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Note

High-performance liquid chromatographic analysis of chlorambucil *tert.*-butyl ester and its active metabolites chlorambucil and phenylacetic mustard in plasma and tissue

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Chlorambucil *tert.*-butyl ester (TBC), *tert.*-butyl 4-[4-bis(2-chloroethyl)aminophenyl]butyrate hydrochloride, is a lipophilic analogue of the widely used anticancer drug chlorambucil, 4-[4-bis(2-chloroethyl)aminophenyl]butyric acid, recently developed for the treatment of primary and metastatic malignant brain tumors [1,2] (Fig. 1). TBC possesses intrinsic alkylating activity and, as a consequence of steric hindrance around its ester link, is sufficiently stable *in vivo* to allow its significant accumulation in brain following its intravenous (i.v.) administration [1,2]. Extensive preclinical pharmacological testing of the compound is presently being undertaken prior to its expected use in humans. To facilitate this and to relate pharmacological effects to drug concentrations, a sensitive and simple high-performance liquid chromatographic (HPLC) assay was developed for the rapid quantitation of TBC, of its active product of ester hydrolysis, chlorambucil, and of the subsequent active product of β-oxidation, phenylacetic mustard, 2-[4-bis(2-chloroethyl)aminophenyl]acetic acid (Fig. 1), from a single plasma or tissue sample. Quantitation of each is essential as one would expect the

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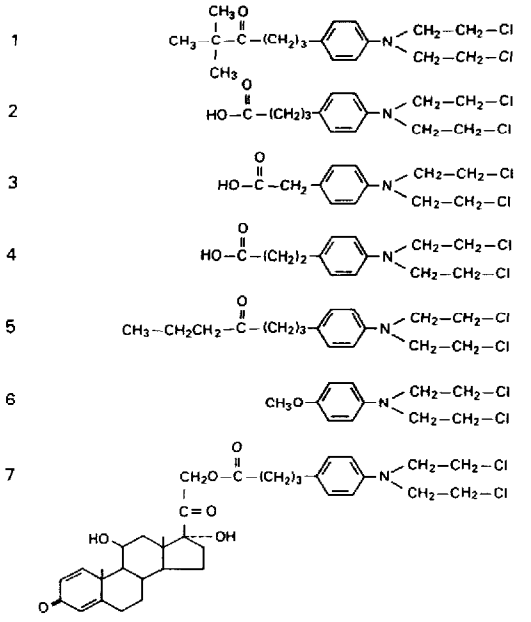

Fig. 1. Chemical structures of chlorambucil *tert* -butyl ester (1), chlorambucil (2), phenylacetic mustard (3), phenylpropionic mustard (4), chlorambucil propyl ester (5), methoxyphenyl mustard (6) and prednimustine (7)

biological activity of the compound to derive from the combined actions of TBC itself and of its transitory alkylating metabolites. As the physicochemical properties of these agents are different, one would predict their distribution and elimination *in vivo* to be heterogeneous.

In this report, we describe an HPLC technique for co-determination of TBC and its active metabolites, from the same biological sample, based on an assay by Newell *et al.* [3] for prednimustine and metabolites (Fig. 1), with several modifications. The preliminary pharmacokinetics of TBC and metabolites in brain and plasma following its i.v. administration to rats are additionally described.

EXPERIMENTAL

Chemicals

Chlorambucil was purchased from Sigma (St. Louis, MO, U S.A.). Phenylacetic mustard and phenylpropionic mustard, 4-[4-bis(2-chloroethyl)aminophenyl]propionic acid, were obtained from the Pharmaceutical Resources Branch of the National Cancer Institute (Bethesda, MD, U.S.A.). The latter is not a metabolic product of TBC or of chlorambucil and was used as an internal standard for quantitation of chlorambucil and phenylacetic mustard. TBC orginally was syn-

