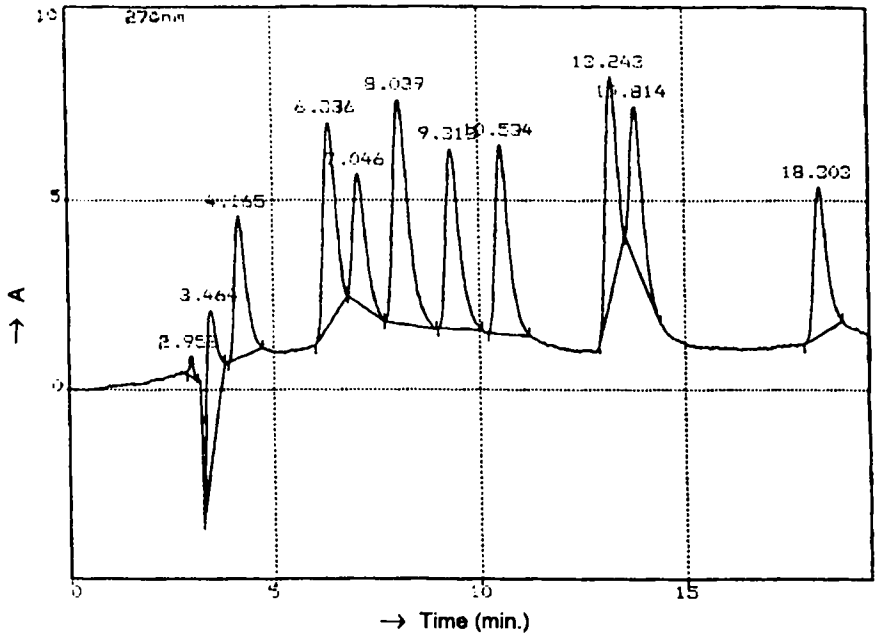

Figure 2. High performance Liquid Chromatogram of Xanthine Derivatives. Peaks: (4.165) = XA, (6.336) = 7MX, (7.046) = 3MX, (8.039) = 1MX, (9.318) = 1C, (10.534) = TB, (13.243) = PX, (13.243) = TP and (18.303) = CA. At a concentration of 3.0 ppm.

RESULTS AND DISCUSSION

Optimisation of the Chromatographic System

The chromatographic system for the separation of the nine xanthine derivatives was chosen among others, regarding the gradient time and eluent consistence, in order to result in optimum separation with high selectivity and detection effectiveness in as shorter analysis time as possible.

A variety of mobile phases was tested in order to find out the optimum chromatographic system for the analysis of xanthine derivatives. The mobile phases, were in principle, binary mixtures of an aqueous solution of ammonium acetate with methanol in several ratios. The final mobile phase is chosen in terms of peak shape, column efficiency and chromatographic analysis time,

selectivity and resolution. An increase in organic solvent percentage yielded a deterioration of chromatogram shape. The optimum conditions are reported in Table 1. Figure 2, shows the chromatogram obtained during the separation of the xanthine derivatives by means of the chromatographic system developed at the present study.

The R_s values for the nine compounds: xanthine (XA), 7-methylxanthine (7MX), 3-methylxanthine (3MX), 1-methylxanthine (1MX), isocaffeine (1,3,9-trimethylxanthine, IC), theobromine (3,7-dimethylxanthine, TB), paraxanthine (1,7- dimethylxanthine, PX), theophylline (1,3- dimethylxanthine, TP) and caffeine (1,3,7- trimethylxanthine, CA) are: 3.11, 1.22, 1.31, 1.37, 1.31, 4.24, 1.16 and 7.02 respectively per couple of compounds.

Performance Characteristics of the Proposed Method

The system described here was used for the simultaneous determination of nine xanthine derivatives: XA, 7MX, 3MX, 1MX, IC, TB, PX, TP and CA.

Optimised chromatographic conditions were set and the following analytical characteristics were evaluated:

- Precision and accuracy.
- Analysis time.
- Calibration data.
- Selection of wavelength.
- Solid-phase extraction and
- Real sample analysis.

