Published in final edited form as:

J Feline Med Surg. 2018 February ; 20(2): 83–90. doi:10.1177/1098612X17693523.

Previously Undescribed Vitamin D C-3 Epimer Occurs in Substantial Amounts in the Blood of Cats

MC Sprinkle¹, SE Hooper², and RC Backus¹

¹Department of Veterinary Medicine & Surgery, University of Missouri, Columbia, MO, USA

²Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, USA

Abstract

Objectives—The aim of this report is to describe the identification of a novel vitamin D metabolite, a 3-C, β -epimer of 25-hydroxycholecalciferol [3-epi-25(OH)D₃], in serum and plasma extracts of cat blood and compare its abundance in cat, dog, and rat serum to 25-hydroxycholecalciferol [25(OH)D₃], a conventional marker of vitamin D status.

Methods—Serum 25(OH)D₃ and 3-epi-25(OH)D₃ concentrations were measured in healthy cohorts of cats (n = 8), dogs (n = 8), and rats (n = 17) using validated reverse and normal-phase high-performance liquid chromatography (HPLC) methods. The methods were verified using liquid chromatography-tandem mass spectrophotometry (LC-MS/MS). Dietary intake and dietary concentrations of vitamin D were also measured for evaluation of species differences and effect of dietary change on vitamin D metabolite concentrations. Differences between cat serum and plasma metabolite concentrations were determined.

Results—Detectable concentrations of 3-epi-25(OH)D₃ were observed in all cats and rats. No 3-epi-25(OH)D₃ was detected in dogs, where our limit of detection was 5 ng/mL. There were differences (P < 0.05) in serum concentrations of 25(OH)D₃ and 3-epi-25(OH)D₃ among species with cats having the greatest concentrations of both metabolites. Serum and plasma results were not significantly different. A diet change, which resulted in an increase in vitamin D intake among the cats, affected serum concentration with an increase (P = 0.004) in 3-epi-25(OH)D₃, but no significant change in 25(OH)D₃.

Conclusions and relevance—Serum and plasma of cats contain 3-epi-25(OH)D₃ in varied and extraordinary concentrations, much greater than in rats and certainly than that of dogs, a species for which the metabolite was not detected. Importantly, this finding indicates a C-3 epimerization pathway is quantitatively significant for vitamin D metabolism in domestic cats, making 3-epi-25(OH)D₃ assays essential for the evaluation of vitamin D status in cats and positioning the cat as a novel model for study of this pathway.

Corresponding author: Megan Sprinkle DVM, Veterinary Health Center, 900 East Campus Drive, University of Missouri, Columbia, MO, USA. sprinklem@missouri.edu. Phone: (256)566-0823.

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Keywords

Feline; Diet; Vitamin D; Chromatograph; High Pressure Liquid; HPLC; Tandem Mass Spectrometry; LC-MS; serum

Introduction

Vitamin D is an essential nutrient that has diverse physiological roles in people and animals well beyond recognized functions in mineral metabolism and skeletal health.^{1, 2} Therefore, food fortification of vitamin D is an important health issue for people and companion animals. Dietary fortification is especially important for cats since cats rely solely on dietary sources of vitamin D. Vitamin D produced in skin as a result of ultraviolet radiation exposure is negligible in cats,³ presenting cats as an attractive model in which to efficiently evaluate relationships between vitamin D and health. Low vitamin D status in cats, as determined from measured serum 25-hydroxycholecalciferol [25(OH)D₃] concentrations, has been observed in cats with inflammatory bowel disease, intestinal small cell lymphoma, and infections.^{4,5} A recent study also found serum 25(OH)D₃ measurements were a predictor of mortality in hospitalized cats.⁶ Despite the recent increase in research investigating the relationship between vitamin D metabolite concentrations and health, comparatively little is known about vitamin D functionality and metabolism in cats.

