

Degradation of Chlorambucil in Aqueous Solution

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Abstract □ The stability of chlorambucil and its degradation product 4-[p-(2-chloroethyl-2-hydroxyethylamino)phenyl]butyric acid (I) was studied using reversed-phase high-pressure liquid chromatography. The degradation rate of chlorambucil was unaffected by pH between pH 5 and 10 but decreased at lower pH. The degradation rates of chlorambucil and I differed only slightly (pH > 6). The proteolytic properties of the compounds were studied using spectrophotometric and partition techniques.

Keyphrases □ Chlorambucil—degradation in aqueous solution, influence of pH and temperature □ Degradation—chlorambucil and metabolites, in aqueous solution, effect of pH and temperature □ Antineoplastic agents—chlorambucil, degradation in aqueous solution, influence of pH and temperature

Nitrogen mustards are important drugs in the treatment of neoplastic diseases. A detailed and accurate knowledge of their chemical properties is important for an understanding of their behavior *in vivo*.

BACKGROUND

The nitrogen mustards are believed to react *via* an intermediate aziridinium ion (1) according to Scheme I. The aliphatic nitrogen mustards given an accumulation of the aziridinium ion, B, during the initial phase of degradation since Step I is faster than Step II (2). The aromatic nitrogen mustards differ in this respect from the aliphatic compounds (3). However, chlorambucil was proposed to follow a reaction profile similar to that of the aliphatic nitrogen mustards based on results obtained using a spectrophotometric procedure involving the reaction of the alkylating agents with 4-(p-nitrobenzyl)pyridine (4). However, this technique is not selective since both the parent compound and the degradation product, C (Scheme I), are derivatized and codetermined (5).

The influence of pH on the degradation rate of chlorambucil was studied previously (6), and a continuous increase in the stability of chlorambucil from pH 7 to 4.5 was observed. However, this study was performed at such a high concentration that the results might have been affected by aggregation phenomena (*cf.*, 7).

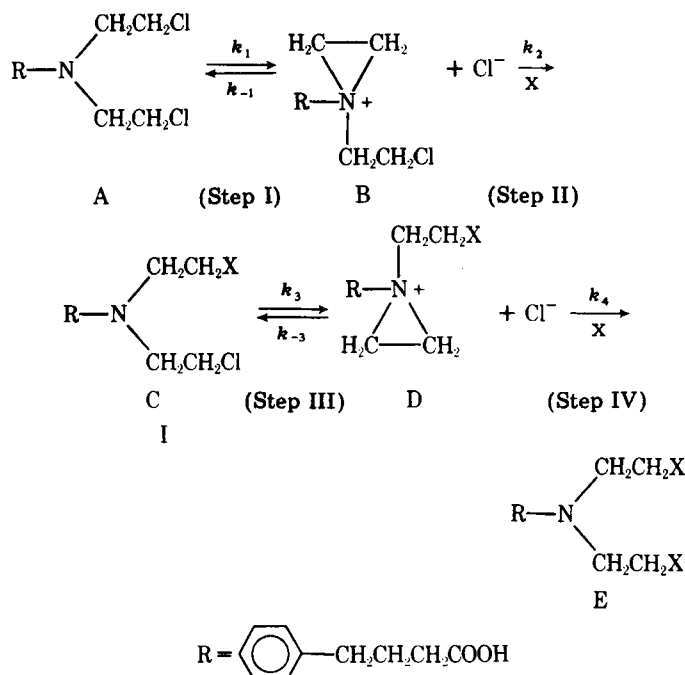
The present investigation considered the stability of chlorambucil and its degradation product 4-[p-(2-chloroethyl-2-hydroxyethylamino)-phenyl]butyric acid between pH 1.5 and 10 using a selective and sensitive high-pressure liquid chromatographic (HPLC) technique. The sensitivity of the technique made study of low concentrations possible, where chlorambucil is present only as the monomer.

EXPERIMENTAL

All experiments were carried out at $25.00 \pm 0.05^\circ$ and at the same ionic strength (0.1) unless otherwise stated.

