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Use of novel solid-phase extraction sorbent materials for high-performance liquid chromatography quantitation of caffeine metabolism products methylxanthines and methyluric acids in samples of biological origin

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Abstract

An automated reversed-phase high-performance liquid chromatographic (RP-HPLC) method, using a linear gradient elution, is described for the simultaneous analysis of caffeine and metabolites according to their elution order: 7-methyluric acid, 1-methyluric acid, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, theobromine, 1,7-dimethyluric acid, paraxanthine and theophylline. The analytical column, an MZ Kromasil C_4, 250×4 mm, 5 μm, was operated at ambient temperature with back pressure values of 80–110 kg/cm^2. The mobile phase consisted of an acetate buffer (pH 3.5)–methanol (97:3, v/v) changing to 80:20 v/v in 20 min time, delivered at a flow-rate of 1 ml/min. Paracetamol was used as internal standard at a concentration of 6.18 ng/μl. Detection was performed with a variable wavelength UV–visible detector at 275 nm, resulting in detection limits of 0.3 ng per 10-μl injection, while linearity held up to 8 ng/μl for most of analytes, except for paraxanthine and theophylline, for which it was 12 ng/μl and for caffeine for which it was 20 ng/μl. The statistical evaluation of the method was examined performing intra-day (n=6) and inter-day calibration (n=7) and was found to be satisfactory, with high accuracy and precision results. High extraction recoveries from biological matrices: blood serum and urine ranging from 84.6 to 103.0%, were achieved using Nexus SPE cartridges with hydrophilic and lipophilic properties and methanol–acetate buffer (pH 3.5) (50:50, v/v) as eluent, requiring small volumes, 40 μl of blood serum and 100 μl of urine.

Keywords: Caffeine; Methylxanthine; Methyluric acid

1. Introduction

As extensively reported in literature caffeine (CA) is ubiquitous in the human diet. Major caffeine metabolites include *N*-demethylated derivatives which are pharmacologically active possessing a wide range of therapeutic activity. Theophylline (TP), acts as a powerful bronchodilator, within a narrow serum therapeutic range. Similarly to theophylline, 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

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than caffeine and it is on the list of forbidden doping substances. Methylxanthines (MX) undergo further metabolism by the hepatic microsomal mixed function oxidase (cytochrome P450, CYP) system so that less than 10% of TP and less than 2% CA are excreted unchanged in the urine.

Caffeine metabolism is complex with significant differences between species as well as between individuals in different clinical situations. In healthy human subjects, caffeine 3-*N*-demethylation producing 1,7-dimethylxanthine (1,7-DMX, other name paraxanthine: PA) is the dominant (80%) metabolic pathway. This reaction is mediated by enzyme CYP1A2. It also undergoes 1- and 7-demethylation to form theobromine (3,7-DMX) (12%) and theophylline (1,3-DMX) (7%) mediated by enzymes CYP1A2 and CYP2E1, respectively. Further 7-*N*-demethylation (CYP1A2 dependent) produces 1-methylxanthine (1-MX). C-8-hydroxylation of 1,7-DMX and 1-MX leads to the corresponding methyluric acids 1,7-DMU and 1-MU. The reactions depend on CYP2A6 and xanthine oxidase, respectively. The three dimethylaxanthine metabolites are measurable in plasma and saliva and the ratio of paraxanthine to caffeine at selected time points have shown a high correlation with the total plasma clearance of caffeine and thus may serve as surrogate measurer 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 are suppressed. Thus caffeine can be used as a probe drug to assess the metabolic activity of cytochrome P450 and other drug metabolizing enzymes by measuring urinary elimination of drug metabolites [1–9].

As shown in Fig. 1, methyluric acids (MU), mono-, di- and tri-methylated derivatives of uric acid, are the final metabolic products of caffeine. The main excretory methylurate products after TP and CA ingestion are 1,3-di-methyluric acid (1,3-DMU) and 1-MU, while the major urinary metabolite of TB is 7-MU. As a result MX and MU coexist in biological fluids as primary and final caffeine metabolites. Patients consuming caffeine-containing beverages excrete the whole spectrum of metabolites in the urine. Thus, it is obvious that their simultaneous

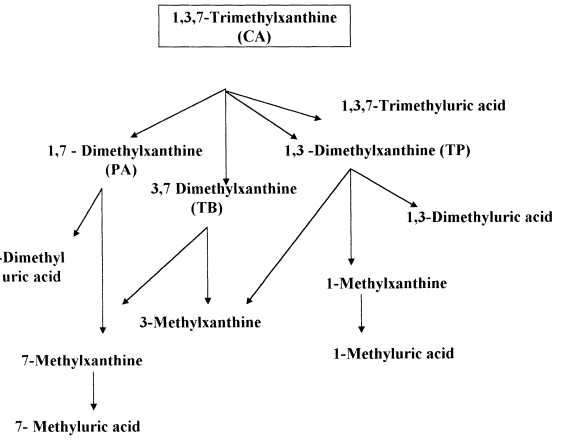

Fig. 1. The interrelationships of the xanthine derivatives and their metabolic products: primary (methylxanthines) and final (methyluric acids) expected to occur in blood serum and urine after caffeine administration.

determination is of paramount interest. However, the determination of CA 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 [4,10,11].