Dietary vitamin D can be acquired through ingestion of two chemical forms: cholecalciferol (vitamin D₃) and ergocalciferol (vitamin D₂). The diet of companion animals contains almost exclusively vitamin D₃, the form of vitamin D synthesized from 7-dehydrocholesterol in the skin of some animals and subsequently transported to the liver. Vitamin D₂ is synthesized in plants from ergosterol by ultraviolet radiation and may be found in animal tissue in proportion to that animal's dietary intake. The bioavailability of vitamin D₂ as compared with vitamin D₃ is reportedly lower in cats as compared with other species, with the bioavailability difference of the vitamin D forms appearing to depend on the mode and matrix of administration. Cats given a diet containing equal concentrations of vitamin D₃ and D₂ used vitamin D₂ with 31% less efficiency for production of the 25-hydroxyvitamin D metabolites.⁷ In clinical nutrition, supplemental vitamin D₂ is sometimes utilized in place of D₃ during dietary antigen elimination trials to avoid animal sources of vitamin D (ie, wool lanoline). For this and other reasons, a vitamin-mineral mix for balancing home-prepared diets, used widely by veterinary clinical nutritionists, underwent a formulation change including the substitution of vitamin D₃ with vitamin D₂ (Balance IT Feline, DVM Consulting, Inc., Davis, CA).

Given the reputed bioavailability differences of vitamin D forms and the increasing use of vitamin D₂, we began researching potential effects of the dietary matrix on vitamin D₂ bioavailability in cats. A methodological difficulty encountered early in this undertaking serendipitously revealed substantial occurrence of a serum vitamin D metabolite that to our knowledge has not been previously reported in cats. The present report describes the identification of the metabolite as a 3-C epimer and its abundance in cats as compared with dogs and rats.

Materials and methods

Animals

All animals were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Care and Use Committee of the University of Missouri.

Serum from jugular venous blood was analyzed from eight university-owned purpose-bred healthy male neutered domestic shorthair cats, approximately 5–10 years of age and lean body condition (ie, 5 on a scale of 1 to 9). The cats were group-housed except for daily periods (six to eight hours) of single housing for monitoring of food intake. Water was continuously available. Body weights were recorded weekly.

Serum from rats and dogs was also collected for determination of vitamin D metabolite concentrations. The rats were obtained from another University investigator whom had completed a study and intended to euthanize them. The rats were wild-type Wistar females (n = 8, 4–5 months of age, Charles River Laboratories, Raleigh, NC, USA) and selectively bred high-runner Wistar males⁸ (n = 9, 3 months old). For 24 days prior to euthanasia, the rats were socially housed in pairs except for four hours of individual housing during diet availability to allow for the collection of diet intake data. Body weights were monitored every two days to ensure weight stability. Two rats could not maintain body weight on this schedule, so the duration of available diet was extended to eight to nine hours to maintain body weights of these rats.

Female (n = 3) and male (n = 5) intact purpose-bred healthy adult (5-year-old) Chinese-Crested/Beagle dogs of ideal body conditions and stable body weights (6–11 kg) were also included in the study. The dogs were housed individually during diet presentation, walked daily for socialization, and weighed weekly.

Diets

The cats exclusively consumed an extruded, dry-expanded commercial diet (“diet A”. Pro Plan FOCUS Adult Urinary Tract Health Formula, Nestle Purina, St Louis, MO, USA) for more than three years in amounts that maintained their body weights (4.3–7.0 kg) at healthy body conditions prior to initial blood collection for vitamin D metabolite analysis. Blood of seven of the eight cats was sampled again after the cats were transitioned to and maintained on a second extruded commercial diet (“diet B”. ProPlan FOCUS Adult Weight Management Formula canine, Nestle Purina, St Louis, MO, USA) supplemented with taurine to 0.7%. Though marketed as a canine product, the nutrient profile of the second diet met or exceeded the profile recommendations for maintenance of adult cats by the Association of Animal Feed Control Officials (2016).⁹ The cats readily consumed this diet and maintained their body weights for more than six weeks before blood was collected for vitamin D metabolite analysis.

Life-long, the rats consumed an extruded pellet diet (Laboratory Rodent Diet 5001, LabDiets, St Louis, MO, USA). Daily dietary intake was recorded for the cats and rats by

calculating the difference between the weighed food offered and food unconsumed after each daily feeding period.

At the time of blood sampling, the dogs had exclusively consumed an extruded, dry-expanded commercial diet (Laboratory Canine Diet 5006, LabDiet, St Louis, MO, USA) in amounts sufficient to maintain stable body weights and ideal body conditions. Diet intake data were unavailable.

