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RAPID ASSAY FOR PLASMA CHLORAMBUCIL AND PHENYL ACETIC MUSTARD USING REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid assay for chlorambucil, a drug used for the treatment of chronic lymphocytic leukemia, and its major metabolite is described. Chromatographic behaviour of the two compounds on two different reversed-phase columns is discussed, as well as the kinetics of their hydrolysis in aqueous medium. The developed analysis can be applied to the determination of the plasma levels of the drug and its metabolite. No sample preparation is required and the spectrophotometric detection affords the sensitivity in the picomole range. Total analysis time is between 10 and 15 min.

INTRODUCTION

Chlorambucil (Leukeran®) is a nitrogen mustard derivative administered in the treatment of chronic lymphocytic leukemia (CLL), ovarian and breast carcinomas, malignant lymphomas and Hodgkin's disease [1—3]. The structure of this drug (Fig. 1), in which the reactive bis(2-chloroethyl)amino group is substituted *para* to the carboxylic acid group on the aromatic ring, ensures the penetration of the alkylating function through cellular membranes [4].

The various analytical procedures reported for the quantitative assay of chlorambucil include the colorimetric determination of 4-(*p*-nitrobenzyl)pyridine derivatives, UV spectrophotometric, and chlorine titrimetric methods [2]. However, these procedures do not provide the sensitivity and accuracy needed to study the pharmacokinetics of the drug or its metabolism in humans [2]. Whereas the tandem operation of gas chromatography (GC) and mass spectrometry (MS), per se, satisfies such analytical criteria, the need for an ethyl acetate extraction procedure prior to the GC—MS assay limits the possible advantages

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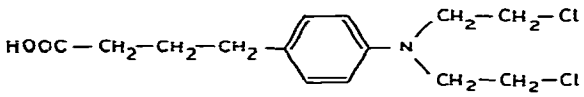
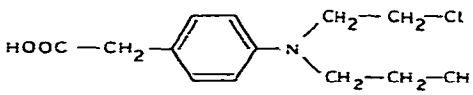
CHLORAMBUCIL (LEUKERAN ^{R})



4 [4-bis(2-chloroethyl) aminophenyl] butyric acid

PHENYL ACETIC MUSTARD (metabolite)



2 [4-bis(2-chloroethyl) aminophenyl] acetic acid

Fig. 1. Structures of chlorambucil and phenyl acetic mustard metabolite.

of the method: this extraction requires the use of a large volume of blood [5]; more importantly, chlorambucil itself is unstable in the ethyl acetate medium [2].

Currently available high-performance liquid chromatographic (HPLC) assays also necessitate several extraction steps [4], which impoverishes the recovery of the compounds of interest, and lack the sensitivity needed for the quantification of the drug or its main metabolite, phenyl acetic mustard (Fig. 1), which precludes their pharmacokinetic study [1].

Described in this paper is a reversed-phase liquid chromatographic assay for the sensitive and accurate quantification of the drug and its metabolite in plasma. The blood fluid is directly injected into the chromatographic system. Sample clean-up is achieved by means of a guard column and the recovery of the two mustards is high. The stability of the drug in aqueous medium and the kinetics of its hydrolysis are also discussed.

EXPERIMENTAL

Apparatus

A Waters ALC 204 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) was used in the course of this study. Simultaneous monitoring of the UV-absorbing compounds at 254 nm and 280 nm was accomplished by means

of a Waters 440 dual-wavelength detector and the signals were recorded on a dual-pen recorder (Omniscribe, Houston Instrument, Austin, TX, U.S.A.). Chromatographic solutes were characterized by their stopped-flow UV spectra, obtained with a Schoeffel SF 770 Spectroflow Monitor, equipped with an MM 700 Memory Module and an SFA 339 Wavelength Drive, all from Kratos (Schoeffel Instrument Division, Westwood, NJ, U.S.A.). Simultaneous fluorometric detection was achieved using an SF 970 fluorescence monitor (Kratos, SID) set at an excitation wavelength of 285 nm and a 320-nm emission cut-off filter. Areas of the peaks detected at 254 nm were computed by a Hewlett-Packard 3380A integrator (Avondale, PA, U.S.A.).

Centrifugation of blood samples was performed with a Dynac centrifuge (Clay-Adams, Parsippany, NJ, U.S.A.). A Vortex Genie K 550-G (Scientific Industries, Springfield, MA, U.S.A.) was used to vortex the samples prior to injection. Eppendorf pipettes (Brinkman Instruments, Westbury, NY, U.S.A.) were also utilized for the sequential dilution of the reference solutions.

