Diphenhydramine Determination in Human Plasma by Gas–Liquid Chromatography using Nitrogen–Phosphorus Detection: Application to Single Low-Dose Pharmacokinetic Studies

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Received April 5, 1982, from the *Division of Clinical Pharmacology, Departments of Psychiatry and Medicine, Tufts University School of Medicine and New England Medical Center Hospital, Boston, MA 02111.* Accepted for publication July 23, 1982.

Abstract □ A highly sensitive method (≤1 ng/ml) for single-dose pharmacokinetic studies of diphenhydramine which utilizes GLC with nitrogen–phosphorus detection is described. Standard curves, using orphenadrine as the internal standard, were linear for diphenhydramine concentrations from 1.0 to 300 ng/ml. Applicability of the method was demonstrated by a pharmacokinetic study in a normal volunteer who received 25 mg iv of diphenhydramine.

Keyphrases □ Diphenhydramine—determination in human plasma using GLC with nitrogen–phosphorus detection, application to single low-dose pharmacokinetic studies □ GLC—with nitrogen–phosphorus detection, determination of diphenhydramine in human plasma, single-dose pharmacokinetic study □ Pharmacokinetics—of diphenhydramine in the human, detection after a single dose using GLC with nitrogen–phosphorus detection

Diphenhydramine, widely used in the treatment of allergic conditions (1) and as a sedative–hypnotic in the elderly (2), has been measured by GLC using flame-ionization detection (3), GLC–MS (4), UV spectrophotometry (5), and by a fluorescent dye procedure (6). With the exception of GLC–MS, these methods lack adequate specificity and sensitivity. A method using GLC with nitrogen–phosphorus detection that is rapid, specific, and sensitive to 1 ng/ml is described. Following a straightforward plasma extraction requiring 3–4 hr, up to 150 samples per 24 hr can be analyzed if an automated injection system is used. This method is valuable since, in some subject populations (particularly the elderly), a diphenhydramine dose small enough to cause little sedative or anticholinergic effect but large enough to permit a blood level determination for the time needed to complete a single-dose pharmacokinetic study is necessary.

EXPERIMENTAL

Apparatus and Chromatographic Conditions—A gas chromatograph[1] equipped with a nitrogen–phosphorus detector and an electronic integrator was used. The column was coiled glass (1.83 m × 2-mm i.d.) packed with 3% SP-2250 on 80/100 mesh Supelcoport[2]. The carrier gas was ultra-high purity helium[3] at a flow rate of 30 ml/min. The detector purge was ultra-high purity hydrogen[3] at 3 ml/min mixed with dry air[3] at 50 ml/min. Operating temperatures were: injection port, 310°; column, 205°; and detector, 275°. Before being connected to the detector, new columns were conditioned at 270° for 48 hr with a carrier flow of 30 ml/min. At the beginning of each work day, the column was primed with 2 μg of phospholipid (asolectin) in benzene.

Reagents—Certified *n*-hexane[4] (99% pure) and isoamyl alcohol[4] were used. The toluene[5], methanol[4], concentrated hydrochloric acid[5], sodium hydroxide[5], sodium carbonate[5], and sodium bicarbonate[5] were analytical reagent grade. The isoamyl alcohol was glass-distilled prior to use; other organic solvents were used without further distillation. All aqueous solvents (0.25 *M* NaOH, 0.1 *M* HCl, and 1 *M* carbonate–bicarbonate buffer) were washed five times with hexane–isoamyl alcohol (98:2) prior to use.

Reference Standards—Pure standards of diphenhydramine hydrochloride[6] and orphenadrine citrate[7] were provided by the manufacturers. Standard solutions of each were prepared by dissolving the appropriate quantity of the salt to yield 100 mg of base in 100 ml of methanol. Sequential dilutions to 1 μg/ml were made. The solutions were stored in the dark in glass-stoppered bottles at 4° and were stable for at least 4 months.

