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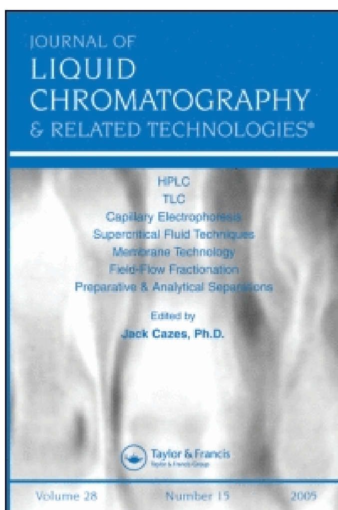

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ABSTRACT

An automated reversed phase high performance liquid chromatographic (RP-HPLC) method, using a multi-linear gradient elution, is described for the simultaneous analysis of five methyluric acids: 7-methyluric acid (7-MU), 1-methyluric acid (1-MU), 1,3-di-methyluric acid (1,3-DMU), 1,7-di-methyluric acid (1,7-DMU), and 1,3,7-tri-methyluric acid (1,3,7-TMU).

The separation method was based on mobile-phase optimization and off-line solid-phase extraction (SPE) from human biological fluids: blood serum and urine. The analytical column used was octylsilica, MZ-Kromasil, 5 μm, 250 x 4 mm i.d. Elution was performed at ambient temperature with a mixture of acetate buffer (pH = 3.5) and methanol at a volume ratio 95:5, changing to 30:70 v/v, over a period of 15 min. Isocaffeine was used as internal standard at a concentration of about 8 ng/μL.

Detection was performed with a variable wavelength UV-visible detector at 280 nm, resulting in detection limits of 0.1 ng per 10 μL injection, while linearity held up to 5 ng/μL for each compound.

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The statistical evaluation of the method was examined performing intra day (n=8) and inter day calibration (n=8) and was found to be satisfactory, with high accuracy and precision results.

High extraction recoveries, ranging from 89% to 106 %, were achieved using Oasis HLB cartridges with methanol as eluent, requiring small volumes, 40 μL of blood serum and 100 μL of urine.

INTRODUCTION

The term methyluric acids, refers to mono - di and tri-methylated derivatives of uric acid, the final products of a group of methylxanthines.

Caffeine (1,3,7-tri-methylxanthine, 1,3,7 TMX) and its di-methylated metabolites: theophylline (1,3-di-methylxanthine, 1,3 DMX) and theobromine (3,7-di-methylxanthine, 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. However large amounts of these xanthines may also be absorbed by consumption of caffeine containing analgesics and antacids. Additionally caffeine and theobromine are present due to chocolate consumption.[1]

Theophylline and caffeine are potent bronchodilators and widely used in the treatment of asthma and neonatal apnoea. Theophylline is a mild diuretic agent, a moderate myocardial and central nervous stimulant, and a powerful bronchodilator.[2-4]

The methylxanthines are extensively metabolised by the hepatic microsomal mixed function oxidase (cytochrome P450) system so that less than 10% of theophylline and less than 2% caffeine are excreted unchanged in the urine. Many of these metabolites are pharmacologically active and the importance of considering their contribution to the total biological effect in therapeutic drug monitoring (TDM) has been suggested especially in cases of renal insufficiency or failure. Additionally the activity of the enzyme can be assessed after caffeine ingestion by determining the ratio of the metabolites.[5,6]

The interrelationships of administered methylxanthines and their metabolic products methyluric acids that can be expected to occur in plasma are indicated in Figure 1.

