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CHLORPHENIRAMINE

I. RAPID QUANTITATIVE ANALYSIS OF CHLORPHENIRAMINE IN PLASMA, SALIVA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method was developed for the rapid quantitative analysis of chlorpheniramine in plasma, saliva and urine using high-performance liquid chromatography. A diethyl ether or hexane extract of the alkalinized biological samples was extracted with dilute acid which was chromatographed on a reversed-phase column using mixtures of acetonitrile and ammonium phosphate buffer as the mobile phase. Ultraviolet absorption at 254 nm was monitored for the detection and brompheniramine was employed as the internal standard for the quantitation. The effects of buffer, pH, and acetonitrile concentration in the mobile phase on the chromatographic separation were investigated. A mobile phase 20% acetonitrile in 0.0075 *M* phosphate buffer at a flow-rate of 2 ml/min was used for the assays of plasma and saliva samples. A similar mobile phase was used for urine samples. The drug and internal standard were eluted at retention volumes of less than 17 ml. The method can also be used to quantify two metabolites, didesmethyl- and desmethylchlorpheniramine, in the urine. The method can accurately measure chlorpheniramine levels down to 2 ng/ml in plasma or saliva using 1 ml of sample, and should be adequate for biopharmaceutical and pharmacokinetic studies. Various precautions for using the assay are discussed.

INTRODUCTION

Chlorpheniramine maleate, a potent antihistamine with little side-effect of somnolence, is incorporated in a variety of pharmaceutical preparations for the symptomatic alleviation of the common cold and various allergic diseases [1]. However, despite its widespread use, surprisingly little has been reported

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about its pharmacokinetic profiles in man. Most of the reported studies were confined to the estimation of renal excretion of the drug [2—4] or the drug and its metabolites [5—7]. These studies showed a large variation in the amount of the unchanged drug excreted in urine, as pointed out by Kamm et al. [7].

In some earlier reports, spectrophotometric methods were used to assay chlorpheniramine in urine for urinary excretion studies [2, 3]. More specific gas—liquid chromatographic (GLC) methods for the assay of chlorpheniramine in biological fluids have since appeared in the literature [4—12]. Beckett and Wilkinson [4], using a GLC method for the assay of drug in urine samples, showed that the renal excretion of chlorpheniramine in man was dependent on the urinary pH and flow-rate. The same general assay procedure was employed in the subsequent studies on the renal excretion of chlorpheniramine and its metabolites in man by Kabasakalian and coworkers [5, 6]. Kamm et al. [7] studied the metabolism of chlorpheniramine in rats and dogs using another GLC method. These investigators showed that didesmethyl- and desmethylchlorpheniramine were the major metabolites of chlorpheniramine [7]. Albert and Windheuser [8] used ion-pair column extraction to separate chlorpheniramine and its metabolites from the biological samples in their GLC assays. All the above-mentioned GLC assays, as well as the methods of Kazyak and Knoblock [9], and Street [10], were developed only for the quantitation of drug and/or metabolites in urine.

Townley et al. [11] reported a GLC method that can measure nanogram quantities of chlorpheniramine in plasma. However, their procedure is quite complicated, involving six replicates of extractions between plasma and solvent and between solvent and aqueous acid or base, and requires up to 5 ml of plasma for each assay [11]. More recently, another GLC method was reported for the assay of plasma chlorpheniramine in a study to investigate relative bioavailability among dosage forms [12]. This method requires 1 ml of plasma to detect 100 ng of drug. It is quite doubtful that such sensitivity is suitable for the measurement of chlorpheniramine concentration in plasma after normal dosing. Lange et al. [13] reported another method for the assay of chlorpheniramine in plasma. These authors used thin-layer chromatography (TLC) to separate the drug from the constituents of plasma supernate, extracted the drug from the adsorbant on the developed TLC plates, and measured the fluorescence generated after reaction with rose bengal [13]. This method is obviously too elaborate and lengthy.

The purpose of this paper is to describe a simple and sensitive high-performance liquid chromatographic (HPLC) method for the quantitation of chlorpheniramine in plasma, saliva and urine after a single dose. In addition, a simpler procedure is also developed for the simultaneous determination of chlorpheniramine and its two N-demethylated metabolites in urine.

