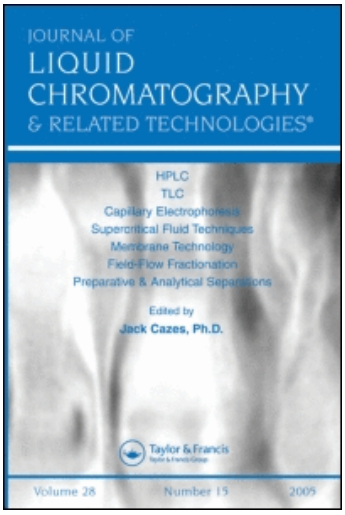

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Determination of Caffeine in Human Urine Samples Free of the Interference of its Metabolites by Reversed-Phase Liquid Chromatography Using Solid-Phase Extraction from Sample Clean-Up

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DETERMINATION OF CAFFEINE IN HUMAN URINE SAMPLES FREE OF THE INTERFERENCE OF ITS METABOLITES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY USING SOLID-PHASE EXTRACTION FROM SAMPLE CLEAN-UP

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ABSTRACT

This study describes a rapid assay for caffeine in urine samples combining solid-phase extraction techniques for sample clean-up, and conventional reversed-phase conditions for elution. The samples are previously conditioned using C18 solid-phase extraction columns, and chromatographed on an HP-Hypersil ODS-C18 column with a mobile phase consisting of acetonitrile-phosphate buffer (pH=3) in gradient elution mode. The detection is set at 275 nm. These conditions provide a complete resolution of caffeine in c. a. 6 min. The described system can be applied to the simultaneous determination of caffeine and its main metabolites. The selectivity, sensitivity and precision at therapeutic levels make it suitable for caffeine monitoring.

INTRODUCTION

Caffeine is a widely consumed substance because it is present in coffee, tea and many soft drinks. Moreover, caffeine is commonly used in the treatment of apnea in newborns [1]-[3].

Caffeine is extensively metabolized into predominantly paraxanthine (1,7-dimethylxanthine), and to a minor extent to theophylline and theobromine [4]. However, the metabolism and pharmacokinetic parameters of this drug in neonates can markedly differ from those observed in older children and adults [5]. Since these metabolites are also pharmacologically active, methods that enable the separation and identification of these compounds in urine samples are required for caffeine drug monitoring [1, 6]. The determination of caffeine clearance may also provide clinically valuable information for assessing the severity of hepatic dysfunction [6, 7]. Furthermore, its bonchodilating activity has been confirmed in adult asthmatics [8, 9]. On the other hand, because of its stimulant properties, caffeine-containing formulations have been used as doping agents in sportsmen [10, 11]. In order to detect the abuse of caffeine in human sports, the rapid determination of caffeine in urine samples is essential.

Although a wide variety of techniques have been proposed many reported studies stress the advantages of HPLC procedures in the determination of caffeine and its main metabolites in biological fluids [1, 2][11]. The major disadvantage of many of these methods, is that long retention

times are usually required for the separation of caffeine and its metabolites, and the sample preparation is very time-consuming, as the vast majority involve a liquid-liquid extraction. Moreover, sample pre-treatment seems to be a critical step [10] probably due to the limited solubility of caffeine in many solvents [12]. Therefore, the determination of caffeine by HPLC procedures generally shows worse reproducibility than the determination of comparable concentrations of theophylline, theobromine or paraxanthine [1, 12]. Solid-phase extraction techniques become a suitable alternative to liquid-liquid extraction of caffeine, as they require minor sample manipulation. Then, the precision is improved. Moreover, the sample clean-up is minor time-consuming.

This paper describes a rapid assay for caffeine determination in urine samples combining solid-phase extraction techniques for sample clean-up, and conventional reversed-phase conditions for elution. The described procedure has also been applied to the determination of theobromine, and can be applied to the determination of other caffeine metabolites .