The main excretory methylurate products after theophylline and caffeine ingestion are 1,3-di-methyluric acid and 1- methyluric acid, while the major urinary metabolite of theobromine is 7-MU.[5,7]

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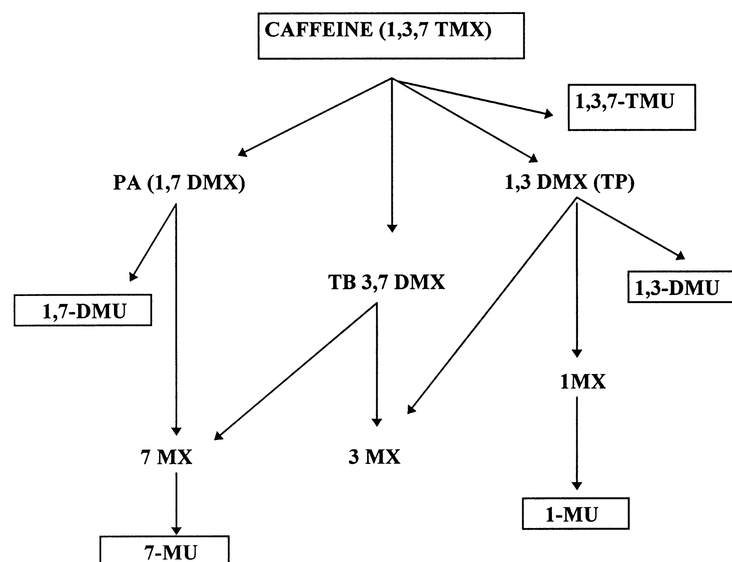

Figure 1. The interrelationships of the xanthine derivatives and their metabolic products (methyluric acids) expected to occur in blood serum and urine.

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The pharmacological activity of theophylline is highly correlated with its concentration in serum and inter-individual variations are considerable during drug clearance. Various environmental factors and genetic polymorphism influence the clearance of methylated xanthine derivatives from plasma.

Owing to the pharmacological activity of these metabolites it is important to measure their concentration, when examining the relationships of the pharmacokinetics of theophylline to its pharmacological effect.[7,8]

The determination of the relationships between the drug and its metabolites permit identification of abnormal metabolism with more precision and provide an explanation for toxic symptoms not resolved by results from less comprehensive routine methylxanthine TDM assays. However, the determination of caffeine and its metabolites in biological fluids poses analytical problems such as: the presence of structurally similar metabolites and the individual variability in the metabolic pattern, in conjunction with the fact that these compounds may be in low concentrations in a small sample size available that can be very hardly extracted from matrices containing a variety of chemically different compounds.[9]

There are very few HPLC methods for the determination of xanthine related compounds and their major metabolites. Among them there is no method dealing solely with the analysis of methyluric acids.

Procedures for the preparation of biological samples reported include liquid-liquid extraction with organic solvent followed by evaporation and reconstitution of the residue, solid phase extraction, and the formation of an ion-pair complex with tetrabutylammonium ion.[2,8,10]

Ion - pair chromatographic systems are also described using tetrabutylammonium or decylammonium ion and Tris buffer, to determine methyluric acid derivatives among other methylxanthines. Ion - pair inclusion in the solvent mixture is used in order to increase the retention times of the polar and poorly related uric acid metabolites.[2,11-12]

Most of the assays developed to measure the concentrations of methylxanthine metabolites in serum have required a large volume of sample, tedious sample preparation and long chromatogram run times.

The present method deals with the simultaneous determination of methyluric acids in human biological fluids, blood serum, and urine, in approximately 15 minutes, 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 pediatric research. It is sensitive and rapid providing high selectivity, satisfactory reproducibility, and high accuracy.

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EXPERIMENTAL

Equipment/Instrumentation

The chromatographic system used for the analysis of methyluric acids consists of the commercial components: a Spectra Physics SP 8800 HPLC ternary pump (Spectra Physics, California, U.S.A.), a Spectra Chrom 100 UV/Vis detector, operated at 280 nm, at a sensitivity setting of 0.002 AUFS. A Spectra Physics SP 4290 integrator was used, for quantitative determination of eluted peaks. The analytical column, was octylsilica, Kromasil, 5 µm, 250 x 4 mm i.d (MZ Analysentechnik , Mainz, Germany).

Sample injection was performed via a Rheodyne 7125 injection valve (Rheodyne, Cotati California, U.S.A), with a 10 mL loop. 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).