Preparation of Samples—Orphenadrine was used as the internal standard for all analyses. A 100-μl volume of stock solution (1 μg/ml), containing 100 ng of orphenadrine, was added to 15-ml tubes, with poly-

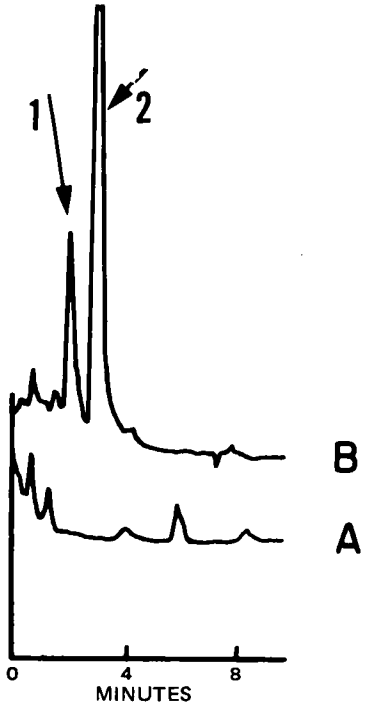

Figure 1—*Chromatogram of the extract of a 1-ml plasma blank (A) and plasma with 25 ng/ml of diphenhydramine and 100 ng of orphenadrine added (B). Key: (1) diphenhydramine; (2) orphenadrine.*

[1] Hewlett-Packard Model 5840A.
[2] Packing 1-1767, Supelco, Bellefonte, Pa.
[3] Matheson Gas Products, Gloucester, Mass.
[4] Fisher Scientific, Fair Lawn, N.J.
[5] Mallinckrodt, St. Louis, Mo.
[6] Benadryl; Parke-Davis Div., Warner-Lambert Co., Ann Arbor, Mich.
[7] Riker Laboratories, Inc., Northridge, Calif.

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Table I—Representative Calibration Data

Diphenhydramine Concentration, ng/ml	Peak Height Ratio[a]
1.0	0.00615 ± 0.00025
5.0	0.03831 ± 0.00354
10.0	0.07177 ± 0.00372
25.0	0.30977 ± 0.01362
50.0	0.64632 ± 0.01118
200.0	2.5786 ± 0.0341
Correlation coefficient	0.9998
Slope	0.01303
Intercept	−0.02341
Slope corrected through origin	0.01287

[a] Mean ± *SD*; $n = 5$.

tef-faced, rubber-lined, screw-top caps. A 0.25–2.0 ml test sample of plasma was added to each tube. Calibration standards for diphenhydramine were prepared by adding 1, 2.5, 5, 10, 25, 50, 100, 200, and 300 ng of drug to consecutive tubes. Drug-free control plasma was added to each of the calibration tubes. One plasma sample, taken from the subject prior to drug administration, was analyzed with the calibration standards and each set of unknown samples as a blank.

Extraction Procedure—One milliliter of 0.25 *M* NaOH solution was added to each tube. To this was added 5 ml of hexane–isoamyl alcohol (98:2), and the tubes were agitated gently in the upright position on a vortex mixer for 15 min. The samples were centrifuged at room temperature for 5 min at 400×*g*[8]. The organic layer was transferred to another 15-ml tube that contained 1.2 ml of 0.1 *M* HCl. This mixture was agitated gently in the upright position on a vortex mixer for 10 min. The samples were centrifuged again at room temperature for 5 min at 400×*g*, and the upper, organic layer was discarded. The aqueous layer was carefully transferred to another tube, and to this was added 0.5 ml of 1 *M* carbonate–bicarbonate buffer (pH 11.5). To the mixture was added 200 μl of toluene–isoamyl alcohol (85:15). This mixture was agitated gently in the upright position on a vortex mixer for 15 min, and the samples were centrifuged again at room temperature for 5 min at 400×*g*. Using a 23-cm disposable pipet passed through the organic layer, the entire aqueous layer was removed leaving the organic phase (150 μl) containing diphenhydramine and the internal standard. This was transferred to a 2-ml automatic sampling vial.[9] Six microliters was injected into the gas chromatograph using the automatic-injection sampling system.

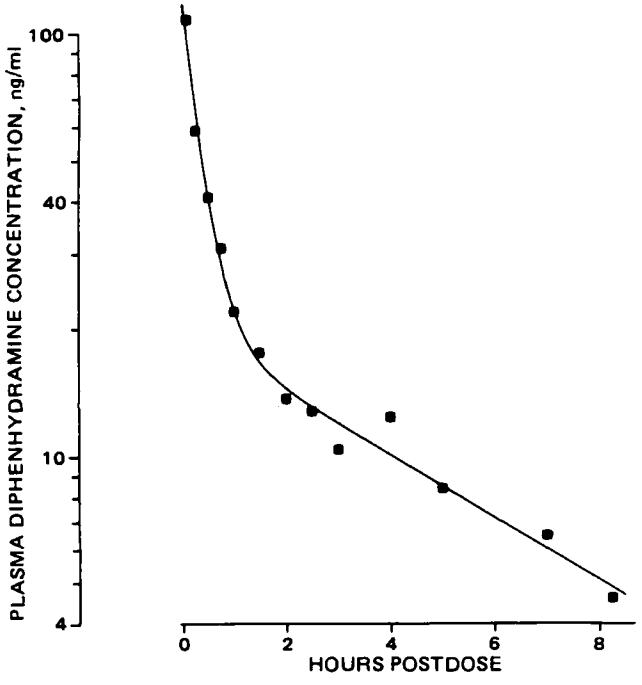

Figure 2—*Plasma diphenhydramine concentrations and the derived pharmacokinetic function following intravenous diphenhydramine administration to a healthy 32-year-old male subject. The elimination half-life is 4.1 hr (see Table I).*