Based on manufacturer claims and/or reported nutrient contents all diets were assumed to be nutritionally complete and balanced for the maintenance condition. The vitamin D content of these diets was determined by a commercial laboratory (Official Methods of Analysis of AOAC INTERNATIONAL, Current Ed., Method 2011.11, AOAC INTERNATIONAL, Gaithersburg, MD, USA).¹⁰

Blood sampling

Jugular venepuncture with 22 gauge needles and 5 ml syringes was utilized for blood collection (5mL) in the cats and dogs after an overnight food withholding. Also after overnight food withholding, the rats were exsanguinated under deep anesthesia using cardiac puncture immediately prior to their euthanasia. All blood samples were transferred into 10 mL BD Vacutainer Red-Top collection tubes and centrifuged at 2000g for 15 min at 4°C. Serum was collected and stored at -20°C.

Experimental Design

Several experiments were conducted to quantify serum vitamin D metabolites.

Experiment 1 – HPLC quantification of vitamin D metabolites—Serum concentrations of 25(OH)D₃, 25-hydroxyergocalciferol [25(OH)D₂], and the C-3,β-epimer of 25(OH)D₃ [3-epi-25(OH)D₃] in cats, rats, and dogs were determined using a previously published high-performance liquid chromatography (HPLC) method.¹¹ In brief, 1.0 mL of serum from each animal was extracted with an equal volume of acetonitrile (CH₃CN) containing internal standard (400mg/L, 1-phenyl-1-dodecanone, Sigma-Aldrich, St Louis, MO). The CH₃CN extract was diluted with two volumes of water and loaded on a conditioned solid-phase extraction (SPE) column (Strata-X 33u, 60mg, Phenomenex, Torrance, CA). After washing with 2 mL of CH₃CN-water (35:65 by volume), vitamin D metabolites were eluted with 2 mL of CH₃CN from the SPE columns and dried by centrifugal evaporation for 70 minutes. Sample residues were reconstituted with 75 μL of ethyl acetate-CH₃CN (5:95 by vol.) and diluted with 55 μL of water. A portion (100 μL) was injected on an equilibrated (1.0 mL/min methanol-water [67:33 by volume]), heated (50°C) stable-bond, cyanopropyl, analytical HPLC column [250 × 4.6 mm (i.d.) Zorbax, 5 μm, SB-CN, 80Å, Agilent Technologies, Santa Clara, CA]. Area-under-the-curve (AUC) of standard peaks, which was proportional to concentrations of 25(OH)D₃ (Cerillant, Round Rock, TX, USA) and 3-epi-25(OH)D₃ (IsoSciences, Prussia, PA, USA) peaks were observed and linearly correlated with injected mass in the range of 3–10 ng. Linearity and sensitivity of the HPLC method was determined using a 4-fold dilution series of 25(OH)D₃ (50 ng/mL, 25 ng/mL, 10 ng/mL, and 5 ng/mL).

Identities of the apparent 25(OH)D₃ and 3-epi-25(OH)D₃ peaks in the feline and rat samples were verified by collecting 2-minute mobile phase fractions coinciding with elution times of standards for 25(OH)D₃, 25(OH)D₂ (Santa Cruz Biotechnology, Dallas, TX, USA) and 3-epi-25(OH)D₃ (IsoSciences, Prussia, PA). These fractions were dried with centrifugal evaporation then reconstituted with 150 µL of a second HPLC mobile phase (hexanes-isopropanol-methanol, 88:10:2 by vol). Thereafter, 100 µL of the reconstitute was injected into a silica, analytical, HPLC column [Zorbax-SIL column (4.6 × 250 nm), Dupont Instruments, Wilmington, DE, USA] equilibrated with the mobile phase (1.0 mL/min). The ultraviolet spectra of sample peaks eluting at the retention time of 3-epi-25(OH)D₃ was determined with a photodiode array detector (996, Waters, Milford, MA, USA).

Experiment 2 – Verification of vitamin D epimer metabolites with LC-MS—The concentrations of 25(OH)D₃ and 3-epi-25(OH)D₃ in serum evaluated in Experiment 1 were determined by a liquid chromatography-tandem mass spectrophotometry (LC-MS) by Mayo Laboratories (Rochester, Minnesota) utilizing a validated method that detects C-3 metabolites.¹²

Experiment 3 – Dietary change effect on serum and plasma vitamin D metabolites—Blood sampling of the cats was repeated after a diet change and exclusive consumption of diet B for six weeks. A portion of blood from each cat (~1.5mL) was added to a tube containing lithium heparin (56 USP units, D Vacutainer, Franklin Lakes, NJ, USA). Serum and plasma were then extracted from the blood samples and stored at -20°C until later determinations of vitamin D metabolite concentrations by the reverse-phase HPLC analysis method described in Experiment 1.

