Subnanogram Quantitation of Chlorpheniramine in Plasma by a New Radioimmunoassay and Comparison With a Liquid Chromatographic Method

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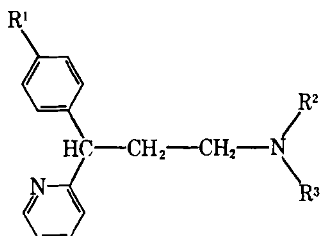
Abstract ☐ A new radioimmunoassay (RIA) procedure for the quantitation of chlorpheniramine in plasma is described. The assay allows the determination of chlorpheniramine levels up to 96 h after oral administration of a single 4-mg tablet to healthy volunteers. This procedure was sensitive to a 156-pg/mL plasma concentration when a 100-μL plasma sample was used. The mean coefficient of variation over the linear range of the assay from 0.156 to 20 ng/mL was 3.79%. The specificity of the assay was investigated, and the antisera showed 7% cross-reactivity with the *N,N*-didemethyl analogue and 17% cross-reactivity with the *N*-demethyl analogue. This high degree of specificity was also evident from the findings that the plasma concentrations determined by this newly described RIA procedure in samples of two healthy male volunteers who were administered 4 mg of chlorpheniramine maleate orally gave a strong correlation ($r^2 = 0.88$) with values obtained by an HPLC-UV procedure. The antiserum cross-reacted 100% with brompheniramine and, thus, can be used for its analysis in plasma. The described RIA procedure is precise, simple, and capable of handling a large number of plasma samples with a minimal turnaround time.

Keyphrases ☐ Radioimmunoassay—chlorpheniramine, plasma, comparison with HPLC-UV ☐ Chlorpheniramine—subnanogram quantitation in plasma, RIA, comparison with HPLC-UV

Chlorpheniramine (2-[*p*- chloro -α-[2-(dimethylamino)ethyl]-benzyl]pyridine), a potent antihistaminic drug widely used for relief of symptoms of the common cold and other allergic reactions, is a common ingredient in nonprescription antihistamine preparations. It is generally recommended for use in relatively low doses (1). Recent studies have established that the assay methods of this drug should be sensitive at least to the low nanogram range to adequately follow the plasma concentrations for thorough, reliable pharmacokinetic investigations (2–6) in children.

The various methods for the quantitation of chlorpheniramine that have been used include TLC with liquid scintillation counting (7), TLC followed by chemical conversion to an azo dye and subsequent colorimetric analysis (8), GC (9–15), HPLC (16, 17), and GC-MS (18, 19). Most of the earlier methods were developed to assay the drug in dosage forms or in urine; only a few procedures (15, 17–19) have the necessary sensitivity to quantitate normal plasma levels of chlorpheniramine after single doses of the drug. The GC procedure using electron-capture detection (15) is complicated because it requires the liquid chromatographic separation of the drug from its metabolites and an oxidation reaction as preliminary steps. The HPLC (17) and GC-MS procedures (18, 19) have adequate sensitivity to determine 1–2-ng/mL plasma concentrations after single doses; however, they all require extraction of the drug from plasma and/or sophisticated instrumentation such as GC-MS and are generally cumbersome for routine analysis. Radioimmunoassay (RIA) procedures, on the other hand, are usually simple, sensitive, and readily applicable to routine analysis. They generally do not require either extraction of the drug from plasma or expensive instrumentation. Such an RIA method for chlorpheniramine, which is sensitive to 0.156 ng/mL and requires only a 100-μL plasma sample, has been developed and is reported here. This report also describes the comparison of the RIA with a new HPLC procedure.



(I): R^1=Br; R^2=R^3=CH_3
(II): R^1=Cl; R^2=R^3=H
(III): R^1=Cl; R^2=CH_3; R^3=H
(IV): R^1=Cl; R^2=R^3=CH_3
(V): R^1=Cl; R^2=CH_3; R^3= $-CH_2CH_2-COOH$

EXPERIMENTAL SECTION

Materials—Chlorpheniramine maleate[1], prochlorperazine dimaleate[2], 2-[*p*-chloro-α-(2-aminoethyl)benzyl]pyridine (II)[3], 2-[*p*-chloro-α-[2-(methylamino)ethyl]benzyl]pyridine (III)[3], and brompheniramine maleate[4] were used. All solvents were HPLC grade, and all other chemicals were commercial analytical reagent grade. The tracer solution was tritiated chlorpheniramine with a specific activity of 14.6 Ci/mmol, prepared by catalytic exchange with tritium gas[5]. The following reagents were used without modification: 0.2 M phosphate buffer (pH 7.2) and dextran-coated charcoal suspension [prepared by the method of Powell *et al.* (20)]. A scintillation fluid[6] was used for liquid scintillation counting.

