Journal of Chromatography, 336 (1984) 293—300
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2303

IMPROVED MICRO-METHOD FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CAFFEINE AND PARAXANTHINE IN BIOLOGICAL FLUIDS

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(First received February 20th, 1984; revised manuscript received July 31st, 1984)

SUMMARY

A high-performance liquid chromatographic procedure is reported for reproducibly and sensitively quantitating caffeine and its N-demethylated metabolite paraxanthine in microsamples. A 5-μm reversed-phase radial compression column and 214-nm fixed wavelength ultraviolet detector were used to attain a sensitivity sufficient to quantitate these compounds at concentrations as low as 80 ng/ml using only 25 μl of sample. The assay is applicable to microliter samples of whole blood, serum, plasma, saliva, amniotic, cerebrospinal and gastric fluids such as might be obtained in studies involving small animals or neonates. The utility of the assay is illustrated with caffeine and paraxanthine levels measured in several maternal and fetal fluids following constant-rate intravenous infusion of caffeine into a rabbit throughout pregnancy.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is ubiquitous in the American diet. It is also utilized in the therapy of neonatal apnea and produced in clinically significant concentrations as a metabolite of theophylline (1,3-dimethylxanthine) which is similarly administered. Its pharmacological and toxicological effects are not fully understood and are of increasing interest to both the scientific and lay communities. While various high-performance liquid chromatographic (HPLC) assays for caffeine have been published [1—3], they generally (1) suffer from worse reproducibility than determinations of comparable concentrations of theophylline and paraxanthine (1,7-dimethylxanthine), its N-demethylated metabolites, (2) poorly resolve paraxanthine from theophylline and (3) provide inadequate sensitivity to accurately quantitate low levels

0378-4347/84/$03.00

of methylxanthines in microsamples such as might be required in studies involving neonates or small animals. Several published caffeine assays [2, 3] involve sample work-ups that fail to take into account the relative insolubility of caffeine during reconstitution steps, resulting in susceptibility to incomplete and undependable recovery. We report assay modifications that result in the dependable, sensitive and selective quantitation of caffeine and paraxanthine in microliter samples of biological fluids.

EXPERIMENTAL

Chemicals

Paraxanthine, caffeine, theophylline, 3-methylxanthine, 1,3-dimethyluric acid, uric acid and β-hydroxyethyltheophylline (Sigma, St. Louis, MO, U.S.A.), 1-methyluric acid (Adams, Round Lake, IL, U.S.A.), 1-methylxanthine (Vega Biochemicals, Tucson, AR, U.S.A.), theobromine (Merck, Rahway, NJ, U.S.A.) and 1-methyl[^{14}C]caffeine (4 mCi/mmol, ICN, Irvine, CA, U.S.A.) were all used as received. Buffer salts were HPLC grade and water was treated with a Milli-Q water purification system equipped with a 0.45-μm final filter (Millipore). Methylene chloride (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), methanol and tetrahydrofuran (Fisher Scientific) were HPLC grade and used without further purification. Tetrahydrofuran was stored tightly sealed under nitrogen.

Apparatus

The HPLC system consisted of a Varian Model 5020 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a WISP autoinjector, a Model 441 fixed 214-nm ultraviolet (UV) detector (Waters Assoc., Milford, MA, U.S.A.) and a 10-mV Varian Model 9176 recorder. Separations were achieved using a radial compression 5-μm C_{18} reversed-phase column protected with a C_{18} Corasil Bondapak® precolumn (Waters Assoc.).

Standard solutions

Stock solutions of caffeine, paraxanthine, theophylline (1 mg/l) and β-hydroxyethyltheophylline (internal standard, 5 mg/l) were prepared in water and stored refrigerated at 4°C. These solutions were found to be stable for at least one month. Working aqueous solutions were prepared from caffeine, paraxanthine and theophylline stocks at concentrations of 0.025—0.5 mg/l. Volumes of 100 μl of each working solution, when added to 25 μl of plasma, resulted in effective standard concentrations of 0.1—2.0 mg/l of plasma. Results for standards prepared in this manner were identical to those obtained for standards prepared directly in plasma. An aqueous working internal standard solution contained 0.25 mg/l.