thesized from chlorambucil and *tert.*-butyl alcohol (Sigma) by reaction with *p*-toluenesulfonic acid (Sigma) in benzene under azeotropic conditions, and was prepared as a hydrochloride salt. Its formation was confirmed by nuclear magnetic resonance (NMR) and infrared (IR) spectral analyses, and the agent was determined to be in excess of 99% purity. Chlorambucil propyl ester, *n*-propyl-4-[4-bis (2-chloroethyl)aminophenyl] butyrate hydrochloride, prepared by similar reaction of propanol (Sigma) with chlorambucil, was of similar purity and was used as an internal standard for quantitation of TBC (Fig. 1). Methanol, methylene chloride, acetone and acetonitrile, HPLC grade, were supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), hexane, HPLC grade, was obtained from Fluka (Ronkonkoma, NY, U.S.A.) and acetic acid, analytical quality, from Mallinckrodt (Paris, KY, U.S.A.). Deionized water was used during HPLC analysis, obtained from a Milli-Q system (Millipore, Milford, MA, U.S.A.), and all solvents were filtered and degassed.

Instrumentation and conditions

HPLC analysis was performed using a Waters dual-pump system (Waters Assoc., Milford, MA, U.S.A.). This consisted of a Model 6000A solvent pump, a Wisp 710B automatic injector and a Model 480 variable-wavelength UV detector, which was set at 254 nm wavelength. Separation was performed on a 5-μm RAC III Partisil 5 ODS 3 column, 100 mm × 4.6 mm I.D. (Ace Scientific, East Brunswick, NJ, U.S.A.) with a guard column packed with pellicular C_{18} material (Waters Assoc.). Chromatograms were recorded on a Waters Model 860 networking computer system. The mobile phase for pump I was water–acetic acid (98:2, v/v); for pump II it was acetonitrile–acetic acid (98:2, v/v). The flow-rate, produced from 30% pump I and 70% pump II, was set at 1.5 ml/min and produced a column pressure of 80 bar. The system was run under curvilinear gradient conditions (curve No. 7) for 17 min to produce 65% pump I and 35% pump II at a flow-rate of 2.5 ml/min. These conditions then were maintained isocratically for 7 min. Retention times were: phenylacetic mustard, 11.3 min; phenylpropionic mustard, 12.6 min; chlorambucil, 13.7 min; chlorambucil propyl ester, 19.0 min; and TBC, 20.0 min (Figs. 2 and 3). Concentrations of TBC, chlorambucil and phenylacetic mustard were calculated from the ratio of the areas under their curves with their appropriate internal standard. These were then quantified from calibration curves of six points, which were run daily and interspersed amongst the unknown samples. Samples were maintained at 4°C to ensure that no ester hydrolysis occurred during the preparation and extraction procedures

Sample preparation and extraction

Aqueous samples, including plasma, were thawed and placed in an ice bath at 0°C. A 100-μl volume of internal standards (freshly prepared in methanol) was added to a final concentration of 2.5 μg/ml for both phenylpropionic mustard and chlorambucil propyl ester in pharmacokinetic studies Acetonitrile (3 ml), 1

ml of methylene chloride and 2 ml of hexane were added to each aqueous sample, which was then shaken and centrifuged (2000 *g* for 10 min at 4°C). The top two phases, containing acetonitrile–methylene chloride and hexane, respectively, were removed, evaporated to dryness, and 200 μl of methanol–hexane (3:1, v/v) were added. Samples were then injected on to the HPLC system. For tissue samples, 5 ml of chilled acetonitrile together with 100 μl of internal standards, to a final concentration of 0.25 μg/g phenylpropionic mustard and 2.5 μg/g chlorambucil propyl ester in pharmacokinetic studies, were added and the sample was sonicated (Model 225, Heat Systems-Ultrasonics, Farmingdale, NY, U.S.A.) for 30 s on ice. The sample was shaken vigorously and centrifuged (2000 *g* for 10 min at 4°C). The top phase was removed, evaporated to dryness, and 200 μl of methanol–hexane (3:1, v/v) were added. Samples then were injected on to the HPLC system.