Apparatus—A liquid chromatographic pump[7] fitted with a valve-loop (500 μL) injector[8] was connected to a variable-wavelength detector[9] operated at 229 nm. A Spherisorb 5-μm cyano column (250 mm × 4.6 mm i.d.)[10] was used at ambient temperature with a flow rate of 3 mL/min. The mobile phase consisted of 30% 0.05 M ammonium acetate in acetonitrile. It was deaerated by filtration[11] before use. Scintillation counting was carried out with a liquid scintillation counter equipped with an automatic quench compensation[12].

Liquid Chromatographic Internal Standard—A stock solution of prochlorperazine dimaleate (100 μg/mL, calculated as free base) was prepared in double-distilled water and stored in the absence of UV light at 4°C; this was prepared fresh monthly. Dilutions of 200 ng/mL were prepared daily.

Preparation of Standard Curves—An aqueous solution of chlorpheniramine maleate (100 μg/mL, calculated as free base) was prepared monthly in double-distilled deionized water and stored at 4°C. Appropriate dilutions of the stock solutions were made in plasma. For RIA, this plasma was obtained from drug-free volunteers. For HPLC, outdated plasma[13] was used.

[1] Schering Corp. Ltd., Pointe Claire, Quebec, Canada
[2] Rhône-Poulenc Ltd., Montreal, Quebec, Canada.
[3] Smith Kline & French Laboratories, Philadelphia, Pa.
[4] A. H. Robins, Canada Ltd.
[5] Nuclear Research Center, Negev, Beer-Sheva, Israel.
[6] Ready-Solve MP; Beckman Instruments, Canada.
[7] Model M-45; Waters Scientific Co., Mississauga, Ontario, Canada.
[8] Model 7125, Rheodyne; Technical Marketing Associates, Calgary, Alberta, Canada.
[9] Waters Scientific Co., Mississauga, Ontario, Canada.
[10] Spherisorb Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Clwyd, U.K.
[11] Millipore Corp., Bedford, Mass.
[12] Model 1215; LKB Rackbeta; Fisher Scientific Co, Canada.
[13] Obtained from the Red Cross.

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Table I—RIA Estimation of Chlorpheniramine Added to Plasma [a]

Concentration, ng/mL	n	Mean, B/B_0, %	SD, ng	RSD^b
0.156	9	93.3	0.0016	1.05
0.313	7	86.8	0.0070	2.24
0.625	7	75.6	0.0137	2.19
1.25	8	59.2	0.0488	3.90
2.5	6	42.7	0.0608	2.43
5	8	25.8	0.2785	5.57
10	7	15.7	0.4560	4.56
20	6	9.7	1.672	8.36

[a] Composite standard curve where logit $y = M \cdot \log_{10} x + I$; $M = -2.329$, $I = 0.6686$, $r^2 = 0.999$. [b] Mean $RSD = 3.79\%$.

For RIA, calibration curves were constructed by the following:

$$\text{Logit } y = \text{Log}_e \frac{B/B_0}{1 - B/B_0} = M \cdot \text{Log}_{10} C + I \quad \text{(Eq. 1)}$$

where B is percentage bound, B_0 is the percentage bound at zero concentrations, M is the slope, C is the concentration (ng/mL), and I is the intercept.

Liquid Chromatographic Extraction of Samples—The extraction procedure was similar to that previously described for chlorpromazine (21, 22). To a 10-mL polytef-lined screw-capped centrifuge tube (13 × 100 mm) were added 2.0 mL of plasma (spiked or from dosed volunteers), 1.0 mL of prochlorperazine internal standard (100 ng/mL), and 0.5 mL of saturated sodium carbonate solution. The tube was mixed for 5 s, and 5 mL of 3% isopropyl alcohol in pentane was added. The tube was tightly capped and mixed[14] for 15 min and centrifuged[15] 10 min at 1725×g at ambient temperature.

