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## Population pharmacokinetics identifies rapid gastrointestinal absorption and plasma clearance of oral chlorambucil administered to cats with indolent lymphoproliferative malignancies

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### Abstract

**OBJECTIVE**—To establish the pharmacokinetics of a single 2-mg oral dose of chlorambucil in cats with indolent lymphoproliferative malignancies.

**ANIMALS**—24 client-owned cats.

**PROCEDURES**—Cats were assigned to 1 of 4 groups, with each group having a total of 3 sample collection time points over 12 hours after receiving a single 2-mg oral dose of chlorambucil. Each time point combined to generate 6 full patient plasma chlorambucil concentration-time curves from the 24 cats. Chlorambucil treatment was continued every other day and a single, variably timed sample collection was obtained on day 14. Population parameter estimates were obtained by nonlinear mixed-effects modeling. Covariates investigated included age, sex, baseline serum cobalamin, study location, weight, and body condition score.

**RESULTS**—Chlorambucil administered orally to cats was found to have a peak plasma concentration of approximately 170 ng/mL (SE, 31.1 ng/mL), percent coefficient of variation (%CV) of 18.4% within 15 minutes, and a terminal half-life of 1.8 hours (SE, 0.21 hour; %CV, 12.4). At the 4-hour mark, a smaller secondary peak in plasma chlorambucil was found. Day 14 samples were similar to those of the initial dose. No covariates showed a significant effect in the population model.

**CLINICAL RELEVANCE**—In these cats, chlorambucil at a 2-mg dose administered every other day undergoes rapid gastrointestinal absorption and plasma clearance with no drug accumulation between doses. These data are critical to inform future work investigating the association of chlorambucil drug exposure with adverse events and outcome of cats with lymphoproliferative diseases.

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Chlorambucil is an oral nitrogen mustard derivative and alkylating agent that is used as both an antineoplastic and immunosuppressant in veterinary medicine. In cancer-bearing cats, chlorambucil in combination with a glucocorticoid (ie, prednisolone) is commonly used to treat indolent lymphoproliferative malignancies, such as low-grade, mucosal, epitheliotropic, small T-cell alimentary lymphoma (LGAL). Chlorambucil, 2 mg, every other day or on a Monday, Wednesday, Friday schedule is commonly prescribed.<sup>1,2</sup> A chlorambucil bolus dose of 20 mg/m<sup>2</sup> administered every 2 weeks has also been reported.<sup>3</sup> These dosages have, in part, been selected empirically based on maintaining a hypothetical dose intensity to match the slow progression of these indolent malignancies<sup>4</sup> and to avoid the splitting of the commercially available tablets as part of a chemotherapy safety protocol. Combination treatment of chlorambucil and prednisolone for indolent lymphoproliferative malignancies in cats has resulted in response rates of greater than 80%, with prolonged median survival times of 1 to 3 years.<sup>1-3,5,6</sup> Despite durable remissions, there is still a subset of cats that lacks a response to this combined therapy, and poor response has been observed as a negative prognostic factor.<sup>1,4,6,7</sup> In addition, some cats receiving treatment may experience clinically significant adverse effects (AEs), including both hematological and gastrointestinal (GI) toxicities, and—less commonly—idiosyncratic liver enzyme elevations, neurotoxicity in form of myoclonus or tonic-clonic seizures, or acquired Fanconi's syndrome.<sup>2-4,6,8,9</sup> Although AEs are uncommon, they may necessitate treatment delays or discontinuation, in addition to requiring supportive medical management. To our knowledge, there are no published data regarding the pharmacokinetics (PK) of chlorambucil in cats to investigate further the relationship between drug exposure and response in terms of treatment efficacy and toxicity. It is possible that some cats that are considered nonresponders with no improvement of their clinical signs could be receiving a subtherapeutic dose or a dose that is not biologically effective to treat the cancer. It also remains unknown whether cats that develop significant AEs to chlorambucil are experiencing greater exposure to the drug after administration. The spectrum of treatment efficacy and toxicity seen in cats may be a result of the variability seen in the PK of chlorambucil in cats, and warrants a foundational investigation and preliminary assessment of this potential variability.

Understanding the PK of chlorambucil in cancer-bearing cats, and analyzing PK data using a population approach allows the investigation of demographic (age, sex), physiologic (weight, body surface area, protein binding), or pathophysiologic (renal and hepatic function) parameters that may influence variability in chlorambucil PK, providing an opportunity for individualized dosing or improving the ability to predict efficacy and toxicity of this important drug in feline cancer therapy. It is essential that chlorambucil PK be evaluated in the population of patients receiving this treatment, because patient characteristics, such as older, cancer-bearing cats that may have other comorbid diseases, can affect PK profiles. Absorption, metabolism, distribution, and elimination may likely be different and more highly variable in this population compared to young, healthy cats. To address these concerns, this study aims to establish the PK of a single oral dose of chlorambucil in cancer-bearing cats for which this treatment is a standard recommendation.

