ELSEVIER

Journal of Chromatography B, 707 (1998) 105–110

JOURNAL OF CHROMATOGRAPHY B

Simple high-performance liquid chromatography method for the simultaneous determination of serum caffeine and paraxanthine following rapid sample preparation

Diane T. Holland[a,*], Kristin A. Godfredsen[a], Theodore Page[b], James D. Connor[a]

[a]*Department of Pediatrics, 0808, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA*
[b]*Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093, USA*

Received 25 April 1997; received in revised form 21 October 1997; accepted 24 November 1997

Abstract

A simple reversed-phase high-performance liquid chromatography (HPLC) method for the simultaneous determination of caffeine and paraxanthine in human serum is described. Serum proteins are precipitated with perchloric acid and the resulting supernatant neutralized for direct injection onto an HPLC column. The method uses a phosphate–methanol mobile phase (85:15, v/v) at pH 4.9 with a flow-rate of 1.75 ml/min and quantitation is by UV absorbance at 274 nm. Elution times are approximately 18 min for caffeine and 8 min for paraxanthine. Theobromine and theophylline have elution times of 5.4 and 9.4 min and do not interfere in the assay. The intra-assay and between-assay means for precision and accuracy for both drugs are: 4.5% C.V. and 3.3% deviation. The sensitivity of the method is 50 ng/ml for each drug.

Keywords: Caffeine; Paraxanthine

1. Introduction

Caffeine (CA) and its metabolites are ubiquitous in human biological fluids due to dietary consumption of coffee, tea, cola drinks etc. The average cup of coffee contains approx 100 mg of caffeine and average coffee consumption is 2–3 cups/day, making caffeine one of the most commonly used of all stimulants. Liver metabolism of caffeine is primarily by demethylation and the three principal metabolites are paraxanthine (PX), theobromine (TB) and theophylline (TP). Studies have shown that 73–80% of CA is metabolized to PX and less than 10% is metabolized to theobromine and theophylline [1,2]. Recent studies show that, in man, PX has a pharmacologic activity similar to CA at doses of 4 mg/kg, although CA was more potent at 2 mg/kg [1]. Therefore, in studies designed to measure the clinical pharmacology or the toxic effects of CA, the activity of PX is also of interest, especially in habitual coffee drinkers.

The most common method used for the determination of serum caffeine and its metabolites is HPLC. The majority of these methods use solvent [3–9], or solid-phase [10], extraction for sample preparation prior to chromatographic separation and detection by either single wavelength UV [3–9] or photodiode array [10]. Solid-phase extraction requires a separate disposable column cartridge for every sample, which increases the cost of the analy-

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00590-2

sis. To process the columns reproducibly and productively it is essential to use a vacuum manifold. Both solvent and solid-phase sample extraction methods result in eluants which require evaporation to dryness and reconstitution in a suitable medium before injection onto the HPLC column. For an efficient throughput of samples this last step requires an evaporator with a stream of nitrogen or a vacuum centrifuge. These extraction procedures are not only time consuming and expensive but losses can occur at each step especially during solvent phase transfer and dry down. To monitor and correct for losses, an internal standard must be extracted with each sample and subsequently used to calculate sample concentrations.

We describe an alternative approach to sample preparation, which involves removal of serum proteins by acid precipitation followed by direct injection onto the HPLC column. We have used this method extensively for sample preparation prior to ganciclovir (DHPG) analysis by HPLC [13]. The method is simple, rapid and inexpensive. A similar method has been used prior to serum CA measurement by HPLC [11,12]. However, the HPLC method could not separate PX and TP and only CA was quantitated. Also our method is six times more sensitive, 50 ng/ml compared to 300 ng/ml.

Using protein precipitation has the added advantage that it does not require the use of an internal standard. The peak areas of external standards are used to plot a standard curve and sample concentration is calculated by reading directly off the standard curve. In this paper we describe validation and application of this method.

2. Experimental

2.1. Chemicals

HPLC grade water, acetonitrile and methanol, and reagent-grade phosphoric acid and sodium hydroxide (50% w/w) were obtained from Fisher Scientific (Fair Lawn, NY, USA). Reagent-grade perchloric acid was obtained from Aldrich (Milwaukee, WI, USA). Potassium hydroxide was purchased as a 50% w/v solution from LabChem (Pittsburgh, PA, USA). Bovine serum albumin (BSA) and normal human serum (NHS) were obtained from ICN (Costa Mesa, CA, USA). Caffeine, paraxanthine, theobromine, theophylline, acetaminophen, phenylbutazone, cortisol, prednisolone, prednisone, phenytoin and acetylsalicylic acid were obtained from Sigma (St. Louis, MO, USA).