Methods for the determination of caffeine and methylxanthines reported in literature include immunoassay techniques (RIA, FPIA), chromatographic techniques GC etc. Reversed-phase and normal-phase HPLC methods with UV detection have been successfully applied for the separation and determination of these compounds in a wide range of samples such as foods, beverages and biological fluids. Ion pair chromatographic systems are also described using tetrabutylammonium or decylammonium ion and Tris buffer, to determine methyluric acid derivatives among other methylxanthines. Some of the reported methods require tedious pretreatment or do not allow separation and quantitation of different derivatives in the same sample. However, the number of HPLC methods for the simultaneous determination of caffeine and its primary and final metabolites in one run is very limited [12–23].

Sample preparation techniques used for the elimination of the matrix such as liquid–liquid extraction, Soxhlet extraction, column chromatography or solid-phase extraction (SPE) are necessary before determination steps. SPE 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. Novel SPE sorbents with hydrophilic–lipophilic properties introduced by different manufacturers include a novel balanced copolymer of *N*-vinylpyrrolidone and divinyl benzene (HLB Oasis by Waters) and Abselut Nexus by Varian (acidic–basic screen). The hydrophilic *N*-vinylpyrrolidone increases the water wettability of the polymer and the lipophilic divinyl benzene provides the reversed-phase retention necessary to retain analytes. It also provides greater pH stability and enhanced retention than C_{18}-bonded silica sorbents. In addition the sorbent preserves analyte retention even if the bed dries out. The acidic–basic screen sorbent used in Nexus designed to extract a wide range of pharmaceuticals from biological fluids using non-conditioned SPE (NC-SPE) technique is a highly cross-linked spherical polymeric sorbent with a combination of hydrophilic and lipophilic moieties. The precise ratio of the dual functionalities enables aqueous samples to be applied directly to a dry sorbent bed. Thus high recoveries can be achieved even though the sorbent does not need conditioning as stated by the manufacturers. These two sorbents are assayed in comparison to silica based C_{18} SPE sorbent.

The authors in previous publications dealt with the simultaneous determination of MU in human biological fluids, blood serum and urine, in approximately 15 min, as well as the simultaneous determination of MX in similar matrices in less than 20 min time. SPE was the pretreatment method of choice in both cases [24–26].

The present paper describes a sensitive method for the simultaneous determination of caffeine, and its primary and final metabolites in biological fluids, blood serum and urine. Caffeine, metabolites examined are according to their elution order: 7-methyluric acid, 1-methyluric acid, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, theobromine, 1,7-dimethyluric acid, paraxanthine and theophylline.

2. Experimental

2.1. Chemicals and reagents

Caffeine, 7-methyluric acid, 1-methyluric acid, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, theobromine, 1,7-dimethyluric acid, paraxanthine and theophylline, were purchased from Sigma (St. Louis, MO, USA). Hydrochloric acid, 37%, glacial acetic acid 100%, and sodium hydroxide were pro analysi grade, from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were obtained from Riedel-de-Haen (AG, Seelze, Germany). Bis de-ionised water was used throughout analysis. Paracetamol was purchased from Merck. Acetate buffer (pH 3.5) was prepared by mixing the appropriate volumes of molar solutions of acetic acid and sodium hydroxide.

2.2. Instrumentation — chromatography

The method was developed on a liquid chromatographic system consisting of the commercial components: a Spectra Physics 8800 HPLC ternary pump (California, USA), a Spectra Chrom 100 UV–VIS detector, operated at 275 nm and a sensitivity setting of 0.002 absorbance units full scale (AUFS), a Rheodyne 7125 (California, USA) injection valve with a 10-μl loop and a Spectra Physics SP 4290 integrator.

The analytical column, a Kromasil C_4, 250×4 mm, 5 μm, for the determination of CA and metabolites was purchased by MZ Analysentechnik, (Mainz, Germany). Column was operated at ambient temperature with backpressure values of 80–110 kg/cm^2. The mobile phase consisted of an acetate buffer (pH 3.5)–methanol (97:3, v/v) changing to 80:20 v/v in 20 min time, delivered at a flow-rate of 1 ml/min. Equilibration time between runs was 10 min. At regular intervals injections of methanol provided the clean up of the injection valve loop and the analytical column protecting from late eluting endogenous peaks.