Precision and Accuracy

Method validation regarding reproducibility was achieved by replicate injections of standard solutions at low and high concentration levels where peak areas were measured in comparison to the peak area of the internal standard.

Statistical evaluation revealed relative standard deviations at different values for eight injections. Results are shown in Table 2.

Long term stability study was conducted during routine operation of the system over a period of eight consecutive days. Results are illustrated in Table 3.

Table 1

Optimum Conditions for the Chromatographic Separation of Xanathine Derivatives

Operation	Parameter	Value
Separation	CH_3COONH_4 (A)	0.05 M
	MeOH (B)	
	pH	7.0
	Flow rate	1 mL/min
	Inlet pressure observed	160 Kg/cm^2
Gradient System	A:B 90-10 (v/v)	0 min
	A:B 80-20 (v/v)	8 min
	A:B 70-30 (v/v)	15 min
	A:B 70-30 (v/v)	20 min
Detection	Photodiode Array	270 nm
	Sensitivity	0.002aufs
	Temperature	32°±0.1° C

Table 2

Within-Day Precision and Accuracy for the Analysis of Xanthine Dervatives (n=8)

Compound	Added (ng)	Found (ng)	SD	RSD(%)
XA	21.6	20.8	0.3	1.44
	40.8	39.9	0.8	2.00
	60.0	58.2	1.3	2.23
	100.8	105.6	3.9	3.69
7MX	20.4	21.2	0.7	3.30
	40.8	39.3	1.5	3.82
	62.2	61.9	2.3	3.72
	102.0	106.6	8.2	7.69
3MX	20.4	21.8	0.9	4.13
	40.8	39.9	0.7	1.74
	61.2	59.8	4.7	7.86
	102.0	101.2	5.6	5.53

(continued)

Table 2 (continued)

Within-Day Precision and Accuracy for the Analysis of Xanthine Dervatives (n=8)

Compound	Added (ng)	Found (ng)	SD	RSD (%)
IMX	20.4	20.7	1.4	6.76
	40.8	40.7	0.8	1.96
	61.2	58.6	4.6	7.85
	102.0	99.9	7.4	7.41
TB	20.0	21.3	1.5	7.04
	40.0	38.7	2.9	7.49
	60.0	57.8	4.7	8.13
	100.0	98.2	5.9	6.01
PX	20.0	21.1	0.6	2.84
	40.0	38.9	2.7	6.94
	60.0	62.3	5.0	8.03
	100.0	101.9	4.7	4.79
TP	20.4	19.2	1.9	9.90
	40.8	41.5	2.2	5.30
	61.2	61.5	4.4	7.15
	102.0	102.5	7.3	7.12
CA	20.2	21.1	1.2	5.69
	40.4	41.4	0.8	1.93
	60.6	61.3	1.9	3.10
	101.0	102.2	4.9	4.79

Working Range and Detectability

For the simultaneous analysis of the nine xanthine derivatives, the term working range is more proper than linear range since column saturation that takes place at high concentrations leads to poor peak shapes.

The upper limit regarding the higher concentration of injected solution assumes the co-existing concentration of other compounds at a similar level. Therefore, higher concentrations could also be injected in case of other compounds' absence. Upper limit reported is 20 ng/μL.

Table 3

Day-to-Day Precision and Accuracy for the Analysis of Xanthine Derivatives over a Period of 8 Consecutive Days.

Compound	Added (ng)	Found (ng)	SD	RSD (%)
XA	21.6	22.4	1.9	8.48
	40.8	39.4	1.0	2.54
	60.0	59.4	2.2	3.70
	100.8	99.2	5.4	5.44
7 MX	20.4	21.6	0.8	3.70
	40.8	40.4	0.5	1.24
	61.2	61.3	0.2	0.33
	102.0	101.8	4.9	4.81
3MX	20.4	21.1	0.2	0.95
	40.8	40.6	2.3	5.67
	61.2	60.6	3.8	6.27
	102.0	103.7	2.3	2.22
1MX	20.4	20.8	0.6	2.89
	40.8	39.8	1.0	2.51
	61.2	60.4	1.3	2.15
	102.0	101.7	5.5	5.41
TB	20.0	22.3	1.8	7.89
	40.0	38.7	1.7	4.39
TB	60.0	61.8	4.9	7.93
	100.0	95.9	4.7	4.90
PX	20.0	21.2	1.1	5.19
	40.0	40.1	0.9	2.24
	60.0	59.8	1.4	2.34
	100.0	100.3	2.0	1.99
TP	20.4	22.1	0.8	3.62
	40.8	40.8	2.1	5.15
	61.2	62.4	4.2	6.73
	102.0	102.7	2.4	2.34

