Biochemical Pharmacology, Vol. 28, pp. 1289–1292.

0006-2952/79/0415–1289 $02.00/0

PHARMACOKINETICS OF CHLORAMBUCIL IN OVARIAN CARCINOMA USING A NEW HPLC ASSAY

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(*Received* 27 *October* 1978; *accepted* 16 *November* 1978)

Abstract—From a study of several ester derivatives of chlorambucil, it is reported that the propyl ester is the best for use in HPLC assay of plasma levels. The pharmacokinetics of elimination following oral doses are reported for five ovarian carcinoma patients and it is concluded that usually peak levels are reached after some thirty minutes while the plasma levels are approaching the limits of detection after two hr.

Chlorambucil is one of a series of nitrogen mustard derivatives [1] in which the chemical combination of the reactive bis-(2-chloroethyl)-amino group with an aromatic carboxylic acid system was intended to facilitate passage of the potent alkylating function through membranes and hence between tissues.

The drug has become a valuable antineoplastic agent, widely used in the treatment of lymphatic disorders and also ovarian cancer chemotherapy [2, 3].

In order to investigate pharmacokinetic and long term aspects of chlorambucil's behaviour in patients in this hospital suffering from the latter condition, we have developed and applied a high-performance liquid chromatographic (HPLC) method for the determination of the drug in plasma. The results of preliminary studies using the method are now reported.

MATERIALS AND METHODS

High-performance liquid chromatography. The apparatus consisted of a Waters Associates M6000 pump, a 10 × 0.46 cm steel column packed with S5 0DS, a Waters Associates Model U6K Injector, a Pye Unicam LC3 Variable wavelength U.V. detector (operating at 254 nm) and a Tekman Series TE 220 Chart Recorder.

The mobile phase was water:methanol, 20:80 (v/v) with 0.1% ammonium acetate (w/v). The flow rate used was 1 ml min^{-1} (≡500 psi).

Esterification studies. These studies concerned the relative ratio of formation of chlorambucil esters using methyl, ethyl, n-propyl and n-butyl alcohols.

Kinetics were investigated at a variety of temperatures using chlorambucil (1 mg) in an excess of alcohol (20 ml) with a catalytic quantity of hydrochloric acid (0.055 M) and the retention times of the esters (whose identities were confirmed by mass spectrometry) were recorded (using the mobile phase given above).

The experiments were repeated using clinical levels of the drug (200 ng ml^{-1}) in the presence of plasma extracts employing the procedure described in the next section.

Analysis of chlorambucil in plasma. Prepared standard solutions of chlorambucil in plasma and clinical specimens were treated in the following way.

A sample (1.0 ml) was shaken manually with chloroform (10 ml) for 2 min, then briefly centrifuged at 1000 *g*. The organic layer was retained and evaporated to dryness at 35° under reduced pressure in a rotary evaporator. The residue was dissolved in n-propyl alcohol (2.0 ml) and a catalytic quantity of hydrochloric acid added (50 μl of concentrated HCl). The mixture was heated at 65° for 80 min then evaporated to dryness at 50° under reduced pressure. The product was dissolved in Tris–HCl (0.01 M):MeOH, 50:50 (v/v), pH 8.4, (2.0 ml) and extracted with cyclohexane (3 × 2.0 ml). The cyclohexane layers containing the esterified material were pooled, evaporated to dryness under a stream of N_2 at 60°, and the residue was dissolved in MeOH (100 μl). 10 μl samples of the final solution were analysed by HPLC.

Areas of the resulting n-propylchlorambucil peaks were determined by cutting and weighing and compared with those of known quantities of chlorambucil processed in the same way.

Chlorambucil was obtained from Burroughs Wellcome & Co. (Wellcome Foundation Ltd.) London. All other chemicals and solvents were of the highest analytical grade obtainable.

Computer fitting of results. Pharmacokinetic data were fitted by a non-linear regression program using a D.G.C. Nova Interactive computer.

RESULTS

The rate constants for the formation of different chlorambucil esters at 65° are shown in Table 1. Experiments using plasma extracts indicated that the n-propyl derivative of chlorambucil was the only one cleanly separated from the extracted plasma constituents (see Fig. 1) by the determination procedure, as is clear from the retention time data given in Table 1. The plasma background was not dependent on the alcohol used.

A typical chromatogram of the n-propylated derivative of chlorambucil against the plasma background is shown in Fig. 1(c) along with the chromatograms of the appropriate standard (b) and control (pre-dose plasma extract) (a). The accuracy and reproducibility of the analysis is dealt with in the next section, but generally the limit of sensitivity is in the order of 10 μg chlorambucil/l plasma.

Table 1. True 2nd order rate constants for the esterification of chlorambucil by various alcohols

	Alcohol	k(M^{-1} min^{-1})	Retention time (min)
Esterification of chlorambucil at 65°	CH_3OH	0.0088	3.4
	CH_3CH_2OH	0.0023	3.9
	$CH_3(CH_2)_2OH$	0.0026	4.9
	$CH_3(CH_2)_3OH$	0.0043	6.1

65 . 55 mM hydrochloric acid according to the scheme chlorambucil + ROH → chlorambucil-ester + H_2O. The retention times of the esters are also given.

