Sensitive GLC–Mass Spectrometric Determination of Chlorpheniramine in Serum

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Abstract □ A new method was developed for the quantitative analysis of chlorpheniramine in serum using the high sensitivity and selectivity of GLC–mass spectrometry with selected-ion monitoring. Intense fragment ions at *m/e* 203 and 207 from the drug and a tetradeuterated analog, respectively, are monitored continuously. The deuterated quantitation standard can be prepared by a simple exchange reaction. A linear calibration curve was obtained over the range of 0–160 ng/ml of serum using 85 ng of the standard/ml. The sensitivity limit is 1–2 ng/ml, and each serum extract can be analyzed in <2 min. The method was applied to a clinical pharmacokinetic investigation and was sufficiently sensitive to measure chlorpheniramine concentrations through at least 1.5 biological half-lives.

Keyphrases □ Chlorpheniramine—GLC–mass spectrometric analysis, pharmacokinetics, human serum □ GLC–mass spectrometry—analysis, chlorpheniramine, pharmacokinetics, human serum □ Pharmacokinetics—chlorpheniramine, GLC–mass spectrometric analysis, human serum □ Antihistaminics—chlorpheniramine, GLC–mass spectrometric analysis, human serum

Chlorpheniramine is an alkylamine antihistamine widely used to alleviate symptoms of the common cold and of allergic reactions. Despite the importance of this drug, little pharmacokinetic data have been published. In three studies using oral dosing with four to six adult subjects, biological half-lives of 12–15 hr (1), 24.5–36.3 hr (2), and 20.6–42.5 hr (3) were reported. The inconsistencies in the available pharmacokinetic data and the unavailability of data in subjects from different age groups with intravenous dosing established the need for further investigation in this area.

BACKGROUND

Chlorpheniramine is a potent antihistamine recommended for use in relatively small doses (4). Previous studies established the need for assay methods that are sufficiently sensitive to measure ~2 ng of drug/ml of serum (1, 2), a fact that contributed to the paucity of pharmacokinetic data. Although several analytical procedures are available to measure chlorpheniramine (1, 2, 5–10), most of the methods were developed to assay the drug in dosage forms or in urine; only a few of these methods have the necessary sensitivity to measure normal serum levels throughout a kinetic investigation.

TLC with liquid scintillation counting (1) cannot be used routinely because it requires administration of radiolabeled drug to human subjects. TLC followed by chemical conversion to an azo dye and subsequent colorimetric analysis (2) is a laborious procedure. The GLC procedure using electron-capture detection is also complicated (9). This method requires the liquid chromatographic separation of the drug from its metabolites and an oxidation reaction as preliminary steps.

Perhaps the best assay available with regard to sensitivity and analysis time involves GLC–mass spectrometry in the selected-ion mode (10). Disadvantages of this procedure for general application include the necessity to synthesize a deuterium-labeled analog of chlorpheniramine for use as the internal standard and the use of chemical-ionization techniques with trimethylamine as the reagent gas. Many analytical facilities are not equipped for chemical-ionization mass spectrometry.

The assay described in this report utilizes the high sensitivity and selectivity of GLC–mass spectrometry operated in the selected-ion mode with electron-impact ionization. A deuterium-labeled internal standard was prepared by a simple exchange reaction. Serum extracts can be analyzed in <2 min to a level of <2 ng of chlorpheniramine/ml. This method was applied to a clinical pharmacokinetic investigation.

EXPERIMENTAL

Materials—Chlorpheniramine[1], monodemethylchlorpheniramine[2], and didemethylchlorpheniramine[2] were obtained as maleate salts. The tetradeuterated analog of chlorpheniramine was prepared by dissolving 50 mg of the free base in 0.5 ml of 98% deuterosulfuric acid in deuterium oxide[3] (99.5 atom % deuterium) and heating the solution in a sealed glass tube at 120° for 6 hr. The contents were cooled and added slowly to 0.5 ml of deuterium oxide[4] (99.8 atom % deuterium).

