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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PAPAVERINE IN PLASMA

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A simple and sensitive assay for papaverine in plasma was developed. Plasma was extracted into n-heptane and back extracted into 0.1 N HCl. An aliquot of 0.1 N HCl was injected onto a reversed-phase column. The mobile phase consisted of 33% acetonitrile in 0.05 M phosphate buffer, pH 3.0, at a flow rate of 1.6 ml/min. The retention times of papaverine and the internal standard (diphenylhydramine hydrochloride) were 4.0 and 7.0 minutes respectively. The coefficient of variation over 2-50 nanogram/ml range for spiked samples (n = 6) was 9.9% suitability of this method for plasma sample analysis from a bioavailability study was demonstrated.

INTRODUCTION

Few methods are available for the measurement of plasma papaverine levels. A turbidimetric method described by Elek *et al.*[1] is not specific for papaverine. A differential spectrophotometric method of Axelrod *et al.*[2] is not sensitive enough for bioavailability

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studies at low drug concentrations below 500 nanograms per ml. A gas-chromatographic method using ion pair extraction has been reported in the literature[3] but requires large sample volumes and numerous extractions and is therefore not practical if a large number of samples are generated by a bioavailability study. A very recent high pressure liquid chromatographic method was reported by Pierson et al.[4] which also requires ion pair formation with sodium heptane sulfonate in the mobile phase. Such a mobile phase system generally requires a long time to condition the column prior to analysis. While this report was being prepared, another method using liquid chromatography appeared in the literature[5]. The lower limit of detection was estimated to be 25 ng/ml and the extraction procedure requires evaporation of the organic phase which causes some error during analysis. This report describes a simple and sensitive high pressure liquid chromatography assay for papaverine with a maximum sensitivity for papaverine detection equal to 2 ng/ml of plasma.

EXPERIMENTAL

Apparatus

The modular high performance liquid chromatograph consisted of a constant flow pump (model 6000A, Water Associates); a valve type injector (sample injection valve, model U6K, Waters Associates); a fixed wavelength UV detector (254 nm., model 440 absorbance detector, Waters Assoc.); a potentiometric recorder, 0.5 cm/minute (Omniscribe, Houston Instrument, Austin, Texas). The column, 3.9 mm (i.d) x 30 cm. was obtained prepacked with reversed-phase material (μ Bondapak

C18, Waters Assoc.); a mechanical vortex mixer (SP deluxe mixer, S8220, Scientific Products, McGraw Park, Illinois); a centrifuge (IEC International centrifuge, Model CS, Int. Equipment Co., Needham Heights, Mass.); a timer (Time it PS, Precision Scientific Co.); 50 ml. centrifuge tubes (pyrex) fitted with glass stoppers were all used in the study.

Reagents

Papaverine hydrochloride (Sigma, let #1160-0063); diphenylhydramine hydrochloride (Parke-Davis); heptane AR (Mallinokrodt, lot #KHKZ); monobasic sodium phosphate (Mallinckrodt, let #WCTD); phosphoric acid (Allied Chemicals lot #W242J); acetonitrile (Fisher, HPLC grade, lot #7513242); methanol (Fisher, lot #796353); iso amyl alcohol AR (Fisher lot #795880); hydrochloric acid (Mallinokrodt, lot #WESD).

Standard Solutions

The following solutions were prepared: 2% iso amyl alcohol (v/v) was added to the normal heptane used for extraction. The 0.1 N hydrochloric acid used for back extraction was saturated with n-heptane. One liter of 5 N sodium hydroxide solution was prepared. Thirty three percent acetonitrile was added to the 0.05 M solution of potassium dihydrogen phosphate and the pH adjusted to 3.0 using phosphoric acid. The mobile phase was filtered (millipore filter) and degassed before use. The internal standard solution was prepared by dissolving 30 mg. of diphenylhydramine hydrochloride in 500 ml of distilled water. Appropriate concentration ranges of papaverine were prepared in plasma for each set of experimental samples and

these standards were extracted and analyzed concurrently with the unknowns.

Chromatographic Conditions

The mobile phase consisted of 33% acetonitrile in 0.05 M phosphate buffer, pH 3.0. A flow rate of 1.6 ml per minutes was established (1100) psi, and the column was equilibrated for 3 hours. The detector was attenuated from 0.02 to 0.005 aufs depending on the plasma concentration of papaverine.