## Materials and Methods

### Study design

This study was designed as a prospective, single-arm, open-label, two-site clinical pilot study. Twenty-four client-owned cats were enrolled from December 2016 through July 2021 at Colorado State University Veterinary Teaching Hospital and the University of California, Davis Veterinary Medical Teaching Hospital. The study protocol was approved by the Institutional Animal Care and Use Committee and/or Clinical Review Board at each institution. All cats were required to have a CBC, chemistry profile, serum cobalamin concentration, and urinalysis performed within 14 days of study initiation for eligibility assessment. To be eligible for enrollment, cats were required to have a cytologic and/or histologic diagnosis of small-cell (low-grade) lymphoproliferative malignancy for which treatment with oral chlorambucil was recommended, age > 1 year, body weight  $\geq 2.5$  kg, adequate organ function as indicated by standard laboratory tests (specifically, hematocrit  $\geq 25\%$ , neutrophil count  $\geq 2,000/\mu\text{L}$ , creatinine  $< 2.5$  mg/dL, total bilirubin to upper limit of normal), and a Veterinary Cooperative Oncology Group-Common Terminology for Adverse Events (VCOG-CTCAE) v1.1 performance score of 0 or 1.<sup>10</sup> Owners provided written informed consent before enrollment. Cats were excluded from the study if they had received previous therapy with chlorambucil, any hematologic or biochemical abnormality grade 2 or greater according to the VCOG-CTCAE v1.1,<sup>10</sup> owners unable or unwilling to administer oral medications to their cat, patient temperament not amenable to repeated blood sampling, and treatment with chemotherapy other than chlorambucil within 14 days prior to day 0 (30 days for lomustine). The extended washout period for lomustine was selected because the time frame for which neutropenia can occur after administration to cats ranges from 7 to 28 days.<sup>11</sup> Concomitant treatment with prednisolone or other corticosteroids and cobalamin supplementation was permissible with documentation.

### Treatment

All cats received chlorambucil 2 mg orally every other day for a duration of 14 days. The FDA-approved formulation of chlorambucil (GlaxoSmithKline) was dispensed from each institutions' pharmacy.

### Study schedule

We predicted that 6 full PK curves would provide preliminary data regarding the PK parameters of chlorambucil in cats. Six samples or patients at each PK time point is generally considered an adequate number to assess the potential PK variability in healthy animals; however, this has not been well investigated for older animals with comorbidities. No information exists regarding the PK and interpatient variability of chlorambucil in cats; thus, it was not possible to assume effect size or identify how many subpopulations (ie, covariate groups) should be included in the final population model.

To collect blood samples safely over the 12-hour period needed to obtain full plasma chlorambucil concentration-time curves, each enrolled cat was assigned to 1 of 4 groups (group A, B, C, or D), with each group having a total of 3 sample collection time points after chlorambucil administration (Table 1). Group assignment occurred sequentially, with the

first cat enrolled in group A. Once assigned, cats returned to the hospital to start treatment; this was considered day 0 of the study. Cats were fasted from midnight the previous day prior to the day 0 visit as to minimize PK variability in rate of absorption.<sup>12</sup> Cats were not required to be fasted for subsequent doses. Physical examination, vital parameters, and body weight were recorded. A chlorambucil 2-mg tablet administered orally to each patient, and the time of administration was recorded. In addition to the baseline plasma sample prior to the chlorambucil administration, 3 additional serial plasma samples were collected throughout the day depending on the group to which each cat was assigned. Documentation of scheduled and actual time of plasma sample collection was recorded. The results at each time point combined to generate 6 full patient plasma chlorambucil concentration-time curves from the 24 participating cats. Owners were instructed to continue administration of 2 mg chlorambucil by mouth at home every other day, and were instructed on proper and safe handling of chemotherapeutics.

The second and last study visit occurred on day 14. The history of the last dose of chlorambucil administered and whether it was given fasted or with food, physical examination, vital parameters, and body weight were recorded. A blood sample was collected for both a CBC and PK analysis. Cats could be removed from the study at the request of the owners at any point during the study period.

### **Blood collection and processing for PK analysis**

At each time point, 1 mL whole blood was collected into an EDTA tube and placed immediately on ice until processing. Within 20 minutes of collection, the blood sample was centrifuged at 1,200 X *g* for 10 minutes in a refrigerated centrifuge kept at 4°C. The plasma sample was aliquoted into 2 polypropylene tubes and stored at -80 °C until analysis. Plasma was also collected on day 14 and processed and stored as just described. The day 14 samples were collected at variable time points after chlorambucil administration earlier that day to compare to the day 0 concentration-time curve and to assess for the potential of drug accumulation over time.

### **Chemicals and reagents**

The analytical reference standard for chlorambucil and the internal standard (melphalan) were obtained from Sigma Aldrich. Calibrator and quality control working solutions of chlorambucil were prepared using 2 different lots of 1 mg/mL standard. Acetonitrile (ACN) was purchased from Fisher Scientific. Formic acid was purchased from Sigma Aldrich. The solvents were high-performance liquid chromatography grade or better.

### **Preparation of calibrators and samples**

Chlorambucil working solutions were prepared by dilution of the stock solutions with ACN to concentrations ranging from 10 to 10,000 ng/mL in ACN with 0.1% formic acid. Feline plasma calibrators were prepared by dilution of the working standard solutions with drug-free plasma to 11 nonzero concentrations ranging from 0.1 to 1,000 ng/mL. In addition, 4 sets of freshly prepared quality control samples (drug-free feline plasma fortified with analyte at 5, 50, and 500 ng/mL) were included. All samples were analyzed during a single run.

For calibrators and quality control samples, 5  $\mu\text{L}$  of working solution was added to 50  $\mu\text{L}$  of drug-free plasma and processed along with unknown samples. Prior to analysis, 50  $\mu\text{L}$  of plasma (calibrator, quality control, and unknown sample) was diluted with 150  $\mu\text{L}$  ACN with 0.1% formic acid to precipitate proteins, containing 200 ng/mL melphalan as an internal standard. The samples were vortexed for 10 minutes to mix and centrifuged at 12,000 X  $g$  for 10 minutes at 4  $^{\circ}\text{C}$ , and 110  $\mu\text{L}$  of supernatant was transferred to autosampler vials containing glass inserts. For analysis, 5  $\mu\text{L}$  was injected into the liquid chromatography tandem mass spectrometry system.