(continued)

Table 3 (continued)

Day-to-Day Precision and Accuracy for the Analysis of Xanthine Derivatives over a Period of 8 Consecutive Days.

Compound	Added (ng)	Found (ng)	SD	RSD (%)
CA	20.2	20.9	0.7	3.35
	40.4	39.0	1.1	2.82
	60.6	57.5	4.2	7.31
	101.0	101.3	5.8	5.73

Detection limits were calculated as a three-fold signal - to - noise ratio at the baseline (S/N = 3) and found to be 2.0 ng for xanthine, 7-methylxanthine and 1-methylxanthine, while 3.0 ng for the rest of the compounds, for 20μL injected sample volume.

Analysis Time

The analysis time in the proposed method is determined by the retention time of the most strongly retained compound in the chromatographic separation, as analytical column is rapidly equilibrated after the last step of gradient system.

As it is shown in Figure 2 the sample analysis time is less than 19 min., as caffeine, the last eluted compound has a retention time of 18.303 min.

Calibration Data

Calibration of the method was performed by injection of mixed standard of xanthine derivatives covering the entire working range. Ten concentrations were used in the range 0.25 - 20 ng/μL.

The sensitivity setting of the photodiode array detector was adjusted to give almost full-scale deflection for the highest standard concentration. Each sample was injected five times.

Linear correlation between absolute injected amount or concentration and peak area ratio was obtained for all xanthine derivatives using isocaffeine as internal standard at a concentration of 3.06 ng/μL. Isocaffeine was selected for two reasons: it is absent in biological fluids as it isn't a caffeine metabolite and it appears in the middle of the chromatographic analysis time. The results of the statistical treatment of calibration data for xanthine derivatives are summarised in Table 4.

Table 4

Calibration Data for Simultaneous Determination of Xanthine Derivatives*

Parameter	Value	XA	7MX	3MX	1MX	TB	PX	TP	CA
Concentration range	mg/L	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20
Slope	$AIU.ng^{-1}$	0.01566	0.02286	0.01743	0.03478	0.04017	0.02809	0.02214	0.02597
Intercept		-0.16856	-0.25512	-0.21823	-0.41312	-0.76511	-0.36104	-0.32832	-0.38750
Correlation coefficient		0.98806	0.99536	0.99426	0.99341	0.99102	0.99550	0.99454	0.99398
Detection limit	ng	2	2	3	2	3	3	3	3
Retention time	min.	4.165	6.336	7.046	9.315	10.534	13.243	13.814	18.303

*Peak area ratio measurement with 3.06ng/μL Isocaffeine as internal standard RT = 8.039 min.

Table 5

Recovery of Xanthine Derivatives after SPE using C_{18} Cartridges with Isocaffeine as a Chromatographic Standard

Compound	Recovery (%)
XA	108.3
7MX	97.2
3MX	101.9
1MX	92.9
TB	91.5
PX	96.5
TP	100.6
CA	94.2

Selection of Wavelength

The analytical wavelength 270 nm was chosen for quantitation since it represents a maximum absorbance as results from xanthine compounds' spectra. Quantitation at this wavelength enhances sensitivity for all compounds.

Solid Phase Extraction of Xanthine Derivatives

Several different solid-phase extraction cartridges provided by different manufacturers were tested for the optimisation of xanthine derivatives' isolation and recovery in human blood serum and urine.

Xanthine derivatives were subsequently analysed by HPLC after separation on a C_8 column with isocaffeine as internal standard in case of blood serum and without internal standard in case of urine.

