Sensitive High-Performance Liquid Chromatographic (HPLC) Determination of Diphenhydramine in Plasma Using Fluorescence Detection

Christine L. Webb[1,3] and Michael A. Eldon[2]

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INTRODUCTION

Diphenhydramine is a competitive histamine H_1 receptor antagonist with anticholinergic, antiemetic, and sedative properties. Previous spectrophotometric methods for analysis of diphenhydramine in biological fluids (1,2) lacked sufficient sensitivity and specificity for pharmacokinetic studies at therapeutic doses. More recently, diphenhydramine has been analyzed by various gas chromatographic methods, using flame ionization detection (3), nitrogen-phosphorus detection (4–6), and mass spectrometry (7). This report describes an HPLC method using fluorescence detection which has sensitivity (0.5 ng/ml), precision, and accuracy comparable to those of published gas chromatographic (GC) methods. The method has been applied to the analysis of diphenhydramine in human plasma samples in a clinical pharmacokinetic study (unpublished data) and in minipig plasma following intravenous administration of diphenhydramine (8).

MATERIALS AND METHODS

Reagents. Acetonitrile and water were HPLC grade. Ethyl ether, phosphoric acid, sulfuric acid, sodium hydroxide, and ammonium phosphate were reagent grade or better. Diphenhydramine hydrochloride (87.5% free base by weight) and orphenadrine hydrochloride (88.1% free base by weight) were USP reference standards. *N*-Desmethyl diphenhydramine was synthesized at Parke-Davis.

Standard Solutions. Stock solutions (100 μg free base/ml) of diphenhydramine and the internal standard, orphenadrine, were prepared daily in 0.005 *N* sulfuric acid. Aliquots of these solutions were diluted wit 0.005 *N* sulfuric acid to prepare stock solutions ranging from 5 ng/ml to 2 μg/ml of diphenhydramine and 250 ng/ml of internal standard. Aliquots (0.1 ml) of these stock solutions were added to plasma (1.0 ml) to make calibration standards ranging from 0.5 to 200 ng diphenhydramine per ml plasma. Diphenhydramine quality control standards of 1, 10, and 100 ng/ml were prepared in human plasma and stored frozen in 1.5-ml aliquots. Diphenhydramine quality-control standards at the same concentrations in minipig plasma were prepared and frozen in the same manner. A stock solution (100 μg free base/ml) of *N*-desmethyl diphenhydramine was prepared in 0.005 *N* sulfuric acid.

Extraction Procedure. All tubes used in the extraction were disposable glass with Teflon-lined screw caps. Plasma samples, quality-control standards (1 ml), and calibration standards were placed in 13 × 100-mm tubes. To each tube was added 0.1 ml of the internal standard solution, 0.1 ml of 6.25 *M* sodium hydroxide, and 3 ml of ethyl ether. After shaking for 15 min on a horizontal shaker, samples were centrifuged for 5 min to separate the phases. The lower aqueous layer was frozen in a dry ice/acetone bath and the ether layer transferred with a Pasteur pipette to a 15-ml tapered centrifuge tube. A 0.2-ml aliquot of 0.5% phosphoric acid was added, and the mixture shaken for 10 min and centrifuged for 5 min. The phosphoric acid layer was frozen in the dry ice/acetone bath, and the ether layer was discarded. Traces of ether were removed with a nitrogen flow for 5 min at room temperature. Thawed phosphoric acid extracts were transferred to injection vials and 0.1 ml was injected.

Chromatographic System. The HPLC system consisted of a Hewlett-Packard 1090A liquid chromatograph and a Perkin-Elmer LS-4 fluorescence detector. Excitation wavelength was 230 nm (slit width, 15 nm) and emission wavelength was 560 nm (slit width, 10 nm). A Waters μBondapak C18 column, 250 mm × 2.0-mm I.D., was used with a guard column, 20 mm × 2.0-mm I.D., hand packed with Waters μBondapak C18. Mobile phase was presaturated by passage through an identical precolumn, 20 mm × 2.0-mm I.D., hand packed with Waters μBondapak C18. The mobile phase consisted of 28% acetonitrile and 72% phosphate buffer (75 m*M* monobasic ammonium phosphate, adjusted to pH 2.6 with phosphoric acid), degassed by constant helium sparging. The flow rate was 0.3 ml/min, and the column temperature was 40°C.