Exchanged chlorpheniramine was isolated by adding the acidic solution dropwise to 4.2 ml of 5 *N* NaOH with ice bath cooling followed by extraction with ethyl acetate. The extract was washed with water and dried over anhydrous magnesium sulfate, and the solvent was evaporated. The resulting product was analyzed by TLC[5], GLC[6], high-performance liquid chromatography[7], PMR[8], and mass spectrometry[9] and was found to be chemically homogeneous. Deuterium was incorporated specifically in the *p*-chlorophenyl ring to >98% of the theoretical amount. Overall yields of tetradeuterated chlorpheniramine (chlorpheniramine-d_4) averaged 50%.

Instrumentation—A gas–liquid chromatograph–quadrupole mass spectrometer–computer system[9] was used. The gas–liquid chromatograph was equipped with a glass column (2 mm × 1.8 m) packed with 3% OV-22 on 80–100-mesh Supelcoport[10] and operated at a helium carrier gas flow rate of 30 ml/min. The injection port was maintained at 250°, the column oven was at 270°, the jet separator interface was at 270°, and the ion source was at 180°. The mass spectrometer was operated in the electron-impact mode with an electron energy of 70 ev.

Analytical Procedure—Serum samples, 1 or 2 ml, were placed in 16 × 125-mm disposable glass culture tubes, and 85 ng of chlorpheniramine-d_4 dissolved in 20 μl of methanol was added with vortex mixing. A 1-ml volume of saturated sodium borate solution and 8 ml of hexane[11] were added to each tube. The serum was extracted on a mechanical mixer[12] for 20 min and centrifuged at 2000 rpm for 3 min. The hexane layer was transferred to another tube and concentrated under a nitrogen stream at 40° to ~1 ml. To this solution was added 0.5 ml of 0.1 *N* HCl, the tubes were vortexed for 2 min and centrifuged, and the hexane layer was discarded.

The aqueous solution was brought to pH >9 with a saturated sodium borate solution and was extracted with 1 ml of ethyl acetate[13] by vortex mixing for 2 min. The mixture was centrifuged, and the ethyl acetate layer was transferred to a 1-ml vial[14]. The solvent was evaporated under a nitrogen stream with warming at 40°. The inner surface of the vial was rinsed with 100 μl of methanol[11] and evaporated to concentrate chlorpheniramine at the bottom. The residue was redissolved in 50 μl of acetonitrile[11], and the volume was reduced again under nitrogen to <5 μl.

Analyses were performed by injecting the entire sample (volumes of

[1] Courtesy of Cord Laboratories, Broomfield, Colo.
[2] Courtesy of Smith Kline and French Laboratories, Philadelphia, Pa.
[3] Merck and Co., Rahway, N.J.
[4] Norell Chemical Co., Landing, N.J.
[5] Silica gel G plates, Analtech, Newark, Del. The solvent system was ethyl acetate–methanol–ammonium hydroxide (45:4:1).
[6] Model 5710 with flame-ionization detector, Hewlett-Packard, Palo Alto, Calif. The column and column packing are described later and were operated at 180° with programming to 260° at 4°/min.
[7] Model 332 with 4.6 × 250-mm Partisil PAC column, Altex Scientific, Berkeley, Calif. The mobile phase was methanol.
[8] Model EM390, Varian, Sunnyvale, Calif.
[9] Model 5984A, Hewlett-Packard, Palo Alto, Calif.
[10] Supelco, Bellefonte, Pa.
[11] HPLC grade, Fisher Scientific Co., St. Louis, Mo.
[12] Labindustries, Berkeley, Calif.
[13] Spectranalyzed, Fisher Scientific Co., St. Louis, Mo.
[14] Reactivial, Pierce Chemical Co., Rockford, Ill.