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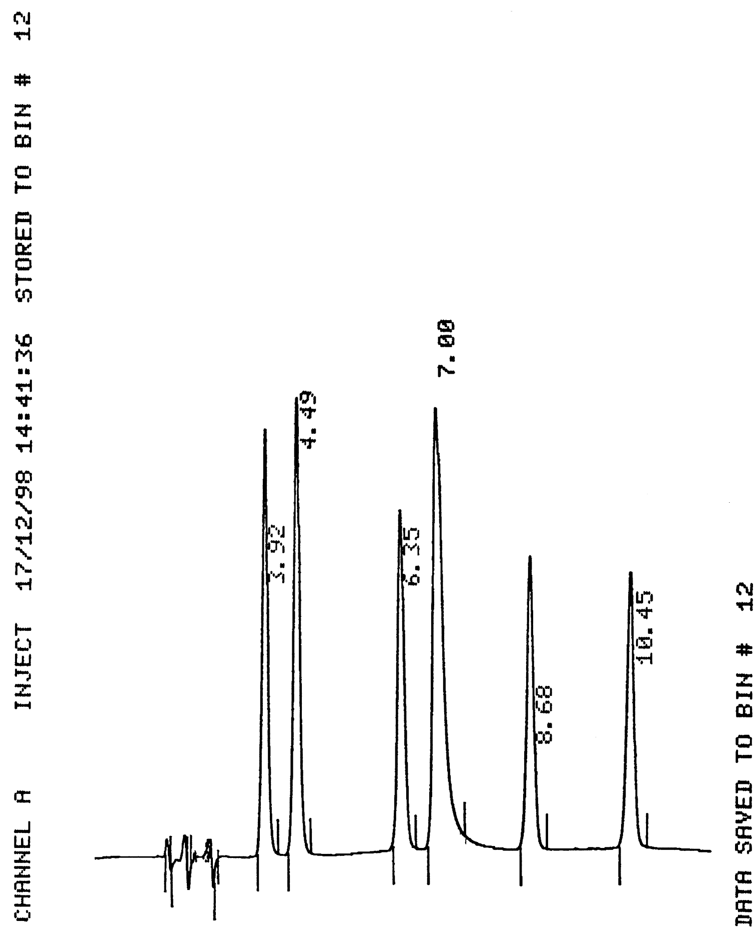

Figure 2. High performance liquid chromatogram of methyluric acids. 7-UM (3.92 min), 1-MU (4.49 min), 1,3-DMU (6.35 min), isocaffeine internal standard (7.00 min). 1,7-DMU (8.68 min), and 1,3,7-TMU (10.45 min). Chromatographic conditions are described in the text.

Degassing of solvents was achieved by continuously sparking with helium (99.999 %). A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the biological samples pre-treatment.

Table 1

Calibration Data for Methyluric Acids Determination with Isocaffeine as Internal Standard

Parameter	Value	7MU	1 MU	1,3-DMU	1,7-DMU	1,3,7-TMU
Concentration range	ng/μL	0.025-3.0	0.025-3.0	0.025-3.0	0.025-3.0	0.025-3.0
Slope	AIU/ng	0.0204 ± 0.00122	0.02527 ± 0.00051	0.02094 ± 0.00100	0.02026 ± 0.00030	0.00186 ± 0.00033
Intercept		0.01314 ± 0.02616	-0.00359 ± 0.00793	0.00643 ± 0.02237	-0.00096 ± 0.00656	-0.00072 ± 0.00760
Correlation Coefficient		0.99806	0.99983	0.99878	0.99806	0.99984

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The SPE assay was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, a division of Varian (Harbor City, USA). Oasis HLB cartridges (3cc, 60 mg) were purchased by Waters (Waters Corporation, Massachusetts, USA).

All evaporations were performed, with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA). UV spectra for selecting the working detection wavelength were taken by a Varian DMS 100S UV/VIS double beam spectrophotometer. All computations were achieved using a VIP 312 computer.

Reagents and Chemicals

Methyluric acids (1-methyluric acid, 7- methyluric acid, 1,3 di-methyluric acid, 1,7-di-methyluric acid, and 1,3,7 tri- methyluric acid) were purchased from Sigma (St. Louis, MO. U.S.A).

Acetate buffer (pH=3.5) constituents and sodium hydroxide were pro analysi grade, from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were obtained from Riedel-de-Haën (AG, Seelze, Germany). Bis de-ionised water was used throughout analysis.

