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## Note

# Determination of chlorambucil in plasma using reversed-phase high-performance liquid chromatography

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Chlorambucil is an alkylating agent which is used to treat chronic lymphocytic leukaemia (CLL) [1], lymphomas and ovarian carcinoma [2]. The major side-effect of this drug is bone marrow suppression. Therefore, a knowledge of the pharmacokinetics of chlorambucil may enable therapy to be optimised whilst safeguarding the patient from toxicity.

The early attempts to quantify chlorambucil were based on colorimetry [3], radiolabelling [4] and gas chromatography–mass spectroscopy [5]. More recently several methods for the determination of chlorambucil, using high-

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performance liquid chromatography (HPLC) have been described [6–9]. Whilst sufficiently sensitive to enable determination of pharmacokinetic data, disadvantages of existing procedures include the use of solvent extraction [6], the need for gradient elution [7] and relatively long retention times [8, 9]. Zakaria and Brown [9] have described a method for chlorambucil measurement by direct injection of plasma onto the column. However, the maximum volume of plasma reported permits detection to only 120 ng/ml.

This paper describes a reversed-phase HPLC assay for chlorambucil in plasma. Sample clean-up involves precipitation of plasma macromolecular components with concentrated perchloric acid, followed by extraction of chlorambucil from the supernatant with $C_{18}$ Sep-Pak. The retention time for the drug is 4.2 min and the limit of detection is 10 ng/ml. In addition, the extraction and modified chromatographic procedures enables simultaneous determination of chlorambucil and melphalan. Although these drugs are rarely combined in therapeutic protocols, the technique may nonetheless facilitate pharmacokinetic studies of these alkylating agents.

## MATERIALS AND METHODS

### *Instrumentation*

HPLC equipment from the Pye Unicam PU 4000 system (Cambridge, U.K.) incorporated a dual reciprocating pulseless pump and variable-wavelength UV detector, set to 260 nm. Detector sensitivity was 0.08 a.u.f.s. Chromatograms were recorded on a Pye CDP 4 computing integrator with 100 mV f.s.d. The column was Spherisorb ODS 5 $\mu$m particle size, 250 × 4.6 mm I.D. (Phase Separations, Queensferry, U.K.). The column, guard column (Spherisorb ODS 5 $\mu$m, 30 × 4.6 mm I.D.), Rheodyne 7125 injection valve (Cotati, CA, U.S.A.) with a 200-$\mu$l loop, were mounted in a block heater (Jones Chromatography, Cardiff, U.K.) and maintained at 40°C.

### *Mobile phase*

The mobile phase consisted of a mixture of methanol (Fisons, Loughborough, U.K.)–water (80:20). All materials and reagents were of HPLC grade and were filtered when appropriate with a 0.2-$\mu$m Millipore filter prior to use. The flow-rate of the mobile phase was 1.3 ml/min (150 bar).

### *Sample preparation and extraction*

Peripheral blood samples (6 ml) were collected in lithium heparin tubes and stored at 2°C. After centrifugation (1300 *g*, 10 min, −6°C), 3-ml plasma aliquots were removed and the macromolecular components precipitated with 132 $\mu$l of cold concentrated perchloric acid (2°C). The mixture was vortex-mixed for 3 min prior to centrifugation (1300 *g*, 15 min, −6°C). The supernatant was removed and passed through a $C_{18}$ Sep-Pak (Waters Assoc., Taunton, MA, U.S.A.). The Sep-Pak was washed with 10 ml of 15% methanol in water (2°C), and chlorambucil then eluted with 2 ml of methanol. The eluate was stored at −20°C prior to chromatography.

## RESULTS AND DISCUSSION

In our assay for melphalan [10], the mobile phase was a mixture of methanol—water (80:20) containing 0.0135% (w/v) sodium dodecyl sulphate (BDH, Poole, U.K.). This was adjusted to pH 3.11, using sulphuric acid. The separation of chlorambucil from remaining plasma components is unaffected by ion-pairing chromatography, thus the mobile phase may be simplified to methanol—water (80:20).

The type of guard column used had a significant effect on drug resolution. A 5-cm guard column packed with Co:Pell ODS (particle size 30—38 $\mu$m) produced inadequate resolution. Effective removal of extraneous plasma contaminants was achieved using a LiChrosorb ODS 10-$\mu$m guard column (30 × 4.6 mm I.D.). However, the best results were obtained by a Spherisorb ODS 5-$\mu$m guard column (30 × 4.6 mm I.D.).

The coefficient of variation for ten concentration duplicates (20– 1200 ng/ml) extracted from plasma was 0.71%; the correlation coefficient for the calibration graph was 0.998. Within-batch variability for standard solutions of 100, 500 and 1000 ng/ml was 1.67%, 0.96% and 0.18%, respectively, for each chromatography ten times. Recovery of drug from plasma was approximately 60%, with a limit of detection of 10 ng/ml.