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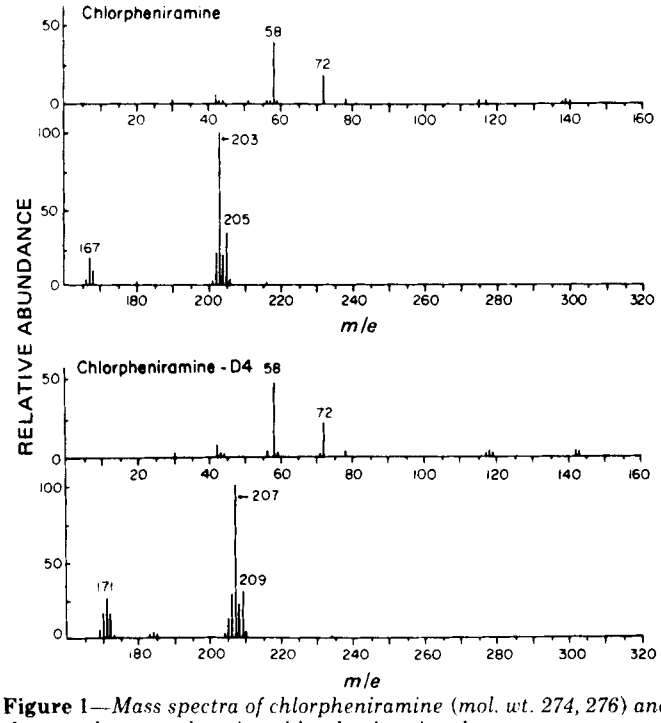
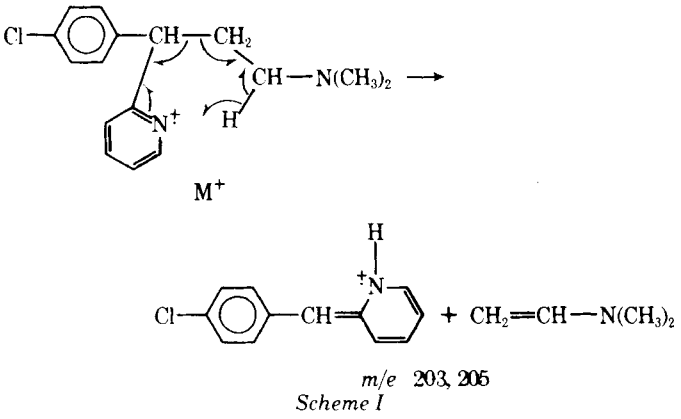


Figure 1—*Mass spectra of chlorpheniramine (mol. wt. 274, 276) and the tetradeuterated analog, chlorpheniramine-*d_4.

2–4 μl) into the gas–liquid chromatograph. After a 30-sec delay, the column effluent was directed into the mass spectrometer, which was operated under computer control in the selected-ion mode. Ions at *m/e* 203 and 207 were monitored continuously with 100-msec dwell times. The chlorpheniramine retention time was 50–60 sec under the conditions employed. Peak areas were determined with the standard system software[9]. Recoveries of chlorpheniramine consistently were in the range from 55 to 70%.

Standard Calibration Curve—Chlorpheniramine maleate was converted to the free base and purified by distillation under reduced pressure. A standard solution was prepared in methanol at a concentration of 1.0 mg/ml. Solutions in methanol were stable for at least 3 months when stored at −20°. Dilutions of the standard solution were prepared at 10, 1.0, and 0.10 μg/ml. Serum samples obtained from a drug-free volunteer were spiked with volumes of the appropriate solution so that no more than 20 μl was added to each milliliter of serum. Chlorpheniramine concentrations were 0, 3, 6, 10, 15, 20, 40, 60, 90, 120, and 160 ng/ml, and each sample contained 85 ng of chlorpheniramine-d_4. Extractions and analyses were performed as described using three or four samples at each concentration. Ratios of *m/e* 203 to 207 peak areas were plotted against known chlorpheniramine concentrations.