Columns

A Partisil PXS-10/25 ODS column (25 cm × 4.6 mm, 10 μm average particle size, Whatman, Clifton, NJ, U.S.A.) and a guard column (5 cm × 3.9 mm), packed with pellicular Co:Pell ODS (octadecyl groups chemically bonded to 30—32 μm glass beads, Whatman), were used. For faster elution of the drug and its metabolite, the Partisil ODS column was substituted with a Chromegabond MC-18 (15 cm × 4.6 mm, 5 μm average particle size, ES Industries, Marlton, NJ, U.S.A.).

Chemicals

Potassium dihydrogen phosphate (KH_2PO_4), HPLC grade, was purchased from Fisher Scientific Company (Fair Lawn, NJ, U.S.A.) and solutions were prepared at a 0.02 *M* concentration with doubly-distilled, deionized water. The aqueous buffer was filtered through a 0.45-μm pore-size Millipore membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum prior to use. The HPLC grade methanol was purchased from Fisher Scientific. The methanol—buffer mixtures were degassed by purging with helium.

Chlorambucil {4[4-bis(2-chloroethyl)aminophenyl] butyric acid} was obtained from Burroughs-Wellcome (Research Triangle Park, NC, U.S.A.) and the phenyl acetic mustard {2[4-bis(2-chloroethyl)aminophenyl] acetic acid} from the Institute of Cancer Research of the Royal Cancer Hospital (Sutton, Great Britain). Since the drug and its metabolite decompose rapidly in water at room temperature, care was taken to chill the glassware as well as the water in which the reference compounds were dissolved, by means of a dry-ice—acetone bath. All reference solution vials were kept on ice during use.

Chromatographic conditions

The chromatographic conditions were optimized for a selective analysis of chlorambucil and its phenyl acetic mustard in the presence of other plasma constituents. The eluent used with the Partisil PXS 10/25 ODS column was a solution (50:50, v/v) of anhydrous methanol—0.02 *M* KH_2PO_4 (natural pH). The temperature was ambient and the flow-rate 1.5 ml/min.

These conditions were slightly modified for faster elution of the drug and its major metabolite on a Chromegabond MC-18 column. The methanol–buffer eluent was a 55:45 mixture and the flow-rate 1.0 ml/min.

Samples

The developed method was tested in the analysis of heparinized plasma samples, obtained from patients on a chlorambucil regimen. The plasma specimens were prepared at the New York University School of Medicine (New York, NY, U.S.A.). Control samples from healthy individuals were obtained from the University of Rhode Island Health Services.

Identification of chlorambucil and its phenyl acetic mustard metabolite

Initial assignment of solute identities in CLL plasma was based on retention times and co-chromatography with the reference compounds. In addition, the ratios of peak heights recorded at 280 nm and 254 nm (Table I), the fluorometric selectivity and stopped-flow UV spectra enabled further characterization of the chromatographic eluites.

TABLE I

SPECTROSCOPIC DATA FOR CHLORAMBUCIL, ITS PHENYL ACETIC MUSTARD METABOLITE AND THEIR HYDROXYLATED DERIVATIVES

	Peak height ratio 280/254 nm	Fluorescence intensity
Chlorambucil	0.095	none
Monohydroxy derivative	0.125	slight
Dihydroxy derivative	0.136	moderate
Phenyl acetic mustard	0.150	none
Monohydroxy derivative	0.214	slight
Dihydroxy derivative	0.250	moderate

RESULTS

Chromatographic assay

Using a Partisil PXS 10/25 ODS (10 μm), the drug and its metabolite were resolved from other plasma constituents. This is illustrated with the chromatogram of a plasma sample to which reference solutions of chlorambucil and phenyl acetic mustard were added (Fig. 2). The blank plasma chromatogram showed no interference which corresponded to the retention of chlorambucil or its metabolite.

The calibration curves for these compounds were linear over the concentration range of interest and the correlation coefficients for chlorambucil and the phenyl acetic mustard were 0.99 and 0.98, respectively. The lower detection limits for the drug and its major metabolite in plasma were 11.9 and 2.07 pmole, respectively. Within-day and day-to-day coefficients of variation for retention times were 1.0% and 1.2%, respectively.

A chromatogram of a plasma specimen, obtained from a CLL patient about

an hour after ingestion of the drug (2 mg Leukeran® tablets from Burroughs-Wellcome), is exemplified in Fig. 3. As little as 10 μl of the blood fluid could be used to detect and quantify the drug and its metabolite.

