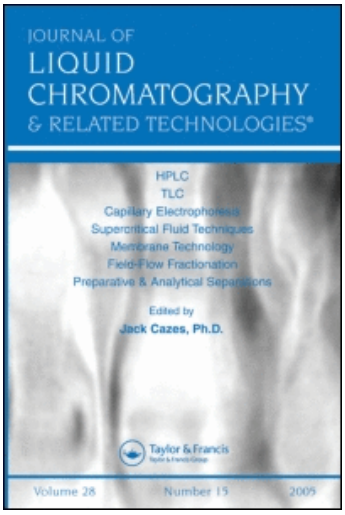

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Determination of Caffeine and Antipyrine in Plasma and Saliva using High-Performance Liquid Chromatography

G. M. De Roche[a]; B. S. Portz[a]; W. G. Rector[a]; G. T. Everson[a]
[a] Department of Medicine, University of Colorado Health Sciences Center, Colorado

To cite this Article De Roche, G. M. , Portz, B. S. , Rector, W. G. and Everson, G. T.(1990) 'Simultaneous Determination of Caffeine and Antipyrine in Plasma and Saliva using High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 17, 3493 — 3505
To link to this Article: DOI: 10.1080/01483919008049117
URL: http://dx.doi.org/10.1080/01483919008049117

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(17), 3493-3505 (1990)

SIMULTANEOUS DETERMINATION OF CAFFEINE AND ANTIPYRINE IN PLASMA AND SALIVA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

G. M. DE ROCHE, B. S. PORTZ,
W. G. RECTOR, AND G. T. EVERSON
Department of Medicine
University of Colorado Health Sciences Center
4200 E. 9th Avenue
Denver, Colorado 80262

ABSTRACT

A rapid and sensitive method of quantitation of caffeine and antipyrine in plasma and saliva is described. Caffeine, antipyrine and phenacetin, the internal standard, are readily extracted from alkalinized plasma and saliva into dichloromethane. After evaporation of the organic solvent, the residue is analyzed by HPLC using a mobile phase of 25% acetonitrile in 0.02 M phosphate buffer at a flow rate of 1.5 ml/min. and a C18 reverse phase column. Baseline separation of all peaks is achieved with retention times for all compounds of less than 10 minutes. There is no interference from endogenous compounds or metabolites of caffeine or antipyrine.

INTRODUCTION

Antipyrine (AP) is used as a model substance for the assessment of hepatic drug metabolism (1). The

clearances of both antipyrine and caffeine have been used to quantitate liver function in healthy controls and patients with a variety of liver disorders (2,3). High-performance liquid chromatography (HPLC) is the most frequently used analytical method for the measuring caffeine (3-8) and antipyrine (9,10) from body fluids or secretions. The goal of this study was to develop a rapid and quantitative method for the simultaneous analysis of caffeine and antipyrine from plasma and saliva. We also sought an alternative to caffeine-free plasma for construction of calibration curves. The use of 3% bovine serum albumin (BSA) for calibration purposes in methods for the determination of caffeine and its metabolites has been advocated by Hartley et.al. (4). We present evidence supporting the use of albumin but also show that water may be used a medium for calibration curves.

MATERIALS AND METHODS

Reagents

Dichloromethane, acetonitrile and methanol (all HPLC grade) were obtained from Burdick and Jackson labs. (Muskegon, MI.) Caffeine, antipyrine and phenacetin were supplied by Sigma. (St. Louis, MO.) Human serum albumin (HSA) was acquired from Alpha Therapeutics Corp. (Los Angeles, CA.) Sodium phosphate, mono and disbasic were obtained from Fischer Scientific. (Denver, CO.)

Instrumentation and Chromatographic Conditions

The liquid-chromatographic system (Waters Assoc., Milford, MA.) consisted of a M-501 pumping system, a Wisp 712 automatic sample injector and a M-484 tunable absorbance detector set at 254 nm. The chromatographic data were processed by a M-745 data module. The system was controlled for overnight runs by a wash and automatic system power down unit. (JANITOR, Jones Chromatography, Littleton, CO.) A 30 cm x 3.9 mm I.D. reverse-phase column (Waters Assoc. μbondapak C-18) was used with a precolumn module containing μbondapak C-18 insert (Waters Assoc. Guard-Pak). A mobile phase of 0.02 M sodium phosphate buffer, pH 6.5-acetonitrile (75:25, v/v) was used at a flow rate of 1.5 ml/min. at ambient temperature. The mobile phase was prepared fresh each day and was filtered and degassed prior to use.