A glass vacuum-filtration apparatus obtained from

Alltech Associates was employed for the filtration of the buffer solutions, 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) are employed for the pre-treatment of biological samples.

The SPE study is performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, a division of Varian (Harbor City, CA, USA). SPE C_{18} cartridges were supplied from Supelco (Bellefonte, PA, USA), Oasis by Waters (Franklin, MA, USA) and Nexus by Varian. All evaporations are performed with a nine-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

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

2.3. Calibration curves

Stock solutions of caffeine and metabolites at concentration of 100 mg/l were prepared using water in the case of MX and 0.01 *M* NaOH in the case of MU and stored refrigerated at 4°C. Working aqueous solutions were prepared from stocks at concentrations: 0.1, 0.25, 0.5, 1, 2, 3, 5 and 8 ng/μl. Paracetamol was used as internal standard at a concentration of 6.18 ng/μl.

2.4. Method validation procedures

2.4.1. Specificity and selectivity

The interference from endogenous compounds was investigated by the analysis of six different blank matrices.

2.4.2. 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 six injections.

Intermediate precision study was conducted during routine operation of the system over a period of 7 consecutive days.

2.4.3. Stability

In deep-frozen biological fluids all compounds were found to be stable for 6 months. Standards stored at 4°C were stable for periods up to 6 months. Even solutions stored at room temperature were quite stable for at least 1 week. Also eluted samples from SPE columns dried on the same day were found to be stable when stored at 4°C for 2–3 weeks. Thus extracted serum and urine samples can be stored at 4°C for subsequent HPLC analysis.

2.5. Sample extraction procedure

Three different sorbents were assayed for the extraction of caffeine and metabolites: Oasis HLB (60 mg) by Waters, C_{18} (500 mg) by Supelco and Abselut Nexus (30 mg) by Varian. Four different extraction protocols were assayed using A: 98% methanol–2% conc. HCl, B: 98% acetonitrile–2% conc. HCl, C: 98% 2-propanol–2% conc. HCl, and D: 50% methanol–50% acetate buffer (pH 3.5), as eluent solvents.

Optimum extraction protocol D was then applied to standard solutions of CA and metabolites.

First, 200 μl of standard solution were applied to the Nexus SPE cartridges, which were conditioned by flushing 3 ml MeOH and 3 ml H_2O prior to the addition of sample. After applying the sample by allowing it to pass through the bed with minimal suction, the cartridge was washed with 2 ml water and cartridge bed was suctioned dry. The caffeine and examined metabolites were eluted using 3 ml methanol–acetate buffer (pH 3.5) (50:50% v/v). The samples were subsequently evaporated to dryness under gentle nitrogen steam in a 45°C water bath and the residue was reconstituted with to 200 μl paracetamol (internal standard) 6.18 ng/μl in 0.01 *M* NaOH. Due to the high aqueous content the evaporation time was approximately 30 min, while total preparation time was about 45 min. Aliquots of 10 μl of the sample were injected onto the HPLC system. For calibration curve construction after SPE

solutions at concentrations 0.25, 0.5, 1, 2, 3 and 5 ng/μl were used.

2.6. Application of the method to biological samples

2.6.1. Blood serum

Aliquots of 40 μl human blood serum were spiked with 200 μl of CA and metabolites solutions at different concentration levels and treated with 200 μl of CH_3CN in order to precipitate proteins. After vortex mixing for 2 min, 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, which was preconditioned with methanol and water.

The sample was subsequently treated according to the procedure described in the solid-phase extraction paragraph.

The same procedure was followed when blank samples were analyzed after CA administration. In this case the reconstitution was made to 40 μl in order to avoid analytes' dilution.

2.6.2. Urine

First, 100 μl of pooled urine sample were spiked with 200 μl of standard solution at concentrations 0.25, 0.5, 1, 2, 3 and 5 ng/μl after addition of 200 μl acetonitrile and centrifugation were extracted according to the procedure described under the SPE paragraph. Matrix interference was removed by washing the Nexus cartridges with 3 ml H_2O After applying the sample, caffeine and metabolites acids were eluted using 3 ml methanol–acetate buffer (pH 3.5) (50:50%, v/v). The samples were subsequently evaporated to dryness under gentle nitrogen steam in a 45°C water bath and diluted to 200 μl internal standard 6.18 ng/μl paracetamol solution in 0.01 *M* NaOH. Aliquots of 10 μl of the sample were injected into the analytical column.

