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High-performance liquid chromatographic method for the separation of chlorambucil and its N-oxide prodrug

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Abstract

A reversed-phase high-performance liquid chromatographic method is described to distinguish chlorambucil N-oxide from the parent chlorambucil and quantitate both after separation from biological samples. The influence of solvent pH, alcohol, acid and ion-pairing agent on the separation is described. The stability of chlorambucil and its N-oxide in buffers and alcohols, as well as stability during filtration is discussed with potential application for metabolic studies.

1. Introduction

Chlorambucil {4-[bis-2-chloroethylamino-phenyl]butyric acid} is an antineoplastic agent used in the treatment of lymphocytic leukemia, ovarian and breast carcinomas, malignant lymphomas and Hodgkin's disease. Due to the bone marrow toxicity of this alkylating agent [1], its N-oxide derivative was developed as a prodrug. It was anticipated that in the hypoxic milieu of solid tumors, the N-oxide would be reduced to the cytotoxic chlorambucil [2]. In order to determine whether this reductive metabolic process actually occurs under these conditions, a method for monitoring the conversion of the N-oxide to chlorambucil was required.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a sensitive technique which has been frequently employed for the quantitative and qualitative analysis of chlorambucil and its metabolites. No methodology was available for the separation and analysis, however, of the N-oxide prodrug and chlorambucil. Therefore, we have developed an RP-HPLC method for quantitative determination of chlorambucil and chlorambucil N-oxide and are currently utilizing it for the examination of enzymatic reduction of chlorambucil N-oxide. This methodology may have future use in synthetic, pharmacokinetic and *in vivo* metabolic studies.

In addition to developing the methodology where the effect of ion-pairing agents and solvent pH on separation were studied, the extraction efficiency from biological preparations and stability of chlorambucil and its N-oxide in buffer and organic solvents were examined.

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2. Experimental

2.1. Chemicals

Chlorambucil N-oxide was synthesized by a modified method of Mann and Shervington [3] and Degutene *et al.* [4]. Chlorambucil was purchased from Sigma (St. Louis, MO, USA). 3-(4-Hydroxyphenyl)propionic acid, 98%, was supplied by Aldrich (Milwaukee, WI, USA). Sodium carbonate anhydrous was purchased from McArthur Chemical Company (Montreal, Que., Canada) and Tris from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Trifluoroacetic acid (Aldrich) was distilled in small quantities before use. Water for HPLC work was doubly-distilled and deionized. All other solvents for chromatography were HPLC grade (BDH, Toronto, Ont., Canada). All solvents were filtered and degassed by vacuum filtration through either 0.45 μm cellulose acetate (Sartorius, Germany) or HVLP Durapore hydrophilic (Millipore, Bedford, MA, USA) filters.

2.2. Instrumentation

Before optimization of the HPLC conditions, UV scans of both drugs and the internal standard 3-(4-hydroxyphenyl)propionic acid were performed on a Hewlett-Packard (Toronto, Ont., Canada) 8452A diode array spectrophotometer processed by the HP Vectra 486s/20 computer with an HP Desk Jet 500 printer. The HPLC system included a Waters 510 dual pump system controlled by a Waters automated gradient controller, a 4-μm Nova Pak C_{18}, 150 × 2 mm I.D. (Waters, Milford, MA, USA), a Kratos Spectroflow 757 Absorbance variable wavelength detector (Ramsey, NJ, USA), and a Shimadzu CR501 Chromatopac integrator (Kyoto, Japan).

2.3. Separation

Attempts were made to produce separating conditions using various ion-pairing agents in combination with methanol. These agents included acetic acid, trifluoroacetic acid (TFA), phosphoric acid, triethylamine (TEA) and trimethylamine (TMA). The effect of pH was examined using trifluoroacetic acid. In addition, replacement of the methanol by acetonitrile, isopropyl alcohol and 1-butanol was investigated using isocratic or gradient conditions. Separation of chlorambucil and chlorambucil N-oxide was achieved by many of the combinations, but the optimal conditions employed for this study were found to be 52% methanol, 48% water–TFA pH 2.25 at a flow-rate of 0.35 ml/min.