Samples for determination of within- and between-day precision were prepared by adding known amounts of AP and caffeine to caffeine-free plasma, 3% HSA and water. Low and high concentrations were used for each compound. Caffeine was tested at 1 ug and 10 ug/ml and antipyrine at 2 ug and 15 ug/ml. These samples were then carried through the analytical procedure. Means, standard deviations and coefficients of variation were determined at each concentration. Extraction recoveries were evaluated by dividing the drug peak areas by the

area obtained by injecting equivalent amounts of unextracted standard solution.

Calibration Curves

Calibration curves were prepared by adding caffeine (1,3,5 and 10 ug/ml) and antipyrine (2,5,10 and 15 ug/ml) to caffeine-free plasma, 3% HSA and water. Evaluation of the calibration curves were obtained by comparing peak area ratios (AUC caffeine/AUC internal standard or AUC antipyrine/AUC internal standard) with actual concentrations of caffeine and antipyrine. These relationships were linear over the working range of 0-10 ug/ml for caffeine and 0-15 ug/ml for antipyrine. Calibration curves were run, in duplicate, with every batch of patient samples.

Extraction Procedure

The extraction procedure was a slight modification of that of Teunissen et.al. (9) and Danhof and colleagues (10). Briefly, to 500 ul of plasma (or saliva) was added 2 ug of phenacetin (in methanol) as the internal standard and the samples were then diluted to 1.0 ml with water. After alkalization (400 ul of 0.1 N sodium hydroxide) the samples were extracted with 6.0 ml of a fresh stock of dichloromethane (25 seconds on a vortex mixer at low speed). Following centrifugation (2200 rpm for 10 min.)

the aqueous layer was aspirated and the organic layer decanted into clean tubes and dried. The residue was dissolved in 400 ul of the mobile phase and 50-80 ul of this was injected into the HPLC system. One caution should be noted. The dichloromethane should be relatively "fresh". Bottles of dichloromethane which had been sitting of the shelf for more than 6-8 months tended to cause the antipyrine peak to split into two separate peaks. The reason for this is unclear, but occurred with different brands of dichloromethane and was independent of whether the bottles had been previously opened or were still sealed. Care should also be taken during the extraction to prevent the formation of large emulsions by using relatively low speeds on a vortex mixer.

RESULTS

The results obtained with saliva were comparable to those obtained with plasma, and only the plasma results are reported here. Fig. 1A, 1B and 1C illustrate chromatograms obtained following the injection of standards (3.0 ug caffeine, 5.0 ug antipyrine and 2.0 ug phenacetin) extracted from caffeine-free plasma, 3 % human serum albumin and water, respectively. All the peaks are comparable. Ratios of area under the curve std / area under the curve of internal standard were 0.565, 0.559 and 0.553 for caffeine in plasma, albumin and water

