Journal of Chromatography, 229 (1982) 387—395
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1200

LIQUID CHROMATOGRAPHIC DETERMINATION OF CAFFEINE IN BIOLOGIC FLUIDS

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(First received August 17th, 1981; revised manuscript received December 2nd, 1981)

SUMMARY

A high-performance liquid chromatographic procedure was developed for the determination of caffeine in various biologic fluids and coffee. A reversed-phase column and UV detection at 254 nm were used to obtain a sensitivity of 0.1 μg/ml caffeine in serum and saliva using a sample volume of 0.1 ml. Caffeine metabolites and commonly ingested xanthines do not interfere with the assay. The within-day coefficients of variation were 9.8 and 9.9% at plasma caffeine concentrations of 2 and 10 μg/ml, respectively. The day-to-day coefficients of variation were 6.8 and 6.6% at plasma caffeine concentrations of 2 and 10 μg/ml, respectively. Serum and saliva caffeine concentrations were determined following a single oral dose of coffee and an intravenous infusion of caffeine in one subject. Computer estimates of caffeine pharmacokinetic parameters in one subject are in excellent agreement with previously published values.

INTRODUCTION

Caffeine is a commonly ingested drug present in foods and many beverages consumed throughout the world. Recently, the drug has been used therapeutically in the management of neonatal apnea [1]. Studies indicate that the

0378-4347/82/0000—0000/$02.75

pharmacologic and toxicologic effects of caffeine are incompletely understood [2,3]. For example, caffeine is teratogenic in rats [2] and as a consequence it has been recommended that pregnant women restrict caffeine intake until further studies can determine the applicability of this finding to humans [3]. Another study has shown that caffeine is a potent cardiovascular stimulant, but it is not known whether caffeine ingestion causes arrhythmias [4]. One study has demonstrated that among regular consumers of caffeine, even a brief abstinence from the drug may produce anxiety [5]. All of these findings suggest that caffeine is not an innocuous compound and that additional studies on the toxicology and pharmacology of caffeine are needed.

Pharmacologic and toxicologic studies of caffeine both in animals and man often require collection of multiple blood samples and detection of caffeine concentrations below the limit of sensitivity for many available assays [4,6]. It may also be necessary to determine the caffeine content of commonly ingested beverages for pharmacologic studies of caffeine. This report describes a sensitive and selective high-performance liquid chromatographic (HPLC) procedure for the analysis of caffeine in various biologic fluids and coffee. Application of the method to pharmacokinetic and protein binding studies of caffeine are demonstrated in one volunteer subject.

MATERIALS AND METHODS

Chemicals and reagents

Caffeine, theophylline and theobromine were purchased from Eastman Kodak (Rochester, NY, U.S.A.). Paraxanthine and 3-methylxanthine were purchased from Adams Chemical (Round Lake, IL, U.S.A.). β-Hydroxyethyltheophylline (internal standard) was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and chloroform (UV grade) were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Phosphoric acid and 2-propanol were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). All reagents and chemicals were reagent grade or better.

Standard solutions

Donor human plasma was obtained from the University Hospital blood bank. Analysis of chromatograms obtained from extracts of blood bank plasma suggested that caffeine and caffeine metabolites were present. Consequently, four 25-ml quantities of plasma were placed in cellulose dialysis membranes (1.75 in. flat width, 24 Å pore size, VWR Scientific, Columbus, OH, U.S.A.) and suspended in 2 l isotonic phosphate buffer, pH 7.4, containing 2.5% activated charcoal. The buffer solution was stirred at 8°C for 40 h to remove any caffeine or caffeine metabolites from plasma. Volumes (10 ml) of plasma containing 0.5, 1.0, 2.0, 2.5, 5.0, 7.5 and 10.0 μg/ml caffeine were prepared. Aliquots of these plasma standards (0.5 ml) were stored in 1.5-ml polypropylene microcentrifuge tubes (VWR Scientific). An aqueous reference solution containing 33 μg/ml theophylline, theobromine, β-hydroxyethyl-theophylline and 20 μg/ml caffeine, and an aqueous internal standard solution containing 5 μg/ml β-hydroxyethyl-theophylline were prepared. Chromatographic resolution of caffeine, caffeine metabolites, and β-hydroxyethyl-theophylline was determined

daily by injecting 25 μl of the reference solution onto the column. All solutions were stored at −4°C.