2.2. Apparatus

The two-pump HPLC system (Model 110B), autosampler (Model 507E), and controller/integrator (System Gold) were obtained from Beckman Instruments (Fullerton, CA, USA). This system was used in conjunction with a Spectroflow 757 ultraviolet detector from Kratos (Foster City, CA, USA). The reversed-phase analytical column [Ultrasphere ODS, C_{18}, 250 mm×4.6 mm (5 μm particle size)] was also from Beckman. Reverse phase guard columns (Adsorbosphere ODS, 10×4.6 mm) were obtained from Alltech Associates (Deerfield, IL, USA).

2.3. Preparation of CA and PX standards and quality controls

The primary aqueous standards of caffeine and paraxanthine were prepared by dilution of a weighed sample of each drug to a defined volume of deionized water to give a concentration of 1 mg/ml.

An intermediate standard containing 100 μg/ml of both CA and PX was prepared by dilution of the primary standards in 5% bovine serum albumin (BSA). The working standards for assay calibration were prepared by serial dilution of the intermediate standard in 5% BSA, to give concentrations of 5.0, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 μg/ml. Unspiked 5% BSA was used as a blank. Aliquots of the working standards were stored frozen and one vial of each concentration thawed immediately before assay.

Quality control (QC) samples, containing both drugs, were prepared at concentrations of 0.1, 0.2, 0.5, 1.0 and 2.0 μg/ml in 5% BSA and stored exactly as standards.

2.4. Sample preparation

Patient samples were stored at −20°C prior to analysis and thawed, along with the standards and

controls, immediately before assay. Samples were deproteinized by mixing 250 μl of serum with 250 μl of 0.8 *M* perchloric acid. After vortex mixing, the proteins were removed by centrifugation at 14 000 *g* (room temperature) for 3–4 min. An aliquot of the supernatant (350 μl) was removed and mixed with approx 27 μl of 4 *M* sodium hydroxide, to bring the sample to around pH 5.0. This was done in a glass HPLC vial ready for direct injection onto the HPLC column.

2.5. High-performance liquid chromatography

Deproteinized sample (100 μl) was injected by autoinjector and eluted isocratically with the elution buffer [15 m*M* potassium phosphate (pH 4.9)–methanol (85:15, v/v)] for 20 min. The column was then flushed with acetonitrile:water (80:20, v/v) for 5 min and then reequilibrated with the elution buffer for 5 min. The flow-rate was constant at 1.75 ml/min and the temperature was ambient (range 21–24°C). Both column solvents were filtered through a 0.45 μm filter and degassed under vacuum before use. Eluted peaks were detected by ultraviolet absorbance at 274 nm and peak areas were used for quantitation using an eight-point standard curve. Standards were run at the beginning of each assay followed by quality control samples in duplicate. A single QC (high, middle or low value) was also run after every nine patient samples.

2.6. Validation

Method validation was based on the recommendations of Shah et al. [16]. The parameters included stability of the drug in normal human serum (NHS) after heating at 56°C for 30 min, after storage at room temperature for 4 nights, after storage at 4°C for four nights and after daily freezing and thawing for four days. Intra- (within-run) and inter- (between-day) assay accuracy and precision was calculated by measuring three or six aliquots of each of the QC samples (2.0, 1.0, 0.5, 0.2 and 0.1 μg/ml) in each of four separate assays on different days. Assay precision was expressed as % coefficient of variation (% C.V.) and assay accuracy by % deviation from the nominal value (% dev). Aliquots of a 0.05 μg/ml control sample were analyzed in quintuplicate on two different days to establish precision and accuracy at the limit of quantitation.

2.7. Specificity and potential drug interferences

Two other caffeine metabolites, theobromine (TB) and theophylline (TP) were tested in the assay along with the following list of prescribed drugs and drug supplements which were tested at physiologically relevant concentrations (1 μg/ml): acetaminophen, phenylbutazone, cortisol, prednisolone, prednisone, phenytoin and acetylsalicylic acid. To determine the range of endogenous concentrations of caffeine and paraxanthine, levels were measured, in duplicate, in nineteen serum samples from normal adults.