The organic layer was transferred to a clean 10-mL tube, and the extraction was repeated with another 5.0 mL of 3% isopropyl alcohol in pentane. The combined organic extracts were evaporated at 65°C in a dry bath[16]. The dried residue was dissolved in 200 μL of acetonitrile, and 100 μL was injected into the chromatograph.

Synthesis of Drug Protein Conjugate and Production of Antisera—Antisera were raised in New Zealand White rabbits to an immunogen synthesized by covalent linkage of bovine serum albumin to N-(2-carboxyethyl)demethylchlorpheniramine employing the mixed anhydride condensation reaction (23, 24). A blank was prepared in the same manner without demethylchlorpheniramine. The number of hapten residues per mole of bovine serum albumin was determined to be 4 by a UV method (23, 24).

The hapten N-(2-carboxyethyl)demethylchlorpheniramine was prepared by alkaline hydrolysis of N-(2-methoxycarbonylethyl)demethylchlorpheniramine, a compound synthesized by refluxing equimolar amounts of methyl acrylate and III (25). The addition of III to methyl acrylate was monitored by GC, in which the absence of the peak due to III indicated completion of addition.

Four rabbits were each given one intradermal injection of 1.0 mg of the immunogen emulsified with 0.5 mL of Freund's complete adjuvant and 0.5 mL of isotonic saline. The rabbits were given four injections at 2-week intervals with the same amount of immunogen emulsified with Freund's incomplete adjuvant. Four intravenous boosters of 1.0 mg of immunogen dissolved in 0.25 mL of isotonic saline were given. After the second booster, titers of 1:500 were produced in three of the rabbits, and titers of 1:1000 were produced in the fourth. Subsequent injections did not increase the titer. The antiserum from each of the rabbits was characterized for its cross-reactivity and sensitivity. The antiserum employed in the present study was lyophilized and stored at −20°C. An aliquot of 25 mg was weighed out and reconstituted with 31.25 mL of phosphate buffer (pH 7.2). The cross-reactions (50% inhibition of binding at zero drug concentration) for the metabolites and other drugs were determined by the criteria of Abraham (26).

Radioimmunoassay—The assay was done in subdued light and an ice water bath so as to maintain the temperature at 4°C. To a 12 × 75-mm polystyrene tube containing 100 μL of plasma sample (standard or from dosed volunteer) was added 250 μL of tritiated chlorpheniramine (22,000 dpm) diluted in 0.2 M phosphate buffer (pH 7.2). The tube contents were mixed[17] for 5 s. An aliquot of 250 μL of the antiserum (80 mg in 100 mL) was added, and the tube contents were mixed[17] once more for 5 s and incubated at 4°C for 60 min. To this incubated solution was added 1.0 mL of a cold dextran-coated charcoal suspension (4°C). The tube contents were mixed[17] again for 5 s and incubated for 30 min at 4°C. The sample was then centrifuged[15] at 1720×g for 10 min at 4 °C. The supernatant was decanted into a scintillation vial containing 10 mL of cocktail[6] and then counted.

[14] SMI, Multitube Shakers; Canlab, Edmonton, Alberta, Canada.
[15] TJ6 Centrifuge; Beckman Instruments, Canada.
[16] Thermolyne Dri-Bath; Fisher Scientific Co.
[17] Vortex Genie; Fisher Scientific Co., Canada.

Table II—Cross-Reactivity of Chlorpheniramine Antiserum

Compound Tested	Cross-Reactivity, %
Brompheniramine (I)	100
2-[p-Chloro-α-(2-amino ethyl)benzyl]pyridine (II)	7
2-[p-Chloro-α-[2-(methylamino)ethyl]benzyl]pyridine (III)	17
Chlorpheniramine (IX)	100
N-(2-Carboxyethyl)demethylchlorpheniramine (V)	—

Plasma Level Study—A 4-mg dose of a commercial preparation of chlorpheniramine maleate[18] was given orally with 250 mL of water to each of two volunteers (weight, 56 and 60 kg, respectively). Blood samples were collected from each of the volunteers over a 96-h period in evacuated glass tubes[19] and centrifuged, and separated plasma was stored at 4°C for a maximum of 7 d. During collection of the venous samples, care was taken to avoid contact of the blood with the rubber stopper of the evacuated tube. For three of these blood samples for each volunteer, one extra tube of blood was collected in the conventional manner in which blood was allowed to come into contact with the rubber stopper.