Statistical Methods

Analyses were performed using proprietary statistical software (Excel 2016, Microsoft; SAS® 9.3, SAS Institute, Cary, NC, USA); a *P* value of <0.05 was considered significant. Percentage concentrations of 3-epi-25(OH)D₃ to total 25(OH)D₃ concentration was calculated for each animal. Serum and plasma concentrations of 25(OH)D₃ and 3-epi-25(OH)D₃ in samples from the cats, dogs, and rats were assessed for normality using four different statistical tests: Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling. Because all observations were normally distributed, central tendency and variance of the observations are presented as mean and ± SD, respectively, unless otherwise stated. The significance of differences in variable observations between species was determined using an ANOVA. Student's *t*-tests were used to determine the significance of differences in 3-epi-25(OH)D₃ concentrations between the rat and cat samples and gender differences among samples from rats and dogs. Paired-*t* tests were used for determining significance of differences between HPLC and LC-MS feline vitamin D metabolite data and between the cat metabolite data after change in diet. Linear regression analysis was performed to determine the significance of variable correlations between analytical methods.

Results

Experiment 1 – HPLC quantification of vitamin D metabolites in cats, rats, and dogs

The SPE extracts of all species samples used for quantification of vitamin D metabolites by reverse-phase analytical HPLC had clear, identifiable chromatographic peaks at retention times of standards for 25(OH)D₃ (~15 minutes) (Figure 1). A chromatographic peak at the retention time of 25(OH)D₂ was not observed in any cat, dog, or rat sample. A substantial peak eluting at the retention time of 3-epi-25(OH)D₃ was found soon after the apparent 25(OH)D₃ peak in all feline and rat samples, but not observed in the dog samples. The apparent 3-epi-25(OH)D₃ peak was not resolvable from 25(OH)D₂ because it occurred at the elution time of the leading shoulder of 25(OH)D₂ standard. A normal-phase chromatogram of the pooled injected fraction revealed peaks eluting at the retention times of 25(OH)D₃ and 3-epi-25(OH)D₃ standards. The peaks had “cis-triene” UV-spectral profiles characteristic of vitamin D structure (ie, maxima and minima at 266 and 228 nm, respectively).¹³ No peak was found to elute at the retention time of 25(OH)D₂ with reverse-phase HPLC. Together these observations indicated that the unidentified peak in all feline serum samples was likely the C-3 epimer of 25(OH)D₃.

Experiment 2 – Verification of vitamin D metabolites with LC-MS

Among the cat samples, a significant positive correlation was found between the HPLC and LC-MS derived values for serum concentrations of 25(OH)D₃ ($r^2 = 0.859$, $P < 0.0001$), but not for the serum concentrations of 3-epi-25(OH)D₃, for which only a non-significant, positive trend was found ($r^2 = 0.469$, $P = 0.061$) (Figure 2). The HPLC-derived values were greater than the LC-MS-derived values by means ($\pm$ SEMs) of $28 \pm 5\%$ for 25(OH)D₃. ($P < 0.001$) and $13 \pm 4\%$ for 3-epi-25(OH)D₃ ($P < 0.037$) (Table 1).

Species differences in serum concentrations of 25(OH)D₃ as determined by the reverse-phase HPLC were significant ($P < 0.0001$) (Table 1). Among dogs, a mean ($\pm$ SEM) serum 25(OH)D₃ concentration of 23.0 ± 4.1 ng/mL was observed. The 25(OH)D₃ concentrations were less ($P < 0.0003$) than those found among the cats (Table 1). Female dogs compared to males had higher ($P = 0.01$) serum 25(OH)D₃ concentrations by a mean of 30.2%. In contrast, the 25(OH)D₃ concentrations among the rats were greater ($P < 0.001$) in males than females. The 25(OH)D₃ concentrations of the rats were the lowest ($P < 0.05$) among the species. There was a much higher concentration of the serum C-3 epimer of 25(OH)D₃ in cats as compared with rats ($P < 0.001$) and a greater ($P < 0.0004$) concentration of 3-epi-25(OH)D₃ in relation to 25(OH)D₃ in cats as compared to rats (Table 1). The mean ratio of C-3 epimer to 25(OH)D₃ among cats was more than twice that observed in rats.