Pharmacokinetic Study—A 12-year-old male subject[15] with a history of allergic rhinitis was administered intravenously a 0.01-mg/kg dose of chlorpheniramine maleate over 5 min after removal of a control blood



Scheme I

[15] Treated in the Clinical Research Unit of the National Jewish Hospital, Denver, CO 80206.

Table I—Hydrogen–Deuterium Exchange in the *p*-Chlorophenyl Group of Chlorpheniramine at 100°

Reaction Time, hr	Deuterium Incorporation[a], % of theoretical
0.17	28
0.33	43
1.0	58
4.0	74
8.5	86
14.6	94
24.0	96
48.0	>98

[a] Determined by PMR spectroscopy.

sample. Blood samples (5–8 ml) were drawn periodically, and the serum was prepared immediately and stored at −70° until it was analyzed. It was established that chlorpheniramine is stable under these conditions for at least 1 year. Analyses were performed as described, and peak ratios were converted to chlorpheniramine concentrations with reference to the standard curve. The plasma concentration *versus* time data were computer fitted (11) to a biexponential equation. After correction for the 5-min infusion period, pharmacokinetic parameters were calculated using standard methods (12).

RESULTS

The electron-impact mass spectrum of chlorpheniramine (Fig. 1) reveals an intense ion at *m/e* 203, which probably is formed by fragmentation of the molecular ion (Scheme I). A complementary ion containing chlorine 37 occurs at *m/e* 205. The relatively high mass and intensity of the *m/e* 203 ion provide an excellent opportunity for quantitation by GLC–mass spectrometry with selected-ion monitoring. The preferred internal standards for this technique are isotopic variants of the compounds to be measured (13). Inspection of the chlorpheniramine spectrum reveals no ions with *m/e* values higher than 206 (except for a small *m/e* 216 ion). Therefore, a deuterated analog should contain at least four deuterium atoms in the part of the molecule that is retained in the fragment ion.

Conditions have been developed for the efficient exchange of the four *p*-chlorophenyl hydrogens for deuterium atoms. The progress of this exchange reaction is shown in Table I. Two hydrogens are exchanged in less than 1 hr at 100°, and the reaction is >98% complete after 48 hr. At 120°, exchange is virtually complete after only 6 hr. A mass spectrum of the tetradeuterated analog of chlorpheniramine (chlorpheniramine-d_4) is shown in Fig. 1. The base peak is shifted to *m/e* 207, and no peak occurs at *m/e* 203 that would interfere with chlorpheniramine quantitation.

The brominated analog of chlorpheniramine is another useful antihistamine that can be quantitated by a similar procedure involving preparation of a tetradeuterated internal standard. Fragmentation of brompheniramine produces a mass spectrum with intense ions at *m/e* 247 and 249 (containing bromine 79 and bromine 81, respectively) with no ions of *m/e* higher than 250. The exchange reaction proceeds in a similar manner to yield brompheniramine-d_4 with intense ions at *m/e* 251 and 253.

A standard calibration curve was developed by adding chlorpheniramine in amounts from 0 to 160 ng together with 85 ng of chlorpheniramine-d_4 to 1.0-ml serum samples and extracting these samples as described under *Experimental*. Extracts were analyzed by monitoring the ion currents for ions at *m/e* 203 and 207. A background signal normally is observed at *m/e* 207 due to bleeding of the silicone rubber septum and of the GLC stationary liquid phase. This relatively small and constant signal easily can be subtracted from the signal of chlorpheniramine-d_4.

With the chromatographic conditions used, the retention time of the drug is 50–60 sec. Typical ion-current profiles obtained with a serum extract containing only the internal standard and with an extract containing both the drug and the internal standard are shown in Fig. 2. Analyses of serum samples yielded data for calculation of a regression line with a correlation coefficient of 0.998. Chlorpheniramine was added to four serum samples (2.0 ml each) at 4.0 ng/ml together with the internal standard. These samples were determined to contain 4.2 ± 0.2 ng of drug/ml (mean ± *SD*). The maximum sensitivity of the method is 1–2 ng/ml using 2 ml of serum.

