MASS SPECTROMETRIC ANALYSIS OF CHLORAMBUCIL,
ITS DEGRADATION PRODUCTS AND METABOLITE IN
BIOLOGICAL SAMPLES

by

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STATEMENT OF AUTHOR

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10/8/1979
To my father, mother, and Merla for their love, support, and encouragement.
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ABSTRACT

A sensitive and specific method for the determination of chlorambucil and its metabolites in biological fluids is reported. The method is based on the selection ion monitoring detection following one-step ethylacetate extraction of the parent compound, its metabolite and an internal standard (chlorambucil-d₈) from plasma and urine samples. After an oral bolus dose of 0.6 - 1.2 mg/kg to three patients its composite half-life was 1.5 hours and 24 hour urinary excretion of chlorambucil administered was 0.56 ± 0.19%. The extractions of chlorambucil at concentrations of 200 and 1,000 ng/ml showed standard deviations of 1.3 percent to 3.0 percent. Recovery of chlorambucil from plasma was temperature dependent. About 90 percent of both concentrations of chlorambucil was recovered after four hours of incubation in plasma at 25°C, whereas only 56 percent of the drug was recovered at 37°C after that time period. The recovery rate constants of chlorambucil at 37°C were k = 0.45 hr⁻¹ for water and k = 0.12 hr⁻¹ for plasma. The β-oxidation product, a metabolite of chlorambucil, was identified as p-[4-N,N-bis (2-chlorambucil)] aminophenylacetic acid, from both plasma and urine. Structures of three degradation products of chlorambucil stored in non-aqueous medium were studied by mass spectrometry. They were proposed as two pyrrol derivatives and p-aminophenylbutyric acid.
INTRODUCTION

Chlorambucil, p-(di-2-chloroethyl) aminophenylbutyric acid, is a bifunctional alkylating agent. Everett, Roberts, and Ross (1953) synthesized the aromatic derivative of nitrogen mustard with the following formula:

\[
\text{HOOC-(CH}_2\text{)_3-} -\text{CH}_2\text{CH}_2\text{Cl} - \text{N} \backslash \text{-CH}_2\text{CH}_2\text{Cl}
\]

Chlorambucil appears as fine white crystals (IARC 1975). At 20°C it is soluble in 1.5 parts ethanol, two parts acetone, 2.5 parts chloroform and two parts ethylacetate (IARC 1975).

The substitution of an electrophilic group at the N-position reduces the basicity and reactivity of the resulting compound. Chlorambucil has a terminal carboxyl group forming a phenylbutyric acid intending to increase the solubility. Haddow (1952) found this compound to be an inhibitor of transplanted Walker rat tumor 256. This warranted clinical investigation which documented its effectiveness against lymphocytic leukemia, Hodgkin's disease and malignant lymphomas (Galton et al. 1955). It was demonstrated during the second World War that nitrogen mustard was active against an experimental lymphosarcoma (Wellcome 1978).

The cytotoxic effects of bifunctional alkylating agents have been attributed to their interaction with DNA (Lawley 1966) with cross-linking the probable mode of action (Brooks and Lawley 1964, and Kohn, Spears, and Doty 1966). In addition, there has been observed alkylation

Chemistry and Mechanism of Action

Alkylating agents can be regarded as electrophilic reactants which combine with nucleophilic or electron rich centers (Ross 1962). Two mechanisms of alkylation are:

1. $\text{R-Y} \overset{\text{slow}}{\longrightarrow} \text{R}^+ + \text{Y}^-$
2. $\text{R}^+ + \text{X}^- \overset{\text{fast}}{\longrightarrow} \text{RX}$
3. $\text{X}^- + \text{R-Y} \longrightarrow [\text{X}----\text{R}----\text{Y}] \longrightarrow \text{R-X} + \text{Y}^-$

These are $S_{N1}$ and $S_{N2}$, respectively. Compounds reacting by $S_{N1}$ mechanism do so at a rate which is largely independent of the concentration of centers capable of alkylation. The rate determining step is the separation of the carbonium ion from the displaced anion. This separation takes place to an extent determined by the dielectric constant of the medium. A feature of $S_{N1}$ reaction is the first order kinetic reaction.

Aromatic nitrogen mustard effectively react by an $S_{N1}$ mechanism. For 2-chloroethylamines:

$$\text{RNCH}_2\text{CH}_2\text{Cl} \longrightarrow \text{RNCH}_2\text{CH}_2\text{A}$$

nitrogen mustard immonium ion

The cyclic ions have extra reactivity due to the strained ring system. In aqueous solution the nitrogen mustard rapidly generates the immonium
ion and this ion will react with a nucleophilic center by a bimolecular
mechanism.

Chlorambucil in a solution in the alkaline or neutral conditions readily forms an anionic species. The anionic form can be activated to give rise to carbonium ion and as a result, the rate of both hydrolysis and alkylation reactions of chlorambucil in an aqueous solution decreases below pH 7.0. Likely reactions of chlorambucil in biological systems will be as follows:

\[
\text{hydrolysis} \quad \begin{cases}
\text{alkylation} R_1 \\
\text{alkylation} R_2
\end{cases}
\]

yielding monohydroxylated, dihydroxylated and alkylated compounds (Chang et al. 1978). The product of alkylation with blood serum proteins is a diester of two carboxyl groups, which may be attached to the same polypeptide chain or cross-link two different chains (Linford 1962).

Three biological effects are observed with clinical usage of alkylating agents: 1) cytostatic effect—mitosis is delayed or entirely prevented in the "resting" cell; 2) mutagenic effect—division proceeds
but the daughter cells have altered properties; 3) cytotoxic effect—cells are severely damaged and cannot survive (Ross 1962). The bifunctional alkylating agents, such as chlorambucil, are very effective in producing small deficiencies in chromosomes.

Since alkylating agents seem to act by the alkylation of DNA, internalization of the drug in an active form is essential for its cytotoxic effect (Guclu, Tai, and Ghose 1975). One possible mechanism might be the facilitation of the transport of chlorambucil across the cell membrane by endocytosis of chlorambucil linked antibody. Such facilitated transport of chlorambucil might explain the increased tumor inhibition by equivalent amounts of tumor antibody or chlorambucil alone (Guclu et al. 1975).