Sample preparation time was approximately one hour including sample evaporation to dryness step. Taking in account that ten samples were simultaneously treated, single analysis time is reduced to six minutes per sample. Extraction efficiency was calculated by extracting standard solutions at six different concentration levels, i.e. 1.0, 2.0, 3.0, 4.0, 5.0, 7.0 ng/μL, of xanthine derivatives.

Recovery of compounds was calculated by comparing peak area ratios against internal standards with those obtained for unextracted methanolic solutions. Results obtained are presented in Table 5.

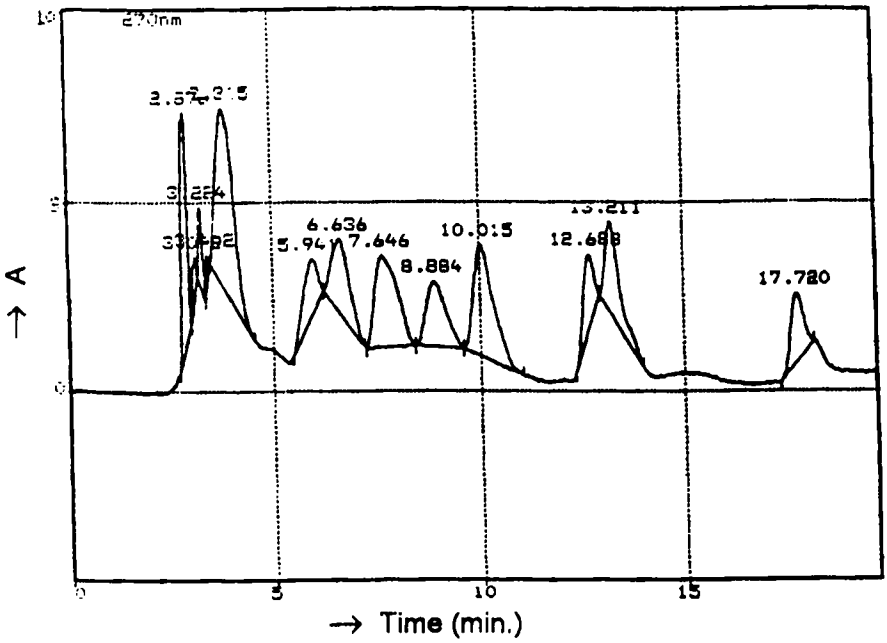

Figure 3. High Performance Liquid Chromatogram of Xanthine Derivatives Extracted from Blood Serum. Peaks: (3.815)=XA, (5.941)=7MX, (6.636)=3MX, (7.646)=1MX, (8.884)=IC, (10.015)=TB, (12.688)=PX, (13.211)=TP and (17.720)=CA.

100 μL of standard solution were applied to the SPE cartridge, which was conditioned by flushing 3 mL MeOH and 3 mL H_2O prior to the addition of sample. After applying the sample, cartridge was dried by sucking air. Xanthine derivatives were eluted using 3 mL 1 % HCl. The sample was subsequently evaporated to dryness under gentle nitrogen steam in a 45 °C water bath and diluted to 100 μL internal standard solution (3.06 ng/μL).

A washing step with water resulted in losses of xanthine derivatives and poor recoveries were noticed. Thus this step was omitted in the present study.

Real Sample Analysis

a. Human Blood Serum

Aliquots of 40 μL of human blood serum were treated with 80 μL of acetonitrile to precipitate proteins in order to release bound xanthine derivatives.