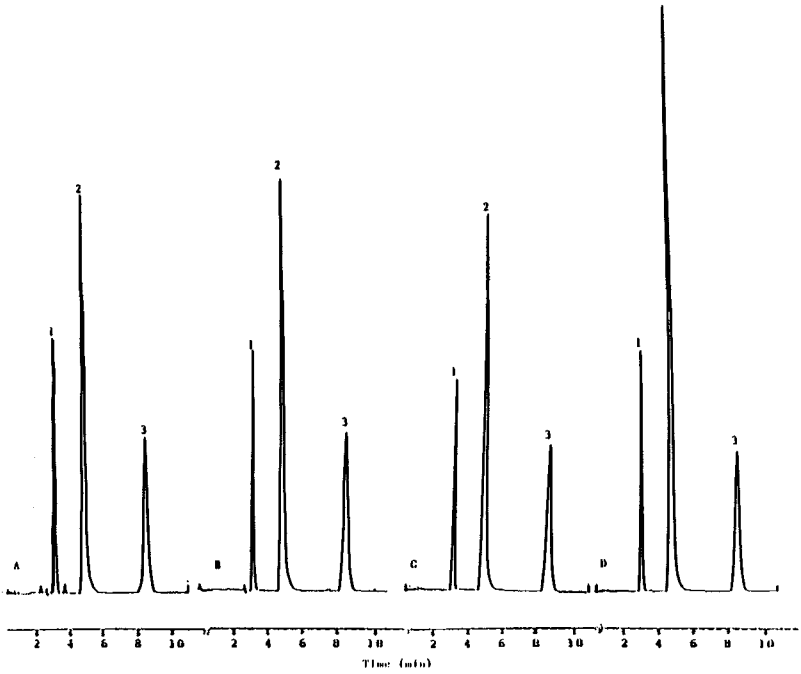

FIGURE 1 Chromatograms of identical amounts of authentic components extracted in (A) caffeine-free plasma, (B) 3% human serum albumin and (C) deionized water. Chromatogram (D) is that of a typical extracted patient plasma 4 hours following an oral dose of caffeine and antipyrine. Conditions as described in Material and Methods section.

Peaks: (1) caffeine; (2) antipyrine; (3) phenacetin

respectively while the ratios for antipyrine were 1.73, 1.75 and 1.7. Retention times were 2.97 for caffeine, 4.73 for antipyrine and 8.66 for phenacetin. Fig. 1D is a typical chromatogram of plasma from a patient 4 hours after receiving an oral dose of caffeine (300 mg) and

TABLE I

Comparison of the Between-day Precision of the Caffeine Assay of Samples Prepared in Caffeine-free Plasma, 3% Human Serum Albumin and Water.

	PLASMA		3% HSA		WATER	
ADDED CONC.	1 ug	10 ug	1 ug	10 ug	1 ug	10 ug
MEASURED MEAN (N=6)	1.05	9.8	1.05	9.9	1.03	9.9
SD	.068	.13	.045	.19	.045	.21
%CV	6.5	1.3	4.3	1.9	4.4	2.2

antipyrine (1.0 g). With the exception of two small residual peaks in the patient's fasting plasma, the chromatograms are free of interference from endogenous compounds.

The between-day precision of caffeine samples prepared in caffeine-free plasma, 3% HSA and water was similar (Table I) with coefficients of variation (C.V.) < 7%. Similar results were found for antipyrine samples in the three mediums (Table II) with coefficients of variation again <7%. Within-day coefficients of variation did not exceed 7% in any of the media and recoveries averaged 91% + 2% in all liquid phases.

TABLE II

Comparison of the Between-day Precision of the Antipyrine Assay of Samples Prepared in Caffeine-free Plasma, 3% Human Serum Albumin and Water.

	PLASMA		3% HSA		WATER	
ADDED CONC.	2 u	15 ug	2 ug	15 ug	2 ug	15 ug
MEASURED MEAN (n=6)	1.9	14.8	2.0	15.1	2.1	15.0
SD	.12	.42	.09	.42	.08	.49
%CV	6.3	2.8	4.2	2.8	4.4	3.3

TABLE III

Between-day Comparison of the Peak Area Ratios of Extracted Caffeine Standards in Caffeine-free Plasma, 3% Human Serum Albumin and Water.

Conc (ug/ml)	Plasma			3% HSA			Water		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
1	.191	.01	4.7	.201	.01	5.0	.188	.012	6.6
3	.533	.03	5.4	.545	.01	1.5	.540	.021	4.0
5	.941	.05	5.2	.935	.02	2.0	.95	.046	4.8
10	1.850	.05	2.8	1.830	.06	3.3	1.840	.043	2.3
	r = .999 slope = 0.184 int = 0.006			r = .999 slope = 0.182 int = 0.013			r = .999 slope = 0.184 int = 0.004		