2.4. Stability testing

The stabilities of chlorambucil and the N-oxide in aqueous buffer and alcoholic solutions were assessed over time and at various temperatures. Preparations of chlorambucil and N-oxide in 0.1 *M* Tris pH 7.5 were incubated at 37°C for 0, 0.5, 1, 4, and 8 h. The N-oxide in Tris (pH 10.2) and in 1% (pH 11.1), 0.175% (pH 10.2), 0.01% (pH 9.9), and $<0.0001\%$ (pH 7.7) sodium carbonate were also analyzed for any breakdown products. Both N-oxide and chlorambucil in methanol were analyzed for stability after 1 h, 1 day, 2 weeks at −20°C and 0, 0.5, 4, 8 h at room temperature. In addition the N-oxide in ethanol stored at room temperature for 24 h was examined for breakdown.

Earlier reports, indicating a loss of chlorambucil by filtration [5], prompted an analysis of the effect of filtration on chlorambucil N-oxide, chlorambucil, and the internal standard. Known concentrations of chlorambucil, N-oxide and internal standard were filtered through 0.2-μm nylon membrane filters (Nalgene, Rochester, NY, USA) and the peak areas were compared to known concentrations of the unfiltered compounds.

2.5. Quantitative analysis

The absorbance spectrum of the internal standard, 3-(4-hydroxyphenyl)propionic acid was measured and a wavelength of 258 nm was chosen for HPLC peak detection. Dilutions of the internal standard were analyzed for optimum concentration, and 50 μg in combination with 40 to 2000 ng of chlorambucil and its N-oxide were used for preparation of standard curves. The

internal standard eluted within the initial 15 min (retention time of 8 min) and was detected at 258 nm after which time the wavelength was changed to 249 nm, an average of λ_{max} for chlorambucil and chlorambucil N-oxide, for analysis of the two compounds. The peak-area ratio of drug to internal standard was calculated and plotted *versus* μg quantity of each drug to produce standard curves.

2.6. Extraction from biological sample

Either chlorambucil or chlorambucil N-oxide was added to rat liver microsomes in Tris (pH 7.5, 37°C). An aliquot of microsomes was removed and two parts methyl ethyl ketone were added. The mixture was vortex-mixed for 45 s then centrifuged at 100 *g* for 5 min. The upper organic layer was removed and dried *in vacuo*. The residue was then dissolved in 1 ml methanol and filtered through a 0.2-μm nylon filter for HPLC analysis.

3. Results

3.1. Separation

The λ_{max} for chlorambucil and chlorambucil N-oxide were 258 and 240 respectively. A wavelength of 249 nm was chosen for analysis and using the optimal separation conditions, the peak areas of chlorambucil and the N-oxide were linear over a range of 40 to 2000 ng drug.

Initial conditions employed for the separation of chlorambucil from chlorambucil N-oxide were 2% acetic acid–methanol (50:50, v/v) based on previous studies with nicotinamide N-oxide (unpublished data). Baseline separation at retention times of 51.55 and 55.12 min for chlorambucil and its N-oxide respectively was achieved (Fig. 1A). Attempts to improve the separation and decrease retention time involved the assessment of other ion-pairing agents, other alcohols and various gradient elutions. The results of these changes are shown in Table 1.

Triethylamine (0.3%) in H_2O–methanol did not improve separation or retention time. Initial

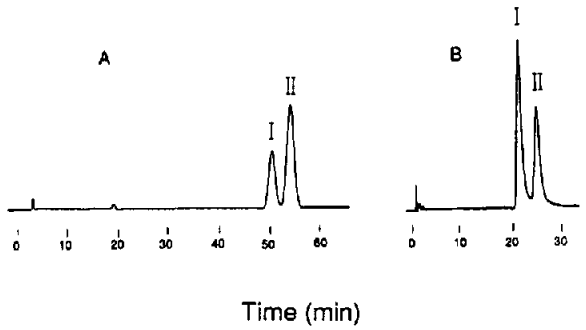

Fig. 1. HPLC analysis of chlorambucil (I) and chlorambucil N-oxide (II) using an isocratic mobile phase of (A) 2% acetic acid buffer–methanol (50:50) pH 2.6 with a flow-rate of 0.4 ml/min, or (B) 0.05% trifluoroacetic acid (pH 2.3)–methanol (48:52) with a flow-rate of 0.35 ml/min and UV detection at 249 nm.

separation, indicated by a very broad band at 53 min occurs when the solvents are mixed in a 50:50 ratio. Although increasing the aqueous component concentration may have completely separated the two peaks, this would also have increased the retention time and therefore would have been counterproductive.