Experiment 3 - repeatability and relationship of dietary change

Vitamin D metabolite concentrations were determined in serum and plasma aliquots obtained from all but two cats. Data from one cat was excluded because it was not transitioned to the same diet as the other cats. Data from another was unavailable due to a technical error during analysis. No significant difference was found between serum and plasma results of HPLC analyses for concentrations of 25(OH)D₃ ($P = 0.655$) or 3-

epi-25(OH)D₃ (P = 0.300) (Table 2). Occurrence of 25(OH)D₂ was not detected in any of these samples.

Serum 25(OH)D₃ concentrations among the cats were not significantly (P = 0.232) different after feeding diet B. However, much greater (P = 0.004) serum concentrations of 3-epi-25(OH)D₃ were observed after feeding the second diet (Table 2). The cats' serum concentrations of 3-epi-25(OH)D₃ were not significantly correlated with serum concentrations of 25(OH)D₃ on either diet.

Analyses of the cats' diets revealed that diet B as compared with diet A had a higher concentration of vitamin D₃, while both diets contained no detectible vitamin D₂, where the limit of detection was reported as 0.02 IU D₂/g (Table 3). From food intake observations, it was determined that energy intakes of the cats were not significantly different between diets (P = 0.013). By combining the food intake and dietary vitamin D concentrations, it was determined that each cat consuming diet B had more than four times the intake of vitamin D₃ per day per kg body weight than when they were consuming diet A (Table 3).

Discussion

The main finding of this study is that a C-3 epimeric form of 25(OH)D₃ occurs in the blood of cats in amounts, that to our knowledge, greater than those reported in the blood of other species.^{14, 15} Various means of study have demonstrated synthesis of C-3 epimeric forms of 25(OH)D₃ and other vitamin D metabolites in cell cultures.¹⁶ These investigations have not fully elucidated tissue sources of the C-3 epimers, but they point to a unique C-3 epimerization pathway that variably occurs in multiple tissues. Findings of the present study indicate that a C-3 epimerization pathway is quantitatively significant for vitamin D metabolism in domestic cats, and that its activity may change because the epimer concentrations were observed to vary among individuals and diets (Table 1). Our analytical methods showed detectable levels of the C-3 epimer in all of cats and rats, but none of the dogs, where our limit of detection was 5 ng/mL (Figure 3). These clearly detectable and wide ranges of C-3 epimer concentrations in the cats are similar findings in adult populations of people.¹⁶ The reverse-phase HPLC method upon which we based our analyses was previously used by investigators to detect the C3-epimer in 99% of their surveyed human being population ranging in age of 1–80+ years. Among those evaluated, a mean 3-epi-25(OH)D₃ concentration of 3.75 nmol/L (1.5 ng/mL) was reported, while the C-3 epimer concentrations ranged from 0.25 to 59.25 nmol/L (0–23.7 ng/mL) and represented up to 25.5% of the total 25(OH)D₃ concentration.¹⁴

The discovery that vitamin D₃ in people can undergo an alternative, unidirectional C-3 epimerization metabolism is a recent finding. While epimerization is an important chemical process for regulating certain steroid hormones, the biological significance of C-3 epimerization of vitamin D has yet to be determined.¹⁵ The limited number of studies investigating the function of the epimerization pathway report that the 3-epi-1,25-dihydroxyvitamin D [3-epi-1,25(OH)₂D] metabolite can bind to the vitamin D receptor (VDR), but with reduced biological activity as compared to its non-epimeric counterpart, calcitriol.¹⁷ Some of these studies further show that 3-epi-1,25(OH)₂D possesses regulatory