### Mass spectrometry

Positive ion electrospray ionization mass spectra were obtained with a 6500+ Q-TRAP triple quadrupole mass spectrometer (AB Sciex) with a turbo ion spray source interfaced to a Sciex ultra-performance liquid chromatography system. Samples were chromatographed with a Kinetex C18, 2.6- $\mu\text{m}$ , 2.1- X 50-mm column (Phenomenex), with a Phenomenex C18 filter frit guard cartridge. A liquid chromatography gradient was used with mobile phase A consisting of milli-Q water with 0.2% formic acid, and mobile phase B consisting of ACN with 0.2% formic acid. Chromatographic separation was achieved by holding mobile phase B steady at 5% for 1 minute, increasing linearly to 95% between 1 and 6 minutes, holding steady at 95% until 6.5 minutes, decreasing linearly to 5% at 6.51 minutes, and re-equilibration at 5% until 8 minutes. The mass spectrometer settings were optimized as follows: turbo ion spray temperature, 350 $^{\circ}\text{C}$ ; ion spray voltage, 3,500 V; source gas, 1 and 2, 30, and 40 U, respectively; curtain gas, 30 U; and collision gas, high. Product masses and collision energies were optimized by infusing the standards into the mass spectrometer.

Concentrations of chlorambucil were quantified by the internal standard reference method in the selected multiple reaction monitoring mode with ion parent ion mass-to-charge ( $m/z$ ) ratios of 304.1 and 305.0 for chlorambucil and the internal standard melphalan, respectively. The response for the product ions for chlorambucil ( $m/z$  168, 192, 241) and the internal standard ( $m/z$  118, 288), were plotted, and peaks at the proper retention times (4.75 minutes for chlorambucil and 3.31 minutes for melphalan) were integrated using Analyst Software version 1.7.1 (Sciex). Analyst was used to generate calibration curves and quantitate analytes in all samples by linear regression analysis. A weighting factor of  $1/y^2$  was used for all calibration curves.

The response was linear and gave a correlation coefficient of 0.999, with accuracies > 90% for all calibrators. The precision and accuracy of the assay was determined by assaying quality control samples in replicates ( $n = 4/\text{concentration}$ ). Accuracy was reported as percent nominal concentration; precision was reported as percent relative SD. For chlorambucil, accuracy was 98.7% for 5 ng/mL, 97.8% for 50 ng/mL, and 97.7% for 500 ng/mL. Precision was 1.8% for 5 ng/mL, 6.8% for 50 ng/mL, and 2.8% for 500 ng/mL. The technique was optimized to provide a limit of quantitation of 0.1 ng/mL (signal-to-noise ratio, > 10) and a limit of detection of approximately 0.03 ng/mL (signal-to-noise ratio, > 3) for chlorambucil.

## PK analysis

Pharmacokinetic parameters were estimated using population PK methods and nonlinear mixed-effects modeling (NLME). A naive pooled analysis using a 2-compartment model was used to obtain initial estimates. From these initial estimates, using a single extravascular input, the NLME model was fitted to the plasma chlorambucil concentration-time data (Phoenix NLME version 8.3; Certara Inc). Compartmental analysis of the data was performed using a 2-compartment model with first-order input, first-order output, no lag time, and micro-constants as primary parameters according to the following formula:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-k_{01}t}$$

where  $C(t)$  is the concentration in the plasma at time  $t$ ,  $A$ ,  $B$  and  $C$  are intercepts of the distribution, elimination and absorption phases, respectively,  $\alpha$  and  $\beta$  are the slopes of the distribution and elimination phases, respectively, and  $k_{01}$  is the rate of absorption.

Estimated primary parameters included volume of the central compartment per fraction absorbed, absorption rate, elimination rate, transfer rate 1 to 2, and transfer rate 2 to 1. Secondary parameters calculated included area under the curve, absorption half-life, elimination half-life, clearance per fraction absorbed, time of maximum plasma concentration, maximum plasma concentration, and the half-life of the initial elimination phase and the terminal elimination phase. Multiple models were tested with various error structures to identify the best-fit base model. The final model was chosen based on examination of the visual predictive plots and an examination of the conditional weighted residual plots, goodness-of-fit plots, plots of residuals, scatterplots of predicted vs observed values, and statistical significance among models using Akaike information criteria and twice the negative log likelihood. Models were run using the Quasi-Random Parametric Expectation Maximization algorithm in Phoenix. Between-subject variability was expressed using an exponential error model according to the equation<sup>13</sup>

$$\pi = tvP \times \exp(\eta P),$$

where  $P$  is the PK parameter of interest for individual  $i$ ,  $tvP$  is the typical value (fixed effect) for the population estimate of the parameter, and  $\eta P$  is the random effect for the between-subject differences of the parameter. A multiplicative error model was used to describe the residual, random intrasubject variability ( $\epsilon$ ), with a mean of zero and a variance of  $\sigma^2$  according to the following equation generated by the modeling software program:

$$C = C_{pred} \times (1 + \epsilon),$$

where  $C$  is the observed chlorambucil concentration for an individual and  $C_{pred}$  is the model predicted concentration plus the random error value ( $\epsilon$ ). Covariate analysis was performed using a forward selection, stepwise covariate search requiring a change in the threshold of the objective function value (twice the negative log likelihood) of 6.64, with a  $P$  value of .01 to add, and a  $P$  value of .001 to remove a covariate. Covariates that were investigated included age, sex, baseline serum cobalamin, study location, weight,

and body condition score. Robustness of the model with respect to predicted parameters and variability was checked by performing a bootstrap analysis with comparison of model-predicted and bootstrap values, and a predictive check with comparison of observed vs predicted quantiles, respectively.