Recovery Study for Liquid Chromatography—For the determination of chlorpheniramine recovery, blank plasma was spiked with 5 or 20 ng of chlorpheniramine/mL. The tritiated chlorpheniramine (10,400 dpm/mL) was also added to the spiked plasma. This brought the total concentration of chlorpheniramine added to 5.25 or 20.25 ng/mL. The samples were extracted as previously described, except that the organic layers were transferred to a 20-mL borosilicate glass scintillation vial and dried. Cocktail[6] (10 mL per vial) was also added, mixed, and then counted. Recovery of chlorpheniramine was determined by counting the radioactivity recovered *versus* the amount added.

Analytical Recovery for Radioimmunoassay—Tritiated chlorpheniramine was added to plasma samples containing chlorpheniramine and incubated with buffer in accordance with the procedure described above. The solutions were then decanted into scintillation fluid, and the radioactivity was measured. The recoveries of 0.156-20 ng were ~95%, which are similar to the values reported for another basic drug, trifluoperazine (27). It should be emphasized that during the decanting technique, small amounts of the sample adhere to the sides of the tube; thus, actual recoveries may be greater. When the tracer was added directly to the polystyrene tube without using plasma, >40% of the tracer was lost, probably due to adsorption onto the surface of the tube. Thus, all standards at a low nanogram range of chlorpheniramine were prepared in plasma.

RESULTS

Radioimmunoassay—The amount bound at zero concentration of chlorpheniramine was determined at incubation times of 30, 60, and 90 min and at temperatures of 4°C and 37°C for the first incubation step in the assay. From the results of these experiments, the optimal conditions for the assay were found to be an incubation time of 60 min at 4°C, and B_0 was 23%. The concentrations of unknown samples were estimated by running a calibration curve with each set of unknown samples.

Table I shows a composite standard curve covering the range of 0.156 to 20 ng/mL, which is definable by:

$$\text{Logit } y = -2.329 \cdot \log_{10} x + 0.6686 \; (r^2 = 0.999) \quad \text{(Eq. 2)}$$

Specificity—The cross-reactivity of the available metabolites of chlorpheniramine, as well as the related drug brompheniramine, as assessed by the criteria of Abraham, is recorded in Table II. Brompheniramine, as expected, did cross-react 100%. Thus, this antiserum can be used for the development of an RIA for brompheniramine. The N-demethyl and N,N-didemethyl metabolites of chlorpheniramine cross-reacted 17 and 7%, respectively. The significant cross-reactivity of the pharmacologically active N-demethyl metabolite (17%) was not surprising since the antisera were raised to a drug-protein conjugate prepared by coupling to this secondary amine. This also may explain the 7% cross-reactivity of the N,N-didemethyl metabolite. This relative cross-reactivity of 7% of the N,N-didemethyl metabolite was the same as that observed in the case of the chlorpromazine RIA (28). In the chlorpromazine RIA, the antisera raised in New Zealand White rabbits to the bovine serum

[18] Chlor-Tripolon; Schering Inc., Canada.
[19] Vacutainers, each containing 143 USP units of sodium heparin; Becton, Dickinson and Co., Mississauga, Ontario, Canada.