3. Results and discussion

3.1. Sample preparation

The sample preparation method using perchloric acid is fast (compared to solvent extraction), highly efficient at removing proteins (99% removal using 1:1 ratio of sample to acid) [13,14] and, because no extraction is involved, does not require the use of an internal standard. The resulting acidic supernatant requires only neutralization before direct-injection onto the HPLC column. Although 250 μl of serum sample was generally used, a sample volume of 100 μl gave comparable results. Samples prepared in a similar way have been used for measuring serum CA by HPLC (no metabolite quantitification) [11,12] and we have used the method extensively for ganciclovir (DHPG) [13].

3.2. Measurement of CA and PX and potential assay interferences

Standard curves and quality controls were prepared in 5% aqueous BSA because trace amounts of caffeine were detected in the batches of commercially prepared NHS which were tested (Fig. 1D). Other investigators have documented similar experiences [3,4,15] even in human subjects who had abstained from methylxanthines for 1 week [15]. Alternatives to the use of human serum for the preparation for standards and controls have included: sheep serum

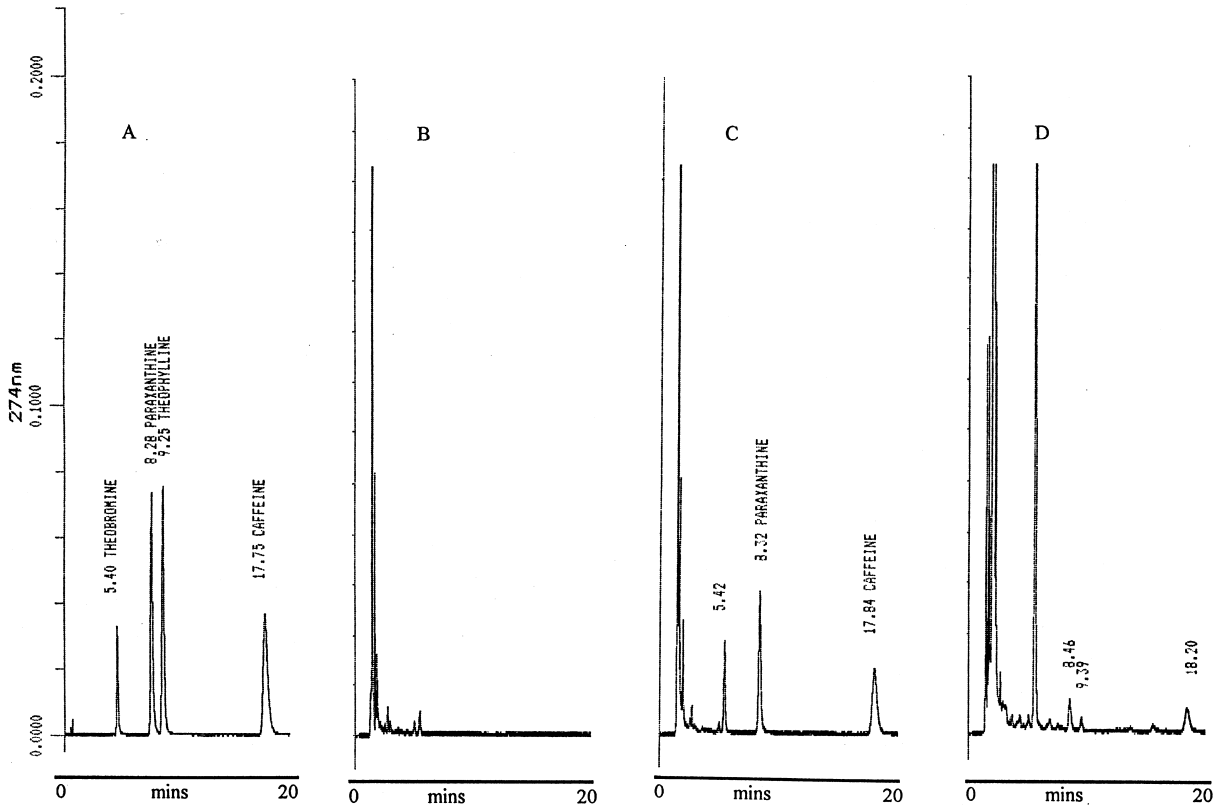

Fig. 1. Chromatograms for caffeine (CA) and paraxanthine (PX). (A) CA, PX, theobromine (TB) and theophylline (TP) in water, 5 μg/ml each. (B) 5% BSA unspiked. (C) 5% BSA spiked with 2 μg/ml amounts of TB, PX and CA. (D) Unspiked commercially available NHS.

