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Note

High-performance liquid chromatography of chlorambucil prodrugs structurally related to lipids in rat plasma

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Chlorambucil (Leukeran®) {4[(bis-2-chloroethyl)aminophenyl]butyric acid} (**1**, Fig 1) is an antineoplastic agent of the nitrogen mustard alkylating type, which is indicated in treatment of chronic lymphocytic leukaemia, lymphosarcoma and Hodgkin's disease

Reversed-phase high-performance liquid chromatography (RP-HPLC) is frequently employed for assaying this drug and structurally related compounds in plasma [1–4] For determining prodrug forms of **1**, radiochemical methods have been applied using either ^{3}H- or ^{14}C-labelled chlorambucil [5,6] These techniques, however, lack specificity with regard to the fate of the prodrug itself More recently, normal-phase (NP) HPLC has been used to separate different classes of lipids and to identity [^{3}H]fenoprofen and other triacylglycerols containing nitrophenylacyl moieties [7–9]

To study the biodistribution of chlorambucil prodrugs **3–6** (Fig. 2), structurally related to natural triacylglycerides, we have investigated their RP-HPLC and NP-HPLC behaviour with either fluorimetric (excitation at 275 nm, emission at 335 nm) or UV detection (264 nm)

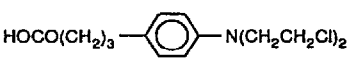

Fig 1 Structure of chlorambucil (**1**)

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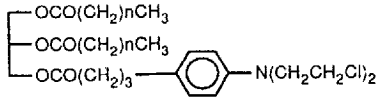

3 6

Prodrug	n
3	10
4	12
5	14
6	16

Fig 2 Prodrugs of chlorambucil (**3–6**) structurally related to natural triglycerides

EXPERIMENTAL

Apparatus

A Model 6000A solvent-delivery system (Waters Assoc, Milford, MA, U S A) equipped with a Model 7125 injection valve and a 20-μl sample loop (Rheodyne, Berkeley, CA, U S A) was used UV monitoring was accomplished by means of a Perkin-Elmer LC-85B variable-wavelength spectrophotometer, the signals were recorded and processed using a Sigma 10 data station (Perkin-Elmer) Fluorimetric detection was carried out with a Perkin-Elmer LS-4 spectrometer, the signals were recorded and processed using an LCI-100 data station (Perkin-Elmer) The data were processed using Comstat-Pharm, a statistical package for pharmacokinetic and dosage data operating on IBM-PC and compatibles

Chemicals

All solvents were of HPLC grade (Lab-Scan, Dublin, Ireland) Chlorambucil was kindly supplied by the Wellcome Foundation Diacylglycerols **2** (Fig 3) were prepared according to Pfeiffer et al [10]

The following procedure [11] was employed to synthesize **3–6** A solution was prepared by dissolving 3 0 mmol of **2**, 3 3 mmol of **1** and 0 3 mmol of 4-dimethylaminopyridine (4-DMP) in 25 ml of anhydrous dichloromethane To this solution, with stirring, was added dropwise 3 5 mmol of dicyclohexylcarbodiimide (DCC) Stirring at room temperature was maintained for 24 h, then the reaction mixture was filtered The filtrate was concentrated in vacuo to a residue which was chromatographed on a silica gel column (silica gel G, 70–230 mesh) using light petroleum (b p 60–80°C)–ethyl acetate (90 10, v/v) (yield, 62–75%)

The structures of **3–6** were confirmed by ^{13}C NMR spectroscopy on a Bruker WM 250 spectrometer operated at 62 3 MHz Typically, these compounds dis-