Once in the cell chlorambucil has been shown to cause approximately a doubling of normal cell levels of c-AMP (cyclic adenosine 3', 5'-monophosphate), and an increase in protein kinase activity corresponding to c-AMP increase (Tisdale and Roberts 1978).

There is also early reduction in d-TMP (Thymidylate monophosphate) synthetase activity. However, drug-resistant tumors remain the same as the control animals after chlorambucil treatment (Srinivasan, Gupta and Howarth 1977). Since d-TMP synthetase is essential for cell division, it is suggested, in part, that tumor regression is due to this loss of enzyme. Inhibition studies, in vivo and in vitro, suggests chlorambucil may inhibit reactions leading to DNA synthesis rather than by alkylation of DNA. Further studies demonstrated that uptake and phosphorylation of thymidine is not impaired by chlorambucil treatment, indicating that inhibitory effect is located at some other step involved
in DNA synthesis than the incorporation of thymidine into DNA (Riches et al. 1975). Chlorambucil has no effect on rate of uptake of cysteine or glutathione and does not alter the pools of low molecular weight thiols (Harrap et al. 1973).

In a drug-sensitive strain of Yoshida ascites sarcoma treated with chlorambucil a decreased histone to DNA ratio has been observed (Riches, Sellwood and Harrap 1977). Such a decrease is accomplished by an early inhibition of histone synthesis. Biochemical and morphological changes in the nucleus of treated sensitive cells might have occurred either from a direct reaction of chlorambucil with nuclear proteins. The overall increase in nuclear protein phosphorylation may correlate with the withdrawal of cells from the division cycle. The alternate hypothesis suggests alkylating agents modify the association between nuclear protein and DNA.

Exposure of cells to bifunctional alkylating agents result in the insertion of inter- and intra-strand cross-links into their DNA (Walker 1971). In resistant cells rapid appearance and removal of inter-strand DNA cross-links have been shown (Harrap and Gascoigne 1976). Chlorambucil has been shown to induce rapid conformational changes in the chromatin, to bind non-covalently to non-histone nuclear proteins and to induce changes in nuclear protein structure (Riches and Harrap 1973). The early association of chlorambucil with nuclear proteins and to induce reorganization of the chromatin may delay the formation of DNA cross-links.

It is proposed that DNA-protein adducts may prevent normal chromosomal transcription, replication and separation by "derailing" the enzymes that use DNA as a template, by creating permanent "repressor" molecules
from normally noncovalently attached chromosomal proteins and eventually by preventing disjunction of the incompletely divided and cross-linked daughter chromosomes (Morin et al. 1977). Final damage is done with the next mitotic division by pulling and breaking the chromosomes. Human lymphocytes from CLL patients treated with chlorambucil showed that the majority of lesions were found to be of the chromatid type compatible with damage in the S or G₂ phase (Reeves and Margoles 1974). Treatment with chlorambucil in G₁ phase does not delay progression into the S phase where there is inhibition of the rate of DNA synthesis (Roberts 1975). Treatment in the G₂ phase does inhibit the rate of DNA synthesis in the following cell cycle.

There is suggested a correlation between DNA repair and the accumulation of sister chromatid exchange. These are greatly increased by alkylating agents, such as chlorambucil, which cause cross-links between the DNA strands (Raposa 1978 and Solomon and Bobrow 1975). The very high levels of chromatid damage which can be induced on chromosomes in vitro, the indication of stimulation followed by cell-killing, and the concentration of the effects on the S phase suggest strongly that the inhibiting effects of chlorambucil on cells of CLL patients act by these mechanisms (Stevenson and Patel 1973).

**Biological Properties**

It has been shown that immunoglobulins can transport chlorambucil selectively to neoplastic cells. However, it has been suggested that the presence of the aggregate may lead to a possible overestimation of the active chlorambucil which can be non-covalently bound to IgG,
immunoglobulin G (Blakeslee, Chen and Kennedy 1975). The conjugation of the drug by a covalent linkage to $\gamma$-globulin must be done by way of the carboxamide linkage (Ross 1975a). This leaves the cytotoxic "mustard" moiety, bis-2-chloroethylamino group, intact. Chlorambucil bound under these conditions is in the active form (Ross 1975b).

A study of the extent to which the alkylating agent reacts with the constituents of blood serum, for example esterification of the carboxyl groups of proteins, was undertaken by Linford (1961). Studies showed no alkylation reaction with the whole red cells incubated with chlorambucil at 37°C for 20 hours. Also, no interaction of chlorambucil with bilirubin, or the lipochrome fractions of serum, could be detected. Measurements did demonstrate a high degree of absorption in the presence of protein and is suggested that the benzene ring is responsible.

A cell surface localizing antibody could be used as a carrier of chlorambucil (Ghose, Path and Nigam 1972). Chlorambucil when bound to this antibody was a more effective tumor inhibitor than chlorambucil by itself, the antibody alone or chlorambucil bound to a nonspecific protein carrier. There has been reported suppression of experimental mouse lymphoma EL4 and human malignant melanoma metastases after administration of chlorambucil-antitumor antibody (Ghose, Norvell et al. 1972).

Chlorambucil has been shown to bind to nuclear protein more in tumor cells sensitive to the alkylating agent than alkylating-agent-resistant cells (Sellwood, Riches and Harrap 1975). The results indicate that the differences in drug binding observed in vivo results from a metabolic interaction of the cell with the drug rather than differences
in nuclear protein constituents. Prior to entering the cell the chloro-ethyl group binds to the cell surface, presumably by virtue of its fat solubility with adsorption taking place independently of alkylation (Linford, Hryniuk and Israels 1969). This disruptive effect on the cell surface is not associate with the binding of the compound by the Van der Waals type of adsorptive forces.

Chlorambucil has been shown to produce necrosis by the formation of lysosomes within dying cells, with activation and subsequent destruction (autolysosomal system), followed by phagocytosis of fragments by neighboring cells (Sadler and Kochhar 1976a). The first changes occur in the cytoplasm and are characterized by the appearance of membrane-bound vacuoles containing cytoplasmic organelles. The cells become fragmented and the resulting debris engulfed by macrophages. Nuclear material does not appear involved until fragmentation of the cells occurs. Although changes in the nucleus may not be apparent, biochemical lesions may exist. The absence of observable alterations would be expected if the drug was affecting transcription of altering DNA.