Apparatus

Chromatography was performed with a Beckman Model 100A HPLC pump equipped with a Beckman Model 210 sample injection valve with a 50-μl sample loop capacity (Beckman, Berkeley, CA, U.S.A.). The mobile phase effluent was monitored with a Beckman Model 153 fixed-wavelength detector (254 nm) equipped with an 8-μl flow cell (Beckman). Specificity studies were performed with a Beckman Model 155-00 variable-wavelength detector equipped with a 20-μl capacity flow cell. A 5-μm particle size ODS column, 25 cm × 4.6 mm I.D. was used (Altex Scientific, Berkeley, CA, U.S.A.).

Extraction procedure

Volumes (0.1 ml) of serum, plasma, or saliva were placed in 16 × 125 mm PTFE-capped borosilicate culture tubes (Kimble, Vineland, NJ, U.S.A.) containing 100 μl 0.2 *N* hydrochloric acid and 75 μl internal standard solution. Twelve ml of 20% 2-propanol in chloroform were added and the mixture was shaken gently for 20 min. After centrifugation for 5 min the aqueous (top) layer was aspirated and the remaining organic layer was transferred to a clean disposable culture tube. The organic phase was evaporated at 50°C under a gentle stream of nitrogen. The residue was reconstituted with 100 μl mobile phase and 25 μl were injected onto the column.

Chromatography and standard curve

The mobile phase was prepared by diluting a mixture of 260 ml acetonitrile and 1 ml of 85% phosphoric acid to a final volume of 2 l with distilled water. The mobile phase flow-rate was 1.0 ml/min and the effluent was monitored at 254 nm with detector output recorded at 10 mV on a Beckman Model BD 40 chart recorder with the chart speed set at 0.25 cm/min. Peak heights of caffeine and internal standard were measured following the analysis of plasma standards and the ratio plotted against the respective plasma caffeine concentrations. Linear regression analysis was used to determine the slope and intercept values of the standard curve. Samples containing caffeine were compared with the standard curve using the caffeine:internal standard peak height ratio.

Recovery studies

Plasma was prepared as described above and spiked with caffeine to concentrations of 1.0 and 10.0 μg/ml. Volumes of plasma (0.1 ml) were extracted as described above with 12 ml 20% 2-propanol in chloroform and 6 ml of the organic phase were transferred to a clean disposable culture tube and evaporated to dryness. The residue was reconstituted with 100 μl mobile phase and 50 μl were injected onto the column. Peak heights of caffeine were measured and compared to those obtained following the injection of 25 μl of 1.0 and 10.0 μg/ml aqueous caffeine solutions.

Five coffee mixtures were prepared by dissolving 2-g quantities of instant coffee (Taster's Choice®, Nestlé, White Plains, NY, U.S.A.) in 200 ml water by gently heating to boiling. The mixtures were cooled to room temperature and

aliquots were diluted 1:100 and analyzed for caffeine as described above for plasma and serum. Recovery of caffeine from coffee was determined by spiking aliquots of each coffee mixture with an additional 100 μg/ml caffeine and then analyzing the resulting mixture to determine the caffeine content.

Precision

Replicate plasma specimens containing 2.0 and 10.0 μg/ml caffeine were analyzed each day for ten consecutive days to determine the day-to-day coefficient of variation of the assay. Ten plasma specimens containing 2.0 and 10.0 μg/ml caffeine were analyzed on the same day to assess the within-day coefficient of variation of the assay. These plasma specimens were stored at −4°C in glass vials between analyses.

