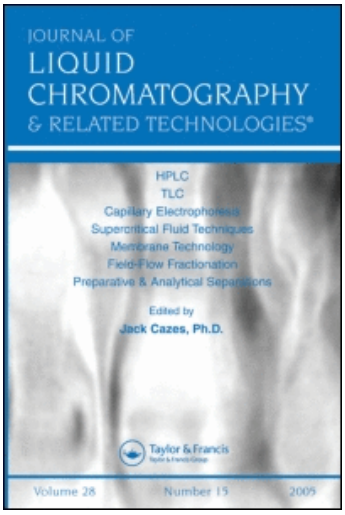

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IMPROVED MICRO-METHOD FOR THE HPLC ANALYSIS OF CAFFEINE AND ITS DEMETHYLATED METABOLITES IN HUMAN BIOLOGICAL FLUIDS AFTER SPE

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ABSTRACT

An improved high performance liquid chromatographic-photodiode array method, using a multi-linear gradient elution, is described for the simultaneous determination of caffeine and its eight primary metabolites: xanthine (XA), 7-methylxanthine (7-MX), 3-methylxanthine (3-MX), 1-methylxanthine (1-MX), isocaffeine (IC), theobromine (TB), paraxanthine (PA) and theophylline (TP).

The separation method Is based on mobile phase optimization and off-line solid-phase extraction (SPE) from human biological fluids: blood serum and urine. The eluting system consisted of ammonium acetate 0.05 M (pH=7) and methanol (90:10 v/v) changing to (40:60 v/v) over a period of 30 min.

Identification of metabolites was achieved by photodiode array detector at 270 nm resulting in 2 ng limit of detection, while linearity held up to 20 ng/μL for each compound.

Lamotrigine was used as internal standard at a concentration of 10.0 ng/μL.

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

High extraction recoveries were achieved from blood serum and Furine using Merck C_8 400 mg and Oasis HLB cartridges respectively, requiring small volumes, 40 μL of blood and 100 μL of urine.

The separation was achieved on octylsilica, using a Silasorb C_8, 10 μm, 250 x 4.6 mm analytical column at ambient temperature and proved to be highly selective, sensitive, reproducible and rapid regarding the nine compounds.

INTRODUCTION

As extensively reported in a previous work of the authors, caffeine (CA) is ubiquitous in the human diet. Major caffeine metabolites include N-demethylated derivatives which are pharmacologically active. Theophylline acts as a powerful bronchodilator, within a narrow serum therapeutic range. Similarly to theophylline (TP), caffeine and theobromine (TB) stimulate the central nervous system (CNS) , gastric acid secretion and cardiac muscle, relax smooth bronchial muscle and enhance diuresis. Theophylline stimulation of CNS is more profound and more dangerous than caffeine and it is on the list of forbidden doping substances. Threshold levels are necessary for doping analysis purposes.[1,2]

Caffeine is predominately (80%) metabolized by 3-demethylation to form paraxanthine. This reaction is mediated by enzyme CYP1A2. It also undergoes 1- and 7- demethylation to form theobromine (12%) and theophylline (7%) mediated by enzymes CYP1A2 and CYP2E1, respectively. The three dimethylaxanthine metabolites are measurable in plasma and saliva and ratio values of paraxanthine (1,7 dimethylxanthine) (PA) to caffeine at selected time points have shown a high correlation with the total plasma clearance of caffeine and, thus, may serve as surrogate measure of cytochrome CYP1A2 activity. Changes in blood concentration of caffeine and its three primary metabolites after caffeine administration in patients with liver cirrhosis can possibly predict the extent of hepatic disorder as production of paraxanthine, theophylline and theobromine is suppressed. Additionally, the ratio TP/PA independent of urinary specific grants is a reliable indicator for the intake of TP.[3-5]