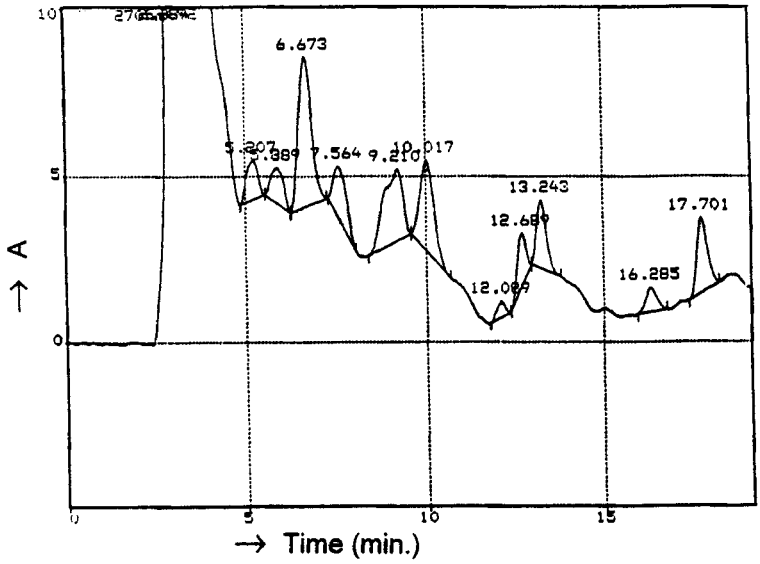

Figure 4. High Performance Liquid Chromatogram of Xanthine Derivatives Extracted from Urine. Peaks: (5.841)=7MX, (7.564)=1MX, (10.017)=TB, (12.689)=PX, (13.243)=TP and (17.701)=CA.

After 2 min. vortex mixing, 100 µL of mixed standard solution were added to the sample at concentrations: 1.0, 2.0, 3.0, 4.0 and 5.0 ng/µL.

The sample was subsequently centrifuged at 3500 rpm for 15 min and the supernatant was transferred to a clean eppendorf tube, where 30 µL of methanol were added. Finally the sample was slowly applied to the SPE cartridge and procedure followed the steps described under solid-phase extraction paragraph.

b. Urine

A similar sample preparation method was followed for urine samples using 100 µL urine sample and 200 µL of acetonitrile for deproteinization. The rest of the procedure is described above. Reconstitution to volume was performed with 100 µl of methanol, as no internal standard was used in case of urine analysis.

The serum and urine samples were pooled samples free from xanthine derivatives and collected from five healthy volunteers. No interference from endogenous compounds from sample matrix was observed in case of blood serum as shown in Figure 3.

Table 6

Calibration Curves for the Analysis of Xanthine Derivatives in Spiked Blood Serum Samples using Isocaffeine (3.06 ng/μL) as Internal Standard

Analyte	Calibration Curve Equation $Y^* = a + bx$	Correlation Coefficient
XA	Y = -0.45033 + 0.02969X	0.99985
7MX	Y = 0.06142 + 0.00611X	0.99507
3MX	Y = 0.00465 + 0.00967X	0.99985
1MX	Y = -0.10697 + 0.01718X	0.99992
TB	Y = 0.06450 + 0.01195X	0.99953
PX	Y = -0.0041 + 0.00582X	0.99195
TP	Y = -0.15740 + 0.01182X	0.99878
CA	Y = -0.09367 + 0.01026X	0.99976

Y^* = Peak area ratio of analyte to internal standard. X=ng of the analyte.

Table 7

Calibration Curves for the Analsis of Xanthine Derivatives in Spiked Urine Samples

Analyte	Calibration Curve Equation $Y^* = a + bx$	Correlation Coefficient
7MX	Y = -23846.3 + 623.3X	0.99807
1MX	Y = 742.8 + 556.6X	0.97658
TB	Y = -12.8 + 892.1X	0.98681
PX	Y = -5811.2 + 410.7X	0.99252
TP	Y = -536.0 + 548.0X	0.99008
CA	Y = 13144.2 + 478.8X	0.98469

Y^* = Peak area, X=ng of the analyte.

While some urine endogenous compounds interfere with some determined xanthine derivatives. Thus xanthine and 3-methylxanthine could not be analysed in urine samples and isocaffeine could not be used as internal standard as shown in Figure 4.