Calculations. The method was calibrated for each run by regressing diphenhydramine/IS peak height ratios against diphenhydramine concentration in the calibration standards. A straight line estimated using linear regression with a weighting factor of 1/concentration^2 was found best to represent the data. Diphenhydramine concentration in quality controls and unknown samples was calculated using the regression equation.

Validation of the method was performed by assaying triplicate sets of calibration and quality-control standards on 3 separate days. The calibration standards from each day were fit with a straight line as described above. The daily calibration curves were used to calculate the concentration of diphenhydramine in standards and controls, and these data were pooled across experimental days to evaluate precision and accuracy. The method was validated in human plasma and in minipig plasma, on two separate occasions.

[1] Pharmacokinetics/Drug Metabolism, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company; Ann Arbor, Michigan 48105.
[2] Clinical Pharmacology Departments, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105.
[3] To whom correspondence should be addressed at Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, Michigan 48105.

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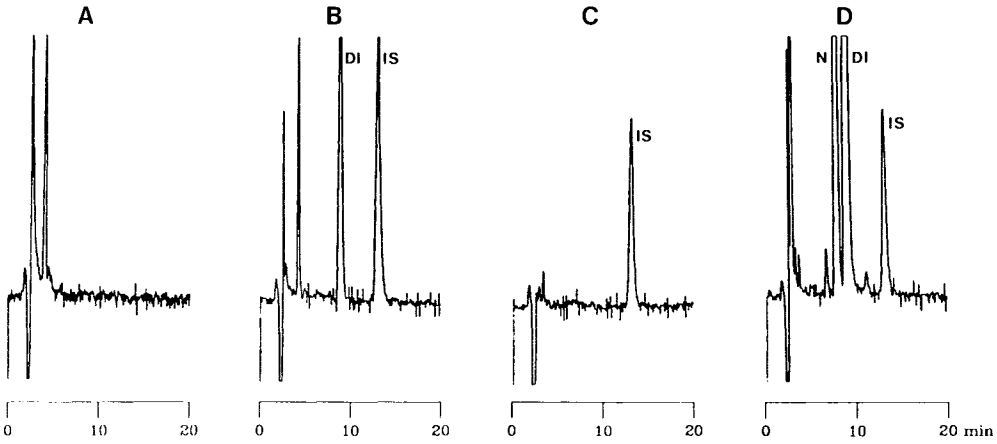

Fig. 1. Chromatograms of diphenhydramine in human plasma: (A) blank plasma, (B) 5 ng/ml calibration standard, (C) predose sample from a pharmacokinetic study (with internal standard), and (D) sample taken 3 hr after a 100-mg oral capsule dose (measured concentration = 97 ng/ml). DI, diphenhydramine; IS, internal standard; N, *N*-desmethyl metabolite.

Extraction recovery of diphenhydramine and internal standard was determined by spiking human plasma to contain 1, 10, and 100 ng/ml diphenhydramine, or 2.5 ng/ml internal standard, and extracting as described above. Peak heights from extracted samples (n = 6) were compared to peak heights from injection of the standard solutions used to prepare the samples. Recovery from minipig plasma was not determined.

Specificity. The major basic metabolite of diphenhydramine, *N*-desmethyl diphenhydramine, was used to test the specificity of the method. Standard solutions (in 0.005 *N* sulfuric acid) of the metabolite alone, and metabolite plus parent drug, were injected onto the column to verify that the compounds were resolved by the chromatographic system. The metabolite was also added to blank human plasma, with and without parent drug, and these samples were extracted following the above procedure.

RESULTS AND DISCUSSION

The extraction procedure and chromatographic method were adapted from a published method for the analysis of chlorpheniramine in plasma (9). The method was initially applied to diphenhydramine using ultraviolet (UV) absorbance detection at 210 nm. Sensitivity of the UV-detection method was increased by using a 2-ml plasma sample and by use of a 2.0-mm-I.D. column, which results in a more concentrated peak due to a smaller elution volume (10). Using this method, diphenhydramine could be measured in plasma at concentrations as lows as 1 ng/ml. However, within-day variability for the UV-detection method was unacceptably high for plasma standards with diphenhydramine concentration less than 10 ng/ml. Fluorescence detection was investigated as a means of improving on these results.

