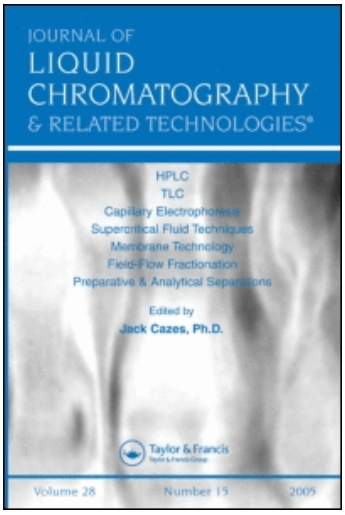

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N. H. Foda[a]; H. W. Jun[a]; J. W. McCall[ab]
[a] Department of Pharmaceutics, College of Pharmacy University of Georgia, Athens, Georgia [b] Department of Parasitology, College of Veterinary Medicine,

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QUANTITATION OF CYPROHEPTADINE IN PLASMA AND URINE BY HPLC

N. H. Foda, H. W. Jun[1], and J. W. McCall[2]
Department of Pharmaceutics
College of Pharmacy
University of Georgia
Athens, Georgia 30602

ABSTRACT

A sensitive, reliable and specific high performance liquid chromatographic procedure has been developed for the quantitation of cyproheptadine in plasma or urine. After extraction of the drug with ethyl acetate from alkalinized samples, the organic extract was evaporated to dryness, reconstituted with acetonitrile and chromatographed using a C_8 reversed-phase analytical column with UV detection at 254 nm. The average recoveries of cyproheptadine from spiked plasma and urine samples in the concentrations ranging from 0.2 - 3 mcg/ml were 95.7 and 100.3%, respectively and their respective CV was 4.1 and 3.9%. Regression analyses for the calibration plots for plasma and urine standards obtained on three different days for the drug concentrations between 0.2 - 3 mcg/ml

1 Communicating Author
2 Department of Parasitology, College of Veterinary Medicine

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indicated excellent linearity ($r > 0.999$) and reproducibility (CV < 2.0%, $p > 0.01$). The limit of sensitivity was 50 ng/ml for both plasma and urine samples. The method was applied to monitor the plasma concentration versus time profile of cyproheptadine following a single bolus IV dose of 1 mg/kg in a dog.

Urine samples taken from a human subject for the duration of 24 hours following a single oral dose of 8 mg showed that the cumulative amount excreted in urine as cyproheptadine was approximately 1% of the dose.

INTRODUCTION

Cyproheptadine is a serotonin and histamine antagonist with moderate anticholinergic and sedative effects. It is often used as an antiallergic and antipruritic agent (1). Cyproheptadine had been quantitated in rat tissues by GC with a sensitivity of around 1 mcg/g (2). Hucker and Hutt (3) developed a GC method with nitrogen detector for the determination of cyproheptadine in dog plasma and urine, and were able to detect as low as 3 ng/ml. Although an HPLC assay for cyproheptadine in pharmaceutical dosage forms with a sensitivity of 20 mcg/ml has been reported (4), no HPLC method for the quantitation of cyproheptadine in biological samples appears to be available. The proposed method is not as sensitive as the GC method (3), but it was adequate to monitor the plasma level vs. time profile of cyproheptadine following a single bolus IV dose of 1 mg/kg in a dog. The method was also applied to determine urinary excretion data following a single oral dose of 8 mg in a volunteer.

In view of its simplicity, reliability and convenience, the method should be useful for pharmacokinetic and bioavailability studies of cyproheptadine in man.

EXPERIMENTAL

Chemicals and Reagents - Cyproheptadine hydrochloride (5) and hydroxyzine hydrochloride (6) were used without further purification. Acetonitrile (7), ethyl acetate (7), methanol (7) and water were HPLC grade. All other chemicals were of U.S.P. of A.C.S. quality, and were used as received.

Instrumentation - The liquid chromatograph equipped with a single wavelength UV detector (8) at 254 nm and fixed volume injector (9) with a 20 µl sample loop was isocratically operated at ambient temperature. A stainless steel reversed-phase octyl column (15 cm x 4.6 mm i.d.) containing 5 µm ultrasphere packing (10) was used with a guard column. Chromatograms were recorded on a strip chart recorder (11) at a speed of 0.5 cm /min.

Mobile Phase - The eluting medium consisting of acetonitrile and 0.05 M acetate buffer, pH 3.5 (20% V/V) was prepared and degassed by bubbling helium gas for 5 min prior to use. Column equilibration with the eluting solvent was established by pumping (12) the mobile phase at a rate of 0.2 ml/min for overnight. The flow rate was set at 1.8 ml/min during analysis.