The principal metabolites of chlorpheniramine that could interfere with the analysis are the products of mono- and di-*N*-demethylation (14,

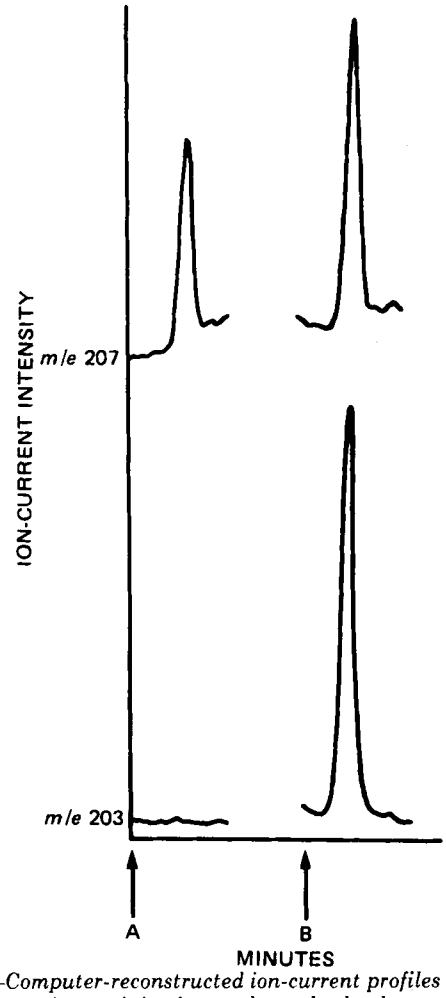


Figure 2—*Computer-reconstructed ion-current profiles of serum extracts. Extract A containing internal standard only was injected at* t = 0. *Extract B containing the internal standard and 10 ng of chlorpheniramine was injected at* t = 2 *min.*

15). The *m/e* 203 ion formed by fragmentation and loss of the amino group appears in the mass spectra of both metabolites. However, with the GLC conditions used in this work, the drug is well separated from its metabolites. The ion-current profiles of chlorpheniramine and monodemethylchlorpheniramine are shown in Fig. 3. The retention time of didemethylchlorpheniramine is greater than that of the metabolite shown, eliminating the possibility of interference with the drug.

The quantitative procedure described here was applied to a pharmacokinetic study of chlorpheniramine in a 12-year-old male subject. The plasma concentration *versus* time curve is shown in Fig. 4, and the selected pharmacokinetic parameters calculated from these data are summarized in Table II.

DISCUSSION

Pharmacokinetic studies of chlorpheniramine require methods capable of producing reliable quantitative data over the range of 2–100 ng/ml of serum. The high sensitivity of the technique involving GLC–mass spectrometry with selected-ion monitoring is particularly well suited to this analysis because of the favorable fragmentation pattern of chlorpheniramine. A high percentage of the total ion current of the compound is due to the *m/e* 203 ion. The selective nature of this analytical technique allows the use of chromatographic conditions with which analyses can be completed rapidly and without interferences from other compounds.

Table II—Pharmacokinetic Parameters for Chlorpheniramine Administered Intravenously to a 12-Year-Old Male Subject

Parameter	Value
Initial dilution volume (V_c)	298.2 ml/kg
Volume of distribution during postdistribution phase (V_β)	562.8 ml/kg
Total body clearance	0.415 ml/min/kg
$t_{1/2}, \alpha$	0.53 hr
$t_{1/2}, \beta$	15.6 hr