Table II—Derived Diphenhydramine Pharmacokinetic Parameters in a Healthy Male Subject[a]

Subject Characteristics	
Age/Sex	32/male
Weight, kg	70.5
Diphenhydramine Kinetic Variables	
Distribution half-life, min	14.3
Elimination half-life, hr	4.06
Central compartment volume, liters/kg	2.66
Total volume of distribution, liters/kg	12.17
Total metabolic clearance, ml/min/kg	34.7

[a] Diphenhydramine dose of 25 mg iv.

Single-Dose Pharmacokinetic Study—A healthy 32-year-old male volunteer participated after giving written informed consent. Diphenhydramine hydrochloride (25 mg) was administered by intravenous bolus injection. The drug solution (50 mg/ml) was administered through a glass syringe. Multiple venous blood samples were drawn into heparin-containing tubes[10] over 8 hr postdose. Concentrations of diphenhydramine were determined by the aforementioned method.

Plasma diphenhydramine concentrations were analyzed by iterative, weighted, nonlinear, least-squares regression analysis (7, 8). After correction of the dose to diphenhydramine base, the following pharmacokinetic variables were determined: distribution half-life, elimination half-life, total volume of distribution, and total clearance.

RESULTS

Under the described conditions, retention times for diphenhydramine and orphenadrine were 3.41 and 4.32 min, respectively. A chromatogram of a typical blank plasma sample compared with a sample containing diphenhydramine and orphenadrine (internal standard) is shown in Fig. 1.

The relationship between diphenhydramine concentration and the area ratio (*versus* internal standard) is linear to at least 300 ng/ml. Analysis of more than 20 standard curves over a month always afforded a correlation coefficient ≥0.99. The day-to-day coefficient of variation in the slopes of the calibration curves was 5.9% (Table I).

The sensitivity limit of the method is 1 ng/ml using a 2-ml extracted plasma sample. The within-day coefficient of variation for identical samples ($n = 5$) were: 1 ng/ml, 4%; 5 ng/ml, 9.2%; 10 ng/ml, 5.2%; 25 ng/ml, 4.4%; 50 ng/ml, 1.7%; 100 ng/ml, 1.7%; 200 ng/ml, 1.3%; and 300 ng/ml, 2.8%. Residue analysis indicated the extraction of diphenhydramine is 97.3% at a plasma concentration of 25 ng/ml.

Figure 2 shows the plasma diphenhydramine concentration and pharmacokinetic function for the test subject. The derived pharmacokinetic parameters are listed in Table II.

DISCUSSION

This report describes a reliable, sensitive, and specific method for the quantitation of diphenhydramine in plasma using GLC with nitrogen–phosphorus detection. This method is an advance over previously reported methods in that the sensitivity is adequate for single-dose pharmacokinetic studies with very low doses, permitting safe study in widely varying subject populations. Addition of a dilute sodium hydroxide–hexane–isoamyl alcohol mixture to the plasma samples followed by an acid extraction of the organic phase, readjustment of the aqueous phase to pH 11.5, and reextraction with toluene–isoamyl alcohol afforded blank samples suitable for direct injection into the GC that were consistently free of contaminants in the area corresponding to the retention time for diphenhydramine. Known basic metabolites will not elute from the GLC column without derivatization, and acidic metabolites are not coextracted by the procedure used (9). The value of this GLC with nitrogen–phosphorus detection method includes the reasonable time required for sample preparation, as well as a sensitivity that is adequate for single low-dose pharmacokinetic studies.

[8] Portable refrigerated centrifuge Model PR-2, Head No. 269; International Equipment, Boston, Mass.

[9] Wheaton Scientific, Millville, N.J.

[10] Venoject.