Several methods for the determination of caffeine and dimethylaxanthines have been reported. These include immunoassay techniques RIA, FPIA, chro-

matographic techniques GC, etc. A common limitation of these is inadequate sensitivity for use in low dose caffeine pharmacokinetic studies. Reversed-phase HPLC methods, with UV detection, have been succesfully applied to the separation and determination of these compounds in a wide range of samples such as foods, beverages, and biological fluids. Some of the reported methods require tedious pretreatment or do not allow separation and quantitation of different derivatives in the same sample.[6-13]

For the elimination of the matrix, different techniques such as liquid-liquid extraction, soxhlet extraction, column chromatography, or SPE are necessary before the determination steps.[2]

In our previous study, a method for determination of eight caffeine and demethylated metabolites was developed using isocaffeine as internal standard. Application of the method to biological fluids revealed some endogenous interferences, especially in the case of urine samples, where some endogenous compounds overlap some determined xanthine derivatives.[1] Thus, xanthine and 3-methylxanthine could not be analysed in urine samples and isocaffeine could not be used as internal standard. For this reason, another substance, lamotrigine, was chosen as internal standard for the present assay.

The present work aims to improve the cited paper by chromatographic system optimization regarding the peak resolution and number of compounds separated, as well as to sample preparation.

This paper describes a sensitive method for the simultaneous determination of caffeine, and its demethylated metabolites in biological fluids, blood serum, and urine. It is an improved method in comparison to our previous work, since the number of compounds resolved is increased and the matrix effect of real samples is eliminated.

EXPERIMENTAL

Instrumentation

A Shimadzu (Kyoto, Japan), quaternary low pressure gradient system was used for chromatographic analysis of caffeine demethylated metabolites. 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.

Sample injection was performed by a SIL-9A autosampler and detection was achieved by an SPD-M6A Photodiode Array Detector, controlled by a Shimadzu Class-LC10/M10A Software.

Chromatograms were stored on the hard disk of a Function 486 PC and printed on a Hewlett-Packard LaserJet 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, 10 μm, 250 x 4.6 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, CA, 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.

Chemicals

Caffeine and demethylated metabolites were purchased from Sigma (St. Louis, MO, USA). Lamotrigine was supplied by Wellcome Foundation (London, U.K.).

HPLC gradient grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Ammonium acetate p. a. was also from Merck. Bis deionised water was used throughout analysis. Solid phase extraction C_8 cartridges were from Merck, while OASIS HLB cartridges were purchased from Waters (Milford, MA. USA).

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

Stock solutions of caffeine and demethylated metabolites were prepared in a 1:1 mixture of methanol-water and stored refrigerated at 4 ^{0}C. These solutions were found to be stable for at least one month.

Working solutions were prepared by diluting stocks with water at concentrations of 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, 10.0, 15.0, and 20.0 ng/μL. Aqueous solution of internal standard lamotrigine was added at a concentration of 10.0 ng/μL.

Chromatography

A variety of binary gradient systems of organic modifier (methanol), in low volume percentage, were tested with ammonium acetate, 0.05 M, in several ratios and different gradient steps to arrive at an 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 75 Kg/cm.^{2}

The optimum multi-linear gradient system has the following shape:

Step 1. Time, 0 min: CH_3COONH_4 90 % - CH_3OH 10 %

Step 2. Time, 8 min: CH_3COONH_4 80 % - CH_3OH 20 %

Step 3. Time, 15 min: CH_3COONH_4 70 % - CH_3OH 30 %

Step 4. Time, 20 min: CH_3COONH_4 50 % - CH_3OH 50 %

Step 5. Time, 30 min: CH_3COONH_4 40 % - CH_3OH 60 %.

Figure 1 shows the chromatogram obtained using the conditions described in text. Resolution factors were found to be satisfactory, indicating sufficient separation. Values for each pair of compound peaks are: 2.52 (XA- 7-MX), 0.90 (7-MX- 3-MX), 1.19 (3-MX- 1-MX), 1.12 (1-MX- IC), 1.12 (IC- TB), 2.44 (TB- PA), 0.60 (PA- TP), 4.79 (TP- CA), and 8.51 (CA- Lamotrigine).