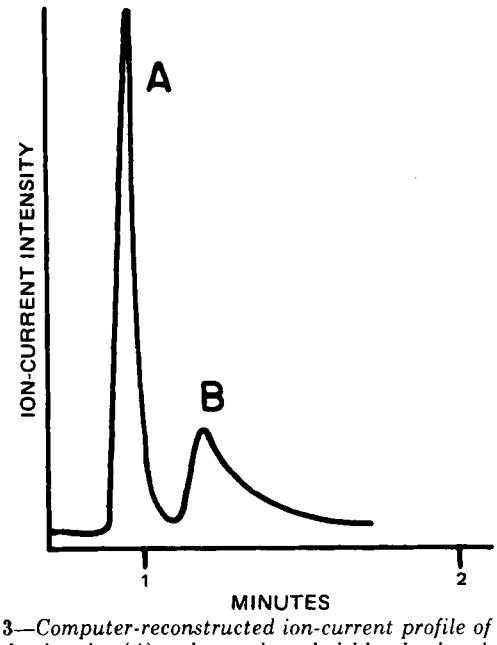


Figure 3—*Computer-reconstructed ion-current profile of a mixture of chlorpheniramine (A) and monodemethylchlorpheniramine (B). The ion current at* m/e *203 was monitored.*

The assay reported here is sufficiently sensitive to measure serum levels 24 hr after a single intravenous dose of 0.01 mg/kg. Each extract can be analyzed in <2 min. The chlorpheniramine assay using GLC–mass spectrometry that was published previously (10) involved the use of chemical-ionization conditions. However, the present results show that

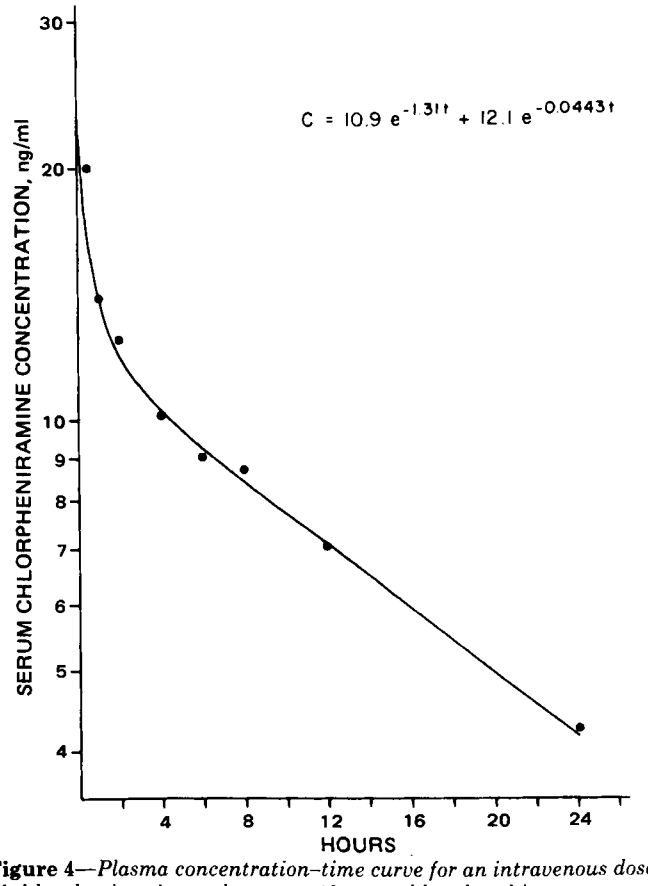


Figure 4—*Plasma concentration–time curve for an intravenous dose of chlorpheniramine maleate to a 12-year-old male subject.*

the more common method of electron-impact ionization is suitable. In addition, the previous investigators used a deuterated analog of chlorpheniramine that must be synthesized. The standard used in the present work can be prepared by a simple exchange reaction and without purification of the deuterated product.

The pharmacokinetic study conducted with one patient demonstrates the utility of the assay. Drug levels during the elimination (β) phase were in the range of 4–10 ng/ml of serum through 24 hr after dosing. These data demonstrate sufficient sensitivity to measure serum levels for a period in excess of 1.5 half-lives of the drug. Biological half-lives reported recently for adults after oral dosing are somewhat higher (2, 3) than the half-life determined in the present study. The young age of the patient and the drug administration route could explain the difference. Work is in progress using the assay described here to obtain pharmacokinetic data for a large group of subjects.