The chromatographic conditions used in this study, are the same utilized in a previous work for the separation and quantification of acetazolamide [13], a diuretic agent which tend to coelute with caffeine and its main metabolites [13, 14]. These conditions permit the complete separation of acetazolamide and the methylxanthines, and avoid its interference in the determination of these compounds.

MATERIALS

Reagents

All the reagents used were of analytical grade. Methanol, acetonitrile and ethyl acetate were of HPLC grade (Scharlau). Water was distilled, deionized and filtered in nylon membranes, 0.45 µm (Teknokroma). Caffeine (Aldrich), paraxanthine (Sigma), theophylline (Fluka) and theobromine (Sigma) standard solutions were prepared by dissolving of pure compounds in methanol. The internal standard was ß-hydroxyethyltheophylline (Sigma). Propylamine hydrochloride (Fluka), sodium dihydrogen phosphate monohydrate (Merck) and, phosphoric acid (Probus), were also used.

Apparatus

A Hewlett-Packard 1040A liquid-chromatography, equipped with a diode array detector linked to a data system (Hewlett-Packard HPLC Chem Station) was used for data acquisition and storage. The system was coupled to a quaternary pump (Hewlett-Packard, 1050 Series) with a 25 µL sample loop injector.

The column was an HP-Hypersil ODS-C18 (5 µm, 250 mm x 4 mm ID). The detector was set to collect a spectrum every 640 ms (over the range 200-400 nm) and all the assays were carried out at ambient temperature.

METHODS

Standard Solutions

Standard solutions of caffeine, paraxanthine, theophylline and theobromine were prepared by dissolving 50 mg of the pure compound in 25 mL of methanol (2000 µg/mL). Working solutions were prepared daily by dilution of these stock solutions with the appropriate volumes of methanol. The internal standard was prepared by dissolving 25 mg of the pure compound in 500 mL of methanol (50 µg/mL). All the solutions were stored in the dark at 2^0C.

Mobile Phase

A gradient phosphate buffer/acetonitrile, with an increasing acetonitrile content from 12 % at zero min to 15 % at min 3 and to 40 % at min 5, was used. After 5 min the acetonitrile content was kept constant. The phosphate buffer was prepared by dissolving 3.45 g of sodium dihydrogen phosphate monohydrate in 500 mL of distilled and deionized water; 0.7 mL of propylamine hydrochloride were added to this solution, and then, the pH was adjusted to 3 by adding the minimum amount of concentrated phosphoric acid (c. a. 0.5 mL). The solution was prepared daily, filtrated with a nylon membrane, 0.45 µm (Teknokroma) and degassed with helium before use. The flow was set to 1 mL/min. The chromatographic signal was monitored at 275 nm.

Sample Treatment

Solid-phase extraction columns (Extra-Sep C18, 200 mg, 3 mL) for sample treatment were previously conditioned by drawing with 1.0 mL of methanol, followed by 0.5 mL of distilled water. Urine samples (2.0 mL) were drawn through the columns (with a flow rate of c. a. 1 mL/min), and washed with 0.5 mL of distilled water to eliminate the biological matrix. Caffeine was then eluted from the column with 0.5 mL of methanol and the resulting extracts were evaporated to dryness with a stream of nitrogen. The residue was reconstituted with 300 µL of the internal standard solution. The resulting solution was finally filtered with nylon filters 15 mm - 0.45 µm (Teknokroma) and 5 µL were injected into the column.

Recovery Studies

Free urine samples of 2.0 mL were spiked with xanthine standard solutions reproducing different concentrations in the therapeutic range (0 - 20 µg/mL). These samples were subjected to the previously described extraction procedure. The percentage of drug recovered for a particular extraction was calculated comparing the peak areas obtained for the xanthine in the spiked samples (referred to the internal standard), with the peak areas obtained for a direct injection 5 µL of methanolic solutions containing an equivalent amount of drug. Each concentration was assayed in duplicate.