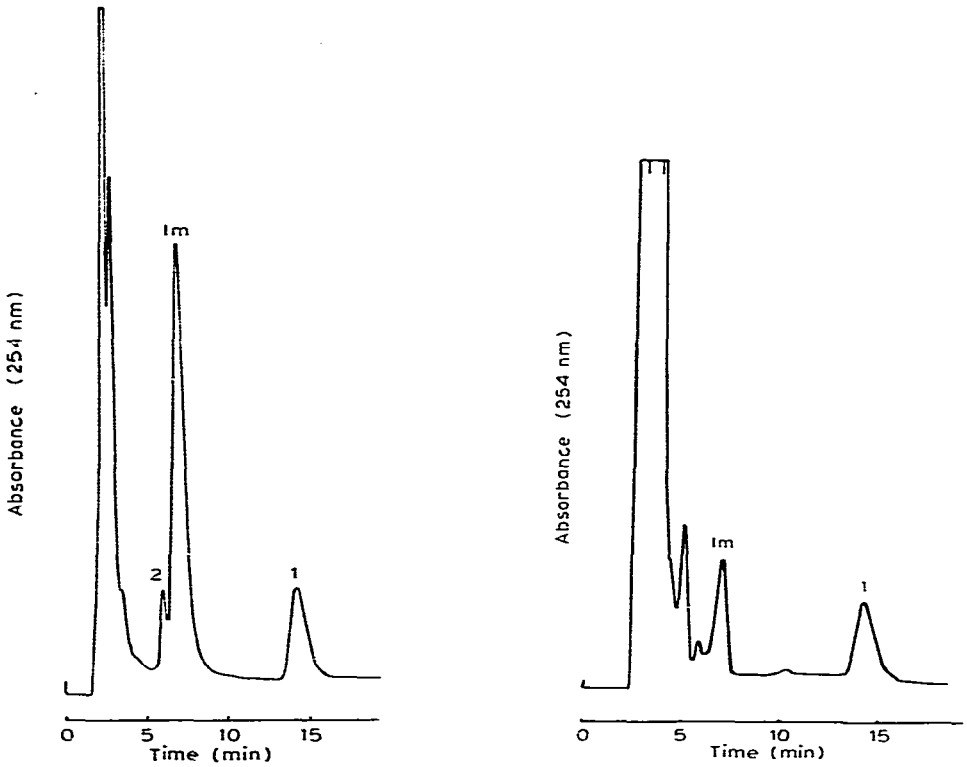


Fig. 2. Chromatogram of a plasma sample spiked with chlorambucil (1), phenyl acetic mustard (1m) and monohydroxy-chlorambucil derivative (2). Volume of plasma injected, 20 μl. Chromatographic conditions: column, Partisil PXS 10/25 ODS (10 μm); eluent, methanol–0.02 *M* KH_2PO_4, (1 : 1, v/v); flow-rate, 1.5 ml/min; temperature, ambient.

Fig. 3. Chromatogram of a plasma specimen obtained from a CLL patient on chlorambucil regimen. Volume of plasma injected, 30 μl. Chromatographic conditions as in Fig. 2. Peaks: 1 corresponds to 42.9 pmole of chlorambucil; 1m, phenyl acetic mustard.

A faster chromatographic analysis was possible using a shorter column, Chromegabond MC-18, with a slight modification of the eluent composition. The average particle diameter of the packing material was 5 μm and thus, adequate resolution of the early eluting solutes was maintained (Fig. 4).

Since chlorambucil is more stable in plasma than in water or ethyl acetate [2], the ethyl acetate extraction procedure was avoided and the removal of proteins and possible interferences was achieved by means of a guard column installed before the analytical column.

Recoveries were determined by adding known volumes of reference solutions to an aliquot of the drug-free plasma. Samples were assayed in triplicate and the average recoveries for different dilutions of the reference compounds were slightly higher than 100% (Table II). This could be attributed to the greater

