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Note

Studies on the quantitation of chlorambucil in plasma by reversed-phase high-performance liquid chromatography

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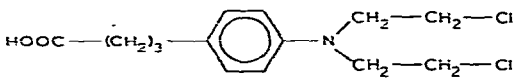
Chlorambucil, 4-{*p*-[bis-(2-chloroethyl)amino]phenyl}butyric acid (Leukeran®), a bifunctional alkylating agent, is an antineoplastic drug widely used in the treatment of chronic lymphocytic leukemia, malignant lymphomas, ovarian carcinomas, and Hodgkins disease [1—4].



Limited pharmacokinetic studies on chlorambucil in humans are primarily due to the lack of a simple, sensitive, yet efficient, means of monitoring changes in concentration of the drug in biological samples. A number of UV spectrophotometric [5, 6] and chlorine titrimetric [5, 7] methods have been described for chlorambucil analysis. More recently, measurement by high-performance liquid chromatography [8—10] and gas chromatography—mass spectroscopy [11—13] has become possible. The high-performance liquid chromatographic (HPLC) method reported by Newell et al. [8] using a methanol gradient has a close retention time for chlorambucil and biological background. Hence, interference occurred at our hands. Leff and Bardsley [9] described an HPLC method which required derivatization of chlorambucil prior to analysis. In addition to being time-consuming, decomposition of chlorambucil may occur under this condition. For the method of Ehrsson et al. [10], there is no description of application to biological samples.

The assay procedure described herein involves a sensitive, rapid, isocratic HPLC analysis of chlorambucil.

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MATERIALS AND METHODS

Reagents

HPLC grade acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and a 0.2% solution of acetic acid (Fisher Scientific, Fair Lawn, NJ, U.S.A.) in water were the two solvents used. Both solvents were glass distilled and were filtered with Gelman 0.2-μm filters and degassed under vacuum prior to use. Chlorambucil was a gift from the Burroughs-Wellcome Co., Research Triangle Park, NC, U.S.A. All other chemicals and reagents were of the highest purity available and were obtained from commercial sources. Human blood plasma was provided by the University of Texas Medical Branch Blood Bank, Galveston, TX, U.S.A.

Instruments

All analyses were run on a Waters Assoc. Model 270 liquid chromatograph with microprocessor-controlled gradient system (Waters Assoc., Milford, MA, U.S.A.). This consisted of the 6000A and M45 solvent delivery systems, Model 720 system controller, Model 730 data module, and Model 710B WISP (Waters Intelligent Sample Processor). Separations were accomplished using Waters Assoc. Radial-Pak C_{18} cartridges containing 10-μm diameter particles. Absorption of the eluents was monitored with a Beckman Model 155 variable-wavelength detector (Altex, Berkeley, CA, U.S.A.).

Centrifugation of samples was achieved at 12,800 *g* with a desk-top, Model 5412 Eppendorf centrifuge (Brinkman Instruments, Westbury, NY, U.S.A.).

Standard curve of chlorambucil

Solutions of chlorambucil in acetonitrile were freshly prepared in concentrations of 0.5, 1, 2, 4, and 5 μg/ml and kept at —20°C. Solvent composition and sample injections were automatically carried out by the system controller and the automatic sample processor. The mobile phase consisted of an isocratic mixture of 65% acetonitrile and 35% dilute (0.2%) acetic acid. The flow-rate was set at 1 ml/min. Absorbance was monitored at 263 nm and recorded on the data module. The module chart speed was 1 cm/min, and quantitation of peak areas was carried out by the data module integrator. A standard curve was developed using the acetonitrile solutions of chlorambucil standards; 50 μl of each were injected.

Standard curve of chlorambucil in human plasma

Appropriate aliquots of the standard chlorambucil solutions were added to human plasma, obtained from the blood bank, to provide concentrations of 0.1, 0.5, 1, 5 and 10 μg/ml of the drug in plasma. Aliquots (100 μl) of chlorambucil-spiked plasma were taken from each tube, mixed with 4 volumes of acetonitrile (i.e. 400 μl of acetonitrile added to 100 μl of plasma), and vortexed. Macromolecular components precipitated by acetonitrile were separated from chlorambucil solution by centrifugation (2 min). To reduce further the concentration of unprecipitated plasma proteins and biological constituents that might interfere with the chromatographic separation of chlorambucil, the samples were rapidly frozen in a solution of dry-ice—acetone and again

centrifuged (2 min). Similar techniques were described by Chang et al. [14] for the analysis of melphalan, another nitrogen mustard used in the treatment of multiple myeloma. Aliquots (200 μl) of the upper, clear supernatants were drawn off, placed in vials and assayed immediately (75-μl injections). Extraction efficiency was 96 ± 8%. Various chlorambucil plasma concentrations were utilized to study the effects of freezing (in the above-mentioned dry-ice—acetone bath), acidification and volume of the extracting solvent on acetonitrile extraction efficiency of chlorambucil. In the above procedure plasma samples are acidified to pH 3.0 prior to extraction.