Specificity studies

Four serum specimens were obtained from a human subject following intravenous caffeine administration. Samples were extracted and injected onto the column and the mobile phase effluent monitored at each of three wavelengths (254, 280 and 290 nm). Caffeine concentrations were determined from standard curves prepared following the analysis of plasma standards with effluent monitoring at the appropriate wavelengths.

Human study

A healthy, non-smoking volunteer received a single, intravenous dose of caffeine followed one month later by a single oral dose of coffee. The subject had refrained from ingesting any xanthine containing foods or beverages for at least one week prior to the studies. An intravenous infusion of caffeine benzoate (equivalent to 280 mg caffeine base, Eli Lilly, Indianapolis, IN, U.S.A.) was administered into an antecubital vein over 5 min with a sage syringe pump (Sage Instruments, Orion Research, Cambridge, MA, U.S.A.). Blood was obtained from a heparin-containing syringe placed in a vein in the contralateral arm. The oral dose consisted of 400 ml instant coffee (Taster's Choice) containing 160 mg caffeine (base). The dose was ingested over 10 min. Five ml blood and mixed saliva samples were obtained at various times over the subsequent 24-h periods. Caffeine concentrations in serum and saliva extracts were determined according to the present procedure. Blank serum for caffeine protein binding determination was dialyzed against an equal volume of phosphate buffer (pH 7.4) containing 2.0 and 10.0 μg/ml caffeine. Equilibrium dialysis was performed at 37°C in a plexiglass two-chambered apparatus of 1-ml capacity (Technilab Instrument Corp., Pequannock, NJ, U.S.A.) separated by a dialysis membrane (Spectrapor, Spectrum Medical Industries, Los Angeles, CA, U.S.A.). After 16 h of mild agitation, 0.1-ml volumes of serum and buffer were extracted for caffeine determination according to the present procedure.

Pharmacokinetics

Serum concentration—time data from the intravenous and oral doses were simultaneously fitted to a one-compartment open pharmacokinetic model with first order absorption following the oral dose [7]. A non-linear regression technique [8] was used to generate the least-squares computer estimates of the

elimination rate constant, K; the volume of distribution, V; the absorption rate constant, K_a; and the fraction of the oral dose absorbed, F. Areas under the total and free concentration—time curves were determined by trapezoidal integration with inclusion of the area from the last measured concentration to infinity. Clearance values were determined from the dose to area under the serum concentration—time curve quotient for free and total drug concentrations following the intravenous dose.

RESULTS

Column life was at least 3.5 months with almost daily use. Slight rises in pressure and loss of resolution were corrected by flushing the column overnight with a solution consisting of 500 ml methanol and 500 ml distilled water at a flow-rate of 0.2—0.3 ml/min. All of the dimethylxanthine metabolites of caffeine were well separated from caffeine and internal standard (Fig. 1). The retention times for caffeine, β-hydroxyethyl-theophylline, theophylline, paraxanthine, and theobromine were 8.8, 5.7, 5.2, and 4.0 min, respectively. Theophylline and paraxanthine elute with identical retention times on this chromatographic system. Caffeine and β-hydroxyethyl-theophylline are well separated from 3-methylxanthine which has a retention time of 2.4 min. Analysis of chromatograms from extracts of blood-bank plasma revealed peaks with retention times identical to those of theophylline, theobromine, and caffeine (Fig. 1). Serum extracts from coffee drinkers resulted in similar chromatograms. These peaks probably represent dietary xanthines and their