Stock Solutions - An appropriate quantity of cyproheptadine hydrochloride and hydroxyzine hydrochloride was accurately weighed and dissolved separately in a 50 ml volumetric flask to prepare a 1

mg/ml solution in methanol. The standard solutions of cyproheptadine in the concentrations between 0.2 and 3 mcg/ml and containing 50 mcg/ml of hydroxyzine hydrochloride as internal standard were prepared by diluting the appropriate quantities of stock solutions with the mobile phase. These solutions were used to compare the recovery of the drug from spiked plasma or urine samples.

Extraction Procedure - To one ml of dog plasma or human urine samples (dosed or spiked) in a 12 ml glass centrifuge tube were added 50 µl of the stock solution of internal standard and 0.1 ml of 3 N NaOH, and briefly vortexed. After addition of 3 ml of ethyl acetate the mixture was vortexed at high speed for 2 min and centrifuged at 3000 rpm for 5 min. The upper organic layer was collected and set aside. The aqueous layer was reextracted with 3 ml of ethyl acetate. Five ml of the combined organic layer was then dried under a nitrogen gas stream at the ambient temperature. The residue was reconstituted in 200 µl of the elution solvent. The sample was then shaken on a vortex mixer for 30 sec, and 20 µl of the resulting solution was injected into the column for analysis.

Calibration Plots - Reconstituted extracts of spiked plasma and urine standard samples were chromatographed, and calibration plots were obtained by plotting the peak height ratios (cyproheptadine/hydroxyzine) versus the concentration of the drug cyproheptadine. An aliquot (1.0 ml) of blank dog plasma or human urine in a 12 ml centrifuge tube was spiked with different amounts of the cyproheptadine stock solution to prepare seven standard solutions in

concentrations ranging 0.2 - 3 mcg/ml containing a fixed amount of the internal standard (50 mcg/ml).

Quantitation - After subjecting unknown plasma or urine samples to the described extraction and chromatographic procedures, the amount of cyproheptadine was determined by comparing the peak height ratios of cyproheptadine to hydroxyzine obtained from unknown samples with the calibration plots prepared from spiked standard samples.

Recovery Studies - The extraction efficiency of cyproheptadine from dog plasma or human urine was determined by comparing the peak height ratios of the drug to the internal standard obtained after direct injection of the solutions containing known quantities of cyproheptadine (0.2, 0.3, 0.5, 0.8, 1, 2 and 3 mcg/ml) dissolved in the mobile phase with those obtained after extracting the drug from spiked plasma or urine samples containing the equivalent amounts of the drug. At each of the seven cyproheptadine concentrations used five replicate samples were measured.

Animal Study - To demonstrate the applicability of the assay to the quantitation of cyproheptadine in plasma, a single bolus intravenous dose of 1 mg/kg in 2 ml of ethyl alcohol/water solution (50% V/V) was administered into the cephalic vein of a mongrel dog weighing 19 kg. Blood samples (5 ml) were withdrawn from the jugular vein using heparinized vacutainer tubes at the intervals of 0, 10, 20, 30, 45, 60 and 1½, 2, 2½, 4 1/3, 6, 8 and 11 hours post-dose. Blood specimens were immediately centrifuged and the plasma (2 ml) was stored in a screw-capped plastic vial at -4°C until assayed within a week.

Urinary Excretion In Man - The assay was applied for the measurement of urinary excretion profile of cyproheptadine in a healthy male subject (68 kg) after receiving a single oral dose of 8 mg in an empty stomach. Urine samples were collected prior to dose and at the intervals of 1, 2, 3, 5, 7, 9, 11, 14 and 24 hours post-dose. Two hundred ml of water was ingested by the subject every two hours during the day.

RESULTS AND DISCUSSION

Chromatograms obtained at the lower limit of sensitivity for drug free plasma or urine extracts showed no interfering peaks at the retention times of cyproheptadine and the internal standard, hydroxyzine. Figure 1 shows a typical chromatogram for the samples prepared from blank dog plasma (1-A), plasma spiked with 1 mcg/ml of cyproheptadine (1-B), and the sample containing the drug and hydroxyzine (1-C). Using the chromatographic conditions described, cyproheptadine and hydroxyzine were well separated and their retention times were 6.5 and 5.2 min, respectively. Both peaks were sharp and symmetrical with good baseline resolution and minimal tailing, which helped the accurate measurement of the peak height ratios. Hydroxyzine was an acceptable internal standard because of its adequate retention time and similar spectral properties to cyproheptadine. No interferences by the metabolites or normal constituents of plasma or urine were observed.