EXPERIMENTAL

Reagents

Chlorpheniramine maleate, brompheniramine maleate, desmethylchlorpheniramine and didesmethylchlorpheniramine were all generously supplied by Schering (Bloomfield, N.J., U.S.A.). Brompheniramine was used as an in-

ternal standard. Glass-distilled acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Most of the other chemicals were of reagent grade.

Source of biological samples

Samples of plasma, saliva and urine were collected at various time intervals for one or two days after single doses of chlorpheniramine maleate administered to healthy subjects either orally or intravenously. Blood samples were collected in heparinized tubes by venipuncture and centrifuged to separate the plasma. Saliva samples were collected in glass vials by mastication and transferred to small culture tubes for centrifugation to remove any particulate debris. The volume and pH of urine from each voidance were measured and aliquots of about 10 ml were preserved. All the biological samples were stored frozen in screw-capped culture tubes until analysis. Blank samples of plasma, saliva and urine were obtained from each subject before drug administration and processed likewise to serve as controls.

HPLC instrumentation

An HPLC solvent delivery pump (Model M-6000A), a sample injector (Model U6K), a 30 cm × 3.9 mm I.D. column prepacked with 10 μm porous silica particles chemically bonded with a monomolecular layer of octadecylsilane (μBondapak C_{18}), and a UV detector with an 8-μl flow-cell and 254 nm filter (Model 440), all from Waters Assoc. (Milford, Mass., U.S.A.), were used in this study.

Selection of mobile phase

Aqueous solutions of chlorpheniramine and the internal standard were initially chromatographed to select a suitable mobile phase. The binary mixtures consisted of various proportions of acetonitrile—water, acetonitrile—phosphoric acid (0.05%, pH 2.5), and acetonitrile—phosphate buffer which were tested as mobile phases in the initial studies. The phosphate buffer was prepared by acidifying ammonium phosphate (monobasic, 0.05 *M*) with phosphoric acid to a pH of about 2.5. All aqueous components of the mixtures were passed through a 0.45 μm HA filter (Millipore, Bedford, Mass., U.S.A.) prior to mixing with acetonitrile. From the chromatograms so obtained, the chromatographic parameters of capacity (k'), selectivity (α), and resolution (R) were calculated. The mobile phase that gave desirable retention and resolution was then tried with any necessary adjustment for the chromatography of biological samples.

Sample preparations

Plasma and saliva samples. Plasma samples in 1-ml aliquots in 13 × 100 mm screw-capped disposable culture tubes were supplemented with 100 μl of brompheniramine maleate (1000 ng/ml) in 0.5% phosphoric acid, basified with 300 μl of 5% KOH to a pH of about 11.5, and extracted with 3 ml of diethyl ether by shaking for 3 min followed by centrifugation at ca. 900 *g* for 2 min. These sample tubes were allowed to stand in a shallow bath of dry-ice—alcohol mixture such that only the lower aqueous layers were allowed to submerge for

freezing. The diethyl ether layers were poured into another set of disposable tubes containing 100 μl of 0.5% phosphoric acid. These mixtures were shaken, centrifuged, and the lower layer frozen as before. The diethyl ether layers were poured out and discarded. The aqueous layers were kept at room temperature in a vortex-evaporator (Buchler Instruments, Fort Lee, N.J., U.S.A.) at reduced pressure for 10—15 min to remove traces of diethyl ether. Most of the remaining aqueous solutions were taken up in a syringe for chromatography. Saliva samples were prepared in the same manner.

Urine samples. Aliquots of 500 μl of urine samples were transferred to 13 × 100 mm screw-capped disposable culture tubes. These tubes were supplemented with 100 μl of aqueous stock solution of brompheniramine maleate, 10 μg/ml, basified with 100 μl of 10% potassium hydroxide, and extracted with 3 ml of hexane (the screw-cap was lined with a piece of aluminum foil to prevent possible leaching of chemicals from the cap and also adsorption of the drugs and metabolites onto the cap) by vortexing for 1 min. After centrifugation at ca. 900 *g* for 3 min, approximately 2 ml of the hexane extract were transferred to another disposable tube containing 200 μl of 0.5% phosphoric acid. After vortexing and centrifugation aliquots of 50 μl of the aqueous acid extracts were injected into the column through the injector for chromatography.