Peak areas of caffeine and demethylated metabolites were measured and the ratio to internal standard was compared for each compound to that of the calibration standards. Chromatographic separation was accomplished at ambient temperature which was chosen among higher values (30, 40, and 50 ^{o}C) as

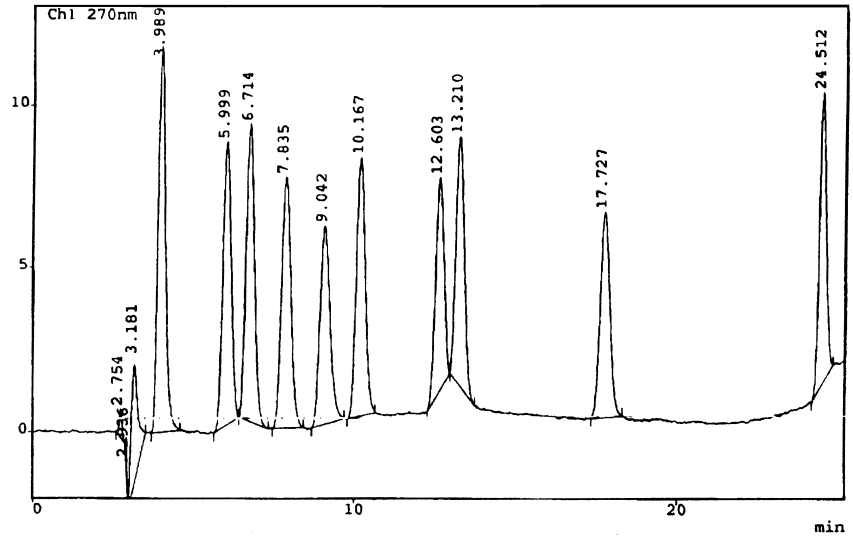

Figure 1. High performance liquid chromatogram of caffeine and its metabolites: XA (3.989 min) , 7-MX (5.999 min), 3- MX (6.71 min), 1-MX (7.835 min), IC (9.042 min), TB (10.167 min), PA (12.603 min), TP (13.210 min), CA (17.727 min), and Lamotrigine internal standard (24.512 min). Chromatographic conditions are described in the text.

separation is slightly deteriorated and retention times become shorter, leading to matrix effects of real samples.

Calibration Data-Analytical Procedure

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

Each sample was injected five times. Monitoring wavelength was 270 nm.

Linear correlation between absolute injected amount or concentration and peak area ratio was obtained for all analytes using lamotrigine as internal standard at a concentration of 10.0 ng/μL. Lamotrigine was selected as internal standard because it elutes after the analytes, providing satisfactory resolution from the last eluting peak.

The results of the statistical treatment of calibration data for caffeine and demethylated metabolites are summarised in Table 1.

Table 1

Calibration Data for Simultaneous Determination of Caffeine and Demethylated Metabolites*

Analyte	R_t (min.)	Slope (AIU. ng^{-1})	Intercept	Correlation Coefficient
XA	3.989	0.01782 ± 0.00087	0.09476 ± 0.06240	0.99531
7-MX	5.999	0.01876 ± 0.00020	-0.00123 ± 0.01827	0.99996
3-MX	6.714	0.02043 ± 0.00029	-0.00039 ± 0.02110	0.99996
1-MX	7.835	0.01892 ± 0.00038	-0.01980 ± 0.00300	0.99984
IC	9.042	0.01584 ± 0.00023	-0.01913 ± 0.02408	0.99989
TB	10.167	0.01796 ± 0.00016	-0.00945 ± 0.01687	0.99996
PA	12.603	0.01403 ± 0.00034	-0.01665 ± 0.02777	0.99978
TP	13.210	0.01551 ± 0.00083	0.01591 ± 006563	0.99892
CA	17.727	0.01614 ± 0.00017	-0.01624 ± 0.01783	0.99994

* Peak area ratio measurement with 10.0 ng/μL lamotrigine as internal standard. RT= 24.512 min.