Using acetonitrile as the organic component resulted in separation of broad peaks at a 65:35 ratio (2% acetic acid–acetonitrile) with a shorter retention time (45 min). Acetonitrile was then mixed with methanol in a 25:75 ratio (acetonitrile–methanol). This resulted in broad peaks separated at 42 min at a 55:45 aqueous–organic ratio, but no separation of the peaks at a 50:50 ratio (aqueous–organic).

In an attempt to decrease the retention time, methanol was replaced by isopropyl alcohol. This change in alcohol decreased retention time but separation of chlorambucil and the N-oxide was not achieved. Triethylamine was used in the aqueous phase (0.3%), and although this decreased the retention time to 14 min, it did not improve the separation even at a 75:25 ratio (aqueous–isopropyl alcohol) and baseline noise made quantitative analysis difficult. Butanol was also used to replace methanol but again, although the retention time was decreased, separation of the components could not be realized.

Utilizing methanol with TFA (0.05%) reduced the retention times to approximately 30 min compared to that obtained with acetic acid (53

Table 1
Attempted HPLC separations of chlorambucil and chlorambucil N-oxide by various mobile phase compositions

Aqueous	Organic	Composition (aqu:org)	Resolution (R)[a]	Separation (α)	Capacity factor (k')[b]	
					Chlorambucil	N-oxide
2.0% AA[c]	MeOH	50:50	1.31	1.07	46	48
1.2% AA	MeOH	50:50	1.31	1.06	30	32
0.5% AA	MeOH	49:51	1.12	1.06	33	36
H_2O	25–75% BuOH/ MeOH	all[c]	0	none	0–37	0–37
2.0% AA	10–25% BuOH/ MeOH	all	0	none	0–26	0–26
0.3% TEA/ PA	MeOH	all	0	none	0–40	0–40
0.3% TEA/ PA	IsOH	all	0	none	0–7	0–7
5 m*M* TMA/ 50 m*M* TFA	MeOH	all	0	none	13–22	14–22
H_2O	AcN	all	0	none	2–5	2–5
2.0% AA	AcN	all	0	none	1–44	1–44
2.0% AA	25% AcN/ MeOH	55:45	U[d]	1.06	35	37
TFA (0.05%)	MeOH	48:52				
pH 2.70			U	1.05	26	27
2.49			U	1.07	25	27
2.52			U	1.07	25	27
2.33			1.81	1.11	24	27
2.19			2.67	1.17	24	28

Abbreviations: methanol (MeOH); isopropyl alcohol (IsOH); acetonitrile (AcN); 1-butanol (BuOH); triethylamine (TEA); trimethylamine (TMA); acetic acid (AA); trifluoroacetic acid (TFA); phosphoric acid (PA).
[a] $R = 1.176 \times$ (retention time peak B − retention time peak A)/peak A width at 1/2 height + peak B width at 1/2 height.
[b] $k' = (t_A - t_0)/t_0$.
[c] All: represents all compositions of mobile phase tested.
[d] U: represents an undetermined value because a peak width could not be calculated.

min) and provided an improved separation at a aqueous–methanol ratio of 48:52 (Fig. 1B). A combination of TMA with TFA/methanol did not give a better result. Gradient elutions under various conditions were studied to improve separation at a shorter retention time. Again, no benefit was observed compared with isocratic conditions. Therefore a simple isocratic elution with TFA (0.05%)–methanol (48:52) was used for subsequent studies.

