Assessment of plasma uracil-to-dihydrouracil concentration ratio as an indicator of dihydropyrimidine dehydrogenase activity in clinically normal dogs and dogs with neoplasia or renal insufficiency

Chad W. Schmiedt, DVM; Corey F. Saba, DVM; Kimberly G. Freeman, MS; Gaylen L. Edwards, DVM, PhD

Objective—To determine and compare the ratio of uracil (U) to dihydrouracil (UH₂) concentrations in plasma as an indicator of dihydropyrimidine dehydrogenase activity in clinically normal dogs and dogs with neoplasia or renal insufficiency.

Animals—101 client- and shelter-owned dogs.

Procedures—Study dogs included 74 clinically normal dogs, 17 dogs with neoplasia, and 10 dogs with renal insufficiency. For each dog, a blood sample was collected into an EDTA-containing tube; plasma U and UH₂ concentrations were determined via UV high-performance liquid chromatography, and the U:UH₂ concentration ratio was calculated. Data were compared among dogs grouped on the basis of sex, clinical group assignment, reproductive status (sexually intact, spayed, or castrated), and age.

Results—Mean ± SEM U:UH₂ concentration ratio for all dogs was 1.55 ± 0.08 (median, 1.38; range, 0.4 to 7.14). In 14 (13.9%) dogs, the U:UH₂ concentration ratio was considered abnormal (i.e., > 2). Overall, mean ratio for sexually intact dogs was significantly higher than that for neutered dogs; a similar difference was apparent among males but not females. Dogs with ratios > 2 and dogs with ratios ≤ 2 did not differ significantly with regard to sex, clinical group, reproductive status, or age.

Conclusions and Clinical Relevance—Determination of the U:UH₂ concentration ratio was easy to perform. Ratios were variable among dogs, possibly suggesting differences in dihydropyrimidine dehydrogenase activity. However, studies correlating U:UH₂ concentration ratio and fluoropyrimidine antimetabolite drug tolerability are required to further evaluate the test’s validity and its appropriate use in dogs. (Am J Vet Res 2012;73:119–124)

In human medicine, 5-fluoropyrimidine antimetabolite drugs, such as 5-FU and the 5-FU prodrug capecitabine, are primarily used by oncologists to treat epithelial tumors. Although 5-FU has been used to treat cancer in dogs,1,2 the effective dosage, treatment schedule, and toxic effects in veterinary patients have not been appropriately investigated in phase I trials. Even less is known about capecitabine; its use as a cancer treatment in a dog with metastatic mesojejunoileal liposarcoma has been reported.3 Veterinary use of capecitabine has primarily been investigated as an immunosuppressant following renal transplantation in dogs.4-6 In those studies,4-6 capecitabine was effective in preventing rejection of renal allografts in dog erythrocyte antigen-mismatched mongrel dogs, but fatal, irreversible neurotoxicosis, which was potentially attributed to the drug, developed in 2 of 8 allograft recipients. This unacceptable severity of toxic effects has prohibited further clinical investigation.4-6 Because the maximally tolerated dosages of these drugs are unknown, it is plausible that some of the reported toxic effects may be alleviated with optimized, 

<table>
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<th>Abbreviations</th>
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
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<tr>
<td>DPD</td>
<td>Dihydropyrimidine dehydrogenase</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>U</td>
<td>Uracil</td>
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<td>UH₂</td>
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From the Departments of Small Animal Medicine and Surgery (Schmiedt, Saba) and Physiology and Pharmacology (Freeman, Edwards), at the College of Veterinary Medicine, University of Georgia, Athens, GA 30602.
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Address correspondence to Dr. Schmiedt (cws@uga.edu).
deficiencies in patients.15–17 Direct methods of identifying enzyme deficiencies have proven unreliable or too time-consuming to perform.9 An alternative approach has been to use an indirect measure as a surrogate estimate of DPD activity. Dihydropyrimidine dehydrogenase activity varies widely among individuals and is linked to polymorphisms in DPD genes.10–12 Influence of age or sex on DPD activity or 5-FU clearance is not entirely known, although some studies13,14 have revealed that 5-FU clearance decreases with age and, compared with findings in men, is reduced in women. Because 5-FU toxicosis can be fatal, there is considerable interest in identifying DPD deficiencies in patients.15–17 Direct methods of identifying enzyme deficiencies have proven unreliable or too time-consuming to perform.9