Preparation of 4-[p-(2-Chloroethyl-2-hydroxyethylamino)-phenyl]butyric Acid (I)—Chlorambucil (10 mg in methanol, 0.5 ml) was mixed with 100 ml of distilled water. The solution was kept at 25° for 2 hr (final pH of 4) and extracted with ethyl acetate (100 ml). The extract was evaporated to 0.2 ml under a nitrogen atmosphere and separated by TLC¹. The silica gel zone containing I was scraped from the plate, packed into a Pasteur pipet, and eluted with methanol (5 ml). The yield was 15% as determined spectrophotometrically. The mass spectrum was consistent with the expected structure. Prominent peaks were located at *m/e* (relative intensity, %) 250 (17), 249 (62), 191 (19), 177 (17), 176 (100), 132 (19), 131 (35), 118 (55), 117 (14), and 91 (18).

¹ Silica gel 60, F₂₅₄, 2 mm, Merck, Darmstadt, West Germany. The eluting solvent was toluene-methanol-heptane-isopropylacetone (8:6:3:4). The spots were 4-[p-(2-hydroxyethylamino)phenyl]butyric acid (*R_f* 0.4), I (*R_f* 0.6), and chlorambucil (*R_f* 0.7).



Scheme I—Degradation of nitrogen mustards

Spectrophotometric Determination of Acid Dissociation Constants—A stock solution of chlorambucil or I in ethanol (3.4×10^{-3} M) was diluted (1:40) with 1 M HCl, phosphate buffer, or citrate buffer. The absorbance was determined at 257 nm². The slit width of the spectrophotometer was kept constant within each experimental series. The absorbance of at least three dilutions was measured at each pH value³.

Determination of Acid Dissociation Constants by Partition Studies—Partition experiments were performed in centrifuge tubes using equal volumes (10 ml) of hexane and buffer and mechanical shaking for 30 (pH < 3.5) or 15 (pH > 3.5) min. Chlorambucil was dissolved initially in the organic phase to a concentration of 1.2×10^{-4} M.

The concentration of chlorambucil was determined spectrophotometrically (257 nm) in both phases. The organic phase was measured directly. Chlorambucil in the aqueous phase was measured by extraction into methylene chloride, buffering to pH 3.4, and mechanical shaking for 30 min.

Degradation Studies—Chlorambucil or I in methanol (0.1 ml) was mixed with the appropriate buffer (10 ml) to give a final alkylating agent concentration of 3×10^{-5} M, unless otherwise stated.

The solutions were analyzed by reversed-phase HPLC⁴.

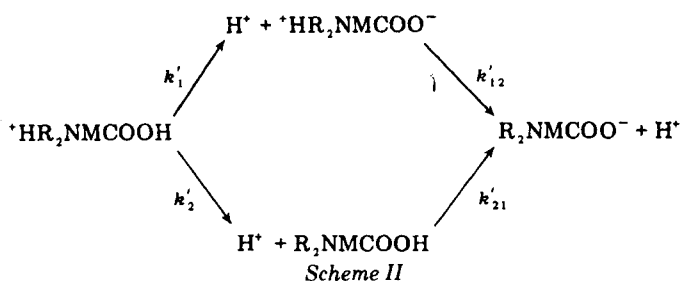
RESULTS AND DISCUSSION

Proteolytic Properties—The general proteolysis of an amino acid is summarized in Scheme II. The compound may occur in four forms: ammonium (⁺HR₂NMCOOH), carboxylate (R₂NMCOO⁻), uncharged (R₂NMCOOH), and zwitterion (⁺HR₂NMCOO⁻). The microscopic constants *k*₁, *k*₂, *k*₁₂, and *k*₂₁ were defined previously (8).

² Zeiss PMQ III spectrophotometer.

³ Orion Research model 701 digital pH meter equipped with an Ingold combined electrode type 401.

⁴ The detector (Altex model 153) measured at 253.7 nm. The cell volume was 8 μl, and the path length was 10 mm. The pump was model LDC 711. A stainless steel 150-mm long × 4-mm i.d. column and a Rheodyne (model 70-10) injection valve with a 300-μl sample loop were used. The chromatographic support, LiChrosorb RP-18 (Merck, Darmstadt, West Germany), had a mean particle diameter of 5 μm. Methanol-water containing 0.01 M phosphoric acid was used as the mobile phase.