## Results

### Study population

Twenty-four cats met the inclusion criteria and were enrolled between December 2016 and July 2021. Thirteen of these cats were enrolled at University of California, Davis Veterinary Medical Teaching Hospital, with the remainder enrolled at Colorado State University Veterinary Teaching Hospital. Their median age was 12.2 years (range, 5.2 to 17.7 years), and there were 13 castrated males (54%) and 11 spayed females (46%). Median body weight was 4.4 kg (range, 2.7 to 7.1 kg) and median body conditional score was 4/9 (range, 3 to 8). Twenty-three cats (96%) were diagnosed with LGAL. Disease was confirmed in the following sites: stomach (n = 4, 17%), duodenum (n = 17, 74%), ileum (n = 12, 52%), colon (n = 2, 9%), and abdominal lymph node (n = 3, 13%). Fourteen cats (61%) had lymphoma confirmed in multiple sites. Chronic lymphocytic leukemia was diagnosed in 1 cat by flow cytometry performed on peripheral blood. This cat was also suspected to have splenic and GI involvement, with splenomegaly and small intestinal diffuse wall thickening observed on abdominal ultrasound. The median dose of chlorambucil was 0.5 mg/kg (range, 0.28 to 0.74 mg/kg).

### Baseline serum cobalamin concentration

Four cats had baseline serum cobalamin concentrations < 400 ng/L. Eleven cats had baseline serum cobalamin concentrations > 1,000 ng/L; 7 of these cats were receiving cobalamin supplementation, 6 of which had serum cobalamin concentrations of < 400 ng/L at the time of their initial LGAL diagnosis.

### Concurrent medications

Twenty-one cats were administered medications within 24 hours of chlorambucil administration on day 0. Sixteen cats were receiving concurrent prednisolone 0.7 to 1.5 mg/kg PO every 24 hours (n = 13), 0.6 to 1.1 PO twice daily (n = 2), or 1.0 mg/kg every 48 hours (n = 1). Two cats received budesonide 0.2 to 0.3 mg/kg PO every 24 hours. Other concurrent medications to manage clinical signs, other comorbidities, and stress of veterinary visits included gabapentin 10 to 24.4 mg/kg PO 1 to 2 hours prior to study visits (n = 4), maropitant 1.4 to 1.6 mg/kg PO every 24 hours (n = 2), ondansetron 0.5 mg/kg PO every 12 hours (n = 1), mirtazapine 0.6 mg/kg transdermal every 24 hours (n = 1), amoxicillin-clavulanic acid 12.5 mg/kg PO every 12 hours (n = 1), enalapril 0.4 mg/kg PO every 24 hours (n = 1), famciclovir 79 mg/kg PO every 24 hours (n = 1), famotidine 1.2 mg/kg PO every 24 hours (n = 1), levothyroxine 0.02 mg/kg PO every 24 hours (n = 1), and L-lysine 119 mg/kg PO every 24 hours (n = 1). Cobalamin (250 µg SC weekly [n = 5], every 2 weeks [n = 1], or monthly [n = 1]) was being administered to 7 cats at the time of study initiation.



## Pharmacokinetics

Chlorambucil plasma concentration-time data were plotted (Figure 1); select population-based chlorambucil PK parameters are depicted in Table 2. After oral administration of a 2-mg dose, chlorambucil reached a maximum concentration of approximately 170 ng/mL within 15 minutes Table 3. This was followed by a biphasic decline in plasma concentrations, with a small secondary peak at approximately 4 hours. There was a substantial amount of variability observed in this study, as evidenced by Figure 1 and Table 2. Although we attempted to identify some of the sources of this variability using covariate analysis that included age, sex, weight, body condition score, baseline serum cobalamin concentration, and study site, none of these covariates showed a significant effect in the population model. Plasma samples collected on day 14 of dosing were similar to those collected on day 0 of dosing (Figure 2), suggesting no accumulation of drug with the dosing regimen used.

## Discussion

The purpose of our study was to evaluate the PK of oral chlorambucil in cats with indolent lymphoproliferative malignancies for which this treatment is the standard recommendation. Plasma chlorambucil concentrations were measured on day 0 during the initial 12-hour administration of a single 2-mg chlorambucil dose using 24 participating cats to generate 6 full patient plasma concentration-time curves, and on day 14 after all cats continued receiving their dose every other day at home. Here we report that oral chlorambucil in cats is absorbed rapidly from the GI tract, with a peak plasma concentration of approximately 170 ng/mL within 15 minutes and a terminal half-life of 1.8 hours. A smaller secondary peak in plasma chlorambucil concentration was found 4 hours after administration. Day 14 samples were found to be similar to those of the initial dose, indicating drug accumulation did not occur with this dosing protocol, which is not surprising given the short half-life (1.8 hours) relative to the dosing interval (48 hours).