Statistical analysis of chlorambucil assay method. Figure 2(a) shows a plot of the efficiency of the drug assay at different plasma concentrations of the drug based on 6 replicate determinations at each concentration. For example, at the 100 ng ml^{-1} level the efficiency of the assay is 80 ± 11 per cent. Figure 2(b) illustrates the dependence of the variance on plasma drug concentrations. A hyperbolic relationship is approximately obeyed in the region illustrated and the line drawn through the monoreciprocal plot is a least-squares regression to the data. This graph has been used as a standard curve for the error on raw data.

Application of assay to clinical specimens. Five patients have been studied, all of whom were ovarian carcinoma sufferers and all but one of whom had received no previous chlorambucil therapy. Pre-dose samples of blood were taken to establish the chromatographic 'base-line' then a single oral dose (10 mg or 5 mg) was administered. Up to five blood samples were collected which were processed as described after removal of red cells. Results are shown in Fig. 3.

Data were fitted by computer according to the model:

$$C = \frac{k_1 D}{(k_2 - k_1) V} (e^{-k_1 t} - e^{-k_2 t})$$

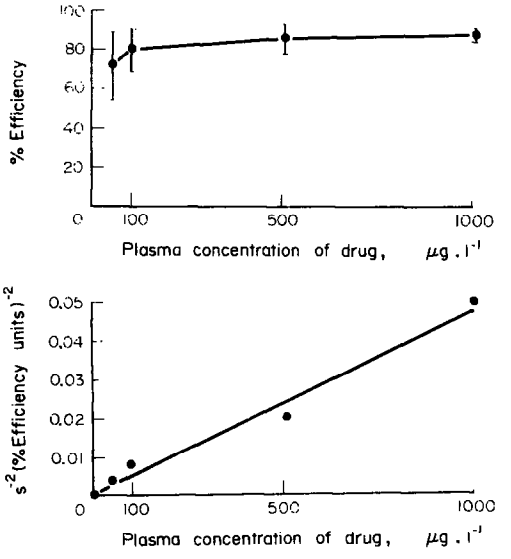

Fig. 2. Statistical analysis of chlorambucil assay. (a) Variation of assay efficiency with plasma concentration. Error bars represent 95 per cent confidence limits for the mean based on six replicate determinations. (b) Dependence of variance [(s)^2, where s is the standard deviation] on plasma concentrations, plotted as a monoreciprocal graph.

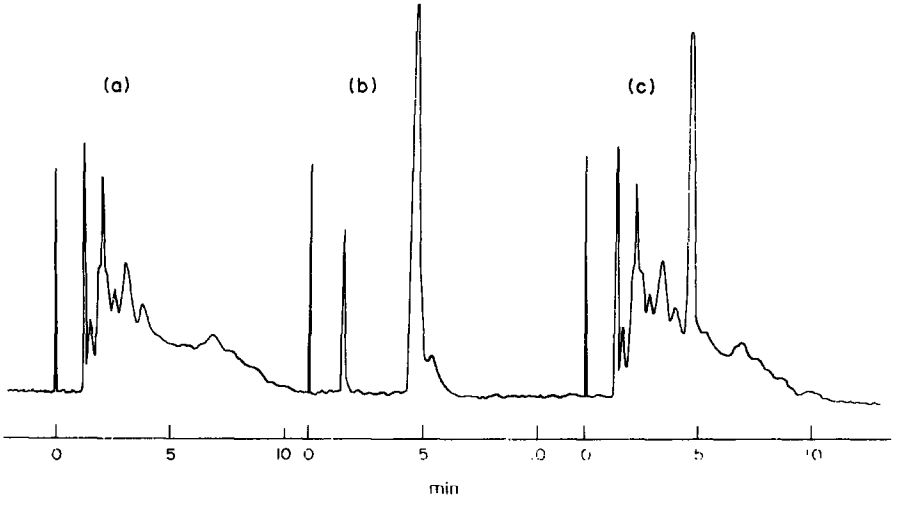

Fig. 1. Chromatograms obtained using the analytical procedure for chlorambucil described in the text. (a) Pre-dose plasma extract from patient (c) in Fig. 3. (b) n-Propyl chlorambucil standard (20 ng). (c) 25 min extract from patient (c). The peak corresponds to approximately 15 ng of the n-propyl ester (160 μg drug/l plasma).