Spectrophotometric Determination of Acid Dissociation Constants—The absorption spectrum of chlorambucil and 4-[p-(2-chloroethyl-2-hydroxyethylamino)phenyl]butyric acid (1) were strongly pH dependent due to proteolysis of the amino group. Spectrophotometric determination of the microscopic constants with graphic evaluation according to a literature method (8) is illustrated in Fig. 1. The fraction of species with an unprotonized amino group, α_{R_2N} , was calculated from:

$$\alpha_{R_2N} = \frac{[\text{R}_2\text{NMCOOH} + \text{R}_2\text{NMCOO}^-]}{C_M} = \frac{A - A_A}{A_B - A_A} \quad (\text{Eq. 1})$$

where C_M is the total concentration and A , A_A , and A_B are the absorbances at 257 nm in buffer, 1 M HCl, and buffer at pH 8.5, respectively. From the spectral data, it was concluded that $C_M = [\text{HR}_2\text{NMCOOH}]$ in 1 M HCl and that $C_M = [\text{R}_2\text{NMCOO}^-]$ in buffer at pH 8.5. Furthermore, M_{R_2N} was defined by:

$$M_{R_2N} = \frac{a_h([\text{R}_2\text{NMCOOH}] + [\text{R}_2\text{NMCOO}^-])}{[\text{HR}_2\text{NMCOOH}] + [\text{HR}_2\text{NMCOO}^-]} \quad (\text{Eq. 2})$$

From Fig. 1 and Ref. 8, it was concluded that the pk'_2 value of chlorambucil is 2.49 ± 0.02 . A similar evaluation for I gave a pk'_2 value of 3.63 ± 0.04 . It was not possible to evaluate the pk'_{12} values from the graphs with sufficient precision, but the most likely pk'_{12} for chlorambucil was >4.2 ; for I, it was >6 . An evaluation of pk'_1 for the two compounds also was not possible. However, since pMR_{2N} was almost constant when α_{R_2N} was within the range of 0.1–0.6, it was concluded that zwitterion formation was negligible, i.e., $\text{pk}_1 \gg \text{pk}_2$ (cf., 9).

Determination of Acid Dissociation Constants for Chlorambucil by Partition Studies—Chlorambucil probably partitions from an aqueous phase into an organic phase in the uncharged form. The distribution ratio, D , then is given by:

$$D = \frac{[\text{R}_2\text{NMCOOH}]_{\text{org}}}{[\text{R}_2\text{NMCOOH}] + [\text{R}_2\text{NMCOO}^-] + [\text{HR}_2\text{NMCOOH}] + [\text{HR}_2\text{NMCOO}^-]} \quad (\text{Eq. 3})$$

which can be rewritten as:

$$D = \frac{k_d}{1 + k_{21}/a_h + a_h/k_2 + k_{21}/k'_{12}} \quad (\text{Eq. 4})$$

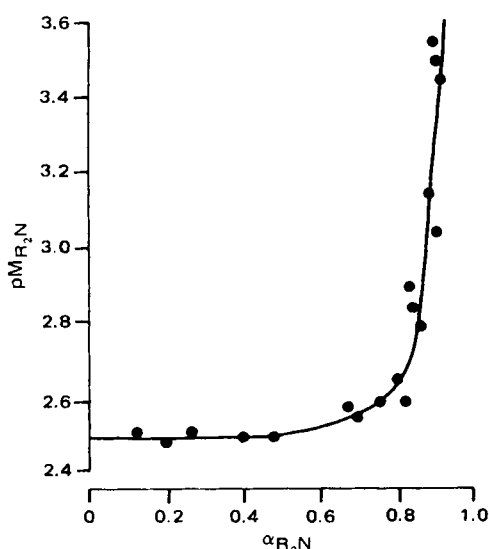


Figure 1—Spectrophotometric determination of microscopic dissociation constants for chlorambucil.

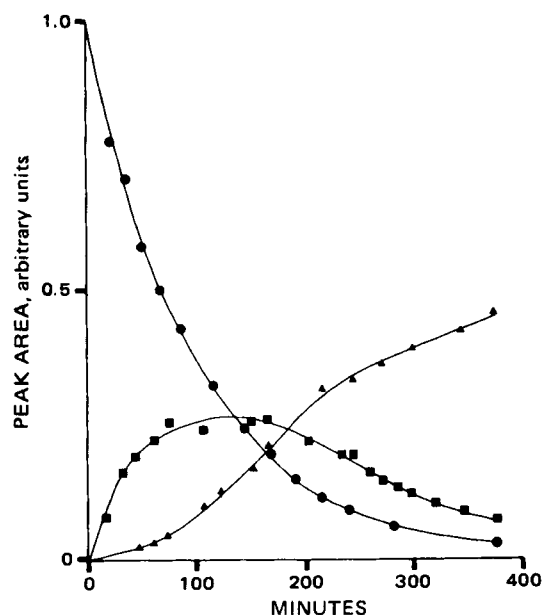


Figure 2—Time course for chlorambucil and degradation products in aqueous solution. Key: ●, chlorambucil (A, Scheme I); ■, I (C, Scheme I); and ▲, 4-[p-[di(2-hydroxyethyl)amino]phenyl]butyric acid (E, Scheme I). The molar absorbance and yield from the chromatographic column were considerably lower for E compared to A and C.