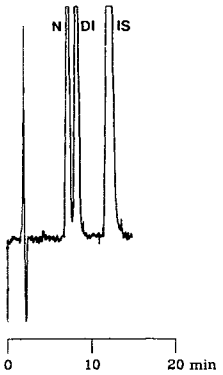

Fig. 2. Chromatographic separation of diphenhydramine (DI), internal standard (IS), and *N*-desmethyl diphenhydramine (N); five nanograms each, in 100 μl of 0.005 *N* sulfuric acid. Integrator chart set at 16-mV full scale.

The fluorescence properties of diphenhydramine were determined empirically. A scan of the UV absorbance of diphenhydramine in mobile phase, from 190 to 360 nm, showed essentially no absorbance above 230 nm. Below 230 nm, absorbance increased as wavelength decreased. With excitation set at 210 nm, the lowest wavelength possible on the detector, the fluorescence emission of diphenhydramine in mobile phase was scanned from 240 to 800 nm. Two emission peaks, of comparable magnitude, were observed at 280 and 560 nm. A similar detector response was observed for the internal standard in mobile phase. We chose to work with the emission at 560 nm because there was less baseline noise at the higher wavelength.

Since 210 nm is at the extreme low end of the detector's capabilities, the method was validated with excitation wavelength at 230 nm, using the widest available slit width (15 nm). These conditions gave a more stable baseline than excitation at 210 nm, with no noticeable decrease in detector response.

To date, only two commercial HPLC fluorescence detectors have proven useful for this assay method. They are the Perkin–Elmer Model LS-4 and the Hewlett–Packard Model 1046 fluorescence detectors. Both detectors are dual monochromator systems, with the Perkin–Elmer Model LS-4 having a deuterium lamp, whereas the Hewlett–Packard Model 1046 used a pulsed-xenon source. The method has been validated as described herein using both detectors. Our early attempts at using various filter-based

Table I. Precision and Accuracy Results from Validation in Human and Minipig Plasma

Human plasma				Minipig plasma			
Diphenhydramine concentration (ng/ml)		Percentage relative error[b]	% RSD[c]	Diphenhydramine concentration (ng/ml)		Percentage relative error[b]	% RSD[c]
Added	Found[a]			Added	Found[a]		
			Calibration standards				
0.5	0.50	0.0	5.6	0.5	0.50	0.0	5.7
1	1.01	1.0	4.4	1	1.00	0.0	4.7
2	1.98	−1.0	3.9	2	1.95	−2.5	1.8
5	4.64	−7.2	4.7	5	4.89	−2.2	1.4
10	9.87	1.3	2.1	10	9.95	−0.5	2.3
50	50.4	0.8	2.1	50	50.2	0.4	4.4
100	102	2.0	2.3	100	101	1.0	1.3
200	211	5.5	2.6	200	205	2.5	1.5
			Quality controls				
1	1.04	4.0	6.5	1	0.80	ND[d]	8.4
10	10.3	3.0	6.0	10	11.0	ND	7.6
100	106	6.0	5.9	100	101	ND	1.6

[a] Mean of nine values pooled from three separate calibration curves.
[b] Relative error = (found − added) ÷ added × 100.
[c] Relative standard deviation.
[d] Not determined (see text).

HPLC fluorescence detectors were unsuccessful in that no or weak signals were obtained.

Figure 1 shows chromatograms of diphenhydramine in human plasma. Retention times were 8.5 and 12.4 min for diphenhydramine and the internal standard, respectively. Blank plasma and predose samples were free of interfering peaks. Separation of the *N*-desmethyl metabolite from the parent compound is shown in Fig. 2. While the metabolite eluted close to the parent drug, it was sufficiently resolved to allow accurate quantitation of diphenhydramine. Resolution (R) was 1.79 and separation (α) was 1.21 for drug and metabolite peaks (11). Capacity (k') and theoretical plates (N) for drug, metabolite, and internal standard are given in Table II. Based on these findings, the method could be used for simultaneous analysis of diphenhydramine and its *N*-desmethyl metabolite in plasma.

