Journal of Chromatography, 325 (1985) 304–308
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 646

Note

Quantitation of caffeine in doping analysis using an automated extractor/concentrator

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(Received February 12th, 1985)

Caffeine (1,3,7-trimethylxanthine) is employed mainly as an ingredient of coffee, cola beverages and tea. Because of its stimulant properties, however, caffeine-containing formulations are also used as doping agents by sportsmen. Therefore the continuing challenge between analysts and athletes is nowadays focused on the misuse either of synthetic anabolics or endogenous substances like testosterone or normal food constituents such as caffeine. Based on a comparative study and taking into account the pharmacological properties of caffeine, a maximum allowable level of 15 $\mu g\ ml^{-1}$ in urine has been proposed[1].

In order to quantitate caffeine, gas chromatography (GC) or high-performance liquid chromatography (HPLC) can be used[2]. The precision and accuracy of any chromatographic analysis, however, is as dependent on the sample preparation as on the separation and identification techniques. Often the sample preparation procedure is the least accurate and the most laborious part of the whole analytical method. However, the use of large volumes of solvents, the emulsion formation and consequently long analysis times in conventional extraction methods in doping analysis can be overcome by the use of solid–liquid extraction procedures with XAD resins for amphetamines[3,4] or Extrelut for stimulant amines[5] and caffeine[2].

Although the use of XAD resins, Kieselguhr sorbents like Extrelut and more recently reversed-phase solid phase extraction are elegant chemical isolation techniques and certainly constitute an improvement in drug analysis, they still remain time consuming as they need a more or less continuous monitoring. However, this inconvenience could be substantially reduced by the use of an automated extractor/concentrator[6].

This paper describes a method for the preparation and quantitative analysis of caffeine in urine using GC and an automated extractor/concentrator.

EXPERIMENTAL

Materials

Caffeine was obtained from Merck (Darmstadt, F.R.G.) and the internal standard, mepivacaine hydrochloride, was supplied by Astra (Södertälje, Sweden). Stock solutions were prepared either in methanol or in double distilled water. The concen-

0021-9673/85/$03.30

tration of the internal standard used was 50 μg ml^{-1}. Animine®, a formulation containing caffeine α-naphthylacetate, was obtained through Alcon-Couvreur (Puurs, Belgium). The ammonium buffer was prepared by adjusting a saturated ammonium chloride solution to pH 9.5 with ammonia.

Analytical-grade (dichloromethane, methanol) and pesticide-quality (ethyl acetate) solvents were obtained from Merck.

The automated extractor/concentrator (PREP^{TM}I, DuPont Instruments) was used with Type W extraction cartridges containing a lipophilic polystyrene resin (DuPont). Dilutions and dispensings were made with a Hamilton diluter/dispenser.

Gas chromatography

A Varian 3700 gas chromatograph equipped with a nitrogen-selective detector was used, connected to a Varian CDS 111 integrator. The glass column (200 × 0.25 cm I.D.) was packed with 3% OV-7 on Chromosorb W HP. Nitrogen was used as carrier gas at a flow-rate of 25 ml min^{-1}. The column, injector and detector temperatures were 180, 230 and 330°C respectively.

Analytical procedure

All urine samples were prepared for chromatography with a microprocessor-controlled, centrifugally based extractor/concentrator using Type W extraction cartridges[6]. A 1-ml volume of urine was pipetted into a reaction tube and made alkaline by adding 0.1 ml of ammonium buffer. After adding 0.1 ml of the aqueous internal standard solution, the contents of the tube were briefly vortexed, centrifuged (2 min) and decanted into the cartridge reservoir. The cartridge components (resin column, effluent cup and recovery cup) were placed into the swinging buckets of the dual rotor of the extractor/concentrator. Up to twelve cartridges could be processed simultaneously.

Subsequently, caffeine was sorbed on to the resin centrifugally. Double distilled water (12 ml) and the elution solvent, dichloromethane–methanol (9:1, 25 ml), were automatically and consecutively dispensed to the hub of the spining rotor and distributed equally to the twelve cartridges in order to wash out contaminants and to elute caffeine respectively. The organic solvent was then automatically evaporated to dryness by a stream of warm air (45°C). Upon completion of the operation (30 min) twelve dry extracts were ready for GC. The residue was redissolved in 0.2 ml of ethylacetate and 1 μl injected into the gas chromatograph.

Investigations on humans

Five healthy volunteers abstained from coffee, tea and other caffeine-containing beverages for at least 1 week. Two tablets of Animine each containing 96.5 mg of caffeine were taken orally followed by the same amount 2 h later. All urine was collected during fixed intervals (0, 1, 2, 3, 4, 5, 6, 9, 12, 24, 30 and 36 h), the volume and pH measured and analyzed immediately or stored deep frozen for later analysis. No caffeine-containing beverages were used during the whole experiment.

RESULTS AND DISCUSSION

Chromatographic analysis

Under the chromatographic conditions described previously, caffeine and the internal standard mepivacaine gave sharp peaks with retention times of 3.93 and 7.27 min respectively.

Recovery

Urine was spiked with small amounts of caffeine to give samples of known concentration (2 and 1 $\mu g\ ml^{-1}$). Volumes (1 ml) of these urines were then processed as described (five samples for each concentration), the internal standard being substituted by 0.1 ml water. After redissolving the residues in 0.2 ml of a methanolic mepivacaine solution, the amount of caffeine was quantified using a calibration graph for pure standards.