For the determination of extraction efficiencies, each agent was added to triplicate samples of plasma and brain (phenylacetic mustard: plasma, 0.25 and 10.0 μg/ml; brain, 0.05 and 1.0 μg/g; phenylpropionic mustard: plasma, 2.5 μg/ml; brain, 0.25 μg/g; chlorambucil: plasma, 0.5 and 20.0 μg/ml; brain, 0.025 and 1 0 μg/g; chlorambucil propyl ester: plasma, 2.5 μg/ml; brain, 2.5 μg/g; TBC: plasma, 0.5 and 20.0 μg/ml; brain, 0.25 and 10 μg/g), maintained on ice. These were then extracted as described, analyzed by HPLC and quantified against individual standard curves prepared in methanol–hexane (3:1, v/v).

Finally, TBC, chlorambucil and phenylacetic mustard were added separately to fresh rat plasma and brain samples maintained at 4°C and the samples were immediately stored at −70°C. At six consecutive weekly intervals, triplicate samples of each were withdrawn and analyzed by HPLC.

Animal studies

Female Wistar rats (Charles Rivers Labs. Wilmington, MA, U.S.A.), between 110 and 140 g weight, were injected i.v. with TBC, 13 mg/kg dissolved in Tween 80–ethanol (3:1, v/v) and diluted in nine volumes isotonic saline. At 5–60 min, two rats were killed, blood was removed, centrifuged (8000 *g* for 45 s), and plasma and a weighed sample of brain were stored at −70°C.

RESULTS

Figs 2 and 3 illustrate typical HPLC resolutions of TBC, its active metabolites and internal standards in plasma and brain, respectively, of a rat 5 min following administration of TBC (13 mg/kg, i.v.). Five separate peaks representing, with increasing retention time, phenylacetic mustard, phenylpropionic mustard, chlorambucil, chlorambucil propyl ester and TBC can readily be delineated in both chromatograms with minimal interference from plasma and brain constituents. Chromatograms of plasma and brain from untreated animals had flat and stable baselines.

A least-squares linear regression between concentration and area under the

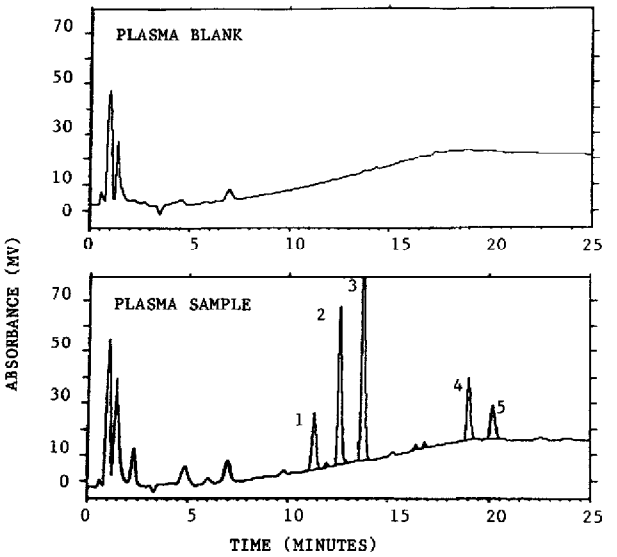

Fig. 2 Chromatograms of untreated blank plasma and of a plasma sample from a rat administered chlorambucil *tert* -butyl ester hydrochloride (TBC), 13 mg/kg i v Peaks· 1 = phenylacetic mustard; 2 = phenylpropionic mustard (internal standard), 3 = chlorambucil; 4 = chlorambucil propyl ester (internal standard); 5 = TBC

