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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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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Table I—Acid Dissociation Constants and Partition Coefficients of Chlorambucil and I

Compound	Dissociation Constants[a]				log Partition Coefficient to Methylene Chloride
	pK_1	pK_2	pK_{12}	pK_{21}	
Chlorambucil[b]	—	2.49 ± 0.02	—	4.46 ± 0.04	4.45 ± 0.04
I	3.68 ± 0.02	4.12 ± 0.02	4.45 ± 0.03	4.00 ± 0.02	3.47 ± 0.04

[a] For definition, see Ref. 3. [b] Microscopic constants from Ref. 3.

Alkylation of I—A solution of the alkylating reagent (I) and the internal standard (decachlorobiphenyl) in methylene chloride (2 ml) was mixed with a solution (2 ml) of 0.1 *M* tetrabutylammonium in 0.1 *M* NaOH. The mixture was shaken at 25°. Part of the organic phase (0.1 ml) was separated and mixed with pH 4 phosphate buffer. The organic phase was analyzed by GLC with flame-ionization detection[4].

Extraction of Chlorambucil from Plasma—Plasma (2.00 ml) containing 8 μg of chlorambucil/ml was mixed with hydrochloric acid, phosphoric acid, or phosphate buffer (0.2 ml). The mixture was extracted with methylene chloride (2.00 ml) for 30 min. An aliquot of the organic phase was separated and evaporated to dryness with nitrogen gas. The residue was dissolved in methanol–water–acetic acid (0.1 *M*) (75:15:10) and analyzed by liquid chromatography[5].

Determination of Chlorambucil in Plasma—Plasma (2.00 ml) was mixed with 0.100 ml of [^{2}H]chlorambucil[6] in ethanol (2.00 μg/ml)[7] and 0.2 ml of 1 *M* phosphoric acid (final pH of ~3) and extracted with 5.00 ml of methylene chloride for 30 min. The organic phase was separated and extracted with 0.5 ml of 0.1 *M* sodium sulfide for 5 min. The aqueous phase was separated and heated for 15 min at 80°. Concentrated phosphoric acid (0.01 ml) was added, and hydrogen sulfide was removed with nitrogen for 5 min. Sodium hydroxide (12 *M*, 0.05 ml), 0.5 *M* tetrabutylammonium (0.2 ml), methylene chloride (0.2 ml), and allyl bromide (0.05 ml) were added, and then the mixture was shaken for 30 min at 25°. The organic phase (1–2 μl) was analyzed by GLC–mass spectrometry[8].

Handling of Blood Samples—Blood samples were obtained by venous puncture using 10-ml glass tubes containing 250 IU of lyophilized heparin. The plasma fraction was separated immediately, and an aliquot was mixed with the internal standard and frozen until it was assayed. Chlorambucil was stable for at least 2 weeks at −20°.

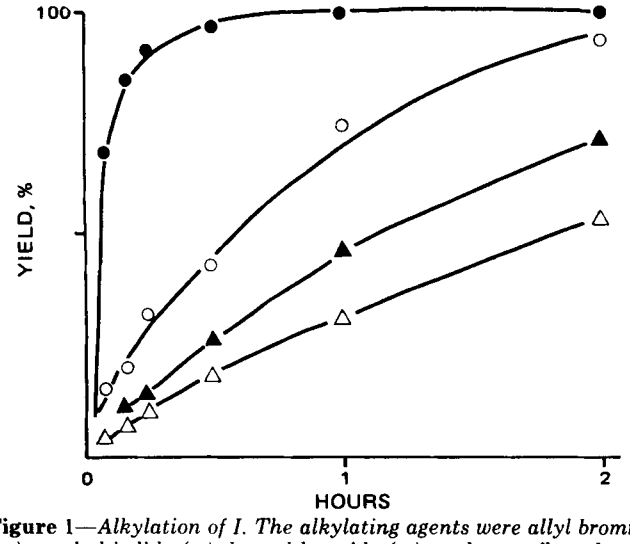

Figure 1—*Alkylation of I. The alkylating agents were allyl bromide (△), methyl iodide (▲), benzyl bromide (○), and pentafluorobenzyl bromide (●); the organic phase was 0.05* M *alkylating agent in methylene chloride; the aqueous phase was 0.100* M *tetrabutylammonium in 0.100* M *NaOH (equal phase volumes); and the temperature was 25.0°.*

[4] Varian 1400 gas–liquid chromatograph. The 1.5-m column was packed with 3% OV-17 on Gas Chrom Q and was operated at 275°.

[5] For the chromatographic equipment and conditions, see Ref. 3.

[6] Kindly supplied by AB Leo, Helsingborg, Sweden. The deuterium labeling was in the bis(2-chloroethyl)amino group.

[7] The internal standard was stable for at least 6 hr at 4° in ethanol.

[8] LKB 2091 with an ionizing energy of 70 ev. The 1.5-m column was packed with 5% OV-17 on Gas Chrom Q and was operated at 250°.