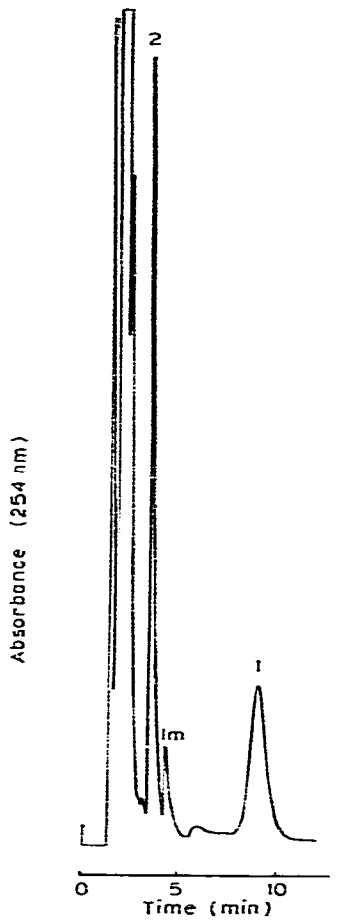


Fig. 4. Chromatogram of a plasma sample spiked with chlorambucil (1), phenyl acetic mustard (1m), and monohydroxy-chlorambucil derivative (2). Volume of plasma injected, 20 µl. Chromatographic conditions: column, Chromegabond MC-18 (5 µm); eluent, 45% of 0.02 *M* KH_2PO_4—methanol; flow-rate, 1.0 ml/min; temperature, ambient.

stability of the drug and its metabolite in plasma than in aqueous medium.

Hydrolysis of chlorambucil

In neutral or basic solutions, chlorambucil is rapidly hydrolyzed into its mono- and dihydroxy derivatives; the latter compound lacks antitumor activity. This decomposition, followed chromatographically as illustrated in Fig. 5, is noticably slower at 4°C than at 25.5°C.

The decomposition of chlorambucil in aqueous solution follows a first-order reaction rate (Fig. 6). Our results support the unimolecular nucleophilic substitution scheme devised by Owen et al. [6], in which the rate-limiting step is the ionization of one chlorine together with the formation of a cyclic ethyleneimmonium ion.

The chromatograms show that as the concentration of chlorambucil decreases, the concentration of the monohydroxy derivative increases initially and sub-

TABLE II

AVERAGE RECOVERIES FOR DIFFERENT DILUTIONS OF CHLORAMBUCIL AND ITS PHENYL ACETIC MUSTARD METABOLITE

	Concentration of reference solution (M)	Recovery (%)
Chlorambucil	$6.57 \cdot 10^{-4}$	106
	$1.32 \cdot 10^{-4}$	103
	$2.63 \cdot 10^{-5}$	104
Phenyl acetic mustard	$5.21 \cdot 10^{-4}$	98.4
	$1.04 \cdot 10^{-4}$	100
	$4.17 \cdot 10^{-6}$	106

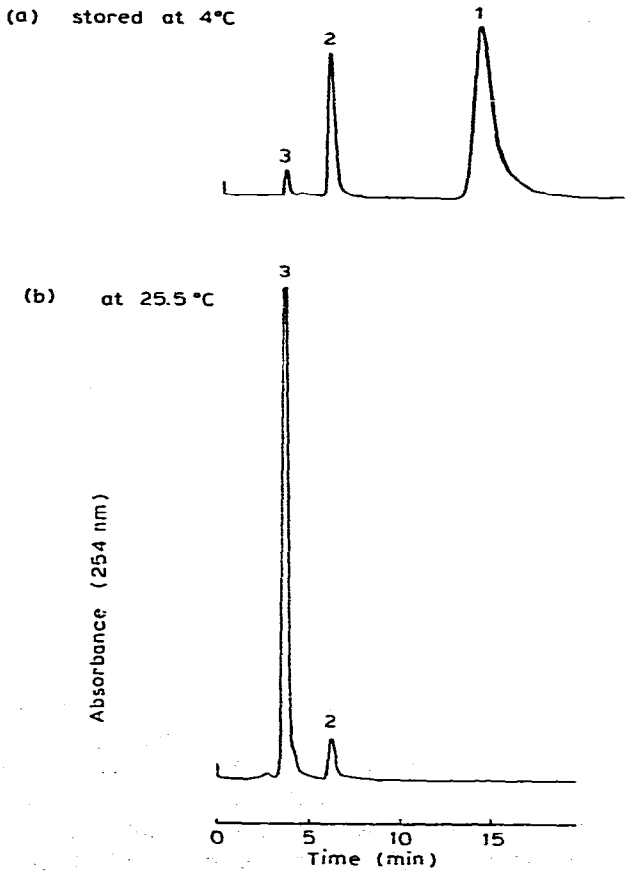


Fig. 5. Chromatograms illustrating the extent of hydrolysis of a reference solution of chlorambucil stored for 7 h at 4°C (a) and 25.5°C (b). Peaks: 1, chlorambucil; 2, monohydroxy-chlorambucil derivative; 3, dihydroxy-chlorambucil derivative. Chromatographic conditions as in Fig. 2.