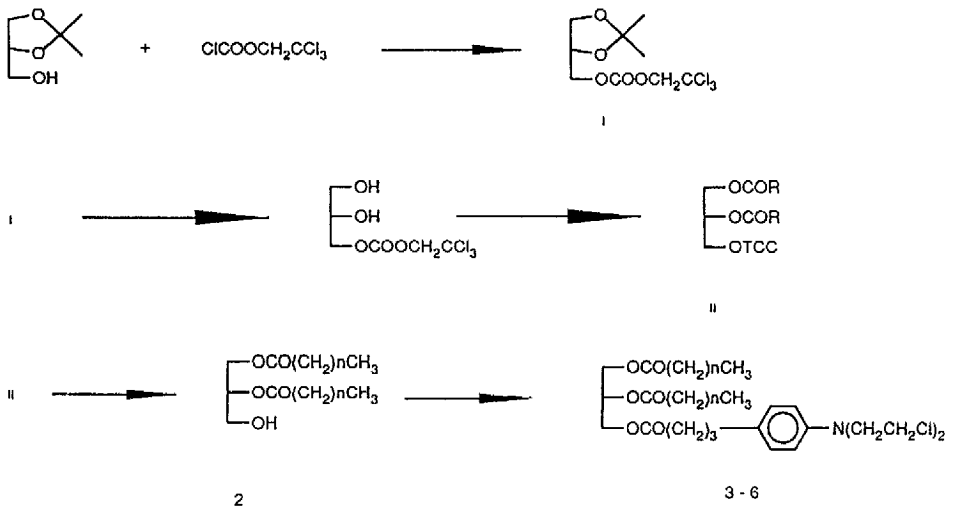

Fig 3 Synthetic method for preparation of chlorambucil prodrug forms **3–6**

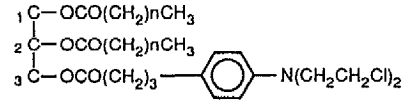

Fig 4 The glyceridic carbons C-1, C-2 and C-3 of chlorambucil prodrug forms **3–6**

play three signals for the glyceridic carbons, C-1 at 62 282 ppm, C-2 at 62 188 ppm and C-3 at 69 864 ppm (Fig 4), in analogy with other glyceridic compounds [9]

Mass spectra, recorded on an LKB 900 gas chromatograph–mass spectrometer using direct sample introduction, showed the peak of m/z corresponding to the molecular ion (M^+) of both prodrugs with a characteristic accompanying ($M^+ + 2$) peak Another characteristic signal in the mass spectrum of prodrugs **3–6** was an ($M^+ - 302$) peak, attributed to fragmentation of the acyl group of the drug moiety

The melting points (uncorrected) of the prodrugs, determined in open capillaries with a Thomas Hoover Uni-Melt apparatus, were 28–30 °C for **4**, 36–38 °C for **5** and 43–45 °C for **6**

*Administration of the prodrugs **3–6** and preparation of the samples*

Male rats (Wistar strain, 270–290 g) were canulated at the level of the right jugular vein Prodrugs were administered intravenously (60 mmol/kg) in 0 5 ml sodium chloride solution containing 5% Tween 20 by means of an infusion pump Blood was removed at the level of left jugular vein by preheparinized

syringes and centrifuged (2500 *g*, 10 min, 5°C) using a BHG-Hermle Z320K centrifuge

Plasma samples (50 μl) were extracted with 100 μl of hexane by vortex mixing (2 min) After centrifugation (2500 *g*, 5 min, 0°C) in closed tubes, the supernatant was concentrated under a stream of nitrogen The residue was taken up in 100 μl of mobile phase and injected via a 20-μl loop

Chromatographic conditions

Under NP-HPLC conditions, the eluent was *n*-hexane–2-propanol (98 2, v/v) at a flow-rate of 1 8 ml/min A Zorbax-CN column (250 mm×4 6 I D ×6 mm O.D) (DuPont, Wilmington, DE, U S A) fitted with a guard column (3 cm×4 6 mm I D.) packed with 10-μm Cyano-Cn-GU particles (Pye Unicam, Cambridge, U K) was used

Under RP-HPLC conditions, the mobile phase was tetrahydrofuran (THF)–water (73 27, v/v) at a flow-rate of 1 2 ml/min. A CP-Spher-C_{18} column (100 mm×3 mm I D ×6 mm O D) (Chrompack, Middelburg, The Netherlands) fitted with a guard column (10 mm×2 mm I D) with the same stationary phase was employed Elution was always carried out at room temperature under isocratic conditions

Construction of calibration graphs

Two standard solutions were prepared by dissolving accurately weighed amounts of 150 nmol of prodrugs **3** (standard solution A) and **5** (standard solution B), both in 10 ml of *n*-hexane In the analysis of prodrugs **4**, **5** and **6**, standard solution A was used as an internal standard; in the analysis of prodrug **3**, standard solution B was used as an internal standard

The same plasmas (45 μl) were spiked with 5 μl of prodrug solution (sodium chloride solution containing 5% Tween 20) to obtain standard plasma concentrations of 1 16, 3.52, 8 1, 16 2 and 32 4 nmol/ml. The same standard plasma were extracted with 100 μl of standard solution A or B and employed in the

