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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 621–624
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Determination of methylxanthines in urine by liquid chromatography with diode array UV detection

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Received 24 June 2004; received in revised form 6 July 2004; accepted 6 July 2004
Available online 20 August 2004

Abstract

A liquid chromatography–diode array UV detection (LC–UVDAD) method for the simultaneous determination of four methylxanthines (caffeine, theobromine, paraxanthine and theophylline) is described. The chromatographic separation was achieved on a LC-18-DB column using 20:80 methanol:buffer (5 mM citric acid adjusted to pH 5 with triethylamine) as mobile phase. The method has been applied to urine samples. The overall procedure had % recoveries ranging from 81.6 ± 2.6 (theophylline) to 99.3 ± 6.3 (theobromine). The within-day ($n = 5$) and between-days ($n = 5$ over 5 days) coefficients of variation in urine ranged from 2.9% (theophylline) to 3.4% (theobromine) and from 5.2% (theophylline) to 6.2% (theobromine). Estimated LOD and LOQ in urine ranged from 0.15 μg/ml (theophylline) to 0.3 μg/ml (theobromine) and from 0.8 μg/ml (theophylline) to 1.2 μg/ml (theobromine), respectively. Urine samples naturally contaminated with the target analytes were found.
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Keywords: LC; Caffeine; Theobromine; Paraxanthine; Theophylline; Human urine

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is one of the three methylated xanthine alkaloid derivatives that are present in many plant species throughout the world. The other two methylxanthines derivatives are theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthyne). Caffeine is a constituent of coffee and other beverages [1], also included in many drugs. Theobromine and theophylline are presents [2] in cocoa, teas and chocolate products. Theophylline is also a widely used bronchodilating agent [3] with a narrow serum therapeutic range. Paraxanthine (1,7-dimethylxanthine) is not found in foods but is the main metabolite [4] of caffeine. These substances show various physiological effects [5] on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and renal systems. There is also a general consensus by sport investigators that methylxanthines are in some respect ergogenic. Thus, sport federations feel a need to control their use in athletes. A problem arises, however, when trying to prevent their use. They are very common chemicals in the human diet, which makes it difficult to entirely avoid. These aspects forced federations to set limits rather than imposing a complete avoidance rule. For instance, caffeine is present in the Monitoring Program 2004 of the World Anti-Doping Agency (WADA) [6], that regards substances which are not on the Prohibited List but which WADA whishes to monitor in order to detect patterns of misuse in sport; theophylline is on the list of forbidden doping substances as issued [7] by the Flemish Government.

These considerations suggest that a method for the simultaneous determination of methylxanthines in human urine is highly advisable for doping purposes, since analytical screens that can detect multiple analytes in one assay are vital for rapid laboratory response.

Existing papers dealing with the determination of these compounds in urine for doping purposes [8–10] are based on liquid chromatography. Perez-Martinez et al. reported [8] a procedure for the simultaneous determination of caffeine,

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doi:10.1016/j.jpba.2004.07.010

theobromine, and theophylline while other methods were capable to determine caffeine [9,10].

In the present work, a LC–UV/DAD method for the simultaneous determination of the methylxanthines caffeine, theobromine, paraxanthine and theophylline was developed. The eluent composition was properly optimized in order to achieve the highest possible chromatographic resolution. The applicability of the procedure to the analysis of real samples was demonstrated by the analysis of human urine samples.

2. Experimental

2.1. Chemicals

Caffeine, theobromine, paraxanthine and theophylline were obtained from Sigma (St. Louis, MO, USA). Stock solution were prepared in tridistilled water and stored at 4 °C in the dark. Dilute solutions were prepared just before use.

Organic solvents (Carlo Erba, Milan, Italy), were HPLC grade. Mobile phase was filtered through a 0.45 μm membrane (Whatman Limited, Maidstone, UK) before use.

2.2. Apparatus

The HPLC system consisted of a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA), equipped with a Rheodyne injector with a 100 μl loop and a 5 μm Supelcosil LC-18-DB column (250 × 2.1 mm i.d.) (Supelco). A 5 μm Supelguard LC-18-DB precolumn (20 × 2.1 mm i.d.) (Supelco) was used to protect the analytical column. The detector was a photodiode-array (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer.

2.3. Chromatographic and detection conditions

The composition of the optimized buffer 5 mM citric acid adjusted to pH 5 with triethylamine. The optimized mobile phase conditions were 20:80 methanol:buffer with a flow rate of 0.2 ml/min at ambient temperature. Mobile phase was degassed on-line by an SCM1000 Vacuum Membrane Degasser (Thermo Separation Products). Spectra were acquired in the 224–380 nm range using 1 Hz frequency and 5 nm bandwidth. Chromatograms were monitored at 280 nm (10 Hz frequency, 5 nm bandwidth).