The same procedure was followed when blank samples were analyzed after CA administration. In this case the reconstitution was made to 100 μl in order to avoid analytes' dilution.

3. Results

3.1. Chromatography

Chromatogram obtained using the gradient elution program is illustrated in Fig. 2. Resolution factors ranged from 0.96 to 11.42 indicating satisfactory separation of resolved analytes.

3.2. Method validation

Method validation was performed in terms of linearity, precision and accuracy assays, sensitivity and specificity.

Calibration curves were obtained by least-squares linear regression analysis of the peak area ratio of analyte/internal standard versus analyte concentration. The method was linear up to 8 ng/μl for all compounds except for PA and TP for which was 12 ng/μl and for CA that was linear up to 20 ng/μl. Correlation coefficients ranged from 0.99535 to 0.99987.

Table 1 summarizes the results of the method validation regarding accuracy, intra-day and inter-day precision assays. The measured concentrations

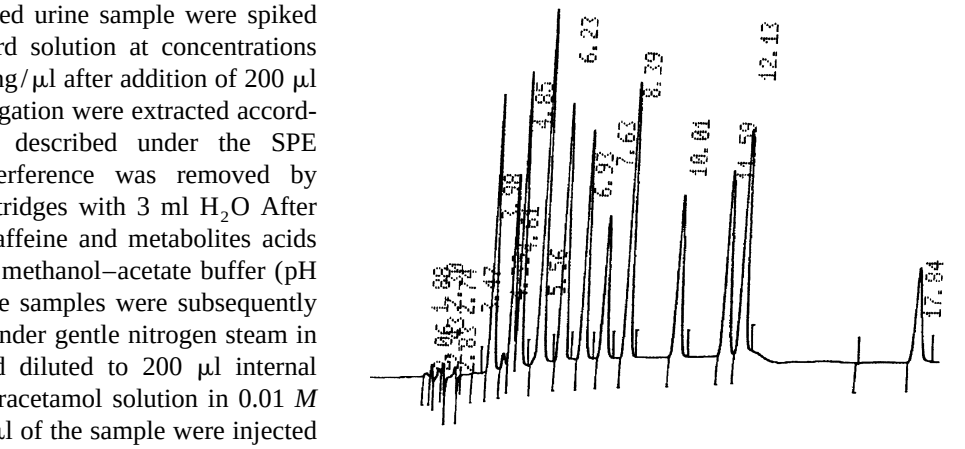

Fig. 2. High-performance liquid chromatogram of primary and final caffeine metabolites: 7-MU (3.98 min), 1-MU (4.61 min), 7-MX (4.85 min), 3-MX (5.56 min), 1-MX (6.23 min), 1,3-DMU (6.93 min), IS (7.63 min), TB (8.39 min), 1,7-DMU (10.01 min) PA (11.59 min), TP (12.13 min) and CA (17.84 min). Chromatographic conditions are described in text.

Table 1
Within-day and between-day precision and accuracy study for caffeine and its primary and final metabolites simultaneous determination in the presence of paracetamol (internal standard)