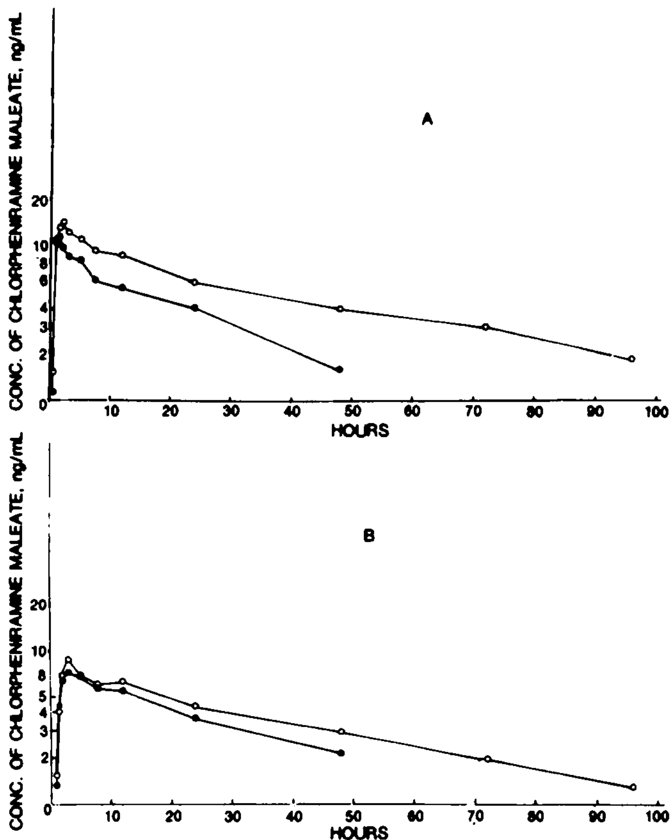

Figure 1—*Plasma concentrations* versus *time profiles obtained by RIA (○) and HPLC (●) for two healthy volunteers (A and B) weighing 56 and 60 kg, respectively, each of whom received a 4-mg dose of chlorpheniramine maleate.*

albumin conjugate of *N*-(2-carboxyethyl)demethylchlorpromazine cross-reacted 7% with the *N,N*-didemethyl metabolite.

The detection limit is <15.6 pg, which corresponds to 0.156 ng/mL if a 100-μL plasma sample is used. This sensitivity is greater than any method reported for chlorpheniramine. Thus, this RIA should be ideal for samples from infants and children, as the plasma volume needed does not exceed 0.2 mL for duplicate analyses of each sample.

Precision—The coefficient of variation for the replicate analyses of various concentrations of chlorpheniramine from 0.156 to 20 ng/mL is shown in Table I. The coefficient of variation of 0.156 ng/mL (n = 9) was ~1.05%.

Influence of Plasma Volume on Standard Curves—Standard curves were prepared by use of 50-, 100-, and 200-μL volumes of plasma. The slopes and intercepts derived were identical, thereby suggesting that plasma volumes did not affect the assay.

Capacity and Application of the Procedure—One technician can assay 50 samples in triplicate in a normal working day. Concentrations in plasma measured in two healthy volunteers (weight, 56 and 60 kg) after oral administration of a single 4-mg dose of the maleate salt of the drug are illustrated in Fig. 1. Note that the assay can easily quantitate the drug and/or its *N*-dealkylated metabolites in specimens collected as late as 96 h after a single oral dose.

Effect of Rubber Stoppers of the Evacuated Glass Tubes on Plasma Concentration Determination—To establish whether the measurement of the weakly basic lipophilic compound, chlorpheniramine [pK_a 8.99, (29)], is affected by contact with the rubber stoppers of the evacuated glass tubes, the duplicate samples of blood from the two dosed subjects were collected at three different times as described above. In each case, the first sample was collected with the evacuated tube held vertically so that the blood did not touch the rubber stopper at any time, whereas the second sample was collected immediately, with the tube held horizontally so that the blood did touch the stoppers. Plasma was prepared from the duplicate samples, which were analyzed in parallel by the RIA procedure. There was substantial reduction (18–30%) of the apparent plasma concentration of chlorpheniramine in all six samples when the blood was allowed to touch the stoppers. This significant lowering of apparent plasma concentration in chlorpheniramine is similar to that reported for other weakly basic lipophilic drugs (30). Therefore, for