[3], water [5] and BSA [15]. The choice of 5% BSA as a matrix was based on studies showing that caffeine was 35% protein bound, primarily, if not exclusively to albumin [11] and normal human levels of albumin are around 4.5% w/v. Furthermore, using BSA there were no interfering peaks in the expected positions of CA and PX and it has been shown that standard curves prepared in BSA resulted in slopes identical to using human plasma [15]. Spiked and non-spiked 5% BSA chromatograms are shown in Fig. 1B,C.

The coefficient of determination for standard curves was always >0.999 and back calculation of standards was within 10% of the nominal value for standards >0.1 μg/ml and within 20% for standards <0.1 μg/ml.

The chromatograms of caffeine (17.75 min) and paraxathine (8.40 min) in water, along with two other caffeine metabolites, theobromine (5.40 min) and theophylline (9.25 min), are illustrated in Fig. 1A. The retention times show very good separation and no interference from theobromine and theophylline.

At concentrations of 1 μg/ml the following drugs all showed blank chromatograms (i.e., retention times of >20 min): phenylbutazone, cortisol, prednisolone, prednisone, phenytoin and acetylsalicylic acid. Acetaminophen had a peak at a retention time of 5.7 min.

When the method was applied to sera from nineteen normal adults the range of CA measured was <0.05 μg/ml in three samples and 0.11–4.94 μg/ml in sixteen samples. The range of PX was <0.05 μg/ml in five samples and 0.06–0.67 μg/ml in fourteen samples. The samples were run in duplicate and the mean concentration for CA was 1.13 μg/ml (±1.25) and PX 0.29 μg/ml (±0.21). The results are shown in Fig. 2.

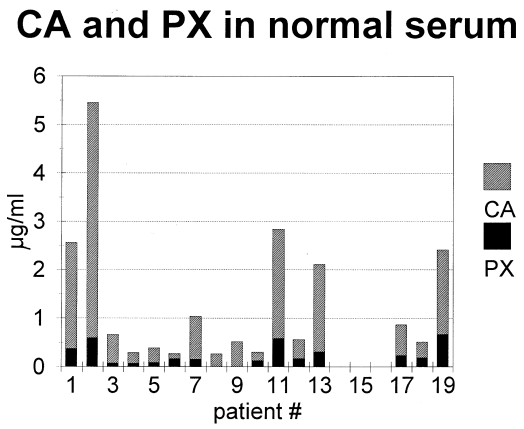

Fig. 2. Each value is a mean of two estimations.

3.3. Accuracy and precision

Intra- and between-assay accuracy and precision results for CA and PX in control samples are shown in Table 1. Precision ranges were 0.4–11.13% C.V. for CA and 1.06–10.6% C.V. for PX. Accuracy ranges are 0.45–10% and 1.3–5% deviation for CA and PX, respectively. These values are well within the acceptance criteria for an assay [16].

3.4. At the limit of quantitation (LOQ)

Precision and accuracy for both drugs at 0.05 μg/ml, which is the LOQ for the assay is shown in Table 2. The % C.V. for CA is 16.4 and PX is 19. The % deviation for both drugs is 8%.

3.5. Stability

There was no loss of either caffeine or paraxanthine when NHS was measured unspiked and spiked with 1 and 2 μg/ml of each drug, after being stored at room temperature and 4°C for four nights and after four freeze–thaw cycles over four days (Table 3). Samples heated at 56°C for 30 min also showed no loss of either drugs (data not shown).

