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Note

Estimation of chlorambucil, phenyl acetic mustard and prednimustine in human plasma by high-performance liquid chromatography

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Chlorambucil {4[4-bis(2-chloroethyl) aminophenyl]butyric acid} is an alkylating agent used in the treatment of human cancer, particularly chronic lymphocytic leukaemia (CLL) [1], Hodgkin's disease [2] and carcinoma of the ovary [3]. Prednimustine {pregna-1,4-diene-11β,17α,21-triol-3,20-dione, 21-(4[4-bis(2-chloroethyl)aminophenyl]butyric acid)}, the prednisolone ester of chlorambucil, has recently been undergoing clinical trial for the treatment of various malignancies including CLL [4].

The estimation of alkylating agents in biological fluids has previously been accomplished by a variety of techniques. These include colorimetry [5], mass spectrometry [6], radiolabelling [7] and gas—liquid chromatography [8]. However, these methods are too complex and time-consuming to allow of their routine use in the clinical laboratory. Recently high-performance liquid chromatography (HPLC) has been employed in the estimation of melphalan {4[bis (2-chloroethyl)amino]-L-phenylalanine}, a chemically related alkylating agent [9, 10]. In the present study a rapid simple HPLC method is reported for the simultaneous estimation of chlorambucil, prednimustine and the chlorambucil metabolite, phenyl acetic mustard, {2[4-bis(2-chloroethyl)-aminophenyl]acetic acid} [11] in human plasma. The HPLC method described has facilitated a study of the fate of chlorambucil and prednimustine in man, the results of which have previously been reported in a preliminary form [12].

EXPERIMENTAL

Apparatus

HPLC separations were carried out on a Waters Assoc. Model ALC/GPC

204 chromatograph (Waters Assoc., Milford, Mass., U.S.A.). This consisted of two Model 6000A solvent pumps, a Model 660 solvent programmer, a Model U6K injector, a Model 440 UV detector, and a Model 450 variable-wavelength detector fitted with a stop/flow spectrum facility. Signals from the detector were recorded on a three-pen Model B-381H Rikadenki recorder (Rikadenki Kogyo, Tokyo, Japan). All separations were accomplished on a Waters Assoc. μBondapak C_{18} column operating in reversed-phase mode. Solvents were filtered and degassed prior to use on a 0.45-μm Millipore filter (Millipore, Bedford, Mass., U.S.A.). Sample injections were made with a 100-μl Pressure Lok Syringe (Precision Sampling, Baton Rouge, La., U.S.A.).

Materials

Glass double distilled water was used in all experiments. Ethyl acetate (Koch-Light, Colnbrook, Great Britain), methanol (James Burroughs, London, Great Britain), acetic acid, sodium sulphate and citric acid (BDH Chemicals, Poole, Great Britain) were all analytical grade. Chlorambucil was a gift from the Wellcome Foundation, Beckenham, Great Britain, and prednimustine was a gift from AB Leo, Helsingborg, Sweden. Phenyl acetic mustard was synthesised by Professor Walter Ross [13] at the Institute of Cancer Research, London, Great Britain.

Patient treatment

Previously untreated patients with Hodgkin's disease requiring chemotherapy were studied immediately before the start of conventional therapy [2]. Following the insertion of an indwelling intravenous cannula patients received either 10 mg of chlorambucil or 20 mg prednimustine, orally.

Extraction procedure

Blood samples were taken into heparinised tubes and centrifuged at 600 *g* for 10 min at 4°. Duplicate 1-ml aliquots of plasma were removed and placed in 15-ml conical glass centrifuge tubes. Two milliliters of ethyl acetate were added to each tube, a fine emulsion was produced by vigorous agitation on a "Whirlimixer", and the tube was immersed in a methanol—carbon dioxide bath at ca. −68°. When completely frozen, the tubes were centrifuged at 600 *g* for 10 min at 4°, during which time the aqueous phase thawed, allowing the organic layer to be removed subsequently. This procedure was repeated with a further 2 ml of ethyl acetate and the pooled ethyl acetate extracts were dried over anhydrous sodium sulphate for 1 h at room temperature. Two milliliters of the dried ethyl acetate extract were removed and evaporated to dryness in a stream of nitrogen at 45°. The residues were redissolved in 100 μl of ethyl acetate and stored in sealed vials at 4° prior to assay.