In humans, deficiencies in DPD can result in altered drug metabolism, accumulation of toxic concentrations of 5-FU, and serious adverse reactions, including death. It is known that DPD is widely distributed throughout the body and found in tissues such as liver, gastrointestinal mucosa, and blood mononuclear cells.9,11 Dihydropyrimidine dehydrogenase activity varies widely among individuals and is linked to polymorphisms in DPD genes.10–12 Influence of age or sex on DPD activity or 5-FU clearance is not entirely known, although some studies13,14 have revealed that 5-FU clearance decreases with age and, compared with findings in men, is reduced in women. Because 5-FU toxicosis can be fatal, there is considerable interest in identifying DPD deficiencies in patients.15–17 Direct methods of identifying enzyme deficiencies have proven unreliable or too time-consuming to perform.9 An alternative approach has been to use an indirect measure as a surrogate estimate of DPD activity. Dihydropyrimidine dehydrogenase is responsible for reduction of U to UH₂, and determination of the plasma U:UH₂ concentration ratio has been highly correlated with DPD activity in people.16–21 A high ratio represents a high plasma concentration of U, compared with that of UH₂, and suggests reduced DPD activity (ie, inadequate reduction of U to UH₂), whereas a low ratio represents adequate DPD activity (ie, sufficient DPD is present to readily reduce U to UH₂). In human medicine, a cutoff ratio > 2 has been adopted on the basis of screening of reference populations and correlation with toxic effects.9,22,23 Individuals with a ratio > 2 have reduced DPD activity; these patients have 5-FU treatment withheld or receive a modified dose.9,24

Because fluorouracil drugs will most likely be used in dogs for cancer treatment or to induce immunosuppression following renal transplantation in dogs, knowledge of potential abnormalities unique to dogs with neoplasia or renal insufficiency is required. Although evidence from human research does not suggest DPD activity will be different in tumor-bearing individuals or those with renal insufficiency, confirmation in the target species is desirable and will complement future studies of the efficacy and toxic effects of such drugs in dogs.

Therefore, the purpose of the study reported here was to determine and compare the plasma U:UH₂ concentration ratio in clinically normal dogs and dogs with neoplasia or renal insufficiency. Additionally, differences in ratios in relation to signalment data for all the study dogs were investigated. On the basis of the results of capecitabine administration as an immunosuppressant following renal transplantation in dogs,4,6 our hypothesis was that approximately 20% of all dogs undergoing assessment would have high plasma U:UH₂ concentration ratios.

**Materials and Methods**

**Dogs**—Client-owned dogs evaluated at the University of Georgia Veterinary Teaching Hospital and dogs used in veterinary student spay and castration teaching laboratories were included in the study. All animal procedures were approved by the Institutional Clinical Research Committee, and written client consent was obtained for all client-owned dogs.

Dogs were classified into 1 of 3 groups: clinically normal dogs, dogs with neoplasia, and dogs with renal insufficiency. Dogs were classified as clinically normal on the basis of historical and physical examination findings. Dogs with neoplasia were classified as such on the basis of a cytologic or histologic diagnosis of any form of cancer; however, dogs that had prior treatment with chemotherapy of any type were excluded. Dogs with renal insufficiency were classified as such on the basis of clinicopathologic findings (ie, serum creatinine concentration ≥ 2 mg/dL or a BUN concentration ≥ 35 mg/dL and urine specific gravity ≤ 1.020). Dogs with neoplasia or renal insufficiency were included in the study because these patients were considered potential recipients of fluoropyrimidine antimetabolite drugs. All dogs were also classified as juvenile (< 1 year old), adult (1 to 10 years old), and geriatric (> 10 years old).

At the time of inclusion in the study, data including age, sex, reproductive status (sexually intact, spayed, or castrated), and clinical group assignment were recorded for each dog. Approximately 2 to 4 mL of blood was collected into an EDTA-containing tube from each dog and centrifuged4 (1,489 g for 10 minutes) within 1 hour after collection. Plasma was collected and immediately frozen at –80°C until analysis.