Table 8

Recovery of Xanthine Derivatives from Human Blood Serum after SPE on C_{18} Cartridges using Isocaffeine as Internal Standard.*

Compound	Added (ng)	Found (ng)	SD	RSD (%)
XA	19.6	19.4 ± 0.5	2.58	99.0
	58.8	59.6 ± 2.1	3.52	101.4
	100.0	100.9 ± 1.2	1.19	100.9
7MX	20.4	19.5 ± 0.3	1.54	95.6
	61.2	65.6 ± 2.5	3.81	107.2
	102.0	94.4 ± 1.8	1.91	92.5
3MX	21.2	21.1 ± 0.8	3.79	99.5
	63.6	63.1 ± 1.5	2.38	99.2
	106.0	107 ± 4.5	4.20	101.0
1MX	19.6	19.9 ± 1.1	5.53	101.5
	57.9	57.9 ± 2.3	3.97	98.5
	98.3	98.3 ± 6.7	6.82	100.3
TB	19.5	19.5 ± 1.6	8.21	96.5
	59.7	59.7 ± 1.5	2.51	98.5
	100.2	100.2 ± 4.1	4.09	99.2
PX	21.2	21.3 ± 0.7	3.29	100.5
	63.6	65.2 ± 1.8	2.76	102.5
	106.0	106.9 ± 6.1	5.71	100.8
TP	20.0	21.2 ± 0.4	1.89	106.0
	60.0	57.8 ± 3.0	5.19	96.3
	100.0	102.1 ±5.1	5.00	102.1
CA	20.4	20.2 ± 0.4	1.98	99.0
	61.2	63.3 ± 2.5	3.95	103.3
	102.0	103.6 ± 2.5	2.41	101.6

* Mean value of six measurements

Table 9

Recovery of Xanthine Derivatives from Human Urine Samples after SPE on C_{18} Cartridges*

Compound	Added (ng)	Found (ng)	SD	RSD (%)
7 MX	40.8	38.4 ± 1.8	4.69	94.1
	61.2	60.4 ± 5.2	8.61	98.7
	102.0	102.5 ± 4.1	4.00	100.5
1MX	39.2	41.6 ± 2.1	5.03	106.1
	58.8	56.9 ± 3.3	5.80	96.8
	98.0	103.7 ± 5.6	5.40	105.8
TB	40.4	41.1 ± 0.8	1.95	101.7
	60.6	58.6 ± 4.1	7.00	96.7
	101.0	98.6 ± 1.7	1.72	97.6
PX	42.4	43.0 ± 0.6	1.40	101.4
	63.6	60.7 ± 2.5	4.12	95.4
	106.0	100.6 ± 1.7	1.68	95.2
TP	40.0	40.2 ± 1.3	3.23	100.5
	60.0	58.6 ± 0.7	1.19	97.7
	100.0	101.7 ± 4.4	4.32	101.7
CA	40.8	38.9 ± 0.8	2.06	95.3
	61.2	63.0 ± 4.0	6.35	102.8
	102.0	98.3 ± 5.5	5.60	96.4

* Mean value of six measurements.

Calibration curves for the analysis of xanthine derivatives in blood serum and urine samples are presented in Tables 6 and 7, respectively. The precision and accuracy studies of solid phase extraction of xanthine derivatives from biological samples were conducted by spiking drug free blood serum and urine samples with known concentrations of the compounds and then by comparing obtained results with those as calculated from regression equations. Results are given in Table 8 for serum samples and Table 9 for urine samples. Each value represents the mean of six measurements carried out.

CONCLUSIONS

Nine xanthine derivatives: xanthine, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, isocaffeine, theobromine, paraxanthine, theophylline and caffeine were separated and analysed by means of HPLC with photodiode array detection.

Isocaffeine as being absent in biological fluids was selected as internal standard.

The binary gradient eluent system used provides good separation, high selectivity, resolution within a minimum analysis time of approximately 18 min. Detection limits are within 3 ng range for 20 μL injected sample volume.

Day-to-day precision was tested over 8 consecutive days and repeatability (within day run) proved to be very satisfactory (RSD < 10 %).

Solid phase extraction was used for the isolation of the analytes from biological fluids. Only a small volume of the sample is required (40 μL in case of serum and 100 μL in case of urine) making the method a valuable tool for clinical paediatric research.

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