Sample Preparation

One ml. serum aliquots were pipetted into 50 ml centrifuge tubes. Volumes of 100 µl of internal standard solution, 0.5 ml of 5 N sodium hydroxide and 25 ml of n-heptane, were added sequentially and vortexed on a mechanical shaker for exactly 5 minutes. After shaking, the samples were centrifuged to separate the layers, subsequently 20 ml of the organic layer was transferred to another 50 ml centrifuge tube containing 0.3 ml of 0.1 N hydrochloride. The tubes were vortexed again on a mechanical shaker for 5 minutes and centrifuged for 10 minutes to separate the layers. Ten to fifty µl of the aqueous layer was injected on the chromatograph.

Quantitation

The ratios of papaverine peak height to the internal standard peak height were calculated for each standard. Each of these ratios were plotted versus concentration and the resulting curves were used to convert peak height ratios of experimental samples to concentrations.

RESULTS AND DISCUSSION

High pressures liquid chromatography, in recent years has proven to be a fast, accurate and sensitive technique for determining drugs in biological fluids. A selective method for determining plasma papaverine levels at suitably low concentrations was desired. Beacuse of the large number of plasma samples expected in a planned bioavailability study, the time required for each analysis was critical. High pressure liquid chromatography was chosen because of its relative ease of operation.

Initial trials to develop the assay were conducted using a mobile phase containing 60% methanol in a 0.05 M phosphate buffer pH 7.4. The chromatograms showed very broad peaks with poor resolution for both papaverine and diethylstilbesterol which was used as an external standard. The system was not very efficient at lower concentrations of papaverine. Since papaverine is a weak base (pKa around 6.4), an acidic system which would allow the protonated species of the molecule to prevail was investigated. The peaks obtained using a mobile phase consisting of 33% acetonitrile in pH 3.0 buffer were well resolved and much sharper. Occasional adjustments of the acetonitrile concentration in the mobile phase between 31 to 34% was necessary due to changes in the column conditions to obtain optimal separation of the internal standard and the drug peaks.

Standard Curve

The relationships between peak height ratios and concentration in spiked plasma samples was linear ($r = 0.999$) over a range of 2

to 50 nonograms per ml. The lowest concentration measued was 1.0 nanogram per ml. of plasma (Table 1). A typical standard curve is shown in Figure 1.

Assay Reproducibility and Recovery Studies

Recovery studies were conducted using actual plasma samples. Known amounts of papaverine were added to blank plasma and extracted

TABLE 1

Assay Sensitivity

Serum Papaverine nanograms/ml	Peak Height Ratio[1] Papavereine/Int. Std.	C.V.
1.0	0.0356 (0.0181)	50.0
2.0	0.0857 (0.0067)	7.8
4.0	0.1880 (0.0169)	9.0
5.0	0.2488 (0.0106)	4.3
8.0	0.3735 (0.0153)	4.1

Linear Regression Parameters:

Number of Points	30
y-intercept	0.0493
Correlation Coefficient	0.992
R Square	0.983
Estimated Sensitivity, ng/ml[2]	2.0

1. Mean of six determinations of each concentration.

2. Sensitivity was defined as the concentration that yielded a coefficient of variation of 10% or less after replicate analysis.

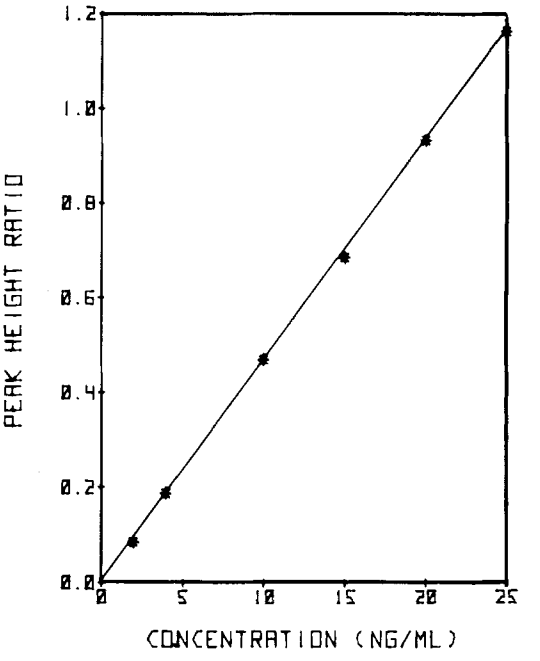

Figure 1. Typical standard curve obtained from spiked plasma samples.