Preparation of Stock and Standard Solutions

Aqueous stock solutions of methyluric acids at concentration of 100 mg/L were prepared using 0.01 M NaOH and stored refrigerated at 4°C.

These solutions were found to be stable throughout experimental analyses. Working aqueous solutions were prepared from stocks at concentrations: 0.025, 0.05, 0.10, 0.25, 0.60, 1.0, 2.0, 3.0 ng/µL. Isocaffeine was used as internal standard at a concentration of 8.08 ng/µL.

RESULTS AND DISCUSSION

A reversed phase C_8 Kromasil 250 x 4 mm, 5 µm, is used for the separation of methyluric acids at ambient temperature.

A variety of binary gradient systems of organic modifier (methanol) in low volume percentage, were tested with acetate buffer (pH 3.5), in several ratios and different gradient steps, to result in optimum chromatographic system.

The final mobile phase was chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution. Inlet pressure observed with the eluent system, at a flow rate 1 mL/min, was starting at 2100 psi and reaching 2700 psi at the final gradient step. The optimum multi-linear gradient system has the following shape:

Step 1. Time 0 min: Acetate buffer 95 % - MeOH 5 %

Step 2. Time 8 min: Acetate buffer 80 % - MeOH 20 %

Step 3. Time 15 min: Acetate buffer 70 % - MeOH 30 %

Figure 2 shows the chromatogram obtained using the conditions described in text. Resolution factors were found to be satisfactory indicating sufficient separation: 1.42 (7-MU and 1-MU), 4.65 (1-MU and 1,3 DMU), 1.30 (1,3 DMU and isocaffeine), 3.36 (isocaffeine and 1,7 DMU) 1,3,7 TMU), and 4.42 (1,7 DMU and 1,3,7 TMU).

Analytical Variables

Optimised chromatographic conditions were set and the statistical evaluation of the proposed method was performed according to the following parameters:

- Calibration data and analysis time.
- Working range and detectability.
- Precision and accuracy.
- Solid-phase extraction.
- Application to biological fluids: blood serum and urine.

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Table 2

Within-Day[a] and Day-to-Day[b] Precision and Accuracy Study for Methyluric Acids Determination in the Presence of Isocaffeine[c]

Analyte Analyte	Added (ng)	Within-Day Found ± SD (ng)	RSD (%)	Recovery (%)	Day-to-Day Found ± SD (ng)	RSD (%)	Recovery (%)
7-MU	2.71	2.35 ± 0.04	1.70	86.72	2.22 ± 0.05	2.25	81.92
	11.28	12.36 ± 0.09	0.73	109.57	12.34 ± 0.14	1.13	109.40
	28.20	27.26 ± 0.12	0.44	96.67	28.70 ± 0.70	2.44	101.77
1-MU	2.88	2.89 ± 0.07	2.42	100.35	2.86 ± 0.10	3.50	99.31
	12.00	12.02 ± 0.16	1.33	100.17	12.09 ±0.20	1.65	100.75
	30.00	27.96 ± 0.36	1.29	93.20	29.33 ± 0.58	1.98	97.77
1,3-DMU	2.82	2.37 ± 0.06	2.53	84.04	2.39 ± 0.10	4.18	84.75
	11.76	12.26 ± 0.24	1.96	104.25	12.17 ± 0.15	1.23	103.49
	29.40	29.90 ± 0.21	0.70	101.70	30.86 ± 0.37	1.20	104.97
1,7-DMU	2.76	2.48 ± 0.09	3.63	89.86	2.62 ± 0.09	3.44	94.93
	11.52	11.70 ± 0.13	1.11	101.56	11.87 ± 0.20	1.68	103.04
	28.80	27.97 ± 0.14	0.50	97.12	29.14 ± 0.42	1.44	101.18
1,3,7-TMU	2.94	2.69 ± 0.08	2.97	91.50	2.63 ± 0.17	6.46	89.46
	12.24	12.42 ± 0.18	1.45	101.47	12.21 ± 0.24	1.97	99.75
	30.60	29.68 ± 0.17	0.57	96.99	30.76 ± 0.62	2.02	100.52

[a] n = 8; [b] over a period of 8 consecutive days; [c] internal standard.