Extraction procedure

To 200 μl of 0.2 *M* phosphate buffer (pH 6.0) in a 13 × 100 mm borosilicate glass tube were added 25 μl of plasma and 100 μl of internal standard solution. Methylene chloride (3 ml) was added and the samples shaken for 20 min (120 oscillations per min) and centrifuged (174 *g*). The lower phase was transferred

to a clean 12 × 75 mm silanized borosilicate glass tube and the solvent removed under a stream of nitrogen at 60° C. Water (230 μl) was then added. The sample was held at 90° C in a heating block for 6 min, vortexed and approx. 180 μl were injected onto the column for chromatographic analysis.

Chromatography

The mobile phase consisted of a ternary mixture of tetrahydrofuran—methanol—0.01 *M* potassium dihydrogen phosphate, pH 3.5 (1:9:90). The mobile phase flow-rate was 2.5 ml/min and the effluent was monitored at 214 nm with the recorder set at 0.2 cm/min. Column temperature was ambient (approx. 23° C) since the radial compression system used as not amenable to further control. Peak heights of the methylxanthines were measured and the drug/internal standard ratios compared to those of the calibration standards. Aqueous stock solutions of uric acid, 1-methyluric acid, 1,3-dimethyluric acid, 3-methylxanthine, 1-methylxanthine and 3,7-dimethylxanthine (theobromine) were injected to check for chromatographic interference and worked up according to the above procedure to determine extraction characteristics.

Recovery studies

Drug-free plasma was equilibrated with methylene chloride containing 1-methyl[^{14}C]caffeine (50 ng per 3 ml, 4 mCi/mmol) using the same conditions and phase ratios as described in the extraction procedure above. The organic phase (2.5 ml) was then evaporated to dryness and the tube in which the drying was performed was tared. The residue was redissolved in approx. 200 μl of water and incubated for 6 min at 90° C. The reconstituted sample was weighed at room temperature and the liquid was transferred to a tared scintillation vial. The vial was reweighed to permit correction for losses due to transfer, then the sample was counted in a liquid scintillation counter (Beckman, Model LS-100). The overall recovery of caffeine was calculated as the ratio of the [^{14}C]caffeine present in the entire reconstituted sample to that present in the aliquot of radiolabelled drug which was originally added to the methylene chloride extractant. Recovery from the dry-down procedure was also determined in an attempt to gauge the contribution of irreversible drug loss to glass surfaces. For these experiments the methylene chloride solution of radiolabelled caffeine was dried without extraction and the quantity of reconstituted caffeine compared to that which had originally been placed in the system. In all cases appropriate quench corrections were performed.

Precision

Plasma specimens containing caffeine at concentrations of 0.3 and 1.7 mg/l and paraxanthine at concentrations of 0.3 and 1.5 mg/l were analyzed on six separate occasions to determine the between-day coefficient of variation of the assay. Ten specimens at each of the same concentrations were analyzed on the same day using a common standard curve to elucidate within-day variability. All plasma specimens were stored at −20° C in polystyrene vials between analyses.

Animal study

A refillable infusion pump (Infusaid, Model 300) was implanted in the infrascapular region of the thorax of a female New Zealand white rabbit. The outlet catheter from the pump was passed subcutaneously to the neck region and inserted into the superior vena cava via the jugular vein. A caffeine benzoate solution (80 mg/ml caffeine + 80 mg/ml sodium benzoate in water) was then infused at a nominal caffeine infusion rate of 20 mg/kg/day. The pump had previously been shown to maintain its nominal rate of delivery within a precision of ± 5% over periods in excess of 100 days. Following a seven-day stabilization period the rabbit was bred, then sacrificed at 29 days gestation. Maternal plasma and cerebrospinal fluid were sampled as well as fetal plasma, cerebrospinal, amniotic and gastric fluids. Aliquots of 25 μl of each fluid were analyzed for caffeine and paraxanthine by the present procedure.