**HPLC and determination of the plasma U:UH₂ concentration ratio**—The HPLC method used in the study was a modification of a previously described technique.23,24 Plasma samples were thawed on ice, and a 500-μL aliquot of each sample was combined with 20 μL of 5% phosphoric acid and vortexed for 5 seconds. Six milliliters of a mixture of n-propanol and ether (16:84 [vol/vol] mixture) was added to the tube containing the sample and vortexed for 5 minutes. Each sample was then centrifuged (6,000 × g) at 4°C for 15 minutes. The organic layer was carefully removed to another test tube and dried under a stream of nitrogen at 23°C. The residue was reconstituted with 100 μL of the mobile phase. Each sample was further centrifuged (24,000 × g) at 4°C to deposit any residual particulates. A final sample volume of 70 μL was injected into the HPLC system for separation and quantitation.

The HPLC system consisted of 2 pumps, an autosampler, and a UV detector. The mobile phase was an aqueous buffer of 0.05M KH₂PO₄ and 0.1% triethylamine. The mobile phase was pumped isocratically at 0.6 mL/min. The sample separation and analysis were performed at 23°C. Samples were injected onto a precolumn filter. Detection of the analytes was performed at a wavelength of 230 nm. The position and height of HPLC peaks of U and UH₂ were compared with a 6-point reference standard.
(diluted in the mobile phase) curve. Peak heights were quantified by software and determined on the basis of the mean of 4 standard curves for each set of processed samples. During each daily run, high (54 ng of U/mL and 86 ng of UH₂/mL) and low (5.4 ng of U/mL and 8.6 ng of UH₂/mL) concentration standards were prepared in a bovine serum albumin solution (80 mg/mL) to imitate the protein concentration of plasma (Figure 1). These standards in bovine serum albumin were processed in duplicate in the manner that the plasma samples were processed. These standards were processed with the sample set. Peak heights were determined and concentrations calculated by use of the standard curve as if they were unknown values. The concentrations were then compared with the known concentrations, and percentage recovery was determined. The mean percentage recovery was 90% for both U and UH₂. Values for samples were corrected for recovery before ratio calculations were made.

A U:UH₂ concentration ratio > 2 was considered abnormal. In instances where U or UH₂ was undetectable, the minimum detectable amount (0.89 ng/mL) was used for statistical analysis.

Statistical analysis—A Kolmogorov-Smirnov test was used to evaluate data for normality. Uracil-to-dihydrouracil concentration ratios were compared on the basis of sex, reproductive status (sexually intact, spayed, or castrated), age (juvenile, adult, or geriatric), and clinical group (clinically normal dogs, dogs with neoplasia, or dogs with renal insufficiency) with a 2-tailed Mann-Whitney U test or 1-way Kruskal-Wallis ANOVA on ranks. A 2-way ANOVA was used to evaluate differences in U:UH₂ concentration ratio on the basis of clinical group and reproductive status. A χ² test was used to compare dog characteristics among categories. Age was correlated with the U:UH₂ concentration ratio by use of the Spearman correlation. Simple logistic regression analysis was used to compare sex, reproductive status, age, and clinical group between dogs with U:UH₂ concentration ratios > 2 to those with ratios ≤ 2. Data are reported as mean ± SEM, median, and range. Values of P ≤ 0.05 were considered significant. Statistical analysis was performed with computer software.

Results

One hundred one dogs were included in the study; other dogs were excluded from the study but the number and reasons for exclusion were not recorded. Dogs included in the study were classified on the basis of reproductive status, age, and clinical group assignment (Table 1). Mean ± SEM age of all dogs was 4.37 ± 0.40 years (median, 3.0 years; range, 0.3 to 14 years). Age and repro-
Plasma U:UH₂ concentration ratios were determined for all dogs overall (Figure 2) and for dogs in each clinical group (Table 3). Among all 101 dogs, 14 (13.9%) had U:UH₂ concentration ratios > 2, which were considered abnormal values. Of these dogs with abnormal ratios, the mean U:UH₂ concentration ratio was 3.09 ± 0.35 (median, 2.69; range, 2.15 to 7.14).

