# Method for the Determination of Diphenhydramine in Rabbit Whole Blood by High-Performance Liquid Chromatography (HPLC) with Ultraviolet (UV) Detection in Conjunction with Gas Chromatography (GC) with Mass Selective Detection (MSD)

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An HPLC/GC-MSD method for the determination of diphenhydramine in rabbit whole blood has been developed and validated. This method is based on a liquid–liquid extraction and reversed-phase chromatography with ultraviolet absorbance detection monitored at 258 nm. HPLC eluant fractions containing diphenhydramine and the internal standard, orphenadrine, were collected, reextracted, then subjected to GC-MSD analysis. Whole blood was utilized, thereby decreasing the required sample volume and increasing the sensitivity of the assay. Diphenhydramine concentrations can be quantitated over a range of 1 to 1000 ng/ml whole blood.

KEY WORDS: diphenhydramine, orphenadrine; high-performance liquid chromatography (HPLC); gas chromatography with mass selective detection (GC-MSD); rabbit whole blood.

## INTRODUCTION

Diphenhydramine, a competitive histamine ($H_1$) receptor antagonist, is often administered as a component of dimenhydrinate, an antinauseant/antiemetic preparation. Earlier diphenhydramine assays in biological fluids (1–3) yielded insufficient sensitivity for pharmacokinetic studies. More recent assays of diphenhydramine in plasma utilized gas chromatography with flame ionization (4–6), nitrogen–phosphorus detection (7–9), and mass spectrometry (10). This report describes an HPLC/GC-MSD procedure for the quantitative determination of diphenhydramine in whole blood. This method has been applied to the analysis of diphenhydramine in rabbit whole blood as part of a pharmacokinetic investigation of dimenhydrinate following intravenous versus intramuscular dosing.

## MATERIALS AND METHODS

### Reagents

HPLC-grade acetonitrile and UV-grade hexane were obtained from Burdick and Jackson (Muskegon, MI). ACS reagent-grade isopropyl alcohol, acetone, hydrochloric and orthophosphoric acid, sodium hydroxide, and sodium hydroxide, and sodium bicarbonate were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium carbonate, potassium hydroxide, and triethylamine were obtained from Mallinckrodt (Milwaukee, WI). Ultrapure helium gas (Specialty Gas) and HPLC/GC-grade methanol (J. T. Baker, distributed by VWR Scientific, Chicago, IL) were used for GC-MSD work. Orphenadrine · HCl was obtained from Sigma Chemical Co. (St. Louis, MO), while diphenhydramine · HCl and its metabolite, mono-*N*-desmethyl diphenhydramine · HCl were obtained from Parke Davis (Morris Plains, NJ). All reagents were used without further purification.

### Apparatus

The HPLC system consisted of a Model 110A solvent delivery pump (Altex), a Model 712 WISP autosampler (Waters Assoc., Milford, MA), a SPD6A ultraviolet detector (Shimadzu), and a 10-mV dual-pen chart recorder (Omniscribe, Houston Instruments, Austin, TX). Quantitative results from peak heights were obtained with an Altex CR1-A integrator. An Altex ultrasphere 5-μm octyl column (distributed by P. J. Cobert, St. Louis, MO), 150 × 4.6-mm i.d., maintained at 37°C was used for this analysis. HPLC peak fractions were collected using an ISCO FOXY fraction collector, Series 2130-001. The GC-MSD system consisted of a Model HP5890A gas chromatograph with a Model HP5979 mass selective detector (Hewlett Packard). The column utilized was a Hewlett Packard HP1 fused silica capillary column cross-linked with methyl silicone, 15 m × 0.2-mm i.d., with a film thickness of 0.11 μm.

### Standard Solutions

Primary stock solutions [100 ng free base/μl (aq)] of diphenhydramine and internal standard, orphenadrine, were prepared. Secondary, tertiary, and quaternary aqueous dilutions of the primary diphenhydramine stock solution were prepared at 10, 1.0, and 0.10 ng/μl, respectively. Calibration standards and method validation pools were then prepared in the following manner. Fresh, heparinized, whole blood was obtained from adult, female, New Zealand, albino rabbits and pooled. The whole-blood pool was stirred constantly on a magnetic stir plate during standard preparation. Appropriate volumes of whole blood were pipetted into small beakers and spiked with one of the diphenhydramine aqueous solutions to yield standard pools at the designated concentrations. Aliquots (1.0 ml) of the standard pools were immediately pipetted into 13 × 100-mm borosilicate disposable culture tubes. One standard, 0.25-ml aliquots, was diluted 1:4 with 0.75 ml whole blood. The tubes were capped and stored at 5°C until analysis. Validation standards were taken from the calibration standard pools and assayed as unknowns. All pipetting was done using Eppendorf adjustable pipettors.