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Calibration Data and Analysis Time

The sample analysis time of methyluric acids, in the proposed method, is approximately 15 min. Calibration of the method was performed by injection of standards, covering the entire working range. Eight concentrations were used in the range 0.025-3.0 ng/μL, namely 0.025, 0.05, 0.10, 0.25, 0.60, 1.0, 2.0, 3.0 ng/μL.

The sensitivity setting of the UV-VIS detector was adjusted at 0.002 AUFS. Each sample was injected six times.

Linear correlation between absolute injected amount or concentration and peak area ratio, with isocaffeine, as internal standard, at a concentration of 8.08 ng/μL was observed. The results of the statistical treatment of calibration data are summarised in Table 1.

Table 3

SPE Recovery Results of Methyluric Acids from OASIS HLB with Different Procedures

Analyte	Recovery (%) A	B	C	D
1-MU	85.45	84.02	87.20	92.78
7-MU	107.05	104.59	82.57	96.95
1,3-DMU	90.92	106.95	86.28	93.75
1,7-DMU	96.39	96.98	90.71	94.38
1,3,7-TMU	93.81	95.19	83.45	97.91

A = Conditioning with MeOH-water. Elution with MeOH; B = Conditioning MeOH-water. Elution with MeOH-2% HCl; C = Conditioning with MeOH-2% HCl. Elution with ACN; D = Conditioning with MeOH-2% HCl. Elution with MeOH.

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Working Range and Detectability

The upper limit was found to be 5.0 mg/L, for all examined methyluric acids, while limit of detection, calculated as a three fold signal to noise ratio, at the baseline (S/N=3), was found to be 0.1 ng, when 10 µL of the sample were injected onto column. Limit of quantitation was found to be 0.25 ng for all compounds.

Isocaffeine was selected as internal standard for the reason that it is normally absent in real samples of biological fluids as not being a caffeine metabolite.

Precision and Accuracy

Method validation regarding reproducibility, was achieved, by replicate injections of standard solutions, at low, medium, 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. Mean values of five injections are presented in Table 2.

Table 4

Recovery of Methyluric Acids from Standard Solutions After SPE on OASIS HLB Cartridges Using Internal Standard

Analyte	Added (ng)	Found (ng)±SD	RSD (%)	Recovery (%)
7-MU	2.71	2.41 ± 0.12	4.98	88.93
	5.64	5.22 ± 0.44	8.35	92.55
	11.28	12.02 ± 1.16	9.64	106.56
	22.56	22.02 ± 1.10	4.99	97.61
	28.20	27.52 ± 1.17	4.25	97.59
1-MU	2.88	2.31 ± 0.07	3.04	80.21
	6.00	5.32 ± 0.06	1.05	88.67
	12.00	12.14 ± 0.08	0.69	101.17
	24.00	23.19 ± 0.70	3.01	96.62
	30.00	29.17 ± 0.53	1.83	97.23
1,3-DMU	2.82	2.31 ± 0.06	2.82	81.91
	5.88	5.48 ± 0.28	5.15	93.20
	11.76	11.64 ± 0.80	6.89	98.98
	23.52	23.18 ± 0.45	1.95	98.55
	29.40	28.25 ± 0.56	1.98	96.09
1,7-DMU	2.76	2.60 ± 0.05	1.82	94.20
	5.76	5.11 ± 0.11	2.09	88.71
	11.52	11.49 ± 0.42	3.66	99.74
	23.04	21.77 ± 0.45	2.08	94.49
	28.80	27.29 ± 0.60	2.20	94.76
1,3,7-TMU	2.94	3.00 ± 0.09	2.87	102.04
	6.12	5.87 ± 0.13	2.16	95.91
	12.24	12.30 ± 0.09	0.76	100.49
	24.48	23.56 ± 0.24	1.01	96.24
	30.60	29.03 ± 0.50	1.73	94.87

Solid Phase Extraction

Solid phase extraction protocol was selected among different solid phase cartridges and different conditioning and elution steps. Oasis HLB cartridges, that presented the highest recoveries, were subsequently tested, using four different protocols as shown in Table 3.