as described above. Corresponding unextracted samples were prepared in 0.1 N hydrochloric acid, so that their concentration would be equal to 100% recovery from the extracted group. Peak height ratios obtained with extracted serum samples and corresponding unextracted standards are shown in Table 2. The table also indicates the reproducibility of the assay by giving means and coefficients of variations for amounts found versus amounts actually added. The results are based on six samples for each unknown concentration. Recovery, expressed as a percent, indicates the drug added to each original plasma sample over a range of concentrations is recovered in quantities between 98 and 107% (mean 101%). These results were

TABLE 2

Assay Reproducibility and Recovery Using Spiked Plasma

Amount Added ng/ml	Peak Height Ratio (a) drug/Int. Std.	Amount Recovered ng/ml	Percent Recovered	C.V.%
2.0	0.0857 (.007)	1.84	92	8.2
4.0	0.1880 (.017)	4.04	101	9.0
10.0	0.4700 (.019)	10.10	101	4.0
15.0	0.6850 (.017)	14.70	98	2.5
20.0	0.933 (.005)	20.10	100	0.5
25.0	1.165 (.014)	25.10	100	1.2

(a) Mean of six determination at each concentration.

TABLE 3

Assay Reproducibility and Recovery Using Spiked Water

Amount Added ng/ml	Peak Height Ratio (a) drug/Int. Std.	Amount Recovered ng/ml	Percent Recovered	C.V.%
2.0	0.0894 (.008)	1.92	96	8.9
4.0	0.1925 (.019)	4.14	103	9.9
10.0	0.4640 (.028)	9.98	100	5.0
15.0	0.6844 (.027)	14.72	98	3.9
20.0	0.9305 (.021)	20.01	100	2.3
25.0	1.164 (.016)	25.03	100	1.4

(a) Mean of six determinations at each concentration.

obtained using a standard curve from the assay of unextracted standards. The same reproducibility and recovery studies were conducted using spiked water samples and the results of this study are shown in Table 3. The mean coefficient of variation was 3.6% using spiked plasma samples and 4.8% using spiked water samples. There were no significant differences in the coefficients of variation of standards prepared in water and plasma.

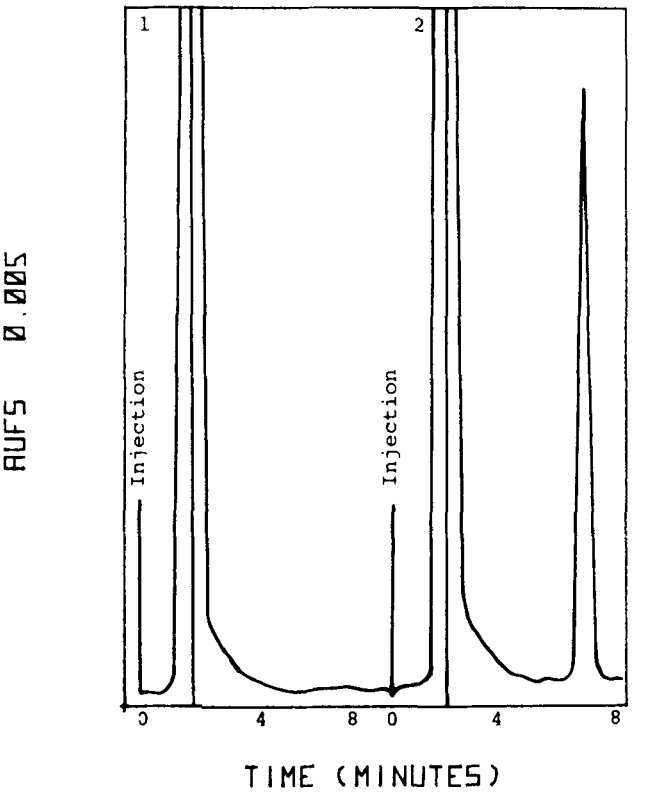

Figure 2. Chromatograms from extracted plasma samples.
Key: 1. Plasma blank
2. Plasma containing internal standard

Typical chromatograms obtained with blank human plasma, plasma to which papaverine (A) and the internal standard (B) have been added and plasma collected eight hours after administration of 5 mg/kg. body weight to a dog are shown in Figures 2-3. Chromatograms from water spiked with A and B, before and after extraction are shown in Figure 4. No endogenous materials interfering with the