Clinical application of the analytical technique

Blood samples from patients undergoing chlorambucil chemotherapy were obtained under the supervision of David Gill, M.D., at the Department of Medicine, Division of Medical Oncology and Hematology of the University of Texas Medical Branch at Galveston. The patients were administered 18—40 mg chlorambucil tablets orally with water following an overnight fast. These patients were receiving chlorambucil for chronic lymphocytic leukemia. Blood samples (400 μl) were placed in heparinized micro-test tubes and centrifuged at 12,800 *g* for 2 min. The plasmas were subsequently prepared for analysis by the previously described procedure.

Time—course of chlorambucil in rats

Male Sprague—Dawley rats, six per group (250—300 g, Charles River, Wilmington, MA, U.S.A.) were treated orally with 20 mg/kg chlorambucil. Blood samples were taken from the supraorbital plexus into heparinized 500-μl microcentrifuge tubes and kept on ice. Blood samples were collected at 5, 15, and 30 min and at 1, 2, 4, 6, 12, and 28 h after treatment. Samples were processed for chlorambucil analysis as described before.

RESULTS AND DISCUSSION

Fig. 1 illustrates the HPLC resolution of chlorambucil in plasma. It shows chromatograms of normal blood plasma supplied by the blood bank (A) and of blank plasma spiked with 10 μg/ml chlorambucil (B). The retention time of chlorambucil by the previously described chromatographic conditions is 7.65 min. Detection of chlorambucil by this method is not accompanied by interference from plasma constituents.

Standard curve

The regression line for the standard curve of chlorambucil in acetonitrile is $Y = 0.00137X + 0.08869$, where Y is the concentration and X the absorbance; the correlation value is 0.9986. The standard curve of chlorambucil in plasma yields a regression line of $Y = 0.00279X + 0.01395$ with a correlation value of 0.9999. This procedure allows the quantification of chlorambucil in plasma with a high degree of accuracy. In separate determinations of the spiked standard chlorambucil sample (1 μg/ml) the range of difference in area of the chlorambucil peak is only ± 6% of the mean.

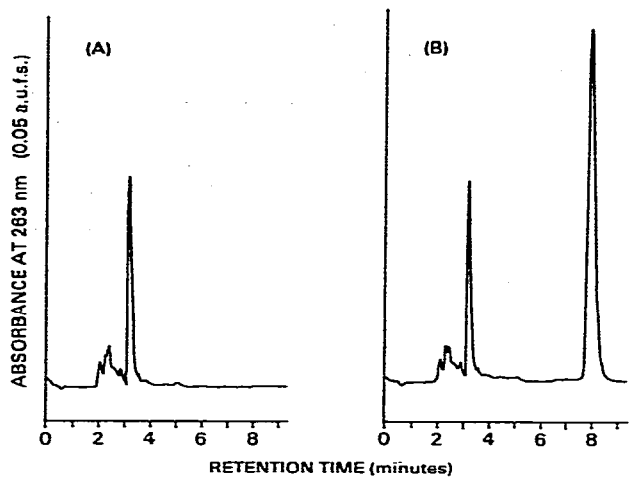


Fig. 1. HPLC analysis of chlorambucil in human plasma using an isocratic mobile phase of acetonitrile and 0.2% acetic acid buffer (65:35, v/v) with a flow-rate of 1 ml/min and UV detection at 263 nm. (A) Blank plasma; (B) plasma spiked with chlorambucil.

Effect of temperature

A major difference in extraction efficiency is observed when the extraction procedure is followed by freezing of the samples at −70°C versus not freezing (room temperature, 27°C). As seen in Fig. 2, failure to freeze the samples prior to centrifugation reduces the recovery of chlorambucil by approximately 60%. Low temperature provides efficient denaturation and precipitation of biological material. Hence, hydrophobic and other physical bonding forces between drug and biological macromolecules decrease and free drug concentration increases.