Preparation of Standards for Calibration

Standards for calibration were prepared by spiking 2.0 mL of the urine samples with the appropriate volumes of caffeine or theobromine methanolic solutions, reproducing six concentrations in the 0 - 20 µg/mL range. These samples were extracted and chromatographed as described above. Peak area ratios of caffeine or theobromine to ß-hydroxyethyl-theophylline, were plotted versus the xanthine concentration, and the resulting calibrations curves were used to calculate the xanthine concentration in the unknown samples. A calibration curve obtained by direct injection of methanolic solution of each xanthine in the same concentration range, was also constructed. Each concentration was assayed in duplicate.

RESULTS AND DISCUSSION

In Figure 1 is shown the chromatogram obtained for a mixture in methanol of caffeine (t_R = 5.8 min), theobromine (t_R = 3. 3 min), theophylline (t_R = 4.1 min) and paraxanthine (t_R = 4.1 min). This figure also shows the chromatographic signal of acetazolamide (t_R = 5.4 min). This agent has been reported to interfere many of chromatographic determinations of the xanthines as they have similar polarities, and tend to coelute in reversed-phase conditions [15]. With the proposed procedure, the resolution between this compound and the main xanthines is complete.

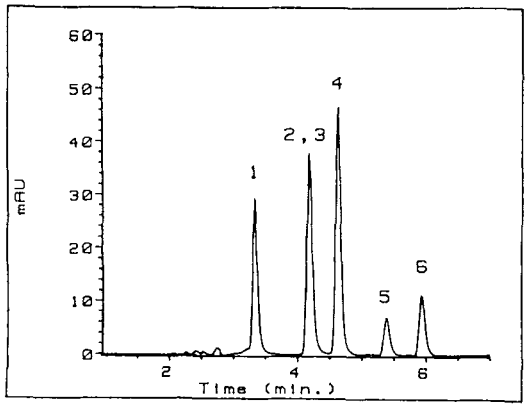

FIGURE 1
Chromatograms at 275 nm of a mixture of theobromine (1), theophylline (2), paraxanthine (3), acetazolamide (5) and caffeine (6) in methanol. The amount injected of each compound was 0.1 µg. (Peak 4 corresponds to the internal standard).

As can be seen, the chromatographic peaks of theophylline and paraxanthine are overlapped. The coelution of these two xanthines has been reported to be the most serious problem of the reversed-phase separations of xanthines. Moreover, the spectra of these compounds are very similar (Figure 2); this makes very difficult the quantification of these compounds by measuring their absorbances at selective wavelengths. Although normal-phase HPLC procedures can provide good resolution of theophylline and paraxanthine, these techniques seem to be inadequate for clinical laboratories, due to their inability to maintain a stable separation; moreover, because of the differences of polarity between caffeine and its metabolites, high flow-rates or long times of analysis are involved, and two different internal standards may be required [3].

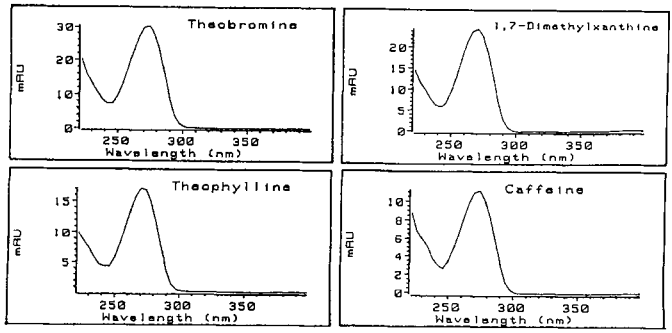

FIGURE 2
Absorption spectra of caffeine and its main metabolites. (The amount injected of each compound was 0.1 µg).