### Extraction Procedure/Sample Preparation for HPLC Analysis

The stored samples were allowed to equilibrate to room temperature, uncapped, and vortexed briefly. Internal stan-

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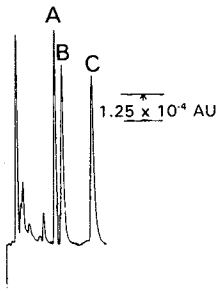


Fig. 1. Typical HPLC chromatogram showing resolution between diphenhydramine, internal standard (orphenadrine), and metabolite (mono-*N*-desmethyl diphenhydramine). Peaks: (A) metabolite, (B) diphenhydramine, and (C) internal standard, 100 ng of each on column.

dard was added to each sample [100 μl of 1000 ng/ml orphenadrine ·HCl (aq)] and each tube was vortexed briefly. The samples were made alkaline by the addition of 1.0 ml of 0.25 *N* sodium hydroxide, again vortexed briefly, and extracted with 5 ml of 98:2 hexane/isopropanol by shaking horizontally for 15 min at 1 oscillations/sec. After centrifugation for 5 min, the aqueous layer was frozen in an acetone/dry ice bath to allow the organic layer to be completely transferred to a clean 15-ml conical glass tube. The drug and internal standard were back-extracted into 1.0 ml of 0.1 *N* hydrochloric acid, vortexed for 10 min, then centrifuged 5 min. The upper organic fraction was discarded and the remaining aqueous layer alkalinized by the addition of 0.5 ml of carbonate/bicarbonate buffer, pH 11.8. The samples were finally extracted into 1.0 ml of 90:10 hexane/isopropanol by vortexing 10 min. After 5 min of centrifugation, the aqueous layer was frozen in an acetone/dry ice bath and the organic layer transferred to a clean 15-ml conical tube. The samples were evaporated to dryness for 15 min at 41°C. The residues were allowed to equilibrate to room temperature, reconstituted in 0.25 ml of HPLC mobile phase, and vortexed for 1 min, and 200 μl of each standard was injected onto the

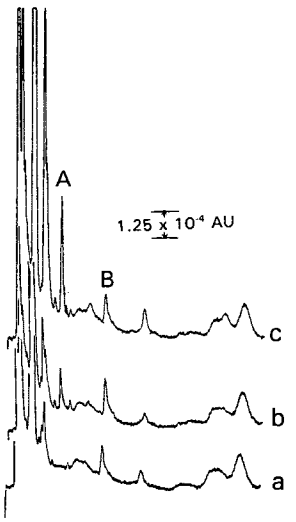


Fig. 2. Typical HPLC chromatograms for rabbit whole-blood extracts: (a) blank whole blood; (b) 25 ng/ml standard; (c) 250 ng/ml standard (1:4 dilution) with internal standard. Peaks: (A) diphenhydramine; (B) internal standard.

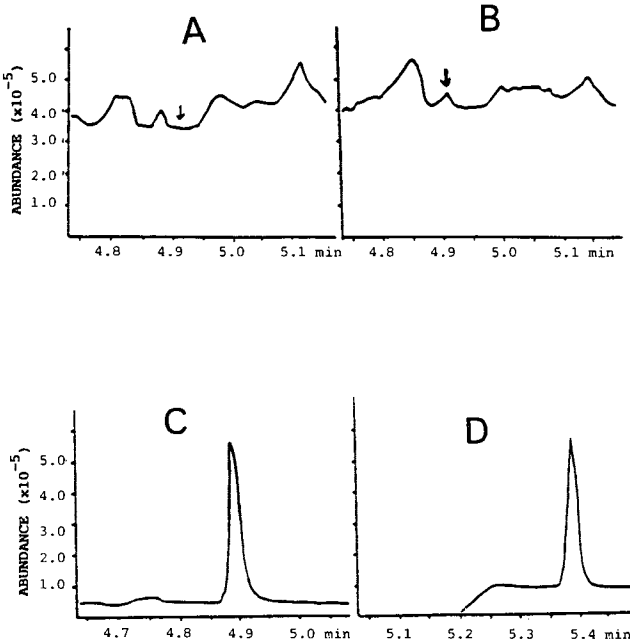


Fig. 3. Typical GC-MSD chromatograms for HPLC fraction extracts of (A) blank, (B) 5.0 ng/ml standard, (C) 25 ng/ml standard, and (D) internal standard. Ions monitored for (a) diphenhydramine (161.0–161.4 amu) and (b) internal standard (181.0–181.4 amu).