Standard plots obtained for plasma and urine samples were both highly linear in the concentration range of 0.2 - 3 mcg/ml. Linear

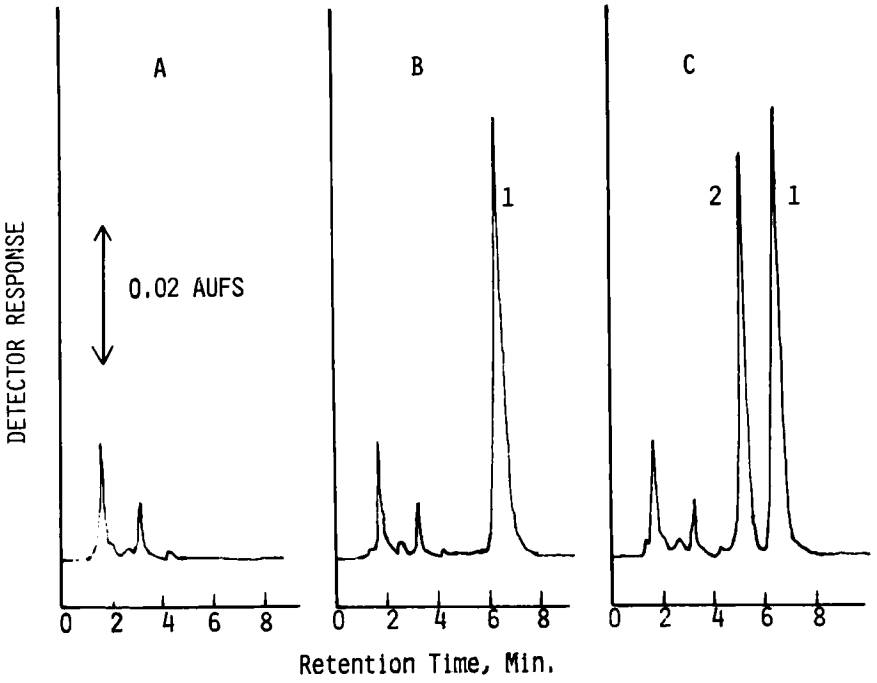

FIGURE 1. Chromatograms of blank dog plasma, A; dog plasma containing cyproheptadine (1 mcg/ml), B; and dog plasma extract containing cyproheptadine and the internal standard. Key: 1 - cyproheptadine, 2 - internal standard

regression analyses of the standard calibration plots for dog plasma and human urine samples were, respectively, $y = 0.566 x - 0.018$ and $y = 0.583 x - 0.011$, where y and x are the peak height ratio and cyproheptadine concentration, respectively. The small negative intercepts indicate that the blank plasma has negligible interferences for the drug. The correlation coefficients of both lines were higher than 0.999.

The day-to-day reproducibility of the assay for plasma or urine samples was evaluated by comparing the least-squares linear

regression analyses of the three standard plots obtained from spiked dog plasma or human urine standards at three different days over one week period. The results of this evaluation are summarized in Tables 1 and 2. The average correlation coefficient was higher than 0.999 and the coefficient of variation of the slopes of the three lines was less than 2%.

Table 1

Regression Analysis of Data for the Standard Plots of Cyproheptadine in Dog Plasma

Standard Plot[a]	Slope[b]	Intercept[b]	Correlation[b] Coefficient
1	0.566	-0.0178	0.9994
2	0.566	-0.020	0.9990
3	0.588	-0.024	0.9992

a - obtained in three different days
b - the mean of 5 determinations at each concentrations used

Table 2

Regression Analysis of Data for the Standard Plots of Cyproheptadine in Human Urine

Standard Plot[a]	Slope[b]	Intercept[b]	Correlation[b] Coefficient
1	0.572	0.017	0.9996
2	0.583	0.011	0.9997
3	0.600	0.006	0.9997

a - obtained in three different days
b - the mean of 5 determinations at each concentration used

Analysis of variance of the data showed no detectable difference in the slopes of the three standard plots ($F = 2.88$, $p > 0.01$) and ($F = 1.8$, $p > 0.01$) for plasma and urine samples, respectively. The results thus confirmed excellent linearity of the calibration lines and high reproducibility of the assay. With little variation in the slopes of the standard plots among multiple determinations, the method should be accurate and precise within the assay day as well as between assay days.