where k_d , the partition coefficient, is defined by:

$$k_d = \frac{[\text{R}_2\text{NMCOOH}]_{\text{org}}}{[\text{R}_2\text{NMCOOH}]} \quad (\text{Eq. 5})$$

Since zwitterion formation was negligible as revealed by spectrophotometric determination of acid dissociation constants, $k_{21}/k'_{12} \ll 1$. At low pH, i.e., $k_{21}/a_h \ll 1$, Eq. 4 can be reduced to:

$$\frac{1}{D} = \frac{1}{k_d} + \frac{a_h}{k_2 k_d} \quad (\text{Eq. 6})$$

A plot of $1/D$ versus a_h for pH 1.2–2.4 gave a straight line with a small positive intercept. However, evaluation of the partition coefficient, k_d , was not possible from the intercept. The value for $-\log(k_d \times k_2)$ was calculated from the slope as 1.04 ± 0.02 ; by substituting k_2 with the value determined spectrophotometrically, $\log k_d$ was evaluated as 1.45 ± 0.04 .

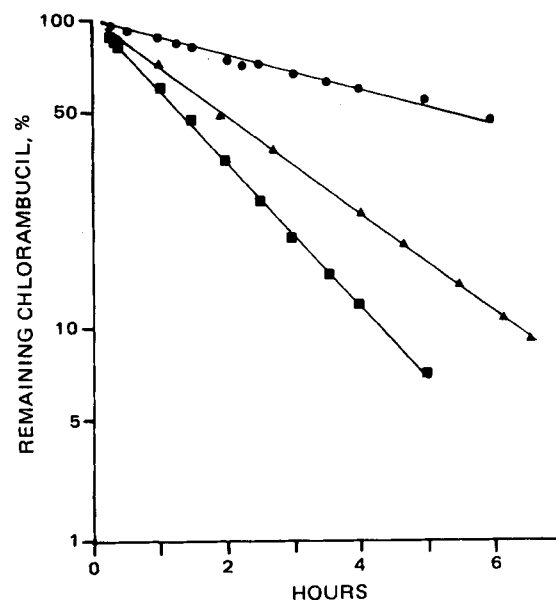


Figure 3—Degradation of chlorambucil in phosphate buffers. Key: ●, pH 1.92; ▲, pH 2.95; and ■, pH 6.99.

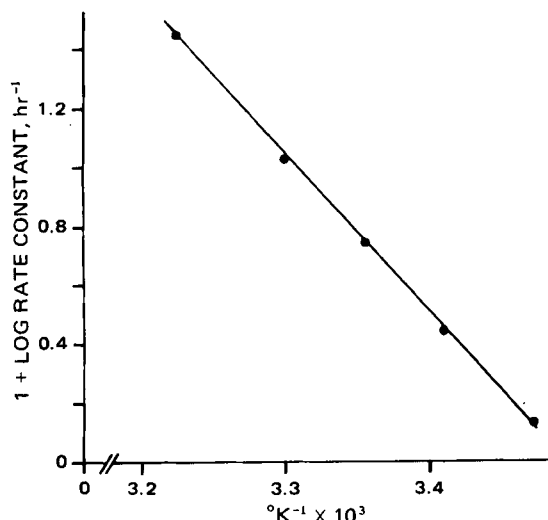


Figure 4—Influence of temperature on the degradation rate of chlorambucil at pH 7.00.

At high pH, i.e., $a_h/k_2' \ll 1$, Eq. 4 can be rewritten as:

$$\frac{1}{D} = \frac{1}{k_d} + \frac{k_{21}'}{a_h k_d} \quad (\text{Eq. 7})$$

A plot of $1/D$ versus $1/a_h$ for pH 4.8–5.9 gave a straight line, indicating the validity of Eq. 7. From the slope and the previously evaluated value of k_d , the pk_{21}' value of 4.46 ± 0.04 was determined.