RESULTS AND DISCUSSION

The chromatographic resolution of paraxanthine from theophylline using the 5-μm Radial-Pak® C_{18} column with UV detection at 214 nm is shown in Fig. 1. For comparison, a chromatogram run on a 30-cm C_{18} steel column (μBondapak C_{18}, Waters Assoc.) under the same conditions is inset. It can be seen from Fig. 1 that baseline resolution is achieved using the Radial-Pak column. A four-fold increase in signal-to-noise ratio was realized by using a detection wavelength of 214 nm instead of 254 nm. Chromatographic parameters are compared in Table I and retention times of all compounds of interest relative to internal standard for the radial compression system are listed in Table II. None of the uric acid and methylxanthine derivatives tested interfered with peaks of interest and only di- and trimethylxanthines were extracted by our procedure. Peak height ratios of paraxanthine and caffeine to internal

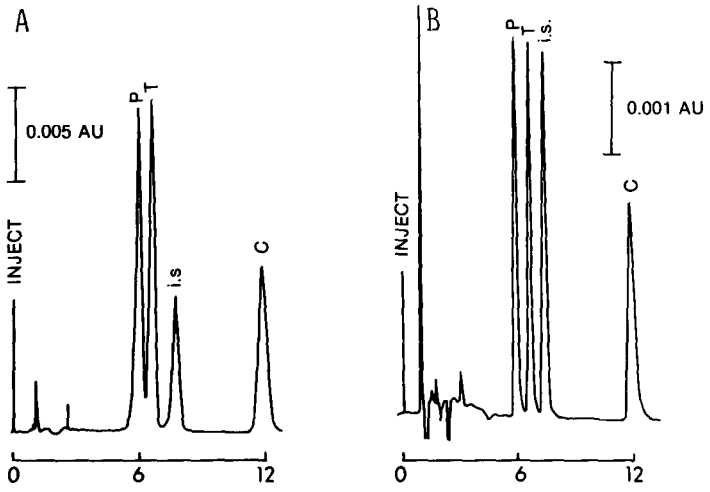

Fig. 1. Comparative resolution of paraxanthine from theophylline. Injected: 35 ng of each component; UV detection at 214 nm. (A) μ-Bondapak C_{18} (10 μm, 30 cm) column. (B) Radial-Pak C_{18} (5 μm) column. Peaks: P = paraxanthine; T = theophylline; C = caffeine; i.s. = internal standard = β-hydroxyethyltheophylline.

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR METHYLXANTHINE SEPARATIONS

Parameter*	μBondapak C_{18}	Radial-Pak
k'_p	4.08	5.78
k'_t	4.50	6.78
k'_β	5.17	7.67
k'_c	8.25	12.9
$R_{p,t}$	0.71	2.21
$\alpha_{p,t}$	1.07	1.68

*p = paraxanthine; t = theophylline; β = β-hydroxyethyltheophylline; c = caffeine; k' = number of column volumes required to elute compound; R = resolution = distance between two band centers/average band width; α = ratio of column volumes required to elute two compounds.

TABLE II

RETENTION CHARACTERISTICS OF VARIOUS XANTHINES USING THE RADIAL-PAK SYSTEM

Compound	Retention time (min)	Relative retention*	Extracted during work-up
Uric acid	1.4	0.197	No
1-Methyluric acid	2.5	0.352	No
3-Methylxanthine	2.7	0.380	No
1-Methylxanthine	3.2	0.451	No
1,3-Dimethyluric acid	3.5	0.493	No
3,7-Dimethylxanthine	3.8	0.535	Yes
1,7-Dimethylxanthine	5.8	0.817	Yes
1,3-Dimethylxanthine	6.4	0.901	Yes
β-Hydroxyethyltheophylline	7.1	1.000	Yes
1,3,7-Trimethylxanthine	11.8	1.660	Yes

*Relative to β-hydroxyethyltheophylline as internal standard.

standard were found to be linear over the concentration range 0.1—2.0 mg/l ($r^2 = 0.999$).