In the dogs with renal insufficiency, mean BUN concentration was 108.4 ± 23.1 mg/dL (median, 114 mg/dL; range, 19 to 274 mg/dL), mean serum creatinine concentration was 6.96 ± 2.43 mg/dL (median, 4.2 mg/dL; range, 1.6 to 26.6 mg/dL), and mean urine specific gravity was 1.015 ± 0.000 (median, 1.014; range, 1.011 to 1.019). In 3 dogs, clinical signs were consistent with acute renal disease, and in the remaining 7 dogs, clinical signs were consistent with chronic renal disease.

Results of a 1-way ANOVA revealed that the plasma U:UH₂ concentration ratio was significantly less in dogs with neoplasia, compared with the value in the clinically normal dogs. However, results of a 2-way ANOVA, which included both clinical group and reproductive status as factors, were not significant.
status, revealed that there was no significant (\( P = 0.49 \)) difference in the U:UH \(_2\) concentration ratio among the 6 classification groups that were based on the combination of those factors.

Further analysis revealed that there was no difference in plasma U:UH \(_2\) concentration ratio between sexes (male vs female) and no association between U:UH \(_2\) concentration ratio and age. However, neutered (spayed or castrated) dogs had a significantly (\( P = 0.01 \)) lower mean U:UH \(_2\) concentration ratio than that for sexually intact dogs (1.33 ± 0.13 and 1.65 ± 0.10, respectively; Figure 3). When reproductive status was compared within sexes, castrated males had a significantly (\( P = 0.02 \)) lower mean U:UH \(_2\) concentration ratio than that for sexually intact males (1.17 ± 0.1 and 1.70 ± 0.15, respectively). However, there was no difference in U:UH \(_2\) concentration ratio between spayed (1.44 ± 0.21) and sexually intact (1.38 ± 0.12) females (\( P = 0.19 \)).

Logistic regression analysis was used to compare data for the 14 dogs with plasma U:UH \(_2\) concentration ratios \( > 2 \) and the 87 dogs with ratios \( \leq 2 \). These 2 subsets of dogs did not differ significantly with respect to age, sex, reproductive status, or clinical group.

**Discussion**

Results of the present study have indicated the feasibility of determining the plasma concentrations of U and UH \(_2\) by use of HPLC and calculation of the U:UH \(_2\) concentration ratio in dogs. Collection of blood samples was simple and safe, and analysis was easy to perform with appropriate equipment and expertise. Mean and median plasma U:UH \(_2\) concentration ratios in all dogs were 1.55 and 1.38, respectively, comparable to reported mean and median ratios for humans (1.49 and 1.5, respectively). Approximately 14% of all 101 dogs in the present study had an abnormally high U:UH \(_2\) concentration ratio (ie \( > 2 \)), suggesting that DPD deficiency is present in a small but not negligible percentage of the canine population.

Definitive correlations of the plasma U:UH \(_2\) concentration ratio with DPD activity and 5-fluoropyrimidine antimetabolite tolerability in dogs are needed. In humans, these relationships are strong and consistent, but similar studies in dogs, including confirmation that a U:UH \(_2\) concentration ratio of 2 is an appropriate cut-off value, are necessary. If a dog has a U:UH \(_2\) concentration ratio \( > 2 \), there may still be benefit in treating that individual with a modified dose of 5-FU. Results of a study\(^{25}\) in humans indicated that DPD-based adaptive dosing reduced the incidence of 5-FU-induced toxicoses, allowing for optimized treatment administration. In that study,\(^{25}\) patients with a plasma U:UH \(_2\) concentration ratio of 2.1 to 3 were classified as having mild DPD deficiency and received a 5-FU dosage reduction of 10% to 20%; patients with a U:UH \(_2\) concentration ratio of 3.1 to 5 were classified as having severe DPD deficiency and received a dosage reduction of 20% to 50%, and patients with a U:UH \(_2\) concentration ratio \( > 5.1 \) were classified as having extremely severe DPD deficiency and received a dosage reduction of 50%.\(^{14}\)