The metabolism of chlorambucil in man is not very well documented. McLean, Newell and Baker (1976) identified both α and β unsaturated compounds, [4-N,N bis (2-chloroethyl) aminophenyl] 2-butenoic acid in the rat. In rats ten percent of the administered chlorambucil is bound to the plasma proteins during the first few hours. By 24 hours, less than one percent remains in the plasma, indicating the reversible nature of the binding (Mitoma et al. 1977). Autoradiography of mice by organ fixation demonstrates that chlorambucil is retained by the liver (Sauvezie et al. 1976).
Suavezie et al. have demonstrated β-oxidation of chlorambucil. The oxidized form becomes localized in two potential target organs: the thymus and the bone marrow, indicating that the action is primarily immunodepressive. Orally administered $^{14}$C-chlorambucil (labeled at the chloroethyl moiety) to rats showed liver and kidney had high concentrations, with approximately 30 percent of the dose found in the gastrointestinal tract and less than ten percent in the feces after 24 hours (Mitoma et al. 1977). Godeneche et al. (1975) found after administration of chlorambucil labeled either on the carboxyl group or the carbon next to the carboxyl group on the butyric acid side-chain, that 37-39 percent of the dose was found in the expired CO$_2$. Therefore, modification of the butyric acid side-chain of chlorambucil results from enzymatic reaction primarily in the liver by β-oxidation.

**Chemotherapeutic Applications**

Chlorambucil can be administered orally (0.1 – 0.2 mg/kg) to outpatients with weekly visits for blood counts (Huff 1974). Intermittent therapy has the advantage of allowing bone marrow recovery between courses (Knospe et al. 1974). Over half of the CLL patients on chlorambucil therapy show relief of symptoms and resume normal activities (Galton et al. 1961). The response to chlorambucil can be very slow and the reduction of lymphocyte infiltration of the bone marrow may take several months (Spiers 1974). Continuous daily alkylating agent therapy is not necessary in CLL since there is an accumulation of long-lived, non-dividing lymphocytes (Knospe et al. 1974).
In the treatment of advanced ovarian cancer, Stage III and IV, there is effective production of sustained palliation with an initial dosage of 0.3 mg/kg (Masterson and Nelson 1965). In the case of advanced breast cancer chlorambucil has been shown to be active both alone and with steroids (Freckman et al. 1964 and Goldenberg et al. 1973). Treatment of Hodgkin's disease with chlorambucil may be palliative or in combination chemotherapy with regimens such as MOPP (nitrogen mustard, vincristine, procarbazine and prednisone) with 75 percent remission (McElwain et al. 1977).

**Toxicology**

Pharmacokinetic studies on rats demonstrated oral absorption of chlorambucil to slightly less than after intraperitoneal administration (Hagan et al. 1957). The LD$_{50}$ of an intraperitoneal dose is 18.5 mg/kg with the development of convulsions. The oral LD$_{50}$ for chlorambucil in rats and guinea pigs is 31 and 16.5 mg/kg, respectively. Apart from the depressant action on the bone marrow, side effects rarely occur with therapeutic doses. In human subjects gastric discomforts, mainly nausea and vomiting, may occur at single bolus doses of 20 mg or more. Patients treated with chlorambucil exhibit immunosuppression (Fernandez, MacSween and Langley 1978, Morrison and Yon 1978, and Westin 1976). Fatal irreversible bone marrow aplasia has been reported after treatment of non-malignant conditions (Rudd, Fries and Epstein 1975). However, the bone marrow depression caused by chlorambucil is usually readily reversible.

Cases have been reported of a toxic syndrome, said to resemble busulphan toxicity. The prominent features are pulmonary fibrosis and
profound weakness (Rose 1975). Pulmonary toxicity is a known complica-
tion of alkylating agents with the lungs showing interstitial
infiltration of lymphocytes, histocytes and plasma cells (Cole, Myers,
and Katsky 1978). A chronic lymphocytic leukemia patient treated with
chlorambucil demonstrated fatal lung disorders with diffuse interstitial
(not intra-alveolar) pulmonary fibrosis and scattered intra-alveolar
reticular fibrosis (Refvem 1977). Further case studies report occasional
incidences of skin rash (Knisley, Settipane and Albala 1971). One such
patient after fourteen days of chlorambucil treatment had a generalized
nonprurritic, morbilliform rash appear (Koler and Forsgren 1958). The
treatment was stopped and the rash cleared. However, upon resuming
therapy three months later a red morbilliform rash was observed within
three days. The rashes were followed by abdominal pain, nausea and
vomiting and clinical jaundice. Jaundice has been seen in other chlor-
ambucil patients as well (Robert et al. 1968). A single case (CLL
patient) of drug fever, occurred after repeated exposure over a period
of several months. Sawitsky, Bokland, and Benjamin (1971) suggested
that antibody formation had altered the patient's reactivity to chlor-
ambucil. It is hypothesized that the antibody or its complex with
antigen activate the lymphocytes which produce the (penultimate) pyrogen
conveyed to the hypothalamus.

Chlorambucil treatment has also been shown to exhibit neuro-
toxicity. For examples, peripheral sensorimotor neuropathy has been
reported by Sandler and Gonsalkorale (1977). Nitrogen mustard and its
derivatives induce neurotoxicity in both man and experimental animals
(Donelli, Rasso and Garattini 1966). Chlorambucil has been administered
to experimental animals to induce reproducible electroencephalogram changes similar to petit mal (Mirsky, Block-Rogas and McNary 1966 and Pinel and Chorover 1976). It has been suggested that chlorambucil causes a chemical rather than structural lesion and clinical studies showed seven of 91 children treated with oral chlorambucil had seizures during and immediately followin therapy (Williams, Makker and Grupe 1978).