Analyte	Added (ng)	Within-day (n=6) (repeatability and accuracy)			Between-day (n=7) (intermediate precision and accuracy)		
		Measured±SD (ng)	RSD	Recovery (%)	Measured±SD (ng)	RSD	Recovery (%)
7-MU	4.70	4.27±0.14	3.3	90.8	4.46±0.21	4.7	94.9
	9.40	8.88±0.35	3.9	94.5	9.03±0.39	4.3	96.1
	18.80	18.04±0.11	0.6	96.0	18.74±0.56	3.0	99.7
1-MU	5.00	4.66±0.08	1.7	93.2	4.51±0.10	2.2	90.2
	10.00	9.03±0.33	3.6	90.3	9.63±0.40	4.2	96.3
	20.00	19.53±0.52	2.7	97.7	19.39±0.39	2.0	97.0
7-MX	4.85	4.52±0.14	3.1	93.2	4.42±0.12	2.7	91.1
	9.70	8.79±0.32	3.6	90.6	9.10±0.31	3.4	93.8
	19.40	19.05±0.45	2.4	98.2	18.72±0.18	1.0	96.5
3-MX	5.10	4.66±0.13	2.8	91.4	4.65±0.10	2.2	91.2
	10.20	9.73±0.39	4.0	95.4	9.45±0.24	2.5	92.6
	20.40	19.98±0.74	3.7	97.9	19.39±0.32	1.6	95.0
1-MX	5.25	5.05±0.14	2.8	96.2	4.98±0.17	3.4	94.9
	10.50	10.12±0.31	3.1	96.4	10.10±0.29	2.9	96.2
	21.00	20.84±0.38	1.8	99.2	19.90±0.61	3.1	94.8
1,3-DMU	4.90	4.91±0.02	0.4	100.2	4.83±0.18	3.7	98.6
	9.80	9.54±0.14	1.5	97.4	9.39±0.20	2.1	95.8
	19.60	18.64±0.70	3.8	95.1	18.97±0.26	1.4	96.8
TB	5.10	4.85±0.07	1.4	95.1	4.55±0.18	4.0	89.2
	10.20	9.65±0.18	1.9	94.6	9.46±0.26	2.8	92.7
	20.40	19.24±0.17	0.9	94.3	19.18±0.17	0.9	94.0
1,7-DMU	4.80	4.81±0.04	0.8	100.2	4.67±0.13	2.8	97.3
	9.60	9.33±0.25	2.7	97.2	9.32±0.07	0.8	97.1
	19.20	18.50±0.15	0.8	96.4	18.21±0.42	2.3	94.8
PA	5.00	4.95±0.20	4.0	99.0	4.74±0.19	4.0	94.8
	10.00	9.48±0.43	4.5	94.8	9.18±0.32	3.5	91.8
	20.00	19.47±0.72	3.7	97.4	19.04±0.59	3.1	95.2
TP	5.05	4.54±0.07	1.5	89.9	4.78±0.15	3.1	94.6
	10.10	9.29±0.61	6.6	92.0	9.51±0.32	3.4	94.2
	20.20	19.03±0.45	2.4	94.2	18.98±0.56	3.0	94.0
CA	5.00	4.91±0.15	3.0	98.2	4.77±0.19	4.0	95.4
	10.00	9.70±0.28	2.9	97.0	9.13±0.45	4.9	91.3
	20.00	20.15±0.63	3.1	100.8	19.25±0.55	2.9	96.2

had RSD values <7%, with recoveries in the range of 89.2–100.8%.

The limit of detection of the assay was 0.3 ng. A signal-to-noise ratio of approximately 3:1 was observed for ten samples. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal-to-noise ratio and found to be 1 ng.

Using the method described above non-interference has been observed in real samples either blood serum or urine.

3.3. Solid phase extraction

Recovery rates using different sorbents and elution protocols are summarised in Table 2.

As it can be seen from the results of Table 2 among the four different extraction protocols assayed, using A: 98% methanol–2% conc. HCl, B: 98% acetonitrile–2% conc. HCl, C: 98% 2-propanol–2% conc. HCl and D: 50% methanol–50% acetate buffer (pH 3.5), as eluent solvents, the latter yielded higher recovery rates. It should be noticed

Table 2
Caffeine and metabolites recovery rates after solid-phase extraction using different sorbent materials and extraction protocols

Analyte	Percentage recovery					
	Nexus A	Nexus B	Nexus C	Nexus D	C_{18} D	Oasis D
7-MU	79.4	85.5	74.6	93.0	86.4	91.06
1-MU	68.3	75.3	68.2	90.4	83.5	90.0
7-MX	65.4	68.5	62.5	91.5	86.0	100.8
3-MX	69.2	63.5	56.2	95.6	89.8	96.0
1-MX	56.8	68.7	66.4	92.5	88.0	99.8
1,3-DMU	76.5	77.0	79.3	87.0	83.9	84.4
TB	69.9	64.6	82.5	92.6	85.4	92.7
1,7-DMU	72.3	91.2	89.5	92.0	88.0	90.0
PA	60.4	64.9	68.5	92.9	87.3	89.0
TP	73.6	84.3	90.3	92.2	86.8	93.2
CA	78.4	81.7	84.5	94.6	86.5	92.2

A: 98% methanol–2% conc. HCl; B: 98% acetonitrile–2% conc. HCl; C: 98% 2-propanol–2% conc. HCl; D: 50% methanol–50% acetate buffer (pH 3.5).

that acidified organic solvents were selected for further optimization, as the use of pure organic solvents leaded to low recoveries in the range of 50–70%. Oasis and Nexus cartridges with similar extraction properties have no significantly different recovery rates, however, the latter were chosen for usage in this study as they have smaller bed volume thus providing higher flushing rates. However, conditioning was found to be unavoidable in spite of manufacturer's instructions that this step can be omitted.

Eluent D was finally selected as provided enhanced recoveries in comparison to the other examined protocols, though it increased sample preparation time and thus total analysis time.

Extraction efficiency with optimum protocol was calculated by extracting standard solutions of all compounds, at six different amount levels, i.e. 0.25, 0.5, 1.0, 2.0, 3.0 and 5.0 ng/μl. Recoveries were determined by comparing observed analyte concentration in extracted sample to those of non-processed standard solutions. The reproducibility and accuracy of solid-phase extraction are shown in Table 3.