In the present study, the plasma U:UH \(_2\) concentration ratio only differed significantly among dogs grouped on the basis of reproductive status. Overall, the mean ratio for sexually intact dogs was significantly higher than that for neutered dogs; although a similar difference was apparent among males, no such difference was identified for females. In the small number of dogs with ratios \( > 2 \), being sexually intact was not identified as a predictive risk factor. Assuming that the plasma U:UH \(_2\) concentration ratio is inversely correlated with DPD activity, as it is in people,\(^{16–20}\) these data suggest that sexually intact dogs may have reduced DPD function. However, whether being sexually intact increases the U:UH \(_2\) concentration ratio within the range that can be considered normal or predisposes to high values outside the normal range remains unknown. The reason for the increase in U:UH \(_2\) concentration ratio in sexually intact dogs has not been determined. Interestingly, in mice, castration resulted in increased susceptibility to toxic effects (leucopenia and death) of 5-FU and treatment with testosterone reversed those effects.\(^{21}\) Dihydropyrimidine dehydrogenase function was not considered in that study,\(^{22}\) but the authors postulated that at least some of the effect was a result of testosterone-mediated stimulation of the bone marrow.

In addition to use of the plasma U:UH \(_2\) concentration ratio to estimate DPD activity, the application of other assays for the detection of DPD deficiency have been reported and compared.\(^{9}\) Genotyping has been used to detect polymorphisms in DPYD, the gene which encodes for DPD. There are at least 30 known variants of DPYD. However, genetic variability does not always correlate with reduced function;\(^{2}\) this assay is cumbersome and costly, and relevant mutations have not been identified in dogs. Quantification of DPD mRNA in circulating lymphocytes by use of a real-time reverse-transcription quantitative PCR assay has also been reported. However, in people, no correlation of the amount of DPD mRNA to DPD function or drug-induced toxicoses has been found.\(^{8}\) An indirect radioenzymatic assay of DPD activity in blood mononuclear cells has been described and is an accepted method of identifying DPD-deficient patients; however, the assay is complicated and large volumes of blood are required.\(^{28}\) Identification of abnormally high plasma or urine U concentration has not proven to be an accurate predictor of abnormal pyrimidine metabolism.\(^{8}\) Uracil concentrations may be affected by nutrition or non-pathological disturbances in pyrimidine production and evaluation of those values in absence of UH \(_2\) data may not be reliable. Measurement of the plasma U:UH \(_2\) concentration ratio allows quantification of DPD activity independent of such factors,\(^{8}\) and the ratio has been found to be highly correlated with DPD activity, 5-FU plasma clearance, and toxic events in people.\(^{16–20}\)

Studies in which DPD activity in dogs has been assessed are sparse; to our knowledge, this is the first investigation (albeit indirect) of DPD activity in healthy and ill dogs. Comparison of hepatic cytosol DPD activity among several species, including humans and dogs, has been undertaken and revealed interspecies variability.\(^{8}\) However, canine DPD activity compared more favorably with human DPD activity than with activities in other evaluated species, suggesting that data from studies, clinical or otherwise, of DPD function or DPD-
dependent drugs in dogs may translate well to human medicine and vice versa.

One limitation of the present study was the high proportion of sexually intact dogs (n = 62), compared to neutered dogs (31); this imbalance of the numbers of sexually intact and neutered dogs was especially evident when compared between and within each clinical group. This may have caused type I error in the data analyses, inaccurately suggesting a difference in U:UH2 concentration ratios on the basis of reproductive status. Most of the clinically normal dogs were from shelters and were being used for veterinary student spay and neuter teaching laboratories; therefore, they were still sexually intact at the time of study enrollment. However, when the population is considered en toto, we believe sufficient numbers of spayed or castrated dogs were included to conduct appropriate data analysis.

Without doubt, determination of plasma U:UH2 concentration ratios in dogs is feasible but more studies are required to definitively associate this ratio with concentration ratios in dogs. When the population is considered en toto, we believe sufficient numbers of spayed or castrated dogs were included to conduct appropriate data analysis. Nevertheless, sexually intact dogs appeared to have higher but not necessarily abnormal ratios. Until a screening test to accurately measure DPD activity is validated in dogs, clinicians should use caution when administering 5-fluoropyrimidine antimetabolite drugs, especially capicitabine, to dogs.

References


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