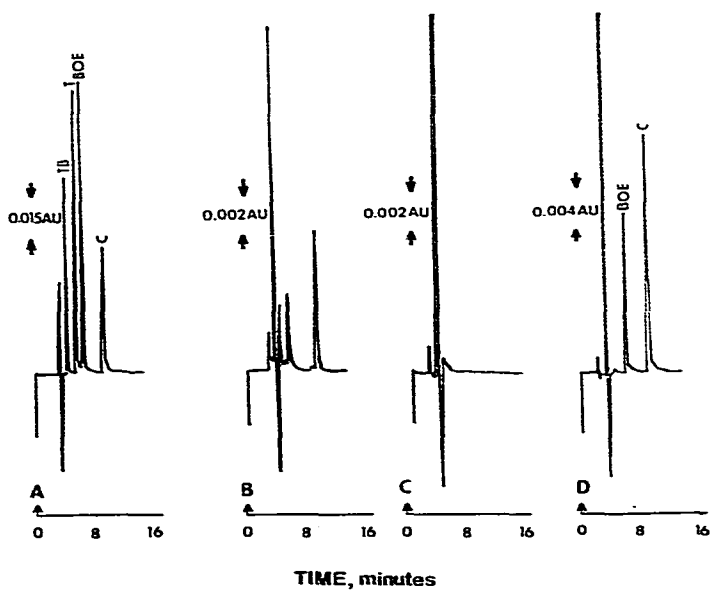

Fig. 1. Chromatograms of an aqueous standard mixture and donor plasma. (A) Standard mixture of the xanthines theobromine (TB), theophylline (T) and β-hydroxyethyl-theophylline (BOE) (0.825 μg), and caffeine (C) (0.5 μg); (B) extract of donor human plasma; (C) extract of stripped donor plasma; (D) extract of human plasma containing caffeine (C) (0.5 μg) with β-hydroxyethyl-theophylline (BOE) as the internal standard.

metabolites. Non-coffee drinkers had serum extracts which were free from any interfering substances. Dialysis of blood-bank plasma removed any interfering peaks (Fig. 1).

The caffeine:internal standard peak height ratios determined from the analysis of extracted samples were linearly related to caffeine concentration over the range of 0.5—50.0 μg/ml. The lower limit of detection for caffeine was 0.1 μg/ml allowing a signal-to-noise ratio of 4 and a sample volume of 0.1 ml.

The analytical recoveries of caffeine from five plasma specimens averaged 102 and 95% at plasma caffeine concentrations of 1.0 and 10.0 μg/ml, respectively. These were not significantly different using an unpaired *t*-test. Caffeine concentrations in five samples of instant coffee ranged from 238 to 258 μg/ml. The recovery of caffeine from spiked coffee averaged 92% for these five samples.

The day-to-day coefficients of variation of the assay were 6.8 and 6.6% at measured plasma caffeine concentrations of 1.98 and 10.1 μg/ml, respectively. The within-day precisions of the assay were 9.8 and 9.9% at measured plasma caffeine concentrations of 2.09 and 9.50 μg/ml, respectively. Serum specimens ranging in concentration from 0.6—5.5 μg/ml obtained following the intravenous dose were analyzed with mobile phase effluent monitoring at each of three different detection wavelengths. There was excellent agreement between the values obtained at 254, 280 and 290 nm (Table I). Linear regression analysis of concentrations determined at 290 and 280 nm (dependent variable) against concentrations determined at 254 nm (independent variable) resulted in slope and *y*-intercept values of 0.95 and 0.04 μg/ml, respectively. These values were not significantly different from unity (slope) and zero (intercept).

TABLE I

SERUM CAFFEINE CONCENTRATIONS (μg/ml) DETERMINED IN FOUR DIFFERENT SERUM SPECIMENS MEASURED WITH HPLC EFFLUENT MONITORING AT THREE DETECTION WAVELENGTHS

Similar concentration values at each detection wavelength indicate assay specificity.