HPLC analysis

Fifty microliters of the ethyl acetate concentrate (representing 0.25 ml plasma) were injected. Samples were eluted by running a linear gradient from methanol—0.175 *M* acetic acid (60:40, v/v) to 100% methanol over 10 min, commencing at injection time. A flow-rate of 2 ml/min was used on all occasions. The absorbances at 254 nm and 280 nm of the eluate were recorded

simultaneously (0.02 or 0.05 a.u.f.s., chart speed 2 cm/min). At the end of each separation the column was returned to the starting solvent over a 5-min linear gradient. The area under each peak was determined by Xeroxing, cutting out and weighing the relevant peaks. Quantitation was then achieved by comparison with the areas obtained following the injection of reference compounds.

RESULTS AND DISCUSSION

Quantitative aspects of the method

A typical separation of chlorambucil, phenyl acetic mustard and prednimustine is illustrated in Fig. 1. Model 440 UV detector response was linear over the range 5—1000 ng for chlorambucil, phenyl acetic mustard and prednimustine, with a correlation coefficient (r) = 0.999 in each case. The recoveries of chlorambucil, phenyl acetic mustard and prednimustine, from plasma, were investigated by the addition of these compounds to 1-ml aliquots of plasma from healthy human donors. Compounds were added in 10 μl of methanol so as to produce plasma concentrations ranging from 0.05 μM to 10 μM. Samples were assayed in quadruplicate and analysed immediately.

Chlorambucil recovery was linear over the range 0.05—10 μM (r = 0.988) with an extraction efficiency of 77.5%. Prednimustine recovery was linear over the range 0.05—10 μM (r = 0.997) with an extraction efficiency of 95.4%. Phenyl acetic mustard recovery, however, was only linear over the range 0.5 –10 μM (r = 0.948) with an extraction efficiency of 32.0%. The poor extraction efficiency of phenyl acetic mustard probably contributed to its higher limit of detection. Attempts to improve the extraction efficiency of phenyl acetic mustard were made by reducing the plasma pH to 3.0 with 1 M citric acid. However this resulted in irreversible protein precipitation during the

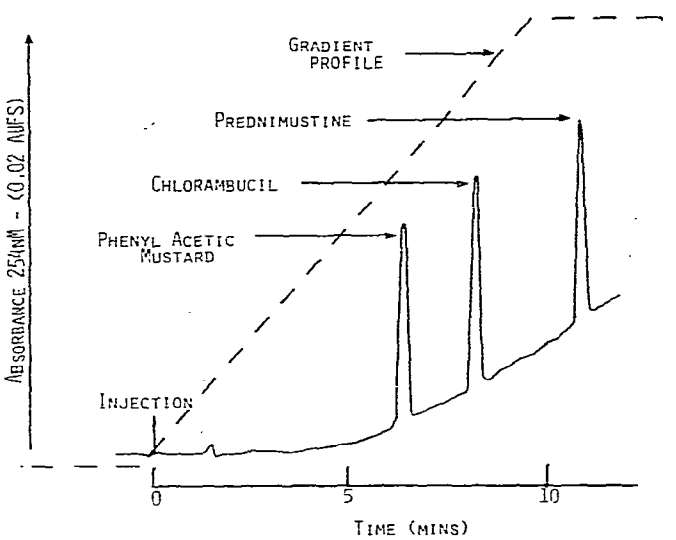

Fig. 1. HPLC separation of phenyl acetic mustard, chlorambucil and prednimustine.

first extraction which prevented the estimation of drug concentrations lower than 3 μM. Hence in all experiments the pH of the plasma samples was left unchanged and relevant calibration factors were used in the calculation. Storage of plasma samples for three weeks at −28° prior to analysis did not affect recovery.

Pharmacokinetic studies

Chlorambucil. The pharmacokinetics of chlorambucil following a 10-mg oral dose were investigated in six patients. Peak levels of chlorambucil were detected 30 min or 1 h after administration, subsequently decaying with a $t_{½}$ of 1.7 h (Fig. 2). Examples of the chromatograms obtained before and 2 h after the oral administration of 10 mg of chlorambucil are shown in Fig. 3. In addition to the chlorambucil peak, a peak with a retention time similar to that of phenyl acetic mustard was detected in the plasma extracts of three patients 2 h after oral administration of 10 mg chlorambucil. The identities of the chlorambucil and phenyl acetic mustard peaks in plasma extracts were verified by retention time, by λ_{max} determinations from stop/flow spectra (Table I), and in two patients by mass spectrometry. Samples for mass spectrometry were obtained by peak collection from the effluent of the HPLC, methylated, and the mass spectra obtained compared with those produced by