Using TFA the effect of pH was assessed to give a consistent optimal separation (Fig. 2). At

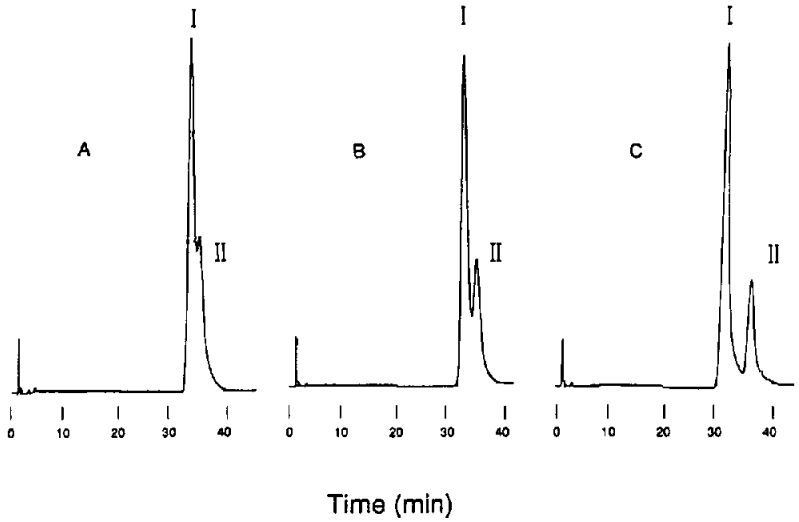

Fig. 2. Chromatograms of chlorambucil (I) and chlorambucil N-oxide (II) using an isocratic mobile phase of trifluoroacetic acid buffer pH (A) 2.7; (B) 2.5; (C) 2.2: MeOH (48:52).

a pH of 2.7 the two peaks could not be resolved. By decreasing the pH to 2.5 separation improved and at pH 2.1 baseline separation was achieved. Because of the detrimental effect of this acidic pH on reversed-phase HPLC columns, a pH of 2.25 was used for analysis.

3.2. Stability

Chlorambucil and chlorambucil N-oxide were found to be stable in both methanol and ethanol over a 8-h, 24-h, and 2-week period at room temperature and −20°C. Chlorambucil was hydrolyzed in 0.1 *M* Tris (pH 7.5) over time with 19.7% remaining after a 1-h incubation at 37°C and 0.001% remaining after 4 h. This is in contrast to chlorambucil N-oxide in Tris (0.1 *M*, pH 7.5) which remained stable for 8 h at 37°C. If the pH of the buffer was made more alkaline (Tris pH 10.2), immediate decomposition occurred. A similar effect was observed using sodium carbonate where at pH 7.7 the N-oxide was stable but at higher concentrations and more alkaline pH, the N-oxide broke down immediately (Fig. 3).

Filtration through nylon filters of the three compounds in either alcoholic or aqueous (Tris) solutions provided recovery of 100% in all cases.

3.3. Quantitative analysis

Using optimum separation conditions and a detection wavelength of 249 nm, the plots of the peak areas of nanogram quantities of chlorambucil and chlorambucil N-oxide were found to be linear. Using an internal standard and a wavelength switch at 15 min from 258 to 249 nm, the peak-area plot of drug/internal standard concentrations was linear from 40 to 2000 ng. The regression line for the chlorambucil concentration curve (using an internal standard) was calculated to be $y = 0.061063x + 0.026174$ with a correlation coefficient of 0.9986 over 3 trials, and for the N-oxide concentration curve (using an internal standard) was calculated as $y = 0.181867x + 0.019362$ with a correlation coefficient of 0.9982. Both showed high accuracy and precision at the lower concentrations but an increased deviation between injections at the