HPLC system. Effluent fractions segmented to contain both diphenhydramine and internal standard were collected into 13 × 100-mm disposable borosilicate culture tubes. All samples found to have concentrations less than or equal to 25 ng/ml by HPLC analysis were extracted and reanalyzed on the GC-MSD system as per the following procedure.

### Extraction Procedure/Sample Preparation for GC-MSD Analysis

Collected HPLC fractions were allowed to equilibrate to room temperature (if refrigerated), and 0.5 ml of 1.0 *N* sodium hydroxide was added to each tube. The contents were transferred to clean 15-ml conical tubes, the culture tubes were rinsed with 1.0 ml of 90:10 hexane/isopropanol to ensure complete transfer of drug and internal standard, and the rinse volume was transferred to the respective conical tubes. Extraction solvent (5.0 ml 98:2 hexane/isopropanol) was added to each tube and extraction effected by shaking horizontally for 10 min at 5 oscillations/sec. After 5 min of centrifugation, the lower "aqueous" layer was frozen in an acetone/dry ice bath and the organic layer transferred to clean 15-ml conical tubes. The samples were then evaporated to

Table I. Regression Statistics for Calibration Curves for Diphenhydramine in Rabbit Whole Blood

| No. of curves | Range (ng/ml) | Slope (ng/ml) | Y-Intercept (ng/ml) | SE est. | Corr. |
|---|---|---|---|---|---|
| 6 | 1.00–25.0 | 74.52 ±7.93 | (0.245 ±0.0459) | 0.0743 ±0.0339 | 0.997 ±0.002 |
| 6 | 25.0–250 | 10.03 ±5.96 | −0.512 ±0.770) | 0.0631 ±0.506 | 0.997 ±0.004 |

Table II. Accuracy and Precision of Diphenhydramine Assay: Low Curve

| | Validation pool concentration (ng/ml) | | | |
|---|---|---|---|---|
| Batch | 1.00 | 5.00 | 10.00 | 25.00 |
| 200/210 | | | | |
| *N* | 2 | 3 | | |
| Mean | 0.985 | 5.35 | | |
| SD | 0.078 | 0.469 | | |
| % CV | 7.92 | 8.77 | | |
| % AR | 98.5 | 107.0 | | |
| 201/210 | | | | |
| *N* | | 3 | 3 | 6 |
| Mean | | 4.68 | 10.05 | 23.29 |
| SD | | 0.552 | 0.259 | 2.82 |
| % CV | | 11.8 | 2.58 | 12.1 |
| % AR | | 93.6 | 100.5 | 93.2 |
| 202/212 | | | | |
| *N* | | 6 | | |
| Mean | | 4.62 | | |
| SD | | 0.314 | | |
| % CV | | 6.88 | | |
| % AR | | 92.4 | | |
| 203/213 | | | | |
| *N* | | | 6 | 3 |
| Mean | | | 9.84 | 23.22 |
| SD | | | 0.517 | 1.11 |
| % CV | | | 5.25 | 4.77 |
| % AR | | | 98.4 | 92.9 |
| 204/214 | | | | |
| *N* | 3 | | 3 | |
| Mean | 1.06 | | 10.25 | |
| SD | 0.144 | | 0.331 | |
| % CV | 13.5 | | 3.23 | |
| % AR | 106.0 | | 102.5 | |
| 206/216 | | | | |
| *N* | 5 | | | 5 |
| Mean | 1.01 | | | 23.02 |
| SD | 0.043 | | | 2.81 |
| % CV | 4.26 | | | 12.2 |
| % AR | 101.0 | | | 92.1 |
| Total | | | | |
| *N* | 10 | 12 | 12 | 14 |
| Mean | 1.02 | 4.82 | 10.00 | 23.18 |
| SD | 0.068 | 0.495 | 0.431 | 2.39 |
| % CV | 6.68 | 10.3 | 4.31 | 10.3 |
| % AR | 102.0 | 96.4 | 100.0 | 92.7 |