Table II—Extraction of Chlorambucil from Human Plasma

pH	log Distribution Ratio (Yield, %)	
	Calculated	Found
1	2.94 (99.9)	0.18 (60 ± 4)
3.2	4.35 (100.0)	>2 (102 ± 4)
7.0	1.91 (98.8)	0.10 (56 ± 2)

RESULTS AND DISCUSSION

Extraction from Plasma—A quantitative extraction of chlorambucil (log $D \sim 2$) theoretically should be obtained within the pH range of 1–7, as revealed by calculations based on the partition coefficient and the acid dissociation constants (Table I). However, the extraction of chlorambucil from plasma was quantitative only at pH ~3, while the yields at pH 1 and 7 were considerably lower (Table II). The extraction yields were unaffected by extraction times of 10–60 min. Obviously, a considerably higher extraction capacity is needed to obtain a quantitative recovery from plasma as compared to pure aqueous solutions. This effect might be due to the fact that chlorambucil is bound extensively to plasma albumin (4), giving a concomitant reduction in the partitioning to the organic phase.

Thiazane Formation—Nitrogen mustards previously were converted to thiazanes by reaction with sodium [^{35}S]sulfide for qualitative metabolic studies (2), but so far the reaction has not been studied quantitatively. The influence of the sodium sulfide concentration on the yield of thiazane was studied by liquid chromatography (Table III). The reaction was complete within 15 min at 80°. Hydrolysis of chlorambucil to 4-[*p*-[di(2-hydroxyethyl)amino]phenyl]butyric acid [*cf.*, Ref. 3] was insignificant, at least in 0.1 or 1 *M* sodium sulfide, reflecting the high nucleophilicity of the sulfide ion. The thiazane yield was only ~25% with a reaction time of 1 hr at 25° (0.10 *M* sodium sulfide).

Protolytic and Partition Properties of I—The protolytic properties of I were evaluated by a photometric technique described previously (3). The base strength of the amino group was considerably higher for I compared to chlorambucil (pK_2 = 4.12 and 2.49, respectively, Table I).

The lipophilic character of chlorambucil was decreased by the thiazane formation, as revealed by a decrease in the partition coefficient to methylene chloride by ~1 log unit (Table I).

Extractive Alkylation of I—The extractive alkylation of I was performed using methylene chloride as the organic phase and 0.1 *M* tetrabutylammonium in 0.1 *M* NaOH as the aqueous phase. More than 90% of I was extracted as an ion-pair into the organic phase, as calculated from the extraction constant ($\log E = 2.17 \pm 0.02$ and $-\log k_{diss} = 4.19 \pm 0.04$, determined according to Ref. 5). Therefore, an increase in the lipophilic character or concentration of the counterion should only slightly affect the reaction rate (6).

The alkylation rates using different alkylating agents are given in Fig. 1. Pentafluorobenzyl bromide showed the highest reactivity, while the lowest one was obtained with allyl bromide. By increasing the concen-

Table III—Influence of Sodium Sulfide Concentration on Yield of I[a]

Minutes	Sodium Sulfide Concentration, *M*		
	0.01	0.10	1.00
	Yield of I, %		
1	46	44	60
5	82	90	94
15	84	92	97
30	87	99	93
60	87	95	97

[a] The temperature was 80°.

Table IV—Relative Retention of Alkyl Derivatives of I [a]

Derivative	Relative Retention
Methyl	1.00
Allyl	1.46
Pentafluorobenzyl	3.50
Benzyl	7.25

[a] The column was packed with 3% OV-17 and was operated at 275°.

tration of allyl bromide to 1 *M*, a quantitative reaction was obtained within 30 min.

GLC Properties of Alkyl Derivatives of I—All of the alkyl derivatives chromatographed with excellent peak symmetry on an OV-17 column. The relative retentions of the derivatives are given in Table IV. The retention increased in the order methyl, allyl, pentafluorobenzyl, and benzyl. Due to their higher volatility, the methyl and allyl derivatives were selected for further studies regarding their selected-ion monitoring properties.

Selected-Ion Monitoring Properties of Methyl and Allyl Derivatives of I—The mass spectra of the allyl and methyl derivatives of I are given in Fig. 2. By focusing the mass spectrometer at the molecular ions of the compounds, a higher selectivity was obtained with respect to the metabolites formed by enzymatic transformation of the butyric acid side chain of chlorambucil as compared to focusing at *m/e* 192 [4-(thiazane-4-yl)benzyl fragment]. Derivatization with allyl bromide and focusing at *m/e* 305 were used in the analytical method since derivatization of plasma blanks with methyl iodide and detection at *m/e* 279 gave an interfering peak with a retention time close to that of the methyl derivative of I.

Internal Standard—Chlorambucil labeled with eight deuterium atoms in the nitrogen mustard moiety was used as the internal standard. It contained <1% of the unlabeled compound. The degradation rate of the internal standard did not differ significantly from that of chlorambucil.

Selectivity—No peaks interfering with the determination of chlorambucil or the internal standard were observed with blank plasma. The thiazane derivative of chlorambucil theoretically could be formed by the reaction of chlorambucil with endogenous sulfur-containing compounds and, if present, be codetermined with chlorambucil. Plasma samples (1, 2, 5, and 6 hr after an oral dose of 10 mg of chlorambucil) were analyzed according to the described procedure, except that the sodium sulfide extraction step was substituted with extraction with 0.1 *M* NaOH. In all cases, the thiazane concentration was below the detection limit (<1 ng/ml of plasma).