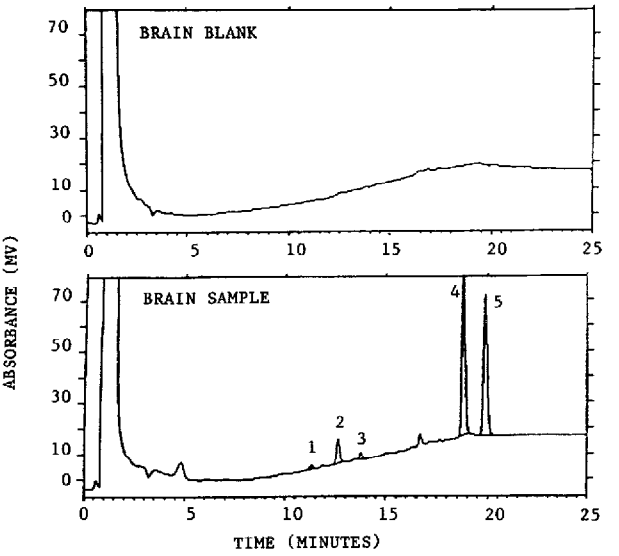

Fig. 3 Chromatograms of untreated blank brain and of a brain sample from a rat administered chlorambucil *tert* -butyl ester hydrochloride (TBC), 13 mg/kg i.v. Peaks· 1 = phenylacetic mustard; 2 = phenylpropionic mustard (internal standard); 3 = chlorambucil; 4 = chlorambucil propyl ester (internal standard); 5 = TBC

curve of appropriate sample peaks was calculated for TBC, chlorambucil and phenylacetic mustard over a 2.5 log concentration range. The mean correlation coefficients of six curves in plasma, brain and methanol–acetonitrile (1:1, v/v), run over a one-month period, were all in excess of 0.999. Additionally, intercepts for each were negligible. The reliable lower limit of detection was 100 ng/ml or ng/g for TBC, 50 ng/ml or ng/g for phenylacetic mustard and 25 ng/ml or ng/g for chlorambucil, in plasma or brain, respectively. Assay reproducibility was assessed by quantifying two reference standards for each compound in plasma and brain on five separate days over a period of one month. The coefficients of variation (C.V.) of TBC in plasma at 2.5 and 10.0 μg/ml were 1.5 and 6.6%, respectively. Those for TBC in brain at 1.0 and 5.0 μg/g were 2.7 and 4.0%, respectively. The coefficients of variation of chlorambucil in plasma at 2 5 and 10.0 μg/ml were 3.6 and 5.3%, respectively. Those of chlorambucil in brain at 0.1 and 0.5 μg/g were 8.5 and 2.3 %, respectively. The coefficients of variation of phenylacetic mustard in plasma at 1.0 and 5.0 μg/ml were 3 0 and 3.7 %, respectively. Those of phenylacetic mustard in brain at 0.1 and 0.5 μg/g were 3.9 and 2.5%, respectively.

Samples of TBC, chlorambucil and phenylacetic mustard were stable over a period of six weeks when maintained at −70°C. For TBC at 0.75 μg/ml and 0.40 μg/g in plasma and brain, respectively, mean concentrations over the six weeks were 100.6 ± 6.6 and 105.1 ± 5.7% of the original levels. For chlorambucil at 1.0 μg/ml and 0.05 μg/g in plasma and brain, respectively, concentrations were 107.5 ± 6.1 and 92.1 ± 24% of the original levels. For phenylacetic mustard at 0.40 μg/ml and 0.075 μg/g in plasma and brain, respectively, concentrations were 96.0 ± 8.1 and 94.4 ± 12.3% of the original levels. Extraction efficiencies from plasma and brain were 77 and 94% for TBC, 59 and 87% for chlorambucil propyl ester, 85 and 88% for chlorambucil, 82 and 99% for phenylpropionic mustard and 63 and 92% for phenylacetic mustard, respectively.

Fig. 4 illustrates plasma and brain concentration profiles of TBC, chlorambu-

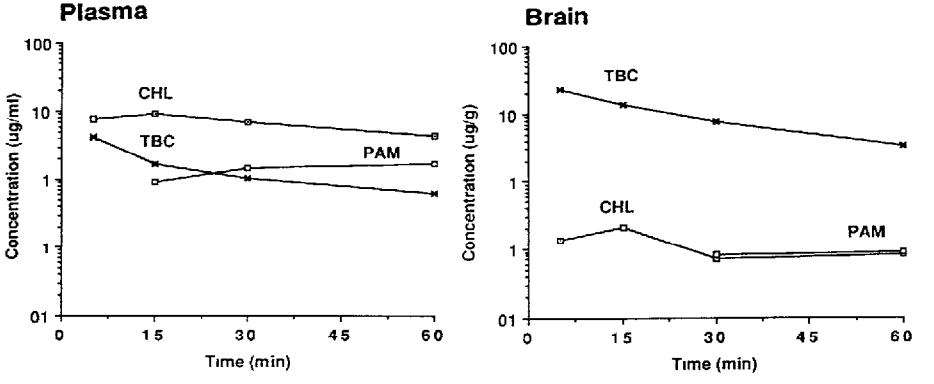

Fig. 4. Time-dependent concentration profiles of chlorambucil-*tert*-butyl ester (TBC), chlorambucil (CHL) and phenylacetic mustard (PAM) in plasma and brain, following i v administration of TBC, 13 mg/kg, to rats. Each time point represents the mean of two animals