TABLE I

AVERAGE RECOVERY YIELD IN THREE ASSAYS OF PLASMA SAMPLES FOR PRODRUGS **3**–**6** IN THE EXTRACTION STEP WITH *n*-HEXANE AND THE RETENTION TIMES OF THE PRODRUGS USING BOTH RP- AND NP-HPLC

Values in parentheses are mean standard errors

Prodrug	Yield (%)	Retention time (min)	
		RP-HPLC	NP-HPLC
3	81 0 (2 02)	3 5	5 8
4	78 2 (2 35)	4 4	5 4
5	67 1 (2 02)	5 5	5 0
6	70 0 (1 97)	7 2	4 8

TABLE II

MODIFIED REGRESSION ANALYSIS OF THE CALIBRATION GRAPHS FOR DETERMINATION OF PLASMA CONCENTRATIONS OF THE PRODRUGS

$y' = a'x' + b$, where y' = plasma concentration of the prodrug, x' = ratio of peak areas of the prodrug to that of the standard, $a' = 1/a$ and $b' = -b/a$ (a and b are coefficients calculated in regression analysis from calibration graphs) UV detection was used

Prodrug	a'	b'
3	14 7636	−1 9588
4	13 6331	−2 8036
5	12 0256	−1 1923
6	11 6956	−0 9923

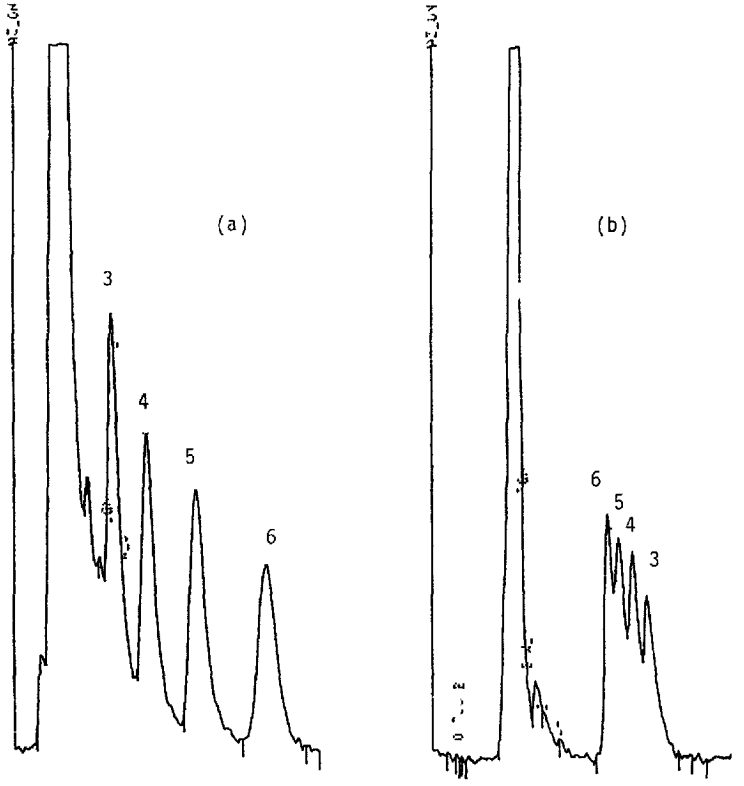

Fig 5 Extracts of standard plasma spiked with prodrugs **3**–**6** at 13 6 nmol/ml (a) RP-HPLC, mobile phase THF–water (73 27, v/v), flow-rate 1 2 ml/min, (b) NP-HPLC, mobile phase *n*-hexane–2-propanol (98 2, v/v), flow-rate 1 8 ml/min Fluorimetric detection (λ_{exc} 275 nm, λ_{em} 335 nm) in both methods

construction of calibration graphs by plotting the ratio of the peak area of the prodrug to that of the standard ratio against the standard plasma prodrug concentration The calibration data for each prodrug were well described by linear equations (r^2=0 9981 for **3**, 0 9993 for **4**, 0 9979 for **5** and 0 9887 for **6**) The recovery yield in the extraction step with *n*-hexane was determined for each prodrug on plasma samples at concentrations of 5 and 15 nmol/ml Samples were assayed in triplicate and the average recoveries for different prodrugs were calculated

RESULTS AND DISCUSSION

Peaks were identified by their retention times (Table I) Quantification of each prodrug was performed from the peak-area ratio using the modified linear regression equation (Table II) calculated from the calibration graphs.