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Table 5

Cartridge-to-Cartridge Reproducibility of Methyluric Acids Recovery After SPE*

Analyte	Added (ng)	Found (ng)±SD	RSD (%)	Recovery (%)
7-MU	5.64	5.56 ± 0.08	1.44	98.58
	11.28	10.40 ± 0.27	2.60	92.20
	28.20	27.33 ± 0.58	2.12	96.91
1-MU	6.00	5.72 ± 0.08	1.40	95.33
	12.00	12.16 ± 0.34	2.89	101.33
	30.00	28.10 ± 0.65	2.31	93.67
1,3-DMU	5.88	5.62 ± 0.08	1.42	95.8
	11.76	11.33 ± 0.48	4.24	96.34
	29.40	27.69 ± 0.39	1.41	94.18
1,7-DMU	5.76	5.71 ± 0.04	0.79	99.13
	11.52	11.34 ± 0.30	2.64	98.44
	28.80	26.70 ± 0.67	2.52	92.71
1,3,7-TMU	6.12	5.78 ± 0.09	1.56	94.44
	12.24	11.61 ± 0.17	1.46	94.85
	30.60	27.75 ± 0.20	1.87	90.69

* n = 24.

Optimum protocol was then applied to standard solutions of methyluric acids, as follows. 200 μL of standard solution were applied to the Oasis HLB SPE cartridges, which were conditioned by flushing 3 mL MeOH and 3 mL 2% HCl prior to the addition of sample. After applying the sample, methyluric acids were eluted using 3 mL MeOH. The samples were subsequently evaporated to dryness under gentle nitrogen steam in a 45°C water bath and diluted to 200 μL internal standard isocaffeine solution in 0.01 N NaOH.

10 μL of the sample were injected into the analytical column. Extraction efficiency was calculated by extracting standard solutions of methyluric acids, at five different amount levels i.e. 0.25, 0.60, 1.0, 2.0, and 3.0 ng/μL. Regression equations after SPE revealed correlation coefficient values ranging from 0.99881 to 0.99971. The reproducibility and accuracy of solid phase extraction of methyluric acids were investigated. Results of recovery are shown in Table 4.

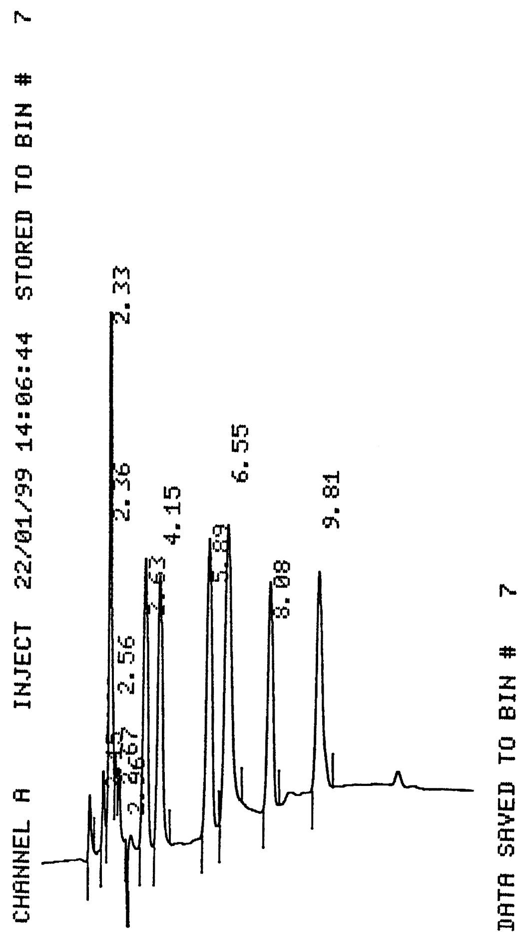

Figure 3. High performance liquid chromatogram of analysis of methyluric acids in spiked human blood samples. 7-MU (3.63 min), 1-MU (4.15 min), 1,3-DMU (5.89 min), isocaffeine internal standard (6.55 min), 1-7-DMU (8.08 min), and 1,3,7-TMU (9.81 min). Chromatographic conditions are described in the text.