On the other hand, several modifications for reversed-phase chromatographic conditions based on the employment of an organic modifier or an ion-pairing reagent have been suggested to improve the resolution between these compounds. These modifications can provide an almost complete resolution between theophylline and paraxanthine but then, the elution of caffeine becomes too slow (with retention times higher than 20 min) [8]. Elevate flow-rates or modificated columns may be required to elute caffeine in acceptable times of analysis [3][16-18]. Moreover, experimental conditions (pH, ionic strength, temperature) must be strictly controlled if reproducible results are to be obtained. Rapid degeneration of column packing due to ion-pairing reagents is also a problem [16, 19].

As an alternative to the modifications in the chromatographic conditions, we have recently applied the H-point standard additions method [20] for the simultaneous analysis of theophylline and paraxanthine. The basis of the

H-point standard additions method (HPSAM) for the treatment of chromatographic data [21] requires fixing two wavelengths λ_1 and λ_2, at which the interferent specie (Y) should have the same absorbance. The chromatographic variable used to apply the HPSAM method will be peak height (absorbance values) at λ_1 y λ_2, registered at the retention time of the analyte (X). Application of the HPSAM method for the injected solutions containing sample alone and sample plus added analyte concentration according to the standard additions method, will yield two straight lines which intersect at point H ($-C_H$, A_H) = ($-C_X$, A_Y). C_H is the existing concentration of the analyte in the sample (C_X) and A_H, the ordinate of the H point, is the analytical signal due to the interferent (A_Y) corresponding to λ_1 and λ_2. This value makes possible the interferent determination from a calibration graph or even from the calibration method with a single standard.

The concentration of theophylline and paraxanthine can be determined by the HPSAM method, considering anyone as analyte or interferent with suitable accuracy and precision, even when the concentration of one of them is many times higher than the other one. Therefore, the simultaneous determination of caffeine and its metabolites should not require any modification in the usual chromatographic conditions, and can be achieved in a few minutes.

In a previous test, we initially have compared the efficiency of a solid-phase extraction using a C18 packing with a liquid-liquid extraction in ethyl acetate according to the procedure described in [22]. In all cases tested, the solid-phase extraction techniques provides higher recoveries than the extraction in ethyl acetate. Figure 3 shows the

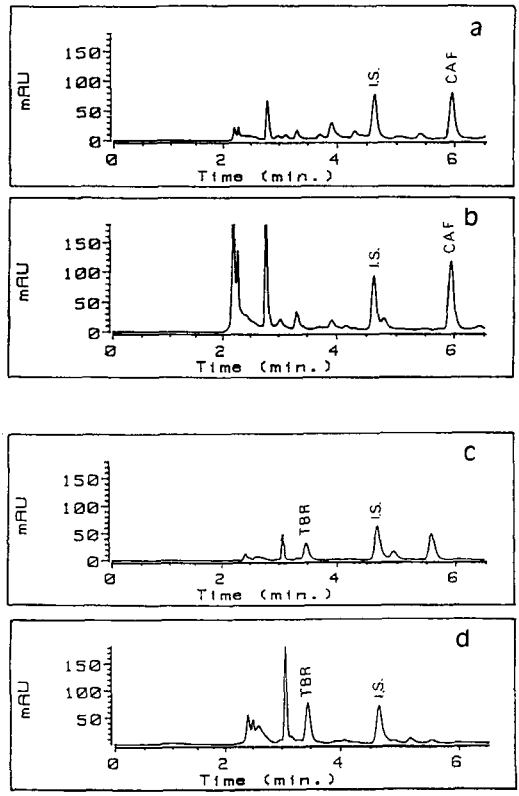

FIGURE 3
Chromatograms at 275 nm of urine samples spiked with caffeine after a liquid-liquid extraction (3a) and a solid-phase extraction using C18 columns (3b), and theobromine after a liquid-liquid extraction (3c) and a solid-phase extraction using C18 columns (3d). The concentration of each xanthine in the samples was 15 µg/mL. Peaks: caffeine (CAF), theobromine (TBR), internal standard (I.S.).

chromatograms obtained for urine samples spiked with caffeine and theobromine.