254 nm	280 nm	290 nm
5.5	5.2	5.5
4.4	4.0	4.2
2.0	1.8	2.1
0.5	0.4	0.6

Fig. 2 shows the serum caffeine concentrations following the intravenous infusion and the oral dose. Serum caffeine concentrations continued to rise slightly for 1 h following the termination of the intravenous infusion due to some extravasation of the dose. The subject complained of some mild discomfort at the injection site at the end of the infusion. Serum caffeine concentrations declined in a log—linear fashion 1 h following the infusion. A peak serum caffeine concentration of 3.2 μg/ml occurred 1 h following the oral dose. There was an excellent correlation between observed and computer-predicted serum concentration—time values. The largest deviation between observed and computer-predicted values was only 13.6% and averaged 5.6% for all the experimental data. The fraction of free drug (unbound) in serum was 0.74 at total

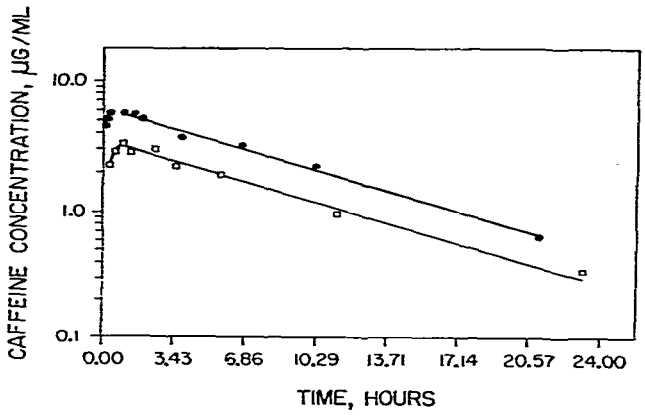

Fig. 2. Measured and computer predicted serum caffeine concentrations following an intravenous infusion of caffeine benzoate (●), and an oral dose of coffee (□).

(free and bound) post-equilibrium caffeine concentrations of 5.7 and 1.2 µg/ml.

The non-linear least-squares computer estimates of k_a, F, K and V were 4.21 h^{-1}, 0.93, 0.106 h^{-1} and 0.58 l/kg, respectively. Clearance of total (free and bound) and clearance of free (unbound) caffeine from serum was 1.0 ml/min/kg and 1.4 ml/min/kg, respectively.

There was considerable variability in the saliva caffeine concentration—time profile particularly after the oral dose. The saliva:unbound serum caffeine concentration ratios ranged from 0.93 to 1.46 and averaged 1.18 with a standard deviation of 0.16. Linear regression analysis of saliva (dependent variable) and unbound serum (independent variable) caffeine concentrations obtained from 1—24 h following the oral and intravenous doses gave a slope of 1.27 and a y-intercept value of −0.18 µg/ml with a correlation coefficient of 0.97 (n = 14).

DISCUSSION

The present procedure for the analysis of caffeine in biologic fluids and coffee is rapid, sensitive, and selective. The method employs a single extraction step suitable for detection of 0.1 µg/ml caffeine in 0.1 ml serum, plasma or saliva and can be used for caffeine determination in solutions of coffee. Previous gas—liquid chromatographic and HPLC methods for the analysis of caffeine in biologic fluids or beverages have been proposed [4,6,9—15]. This method offers some advantages over previously reported procedures. The small sample volume required and good sensitivity of the present method are suitable for pharmacologic studies requiring multiple blood samples in neonates and small laboratory animals. Other methods employ larger sample volumes (0.5—1.0 ml serum or plasma) to obtain similar sensitivity [4,6]. Some gas—liquid chromatographic procedures involve multiple extraction steps or lengthy derivatization steps for caffeine quantitation [12,13]. In addition, one HPLC procedure does not employ an internal standard making sample preparation, extraction, and sample reconstitution potential sources of variability [14]. Another HPLC method employs theobromine as an internal standard [15].

Theobromine is a dimethylxanthine metabolite of caffeine in dogs [14] and would be expected to interfere with the assay in this species. Furthermore, results from the present investigation indicate that theobromine is often present in human specimens due to dietary xanthine intake. The present procedure employs β-hydroxyethyl-theophylline as an internal standard. This compound is well resolved from caffeine and the dimethylxanthine metabolites of caffeine.