3.3.1. Biological fluids: blood serum and urine

The precision and accuracy studies of SPE of caffeine and primary and final metabolites from biological samples were conducted by spiking blood serum and urine samples, with three known concentrations of the compounds and then by comparing obtained results, with those as calculated from regression equations of SPE processed samples. Regression equations are summarized in Table 4. Results of recovery studies for serum and for urine samples are given in Table 5. Each value represents the mean of six measurements carried out. High performance liquid chromatogram of caffeine and metabolites, extracted from human blood serum and urine are shown in Fig. 3 and Fig. 4, respectively. No

Table 3
Recovery of caffeine and metabolites from standard solutions after SPE on Nexus cartridges using chromatographic internal standard ($n=6$)

Analyte	Added (ng)	Measured±SD (ng)	RSD (%)	Recovery (%)
7-MU	2.35	2.21±0.08	3.6	94.0
	9.40	8.74±0.07	0.8	93.0
	28.20	25.78±0.82	3.2	91.4
1-MU	2.50	2.34±0.08	3.4	93.6
	10.00	9.36±0.17	1.8	93.6
	30.00	27.60±0.56	2.0	92.0
7-MX	2.43	2.22±0.11	4.9	91.4
	9.70	9.37±0.16	1.7	96.6
	29.10	28.45±0.08	0.3	97.8
3-MX	2.60	2.47±0.04	1.6	95.0
	10.20	9.59±0.13	1.36	94.0
	30.60	29.88±1.33	4.4	97.6
1-MX	2.63	2.50±0.03	1.2	95.1
	10.50	9.72±0.09	0.9	92.6
	31.50	27.78±0.51	1.8	88.2
1,3-DMU	2.45	2.28±0.10	4.4	93.1
	9.80	9.42±0.11	1.2	96.1
	29.40	28.36±0.53	1.9	96.5
TB	2.60	2.50±0.05	2.09	96.2
	10.20	9.68±0.12	1.2	94.9
	30.60	27.04±1.11	4.1	88.4
1,7-DMU	2.40	2.27±0.07	3.1	94.6
	9.60	9.29±0.10	1.1	96.8
	28.80	26.90±1.06	3.9	93.4
PA	2.50	2.33±0.03	1.3	93.2
	10.00	9.39±0.08	0.8	93.9
	30.00	27.53±0.44	1.6	91.8
TP	2.53	2.29±0.07	3.1	90.5
	10.10	9.79±0.09	0.9	96.9
	30.30	28.10±0.47	1.7	92.7
CA	2.50	2.27±0.04	1.8	90.8
	10.00	9.54±0.06	0.6	95.4
	30.00	28.83±0.62	2.2	96.1

Table 4
Calibration data for simultaneous determination of caffeine and its primary and final metabolites after solid-phase extraction in blood serum and urine (Peak area ratio measurement with 6.18 ng/μl paracetamol as internal standard)

Compound	Blood serum			Urine		
	Slope (AIU/ng)	Intercept	*r*	Slope (AIU/ng)	Intercept	*r*
1,3-DMU	0.04524±0.00104	−0.03979±0.02610	0.99894	0.04678±0.00117	−0.06854±0.02934	0.99875
TB	0.05044±0.00088	0.01548±0.02284	0.99940	0.05199±0.00082	0.01198±0.02155	0.99950
1,7-MU	0.04252±0.00122	0.00047±0.03000	0.99836	0.04354±0.00081	−0.02035±0.01990	0.99931
PA	0.04422±0.00101	−0.01846±0.02590	0.99895	0.04398±0.00123	−0.02336±0.03143	0.99844
TP	0.04770±0.00086	−0.00866±0.02223	0.99935	0.04654±0.00105	−0.00219±0.02720	0.99898
CA	0.02252±0.00077	0.01901±0.01970	0.99767	0.02230±0.00063	0.00240±0.01603	0.99843
7-MU	0.04316±0.00126	0.02278±0.03025	0.99831	0.04272±0.00045	0.02804±0.01091	0.99978
1-MU	0.04266±0.00070	0.00982±0.01789	0.99946	0.04189±0.00105	0.02289±0.02676	0.99876
7-MX	0.04523±0.00169	0.06576±0.04200	0.99721	0.04907±0.00049	0.01657±0.01219	0.99980
3-MX	0.05607±0.00169	0.07508±0.04420	0.99818	0.05612±0.00207	0.07180±0.05393	0.99730
1-MX	0.04876±0.00099	−0.03633±0.02665	0.99917	0.04870±0.00095	−0.03926±0.02560	0.99924

Table 5
Recovery of caffeine and metabolites recovery from biological samples after SPE on Nexus cartridges ($n=6$)