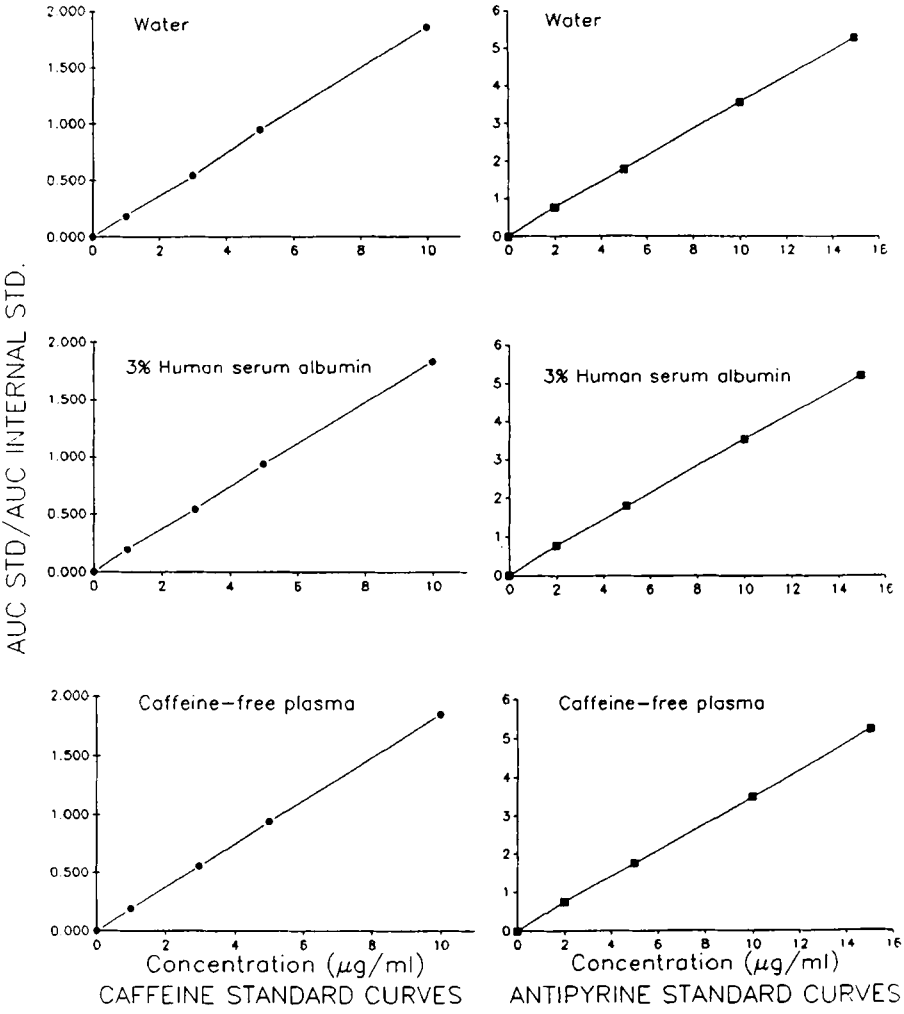

FIGURE 2. Calibration curves for caffeine and antipyrine from extracted samples of authentic components prepared in caffeine-free plasma, 3% human serum albumin and deionized water.

TABLE IV

Between-day Comparison of the Peak Area Ratios of Extracted Antipyrine Standards in Caffeine-free Plasma, 3% Human Serum Albumin and Water.

Conc ug/ml	Plasma			3% HSA			Water		
	Mean*	SD	%C	Mean	SD	%CV	Mean	SD	%CV
2	.765	.02	2.1	.767	.03	4.3	.770	.03	4.0
5	1.750	.03	1.9	1.780	.04	6.6	1.770	.04	4.3
10	3.490	.12	2.9	3.530	.19	5.4	3.570	.20	6.4
15	5.240	.30	6.3	5.210	.29	2.4	5.301	.23	4.3
	r = .999 slope = 0.345 int = 0.055			r = .999 slope = 0.343 int = 0.079			r = .999 slope = 0.350 int = 0.051		

The between-day comparisons of the peak area ratios for caffeine in the three mediums is shown in Table III while the actual calibration graphs are shown in Fig. 2. The curves are linear over the working range (0-10 ug caffeine/ml, sensitivity approximately 150 ng/ml) with almost identical slopes and correlation coefficients from the three different solutions. Similar results are shown in Table IV for antipyrine with calibration graphs appearing in Fig.2. The curves are linear over the working range (0-15 ug antipyrine/ml, sensitivity

approximately 100 ng/ml) and have nearly identical slopes and correlation coefficients with the different liquid phases.