cil and phenylacetic mustard, following administration of TBC (13 mg/kg, i.v.) to rats. A peak level of 4.2 μg/ml TBC was achieved in plasma at 5 min. Thereafter TBC concentrations rapidly declined. Large amounts of chlorambucil were present throughout the study, with a peak level of 9.3 μg/ml occurring at 15 min. Low and increasing concentrations of phenylacetic mustard were detected after 15 min.

Large amounts of TBC were detected in brain. A peak concentration of 23 μg/g was achieved at 5 min, and thereafter TBC levels in brain gradually declined. Additionally, low concentrations of chlorambucil and phenylacetic mustard were detected throughout the study and after 15 min, respectively.

DISCUSSION

The described HPLC assay allows rapid and sensitive analyses of TBC and its active metabolites, chlorambucil and phenylacetic mustard, from the same plasma or tissue sample. During analysis, there is minimal hydrolysis of drug. Therefore the technique is of significant value for the determination of the physicochemical properties and quantification of the *in vivo* distribution/metabolism/elimination of these agents. In accord with this, TBC and its active metabolites were detected in plasma and brain after its i.v. administration to rats. Whereas high concentrations of TBC and low levels of active metabolites were present in brain, the reverse was found in plasma.

Our assay, unlike those for prednimustine [3,4], utilizes two internal standards, as there is significant separation between lipophilic TBC and its hydrophilic active metabolites. Phenylpropionic acid is water-soluble and has physicochemical properties and a retention time close to those of chlorambucil and phenylacetic mustard, for which it was used as an internal standard. Conversely, chlorambucil propyl ester is lipophilic with physicochemical properties and a retention time close to that of TBC. We considered the use of two internal standards rather than one of intermediate physicochemical properties, such as methoxyphenyl mustard, 4-[methoxy-N,N-bis(2-chloroethyl)]aniline (Fig. 1), used by Oppitz *et al.* [4] during HPLC analysis of prednimustine and chlorambucil in plasma, to be preferential for reliable quantitation of all peaks at low concentrations. The use of a single internal standard by Oppitz *et al.* [4], however, is an improvement over many previous assays for prednimustine and chlorambucil that do not include an internal standard [3,5–9]. Additionally, we believe that our same assay procedure, utilizing either TBC or chlorambucil propyl ester as an internal standard, would be of value for rapid and sensitive quantification of prednimustine in clinical plasma and tissue samples. Under similar conditions as those described for TBC, prednimustine has a retention time of 23 min with an extraction efficiency, lower limit of reliable detection and coefficient of variation similar to those of TBC in both plasma and tissue samples. An in depth analysis of the plasma and brain pharmacokinetics and anticancer activities of TBC is presently being undertaken [10].

Interestingly, Leff and Bardsley [11] esterified chlorambucil with propanol, to form its propyl ester as the basis for the quantification of chlorambucil in plasma from patients with ovarian cancer. Conversion of chlorambucil to its propyl ester, rather than to another *n*-alkyl ester, was undertaken as chlorambucil propyl ester forms easily with consistent high yield, is extracted with high efficiency and is well separated from other plasma constituents during chromatography. Our studies, using chlorambucil propyl ester as an internal standard, are in accord with this. However, with our extraction procedure and HPLC conditions, chlorambucil proved to have a higher extraction efficiency and a lower reliable limit of detection than had chlorambucil propyl ester in both plasma and brain samples. Therefore, esterification during the analytical procedure was not required, simplifying the assay. Further, our lower limit of reliable detection for chlorambucil and phenylacetic mustard and our extraction efficiencies are similar to or better than recently developed assays for these agents employing HPLC [3–9,11], fluorescence [12] and gas chromatography with mass spectrometry [13,14].

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