Validation results are presented in Table I. Precision, based on relative standard deviation (RSD) of calculated diphenhydramine concentrations, ranged from 2.1 to 6.5%. Mean calculated concentrations of quality-control standards were within ±6% of theoretical values. The method was linear over the calibration range of 0.5 to 200 ng/ml.

Mean extraction recovery of diphenhydramine from human plasma was 91.3, 94.6, and 95.4% at concentrations of 1, 10, and 100 ng/ml, respectively. Recovery of internal standard was 98.1% at 2.5 ng/ml.

The method was used to determine diphenhydramine concentrations in plasma samples from three subjects participating in a clinical pharmacokinetic study. Subjects had received a single 100-mg oral capsule dose of diphenhydramine HCl. Results are shown in Fig. 3.

The method was also validated in minipig plasma, with results comparable to those achieved for human plasma. This validation was performed using a new column, 8 months after the initial validation in human plasma, indicat-

Table II. Chromatographic Parameters

Compound	Capacity (k')	Theoretical plates (N)[a]
N-Desmethyl diphenhydramine	2.57	2332
Diphenhydramine	3.11	2249
Internal Standard	5.09	2279

[a] $N = 5.54(t_R/w_h)^2$, where t_R = retention time and w_h = peak width at half-height.

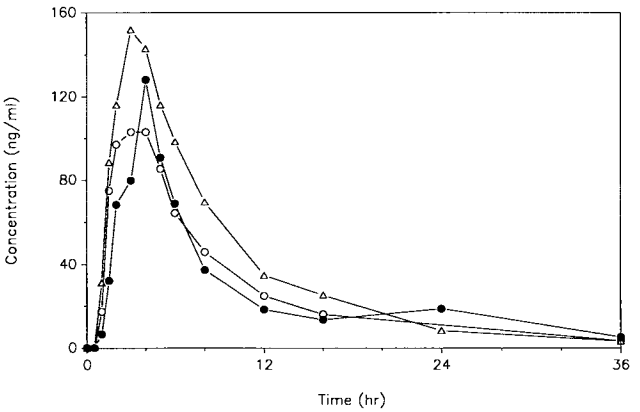

Fig. 3. Plasma diphenhydramine concentrations in three human subjects following a single 100-mg oral capsule dose.

ing that the method is reproducible. Chromatograms of diphenhydramine in minipig plasma were essentially identical to those shown in Fig. 1 for human plasma. Retention times of diphenhydramine and internal standard were 8.6 and 12.7 minutes, respectively.

Validation results for diphenhydramine in minipig plasma are presented in Table I. Precision (%RSD) of calculated diphenhydramine concentrations ranged from 1.3 to 8.4%. Mean back-calculated concentrations of calibration standards were within ±3% of theoretical values. Due to the limited amount of blank minipig plasma available, plasma for quality control standards was measured in a graduated cylinder rather than a volumetric flask, to allow for smaller volumes. Therefore accuracy based on concentrations found for quality control standards was not determined.

The method was used to determine diphenhydramine concentration in plasma samples from four Hanford minipigs following a 50-mg intravenous dose (8); concentrations found are shown in Fig. 4.

In conclusion, the method is precise, accurate, and reasonably simple to perform. It has been validated on two different columns at two separate times, indicating that the method is reproducible, and has been used to analyze post-dose plasma samples from two different species. The method has a sensitivity of at least 0.5 ng/ml (the lowest calibration standard used here) and is suitable for analysis of diphenhydramine in plasma samples from pharmacokinetic studies.

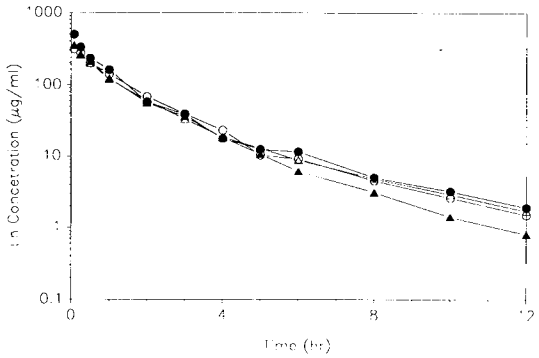

Fig. 4. Plasma diphenhydramine concentrations in four Hanford minipigs following a 50-mg intravenous dose.

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