Chromatographic separation and quantitation

For plasma and saliva samples the mobile phase used was acetonitrile—phosphate (0.075 *M* $NH_4H_2PO_4$ in 0.16% H_3PO_4) solution (20:80, v/v). For urine samples the phosphate solution was made up of 0.05 *M* $NH_4H_2PO_4$ in 0.11% H_3PO_4. The flow-rate was set at 2 ml/min. The effluent from the column was monitored by UV absorption at 254 nm with a 0.002 a.u.f.s. sensitivity setting. The chart speed of the recorder was set at 10, 15, or 20 cm/h. Peak height ratios were used for the quantitation based on the calibration curves established on the same day. The calibration curves were prepared from the results of assays on biological fluids supplemented with known quantities of the drug, metabolites and the internal standard. All the chromatographic separations were carried out at ambient temperatures.

RESULTS AND DISCUSSION

Octadecylsilane-bonded microparticulate silica is one of the most commonly used column packings in reversed-phase HPLC. In this type of chromatography, not only is the column efficiency extremely high but also the chromatographic behavior is usually quite predictable on the basis of pH of the eluent and the pK_a and partition coefficient of the eluate [14]. When the pure chlorpheniramine and internal standard were chromatographed on a reversed-phase column using nonacidified mobile phase, they were strongly retained. For example, their retention volumes were greater than 90 ml using an eluent of 40% acetonitrile in water. When the mobile phase was acidified, these compounds were eluted in much smaller retention volumes. This is probably due to the basic property of the compound. Furthermore, with the acidic mobile phase (pH 2.5) the peaks obtained were more symmetrical than the

peaks obtained with a higher pH of the mobile phase.

Chromatograms with differing degrees of resolution were found from the mobile phases with different acetonitrile composition (ranging from 20 to 70%) and dilute phosphoric acid. The chromatographic parameters, k', α, and R, calculated from these chromatograms, are summarized in Table I. Both the α and R values increased as the acetonitrile content was reduced. However, the k' values were found to decrease initially with the reduction in acetonitrile content in the mobile phase system containing more than 40% of acetonitrile. The k' values then gradually increased as the acetonitrile content was further reduced. The above biphasic phenomenon can probably be rationalized by the opposing effect of acidity (amount of phosphoric acid) and water content in the mobile phase. The higher the acidity of the mobile phase, the higher the solubility of chlorpheniramine in the mobile phase and hence the lower the capacity factor. The opposite is true for the water content. The above results also show that a better resolution of chromatograms could be obtained with a higher ratio of the aqueous phase at the expense of longer elution time. With 20% acetonitrile mobile phase, the internal standard had a retention volume of about 24 ml.

Surprisingly, the incorporation of the inorganic salt, ammonium phosphate, in the eluent was found to reduce the retention volumes and improve the separation. The chromatographic behavior of chlorpheniramine and the internal standard was studied using mobile phases containing ammonium phosphate (0.05 *M*, monobasic) with acetonitrile in the useful range of 20—25%. The

TABLE I

EFFECTS OF COMPOSITION OF THE MOBILE PHASE ON CHROMATOGRAPHIC PARAMETERS OF CHLORPHENIRAMINE AND BROMPHENIRAMINE

Capacity factor subscripts 1 and 2 for chlorpheniramine and brompheniramine, respectively. Resolution calculated as quotient of difference of retention times divided by average of peak widths, and not obtainable with acetonitrile 40% or higher in phosphoric acid.

Mobile phase	Capacity factor		Selectivity factor	Resolution
	k'_1	k'_2	(α)	(R)
Acetonitrile—phosphoric acid*				
70:30	2.98	3.09	1.04	—
50:50	2.56	2.71	1.06	—
40:60	2.10	2.25	1.07	—
30:70	2.52	2.95	1.17	0.58
25:75	2.82	3.73	1.32	0.74
20:80	3.46	4.71	1.36	0.85
Acetonitrile—phosphate buffer**				
25:75	1.66	2.15	1.30	0.70
22:78	2.79	3.67	1.31	1.10
20:80	2.80	3.87	1.38	1.12

*0.05% Phosphoric acid.