In Figure 4 can be observed a chromatogram of a blank urine sample obtained after solid-phase extraction from a subject abstained from foods and beverages containing

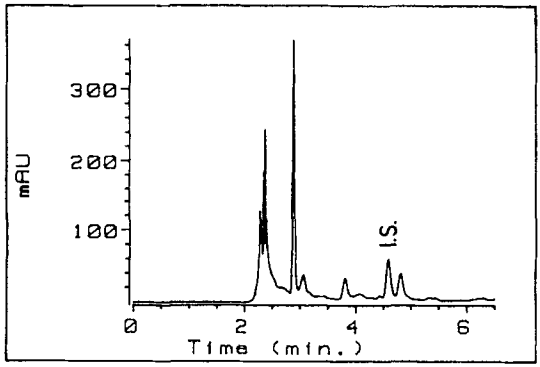

FIGURE 4
Chromatogram at 275 nm obtained from a blank urine sample after a solid-phase extraction using C18 columns.(Internal standard, I.S.).

methylxanthines. By comparing Fig 3 and 4 can be derived than the determination of caffeine is free from the interference of urinary endogenous compounds. The sample treatment described can also be applied to the determination of caffeine metabolites, as any endogenous compounds are eluted at the same retention time than these metabolites.

The recoveries obtained for the different xanthines tested, at different concentrations in the therapeutic range, appear in Table 1. In all the cases tested, the recovery is not dependent on the drug concentration. The recovery of caffeine is higher than those previously reported using solid-phase extraction techniques [15], and similar than that obtained by direct injection of the samples [2], being excellent the efficiency and precision of the extraction for this compound.

On the other hand, with the sample treatment used in this study, there are not significative differences in the recoveries

TABLE 1

Recoveries Obtained for the Main Xanthines with C18 Solid-Phase Extraction Columns.

Xanthine Concentration (µg/mL)	Recovery (%)			
	Theobromine	Paraxanthine	Theophylline	Caffeine
0.75	84	78	79	100
3.75	74	71	74	98
7.50	77	80	69	103
11.25	76	75	67	96
15.00	-	72	76	95
Mean values	(75 ± 8)	(75 ± 4)	(73 ± 5)	(99 ± 3)

and precision for the extraction of caffeine, when for the same set of experiments, the other xanthines are directly added to the urine samples, and used as internal standards for the extraction step (Table 2). Similar conclusions can be derived, when caffeine peak areas are directly utilized in the determination of caffeine recoveries in the same samples. This indicates that the extraction step is reproducible. Dorberecker et al. [12] demonstrated that the recovery of caffeine is strongly dependent on the experimental conditions used for sample treatment. Therefore, the employment of solid-phase extraction columns for extraction of caffeine is clearly advantageous over conventional liquid-liquid extraction because they require minor sample manipulation, resulting in an improvement of the reproducibility. Subsequently, for the quantification of caffeine and theobromine, ß-hydroxyethyltheophylline is added to the samples as an internal standard, to correct sample size fluctuations.

The calibrations graphs are linear in the working interval and their intercept is essentially zero. For caffeine, the slopes of these graphs were 0.153 for standards in methanol and 0.151

TABLE 2

Extraction Recoveries Obtained for Caffeine Using Different Internal Standards: Theobromine (TBR), Paraxanthine (PXT), Theophylline (TFL), β-hydroxyethyltheophylline, and without Internal Standard.