Pregnancy may have complications due to chlorambucil treatment (Shotton and Monie 1963). In rat fetuses of mothers administered chlorambucil the most common abnormality seen was unilateral and bilateral absence of the kidney (Monie 1961). This is similar to the human fetus examined by Shotton and Monie, in which the left kidney and ureter were absent. Other studies have also established teratogenicity of chlorambucil in mouse and rat embryos (Chaube and Murphy 1968; Didock, Jackson and Robson 1956; Murphy 1959; and Sadler and Kochhar 1975). The mice showed numerous deformities and large amounts of necrosis in developing limb buds. Chlorambucil also induced a decrease in the rate of DNA and synthesis at four and eight hours in the mouse embryo without inhibiting RNA and protein synthesis (Sadler and Kochhar 1976b). In addition, in man chlorambucil has been shown to cause serious lesions of the germinal epithelium. Spermatogenesis was inhibited (Miller 1971) and only the sertoli cells and azoospermia were present (Richter et al. 1976).

Analytical Methods

A variety of techniques have been developed for the estimation of alkylating agents in biological fluids. Several of these methods are not sensitive or accurate enough to permit metabolism and pharmacokinetic
studies in humans. Such procedures involve UV spectrophotometric (Linford 1962) and chlorine titrimetric methods (Linford 1961). Recently high pressure liquid chromatographic (Newell et al. 1978) and mass spectrometric determination (Jakhammer, Olsson and Svensson 1977) of chlorambucil in plasma have been reported. However, these methods and procedures are too complex and time consuming to allow routine use in the clinical laboratory.

For detection with the colorimetric method chlorambucil must be reacted with p-nitro benzylpyridine reagent. This along with the chlorine titration technique would not be suitable for the pharmacokinetic or metabolism due to the accuracy and specificity necessary. The HPLC procedure involved an extraction scheme requiring several 4°C centrifugations and freezing the samples with methanol/carbon dioxide at -68°C. In addition, there were poor extraction efficiency of the phenylacetic acid mustard (Newell et al. 1978). The method developed by Jakhammer et al. (1977), using mass spectrometric analysis, requires a back extraction from buffer at a pH of 9.0 and is unsuitable due to the instability of the chloroethyl group of alkaline pH (Linford 1963). Such alkaline exposure at higher temperature could result in increased hydrolysis.

The quantitative method developed here used a deuterated internal standard and a one-step extraction followed by a determination by mass spectrometry of chlorambucil and the metabolite in plasma and urine. The in vitro stability of chlorambucil in plasma and water was studied utilizing this method. This procedure was then applied to quantification of chlorambucil and a metabolite in human plasma and urine samples.
Radiolabeled chlorambucil ($^{14}$C) was used to aid in developing and standardizing the extraction procedure.
STATEMENT OF PROBLEM

Chlorambucil is a useful anticancer drug in the treatment of various cancers, alone and in adjuvant therapy. Only limited data exists concerning its pharmacokinetics in cancer patients.

The objective of this project is to develop a specific analytical procedure for chlorambucil and its possible metabolites in plasma and urine samples. With a fast accurate method pharmacokinetic studies may be carried out. The metabolites are to be extracted and proposed structure given. The kinetics of chlorambucil in vitro in plasma and aqueous solution is to be studied. These studies will expand our knowledge concerning the stability, reactivity and metabolic profile of this useful chemotherapeutic agent.
METHODS AND MATERIALS

Deuterium oxide, acetic acid-d₄, and ethylene-d₄ oxide purchased from Merck and Co., Inc., St. Louis, Missouri. The 4-(p-Nitro-phenyl)-butyric acid and N-methyl-N-nitroso-p-toluene sulfonamide (Diazald) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. Bis-trimethylsilyl trifluoroacetamide (BSTFA), BSTFA-d₁₈ and micro reaction vials (1 ml) with Teflon-lines caps were obtained from Regis Chemical Co., Morton Grove, Illinois. Phosphorousoxy chloride was distilled from its reagent grade chemical and other organic solvents were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Michigan. Chlorambucil was supplied by Burroughs, Wellcome Co., Research Triangle Park, North Carolina. Palladium on alumina was purchased from Ventron Corp., Danvers, Massachusetts. The ¹⁴C-chlorambucil (at the chloroethyl moiety) with 97 percent purity was supplied by Dr. R. Engles, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.

Synthesis of Chlorambucil-d₈
(Labeled at N-chloroethyl Moiety)

The method for synthesis of chlorambucil-d₈:

\[
\text{HOOC-(CH₂)₃} - \text{N} - \text{CD₂CD₂Cl} \quad \text{CD₂CD₂Cl}
\]

was adapted from Bergel and Stock (1954). To 4-(P-nitrophenyl)-butyric acid, etheral diazo methane (with Diazald) was added and allowed to react at room temperature for 30 mintues. The resulting methylnitrophenylbutyrate was dissolved in ethylacetate-methanol (99:1 v/v). Keeping the
solvent at a constant volume, H₂ gas was continuously bubbled through for eight hours yielding the reddish-brown aminophenylbutyric acid methyl-ester. The ester (0.5 μg) was then dissolved in 20 ml of 50 percent acetic acid (CH₂COOD in D₂O). Ethylene oxide-d₄ (2 ml) was then added while the sample was shaking at 4°C. The mixture was allowed to stand at room temperature for 24 hours. The reaction was terminated by the addition of water (~ 10 ml) and NaHCO₃ until no significant CO₂ evolved. Extraction of the yellow solution was done with ethylacetate (8 ml x 5) and dried with anhydrous sodium sulfate.

Upon evaporation of the ethylacetate by N₂ gas and azeotroping the trace of water with methlene chloride, a gummy dark brown product was left. This was then refluxed 45 minutes with 4 ml of freshly distilled phosphorousoxy chloride. Water and methylene chloride were used several times to partition any excess reagent. The nonaqueous layer was evaporated by N₂ gas and then refluxed with concentrated HCl for six hours at 100°C. The chlorambucil was extracted with ethylacetate:hexane (1:1 v/v) and dried with anhydrous sodium sulfate. Mass spectrometry and HPLC were used to examine the chemical purity which was shown to be 85 percent.