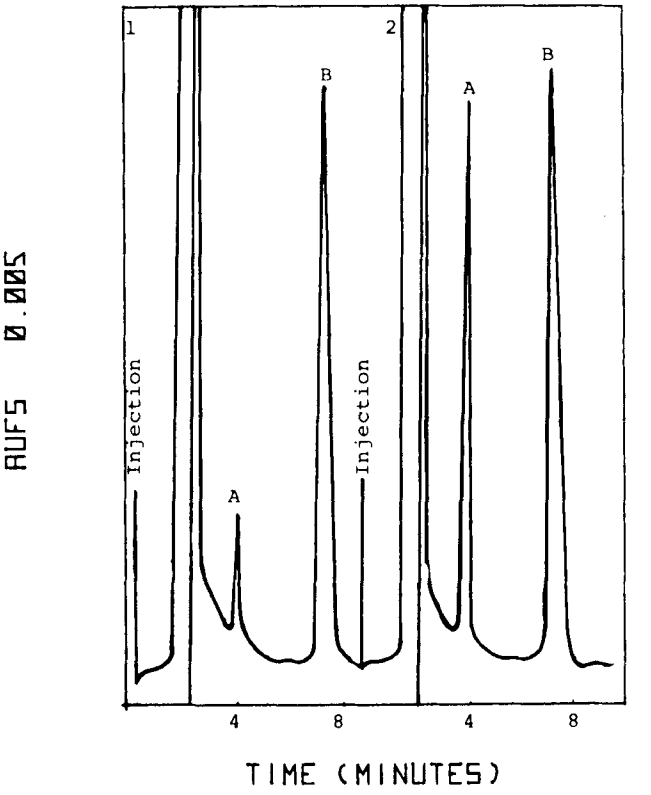

Figure 3. Chromatograms from extracted plasma samples.

Key: 1. Plasma papaverine standard at 4 ng/ml.
2. Dog plasma eight hours after oral administration of 110 mg of papaverine.
A. Papaverine
B. Internal standard

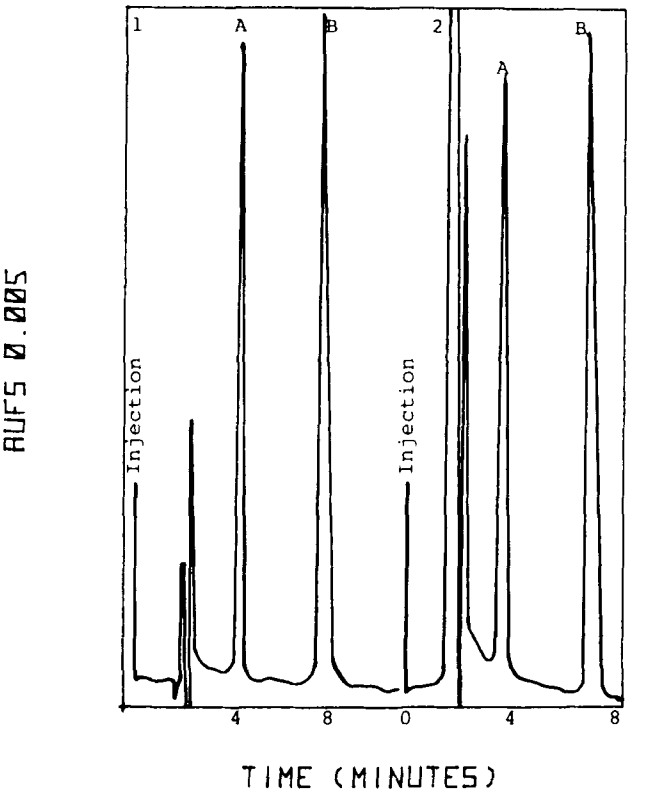

Figure 4. Chromatograms from spiked water samples.

Key: 1. Before extraction.
2. After extraction.

analysis of either component have been seen in the analysis of numerous control serum samples from dogs and humans. Well-resolved, sharp and symmetrical peaks were obtained for both papaverine and internal standard and eluted with retention times of 4.0 and 7.0 minutes respectively. The symmetrical peak shape, peak separation and low noise level may allow a further reduction in sample size which may be useful in small animal studies.

This assay procedure was found to be suitable for the detection of plasma papaverine upto 18 hours after administration of 5 mg of papaverine per kilogram body weight to a dog.

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