Absorption of oral chlorambucil was rapid, with peak concentrations measured at 15 minutes in animals for which this time point was collected. This is similar to humans, with chlorambucil being first detected in the plasma between 15 and 30 minutes, and peak concentrations within 2 hours after oral administration for the treatment of indolent lymphoproliferative malignancies.<sup>14-19</sup> Variability of plasma concentration levels was observed in our study, which has also been observed in human studies.<sup>14,16,18</sup> Factors influencing drug absorption or metabolism could contribute to the variability of drug plasma concentrations. None of our covariates was found to be a significant contributor to the identified variability, including baseline serum cobalamin concentration, which is associated with absorption in the distal intestinal tract. This may be a result of the small sample size and subsequent type II error. Future studies could consider evaluating serum folate as a source of variability because folate is absorbed mainly in the proximal small intestinal tract, and decreased concentrations would indicate malabsorption along this portion of the GI tract. The mechanism of chlorambucil absorption is thought to occur by passive diffusion in the proximal small intestinal tract,<sup>20</sup> which would support the rapid peak plasma concentrations seen here and explain why cobalamin concentrations were not

identified as a covariate. However, passive diffusion also occurs with chlorambucil into malignant lymphocytes,<sup>21,22</sup> and—depending on tumor burden within the GI tract—this may influence the concentration of drug absorbed from the GI tract into the plasma. The PK of chlorambucil in healthy cats has not been performed, and therefore the variability observed in our study cannot be compared to a more homogeneous population; this may have also affected our ability to evaluate more fully the contributions of covariates to the observed variability.

All cats in the study had documented or were suspected to have GI disease involvement. To minimize absorption variability, our patients were fasted, which has been shown to increase oral bioavailability in humans.<sup>17,19,20</sup> Chlorambucil drug elimination was found to be rapid and biphasic in our study. Initial chlorambucil PK studies in humans found the elimination phase to be monophasic, with an elimination half-life between 1 to 2 hours, whereas a more recent study<sup>23</sup> showed chlorambucil having 2 distinct elimination phases, with a distribution half-life of 0.49 hour and a terminal half-life of 2.45 hours, similar to what was found in the cats in our study. The variability seen within the elimination phase of chlorambucil concentration in our study population could indicate variability of drug metabolism. Once absorbed in humans, chlorambucil is metabolized rapidly and extensively in the liver by monodichloroethylation and B-oxidation, forming the metabolite phenylacetic acid mustard (PAAM).<sup>24</sup> Future studies could measure PAAM peak plasma concentration and time to understand more completely the extent of metabolism of the parent drug in a similar study population. Human studies have shown that after oral administration of chlorambucil, metabolism to PAAM is rapid, and exposure to the metabolite is similar to or greater than that of the parent compound.<sup>14,16,18,19</sup> PAAM itself exhibits alkylating activity.<sup>25,26</sup> The contribution of PAAM to the antitumor activity and toxicity of chlorambucil in cats remains to be determined.

A smaller secondary peak in plasma chlorambucil found at the 4-hour mark is the first report of multiple peaks after chlorambucil administration. Previous studies in humans did not report a secondary concentration peak after oral administration.<sup>14-19</sup> Mechanisms by which multiple peak concentrations could occur after oral administration include enterohepatic recycling, the presence of absorption windows along the GI tract, and variable gastric emptying.<sup>27</sup> All of these may be potential causes for the second peak found in our study. There is some evidence that chlorambucil and its metabolites may undergo enterohepatic circulation despite its main elimination via the kidneys.<sup>28</sup> Chronic enteropathies, including LGAL, can affect GI integrity, which may affect absorption rates of drugs and lead to function disturbances, causing delayed gastric emptying or decreased or absent intestinal motility. Alternatively, this second peak may be an artifact generated by our sparse sampling design in which cats at the 4-hour time point may have had greater chlorambucil concentrations than those at the 2-hour time point.

Therapeutic dosages of chlorambucil in cats have been selected empirically and are based on the high percentage of clinical response and acceptable low number of AEs reported. No information is currently available about therapeutic plasma concentrations of chlorambucil in cats. Response rates and outcomes were out of the scope of our study, so it is unknown whether the area under the curve and maximum plasma concentration values predicted

in our study represent optimal therapeutic concentrations. Our study can emphasize that, over a 2-week period, the drug concentration is consistent after repeated administration and is generally well tolerated. The similar plasma concentrations after 2 weeks of dosing demonstrates there is no drug accumulation—a reflection of the rapid terminal elimination rate. Given the predicted half-life, complete elimination of each dose is likely to occur before the next dose is given.

Due to patient safety concerns obtaining multiple blood samples due to blood volume restriction, we used sparse patient sampling to contribute to a population PK approach. The population PK approach in humans has been found to play a pivotal role in the clinical setting by helping to build strategies for optimizing dosage regimens.<sup>29</sup> Currently, there are no guidelines on experimental design with regard to optimal subgroups, and no knowledge regarding the power necessary to detect differences in cats. However, using this approach allowed us to prioritize patient safety to obtain relevant PK parameters and to assess the variability in the target population to be treated, providing preliminary data that can be applied to future population-based approaches.

Correlation of chlorambucil PK parameters with observed AEs was outside the scope of our study, as the duration of the study would have precluded us from accounting for all AEs related to the use of chlorambucil, because some known AEs such as thrombocytopenia occur more often with prolonged therapy over months to years.<sup>6</sup> Such analyses should be considered in the future. Other limitations of our study include concurrent medications, including prednisolone treatment and cobalamin supplementation. Eighteen patients (75%) received one form of a steroid, with prednisolone being the most common. Concurrently starting prednisolone and chlorambucil or starting with prednisolone and adding chlorambucil to the treatment regimen when clinical signs are no longer responding to prednisolone treatment alone are common practices when treating indolent lymphoproliferative diseases in cats. These different treatment practices could be either clinician or client dependent. A human cancer patient chlorambucil PK study<sup>30</sup> with concurrent prednisolone use showed a similar chlorambucil peak concentration and elimination rate as previous studies without concurrent prednisolone. This would suggest that concurrent prednisolone administration may not have influenced the outcome of our study. None of the other concurrent medications given in proximity to day 0 chlorambucil dosing have been reported to alter hepatic metabolism that could potentially affect chlorambucil plasma concentrations.