Precision—The standard curve obtained from plasma samples was linear in the range studied (10–640 ng/ml). A least-squares analysis gave a correlation coefficient of 0.9999, a slope of $7.96 \times 10^{-3} \pm 2.19 \times 10^{-5}$, and an intercept of $6.00 \times 10^{-2} \pm 7.36 \times 10^{-3}$. The relative standard deviation was ±5% ($n = 5$) at the 10-ng/ml level. A chromatogram obtained from the analysis of a sample containing 30 ng/ml is shown in Fig. 3.

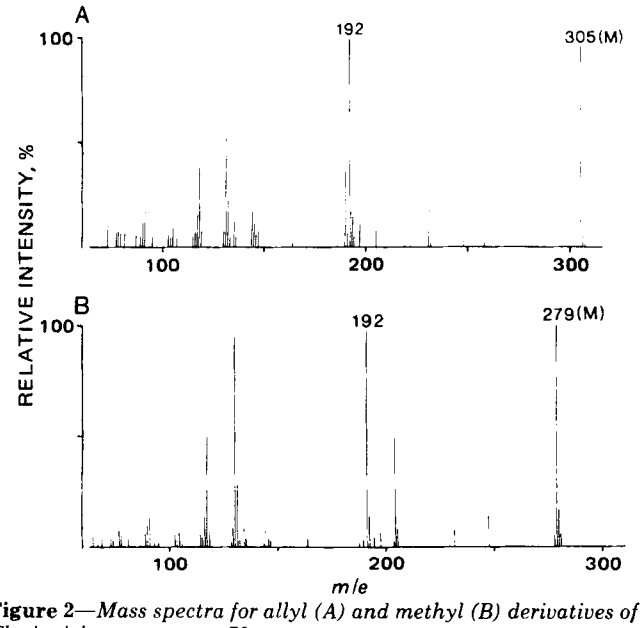

Figure 2—*Mass spectra for allyl (A) and methyl (B) derivatives of I. The ionizing energy was 70 ev.*

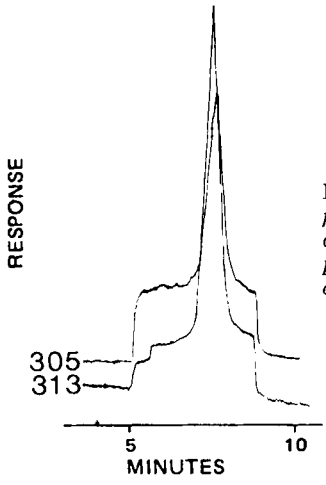

Figure 3—*Chromatogram obtained from patient plasma containing 30 ng of chlorambucil/ml. The chlorambucil peak was at* m/e *305, and the [²H]-chlorambucil was at* m/e *313.*

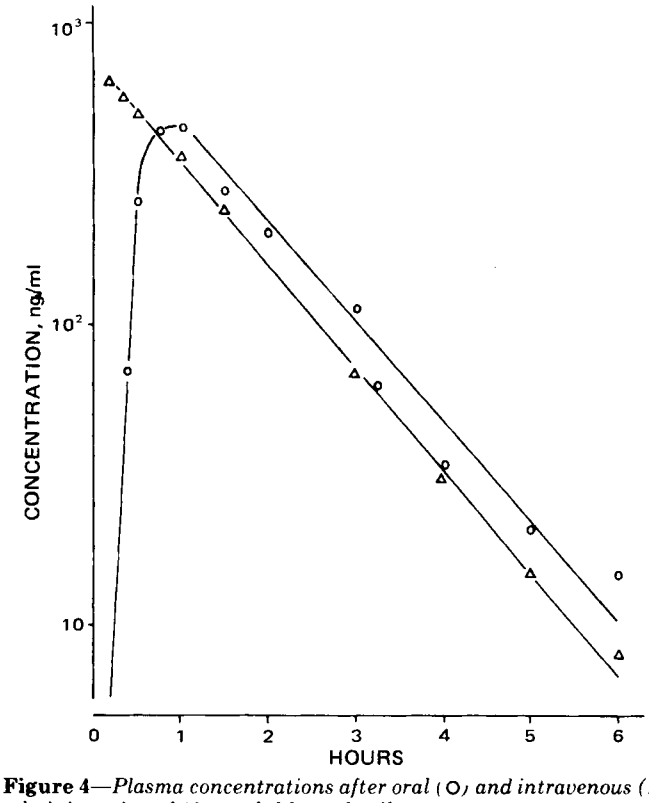

Figure 4—*Plasma concentrations after oral (○) and intravenous (△) administration of 10 mg of chlorambucil.*

Analysis of Plasma from Patients Undergoing Chlorambucil Treatment—The plasma concentration–time profile from a patient receiving 10 mg of chlorambucil orally and intravenously is shown in Fig. 4. A detailed study on the pharmacokinetics of chlorambucil will be published later.

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