**Extraction of Chlorambucil**

Deuterated chlorambucil was added to 0.5 ml sample of plasma or urine followed by mixing and the addition of 0.5 ml of four percent perchloric acid. Ethylacetate:hexane (0.75 ml) (1:1 v/v) was used to extract the chlorambucil from the precipitate. The sample was vortexed well then centrifuged for five minutes at 3000 rpm (GLC-2B, DuPont Instrument Co., Wilmington, Delaware) and the organic layer was transferred into a 1 ml
micro vial with a Teflon-lined cap. The solvent was evaporated at room temperature by a gentle stream of purified N₂ gas. A few drops of methylene chloride were added to the residue and the resulting mixture dried.

Plasma samples (1.0 ml) and purified ¹⁴C-labeled chlorambucil (at the chloroethyl moiety) were used for studies of chlorambucil recovery from plasma. After the first extraction of chlorambucil an additional 1.0 ml of ethylacetate:hexane (1:1 v/v) was added to the perchloric acid precipitate, vortexed, centrifuged and the organic layer removed. This was repeated for a third extraction and ¹⁴C distribution was examined in each extraction. An aliquot of the aqueous layer was removed and counted.

**Derivatization of Chlorambucil**

Dried samples were converted to trimethylsilyl (TMS) derivatives for GC-MS analysis. To the dried residue in the reaction vial 100 µl of Bis-trimethylsilyltrifluoroacetamide (BSTFA):CH₃CN:CH₂Cl₂ (20:10:1 v/v/v) solution were added to the samples and allowed to react at 100°C for 10 minutes. The TMS derivatives of the samples were then analyzed by GC-MS.

**High Pressure Liquid Chromatography (HPLC)**

A high pressure liquid chromatograph (6000 pumps, 660 solvent programmer, 440 detector, Waters Associates, Milford, Massachusetts) with a reverse phase column (Micro C₁₈ Bondapak, Waters Associates, Milford, Massachusetts) was used to determine the purity of ¹⁴C-chlorambucil and synthesized deuterated chlorambucil. The solvent system used was methanol and four percent acetic acid for both compounds. Flow rates were 0.5 ml/min. and 1.0 ml/min. for ¹⁴C-chlorambucil and deuterated chlorambucil, respectively, and they were detected at 254 nm.
Stability of Chlorambucil in Water

Chlorambucil (varied concentrations) was carefully added to a reaction vial. For maximal contact with the water, avoiding as much self-micelle formation as possible, the chlorambucil-ethylacetate solution was evenly coated to the vial wall. The solution was evaporated by a gentle stream of purified N₂ gas. Distilled water (50 μl and 100 μl) was added, any bubble removed, and the vial was then sonicated. The samples were incubated at 37°C for the appropriate time and at each time point the water was evaporated using a N₂ stream and any trace amount of water was azeotroped with methylene chloride. Internal standard was added after the evaporation of the water and the sample kept frozen at -20°C. The samples were derivatized, as described earlier, then analyzed by mass spectrometry.

GC-MS Analysis

A Finnigan 3300-6100 mass spectrometer was interfaced with a Finnigan 9500 gas chromatograph through a glass jet separator. Seventy electron volts were used for all analysis. Derivatized samples were injected onto a GC column (0.5 m x 2mm) packed with three percent OV-17 and the solvent was vented at 190°C for 45 seconds. The temperature programming at 20°C/minute to 310°C was started at that time.

The ions selected for monitoring chlorambucil were m/e 326, corresponding to the molecular ion (M⁺) with a fragment loss of CH₂Cl, and its chlorine isotope ion at m/e 328. This method allows for a great deal of selectivity to the GC elution of chlorambucil, chlorambucil fragmentation pattern, and chlorine isotope ratio. Utilizing these three
parameters contributes to the specificity of this technique. The ion monitored for the internal standard was m/e 383 ($M^+$) rather than m/e 332 ($M-\text{OD}_2\text{Cl}$) due to interference of plasma background. Plasma shows a low level of ions at m/e 332 but not at m/e 383. The fragmentation pattern of chlorambucil has a response for the ion at m/e 326 ($M-\text{CH}_2\text{Cl}$) approximately ten times greater than the response for the molecular ion at m/e 375 ($M^+$). Therefore, in order to obtain an equivalent response for the molecular ion ($M^+$) of the internal standard to the 326 ($M-\text{CH}_2\text{Cl}$) ten times as much deuterated chlorambucil relative to the expected chlorambucil concentration were added.

Decomposed chlorambucil in ethylacetate or hydrolysis products of chlorambucil incubated in water were injected onto a GC column at 150°C as trimethylsilyl derivatives and they were separated by a temperature programed at the rate of 20°C/minute to 310°C. In the case of chlorambucil decomposition products in ethylacetate (three major chromatographic peaks were resolved) and their spectra were obtained. For hydrolysis products partial spectra (100 to 400 atomic mass units, amu), selected ions [m/e 326 for chlorambucil ($M-\text{CH}_2\text{Cl}$) and monohydroxy chlorambucil], and retention times were used for their identification. Monohydroxy and dihydroxy products and chlorambucil were chromatographically resolved.

**Degradation**

The internal standard is stored in a non-aqueous solution, ethylacetate. Therefore, it was necessary to study the stability of chlorambucil in such a solution. Chlorambucil in ethylacetate was left
at room temperature under fluorescent light for two weeks. From the yellow-brown solution an aliquot was taken, dried and converted to the TMS derivative, as described earlier. A second aliquot was taken and deuterated TMS (TMS-d$_{18}$) was used. The deuterated TMS would exhibit a shift in the mass spectra of an eighteen mass increase for every TMS-d$_{18}$ group reacted to the compound(s). The number of TMS groups reacted to the molecule indicates the number of replaceable hydrogens.

Three major decomposition products were observed and assigned probable structures (Figure 1). Two products, compounds A and B (Figure 1A and 1B) had two TMS groups while the third, compound C (Figure 1) had one TMS groups. The mass spectras demonstrated a loss of 131 amu for $^8$TMSOCCH$_2$ indicating the carboxy group of chlorambucil remained intact. The study showed a loss of both chlorines, determined by the absence of chlorine isotope ratios in the mass spectras. Therefore, the chloroethyl group is the reactive site.