Table 1
Precision and accuracy of caffeine and paraxanthine in assay controls

Concentration added (μg/ml)	Within-day (n=6)		Between-day (n=21)	
	mean (μg/ml)	C.V. (%)	mean (μg/ml)	C.V. (%)
Caffeine				
0.1	0.101	4.55	0.110	11.13
0.2	0.208	5.19	0.211	7.31
0.5	0.523	7.53	0.501	7.05
1.0	1.030	2.09	1.004	5.49
2.0	2.060	0.40	2.051	2.57
Paraxanthine				
0.1	0.105	8.41	0.102	9.19
0.2	0.205	4.68	0.213	10.60
0.5	0.507	2.13	0.506	3.95
1.0	1.030	1.90	1.025	2.64
2.0	2.070	1.06	2.068	1.97

Table 2
Precision and accuracy of caffeine and paraxanthine at the limit of quantitation

Concentration added (μg/ml)	Concentration found (mean) (μg/ml)	C.V. (%)	Dev (%)	n
Caffeine				
0.05	0.046	16.4	8	10
Paraxanthine				
0.05	0.054	19.0	8	10

Table 3
Caffeine and paraxanthine stability in NHS

Concentration added (μg/ml)	Concentration found (mean) (μg/ml)	C.V. (%)	*n*
Caffeine			
0	0.682	10.1	15
1	1.658	5.3	12
2	2.629	11.9	12
Paraxanthine			
0	0.454	21	15
1	1.333	7.5	12
2	2.229	12.4	12

Combined mean results from three separate experiments, i.e. stability at RT, at 4°C, and after freeze–thaw cycles (see text).

4. Conclusions

A simple reversed-phase HPLC method, with UV detection, for the simultaneous determination of caffeine and paraxanthine in human serum has been described. The method is accurate and precise over the concentration range 0.039–10.0 μg/ml for both drugs and specific, with no interferences from two other caffeine metabolites, theobromine and theophylline. Both CA and PX were shown to be stable to a wide range of storage and handling conditions likely to be experienced by serum samples obtained from patients during clinical studies.

Sample preparation using protein precipitation is simple, cheap and fast and does not require the addition of an internal standard. The method is routinely used in our laboratory [13]. Samples as small as 100 μl can be used. Quantitation is by use of external standards and the sensitivity is around 50 ng/ml for each drug. The method has been applied to nineteen serum samples and results range from undetectable to 4.94 μg/ml for CA and undetectable to 0.67 μg/ml for PX.

References

[1] N.L. Benowitz, P. Jacob III, H. Mayan, C. Denaro, Clin. Pharm. Ther. 58 (1995) 684.
[2] G.H. Kamimor, S.I. Lugo, D.M. Penetar, A.C. Chamberlain, G.E. Brunhart, A.E. Brunhart, N.D. Eddington, Int. J. Clin. Pharm. Ther. 32 (1995) 182.
[3] C.E. Cook, C.R. Tallent, E.W. Amerson, M.W. Myers, J.A. Kepler, G.F. Taylor, H.D. Christensen, J. Pharm. Exp. Ther. 199 (1976) 679.
[4] H.S. Bada, N.N. Khanna, S.M. Somani, A.A. Tin, J. Pediatr. 94 (1979) 993.
[5] C. Van Der Meer, R.E. Haas, J. Chromatogr. 182 (1980) 121.
[6] D.D.-S. Tang-Liu, R.L. Williams, S. Riegelman, Clin. Pharm. Ther. 31 (1982) 358.
[7] E. Tanaka, J. Chromatogr. 575 (1992) 311.
[8] P.J. McNamara, D. Burgio, S.D. Yoo, Drug Metab. Dispos. 20 (1992) 302.
[9] J.M. Wilkinson, I. Pollard, Dev. Brain Res. 75 (1993) 193.
[10] I.N. Papadoyannis, V.F. Samanidou, K.A. Georga, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 2559.
[11] J. Blanchard, J. Pharm. Sci. 71 (1982) 1415.
[12] J. Blanchard, S.J.A. Sawers, Eur. J. Clin. Pharmacol. 14 (1983) 93.
[13] T. Page, C. Sherwood, J.D. Connor, T. Tarnowski, J. Chromatogr. B. 675 (1996) 342.
[14] J. Blanchard, J. Chromatogr. 226 (1981) 455.
[15] C.P. Denero, C.R. Brown, M. Wilson, P. Jacob III, N.L. Benowitz, Clin. Pharmacol. Ther. 48 (1990) 277.
[16] V.P. Shar, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowell, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.