Analyte	Added (ng)	Serum			Urine		
		Measured±SD (ng)	RSD	Recovery (%)	Measured±SD (ng)	RSD	Recovery (%)
7-MU	2.35	2.03±0.09	4.4	86.4	2.11±0.13	6.2	89.8
	9.40	9.11±0.29	3.2	96.9	9.34±0.35	3.8	99.4
	28.20	24.65±1.15	4.7	87.4	25.83±0.85	3.3	91.6
1-MU	2.50	2.25±0.12	5.37	90.0	2.28±0.12	5.3	91.2
	10.00	9.44±0.14	1.5	94.4	9.73±0.25	2.6	97.3
	30.00	28.82±0.30	1.0	96.1	28.62±1.61	5.6	95.4
7-MX	2.43	2.18±0.11	5.0	89.7	2.32±0.08	3.4	95.5
	9.70	9.17±0.34	3.7	94.5	8.91±0.13	1.5	91.9
	29.10	28.77±0.89	3.1	98.9	28.30±0.14	0.5	97.2
3-MX	2.60	2.51±0.10	4.0	96.5	2.30±0.06	2.6	88.5
	10.20	9.82±0.34	3.5	96.3	9.64±0.11	1.1	94.5
	30.60	31.05±0.42	1.4	101.5	31.53±0.07	0.2	103.0
1-MX	2.63	2.47±0.05	2.0	93.9	2.45±0.12	4.9	93.2
	10.50	9.74±0.45	4.6	92.8	9.41±0.18	1.9	89.6
	31.50	29.91±0.74	2.5	95.0	29.61±0.61	2.1	94.0
1,3-DMU	2.45	2.34±0.10	4.3	95.5	2.22±0.04	1.8	90.6
	9.80	9.37±0.32	3.4	95.6	9.27±0.19	2.0	94.6
	29.40	28.42±0.28	1.0	96.7	25.74±0.44	1.7	87.6
TB	2.60	2.38±0.08	3.4	91.5	2.20±0.05	2.3	84.6
	10.20	9.79±0.15	1.5	96.0	9.91±0.41	4.1	97.2
	30.60	28.13±0.15	0.5	91.9	28.79±0.74	2.6	94.1
1,7-DMU	2.40	2.22±0.12	5.4	92.5	2.20±0.07	3.2	91.7
	9.60	9.40±0.36	3.8	97.9	9.19±0.19	2.1	95.7
	28.80	29.36±0.18	0.6	101.9	28.93±0.70	2.4	100.4
PA	2.50	2.54±0.08	3.2	101.6	2.44±0.11	4.5	97.6
	10.00	9.47±0.21	2.2	94.7	9.48±0.30	3.2	94.8
	30.00	26.24±0.43	1.6	87.5	25.74±0.41	1.6	85.8
TP	2.53	2.49±0.10	4.0	98.4	2.30±0.13	5.6	90.9
	10.10	9.88±0.35	3.5	97.8	9.66±0.40	4.1	95.6
	30.30	26.99±0.86	3.2	89.1	26.20±0.53	2.0	86.5
CA	2.50	2.39±0.03	1.2	95.6	2.17±0.11	5.1	86.8
	10.00	9.14±0.34	3.7	91.4	8.80±0.22	2.5	88.0
	30.00	30.57±0.69	2.3	101.9	25.73±0.47	1.8	85.8