Degradation of Chlorambucil—The degradation of chlorambucil in pure aqueous solution was studied by reversed-phase HPLC. The time course of peak areas for chlorambucil and its degradation products, C and E ($X = OH$, Scheme I), is given in Fig. 2. The concentration of C was ~30% of the initial chlorambucil concentration after 2 hr (assuming the same molar absorbance of A and C).

The rate of loss of the alkylating activity [established by the 4-(*p*-nitrobenzyl)pyridine method] was slower initially, as reported previously by Williamson and Witten (3), who concluded that significant amounts of the aziridinium ion (B, Scheme I) accumulated during the initial degradation of chlorambucil. However, this conclusion might be incorrect since a similar rate profile is observed for a codetermination of chlorambucil and C.

A pseudo-first-order plot of the degradation of chlorambucil in phosphate buffers is shown in Fig. 3. Linear relationships were observed throughout the reaction. No change in degradation rates was observed compared to reactions in pure aqueous solutions, indicating no buffer catalysis at the pH values used. The same rate constant for chlorambucil degradation was obtained within the concentration range of 3×10^{-6} – 3×10^{-5} M, establishing that no aggregation phenomena occurred.

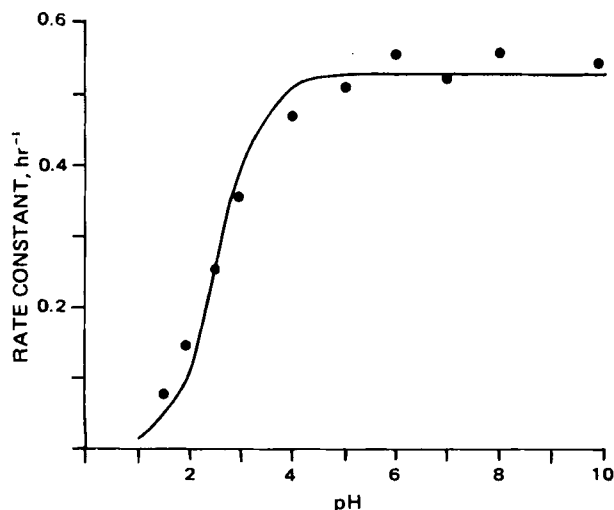


Figure 5—Influence of pH on the degradation rate of chlorambucil. The solid line was calculated from Eq. 8.

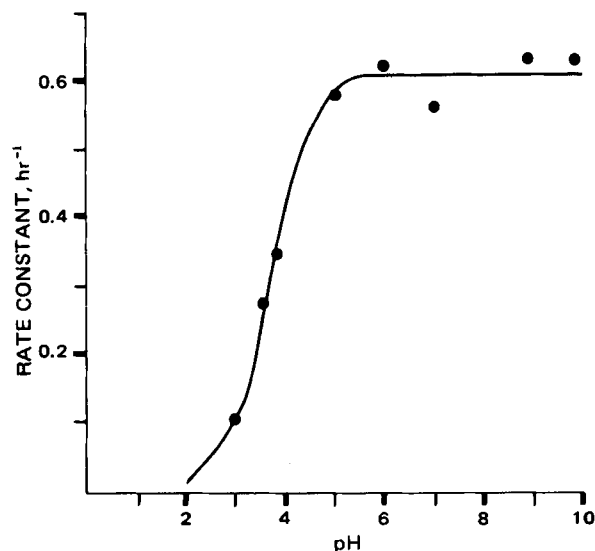


Figure 6—Influence of pH on the degradation rate of I. The solid line was calculated from Eq. 8.

Table I—Pseudo-First-Order Rate Constants for Degradation of Chlorambucil and I

Compound	pk_2'	k_1' , hr^{-1} ^a
Chlorambucil	2.49 ± 0.02	0.54 ± 0.02 ($n = 12$)
I	3.63 ± 0.04	0.61 ± 0.04 ($n = 6$)

^a Determined at pH 6–10.

Influence of Temperature—The relationship between the logarithm of the first-order rate constant and the reciprocal of temperature is given in Fig. 4. The activation energy was 102 kJ/mole, in general agreement with the data given by Linford (10).