Feline chronic enteropathy is a common GI disorder in elderly cats, which mainly includes idiopathic inflammatory bowel disease and LGAL, and hypocobalaminemia is a common finding in both disease entities representing malabsorption.<sup>31</sup> The GI tract's ability to absorb chlorambucil efficiently through enteral administration may be influenced by the malabsorptive nature of either LGAL or cancer-bearing cats with concurrent inflammatory bowel disease. Clinical signs in affected cats are highly variable, and severity of disease can differ greatly among cats. Although there is no validated index to help assess disease activity objectively in cats with LGAL, cobalamin deficiencies have been associated previously with disease severity and chosen in our study as a surrogate for severity of GI signs.<sup>31</sup> Four of the 24 cats in our study were determined to have hypocobalaminemia, with

cobalamin concentrations < 400 ng/L. In addition, 6 cats who were receiving cobalamin supplementation previously were determined to have hypocobalaminemia. This would indicate that nearly half the study population was documented as having or have had malabsorption deficiency along the distal GI tract. Baseline serum cobalamin concentrations were found not to be a covariate in our study. However, this may be a result in part of the inability to assign an exact value in some cats, for which values were reported simply as > 1,000 or > 3,000, instead of discrete values. It is plausible that cobalamin supplementation may have influenced the absorption of chlorambucil and affected our study positively. However, chlorambucil is known to be absorbed by passive diffusion in the GI tract,<sup>20</sup> and cobalamin supplementation may not have had any influence.

To our knowledge, our study is the first to assess the PK of chlorambucil after oral administration in cats with indolent lymphoproliferative malignancies. Our data suggest that oral chlorambucil at a dose of 2 mg administered every other day in cats undergoes rapid GI absorption and plasma clearance with no drug accumulation between doses. These data are critical to inform future work investigating the association of chlorambucil drug exposure with AEs or outcome of cats with lymphoproliferative diseases.

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The authors declare that there were no conflicts of interest.

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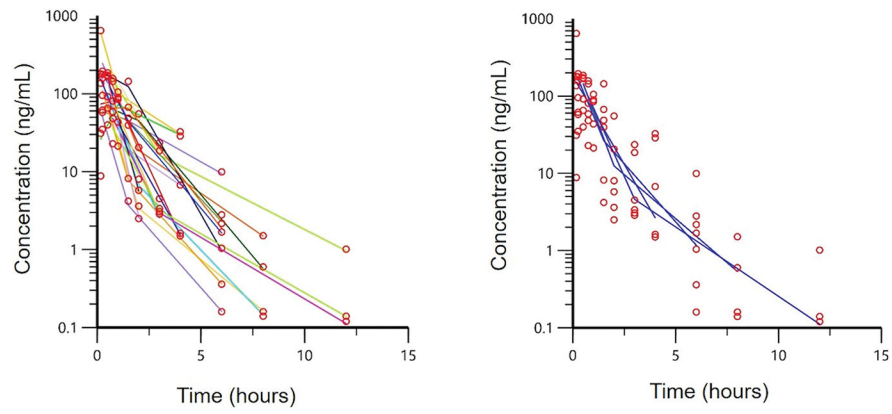
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## References

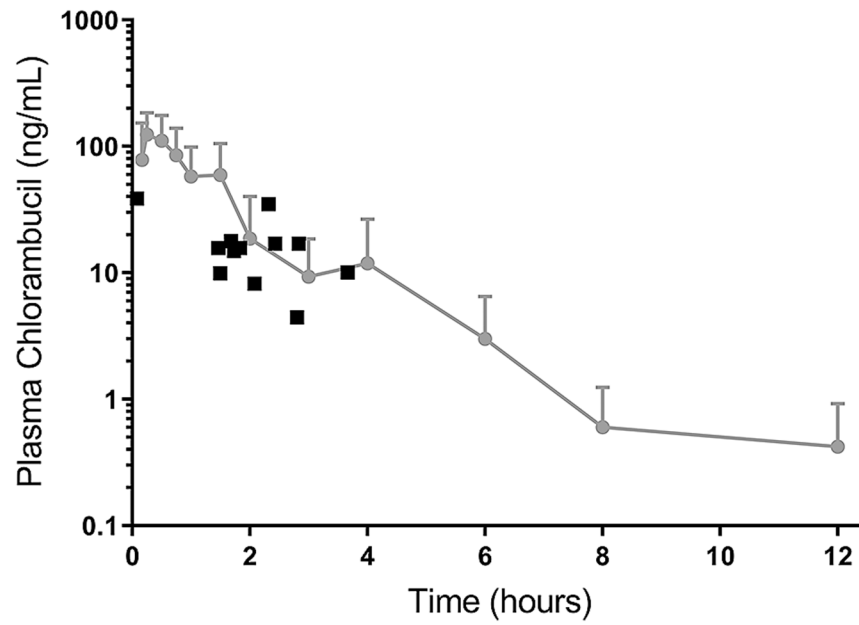
1. Kiselow MA, Rassnick KM, McDonough SP, et al. Outcome of cats with low-grade lymphocytic lymphoma: 41 cases (1995–2005). *J Am Vet Med Assoc.* 2008;232(3):405–410. doi:10.2460/javma.232.3.405 [PubMed: 18241108]
2. Pope KV, Tun AE, McNeill CJ, Brown DC, Krick EL. Outcome and toxicity assessment of feline small cell lymphoma: 56 cases (2000–2010). *Vet Med Sci.* 2015;1(2):51–62. [PubMed: 29067174]
3. Stein TJ, Pellin M, Steinberg H, Chun R. Treatment of feline gastrointestinal small-cell lymphoma with chlorambucil and glucocorticoids. *J Am Anim Hosp Assoc.* 2010;46(6):413–417. doi:10.5326/0460413 [PubMed: 21041334]
4. Fondacaro J, Richter K, Carpenter J, Hart J, Hill S, Fettman M. Feline gastrointestinal lymphoma: 67 cases (1988–1996). *Eur J Comp Gastroenterol.* 1999;4(2):5–11.
5. Campbell MW, Hess PR, Williams LE. Chronic lymphocytic leukaemia in the cat: 18 cases (2000–2010). *Vet Comp Oncol.* 2013;11(4):256–264. doi:10.1111/j.1476-5829.2011.00315.x [PubMed: 22372648]
6. Lingard AE, Briscoe K, Beatty JA, et al. Low-grade alimentary lymphoma: clinicopathological findings and response to treatment in 17 cases. *J Feline Med Surg.* 2009;11(8):692–700. doi:10.1016/j.jfms.2009.05.021 [PubMed: 19576832]