Working Range and Detectability

The upper limit was found to be 20.0 ng/μL for all analytes, while limit of detection, calculated as a three-fold signal-to-noise ratio, at the baseline (S/N=3), was found to be 2 ng, when 20 μL of the sample were injected onto the column. Limit of quantitation was found to be 5 ng for all compounds.

Internal standard, Lamotrigine, was selected, as it elutes after caffeine without interfering either with analytes or with matrix components. This enables its use for the determination of caffeine and its primary metabolites in urine samples, thus improving our previous method.

Selection of Wavelength

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

RESULTS AND DISCUSSION

Method Validation

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.

A long-term stability study was conducted during routine operation of the system over a period of eight consecutive days. Results are illustrated in Table 2.

Solid Phase Extraction Protocol for Caffeine and Demethylated Metabolites Isolation

Several different solid-phase extraction cartridges, provided by different manufacturers, were tested for the optimisation of caffeine and its demethylated metabolites isolation and recovery in human blood serum and urine, prior to HPLC analysis.

Extraction efficiency was calculated by extracting standard solutions at five different concentration levels, i.e., 0.5, 1.0, 2.0, 3.0, and 5.0 ng/μL, of all compounds.

Recovery was calculated by comparing peak area ratios against internal standard with those obtained for unextracted standard solutions.

200 μL of standard solution were applied to the Merck C_8 or OASIS HLB cartridge, which was conditioned by flushing with 3 mL CH_3OH and 3 mL H_2O prior to the addition of sample. After applying the sample, the cartridge was dried by sucking air. Analytes of interest were eluted with 3 mL 2 % HCl in 2-propanol, in the case of C_8 cartridges or with 3 mL 2 % HCl in acetonitrile, when OASIS HLB cartridges were used. The sample was subsequently evaporated to dryness under a gentle nitrogen steam in a 45 ^{0}C water bath and diluted to 200 μL aqueous internal standard solution which, in this case, was used as chromatographic internal standard.

Recovery results are tabulated in Table 3 for both types of sorbent tested for this assay.

Table 2

Within-Day and Day-to-Day Precision and Accuracy Study for Caffeine Metabolite Determination in the Presence of Lamotrigine (Internal Standard)

		Within- Day (n=8)			Day-to- Day (n=8)		
Analyte	Added (ng)	Found ± SD (ng)	RSD (%)	Recovery (%)	Found ± SD (ng)	RSD (%)	Recovery (%)
XA	19.20	19.93 ± 0.34	1.71	103.80	19.92 ± 0.30	1.56	103.75
	38.40	39.21 ± 0.52	1.33	102.11	39.12 ± 0.40	1.02	101.88
	60.00	61.32 ± 0.36	0.59	102.20	60.58 ± 0.65	1.07	100.97
7-MX	20.40	21.82 ± 0.35	1.60	106.96	21.55 ± 0.37	1.72	105.64
	40.80	39.59 ± 0.22	0.56	97.03	39.45 ± 0.40	1.01	96.69
	61.20	59.40 ± 0.54	0.91	97.06	59.57 ± 0.71	1.19	97.34
3-MX	20.00	21.00 ± 0.53	2.52	105.00	20.83 ± 0.43	2.06	104.15
	40.00	38.86 ± 0.30	0.77	97.15	39.20 ± 0.52	1.33	98.00
	60.00	57.99 ± 0.28	0.48	96.65	58.91 ± 0.94	1.60	98.18
1-MX	20.40	21.61 ± 0.42	1.94	105.93	21.35 ± 0.56	2.62	104.66
	40.80	38.97 ± 0.33	0.85	95.51	38.95 ± 0.30	0.77	95.47
	61.20	58.75 ± 0.46	0.78	96.00	59.00 ± 0.57	0.97	96.40
IC	20.40	20.77 ± 0.39	1.88	101.81	20.74 ± 0.48	2.31	101.67
	40.80	39.71 ± 0.69	1.74	97.33	39.65 ± 0.48	1.21	97.18
	61.20	58.90 ± 0.68	1.15	96.24	59.35 ± 0.41	0.69	96.98
TB	20.80	21.11 ± 0.20	0.95	101.49	20.80 ± 0.38	1.83	100.00
	41.60	39.98 ± 0.54	1.35	96.11	40.03 ± 0.50	1.25	96.23
	62.40	61.09 ± 0.88	1.44	97.90	60.69 ± 0.59	0.97	97.26
PA	21.20	22.40 ± 0.18	0.80	105.66	21.87 ± 0.63	2.88	103.16
	42.40	39.84 ± 0.18	0.45	93.96	39.50 ± 0.47	1.19	93.16
	63.60	62.99 ± 0.27	0.43	99.04	63.31 ± 1.33	2.10	99.54
TP	20.60	20.63 ± 0.37	1.79	100.15	20.31 ± 0.44	2.17	98.59
	41.20	41.51 ± 0.64	1.54	100.75	41.02 ± 0.54	1.32	99.56
	61.80	60.19 ± 0.50	0.83	97.39	60.88 ± 0.88	1.44	98.51
CA	20.20	20.50 ± 0.40	1.95	101.48	20.26 ± 0.54	2.66	100.30
	40.40	39.34 ± 0.49	1.24	97.38	36.64 ± 0.38	1.04	90.69
	60.60	59.02 ± 0.35	0.59	97.39	59.62 ± 1.20	2.01	98.38