The recovery and precision of the assay were assessed by comparing the peak height ratios (cyproheptadine/hydroxyzine) obtained from spiked dog plasma samples of different cyproheptadine concentrations (0.2 - 3 mcg/ml) to the peak height ratios for the samples containing the equivalent amounts of the drug and internal standard directly dissolved in the mobile phase. Five replicate samples were assayed at each drug concentration and the results are shown in Table 3. The average recovery of the drug was 95.7% and its coefficient variation was 4.09%. The data indicated that the concentration of the drug in the samples between 0.2 and 3 mcg/ml had no detectable effect on recovery. Table 4 shows the standard calibration and recovery of cyproheptadine from spiked human urine samples between the concentrations of 0.2 - 3 mcg/ml. The average recovery of the drug was 100.3% and its coefficient of variation was 3.88%. The sensitivity of the assay defined as the minimum drug concentration corresponding to two times signal to noise ratio was found to be approximately 50 ng per ml for both plasma and urine samples. The method was applied to monitor the plasma level versus

Table 3

Standard Calibration and Recovery Data of Cyproheptadine from Spiked Dog Plasma

Cyproheptadine added, mcg/ml	Mean Peak Height Ratio	n[a]	Recovery mcg/ml	SD	CV[b] (%)	Recovery[c] (%)
0.2	0.126	5	0.198	0.01	5	99.0
0.3	0.152	5	0.298	0.013	4.4	99.3
0.5	0.266	5	0.463	0.018	3.79	92.6
0.8	0.418	5	0.718	0.037	5.0	87.8
1.0	0.52	5	0.928	0.029	3.1	92.8
2.0	1.116	5	2.030	0.073	3.6	101.5
3.0	1.69	5	2.90	0.105	3.5	96.6

a - Number of replicates
b - Average CV = 4.09%
c - Average recovery 95.7%
r = 0.99947
y = 0.566 x - 0.0178

Table 4
Standard Calibration and Recovery Data of Cyproheptadine from Spiked Urine Samples

Cyproheptadine added, mcg/ml	Mean Peak Height Ratio	n[a]	Recovery mcg/ml	SD	CV[b] (%)	Recovery[c] (%)
0.2	0.127	5	0.199	0.008	3.9	99.8
0.3	0.162	5	0.314	0.014	4.6	104.7
0.5	0.273	5	0.476	0.021	4.4	95.1
0.8	0.474	5	0.813	0.026	3.2	101.7
1	0.547	5	0.977	0.039	4.0	97.6
2	1.13	5	2.056	0.083	4.03	102.8
3	1.76	5	3.018	0.094	3.1	100.6

a - Number of replicates
b - Average CV = 3.88%
c - Average recovery 100.3
y = 0.583 x - 0.011

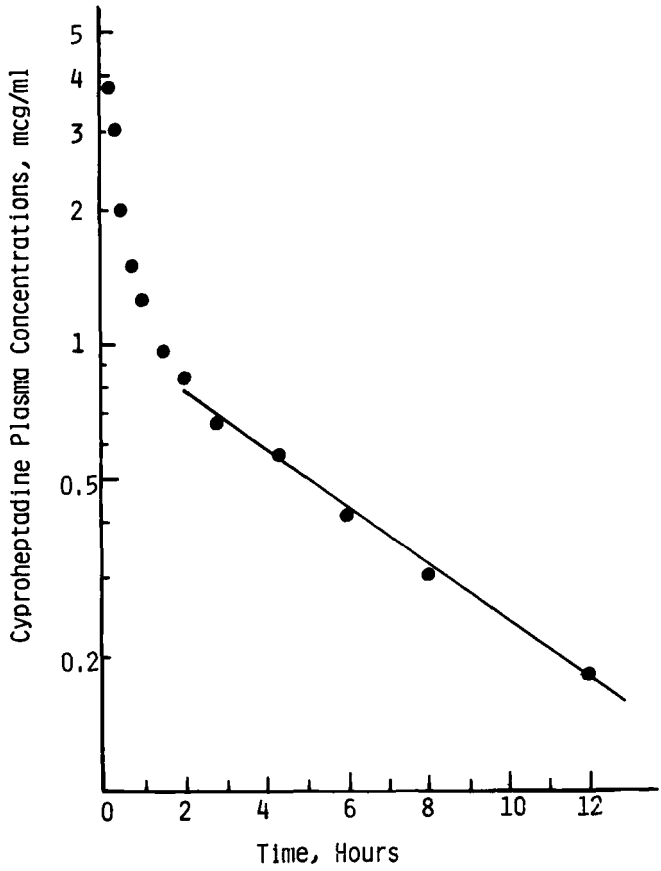

FIGURE 2. Plasma concentration vs. time profile in a dog after bolus IV dose of 1 mg/kg.

time profile of cyproheptadine in a dog after receiving a bolus I.V. dose of 1 mg/kg and the urinary excretion profile in a human subject who received a single oral dose of 8 mg.