sequently decreases due to its further conversion to the dihydroxylated form (Fig. 6). Since the rates of these reactions are temperature-dependent, the de-

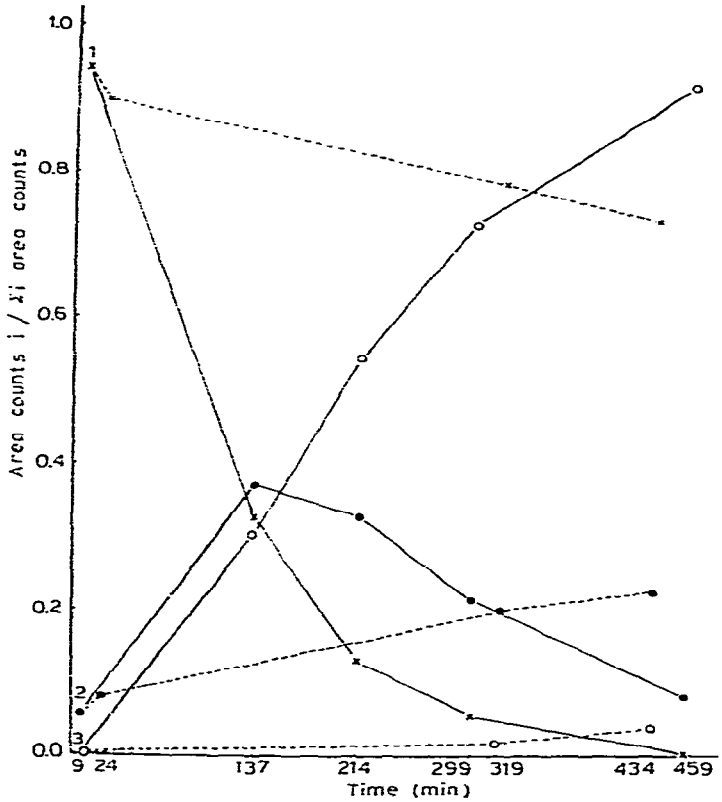


Fig. 6. Kinetic study of the decomposition of chlorambucil (1) into its monohydroxy (2) and dihydroxy (3) derivatives in aqueous medium at 4°C (— — —) and 25.5°C (———).

composition of chlorambucil and its phenyl acetic mustard metabolite can be minimized by chilling the reference solutions. This caution is particularly important in the determination of calibration curves for both the drug and its principal metabolite.

CONCLUSION

An efficient assay for the detection of chlorambucil and its major metabolite in plasma was developed using reversed-phase liquid chromatography. This method is rapid and does not involve any sample preparation. The quantification of picomole amounts of both the drug and its phenyl acetic mustard can be achieved. Recoveries are high, thus permitting accurate assessment of the pharmacokinetics of elimination of chlorambucil from the blood fluid. This is particularly useful in view of the side effects of the drug such as bone marrow suppression [7, 8], fibrosis, increased susceptibility to infections, tuberculosis [9], and seizures in children [10].

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REFERENCES

1 D.R. Newell, L.I. Hart, and K.R. Harrap, J. Chromatogr., 164 (1979) 114.
2 S.Y. Chang, B.J. Larcom, D.S. Alberts, B. Larsen, P.D. Walson and I.G. Sipes, J. Pharm. Sci., 69 (1980) 80.
3 D.S. Alberts, S.Y. Chang, H.-S.G. Chen, B.J. Larcom and S.E. Jones, Cancer Treat. Rev., 6 (Suppl.) (1979) 9.
4 P. Leff and W.G. Bardsley, Biochem. Pharmacol., 28 (1979) 1289.
5 A. McLean, R.L. Woods, D. Catovsky and P. Farmer, Cancer Treat. Rev., 6 (Suppl.) (1979) 33.
6 W.R. Owen and P.J. Stewart, J. Pharm. Sci., 68 (1979) 992.
7 S.D. Gisser and V.B. Chung, Amer. J. Med., 67 (1979) 151.
8 H.M. Golomb and U. Mintz, Blood, 54 (1979) 305.
9 J.W. Millar and N.W. Horne, Lancet, i (1979) 1176.
10 S.A. Williams, S.P. Makker and W.E. Grupe, J. Pediatr., 93 (1978) 516.