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Intestinal Absorption of Amino Acid Derivatives: Structural Requirements for Membrane Hydrolysis

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Abstract □ The intestinal absorption of L-lysine-*p*-nitroanilide, L-alanine-*p*-nitroanilide, and glycine-*p*-nitroanilide was studied in perfused rat intestine in the presence of a variety of potential competitive inhibitors. The results indicate that the hydrolysis site(s) show side-chain specificity, and that inhibitors require a free amino group in the α-position and must be in the L-configuration to be effective. Glycyl-L-proline, a peptide transport inhibitor, had no effect on the absorption rate.

Keyphrases □ Absorption, intestinal—amino acid derivatives, structural requirements for membrane hydrolysis, rats □ Hydrolysis, membrane—structural requirements, intestinal absorption of amino acid derivatives, rats □ Amino acid derivatives—intestinal absorption, structural requirements for membrane hydrolysis, rats

Previous reports (1, 2) have demonstrated that intestinal membrane (or brush-border) enzymes may serve as useful prodrug reconversion sites. For example, compounds that are insoluble, unstable, or have other undesirable pharmaceutical properties may be derivatized so as to improve these properties with the regeneration of the active drug occurring just prior to entry into the systemic circulation. Clearly the specificity of the enzymes in the brush-border region sets a boundary for this strategy. In this report the intestinal absorption of L-lysine-, L-alanine-, and glycine-*p*-nitroanilides is studied in the presence of a variety of potential competitive inhibitors to more clearly define the specificities of the surface peptidases.

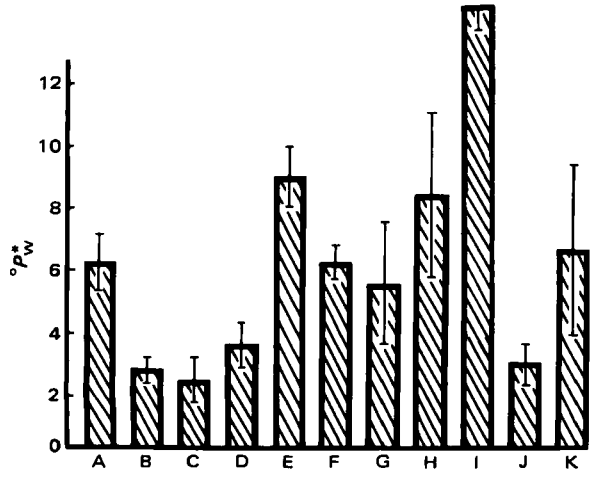

Figure 1—*Intestinal wall permeability (°P^*_w) of L-lysine-p-nitroanilide alone (A) and with L-lysine methyl ester (B), L-arginine-β-naphthylamide (C), L-arginine methyl ester (D), α-N-acetyl-L-lysine methyl ester (E), L-lysine (F), L-phenylalanine methyl ester (G), L-alanine methyl ester (H), glycine methyl ester (I), L-prolylglycine (J), or glycyl-L-proline (K).*

EXPERIMENTAL

Materials—L-lysine-*p*-nitroanilide[1], L-alanine-*p*-nitroanilide[2], and glycine-*p*-nitroanilide[1] were used as received. The inhibitors L-lysine[3], L-lysine methyl ester[2], α-*N*-acetyl-L-lysine methyl ester[3], L-alanine methyl ester[2], L-alanine amide[1], β-alanine methyl ester[1], D-alanine methyl ester[1], L-phenylalanine methyl ester[3], L-phenylalanine amide[1], glycine methyl ester[2], L-arginine methyl ester[1], L-arginine-β-naphthylamide[1], L-prolylglycine[1], and glycyl-L-proline[1] were used as received.

Perfusion Experiments—Rat intestinal perfusion experiments were carried out as previously described (1, 2). Inlet (*Co*) and exit (*Cm*,) concentrations of the perfused segment were measured by determining the *p*-nitroaniline concentration after a 12-hr hydrolysis. The *Cm/Co* ratio was determined using a three-point spectral analysis to account for any background absorbance due to protein in the perfusate. Experiments were carried out with the substrate concentration at 4×10^{-5} *M* and the inhibitor concentration at 4×10^{-3} *M*. Each permeability is the average result from 6–10 rats.

RESULTS AND DISCUSSION

The dimensionless intestinal wall permeability, °P^*_w, was calculated as previously described (3). Tests for significance were done using the two-sample *t* test for samples with unequal variance[4] (95% confidence level). The results are shown in Table I and Figs. 1–3.

L-Lysine methyl ester, L-arginine-β-naphthylamide, and L-arginine

[1] U.S. Biochemical Corp., Cleveland, Ohio
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] MINITAB, University of Pennsylvania, Philadelphia, Pa.

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