effects including anti-proliferative and differentiation activity.¹⁶ The epimer has also been documented to suppress parathyroid hormone secretion equally to the non-epimeric form, but without inducing other classical calcemic effects.¹⁸ Whether 3-epi-1,25-dihydroxyvitamin D₃ occurs in detectable concentrations in cats has yet to be determined. Cats might synthesize 3-epi-1,25-dihydroxyvitamin D₃ from calcitriol or from the abundance of 3-epi-25(OH)D₃ in circulation that we identified in this report. Investigators using recombinant *Escherichia coli* cell systems reported that 3-epi-25(OH)D₃ may be C-1- α -hydroxylated by CYP27B1 to form 3-epi-1,25(OH)₂D₃.¹⁹ Due to possible clinical relevance and the controversies in the correlation of vitamin D on non-calcemic outcomes such as cancer, diabetes, and autoimmune disorders, the discovery of a vitamin D C-3 epimeric pathway in cats is intriguing.

Since the recognition of the epimeric form of 25(OH)D₃ in blood samples of people, analytical chemists have continued to attempt to produce a standardized quantification method for specific vitamin D metabolites.¹⁶ Therefore, it is not surprising for us to have seen a modest variance in our HPLC and LC-MS results (Figure 2). Previous studies performed by the authors have found a similar discrepancy in serum 25(OH)D₃ concentrations in dogs between different analytical methods.²⁰ Utilizing both the HPLC and LC-MS methods allowed verification that the C-3 epimer peak was real and not an artifact of analytical processing, but it also impressed upon us a need for standardization of vitamin D analytical methodology, especially in cats which appear to have substantial concentrations of the C-3 epimer.

In 2011, the International Vitamin D External Quality Assessment Scheme evaluated 14 different methods for their ability to detect the C-3 epimeric and non-epimeric forms of 25(OH)D₃.¹⁶ While HPLC and LC-MS were counted among the five suitably accurate testing methods, Liaison 25 OH Vitamin D Total Assay (DiaSorin, Saluggia, Italy), a commonly used method in analyzing companion animal 25(OH)D concentrations, was among the tests that could not detect the epimer. If 3-epi-25(OH)D₃ has different functional properties from 25(OH)D₃ in cats, the use of analytical methods that do not detect or distinguish the epimer from the native metabolite may misrepresent vitamin D status in domestic cats. Use of such analytical methods could underlie or add to confusion surrounding relationships between circulating apparent 25(OH)D concentrations and clinical outcomes.

Previous studies have suggested that the C-3 epimer of 25(OH)D₃ is produced primarily endogenously, and its production is dependent upon dietary vitamin D intake.¹⁵ Our study showed that despite lower dietary consumption of vitamin D on an IU/kg body weight basis, cats maintained higher serum concentrations of both 25(OH)D₃ and 3-epi-25(OH)D₃ metabolites as compared with rats (Table 1). This species difference could be attributed to dietary factors such as differences in dietary matrices or nutrient bioavailabilities and/or attributed to animal factors such as differences of endogenous vitamin D metabolism or clearance. The significantly higher 3-C epimer concentrations in cat serum, could reflect that domestic cats, compared with rats, utilize the C-3 epimeric pathways more than the traditionally recognized non-epimeric pathways for vitamin D metabolism. Serum concentrations of 25(OH)D₃ were relatively stable within each cat despite increased

ingestion of vitamin D through consumption of diet B. In contrast, with ingestion of the second diet, serum concentrations of 3-epi-25(OH)D₃ were markedly increased among the cats. A readily utilized “detoxification” mechanism could explain why cats show a resistance to vitamin D toxicity. In a study that supplemented dietary vitamin D₃ at 63-times the amount recommended by the National Research Council (NRC, 2006),²¹ after 18 months, cats did not show signs of vitamin D toxicosis despite having a mean ($\pm$ SEM) serum 25(OH)D concentration of $1,071.9 \pm 115.3$ nmol/L (429.4 ng/mL).²² Investigators of the study concluded that cats are very tolerant of high plasma concentrations of 25(OH)D when fed an otherwise nutritionally complete and balanced diet. With respect to 3-epi-25(OH)D₃ as a possible detoxification product, it is important to note that the investigators of that study measured plasma 25(OH)D concentrations by a protein-binding assay, a technique that likely did not distinguish 25(OH)D from its C-3 epimer. In contrast, dogs are more susceptible to hypervitaminosis D where the safe upper limit recommended by the NRC for dietary vitamin D is 2.6 μ g/kg BW^{0.75} or 5.8-times the recommended allowance.²¹