Influence of pH on Degradation Rate of Chlorambucil and I—The degradation rate of chlorambucil was unaffected by pH in the range of pH 5–10 but decreased below pH 5 (Fig. 5). The solid line is calculated using (2):

$$k_1' = \frac{k_2' k_1}{k_2' + a_h} \quad (\text{Eq. 8})$$

where k_1' is the observed pseudo-first-order rate constant, k_1 is the pseudo-first-order rate constant for the alkylating compound with an unprotonized nitrogen atom, and k_2' is the apparent acid dissociation constant for the amino group.

The good agreement between the calculated and experimental values suggests that the nitrogen mustard group is stable when present in the protonized form and that the degree of protonization of the carboxylic group (pk_{21} 4.46) does not affect its stability. These results contradict those of Linford (6) who observed a continuous increase in the stability of chlorambucil in the pH range of 7–4.5. This finding probably was due to the high concentrations used, where the degradation rate was affected by aggregation phenomena (7).

The degradation rates of I and chlorambucil differed only slightly (Table I). These results are in contrast to those found for *N*-ethyl-*N*-(2-chloroethyl)aniline, which has a degradation rate considerably higher than does *N,N*-di(2-chloroethyl)aniline (11).

The influence of pH on the degradation rate of I is given in Fig. 6. The rate profile was similar to that of chlorambucil but the rate declined at higher pH, reflecting the higher base strength of the amino group.

REFERENCES

- (1) M. H. Benn, P. Kazmaier, and C. Watanatada, *Chem. Commun.*, 1970, 1685.
- (2) B. Cohen, E. R. van Artsdalen, and J. Harris, *J. Am. Chem. Soc.*, 70, 281 (1948).
- (3) C. E. Williamson and B. Witten, *Cancer Res.*, 27, 33 (1967).
- (4) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, 27, 1435 (1955).
- (5) M. K. Balazs, C. A. Anderson, R. H. Iwamoto, and P. Lim, *J. Pharm. Sci.*, 59, 563 (1970).

- (6) J. H. Linford, *Biochem. Pharmacol.*, **12**, 317 (1963).
(7) D. Blakeslee, M. Chen, and J. C. Kennedy, *Br. J. Cancer*, **31**, 689 (1975).
(8) J. T. Edsall, R. B. Martin, and B. R. Hollingworth, *Proc. Natl. Acad. Sci. USA*, **44**, 505 (1958).
(9) S. Eksborg, *J. Pharm. Sci.*, **67**, 782 (1978).
(10) J. H. Linford, *Biochem. Pharmacol.*, **8**, 343 (1961).

- (11) W. C. J. Ross, *J. Chem. Soc.*, **1949**, 183.

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GLC Determination of Aprindine in Human Plasma Using a Nitrogen-Phosphorus Flame-Ionization Detector

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Abstract □ A procedure for the determination of aprindine in human plasma was developed. After the addition of *N,N*-diethyl-*N'*-(1,2,3,4-tetrahydro-2-naphthyl)-*N'*-phenyl-1,3-propanediamine as an internal standard, the plasma was buffered to pH 8.0, and the drug and the internal standard were extracted into ethyl acetate-hexane (9:1 v/v). The compounds then were extracted from the organic phase into 0.02 *N* HCl. The acidic solution was made basic with 0.2 *M* tribasic sodium phosphate, and aprindine and the internal standard were extracted into a small volume of hexane. The compounds were analyzed by GLC using a nitrogen-phosphorus flame-ionization detector. The drug concentration and instrument response were linear for 0.10–1.00 µg of aprindine/ml, the slope was 1.1416 (0.0141), the *y* intercept was 0.0096 ± 0.0082, and the correlation coefficient was 0.99960 ± 0.00002. The sensitivity of the method was 0.02 µg of aprindine/ml. The within-day coefficient of variation was 9.50, 3.10, 3.14, and 2.21% for 0.05, 0.20, 0.40, and 0.80 µg of aprindine/ml, respectively. The between-day coefficient of variation was 17.4, 3.40, 2.07, and 1.54% at the same concentrations. Total precision values of 19.9, 4.60, 4.26, and 2.69% were obtained. The overall relative error of the method was +1.33, +2.00, -0.07, and +0.25% at these concentrations.