Sample	Internal Standard				
	TBR	PXT	TFL	β-hydroxyethyl-theophylline	-
1	108	-	108	107	104
2	-	-	101	93	94
3	97	-	108	91	87
4	103	-	91	97	96
5	98	-	102	-	94
6	100	-	-	106	-
7	97	100	-	101	97
8	104	93	-	103	102
9	111	109	-	98	95
10	-	105	-	100	101
Mean value	102±5	102±7	102±6	100±5	97±5

for urine samples, with mean correlation coefficients of 0.9991 and 0.995, respectively. For caffeine, the two calibration graphs are coincident, confirming that the recovery of drug is complete. For theobromine, the slopes of these calibration graphs were 0.134 for standards in methanol and 0.095 for urine samples, with mean correlation coefficients of 0.9998 and 0.996, respectively. As can be seen, the two calibration curves obtained for theobromine, are statistically equivalent, if the mean percentage recovered of drug is considered, therefore the matrix of the sample does not modify the slope of the calibration graph obtained with standards in methanol. Therefore, the determination of both xanthines can be achieved from calibration graphs directly obtained for standard solutions of these compounds.

The described procedure was applied to the determination of caffeine and theobromine in unknown urine samples obtained from

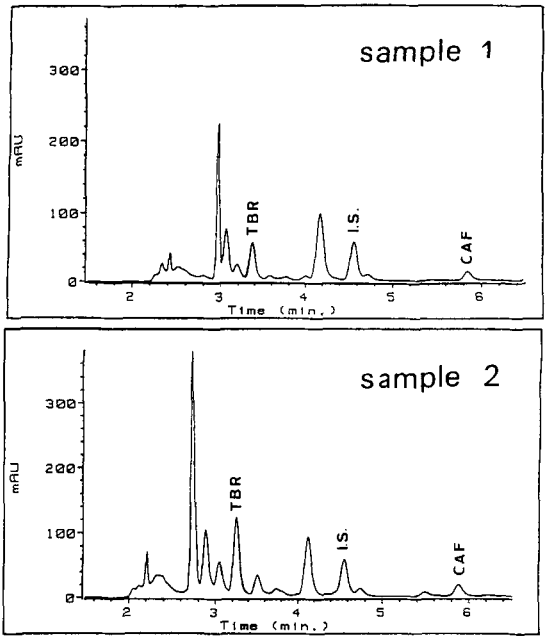

FIGURE 5
Chromatograms at 275 nm for the unknown urine samples tested. Peaks: caffeine (CAF), theobromine (TBR), internal standard (I.S.).

a healthy volunteer after coffee intake. The chromatograms corresponding to these samples appear in Figure 5. For sample 1, the concentrations of caffeine and theobromine found were (1.50 ± 0.07) and (4.3 ± 0.2) µg/mL, respectively (n = 4). For sample 2, the concentrations of caffeine and theobromine found were (1.40 ± 0.07) and (8.9 ± 0.5) µg/mL, respectively (n = 7). In all cases, the concentrations found are in the therapeutic range.

Inter-day assay reproducibility, obtained for sample 2, produced a mean concentration of (1.4 ± 0.1) µg/mL, (C.V. of 7 %, n=12) for caffeine, and (9.4 ± 0.8) µg/mL for theobromine

(C.V. 9 %, n= 12). These values are similar to those previously reported for the studied xanthines.

The limit of detection (for a signal to noise ratio of 3) corresponds to an injected amount of 0.05 ng for caffeine and 0.01 ng for theobromine, which are equivalent to concentrations of 2 ng/mL and 0.4 ng/mL of urine, respectively.

In conclusion, an HPLC assay for the rapid quantitative determination of caffeine and theobromine in urine samples, is described. The employment of solid-phase extraction columns avoids time for sample treatment and provides high recoveries of caffeine and its metabolites. The samples are chromatographed in short times without any modification in the chromatographic conditions usually used in clinical laboratories. Then, the described system can be applied to the simultaneous determination of caffeine and its main metabolites avoiding the interference of acetazolamide. The determination of paraxanthine and theophylline can be achieved by using the H-point standard additions method, as it is described in [20]. The selectivity, sensitivity and precision at therapeutic levels make it suitable for caffeine monitoring or in doping controls.

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