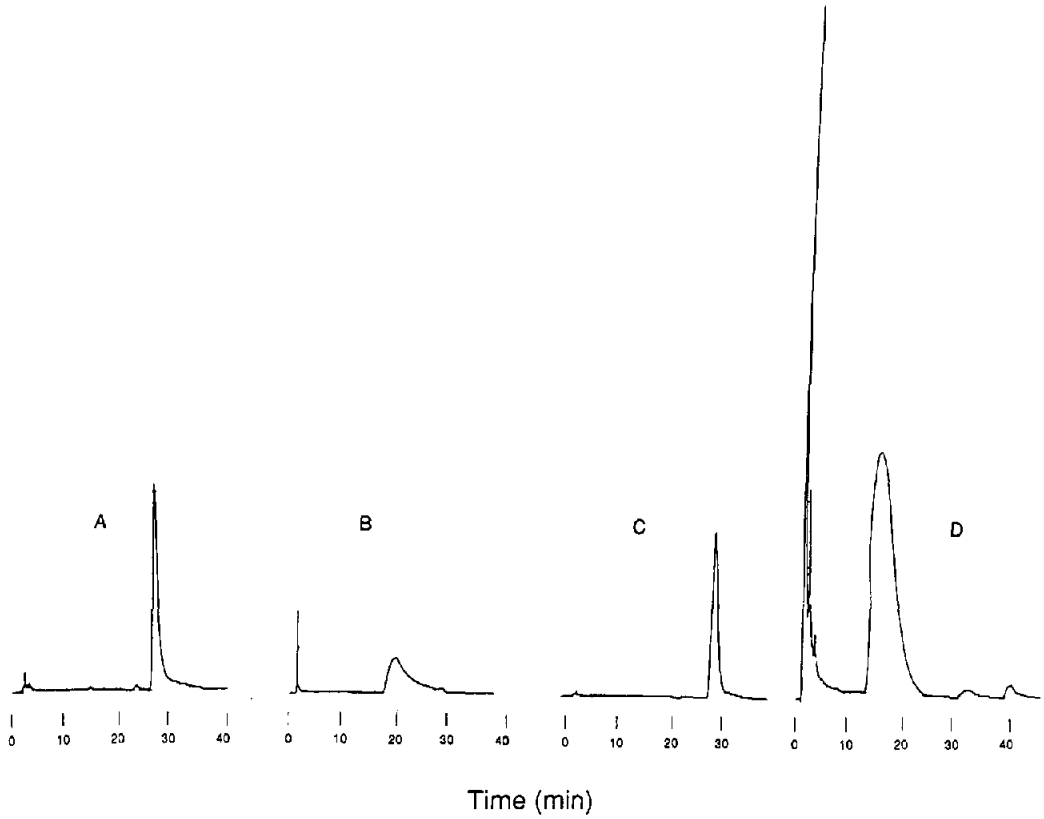

Fig. 3. HPLC analysis of chlorambucil N-oxide immediately after being prepared in (A) Tris buffer pH 7.5; (B) Tris buffer pH 10.2; (C) Sodium carbonate buffer pH 7.7; (D) sodium carbonate buffer pH 11.1.

highest concentration examined (2 μg). The internal standard eluted at approximately 8 min under the chromatographic conditions employed for quantitative analysis (Fig. 4).

3.4. Extraction from a biological sample

HPLC analysis was performed of a methanolic extract from a 1-ml aliquot of a rat liver microsomal extract containing 33.3 μg/ml of chlorambucil N-oxide or 112.0 μg/ml of chlorambucil. Quantitative analysis gave 32.4 μg chlorambucil N-oxide and 111.0 μg chlorambucil giving an extraction recovery of 97.3% and 99.1% respectively.

4. Discussion

A sensitive RP-HPLC method for the codetermination of chlorambucil and chlorambucil N-oxide has been developed. Because the two compounds are very similar in structure, separation was difficult and previously reported HPLC conditions for the separation of chlorambucil from metabolites and prodrugs were not adequate in our studies [6–15]. Separations with various ion-pairing agents, acids and alcohols were performed. The ion-pairing agents TMA and TEA showed no effect on the separation of chlorambucil from its N-oxide. This was also described in a report on the separation of chlorambucil from its metabolites in plasma [6].

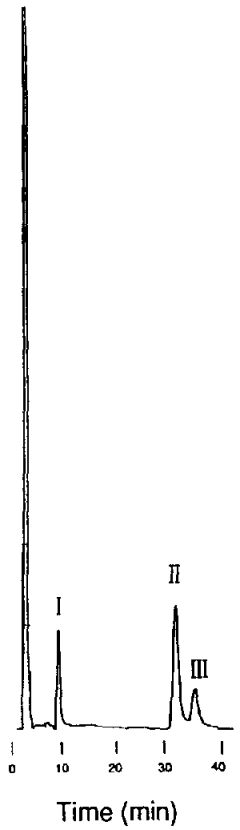

Fig. 4. HPLC analysis of 3-(4-hydroxyphenyl)propionic acid (internal standard) (I), chlorambucil (II), and chlorambucil N-oxide (III) under optimum conditions.