Keyphrases □ Aprindine—GLC analysis in human plasma using nitrogen-phosphorus flame-ionization detection □ GLC—analysis, aprindine, nitrogen-phosphorus flame-ionization detection, human plasma □ Antiarrhythmic agents—aprindine, GLC analysis using nitrogen-phosphorus flame-ionization detection, human plasma

Aprindine¹ (*N,N*-diethyl-*N'*-2-indanyl-*N'*-phenyl-1,3-propanediamine), an oral, long-acting, antidysrhythmic agent, has been used in Europe since the early 1970's. A sensitive (0.1 µg/ml), specific, and precise (coefficient of variation 5%) GLC method was developed (1) and then modified (2) that satisfactorily monitors plasma aprindine levels in patients. Lagerström and Persson (3) reported a sensitive (0.5 µg/ml) high-performance liquid chromatographic assay for monitoring plasma aprindine levels. These methods appear to be suitable for monitoring plasma aprindine levels of ≥0.5 µg/ml. However, for pharmacokinetic and bioavailability studies following unit doses of 10, 25, or 50 mg of aprindine, a more sensitive method was necessary.

This report describes a GLC method using a dual nitrogen-phosphorus flame-ionization detector that optimizes the sensitivity and specificity of the measurement of aprindine and still retains the precision of the assay.

EXPERIMENTAL

Chemicals and Reagents—Ethyl acetate², hexane², and methanol² were distilled in glass; all other chemicals and reagents were analytical reagent grade. Aprindine hydrochloride³, *N,N*-diethyl-*N'*-(1,2,3,4-tetrahydro-2-naphthyl)-*N'*-phenyl-1,3-propanediamine hydrochloride⁴, and [*N*-phenyl-1-¹⁴C]aprindine hydrochloride⁵ with a specific activity of 29.4 µCi/mg were synthesized.

The aprindine standard solution (10 µg/ml) was prepared by weighing accurately 1.12 mg of aprindine hydrochloride into a 100-ml volumetric flask and diluting the solution to volume with distilled water.

The *N,N*-diethyl-*N'*-(1,2,3,4-tetrahydro-2-naphthyl)-*N'*-phenyl-1,3-propanediamine solution (the internal standard) (5 µg/ml) was prepared by weighing accurately 1.11 mg of the hydrochloride salt of the internal standard into a 200-ml volumetric flask and diluting the solution to volume with distilled water.

GLC System—Table I summarizes the GLC equipment, materials, and conditions for the identification and analysis of aprindine in plasma. Disposable 15-ml centrifuge tubes with plastic-lined screw caps⁶ were used throughout the extraction.

Procedure—A plasma calibration curve was prepared by pipetting 1.0 ml of fresh, human, drug-free plasma into each of five disposable 15-ml centrifuge tubes and adding 250 µl of the internal standard solution to give a concentration of 1.25 µg/ml. The first tube served as the blank. Ten microliters of the aprindine standard solution was added to the second tube, 25 µl was added to the third tube, 50 µl was added to the fourth tube, and 100 µl was added to the fifth tube. These plasma standards contained aprindine concentrations of 0.00, 0.10, 0.25, 0.50, and 1.00 µg/ml, respectively.

Borate buffer USP, 0.5 ml, was mixed thoroughly with the plasma standards. Then 10 ml of ethyl acetate-hexane (9:1 v/v) was shaken with each tube of plasma standard for 2 min, and the phases were separated by centrifugation. Approximately 8 ml of the supernate was pipetted into a clean 15-ml centrifuge tube, with care taken not to remove any of the aqueous phase. The compounds were extracted into 5 ml of 0.02 *N* HCl, the contents of the tube were centrifuged, and the organic layer was discarded. The aqueous solution was made basic by mixing with 1.5 ml of 0.2 *M* tribasic sodium phosphate, the drug and the internal standard were extracted into 0.3 ml of hexane, and the phases were separated again by centrifugation. Two microliters of the hexane solution was injected onto the chromatographic column.

When assaying plasma samples, a 1.0-ml sample was placed into a 15-ml disposable centrifuge tube and 250 µl of the internal standard solution was added. After 0.5 ml of borate buffer was added, the plasma samples were handled as were the calibration curve samples.

A least-squares line was prepared by analysis of the calibration curve of the peak height ratio of aprindine to the internal standard *versus*

¹ Christiaens Pharmaceutical Co., Brussels, Belgium.

² Burdick & Jackson, Muskegon, Mich.

³ Fibocil, Eli Lilly and Co., Indianapolis, Ind.

⁴ Eli Lilly and Co., Indianapolis, Ind.

⁵ New England Nuclear, Boston, Mass.

⁶ Corning Glass Works, Corning, N.Y.