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Determination of Chlorambucil in Plasma by GLC with Selected-Ion Monitoring

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Received September 5, 1979, from the **Karolinska Pharmacy, the* ‡*Laboratory for Mass Spectrometry, Karolinska Institut, and the* §*Department of Oncology, Karolinska Hospital, Box 60024, S-104 01 Stockholm, Sweden.* Accepted for publication January 25, 1980.

Abstract □ A GLC technique with selected-ion monitoring is described for chlorambucil determination in plasma using [^{2}H]chlorambucil as the internal standard. Chlorambucil is extracted from plasma with methylene chloride at pH 3 and converted to a thiazane derivative by reaction with 0.1 *M* sodium sulfide at 80°. The carboxylic group of the chlorambucil derivative is derivatized with allyl bromide using extractive alkylation. Analysis by selected-ion monitoring was performed by focusing at *m/e* 305 (M) and 313. The relative standard deviation was ±5% (n = 5) at the 10-ng/ml level.

Keyphrases □ Chlorambucil—GLC determination with selected-ion monitoring, plasma □ GLC—analysis, chlorambucil, selected-ion monitoring, plasma □ Antineoplastics—chlorambucil, GLC determination with selected-ion monitoring, plasma

Chlorambucil, a nitrogen mustard drug, is used for the treatment of neoplastic diseases. The fate of the drug in humans is partially unknown since analytical methods with sufficient sensitivity and selectivity have not been available. A mass fragmentographic method involving derivatization of the carboxylic group of chlorambucil by methylation was published recently (1). However, large variations in the GLC yield were observed in these laboratories after a derivatization involving alkylation, probably as a result of degradation of the nitrogen mustard group in the GLC system.

This paper presents a GLC technique using selected-ion monitoring for the determination of chlorambucil in plasma. The nitrogen mustard group is converted to a thiazane by reaction with sodium sulfide, followed by extractive alkylation of the carboxylic group.

EXPERIMENTAL

Synthesis of 4-[4-(Thiazane-4-yl)phenyl]butyric Acid (I)—Chlorambucil (25 mg) was dissolved in 10 ml of 0.1 *M* sodium sulfide and heated for 1 hr at 80°. The solution was cooled, acidified (pH ~3) with phosphoric acid, and extracted with methylene chloride (10 ml). The organic phase was separated and evaporated to dryness. The residue was recrystallized from methylene chloride–*n*-hexane (mp 81–82°). The purity (~97%) was established by TLC[1] and potentiometric titration with sodium hydroxide.

The mass spectrum[2] was consistent with the expected structure. There were prominent peaks at *m/e* 266 (20%), 265 (100), 218 (13), 193 (14), 192 (77), 191 (59), 132 (24), 131 (52), 130 (14), 118 (52), 117 (13), and 91 (11). No peak was observed at *m/e* 297, thus establishing that no dithiazane was formed (*cf.*, Ref. 2).

Reaction of Chlorambucil with Sodium Sulfide—Chlorambucil in methanol (0.5 ml) was mixed with an aqueous sodium sulfide solution (10 ml), giving a final chlorambucil concentration of 4 μg/ml. The yield of I was established by liquid chromatography[3] after acidifying part of the solution. The derivatization yields were calculated using the synthetically prepared I as a reference compound.

[1] Silica 60, F_{254}, 0.25 mm, Merck, Darmstadt, West Germany; the eluting solvent was methylene chloride–ethyl acetate (8:2).

[2] LKB 2091 with direct inlet; the ionizing energy was 70 ev.

[3] The chromatographic equipment used was described in Ref. 3. The mobile phase was methanol–water–acetic acid (0.1 *M*) (70:20:10). The support was LiChrosorb RP-18 from Merck, Darmstadt, West Germany.

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