Both RP-HPLC (Fig 5a) and NP-HPLC (Fig 5b) can be employed to assay **3–6** However, in RP-HPLC with UV detection at 254 nm, the front of the plasma interfered slightly with prodrug **3** (Fig 6). The detection limits calculated for prodrugs **5** and **6** were 0.53–0 96 nmol/ml The detection limits in both methods using either fluorimetric or UV detection were not significantly different ($p>0$ 05 for $n=11$)

To illustrate the use of the method for in vivo metabolism and pharmaco-

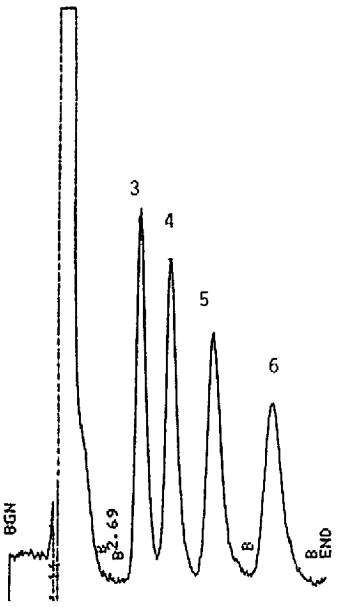

Fig 6 RP-HPLC of extracts of standard plasma spiked with prodrugs **3**-**6** at 13 5 nmol/ml with UV detection at 254 nm

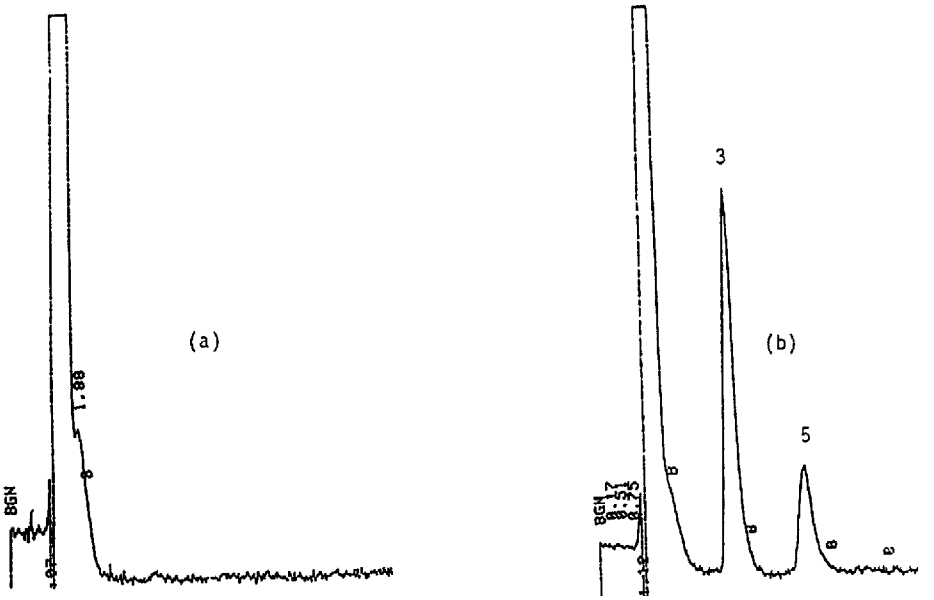

Fig 7 RP-HPLC with UV detection at 254 nm Extracts of a plasma sample from rats (a) injected only with prodrug vehicle or (b) treated with prodrug **5** (60 nmol/g per rat) The sample was prepared from the blood collected at the level of the jugular vein 88 min after prodrug administration The prodrug concentration for the plasma sample was 5 2 nmol/ml

kinetic studies, Fig 7b shows a chromatogram for the RP-HPLC analysis of an extract of plasma from rat injected with the prodrug **5** UV detection (254 nm) was applied No interfering peaks appear in the plasma following administration of the drug vehicle (solution containing 5% Tween 20) (Fig. 4a)

The RP-HPLC method is being applied in our laboratory for in vivo metabolic and enzymatic kinetic studies of the prodrugs **3–6** It was chosen in our assays because of its more efficient separation of the prodrugs; moreover, it is suitable in studies carried out in aqueous media (enzymology and determination of partition coefficients of the drugs)

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