According to Figure 2, the disposition of cyproheptadine in the dog follows the two-compartmental pharmacokinetic model as represented by:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

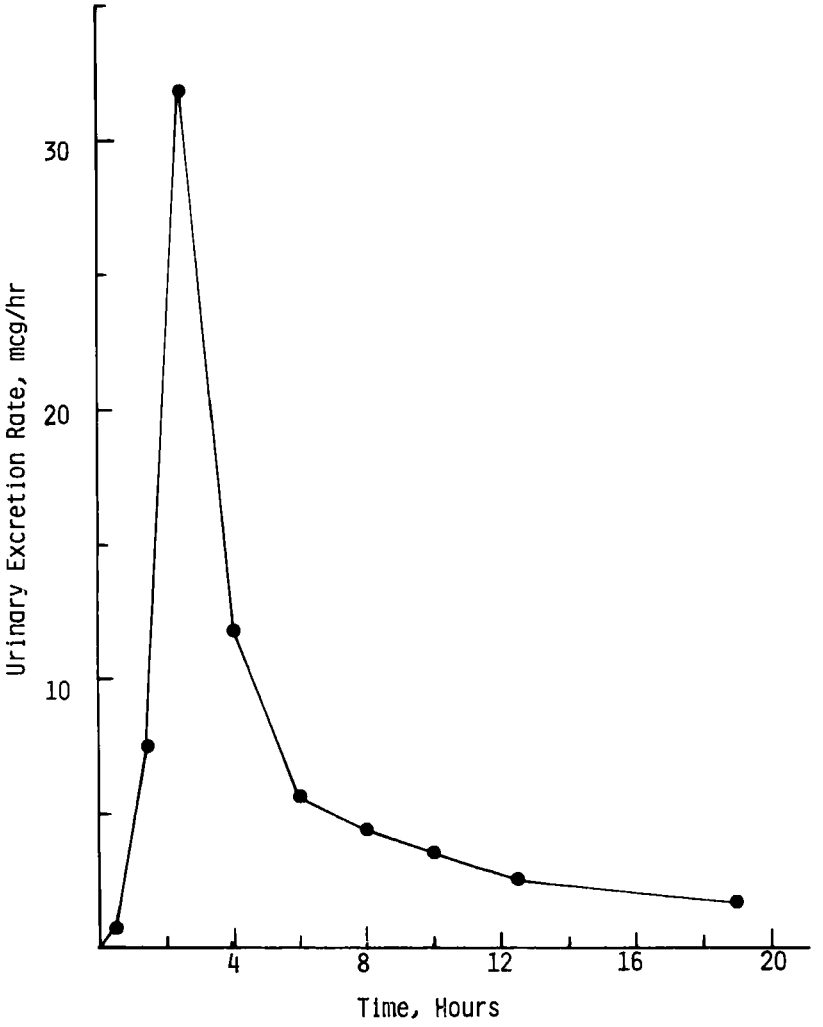

FIGURE 3. Urinary excretion rate vs. time profile of cyproheptadine in a man (68 kg) after a single oral dose of 8 mg.

where C is the plasma cyproheptadine concentration; A and B are the intercepts on the concentration axis; and α and β are the first order hybrid rate constants for the rapid and slow disposition phases, respectively. The apparent half-life for α phase was found to be around 20 min and the half-life for the terminal elimination phase was 4.5 hrs. From 0-7 hours post-dose, the plasma levels of cyproheptadine in the dog were approximately in the range 0.3 - 3.0 mcg/ml. The volume of distribution at steady state (Vd_{ss}) was approximately 17 liters, indicating that the drug was well distributed in the total body space.

Urinary excretion data in a male subject showed that the percentage of dose recovered in urine as cyproheptadine in the interval of 24 hours was approximately one % of the administered dose. Figure 3 shows the time course of urinary excretion rate of the drug in this subject.

In conclusion, the new reversed phase HPLC method developed in this study is simple, sensitive and specific, and is suitable for pharmacokinetic and bioavailability studies of cyproheptadine in man.

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