Cartridge-to-cartridge reproducibility was tested applying the SPE procedure using four OASIS HLB cartridges from the same lot at three different concentration levels. Mean values of six measurements for each cartridge are presented in Table 5.

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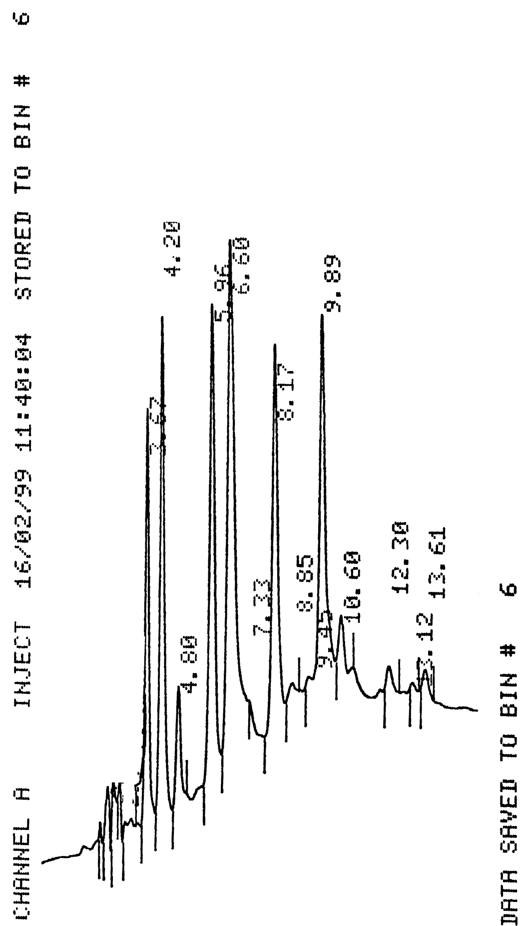

Figure 4. High performance liquid chromatogram of analysis of methyluric acids in urine samples. 7-MU (3.67 min), 1-MU (4.20 min), 1,3-DMU (5.96 min), isocaffeine internal standard (6.60 min), 1,7-DMU (8.17 min), and 1,3,7-TMU (9.89 min). Chromatographic conditions are described in the text.

Application to Biological Fluids: Blood Serum-Urine

Human Blood Serum

Aliquots of 40 μL human blood serum (pooled sample) were treated with 250 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was spiked with 200 μL of methyluric acids solutions, at

concentration levels of 0.25, 0.6, 1.0, 2.0, and 3.0 ng/µL. Then the sample was centrifuged at 3500 rpm for 15 min and the supernatant was evaporated, at 45°C, under nitrogen stream, to remove organic solvents. Subsequently the sample was slowly applied to the solid-phase cartridge.

Then the sample was treated according to the procedure described under solid-phase extraction paragraph. High performance liquid chromatogram of methyluric acids, extracted from human blood serum, is shown in Figure 3. Regression equations revealed correlation coefficient values in the range from 0.99747 to 0.99993.

Urine

100 µL of pooled urine sample were extracted according to the procedure described under the SPE paragraph. Matrix interference was removed by washing the OASIS cartridges with 3 mL H_2O.

No interference from endogenous compounds from sample matrix was observed, as shown in the urine sample chromatogram, in Figure 4.

Extraction efficiency was calculated by extracting standard solutions of methyluric acids, at five different amount levels i.e. 0.25, 0.6, 1.0, 2.0, and 3.0 ng/µL. Correlation coefficients ranged from 0.99867 to 0.99964.

The precision and accuracy studies of SPE of methyluric acids from biological samples were conducted by spiking blood serum and urine samples, with five known concentrations of the compounds and then by comparing obtained results, with those as calculated from regression equations. Results of recovery studies for serum and for urine samples are given in Table 6. Each value represents the mean of six measurements carried out.