DISCUSSION

The technique described provides an excellant tool for studies involving simultaneous analysis of antipyrine and caffeine in plasma and saliva. It is fast, accurate and well suited for automatic analysis. Samples can be prepared and allowed to stand overnight in the hood with no sample deterioration thus allowing for almost continous HPLC analysis of samples. The use of dichloromethane allows excellant recovery of the caffeine and antipyrine without the co-extraction of endogenous substances or metabolites of caffeine. We also tested solid-phase extraction (SPE) but as we found a negligible difference in recoveries between the two methods, more eluted interference compounds with SPE, especially in the caffeine peak area, and a considerable difference in the cost of the assay, we chose to stay with liquid/liquid extraction.

Our results in running calibration curves with 3% human serum albumin agree with those of others [4,11] who used 3% bovine serum albumin in place of the very difficult to obtain caffeine-free plasma. We choose the human albumin only because it was available from other

studies. We now, however, use the inexpensive and readily available deionized water as the medium for standard curves.

ACKNOWLEDGEMENTS

This investigation was supported by Research grant AA07832-03 from the National Institutes of Health, Public Health Service. Reprint requests should be addressed to Dr. W.G. Rector, Department of Medicine, Denver General Hospital, 777 Bannock Street, Box 4001, Denver, Co. 80204.

REFERENCES

1. Vessel, E.S., The Antipyrine Test in Clinical Pharmacology: Conceptions and Misconceptions, Clin. Pharm. Ther.,26, 275, 1979.

2. Renner, E., Wietholtz, H., Huguenin, P., Arnaud, M.J. and Preisig, R., Caffeine: A Model Compound For Measuring Liver Function, Hepatology, 4, 38, 1984.

3. Setchell, K.D.R., Welsh, M.B., Klooster, M.J. and Balistreri, W.F., Rapid High-Performance Liquid Chromatography Assay for Salivary and Serum Caffeine Following and Oral Load: An Indicator of Liver Function, J. Chromatogr-Biomed. Appl. 385, 267, 1987.

4. Hartley, R., Smith, I.J. and Cookman, J.R., Improved High-Performance Liquid Chromatographic Method for Simultaneous Determination of Caffeine and its N-demethylated Metabolites in Plasma Using Solid-Phase Extraction, J. Chromatogr-Biomed Appl. 342, 105, 1985.

5. Alkaysi, H.N., Sheikh Salem, M. and El-Sayed, Y.M., High Performance Liquid Chromatographic Analysis of Caffeine Concentrations in Plasma and Saliva, J. Clin. Pharm. Ther. 13, 109, 1988.

6. Scott, N.R., Chakraborty, J. and Marks, V., Determination of Caffeine, Theophylline and Theobromine in Serum and Saliva Using High-Performance Liquid Chromatography, Ann. Clin. Biochem., 21, 120, 1984.

7. Grant, D.M., Tang, B.K. and Kalow, W., Variability in Caffeine Metabolism, Clin. Pharmacol. Ther., 33, 591, 1983.

8. Biaggioni, I., Paul, S. and Robertson, D., A Simple Liquid-Chromatographic Method Applied to Determine Caffeine in Plasma and Tissues, Clin. Chem. 34, 2345, 1988.

9. Teunissen, M.W.E., Meerburg-Van der Torren, J.E., Vermeulen, N.P.E. and Breimer, D.D., Automated High-Performance Liquid Chromatographic Determination of Antipyrine and its Main Metabolites in Plasma, Saliva, and Urine, including 4,4-Dehydroxyantipyrine, J. Chromatogr-Biomed. Appl. 278, 367, 1983.

10. Danhof, M., DeGroot-Vas der Vis, E. and Breimer, D.D., Assay of Antipyrine and its Primary Metabolites in Plasma, Saliva and Urine by High-Performance Liquid Chromatography and Preliminary Results in Man, Pharmacol. 18, 210, 1978.

11. Wahllander, A., Renner, E. and Karlaganis, G., High-Performance Liquid Chromatographic Determination of Dimethylxanthine metabolites of Caffeine in Human Plasma, J. Chromatogr-Biomed. Appl. 338, 369, 1985.