Although the obtained recoveries of 63.9 ± 3.8 and 66.4 ± 2.2% respectively for 2 and 1 $\mu g\ ml^{-1}$ caffeine were inferior to those obtained in other extraction procedures[2], the purity of the extracts enabled a detection limit of 0.4 $\mu g\ ml^{-1}$ starting with 1 ml urine.

Detection linearity, precision and rapidity of the method

A calibration curve was obtained by adding different amounts of caffeine to caffeine-free urine and treating the samples in triplicate according to the described procedure. The graph was linear in the range 0–20 $\mu g\ ml^{-1}$ caffeine in urine (r = 0.994).

The precision of the method was studied by repeated analysis of urines containing different caffeine concentrations. The data in Table I indicate that the variation (five trials) in the calculated concentrations ranged from 2.2 to 6.3%, lower than the values found with a recently published automated HPLC method[7].

TABLE I

PRECISION OF ASSAYS FOR CAFFEINE IN URINE

Range ($\mu g\ ml^{-1}$ ± *S.D.*)	*C.V. (%)*
0.48 ± 0.03	6.3
0.61 ± 0.03	4.9
1.27 ± 0.03	2.4
1.55 ± 0.03	2.2

Furthermore, it should be noted that the automatic extraction and concentration procedure lasts 30 min for twelve samples and has an organic solvent consumption of *ca.* 2 ml per sample. Although the extraction time is comparable to that in other methods using solid-phase extraction columns, the low solvent volume obviously results in a shortening of the evaporation step. Moreover, other solid extraction methods still need a more or less continuous monitoring of the extraction, washing and evaporation steps, and therefore the fully automated procedure used here is certainly less time consuming and should constitute an improvement in drug analysis.

Excretion study in humans

Following this method, the urinary caffeine content in five subjects was followed for 36 h after the intake of substantial doses of a caffeine-containing formulation. The results given in Fig. 1 indicate that peak values of 6.3 ± 1.5 $\mu g\ ml^{-1}$ were obtained 1 h after the second dose of Animine. In one subject a maximum urinary caffeine concentration of 10.5 $\mu g\ ml^{-1}$ was obtained 2 h after the administration of the second dose.

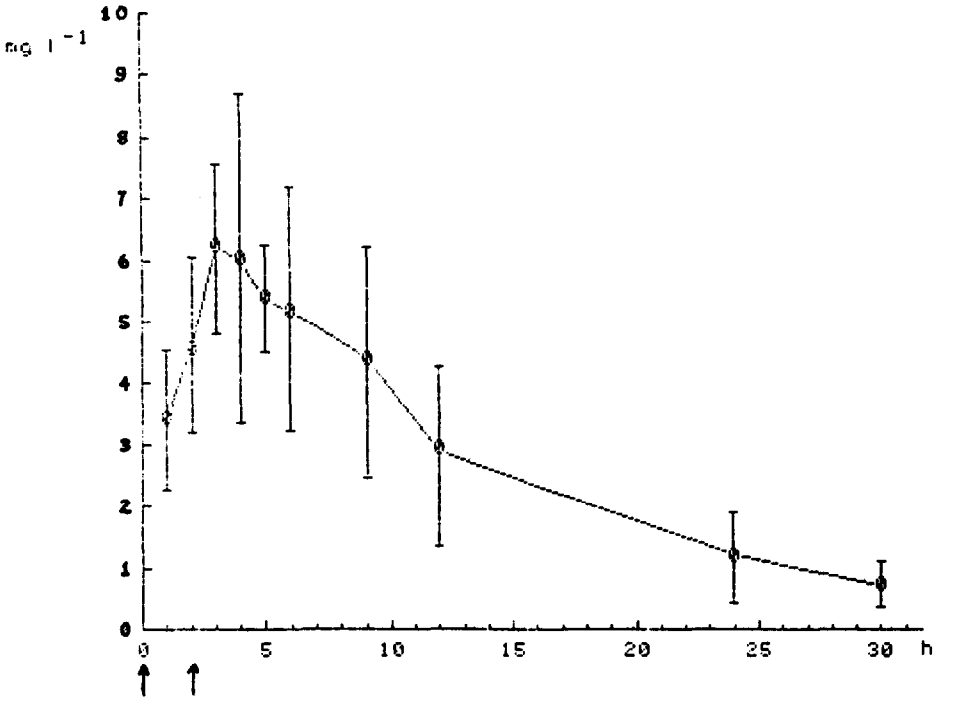

Fig. 1. Mean ± S.D. urinary caffeine concentration in five subjects after the intake of 400 mg Animine at 0 and 2 h.

It should be clear that the intake of two doses each containing 193 mg caffeine in a 2-h period could not be regarded as therapeutic. Even taking into account the fact that the urine from an athlete could be more concentrated after prolonged exercise —but also that substantial quantities of water are often ingested to facilitate urine production for a doping control— the caffeine concentrations obtained here are far below the values found in the urine of some cyclists analysed in 1982 where 1.7% of the samples contained from 15 to 20 $\mu g\ ml^{-1}$ with a maximum value of 39 $\mu g\ ml^{-1}$ (ref. 1).

Therefore, the caffeine contents found during this experiment strongly support the belief that urinary caffeine concentrations of 15 $\mu g\ ml^{-1}$ are indicative of ingestion of caffeine with the intention to improve physical performance and should consequently be regarded as doping.

ACKNOWLEDGEMENT

The technical assistance of Mrs. G. Demey, Mrs. S. Jacobs, Ing. N. Desmet and Ing. F. Maertens is greatly appreciated.

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