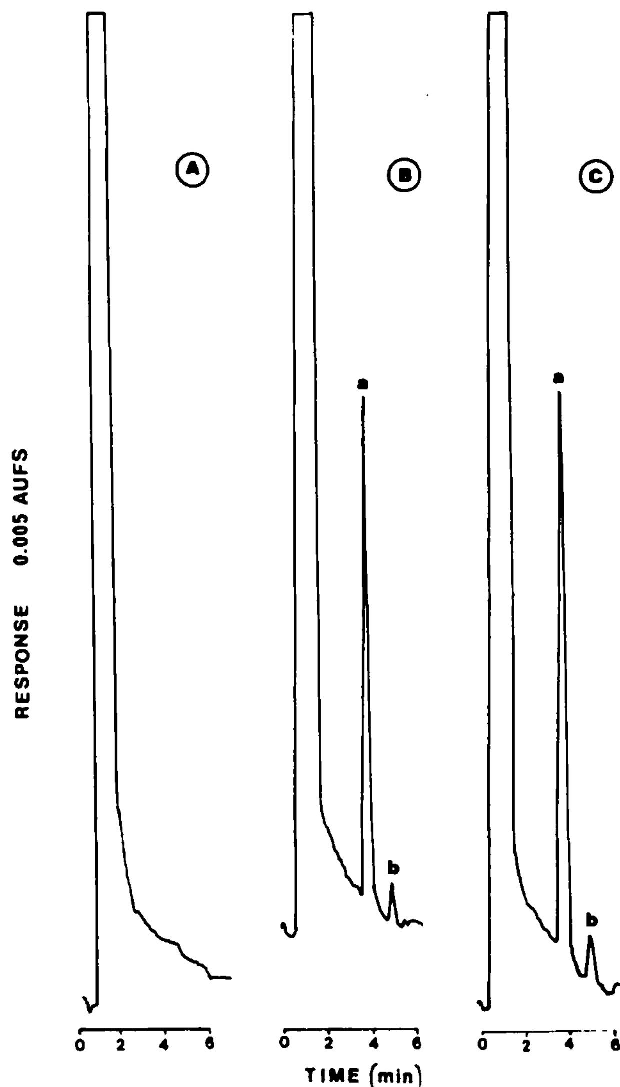

Figure 2—*Chromatograms of extracts from 2.0 mL of plasma. Key; (A) blank plasma; (B) plasma spiked with chlorpheniramine (b, 5.0 ng/mL) and internal standard prochlorperazine (a, 100.0 ng/mL); (C) plasma sample from a volunteer (56 kg) 7.5 h postdose (chlorpheniramine maleate, 4 mg), estimated to contain 6.1 ng of the drug per mL of plasma.*

chlorpheniramine, during blood collection procedures, contact with the rubber stoppers should be avoided.

High-Performance Liquid Chromatography—Under the HPLC conditions described above, chlorpheniramine and the internal standard prochlorperazine gave sharp and symmetrical peaks that eluted in 6 min (Fig. 2). No interference from endogenous plasma constituents was observed. Figure 2B shows a chromatogram of a spiked plasma sample containing 5.0 ng of chlorpheniramine/mL and 100 ng of prochlorperazine/mL. Figure 2C shows a chromatogram of a 7.5-h postdose plasma sample (2 mL) from a volunteer (56 kg) who received 4 mg of chlorpheniramine maleate orally, with the chlorpheniramine concentration estimated as 6.1 ng/mL. *N*-Demethylchlorpheniramine and *N,N*-didemethylchlorpheniramine do not interfere in the assay, as they are eluted at 4.2 and 3.1 min, respectively, as compared with chlorpheniramine and the internal standard, which elute with retention times of 5.7 and 4.7 min, respectively. Brompheniramine may also be amenable to analysis by the described HPLC assay, as it elutes at a retention time of 5.4 min.

Table III shows a composite standard curve for the quantitation of chlorpheniramine by HPLC from plasma. The curve is linear with a negligible intercept over the concentration range of 5–25 ng/mL. The ratios of chlorpheniramine to prochlorperazine peak heights plotted against chlorpheniramine concentrations gave a straight line passing through the origin (r^2 = 0.999). A mean slope value of 0.0102 ± 0.00089 was obtained. The sensitivity at 229 nm for chlorpheniramine (Fig. 2) is such that the HPLC assay can quantitate 2.5 ng/mL of the drug in plasma. This sensitivity is comparable

Table III—HPLC Estimation of Chlorpheniramine Added to Plasma [a]

Concentration, ng/mL	n	Mean Peak Height Ratio	SD	RSD, % [b]
5	6	0.0517	0.0017	3.39
10	6	0.1024	0.0060	5.90
15	6	0.1552	0.0085	5.47
20	7	0.2051	0.0054	2.64
25	8	0.2555	0.0130	5.08

[a] $y = mx$, where $m = 0.0102 \pm 0.00089$ and $r^2 = 0.99996$. [b] Mean $RSD = 4.5\%$.

with that reported for chlorpheniramine by a reverse-phase system with UV detection at 254 nm (17).