Examination of several published HPLC assays for caffeine and theophylline [1—3] and our own attempts to develop an assay of sufficient sensitivity to quantify methylxanthines in microsamples from fetal animal studies brought an unsettling characteristic to our attention. While the ionizable dimethylxanthines paraxanthine and theophylline were generally determined with very low coefficients of variation (less than 5%), the variability in caffeine assays was often considerably higher. This was observed even when sample extraction was not part of the assay procedure as in the direct injection procedure of Tse and Szeto [2]. These authors reported a coefficient of variation for caffeine that was 1—2% at concentrations in excess of 10 mg/l and increased systematically to 12% at 0.5 mg/l. Theobromine, theophylline and paraxanthine showed no such effect. We observed similar results and since the total mass of caffeine in our 25-μl samples was 10—100 times lower than that in the 500-μl samples of Tse and Szeto [2], we felt it was necessary to seek a means of improving on

published caffeine assays. We also observed that the caffeine concentrations we obtained using published techniques were not normally distributed. Outliers unpredictably occurred as if some important parameter in the recovery of caffeine were inadequately controlled.

Caffeine has very limited solubility in many solvents, presenting the possibility that the reconstitution of caffeine extracts following dry-down might have been inefficient and undependable. The use of mobile phase as a reconstitution solvent resulted in caffeine recoveries that were both incomplete (77% overall) and highly variable (14.8% coefficient of variation). The use of any of a number of organic solvents dramatically improved the recovery and reduced the variability (Table III). Hot water was also very effective and was selected because of its compatibility with the chromatography. We postulate that it works well because caffeine is about 30 times more soluble in aqueous systems at 90°C than at 25°C. Using this solvent only about 1% of the dried caffeine residue is lost to the silanized tube.

TABLE III

RECOVERY OF CAFFEINE AS A FUNCTION OF RECONSTITUTION SOLVENT

Solvent	Overall recovery (%)	Coefficient of variation (%)
Water (90°C)	97*	2.5
Tetrahydrofuran	93*	2.9
Ethyl acetate	90*	4.9
Mobile phase	77**	14.8

*Measured by liquid scintillation counting.
**Measured by high-performance liquid chromatography.

Adsorptive loss was also a contributing factor and silanization of the final dry-down tube was found to be essential for precise determination of caffeine concentrations. While the slopes and intercepts of calibration curves derived from standards analyzed with and without silanized tubes were not significantly different for either caffeine or paraxanthine, replicate analyses of unknown plasma samples showed significantly greater coefficients of variation for the determination of caffeine concentrations using untreated glassware. The variability of paraxanthine analyses were unaffected by either reconstitution solvent or dry-down surface and under all experimental conditions were 5% or less.

The combined use of silanized tubes, hot water reconstitution and 214-nm detection has resulted in a very reproducible and sensitive assay for caffeine and its major metabolite paraxanthine. A chromatogram from 25-μl sample of plasma containing 1.0 mg/l caffeine, theophylline and paraxanthine which was analyzed using this method is shown in Fig. 2. Standard curves are linear, independent of matrix for plasma, cerebrospinal, amniotic and gastric fluids and the assay can quantitate 2 ng of each compound (80 ng/ml in a 25-μl sample) with a signal-to-noise ratio greater than 4. Table IV lists within- and between-day coefficients of variation for caffeine and paraxanthine at concentrations near the upper and lower limits of the concentration range studied