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CONCLUSIONS

Five methyluric acids (1-MU, 7-MU, 1,3 DMU, 1,7 DMU, and 1,3,7 TMU) which are the final metabolic products of methylated xanthine derivatives (caffeine and theophylline the most important among them) were isolated from biological fluids by means of solid-phase extraction and subsequently analysed by HPLC.

The binary eluent system used for methyluric acids provide good separation, high selectivity and resolution within a short chromatogram run time of 15 min. The proposed method is very sensitive with 0.1 ng limit of detection for all compounds, when 10 µL are injected onto analytical column.

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Table 6

Recovery of Methyluric Acids from Human Blood Serum and Urine After SPE on OASIS HLB Cartridges

Analyte	Added (ng)	Blood Serum Found ± SD (ng)	Blood Serum RSD (%)	Blood Serum Recovery (%)	Urine Found ± SD (ng)	Urine RSD (%)	Urine Recovery (%)
7-MU	2.71	2.28 ± 0.06	2.63	84.13	2.33 ± 0.01	0.43	85.98
	5.64	5.48 ± 0.08	1.46	97.16	5.09 ± 0.21	4.13	90.25
	11.28	11.36 ± 0.05	0.44	100.71	11.41 ± 0.40	3.51	101.15
	22.56	21.39 ± 0.14	0.65	94.81	20.90 ± 0.50	2.39	92.64
	28.20	27.37 ± 0.30	1.10	97.06	26.65 ± 0.61	2.29	94.50
1-MU	2.88	2.70 ± 0.09	3.33	93.75	2.88 ± 0.05	1.74	100.00
	6.00	5.71 ± 0.05	0.88	95.17	5.66 ± 0.06	1.06	94.33
	12.00	12.54 ± 0.49	3.91	104.50	11.26 ± 0.41	3.64	93.83
	24.00	21.98 ± 0.35	1.59	91.58	23.76 ± 1.06	4.46	99.00
	30.00	29.46 ± 0.16	0.54	98.20	28.87 ± 0.29	1.00	96.23
1,3-DMU	2.82	2.74 ± 0.04	1.46	97.16	2.53 ± 0.06	2.37	89.71
	5.88	5.69 ± 0.03	0.53	96.77	5.83 ± 0.01	0.17	99.15
	11.76	11.14 ± 0.18	1.62	94.73	11.60 ± 0.51	4.40	98.64
	23.52	22.18 ± 0.38	1.71	94.30	22.49 ± 0.52	2.31	95.62
	29.40	28.21 ±0.26	0.92	95.95	26.64 ± 0.50	1.88	90.61
1,7-DMU	2.76	2.65 ± 0.01	0.38	96.01	2.54 ± 0.03	0.01	92.03
	5.76	5.38 ± 0.16	2.97	93.40	5.13 ± 0.03	0.58	89.06
	11.52	11.19 ± 0.33	2.95	97.13	11.86 ± 0.28	2.36	102.95
	23.04	20.95 ± 0.68	3.25	90.93	21.54 ± 0.91	4.22	93.49
	28.80	27.72 ± 0.09	0.32	96.25	27.42 ± 0.38	1.39	95.21
1,3,7-TMU	2.94	2.56 ± 0.07	2.73	87.07	2.75 ± 0.04	1.45	93.54
	6.12	5.84 ± 0.03	0.51	95.42	5.15 ± 0.25	4.85	84.15
	12.24	11.51 ± 1.18	10.25	94.04	11.41 ± 0.79	6.92	93.22
	24.48	23.01 ± 0.62	2.70	94.00	23.45 ± 0.53	2.26	95.79
	30.60	29.10 ± 0.29	1.00	95.10	28.75 ± 0.38	1.32	93.95

Day-to-day reproducibility was tested over eight consecutive days and repeatability (within day assay n=8) proved to be sufficient (RSD_R< 6.46 %, RSD_r < 3.63 %). High recovery rates are accomplished with the SPE protocol developed at this study. No matrix interference was noticed in real sample analysis. Precision and accuracy studies were proven to be satisfactory for biological fluids, blood serum, and urine. The proposed method requires small sample volumes, 40 μL of blood serum and 100 μL of urine, making it a valuable tool for clinical pediatric research, with a relatively short and simple sample preparation protocol.

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