The loss of chlorine leads to cyclization and pyrroll formation (Figure 1B and 1C). The three proposed structures are 4-amino-phenylbutyric acid for compounds 1A, B, and C, respectively. Therefore, storage of stock chlorambucil in ethylacetate solution and chlorambucil tablets should be kept out of fluorescent light.

**Recovery Studies**

Efficiency of extraction was studied with $^{14}$C labeled chlorambucil (505 ng and 3.3 x 10$^4$ cpm). The $^{14}$C-chlorambucil was added to 1.0
Figure 1. Degradation Products of Chlorambucil in Ethylacetate.

A is 4-aminophenylbutyric acid.
Figure 1, continued.

B is 4-[(3-hydroxy) pyrrolyl] phenylbutyric acid.
C

Figure 1, continued.

C is 4-pyrrolylphenylbutyric acid.
ml of plasma and incubated at 37°C or 25°C. One half milliliter aliquots were removed at various time points (0.00, 0.25, 1.00, 3.00, and 6.00 hours) and extracted. At the zero time point 50 μl of plasma with 14C-chlorambucil, 100 μl of the organic layer from the first extraction, and the remainder of the first extraction organic layer combined with the organic layer of the second extraction, and 100 μl of the aqueous layer were counted. The 50 μl of plasma with 14C-chlorambucil gave the standard concentration (count) necessary to establish the basis for proficiency of extraction.

Precision of this recovery was studied by mass spectrometry using 200 and 1000 ng chlorambucil and appropriate amounts of internal standard added to 1.0 ml plasma samples. A single extraction was carried out and the replication of the recovery was examined for the precision of extraction by calculating the ratio of chlorambucil to internal standard.

**Stability Study**

The stability of chlorambucil in plasma (1 μg/ml) at 37°C or 25°C and water (500 ng/50 μl) at 37°C was studied after incubation in each medium. At designated time points an aliquot was taken and the internal standard was added. The samples were then extracted, derivatized and analyzed as described previously.

**Human Studies**

Three chronic lymphocytic leukemia patients were given 0.6 mg/kg or oral chlorambucil (Leukeran). Plasma and urine samples were collected on ice and stored at -70°C until analysis. One half milliliter aliquots were taken at each time point, extracted and derivatized simultaneously.
for chlorambucil and metabolites. They were then analyzed by selected ion monitoring at m/e 298 for p-\([4-N,N\text{-}bis\ (2\text{-}chloroethyl})\] aminophenylacetic acid (M-CH\(_2\)Cl), 326 for chlroambucil (M-CH\(_2\)Cl) and monohydroxy chlorambucil (M-CH\(_2\)OTMS), 380 for dihydroxy chlorambucil (M-CH\(_2\)OTMS), and 383 for the internal standard (M\(^+\)). Possible mono- and dihydroxylated products of the aminophenylacetic acid were looked for in the samples. The selected ions, m/e 401, 352, and 298, correspond to M\(^+\), M-CH\(_2\)Cl and M-CH\(_2\)OTMS) for the dihydroxylated compound.

For two patients given \(^{14}\)C-chlorambucil (100 µCi; 135.5 Ci/mg) samples were collected at various time points and extracted as described previously. The organic layers of the three extraction steps were combined and counted on a liquid scintillation counter (Beckman LS-230). To the aqueous layer 1.0 ml of distilled water was added, followed by centrifugation; for ten minutes at 10,000 rpm, and a 0.5 ml aliquot counted. The percent distribution of \(^{14}\)C-chlorambucil into the organic and aqueous layers was then calculated based on 100 µl aliquots of plasma counted directly.

**Calculations**

The disappearance rate constant, k, of chlorambucil from plasma and water were calculated according to:

\[
k = 2.3 \log \frac{[C]_o}{[C]_t}/t
\]

were \([C]_o\) and \([C]_t\) are the concentrations of chlorambucil in plasma or water at times zero and t. The concentrations of chlorambucil in plasma and urine were calculated using:

\[
\text{ng Chlorambucil/ aliquot} = \frac{\text{Response (chlorambucil) sample}}{\text{Response (IS) sample}} \times \frac{1}{\text{RWR}} \times \text{ng IS}
\]
where RWR standard of equal weight is:

\[
\text{RWR} = \frac{\text{Response (chlorambucil)}}{\text{Response (IS)}}
\]

For patient studies, oral chlorambucil pharmacokinetics data were calculated according to:

\[
C = A(e^{-\alpha(t-t_{lag})} - e^{-\beta(t-t_{lag})})
\]

C is the concentration time t after dose administration (ng/ml), A is a coefficient (ng/ml) $t_{lag}$ is a lag time for drug appearance in plasma and $\beta$ is the apparent first order elimination rate constant. Thus, $\alpha = k_a$ the absorption rate constant for the parent compound, chlorambucil.
RESULTS AND DISCUSSION

Recovery and Precision of Method

The yield and precision of chlorambucil extraction from plasma are shown in Tables 1 and 2. Studies with $^{14}$C-labeled chlorambucil indicated greater than 70 percent recovery with a single extraction and 84 percent when the extraction steps were repeated three times. Table 2 shows the precision of a single extraction for 200 ng/ml and 1000 ng/ml to be 94.3 percent and 90.5 percent, respectively. The precision and yield of a one step extraction is sufficient for removal of chlorambucil from clinical samples. Recovery was shown to not be dependent on the concentration of chlorambucil in the plasma sample.

Stability of Chlorambucil

The disappearance of chlorambucil in water (500 ng/50 μl) incubated at 37°C is shown in Figure 2. The stability data for chlorambucil in plasma incubated at 25°C and 37°C are presented in Table 3. After four hours at 25°C about 90 percent of the chlorambucil was recovered with the decrease at 30 minutes due to experimental error. Figure 3 shows the disappearance of chlorambucil incubated in plasma (1 μg/ml). The $k = 0.43 \text{ hr}^{-1}$ for water (Figure 1) and $k = 0.12 \text{ hr}^{-1}$ for plasma (Figure 3).