**Ammonium phosphate (monobasic) 0.05 *M* with the pH adjusted to 2.5 by addition of phosphoric acid.

results of this study are also summarized in Table I. All the parameters, k', α, and R, were found to increase with reduction of the proportions of the organic solvent in this range. When the ammonium phosphate concentration was increased to 0.075 M, there was a slight improvement in the chromatography. Thus, the mobile phases consisting of 20—25% acetonitrile and acidified phosphate buffer (0.05 or 0.075 M) appears to give the best results as regards resolution and elution time. In our study on plasma, saliva and urine samples, a mobile phase containing 20% acetonitrile was chosen due to a better separation of chlorpheniramine peak from the interfering peaks of endogenous substances.

Typical chromatograms from plasma and urine samples of normal adult subjects after receiving chlorpheniramine maleate, together with those from their blank samples, are shown in Figs. 1 and 2. A typical plasma level profile is shown in Fig. 3. Chromatograms from saliva samples are similar to the plasma samples. No interferences with peaks of the drug and internal standard

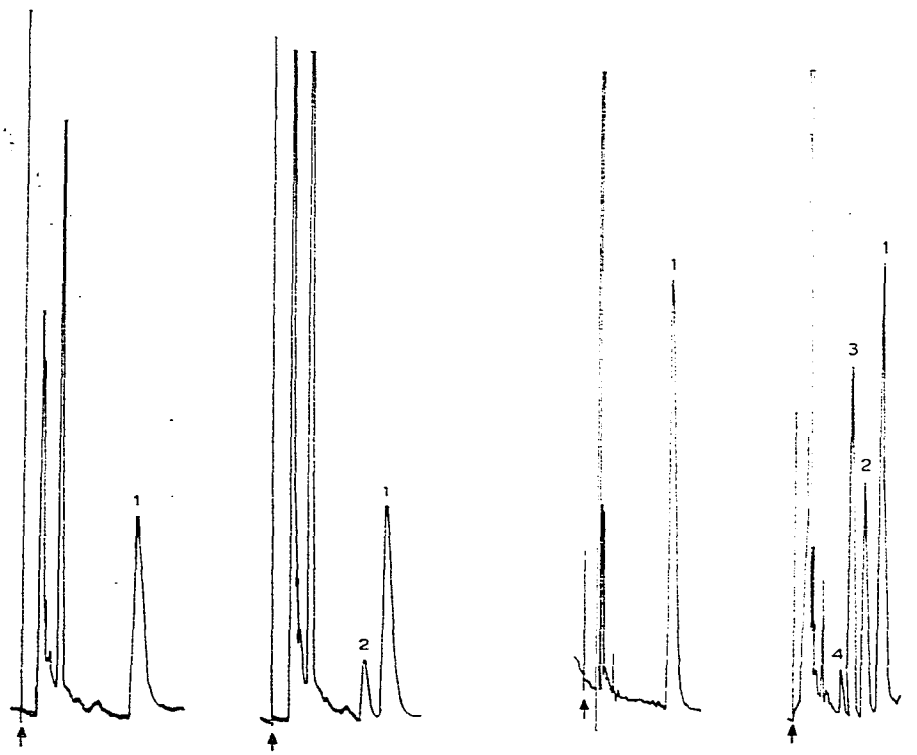

Fig. 1. Chromatograms from a control plasma sample (left) and a plasma sample (right) collected 4 h after an oral dose of 10 mg chlorpheniramine maleate solution to a normal subject. Peaks: 1, brompheniramine; 2, chlorpheniramine (15.4 ng/ml). The arrows mark the point of injection.

Fig. 2. Chromatograms from a control urine sample (left) and a urine sample (right) collected between 24 and 36 h after an oral dose of two 4-mg chlorpheniramine maleate tablets to a normal subject. Peaks: 1, brompheniramine; 2, chlorpheniramine (394 ng/ml); 3, desmethylchlorpheniramine (568 ng/ml); 4, didesmethylchlorpheniramine (62 ng/ml). The arrows mark the point of injection.