Although the addition of acetic acid resulted in separation, the retention time using this ion was unacceptably long. Trifluoroacetic acid improved separation and peak shape, and reduced retention time. Separation was also found to improve with a decrease in solvent pH. Final conditions employed a pH of 2.25 to allow for a balance of optimal separation and column care.

Isopropyl alcohol and 1-butanol decreased the retention time dramatically but also inhibited efficient separation and good peak shape even when the amount of aqueous component was greatly increased. Methanol was therefore employed as the organic component of choice.

Although a previous publication [5] reported a loss of chlorambucil by filtering through polytetrafluoroethylene, cellulose acetate and polysulphone filters, this was not found when nylon filters were used.

The stability of chlorambucil N-oxide in buffer, the study of which was critical for future metabolic and animal studies, was found to be pH dependent. Chlorambucil N-oxide is stable in Tris and sodium carbonate buffer at pH 7.5–7.7, whereas it is immediately degraded in more basic solutions. Chlorambucil, on the other hand, was found to decompose with time in 0.1 *M* Tris (pH 7.5). Zakaria and Brown [16] also report rapid hydrolysis of chlorambucil to its mono- and dihydroxy derivatives in neutral or basic aqueous solutions. The present study showed that both drugs are more stable when stored in methanol. The N-oxide is stable for a longer period of time than chlorambucil, showing no degradation after 2 weeks while chlorambucil was stable for only 2 days.

Both chlorambucil and the N-oxide were found to be easily extracted from aqueous biological homogenates. The RP-HPLC method described in this paper, which assisted in monitoring the stability of our drug preparations, and the extraction procedure are presently being employed by our laboratory for the study of *in vitro* enzymatic activation of chlorambucil N-oxide.

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6. References

[1] F. Bergel, *Ann. NY Acad. Sci.*, 68 (1958) 1238.
[2] I.N.H. White, A. Cahill, A. Davies and P. Carthew, *Arch. Toxicol.*, 66 (1991) 100.
[3] J. Mann and L.A. Shervington, *J. Chem. Soc. Perkin Trans.*, I (1992) 2961.
[4] A. Degutene, D. Dzhyuvene, D. Shukyalene and Yu. Degutis, *J. Org. Chem. USSR*, 22 (1986) 2191.

[5] A.G. Bosanquet and H.E. Clarke, *Cancer Chemother. Pharmacol.*, 18 (1986) 176.
[6] C.G. Adair, D.T. Burns, A.D. Crockard and M. Harriott, *J. Chromatogr.*, 342 (1985) 447.
[7] C.G. Adair and J.C. McElnay, *Cancer Chemother. Pharmacol.*, 17 (1986) 95.
[8] D.C. Chatterji, R.L. Yeager and J.F. Gallelli, *J. Pharm. Sci.*, 71 (1982) 50.
[9] F.Y.F. Lee, P. Coe and P. Workman, *Cancer Chemother. Pharmacol.*, 17 (1986) 21.
[10] N.H. Greig, D.J. Sweeney and S.I. Rapoport, *Cancer Chemother. Pharmacol.*, 21 (1988) 1.
[11] N.H. Greig, D.J. Sweeney and S.I. Rapoport, *Cancer Chemother. Pharmacol.*, 25 (1990) 311.
[12] M.M. Oppitz, E. Musch, M. Malek, H.P. Rub, G.E. von Unruh, U. Loos and B. Muhlenbruch, *Cancer Chemother. Pharmacol.*, 23 (1989) 208.
[13] C.G. Adair, D.T. Burns and M. Harriott, *Anal. Proc.*, 23 (1986) 30.
[14] H. Ehrsson, S. Eksborg, I. Wallin and S.-O. Nilsson, *J. Pharm. Sci.*, 69 (1980) 1091.
[15] P. Leff and W.G. Bardsley, *Biochem. Pharmacol.*, 28 (1979) 1289.
[16] M. Zakaria and P.R. Brown, *J. Chromatogr.*, 230 (1982) 381.