Table 3

Mean Recovery* of Caffeine Metabolites from Standard Solutions After SPE

Analyte	Merck C_8 Cartridges Recovery ± SD (%)	RSD (%)	OASIS HLB Cartridges Recovery ± SD (%)	RSD (%)
XA	92.57 ± 2.29	2.47	100.42 ± 3.19	3.18
7-MX	92.53 ± 6.27	6.78	86.04 ± 6.85	7.96
3-MX	93.94 ± 3.82	4.07	86.58 ± 3.71	4.28
1-MX	93.99 ± 6.68	7.11	89.50 ± 8.10	9.05
IC	93.34 ± 2.67	2.86	88.79 ± 6.15	6.92
TB	89.53 ± 5.06	5.65	88.81 ± 8.52	9.59
PA	93.21 ± 2.47	2.65	80.54 ± 6.76	8.39
TP	96.78 ± 2.82	2.91	85.96 ± 2.75	3.20
CA	96.47 ± 6.42	6.65	94.98 ± 6.45	6.79

* Mean values from five concentration levels (0.5-5.0 ng/μL), six measurements.

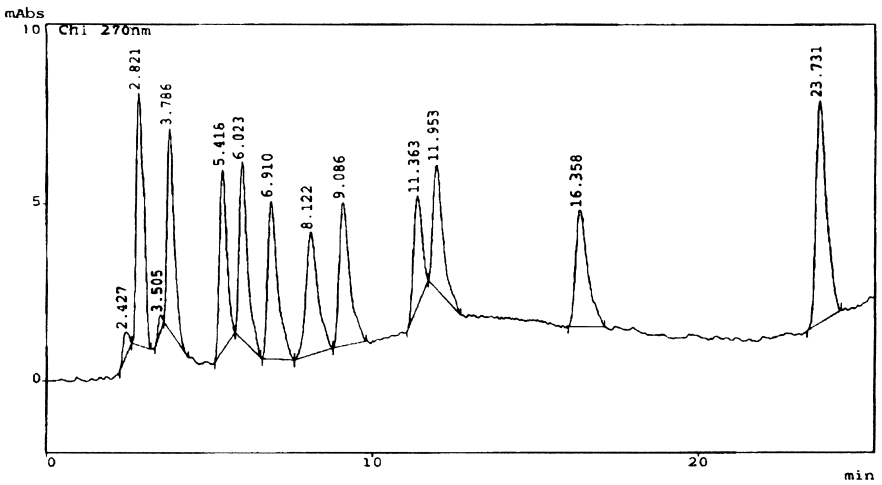

Figure 2. High performance liquid chromatogram of analysis of caffeine and its metabolites in spiked human blood serum samples. XA (3.786 min) , 7-MX(5.418 min), 3- MX (6.023 min), 1-MX (6.910 min), IC (8.122 min), TB (9.086 min), PA (11.363 min), TP (11.953 min), CA (16.358 min), and Lamotrigine internal standard (23.731 min). Chromatographic conditions are described in the text.