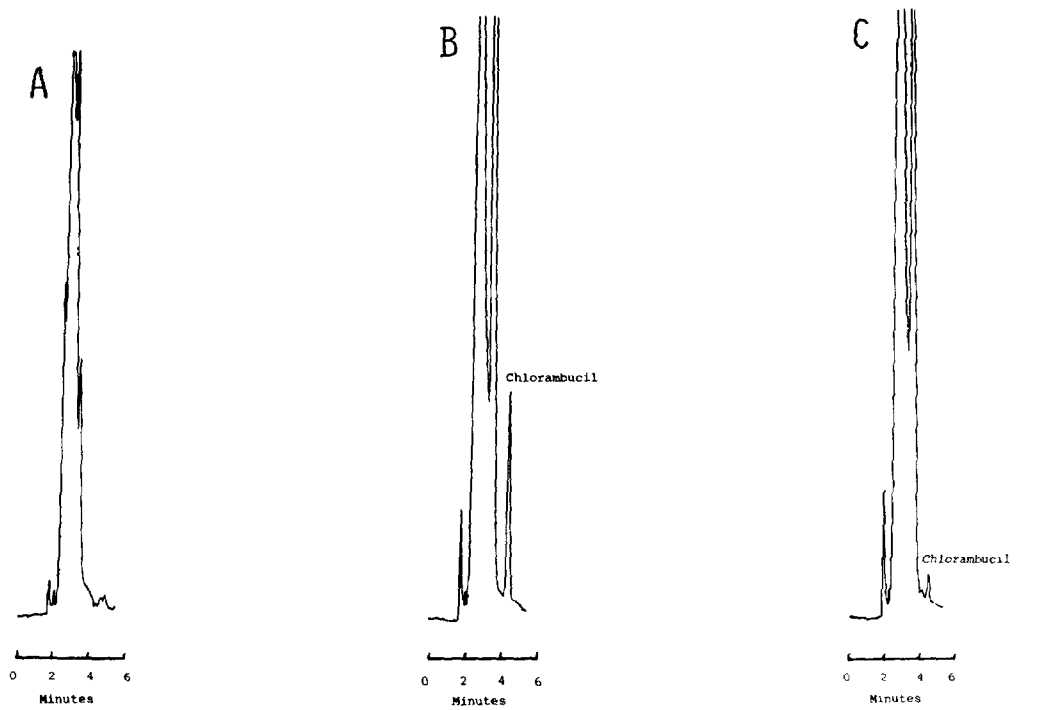


Fig. 1. Chromatograms of plasma samples from a patient (A) before drug administration, (B) 70 min after oral administration of 30 mg chlorambucil (plasma concentration 272 ng/ml), (C) 270 min after drug administration (plasma concentration 40 ng/ml). Samples were separated on a Spherisorb ODS 5-$\mu$m column (250 × 4.6 mm I.D.) with a Spherisorb ODS 5-$\mu$m (30 × 4.6 mm I.D.) guard column at 40°C. The mobile phase (flow-rate 1.3 ml/min) was a mixture of methanol—water (80·20). Detection was by UV absorption at 260 nm.

Acetonitrile and trichloroacetic acid (BDH) were examined as alternative precipitating reagents in an attempt to improve extraction efficiency and reduce plasma interference. Recovery of drug from plasma was 84% and 30%, respectively. However, both reagents increased plasma background. Thus, the best compromise between resolution and recovery was achieved using concentrated perchloric acid (Fig. 1).

Zakaria and Brown [9] noted the potential degradation of chlorambucil, in previous reports, during collection of plasma samples and storage of extracts prior to chromatography. In view of this we investigated the stability of the drug in methanol and plasma. Solutions were incubated at −20°C, 2°C and 37°C over a 6-h period, and samples removed and chromatographed at intervals. Chlorambucil was found to be stable in methanol at all temperatures investigated. In plasma (Fig. 2) the drug was not hydrolysed at −20°C and 2°C. However, at 37°C hydrolysis occurred, which has also been described by Ehrsson et al. [11]. Therefore, loss of chlorambucil by hydrolysis does not occur during the conditions reported here for collection of plasma samples and storage of methanolic extracts.

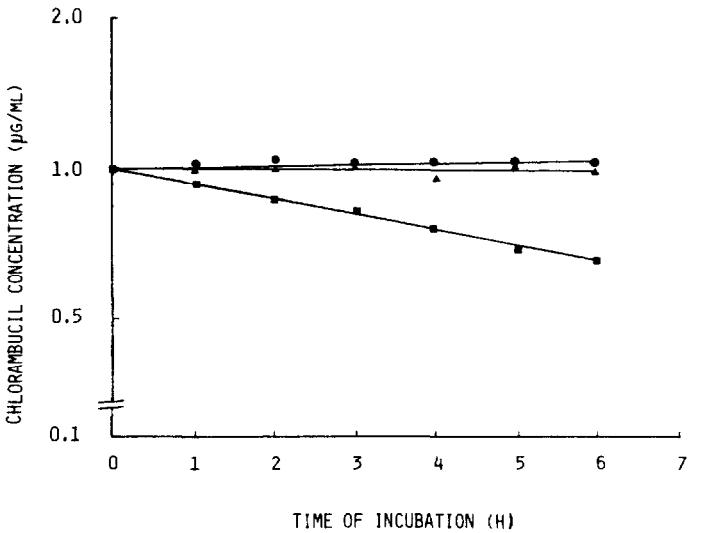


Fig. 2. Stability of chlorambucil in plasma over a 6-h period at −20°C (•), 2°C (▲), and 37°C (■).

We have determined plasma levels of chlorambucil in five patients (two CLL and three non-Hodgkin's lymphoma) receiving high-dose chemotherapy (25–40 mg daily for three days). Samples of peripheral blood were taken over a 6-h period and stored in ice (2°C) prior to analysis. Peak plasma concentrations ranged from 703 to 909 ng/ml occurring 25–120 min after drug administration. Methanolic solutions of prednisolone and prednisone were chromatographed to confirm that concomitant medication did not affect chlorambucil resolution.

## CONCLUSION

In this paper we have described a rapid and simple assay for chlorambucil in plasma, with a limit of detection of 10 ng/ml (based on a peak height of twice baseline noise). We have shown that plasma samples may be collected in ice (2°C) over a period of 6 h without hydrolysis of drug. In addition, the methanolic extract of chlorambucil is stable under storage. We are currently using this assay to study the pharmacokinetics of chlorambucil in patients with CLL and lymphoma.

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