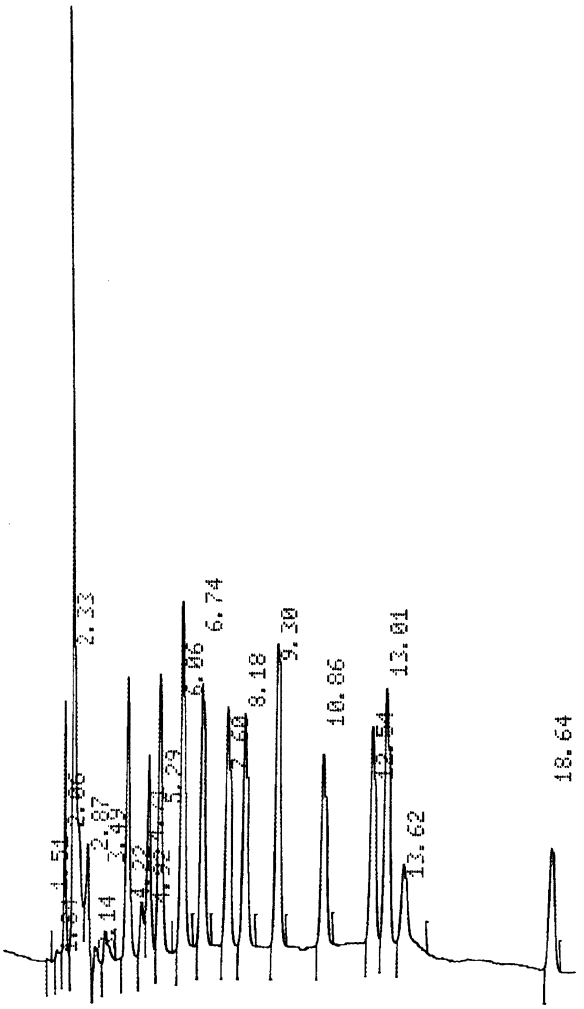

Fig. 3. High-performance liquid chromatogram of analysis of primary and final caffeine metabolites in spiked human blood samples: 7-MU (4.22 min), 1-MU (4.92 min), 7-MX (5.29 min), 3-MX (6.06 min), 1-MX (6.74 min), 1,3-DMU (7.60 min), IS (8.18 min), TB (9.30 min), 1,7-DMU (10.86 min) PA (12.54 min), TP (13.01 min) and CA (18.64 min). Chromatographic conditions are described in text.

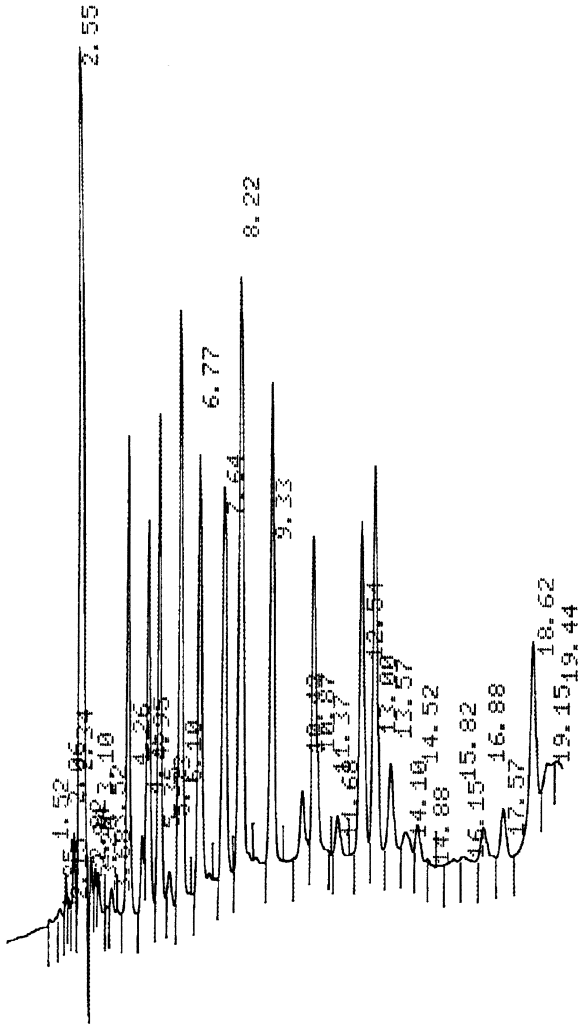

Fig. 4. High-performance liquid chromatogram of analysis of primary and final caffeine metabolites in urine samples: 7-MU (4.26 min), 1-MU (4.95 min), 7-MX (5.53 min), 3-MX (6.10 min), 1-MX (6.77 min), 1,3-DMU (7.64 min), IS (8.22 min), TB (9.33 min), 1,7-DMU (10.87 min) PA (12.54 min), TP (13.00 min) and CA (18.62 min). Chromatographic conditions are described in text.

interference from endogenous compounds from sample matrix was observed.

4. Conclusion

The method described herein is a simple validated assay that can readily be used in any laboratory for the quantitative determination of caffeine and metabolites methylxanthines and methyluric acids in one run in under 19 min. Taking into account the program gradient time, equilibration time and sample pre-treatment step, ten samples can be treated and analyzed, per day, using the SPE manifold. The application of the method was investigated in blood serum and urine after SPE using novel sorbent materials with hydrophilic and lipophilic moieties

with high percentage recoveries. The assay procedures are simple with satisfactory precision and accuracy with RSD <7%. It covers the concentration range of interest and is suitable for monitoring caffeine metabolites concentration levels in matrices of biological origin in the case that caffeine is used as a probe drug to assess the metabolic activity of cytochrome P450 and other drug metabolizing enzymes by measuring urinary elimination of drug metabolites. Reduced sample volumes of biological samples required render the method applicable in subjects of any age.

Novel SPE sorbent exhibit higher recovery rates than conventional C_{18} materials, though conditioning of sorbent bed was necessary.

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