2.4. Sample collection and pre-treatment

Seven urine samples were collected from healthy non-smoking donors with ages in the range 10–40; five of them were regular caffeine consumers. Samples were stored at 4 °C for not more than 2 days before analysis.

To a 100 μl of urine sample in 20 ml glass tube were added 900 μl of saturated ammonium sulphate solution followed by a brief vortex mixing. About 7 ml of an chloroform–isopropylalcohol (85/15, v/v) mixture were successively added and the resulting mixture was vigorously shaken for 10 min, centrifuged at 2000 rpm for 10 min and the organic phase carefully transferred into a tapered tube. The extract so obtained was evaporated to dryness at room temperature under a gentle stream of nitrogen. Finally, the extract was simply reconstituted with 7.5 ml of HPLC water, filtered through a 0.45 μm Millex-HV type filter (Millipore) and 20 μl were injected.

Recoveries were calculated as peak area ratio of analyte (standard)/analyte (spiked urine samples). Urine samples were spiked with the analytes at 1.5, 5, 15, and 50 μg/ml concentration levels, equilibrated at room temperature for at least 1 h and then analyzed as described above.

Quantitation was performed with the standard addition method. Calibration curves in urine were constructed spiking analyte free urine samples with caffeine, theobromine, paraxanthine and theophylline in order to cover the range from 1.5 to 150 μg/ml; five replicates for each concentration (1.5, 5, 10, 30, 75, and 150 μg/ml) were performed.

The within-day (n = 5) and between-days (n = 5 over 5 days) coefficient of variation in urine were calculated on analyte free urine samples spiked with variable amounts of the target analytes in order to obtain the following concentration levels:1.5, 5, and 150 μg/ml.

3. Results and discussion

The first step of the work consisted in the optimization of the chromatographic conditions in order to optimize the separation and the chromatographic efficiency. Fig. 1 reports the LC–UV chromatograms obtained by direct injection of a standard solution of caffeine, theobromine, paraxanthine and theophylline at a concentration level of 0.1 μg/ml using the optimised mobile phase composition, i.e., 20:80 methanol:buffer (5 mM citric acid adjusted to pH 5 with triethylamine) and shows its capability to resolve the target compounds.

Calibration curves resulted linear in the range 2 ng/ml to 2 μg/ml with correlation coefficients better than 0.999 and intercept not significantly different from zero at 95% confidence level.

The within-day (n = 5) and between-days (n = 5 over 5 days) coefficients of variation, estimated by an ANOVA test, ranged from 1.6% (theobromine) to 2.6% (caffeine) and from 3.2% (theobromine) to 4.1% (caffeine), respectively, and remained practically unchanged at 0.01, 0.5–1 μg/ml level.

LOD and LOQ were calculated as signal to noise ratio of 3 and 10, respectively. The estimated LOD were in the ranges 0.66 ng/ml (theobromine) and 0.8 ng/ml (paraxanthine), respectively, while LOQ were in the range 2 ng/ml (theobromine) and 2.5 ng/ml (paraxanthine), respectively.

Once the ideal chromatographic conditions were reached, the method was applied to the determination of caffeine, theobromine, paraxanthine and theophylline in human urine samples.

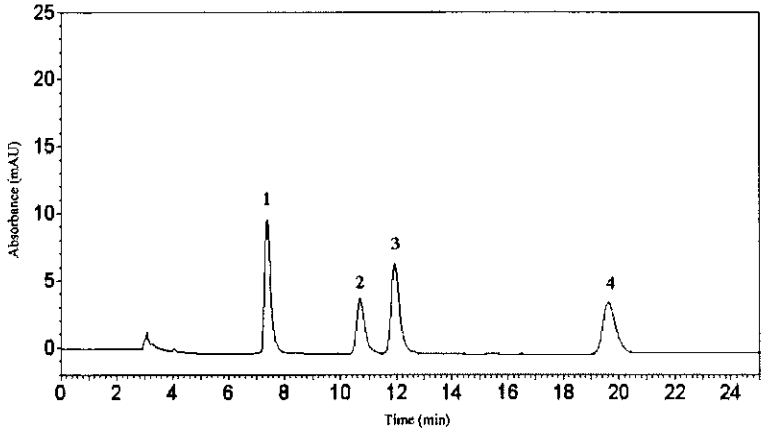

Fig. 1. LC–UV chromatograms obtained by direct injection of a standard solution of the target analytes at a concentration level of 0.1 μg/ml. Peak legend: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = caffeine.

Fig. 2 shows, for instance, the LC–UV chromatograms relevant to (A) a analyte free urine sample and (B) the same sample spiked with known amounts (7.5 μg/ml) of the target analytes, respectively, and treated as described in the experimental section. As apparent, the optimized mobile phase permitted to resolve the target compounds with no significance interference from urine endogenous components.

Calibration curves resulted linear in the range 1.2–150 μg/ml with correlation coefficients better than 0.999 and intercept not significantly different from zero at 95% confidence level.