At room temperature over a 30 minute period there is about 13 percent hydrolysis of chlorambucil in aqueous solution at pH 9.0 (Jakhammer et al. 1977). However, the chloroethyl group in an alkaline solution is
Table 1: Yield of Chlorambucil Extracted from Plasma

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Extractable $^{14}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>74.0 ± 1.3$^a$</td>
</tr>
<tr>
<td>2nd</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>3rd</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$ Recovery and % R. S. D. of triplicate samples

Table 2: Precision of Chlorambucil Extraction from Plasma

<table>
<thead>
<tr>
<th>ng/ml</th>
<th>Recovery$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>94.3 ± 1.3</td>
</tr>
<tr>
<td>1000</td>
<td>90.5 ± 3.0</td>
</tr>
</tbody>
</table>

$^a$ % Recovery and % R. S. D. of triplicate (200 ng level) and quadruplicate (1000 ng level)
Figure 2. Recovery of Chlorambucil (500 ng) from Water (50 μl) Incubated at 37°C.

Internal standard was added at the end of incubation, water was removed by nitrogen, and chlorambucil content was analyzed at the designated time points.
Table 3: Effect of Temperature and Time on Chlroambucil Recovery from Plasma

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Recovery &amp; % R. S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90.5 ± 3.0</td>
<td>80.4 ± 3.0</td>
</tr>
<tr>
<td>0.5</td>
<td>82.5 ± 4.3</td>
<td>76.6 ± 3.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.0</td>
<td>91.7 ± 2.6</td>
<td>55.6 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> % Recovery and % R. S. D. of quadruplicate
<sup>b</sup> % R. S. D. of minimum of five samples
Figure 3. Recovery of Chlorambucil (1.0 g) from 1.0 ml of Plasma and Incubated at 37°C.

At designated time points aliquots were taken, extracted and analyzed.
unstable (Linford 1963) and results in an increased hydrolysis. Mass spectrometric studies demonstrated hydrolysis of chlorambucil in water incubated at 37°C to form dihydroxylated chlorambucil.

There is probably both alkylation and hydrolysis resulting in a lower recovery. The in vivo disappearance of chlorambucil at 37°C (Figure 3) is slower than in water. Plasma proteins may exhibit a protective effect against hydrolysis (Linford 1962).

**In Vivo Studies**

In Figures 4 and 5, plots of plasma levels for chlorambucil and phenylacetic acid—mustard of three chronic lymphocytic leukemia patients indicate rapid absorption of the parent drug. The study involving the urinary excretion of two patients given $^{14}$C-chlorambucil had a distribution of the chlorambucil, hydroxylated chlorambucil and phenylacetic acid mustard into the organic and aqueous layers, as shown in Table 4. In 24 hours 55.5 percent and 60.7 percent of the total administered $^{14}$C, with only 2.8 percent and 3.3 percent of the $^{14}$C being in the organic layer, was excreted. The urinary excretion of chlorambucil and metabolite, which are extracted in the organic layer, were shown to be less than one percent and 0.2 percent of the administered dose, respectively.

**Mass Spectral Identification**

The selected ion monitoring at m/e 326 (M-CH$_2$Cl for chlorambucil) and at m/e 383 (M$^+$ for internal standard) allows for specific and accurate determination of chlorambucil in plasma and urine. Figure 6 shows the ion current tracing used for quantitation of m/e 383 and 326 and the chlorine isotope ions at 385 and 328. Figure 7A is a mass spectra of
Figure 4. Plasma Chlorambucil Disappearance in Three Patients with CLL.

Dose of chlorambucil was 0.55 mg/kg p.o. One standard deviation:---.
Figure 5. Plasma Phenylacetic Acid Mustard Disappearance in Three Patients with CLL.

Dose of chlorambucil was 0.55 mg/kg p.o. One standard deviation: ---.
Table 4: Urinary Excretion of Chlorambucil and Metabolite

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chlorambucil</th>
<th>Phenylacetic acid mustard&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.710</td>
<td>0.376</td>
</tr>
<tr>
<td>B</td>
<td>0.349</td>
<td>0.099</td>
</tr>
<tr>
<td>C</td>
<td>0.600</td>
<td>0.132</td>
</tr>
<tr>
<td>D</td>
<td>55.50</td>
<td>2.87</td>
</tr>
<tr>
<td>E</td>
<td>60.73</td>
<td>3.31</td>
</tr>
</tbody>
</table>

<sup>a</sup> % of chlorambucil and phenylacetic acid extracted based on total administered chlorambucil. Both were removed in the organic layer of one extraction.

<sup>b</sup> These patients were administered <sup>14</sup>C-chlorambucil. The percentage of the total administered <sup>14</sup>C is shown to be excreted in the urine (total <sup>14</sup>C) and in each layer (organic and aqueous). This is based on one extraction.
Figure 6. Ion Chromatograms of Chlorambucil in Plasma and Plasma Background.

A is two hundred nanograms of chlorambucil and 2.0 g of the internal standard added to 10. ml plasma. After extraction and derivatization, 0.1 ml equivalent plasma was analyzed.
Figure 6, continued.

B is ion chromatograms of 0.5 ml equivalent plasma after extraction and derivatization.
Figure 7. Mass Spectra of Chlorambucil and Deuterated Chlorambucil.  
A is the trimethylsilyl chlorambucil derivative.
Figure 7, continued.

B is methylester of deuterated chlorambucil.
trimethylsilyl chlorambucil derivative as seen in plasma samples and 7B is the methyl ester of deuteration chlorambucil. For the internal standard the molecular ion (in Figure 7B m/e 325) is monitored rather than the primary peak of abundance (in Figure 7B m/e 274). The molecular ion is approximately ten times less, therefore, a ten-fold excess of internal standard over the expected chlorambucil level was added. Also, the addition of excess internal standard increased the percent of chlorambucil which is extracted from the solution.

Three major decomposition products in ethylacetate were assigned probable structures (Figure 1). Two products, compounds A and B (Figure 1A and 1B) had two TMS groups while the third, compound C (Figure 1C) had one TMS group. The GC elution time was shortest for A and longest for B.

Chlorambucil was observed to undergo hydrolysis. Mass spectrometric studies demonstrated that chlorambucil in water forms first a monohydroxy compound and then the dihydroxy compound, p-(bis-2-hydroxyethyl) aminophenylbutyric acid, the predominant degradation product in water.