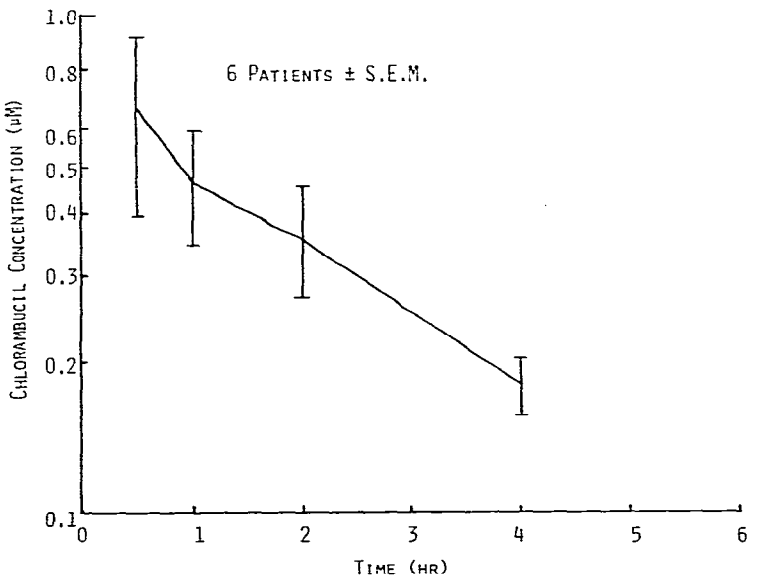

Fig. 2. Concentration of chlorambucil in human plasma following a 10-mg oral dose.

synthetic standards. Hence it has been demonstrated that chlorambucil is metabolised to phenyl acetic mustard in man. However, insufficient assay sensitivity precluded a pharmacokinetic study of this metabolite.

Prednimustine. Twenty milligrams of prednimustine were given orally to six patients and blood samples taken at 0.5, 1, 2, 4 and 6 h and thereafter at six-hourly intervals up to 48 h.

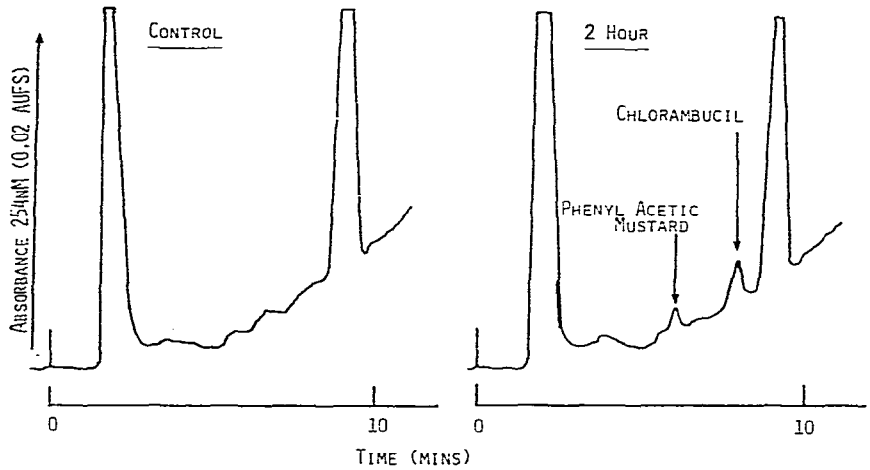

Fig. 3. Chromatograms of extracts from plasma obtained before and 2 h after a 10-mg oral dose of chlorambucil.

TABLE I

PLASMA COMPONENT IDENTIFICATION BY HPLC

	Chlorambucil		Phenyl acetic mustard	
	Standard	Plasma component	Standard	Plasma component
Retention time (min)	7.83 ± 0.07*	7.88 ± 0.12*	5.95 ± 0.07*	6.01 ± 0.09*
λ_{max} (nm)	254—256	254—256	256—259	257—260

*Mean of 6 determinations ± S.E.M.

At no time could prednimustine, chlorambucil or phenyl acetic mustard be detected in the plasma. In all patients it had previously been demonstrated that chlorambucil was absorbed following oral administration. Hence these results indicate that the bioavailability of orally administered prednimustine is lower than that of chlorambucil.

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