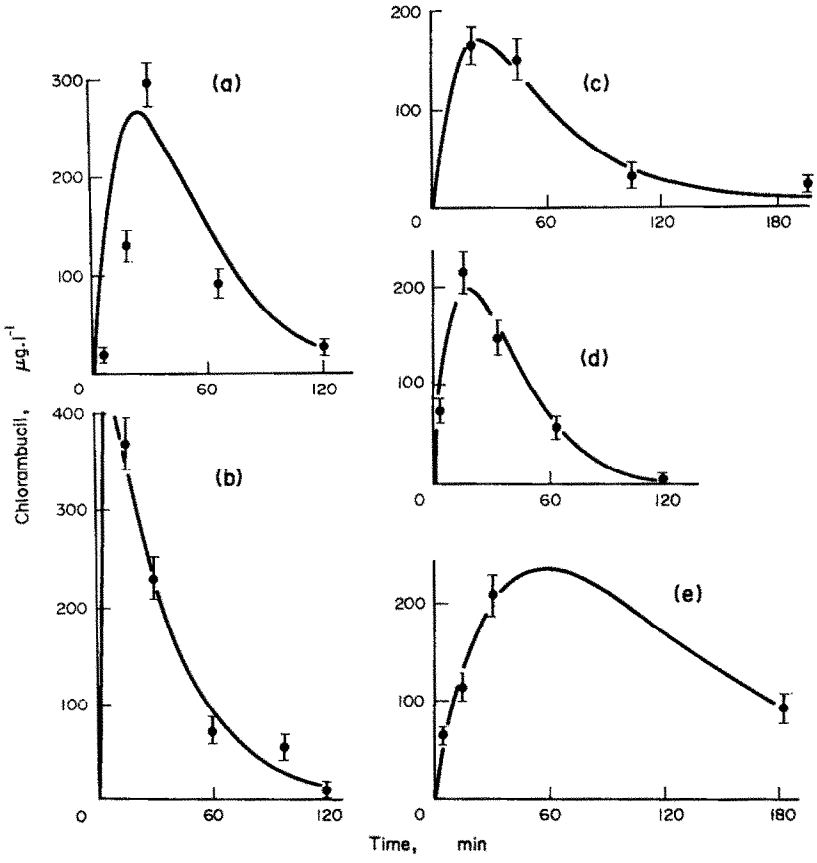

Fig. 3. Concentration/time profiles for chlorambucil in five patients after a single oral dose. Patients (a), (b) and (d) received 10 mg; patients (c) and (e) 5 mg. Error bars represent 95 per cent confidence limits for the mean, calculated from the error curve (Fig. 1(b)). Lines through the data are computer-generated fits using a two-exponential model (see text).
The calculated parameter values in each case are: (a) $k_1 = 0.04183$ min^{-1}; $k_2 = 0.04171$ min^{-1}; (b) $k_1 = 0.6708$ min^{-1}; $k_2 = 0.3094$ min^{-1}; (c) $k_1 = 0.05397$ min^{-1}; $k_2 = 0.01986$ min^{-1}; (d) $k_1 = 0.05104$ min^{-1}; $k_2 = 0.0536$ min^{-1}; (e) $k_1 = 0.02242$ min^{-1}; $k_2 = 0.01263$ min^{-1}.

where C is the concentration of the drug in the plasma compartment (μg l^{-1}), V the volume of that compartment [1], D is the dose in μg, and k_1 and k_2 the rate constants for the absorption and elimination processes respectively.

Although it may be that the pharmacokinetics of the system obey a higher order model, as possibly indicated by profile 3(a), with the number of data points concerned it would not be reasonable to apply such a model at this stage.

DISCUSSION

We have studied the rate of reaction of chlorambucil with several alcohols to form the corresponding esters in order to develop a method for the quantitative assay of plasma levels. The relative rate of formation of the esters is methyl- > n-butyl- > ethyl- > n-propyl-; but of more importance is the ease of extraction and chromatographic separation. From this study we conclude that the propyl ester is the most appropriate since it forms easily, extracts with high efficiency and is well separated from other plasma constituents on chromatography as is apparent from Fig. 1.

The limited number of patients and blood samples taken per patient does not allow more than a general discussion of the pharmacokinetic data. Of prime importance, however, is the successful application of the HPLC method to clinical specimens from individuals with ovarian carcinoma and the possibility now of proper pharmacokinetic and long term analysis of chlorambucil in human plasma. What is already apparent from these initial studies is the swift elimination of the drug from the plasma (chlorambucil is undetectable after 2 hr in four of the five patients studied). Although the drug is susceptible to hydrolysis in aqueous solutions [1], we have shown that it is stable in the presence of plasma proteins at 37° and therefore the removal of chlorambucil from the plasma as demonstrated here can be attributed to metabolism and excretion [4].

Acknowledgements—We thank the University of Manchester, Department of Chemistry for facilities and, in particular, G. N. Smith, G. F. Smith and P. B. McGinlay for help and advice. Also we thank V. R. Tindall, P. Hirsch and the Medical Staff of St. Mary's Hospital for arranging the clinical experiments and R. F. Jennison and staff for experiments using whole blood. Gratitude is expressed to B. R. Pullen and R. W. Foster for computer facilities and advice, to the Burroughs Wellcome Co. Ltd. for technical details concerning the chemistry and synthesis of chlorambucil, to the

M.R.C. for provision of a Cary 118C spectrophotometer and the North West Regional Health Authority for a grant to one of us (PL).

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