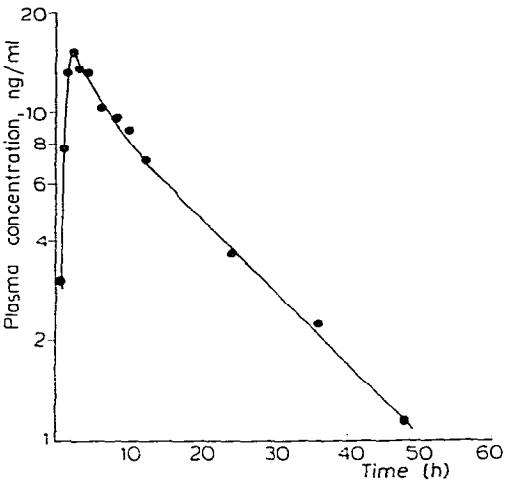

Fig. 3. Chlorpheniramine plasma concentration profile following an oral dose of 10 mg chlorpheniramine maleate in aqueous solution to a normal subject.

were found in most plasma, saliva and urine samples obtained from numerous normal subjects participating in the oral absorption study. For the plasma and saliva samples the retention times for the drug and internal standard were 6.6 and 7.8 min, respectively. For the urine samples the retention times for didesmethylchlorpheniramine, desmethylchlorpheniramine, chlorpheniramine and brompheniramine were 4.4, 5.24, 6.6, and 8 min, respectively. Chlorpheniramine and its metabolites were found in all urine samples collected up to 48 h after an oral dose of 8 mg of chlorpheniramine maleate. No metabolites could be detected in plasma and saliva by the present method.

Within the concentration range of 3.52—25.16 ng/ml (equivalent to 5—40 ng/ml of maleate salt) for both plasma and saliva samples, the drug—internal standard peak height ratios were found to be linear. This is illustrated in the response factor data for plasma samples as shown in Table II. The coefficient of variation of the response factors studied in the same day is usually between 5 and 8% for plasma samples. For the same plasma sample supplemented to yield 3.52 ng/ml of chlorpheniramine the coefficient of variation of the response factors obtained on 10 days over a three-week period is 10.5%. Excellent reproducibility of plasma assay was demonstrated with two HPLC units from our laboratory and four HPLC units from ArnarStone Labs. (Mount Prospect, Ill., U.S.A.) involving several thousands of sample analyses. The over-all extraction recoveries of the drug and internal standard from plasma and saliva samples were about 85%.

For urine samples the detector responses were found to be linear for both the drug and its two metabolites in the range 100—1500 ng/ml. The results are summarized in Table III. In one replicate study (n = 5) on a urine sample collected between 24 and 34 h after an 8 mg oral dose in an adult, the coefficients of variation for didesmethylchlorpheniramine (43.5 ng/ml), desmethylchlorpheniramine (335 ng/ml) and chlorpheniramine (205.6 ng/ml) were 6.62, 9.11 and 4.24%, respectively. The extraction efficiencies for urine samples

TABLE II

TYPICAL RESPONSE FACTORS OF CHLORPHENIRAMINE AT VARIOUS CONCENTRATIONS IN PLASMA

Chlorpheniramine concentration (ng/ml)		Response factor* (ml/ng)
3.5		0.0156
7		0.0167
14		0.0167
21		0.0181
28		0.0174
	Mean ± S.D.	0.0169 ± 0.00096
	C.V. (%)	5.68%

*Drug–internal standard peak height ratio divided by the drug concentration.

TABLE III

RESPONSE FACTORS OF CHLORPHENIRAMINE AND ITS TWO METABOLITES AT VARIOUS CONCENTRATIONS IN URINE

Response factor calculated as drug or metabolite–internal standard peak height ratio × 10^3, divided by the concentration of drug or metabolite based on the average of two determinations.

Spiked concentration (ng/ml)	Chlorpheniramine (ml/ng)	Didesmethyl chlorpheniramine (ml/ng)	Desmethyl chlorpheniramine (ml/ng)
100	1.364	1.799	1.504
250	1.291	1.734	1.321
500	1.281	1.589	1.316
1000	1.309	1.744	1.355
1500	1.426	1.701	1.402
Mean ± S.D.	1.334 ± 0.061	1.713 ± 0.078	1.380 ± 0.078
C.V. (%)	4.54	4.55	5.62

were found to be 66, 76, 98 and 84% for didesmethylchlorpheniramine, desmethylchlorpheniramine, chlorpheniramine and brompheniramine, respectively.