The within-day (n = 5) and between-days (n = 5 over 5 days) coefficients of variation, estimated by an ANOVA test, ranged from 2.9% (theophylline) to 3.4% (theobromine) and from 5.2% (theophylline) to 6.2% (theobromine), respectively, and remained practically unchanged passing from 1.5 to 15 μg/ml level.

The obtained % recoveries ± standard deviation (n = 3) were 85.0 ± 3.1 (caffeine), 99.3 ± 6.3 (theobromine), 81.6 ± 2.6 (theophylline) and 83.1 ± 2.4 (paraxanthine), respectively, and remained practically unchanged passing from 1.5 to 50 μg/ml level.

Table 1
Methylxanthines concentrations estimated in the considered urine samples

Compound	Concentration (μg/ml)				
	Urine 1	Urine 2	Urine 3	Urine 4	Urine 5
Caffeine	1.9	–	5.1	1.5	–
Theobromine	11.5	11.5	2.8	13	–
Paraxanthine	6.7	6.4	34.2	6.9	2.5
Theophylline	0.3	–	0.3	1.5	–

LOD and LOQ in urine were calculated as signal to noise ratio of 3 and 10, respectively. The estimated LOD were in the ranges 0.15 μg/ml (theophylline) and 0.3 μg/ml (theobromine), respectively, while LOQ were in the range 0.8 μg/ml (theophylline) and 1.2 μg/ml (theobromine), respectively.

Five of the analyzed samples were found to be contaminated with variable quantities of the analytes. Fig. 3 shows, for instance, an LC–UV chromatogram relevant to a naturally contaminated urine sample. Peak identity was confirmed on the basis of retention times and by UV spectra overlay. Table 1 reports the relevant results.

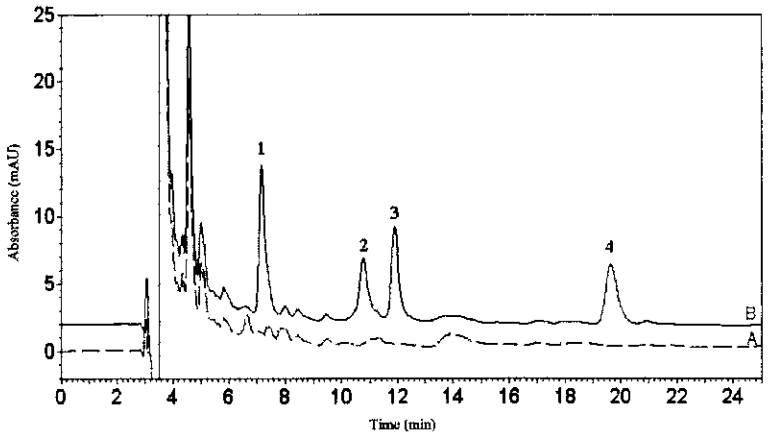

Fig. 2. LC–UV chromatograms relevant to (A) a analyte free urine sample and (B) the same sample spiked with known amounts (7.5 μg/ml) of the target analytes, respectively, and treated as described in the experimental section. Peak legend: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = caffeine.

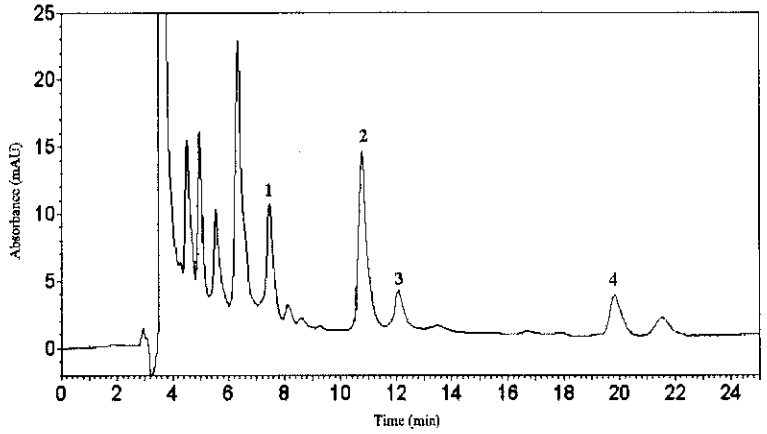

Fig. 3. LC–UV chromatogram relevant to the analysis of a urine sample (urine 1) naturally contaminated with the target methylxanthines (see Table 1 for the estimated concentration). Peak legend: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = caffeine.

4. Conclusions

A LC–UV method for the simultaneous determination of caffeine, theobromine, paraxanthine and theophylline has been developed for the first time. The LC separation of the target compounds could be achieved on a LC-18-DB column using 20:80 methanol:buffer (5 mM citric acid adjusted to pH 5 with triethylamine) as mobile phase. The potential of the described procedure was demonstrated by the application to human urine samples naturally contaminated with the target analytes.

Acknowledgments

Work carried out with financial support from University of Bari and National Research Council.

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