7. Paulin MV, Couronne L, Beguin J, et al. Feline low-grade alimentary lymphoma: an emerging entity and a potential animal model for human disease. *BMC Vet Res.* 2018;14(1):306. doi:10.1186/s12917-018-1635-5 [PubMed: 30305106]
8. Benitah N, de Lorimier L-P, Gaspar M, Kitchell BE. Chlorambucil-induced myoclonus in a cat with lymphoma. *J Am Anim Hosp Assoc.* 2003;39(3):283–287. [PubMed: 12755202]
9. Reinert NC, Feldman DG. Acquired Fanconi syndrome in four cats treated with chlorambucil. *J Feline Med Surg.* 2016;18(12):1034–1040. doi:10.1177/1098612X15593108 [PubMed: 26170278]
10. No authors. Veterinary Cooperative Oncology Group: common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Vet Comp Oncol.* 2016;14(4):417–446. [PubMed: 28530307]
11. Rassnick KM, Gieger TL, Williams LE, et al. Phase I evaluation of CCNU (lomustine) in tumor-bearing cats. *J Vet Intern Med* 2001;15(3):196–199. doi:10.1111/j.1939-1676.2001.tb02311.x [PubMed: 11380027]
12. Ehrsson H, Wallin I, Simonsson B, Hartvig P, Oberg G. Effect of food on pharmacokinetics of chlorambucil and its main metabolite, phenylacetic acid mustard. *Eur J Clin Pharmacol.* 1984;27(1):111–114. doi:10.1007/BF02395216 [PubMed: 6489421]
13. Mould D, Upton R. Basic concepts in population modeling, simulation, and model-based drug development—part 2: introduction to pharmacokinetic modeling methods. *CPT Pharmacometrics Syst Pharmacol.* 2013;2(4):e38. doi:10.1038/psp.2013.14 [PubMed: 23887688]
14. Alberts DS, Chang SY, Chen HS, Larcom BJ, Jones SE. Pharmacokinetics and metabolism of chlorambucil in man: a preliminary report. *Cancer Treat Rev.* 1979;6(suppl):9–17. doi:10.1016/S0305-7372(79)80005-5 [PubMed: 498174]
15. McLean A, Woods RL, Catovsky D, Farmer P. Pharmacokinetics and metabolism of chlorambucil in patients with malignant disease. *Cancer Treat Rev.* 1979;6(suppl):33–42. doi:10.1016/S0305-7372(79)80008-0 [PubMed: 498170]
16. Ehrsson H, Wallin I, Nilsson SO, Johansson B. Pharmacokinetics of chlorambucil in man after administration of the free drug and its prednisolone ester (prednimustine, Leo 1031). *Eur J Clin Pharmacol.* 1983;24(2):251–253. doi:10.1007/BF00613827 [PubMed: 6840176]
17. Newell DR, Calvert AH, Harrap KR, McElwain TJ. Studies on the pharmacokinetics of chlorambucil and prednimustine in man. *Br J Clin Pharmacol.* 1983; 15(2):253–258. doi:10.1111/j.1365-2125.1983.tb01494.x [PubMed: 6849759]
18. Hartvig P, Simonsson B, Oberg G, Wallin I, Ehrsson H. Inter- and intraindividual differences in oral chlorambucil pharmacokinetics. *Eur J Clin Pharmacol.* 1988;35(5):551–554. doi:10.1007/BF00558252 [PubMed: 3069479]
19. Oppitz MM, Musch E, Malek M, et al. Studies on the pharmacokinetics of chlorambucil and prednimustine in patients using a new high-performance liquid chromatographic assay. *Cancer Chemother Pharmacol.* 1989;23(4):208–212. doi:10.1007/BF00451643 [PubMed: 2924378]
20. Adair CG, Bridges JM, Desai ZR. Can food affect the bioavailability of chlorambucil in patients with haematological malignancies? *Cancer Chemother Pharmacol.* 1986;17(1):99–102. doi:10.1007/BF00299876 [PubMed: 3698184]
21. Hill BT, Harrap KR. The uptake and utilization of chlorambucil by lymphocytes from patients with chronic lymphocytic leukaemia. *Br J Cancer.* 1972;26(6):439–443. doi:10.1038/bjc.1972.60 [PubMed: 4647395]
22. Bank BB, Kanganis D, Liebes LF, Silber R. Chlorambucil pharmacokinetics and DNA binding in chronic lymphocytic leukemia lymphocytes. *Cancer Res.* 1989;49(3):554–559. [PubMed: 2910477]
23. Silvennoinen R, Malminiemi K, Malminiemi O, Seppälä E, Vilpo J. Pharmacokinetics of chlorambucil in patients with chronic lymphocytic leukaemia: comparison of different days, cycles and doses. *Pharmacol Toxicol.* 2000;87(5):223–228. doi:10.1034/j.1600-0773.2000.d01-78.x [PubMed: 11129502]
24. Highley MS, Landuyt B, Prenen H, Harper PG, De Bruijn EA. The nitrogen mustards. *Pharmacol Rev.* 2022;74(3):552–599. doi:10.1124/pharmrev.120.000121 [PubMed: 35710137]
25. McLean A, Newell D, Baker G, Connors T. The metabolism of chlorambucil. *Biochem Pharmacol.* 1980;29(14):2039–2047. doi:10.1016/0006-2952(80)90489-X [PubMed: 7406916]