The overall recoveries of chlorpheniramine obtained from seven determinations, each at two concentrations (5.25- and 20.25-ng/mL of plasma) were 78.15 ± 2.71 and 78.43 ± 5.92, respectively. A mean percent recovery of 78.29 ± 4.32 was considered satisfactory.

Application of the described HPLC procedure to plasma concentration determinations in two healthy volunteers (56 and 60 kg), each of whom received a single oral dose of a commercial preparation[19] containing 4 mg of chlorpheniramine maleate, is shown in Fig. 1. As can be seen, the method is of sufficient sensitivity to analyze specimens obtained up to 48 h after this low oral dose, whereas the RIA procedure could measure the plasma concentrations in the specimens up to 96 h.

DISCUSSION

Sensitive, specific, and reproducible procedures are essential in order to carry out pharmacokinetic investigations of drugs which are given in small doses. Rapidity, high sample turnover, and low cost would be advantageous in encouraging clinicians to monitor plasma levels in individuals who do not respond to a certain dose of a drug.

For chlorpheniramine, two such methods are described here. The RIA procedure has the simplicity and sensitivity to measure plasma concentrations of chlorpheniramine and/or its *N*-dealkylated metabolites in specimens collected as late as 96 h after administration of single low doses, such as 4 mg. Because of its sensitivity, the RIA procedure is well suited to determine plasma chlorpheniramine levels in infants and children where the available plasma volume may not be large. The described HPLC procedure could measure concentrations up to 48 h after administration of single low doses in the two volunteers studied here. The plasma half-lives of chlorpheniramine after the single oral dose of 4 mg of the maleate salt in these two volunteers were estimated by the HPLC method to be 17 and 23 h, which are comparable with previous reports (2, 3). However, these half-lives were estimated by the RIA procedure for these volunteers as 35 h in each case. This over-estimation in half-lives is probably due to the cross-reactivity (17%), especially of the active *N*-demethyl metabolite with the antiserum used. Similarly, area under the plasma concentration time curves up to 48 h (AUC_0^{48}) by RIA for the two subjects were 330 and 222 ng·h/mL, respectively, whereas these values by HPLC were 205 and 187 ng·h/mL, respectively. This overestimation of AUC_0^{48} and 1.5-2.0-fold increase in half-life values between RIA and HPLC suggest that the RIA may not be useful in pure pharmacokinetic studies of chlorpheniramine.

The increases of 61 and 19% in the AUC_0^{48} value when measured by RIA probably represent the contributions due to the *N*-demethyl and, to a lesser extent, the *N,N*-didemethyl metabolites. The *N*-demethyl and *N,N*-didemethyl metabolites could not be detected by Huang *et al.* (2) in plasma after single oral doses. After both single and multiple doses of chlorpheniramine, the *N*-demethyl and *N,N*-didemethyl metabolites constitute 22 and 3%, respectively, of the total administered dose of the drug excreted in urine. This raises the possibility that there may be other unknown metabolites, which are responsible, at least in part, for the difference in results from RIA and HPLC. In spite of the cross-reactivity of the antiserum to the *N*-demethyl metabolite (17%), the RIA procedure, when compared with the HPLC method, gave a slope of 1.21 with a positive intercept of 0.46 and a correlation coefficent of 0.88 in plasma samples from the two healthy volunteers, who were each given orally a single dose of chlorpheniramine maleate.

The described RIA procedure is simple, precise, and capable of handling a large number of samples. This RIA may be useful for comparative bioequivalency studies of different formulations and for monitoring compliance. The HPLC method is reliable and should be well suited for pure pharmacokinetic studies.

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