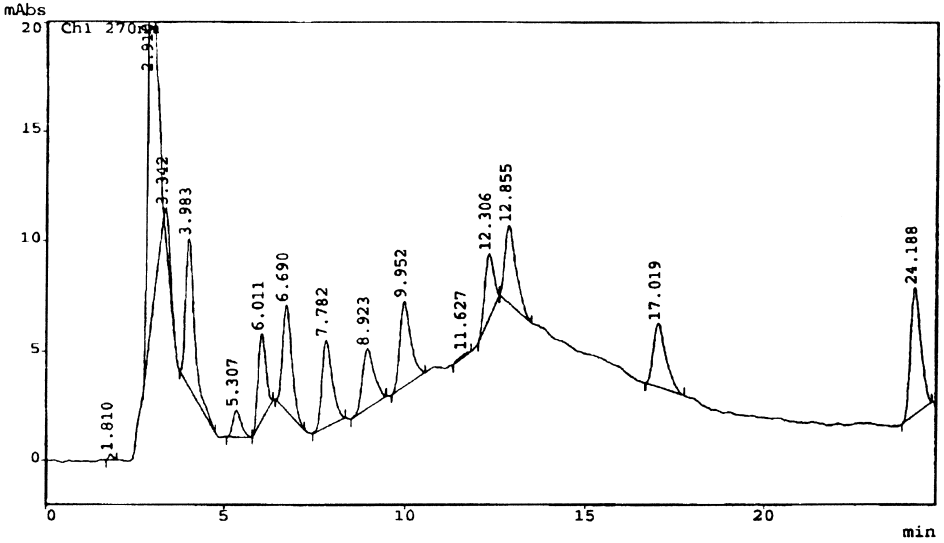

Figure 3. High performance liquid chromatogram of analysis of caffeine and its metabolites in urin samples. XA (3.983 min) , 7-MX(6.011 min), 3- MX (6.690 min), 1-MX (7.782. min), IC (8.923 min), TB (9.952 min), PA (12.306 min), TP (12.855 min), CA (17.019 min), and Lamotrigine internal standard (24.188 min). Chromatographic conditions are described in the text.

Application to Real Sample Analysis

Human Blood Serum

Aliquots of 40 µL of human blood serum were treated with 200 µL of acetonitrile to precipitate proteins, in order to release bound caffeine metabolites.

After 2 min. vortex mixing, 200 µL of mixed standard solution were added to the sample at concentrations: 0.5, 2.0, 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 C_8 cartridge and the procedure followed the steps described under the solid-phase extraction paragraph.

Table 4

Recovery of Caffeine and Metabolites from Spiked Biological Fluids After SPE Using Internal Standard

		Serum Using Merck C_8 Cartridges			Urine Using OASIS HLB Cartridges		
Analyte	Added (ng)	Found ± SD (ng)	RSD (%)	Recovery (%)	Found± SD (ng)	RSD (%)	Recovery (%)
XA	9.60	8.20 ± 1.02	12.34	85.42	9.1 ± 0.75	8.24	94.79
	38.40	34.70 ± 2.15	6.20	90.36	37.8 ± 0.46	1.22	98.44
	97.40	84.26 ± 0.96	1.14	86.51	96.2 ± 0.65	0.68	98.77
7-MX	10.20	9.31 ± 0.52	5.58	91.27	9.40 ± 0.21	2.23	92.16
	40.80	36.47 ± 1.72	4.72	89.39	38.6 ± 0.95	2.46	94.61
	100.00	93.98 ± 1.97	2.10	93.98	96.7 ± 1.46	1.51	96.70
3-MX	10.00	9.50 ± 0.62	6.53	95.00	8.4 ± 0.82	9.76	84.00
	40.00	36.40 ± 1.83	5.03	91.00	36.8 ± 1.21	3.29	92.00