Table III. Accuracy and Precision of Diphenhydramine Assay: High Curve

| | Validation pool concentration (ng/ml) | | |
|---|---|---|---|
| Batch | 25.00 | 100.00 | 250.00 |
| 200/210 | | | |
| *N* | | 6 | |
| Mean | | 97.79 | |
| SD | | 7.66 | |
| % CV | | 7.83 | |
| % AR | | 97.8 | |
| 201/211 | | | |
| *N* | 6 | | |
| Mean | 26.87 | | |
| SD | 2.20 | | |
| % CV | 8.19 | | |
| % AR | 107.5 | | |
| 202/212 | | | |
| *N* | | 3 | 3 |
| Mean | | 99.00 | 237.90 |
| SD | | 10.81 | 9.16 |
| % CV | | 10.9 | 3.85 |
| % AR | | 99.0 | 95.2 |
| 203/213 | | | |
| *N* | 3 | 2 | |
| Mean | 24.91 | 100.66 | |
| SD | 3.39 | 10.86 | |
| % CV | 13.6 | 10.8 | |
| % AR | 99.6 | 100.6 | |
| 204/214 | | | |
| *N* | | | 6 |
| Mean | | | 254.56 |
| SD | | | 26.78 |
| % CV | | | 10.5 |
| % AR | | | 101.8 |
| 206/216 | | | |
| *N* | 5 | | 3 |
| Mean | 25.28 | | 246.34 |
| SD | 2.99 | | 16.0 |
| % CV | 11.8 | | 6.48 |
| % AR | 101.1 | | 98.5 |
| Total | | | |
| *N* | 14 | 11 | 12 |
| Mean | 25.88 | 98.64 | 250.60 |
| SD | 2.68 | 8.11 | 18.28 |
| % CV | 10.4 | 8.22 | 7.30 |
| % AR | 103.5 | 98.6 | 100.2 |

dryness for 35 min at 41°C. The residues were reconstituted in 200 μl of Baker HPLC-grade methanol and vortexed for 1 min. The samples were then carefully transferred to 12 × 33-mm borosilicate GC vials fitted with 200-μl borosilicate pulled point inserts with springs. The samples were concentrated in the vials under vacuum at ambient temperature using a Savant Speed Vac Concentrator centrifuge for 15 min, after which 20 μl of HPLC-grade methanol was added to each, capped, and vortexed for 1 min. Three microliters of each standard was injected onto the GC-MSD system.

### Validation Study Design

A total of six validation batches was run. Each batch was set up to contain a total of nine calibration standards assayed singly, one of the six validation pools assayed six times to assess intrabatch variability, and two of the remaining four validation standards assayed three times each to assess interbatch variability. The calibration standards and validation pools were assayed in random order. Specificity of this assay was determined by evaluating whole-blood standards containing no drug to verify the absence of interfering substances unresolvable by retention time from diphenhydramine or the internal standard. The major basic metabolite of diphenhydramine, mono-*N*-desmethyl diphenhydramine, was also chromatographed on this system to further verify specificity. An HPLC chromatograph showing

resolution of the analyte, internal standard, and metabolite is shown in Fig. 1.

### Chromatography

A mobile phase consisting of 27/73 (v/v) acetonitrile/50 m*M* phosphate buffer containing 7 m*M* TEA, pH 3.0, was employed at a flow rate of 2.0 ml/min for the HPLC analysis. This provided about 300 and 470 theoretical plates for diphenhydramine and internal standard per cm of column, respectively. With capacity factors of 6.70 and 10.9 for the analyte and internal standard, good resolution from coextracted compounds was observed. Detection was monitored by ultraviolet absorbance at 258 nm at a range of 0.001 AU/10 mV. All extractions were carried out at ambient temperature (20–30°C).

Helium, maintained at 13 psi and a flow rate of 1.0 ml/min, was utilized as the carrier gas on the GC-MSD system with the following temperature program. The injector was maintained at 200°C, and the transfer line at 230°C. The oven was initialized at 50°C for 1 min, then ramped to 180°C at a rate of 65°C/min and held at this temperature for 5 min, after which the temperature was increased to 210°C at a rate of 30°C/min. This program was applied for each run.

### Data Analysis

Calibration curves were generated by linear regression: a high curve to include a blank and 25.0, 50.0, 100.0, 175.0, and 250.0 ng/ml standards and a low curve to include a blank and 1.00, 5.00, 10.0, and 25.0 ng/ml standards. Computed concentrations for each standard were obtained from regression analysis. The percentage coefficient of variation (%CV) was used as a measure of precision. The analytical recovery (%AR) was used to assess accuracy and was calculated as %AR = average concentration/amount analyte added $\times 100$. The limit of quantitation was defined as the smallest detectable concentration with sufficient precision to yield a %CV less than 25%. The limit of detection was set at the lowest concentration level that could be determined to be statistically different from an analytical blank (0.00 standard), i.e., three times the standard deviation of the blank (in units of concentration, ng/ml). In accordance with our acceptance criterion, the computed concentration for each standard was within 15% of the theoretical concentration, while the mean computed concentration and the standard deviation for the validation standards were within 15% of the theoretical concentrations, with 15% or less variation.