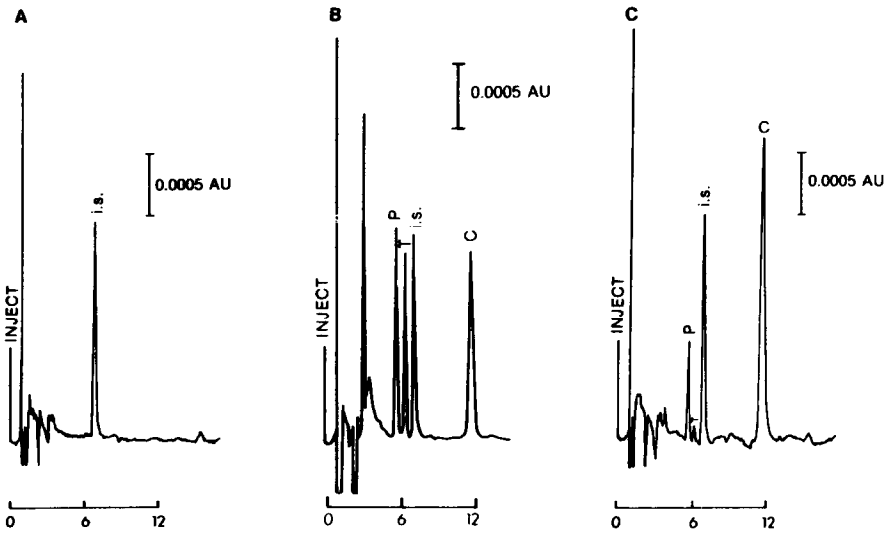

Fig. 2. Chromatograms of extracts of (A) blank rabbit plasma; (B) rabbit plasma spiked with paraxanthine (P), theophylline (T), β-hydroxyethyltheophylline (i.s.) and caffeine (C) all at 1.0 mg/l; (C) plasma from rabbit receiving caffeine (C = 1.72 mg/l, P = 0.48 mg/l).

TABLE IV

INTRA- AND INTER-DAY VARIABILITY IN THE ANALYSIS OF PARAXANTHINE AND CAFFEINE

	Concentration (mg/l)			
	Paraxanthine		Caffeine	
Actual	0.25	1.48	0.30	1.68
Calculated within-day (n = 10)				
Mean	0.25	1.43	0.31	1.65
S.D.*	0.01	0.03	0.02	0.09
C.V.**	5.2%	2.3%	4.8%	5.5%
Dev.***	+1.6%	−3.7%	+5.1%	−1.8%
Calculated between-day (n = 6)				
Mean	0.25	1.47	0.30	1.74
S.D.	0.01	0.03	0.01	0.06
C.V.	3.5%	2.1%	4.3%	3.3%
Dev.	+0.8%	−1.3%	+0.7%	+3.7%

*S.D. = standard deviation.
**C.V. = coefficient of variation (%).
***Dev. = percent deviation from actual.

(0.1—2.0 mg/l). They are about 5% or less in all instances. By comparison, one published assay [1] reports within-day coefficients of nearly 10% for 0.1-ml samples containing 200 and 100 ng of caffeine. We found a within-day coefficient of variation of 4.8% when we quantitated only 7.5 ng in a 0.025-ml sample. We have used a single column for over 1000 injections spanning months while maintaining good resolution and sensitivity.

TABLE V

MEAN FETAL FLUID/MATERNAL PLASMA CONCENTRATION RATIOS AT 29 DAYS GESTATION

Fetal fluid	Concentration ratio (mean ± S.D.)	
	Paraxanthine	Caffeine
Amniotic	0.68 ± 0.05*	0.89 ± 0.09
Gastric	0.70 ± 0.11	0.92 ± 0.11
Plasma	0.68 ± 0.07	0.84 ± 0.06
Cerebrospinal	0.62 ± 0.10	0.94 ± 0.06
	F = 1.06 ns**	F = 1.42 ns

*n = 6.

**ns = not significant.

Table V lists mean fetal fluid/maternal plasma concentration ratios for a variety of fetal fluids taken from a pregnant rabbit following caffeine infusion (20 mg/kg/day) throughout pregnancy. Samples were taken at 29 days gestation which is two days before normal delivery for these animals. There were no significant differences in either paraxanthine or caffeine fetal/maternal ratios among the various fluids although caffeine did exhibit higher ratios than its ionizable (pK_a = 8.7) and more polar metabolite.

The method described herein is well suited to performing drug disposition or pharmacokinetic studies in systems where sample volume is severely restricted. It is also of potential usefulness for the analysis of low levels of the methylxanthines in microliter samples of ultrafiltrate such as those obtained when protein binding is being characterized.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. John R. Raye for his assistance with the surgical aspects of this study. Supported in part by a grant from the Hood Foundation and by Grant No. HD16900 from the National Institutes of Health.

REFERENCES

1 D.B. Haughey, R. Greenberg, S.F. Schaal and J.J. Lima, J. Chromatogr., 229 (1982) 387.
2 F.L.S. Tse and D.W. Szeto, J. Chromatogr., 226 (1981) 231.
3 M. Bonati, D. Castelli, R. Latini and S. Garattini, J. Chromatogr., 164 (1979) 109.