Although the UV absorption maximum of chlorpheniramine in the mobile phase was found to be at about 263 nm, the detection at 254 nm used in this study did give satisfactory sensitivity. The excellent signal-to-noise ratio using the 254 nm fixed wavelength detector (mercury lamp) allowed use of a 0.002 a.u.f.s. sensitivity setting for plasma, saliva and urine samples. A higher sensitivity can also be obtained by using 2 or 3 ml of plasma or saliva samples.

A slow chart speed of 10 cm/h was employed in most studies which would not only reduce the cost of assay but also make the peak height measurement easier and more consistent. The accuracy of the measurement of peak heights, especially for those smaller than 2 cm, appears to be enhanced greatly by the

use of a micrometer (Vernier Caliper from Fisher Scientific, Chicago, Ill., U.S.A.) as opposed to the conventional ruler.

Somewhat different extraction procedures and chromatographic conditions are required for plasma/saliva samples and for urine samples. These modifications were made to accommodate the large differences in the concentrations of chlorpheniramine in these samples and to avoid interferences by the normal constituents of these biological fluids. In general, the concentrations of chlorpheniramine in urine were found to be as much as 50—100 times higher than in plasma and saliva. The flow-rate of the mobile phase had an effect on the peak height obtainable at a given concentration of drug. A flow-rate of 2 ml/min appeared to give peaks about 5—10% higher with slightly slower or faster flow ratios. This flow-rate was used for all samples studied. The extraction and chromatographic procedures for the plasma/saliva samples are also suitable for urine samples. The procedure outlined for the urine samples, however, is simpler and faster, and yet with sufficient sensitivity for the concentrations of the drug and its two metabolites normally encountered in urine.

There was found to be no interference in the HPLC assay reported by the presence of the following commonly used drugs in the same sample: aspirin, salicylic acid, acetaminophen, phenacetin, theophylline, phenobarbital, dilantin, procainamide, propranolol, and ephedrine. This specificity study was carried out on biological samples supplemented with these drugs. No in vivo study of concomitant administration of these drugs with chlorpheniramine was made. Biological samples taken from subjects who took pseudoephedrine, atropine, scopolamine, hysocycamine, and phenylpropanolamine showed no interference with the assay.

In the assay development a significant fraction of chlorpheniramine from its aqueous solutions at low concentrations (5—100 ng/ml) was found to be adsorbed by various types of glassware. This problem was overcome by preparing stock solutions in dilute (0.05%) phosphoric acid solution. It is of interest to note that the source of disposable glass culture tubes used in the extraction could markedly affect the reproducibility and efficiency of the extraction. Those from Fisher Scientific (Cat. No. 14-959-25A) were found to be superior to the Kimble brand (Cat. No. 73750; Vineland, N.J., U.S.A.). A much better recovery of chlorpheniramine was obtained using 0.5% phosphoric acid rather than dilute sulfuric acid with a similar pH for the last extraction step in the plasma assay.

In plasma and saliva assays a freezing technique using the dry-ice—alcohol mixture was employed twice for phase separation. It is important to note that either over-freezing or under-freezing may result in poor quantitation.

Leaching of chemicals from some syringes used for the collection of blood samples may seriously interfere with the assay. In the present study the 6-ml Monoject sterile disposable syringe from Sherwood (Deland, Fla., U.S.A.) was found to leach out various interfering chemicals into blood or water. Syringes from Becton—Dickinson (Rutherford, N.J., U.S.A.) were found to be acceptable.

An unknown impurity from the ultraviolet-grade hexane from the Burdick & Jackson Labs. was found to interfere with the urine assay as it had the same retention time as the internal standard. The pesticide-grade hexane from Fis-

her Scientific contained an impurity with a retention time of 20.8 min. Therefore, caution should be taken when using this hexane for extraction in the urine analysis. For example, it is recommended that a second injection can be started after the appearance of the internal standard peak from the first injection and the third injection should await the appearance of this interfering peak from the first injection.

In summary, simple, rapid, and sensitive high-performance liquid chromatographic methods were developed for the determination of chlorpheniramine in plasma and saliva, and also for the simultaneous determination of chlorpheniramine and its two N-demethylated metabolites in urine. These methods should be useful in biopharmaceutical and pharmacokinetic studies in humans after single or multiple doses. The results of our preliminary pharmacokinetic study in humans will be reported elsewhere.

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