## RESULTS AND DISCUSSION

The extraction procedure and chromatographic conditions were adapted from published methods for the GC analysis of diphenhydramine in human plasma (7) and the capillary GC analysis of diphenhydramine in human serum (4).

Typical HPLC chromatograms following 200-μl injections of extracted standards prepared to contain 250 (1:4 dilution), 25.0, and 0.00 ng/ml diphenhydramine and 100 ng/ml internal standard are presented in Fig. 2. Diphenhydramine was found to have a retention time of 4.61 min (9.22 ml), while the internal standard was found to elute at 7.15 min (14.3 ml). Representative GC-MSD chromatograms for the 5.00 and 25.0 ng/ml standards and the blank are shown in Fig. 3. Diphenhydramine was found to elute on this system in 4.8 min, and the internal standard in 5.2 min.

Linearity from 1.00 to 25.0 and from 25.0 to 250 ng/ml was reproducibly demonstrated. A 1:4 dilution procedure was also validated within the acceptance criterion for this assay. The mean regression statistics from the validation standard curves are presented in Table I. Tables II and III present the intra- and interbatch precision and accuracy as determined by the %CV and %AR of the validation pools for the low and high curves, respectively. The minimum quantifiable concentration was determined to be 1.00 and 25.0 ng/ml for the low and high curves, respectively. The within-batch precision (%CV) of the validation pools ranged from 2.58 to 13.6%, while the batch-to-batch precision (%CV) of the validation pools ranged from 4.31 to 10.4%. The within-batch accuracy (%AR) of the validation pools ranged from 92.1 to 107.5%, while the batch-to-batch analytical recovery for the validation pools covered a range of 96.4 to 103.5%. The limit of detection was determined to be 0.77 and 13.74 ng/ml for the low and high curves, respectively.

This method is suitable for the whole-blood analysis of diphenhydramine within a concentration range of 1.0 to 250 ng/ml and can be extended to quantitate levels up to 1000 ng/ml.

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## REFERENCES

1. J. E. Wallace, J. D. Biggs, and E. V. Dahl. Determination of diphenhydramine and certain related compounds by ultraviolet spectrophotometry. *Anal. Chem.* **38**:831–833 (1966).
2. S. L. Tompsett. The detection of bases and other drugs in urine by distillation and UV spectrophotometry. *Clin. Chem.* **15**(7): 591–599 (1969).
3. K. W. Hindmarsh, N. W. Hamon, and D. F. LeGatt. Simultaneous identification and quantitation of diphenhydramine and methaqualone. *Clin. Toxicol.* **11**(2):245–255 (1977).
4. D. Lutz, W. Gielsdorf, and H. Jaeger. Quantitative determination of diphenhydramine and orphenadrine in human serum by capillary gas chromatography. *J. Clin. Chem. Clin. Biochem.* **21**:595–597 (1983).
5. K. S. Albert, E. Sakmar, J. A. Morris, M. R. Hallmark, and J. G. Wagner. Determination of diphenhydramine in plasma by gas chromatography. *Res. Comm. Clin. Pathol. Pharmacol.* 7(1):95–103 (1974).
6. R. Baugh and R. T. Calvert. A rapid method for the determination of diphenhydramine in plasma. *Br. J. Clin. Pharmac.* **3**:1062–1064 (1976).
7. D. R. Abernathy and D. J. Greenblatt. Diphenhydramine determination in human plasma by gas-liquid chromatography using nitrogen-phosphorus detection: Application to single low-dose pharmacokinetic study. *J. Pharm. Sci.* **72** (8):941–943 (1983).
8. R. C. Meatherall and D. R. P. Guay. Isothermal gas chromatographic analysis of diphenhydramine after direct injection onto a fused-silica capillary column. *J. Chromatogr.* **307**:295–304 (1984).
9. C. Giachetti, P. Poletti, and G. Zanolo. HRGC assay of antihistaminic drugs in plasma samples using a nitrogen-phosphorus detector. *J. High Resol. Chromatogr. Chromatogr. Comm.* **11**:525–527 (1988).
10. S. G. Carruthers, D. W. Shoeman, C. E. Hignite, and D. L. Azarnoff. Correlation between plasma diphenhydramine level and sedative and antihistamine effects. *Clin. Pharmacol. Ther.* **23**:375–382 (1978).