	99.00	90.09 ± 2.74	3.04	91.00	95.2 ± 0.80	0.84	96.16
1-MX	10.2	9.38 ± 0.82	8.74	91.96	8.7 ± 0.51	5.86	85.29
	40.8	35.10 ± 3.39	9.66	86.03	38.4 ± 2.27	5.91	94.12
	100	96.83 ±1.11	1.15	96.83	98.6 ± 0.83	0.84	98.60
IC	10.2	11.15 ± 0.42	3.77	109.31	8.3 ± 0.37	4.46	81.37
	40.8	39.08 ± 1.24	3.17	95.78	39.1 ± 1.44	3.68	95.83
	100	83.55 ± 3.57	4.27	83.55	98.6 ± 1.23	1.25	98.60
TB	10.4	8.34 ± 0.74	8.87	80.19	8.9 ± 0.68	7.64	85.58
	41.6	37.74 ± 0.96	2.54	90.72	39.7 ± 0.74	1.86	95.43
	103	95.28 ± 2.13	2.24	92.50	99.2 ± 1.34	1.35	96.31
PA	10.6	10.88 ± 0.82	7.54	102.64	9.3 ± 0.34	3.66	87.74
	42.4	39.24 ± 0.68	1.73	92.55	41.7 ± 1.18	2.83	98.35
	105	88.30 ± 0.71	0.80	84.10	102.3 ± 0.31	0.30	97.43
TP	10.3	9.61 ± 0.70	7.28	93.30	9.1 ± 0.61	6.70	88.35
	41.2	35.00 ± 1.04	2.97	84.95	39.7 ± 1.27	3.20	96.36
	102	92.46 ± 1.04	1.12	90.65	101.4 ± 0.98	0.97	99.41
CA	10.1	10.30 ± 1.00	9.71	101.98	9.3 ± 0.44	4.73	92.08
	40.4	38.35 ± 2.01	5.24	94.93	39.4 ± 1.17	2.97	97.52
	100	89.58 ± 2.10	2.34	89.58	98.5 ± 1.06	1.75	98.50

Urine

A similar sample preparation method was followed for urine samples using 100 μL urine sample. OASIS HLB cartridges were used for analyte recovery, as they provided better clean up for the difficult matrix of urine samples, than C_8 cartridges. After cartridge conditioning with 3 mL methanol and 3 mL water, urine sample was applied and interference was washed out with water. Caffeine and metabolites are recovered by elution with 2% HCl in acetonitrile. The sample was subsequently evaporated to dryness under a gentle nitrogen steam in a 45 ^{0}C water bath and diluted to 200 μL aqueous internal standard solution.

No interference from endogenous compounds in the sample matrix was observed, either in blood serum or urine samples, as shown in Figures 2 and 3.

Table 4 presents recovery results of caffeine and metabolites from biological fluids.

CONCLUSIONS

Caffeine and its major demethylated metabolites were isolated from biological fluids by means of solid-phase extraction and subsequently analysed by HPLC.

The binary eluent system used provides good separation, high selectivity, and resolution within a run time of 25 min.

The proposed method is very sensitive, with 2 ng limit of detection for all compounds, when 20 μL are injected onto the analytical column.

Day-to-day reproducibility was tested over eight consecutive days and repeatability (within-day assay, n=8) proved to be sufficient ($RSD_R < 2.88$ % and $RSD_r < 2.52$ %).

High recovery rates are accomplished with the SPE protocol developed in this study. No matrix inrerference was noticed in real sample analysis.

The assay is applicable to microliter samples of blood serum and urine: 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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