26. Goodman GE, McLean A, Alberts DS, Chang SY. Inhibition of human tumour clonogenicity by chlorambucil and its metabolites. *Br J Cancer*. 1982;45(4):621–623. doi:10.1038/bjc.1982.101 [PubMed: 7073953]
27. Godfrey KR, Arundel PA, Dong Z, Bryant R. Modelling the double peak phenomenon in pharmacokinetics. *Comput Methods Programs Biomed*. 2011;104(2):62–69. doi:10.1016/j.cmpb.2010.03.007 [PubMed: 20381191]
28. Mitoma C, Onodera T, Takegoshi T, Thomas DW. Metabolic disposition of chlorambucil in rats. *Xenobiotica*. 1977;7(4):205–220. doi:10.3109/00498257709035779 [PubMed: 868079]
29. Guidi M, Csajka C, Buclin T. Parametric approaches in population pharmacokinetics. *J Clin Pharmacol*. 2022;62(2):125–141. doi:10.1002/jcph.1633 [PubMed: 33103774]
30. Bastholt L, Johansson CJ, Pfeiffer P, et al. A pharmacokinetic study of prednimustine as compared with prednisolone plus chlorambucil in cancer patients. *Cancer Chemother Pharmacol*. 1991;28(3):205–210. doi:10.1007/BF00685510 [PubMed: 1855277]
31. Marsilio S Feline chronic enteropathy. *J Small Anim Pract*. 2021;62(6):409–419. doi:10.1111/jsap.13332 [PubMed: 33821508]



**Figure 1—.** Plasma concentration-time plots of chlorambucil in cats after oral administration of 2 mg every 48 hours. Left—Individual cats with the model fitted to the data. Right—Cats fitted to the model after incorporation of random effects.



**Figure 2—.**

Plasma concentration-time plot of chlorambucil in cats on day 0 and day 14 of oral administration. Light-gray circles and lines represent mean plasma chlorambucil (with SD) on day 0 of dosing; black squares represent individual plasma concentrations measured on day 14 of dosing.



**Table 1—**

Serial plasma sample collection times on day 0 by group.

<b>Group</b>	<b>Sample collection times</b>
A	Baseline 10 minutes 60 minutes 4 hours
B	Baseline 15 minutes 90 minutes 6 hours
C	Baseline 30 minutes 2 hours 8 hours
D	Baseline 45 minutes 3 hours 12 hours

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**Table 2—**

Median and range of plasma chlorambucil concentrations at each time point after oral administration of a 2-mg dose in cats.

<b>Time (hours)</b>	<b>n</b>	<b>Plasma chlorambucil, ng/mL; median (range)</b>
0.167	5	34.9 (8.8–174.9)
0.250	6	126.6 (57.6–194.5)
0.5	6	91.7 (39.8–185.3)
0.75	6	69.6 (22.8–157.0)
1.0	6	64.1 (4.2–105)
1.5	6	48.6 (8.2–144.0)
2.0	6	8.0 (3.6–55.1)
3.0	6	3.9 (2.9–23.5)
4.0	6	4.2 (0.2–32.5)
6.0	6	1.9 (0.4–9.9)
8.0	5	0.4 (0–1.5)
12.0	6	0.1 (0–1.0)

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**Table 3—**

Pharmacokinetic parameters of chlorambucil in cats as determined by nonlinear mixed-effects modeling.

Parameter	Units	Estimate	SE	CV%
$\theta K_a$	1/h	5.40	2.19	40.5
$\theta V$	L	6.52	1.14	17.5
$\theta K_e$	1/h	1.84	0.25	13.6
$\theta K_{12}$	1/h	0.246	0.066	26.9
$\theta K_{21}$	1/h	0.469	0.060	12.7
AUC	ng*h/mL	166.9	23.9	14.3
$C_{max}$	ng/mL	169.1	31.1	18.4
$T_{max}$	h	0.289	0.059	20.3
Cl/F	L/h	11.9	1.7	14.3
Alpha $T_{1/2}$	h	0.322	0.044	13.6
Beta $T_{1/2}$	h	1.73	0.21	12.4

$K_a$  = absorption rate constant,  $V$  = volume,  $K_e$  = elimination rate constant,  $K_{12}$  = Transfer rate 1 to 2.  $K_{21}$  = Transfer rate 2 to 1.

Typical values for the population ( $\theta$ ) are depicted along with the percent coefficient of variation (%CV). Additional selected secondary parameters include area under the drug concentration-time curve (AUC), maximum plasma chlorambucil concentration ( $C_{max}$ ), time of maximum plasma chlorambucil concentration ( $T_{max}$ ), clearance per fraction absorbed (Cl/F), and half-lives of the initial and terminal elimination phases (Alpha  $T_{1/2}$  and Beta  $T_{1/2}$ , respectively).