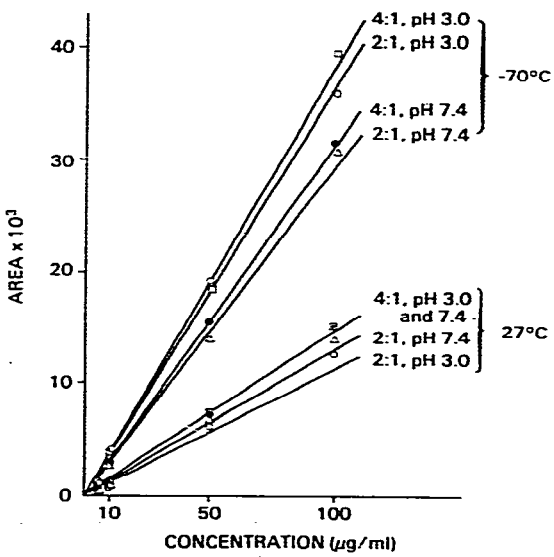


Fig. 2. Effect of pH, volume of extractant, and temperature on acetonitrile extraction efficiency of chlorambucil. (□) 4:1 acetonitrile/plasma ratio, pH 3.0; (○) 2:1 acetonitrile/plasma ratio, pH 3.0; (●) 4:1 acetonitrile/plasma ratio, pH 7.4; (△) 2:1 acetonitrile/plasma ratio, pH 7.4.

Effect of pH

Plasma samples spiked with various amounts of chlorambucil were acidified to pH 3.0 by adding 0.1 ml of 0.2 *N* hydrochloric acid to the sample prior to its extraction with acetonitrile. Acidification followed by extraction at 27°C had no effect on the extractability of chlorambucil from biological fluids as compared to unacidified plasma at this temperature. However, acidification followed by extraction and cooling of the mixture to −70°C enhanced the extraction efficiency of chlorambucil 15% over the unacidified samples (Fig. 2).

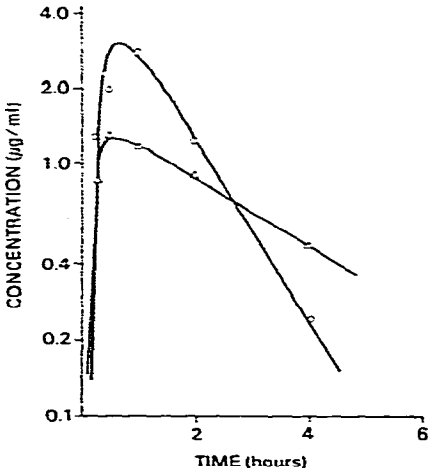
The nitrogen atom in the bis-2-haloethylamine moiety of chlorambucil is not strongly basic [15]. Ionization at pH 3 may occur, although it will be minimal, particularly at low temperatures. This minimal ionization is compensated by the unionized form of the carboxyl group of the butyric acid moiety of the compound, thus slightly enhancing its extractability in organic solvents.

Effect of solvent/plasma ratio

The effect of using various acetonitrile/plasma ratios for extraction of chlorambucil is also seen in Fig. 2. Using a 4:1 acetonitrile/plasma ratio resulted in an insignificant increase in the efficiency of chlorambucil extraction over the corresponding 2:1 ratio.

Application

The results of the analyses of chlorambucil concentration versus time in the plasma of two cancer patients receiving chlorambucil chemotherapy are shown in Fig. 3. Essentially, both patients showed a rapid attainment of peak drug concentration followed by a rapid decline in drug plasma levels. These data are in agreement with previous studies [9, 12]. A similar pattern is also observed in the analysis of rat data depicted in Fig. 4 (pooled results). The plasma levels of chlorambucil peak in less than 2 h, then decline rapidly with an approximate half-life of 1.79 h.



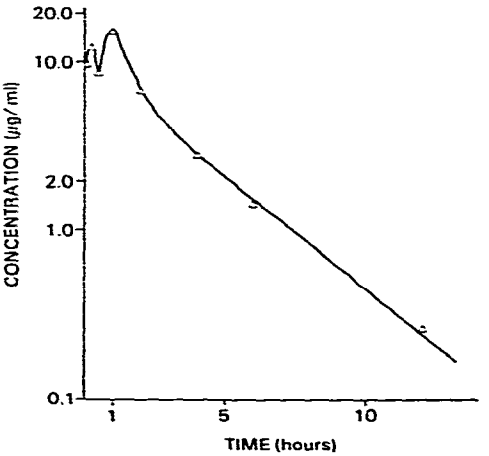


Fig. 3. Plasma levels of chlorambucil in two patients receiving chlorambucil chemotherapy.

Fig. 4. Concentration–time profile of chlorambucil in rats. Each point represents the mean of six animals. The standard error ranges from 0.69 to 2.61 µg/ml.

Elucidation of the pharmacokinetics of chlorambucil, hence its chemotherapeutic efficiency in patients, can be greatly enhanced by the use of a simple, yet efficient method of chlorambucil analysis. As such, significant advantages of the technique reported here can be readily seen. This technique allows quantification with a high degree of accuracy. Sample preparation is rapid and efficient. Clear resolution of the drug in biological samples is achieved with no background interference. Application of this technique toward patient studies is now in progress in our laboratory and should yield a better understanding of chlorambucil pharmacokinetics and the mechanisms of its chemotherapeutic and toxic actions in cancer patients.

ACKNOWLEDGEMENT

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