A metabolite, p-[4-N, bis (2-chloroethyl)] aminophenylacetic acid isolated from human plasma and urine is shown in Figure 8, where 298 m/e (M-CH₂Cl) was used for the selection ion monitoring. Plasma disappearance of the metabolite is seen in Figure 5. The phenylacetic acid mustard was also isolated from human urine. There was no indication of hydroxylated phenylacetic acid mustard isolated in the organic layer of urine or plasma samples.
Figure 8. A Chlorambucil Metabolite, p-[4-N, N bis (2-chloroethyl)] amino-phenylacetic acid, isolated from Human Plasma and Urine.
Human Pharmacokinetics

Table 5 shows the pharmacokinetic data of orally administered chlorambucil and a metabolite, p-[4-N, bis (2-chloroethyl)] aminophenylacetic acid. The mean plasma half-life for chlorambucil and its metabolite were 98 and 158 minutes, respectively.

The patient studies of chlorambucil and the metabolite suggest that orally administered chlorambucil is rapidly absorbed from the gastrointestinal tract and undergoes relatively rapid metabolism to the phenylacetic acid mustard (Figure 9). The calculation of the area under the chlorambucil plasma concentration versus time curve (CXT), used clinically for availability analysis, and a mean for chlorambucil of 121.6 μg min/ml and for the metabolite a value almost twice that amount.

Chlorambucil undergoes fatty acid metabolism (Figure 9) forming the phenylacetic acid mustard through β-oxidation. The unsaturated intermediate, 2-[4-bis (2-chloroethyl) aminophenyl]-2-butenic acid, and the α and β unsaturated metabolites, identified in the rat (McLean, Newell and Baker 1976) were not detected to be present in man.
Table 5: Pharmacokinetics of Chlorambucil and Metabolite

<table>
<thead>
<tr>
<th>Patient</th>
<th>α (min⁻¹)</th>
<th>T¹/₂ min</th>
<th>CXT (μg·min/ml) for 0.6 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.026345</td>
<td>97.7</td>
<td>86.1</td>
</tr>
<tr>
<td></td>
<td>±0.016123</td>
<td>±20.6</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.036261</td>
<td>80.6</td>
<td>73.2</td>
</tr>
<tr>
<td></td>
<td>±0.011130</td>
<td>±9.5</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.020287</td>
<td>116.0</td>
<td>205.5</td>
</tr>
<tr>
<td></td>
<td>±0.012211</td>
<td>±18.2</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.027631</td>
<td>98.1</td>
<td>121.6</td>
</tr>
<tr>
<td></td>
<td>±0.008004</td>
<td>±17.7</td>
<td></td>
</tr>
</tbody>
</table>

Metabolite

<table>
<thead>
<tr>
<th>Patient</th>
<th>C=α(t-t₀) - e⁻β(t-t₀)</th>
<th>CXT (μg·min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.016918</td>
<td>139.6</td>
</tr>
<tr>
<td></td>
<td>±0.009808</td>
<td>±53.8</td>
</tr>
<tr>
<td>B</td>
<td>0.010403</td>
<td>170.9</td>
</tr>
<tr>
<td></td>
<td>±0.006480</td>
<td>±38.4</td>
</tr>
<tr>
<td>C</td>
<td>0.012082</td>
<td>163.6</td>
</tr>
<tr>
<td></td>
<td>±0.007570</td>
<td>±33.6</td>
</tr>
<tr>
<td>mean</td>
<td>0.013136</td>
<td>158.0</td>
</tr>
<tr>
<td></td>
<td>±0.003383</td>
<td>±16.4</td>
</tr>
</tbody>
</table>

*a* Calculations based on:

\[ C = A(e^{-\alpha(t-t_{lag})} - e^{-\beta(t-t_{lag})}) \]

\[ CXT = \left( \frac{1}{\alpha} - \frac{1}{\beta} \right) \]

Where \( \alpha \) is the rate constant for the appearance of the drug in the plasma, \( \beta \) is the elimination rate constant, \( T^{1/2} \) is the half-life, and CXT is the concentration versus time used to study availability.
Figure 9. Metabolism of Chlorambucil.

R is ClCH₂CH₂_\text{N} \text{ClCH₂CH₂}
CONCLUSION

The method presented here employs a one step extraction from urine and plasma and the selected ion monitoring method is used for the determination of chlorambucil and a metabolite. This procedure can be used to study pharmacokinetics and metabolism in cancer patients. The one step extraction yield and precision were high enough to allow routine analysis.

The synthesis of deuterated chlorambucil was carried out. The procedure yielded an 85 percent purity of chlorambucil-d$_8$. Use of deuterated chlorambucil as the internal standard and chlorine isotope ratio allows for greater selectivity of the method.

The in vitro studies demonstrated the chemical stability of chlorambucil in plasma. The disappearance rate constant for chlorambucil in water is approximately four times greater than that of plasma. Plasma proteins probably play a role in protection from hydrolysis. It is likely that there is some irreversible covalent binding of chlorambucil to plasma proteins. The incubations of chlorambucil in plasma at 25°C and 37°C indicate that the rate of parent compound disappearance is temperature dependent. The plasma samples containing chlorambucil are stable at room temperature during the extraction procedure.

Use of mass spectrometric analysis allows for very specific and selective studies. Nanogram quantities of chlorambucil and metabolite may be separated through the GC and quantitated using selected ion monitoring. Unknown compounds are identified by studying the elution times
and fragmentation patterns, as done with the three degradation products. However, for quantitation of the hydroxylated chlorambucil an adequate separation method must be developed. This method for studying chlorambucil and a metabolite is the first one developed to observe human plasma and urine levels, as well as pharmacokinetics of oral administration.

Chlorambucil is rapidly absorbed from the gastrointestinal tract and has a plasma half-life of approximately 1.5 hours. There is relatively rapid metabolism to the phenylacetic acid mustard, which has a half-life of about 2.5 hours. The urinary excretion over a 24 hour period is less than one percent for both chlorambucil and the phenylacetic acid mustard. The stability of chlorambucil in plasma, nearly complete gastrointestinal absorption, and low urinary excretion makes it a beneficial drug for oral usage in cancer chemotherapy.
LIST OF REFERENCES