The results of the single-dose caffeine study agree well with the findings reported by other investigators. Desmond et al. [16] reported similar caffeine distribution volumes and slightly higher clearance values for caffeine compared to the values obtained in the present study, probably due to inclusion of smoking subjects in their study. Cigarette smoking can induce drug metabolising enzymes and as a result increase caffeine clearance [17].

Considerable variability was observed in the ratio of saliva to free (unbound) serum concentrations following the oral and intravenous doses. The saliva to unbound serum caffeine concentration ratio ranged 1.5-fold in the present study. Caffeine is a neutral compound and partitioning across biologic membranes should not be influenced by pH differences between blood and saliva. The drug is only 26% bound to plasma proteins and yet the saliva to unbound serum caffeine concentration ratio averaged 1.18. Active secretion of caffeine into saliva or non-specific binding of caffeine to the buccal mucosa would account for the high saliva to serum concentration ratio observed in the present study. The correlation between serum and saliva caffeine concentrations is much higher however than previously reported values in neonates [18]. Saliva caffeine concentrations obtained within 1 h of the oral dose were not included in the analysis because those values were several-fold higher than the corresponding serum concentrations, probably due to buccal adsorption of caffeine. A previous study of another xanthine compound, theophylline, has shown that drug concentrations in saliva are relatively higher than drug concentrations in serum 10—30 min following ingestion of a theophylline solution [19].

ACKNOWLEDGEMENTS

Sponsored by Grant GM-28424-01 from the National Institutes of General Medical Sciences, National Institutes of Health and a grant from the Central Ohio Heart Association.

REFERENCES

1 J.V. Aranda, W. Gorman, H. Bergsteinsson and T. Gunn, J. Pediatr., 90 (1977) 467.
2 T.F.X. Collins, J.J. Welsh, T.N. Black and E.V. Collins, J. Environ. Pathol. Toxicol., in press.
3 Fed. Reg., 45 (1980) 69817.
4 D. Robertson, J.C. Frolich, R.K. Carr, J.T. Watson, J.W. Hollifield, D.G. Shand and J.A. Oates, N. Engl. J. Med., 298 (1978) 181.
5 B.C. White, C.A. Lincoln, N.W. Pearce, R. Reeb and C. Vaida, Science, 209 (1980) 1547.
6 M. Bonati, D. Castelli, R. Latini and S. Garattini, J. Chromatogr., 164 (1979) 109.
7 M. Gibaldi and D. Perrier, Pharmacokinetics: Drugs and the Pharmaceutical Sciences, Marcel Dekker, New York, 1975, p. 33.

8 C.M. Metzler, G.L. Elfring and A.J. McWen, Biometrics, 30 (1974) 562.
9 C. van der Meer and R.E. Haas, J. Chromatogr., 182 (1980) 121.
10 J. van Duijn and G.H.D. van der Stegen, J. Chromatogr., 179 (1979) 199.
11 J. Blanchard, J.D. Mohammadi and K.A. Conrad, Clin. Chem., 26 (1980) 1351.
12 J.L. Brazier, J. Descotes, N. Lery, M. Ollagnier and J.-Cl. Evreux, Eur. J. Clin. Pharmacol., 17 (1980) 37.
13 E.E. Tyrala and W.E. Dodson, Arch. Dis. Child., 54 (1979) 787.
14 A. Aldridge and A.H. Niems, Biochem. Pharmacol., 29 (1980) 1909.
15 A.A. Tin, S.M. Somani, H.S. Bada and N.K. Khanna, J. Anal. Toxicol., 3 (1979) 26.
16 P.V. Desmond, R.V. Patwardhan, R.F. Johnson and S. Schenker, Dig. Dis. Sci., 25 (1980) 193.
17 W.D. Parsons and A.H. Niems, Clin. Pharmacol. Ther., 26 (1978) 40.
18 N.N. Khanna, H.S. Bada and S.J. Somani, J. Pediatr., 96 (1980) 494.
19 R. Koysooko, E.F. Ellis and G. Levy, Clin. Pharmacol. Ther., 15 (1974) 454.