While the C-3 epimer was not detected in canine samples, it does not eliminate the possibility that dogs are capable of utilizing the epimeric pathway. Most survey studies in people do not find detectable serum concentrations of 3-epi-25(OH)D₃ in all participants.¹⁶ If 3-epi-25(OH)D₃ is a means of removal of excess 25(OH)D₃, dogs may preferentially use the conventionally recognized metabolism of 25(OH)D to 24,25-dihydroxyvitamin D [24,25(OH)D] for vitamin D clearance. Circulating concentrations of 24,25(OH)D₃, are reportedly higher in dogs than in people, pigs, and rodents.² We were unable to find reports of 24,25(OH)D₃ concentrations in cats. One might expect serum 24,25(OH)D₃ concentrations to be low in cats if production of 3-epi-25(OH)D₃ is a principal means of 25(OH)D₃ clearance for the species.

Our study found significantly higher 25(OH)D₃ concentrations in female dogs and male rats as compared with the opposing gender. Most studies do not observe a gender difference in vitamin D metabolites, but few studies in dogs and people compare populations consuming identical diets as in our study. In 2014, Bianchini et al¹⁵ studied 36 female and 36 male rats, fed a single diet. They observed no gender difference in initial 25(OH)D₃ concentrations, but female rats had a more prominent serum 25(OH)D₃ and 3-epi-25(OH)D₃ response when supplemented with exogenous vitamin D and/or C-3 epimer. Greater numbers of dogs and rats of both genders, in which diet, age, and body condition is controlled is needed before a definitive conclusion on gender influence can be determined.

The small sample size is a limitation of this study, and only male cats were represented, so future studies should survey both genders of a larger population of cats. Our investigations of the occurrence of 3-epi-25(OH)D₃ in the serum of rats served primarily to validate that our methodology detected the epimer in a species in which its existence was previously described. A more definitive description of the epimer in rats would investigate strain differences and control for differences in conditioning. The sample population of dogs was also limited in number and to a single genetic background.

To better assess metabolism and source of C-3 epimer, an exogenous vitamin D supplemental trial using identical diets can be performed. In all species, further studies are

also required to explain the organ or tissue source(s) and enzyme(s) responsible for epimerization.

Conclusion

To our knowledge, this is the first report of C-3 epimer measurements in cats. Our findings indicate that cats utilize an epimeric pathway for vitamin D metabolism. The function and health significance of the previously unrecognized metabolite is unknown. Because of its apparent abundance in circulation, concentrations of the epimer should be determined in feline research evaluating vitamin D status as an outcome or predictor. A current challenge for people and companion animal research is to clarify whether vitamin D is causally linked to the numerous non-skeletal disorders where correlations of vitamin D status and disease have been identified or if vitamin D status can be used as a marker. Based on our findings, it is reasonable to conclude that prominent species differences exist for vitamin D metabolism. Due to an apparently high utilization of a C-3 epimerization pathway for vitamin D in cats, it would appear that a feline model may be beneficial for use in future studies exploring the C-3 epimer's role in the relationship between vitamin D and health.

Acknowledgments

We wish to thank Dr. Leslie Hancock with Big Heart Pet Labs for her assistance in diet analysis, Dr. Ravinder J. Singh for conducting the LC-MS analyses, and the NIH T32OD011126 grant stipend supporting SEH.

Funding: This work was funded, in part, by the Phi Zeta Veterinary Honor Society at the University of Missouri and, in part, by the Nestle Purina Endowment for Small Animal Nutrition at the University of Missouri.

References

1. Stöcklin E, Eggersdorfer M. Vitamin D, an Essential Nutrient with Versatile Functions in Nearly all Organs. *International Journal for Vitamin and Nutrition Research*. 2013; 83:92–100. [PubMed: 24491882]
2. Mellanby RJ. Beyond the skeleton: the role of vitamin D in companion animal health. *Journal of Small Animal Practice*. 2016; 57:175–80. [PubMed: 27000647]
3. Morris JG. Ineffective Vitamin D Synthesis in Cats Is Reversed by an Inhibitor of 7-Dehydrocholesterol- 7-Reductase. *The Journal of Nutrition*. 1999; 129:903–8. [PubMed: 10203568]
4. Lalor S, Schwartz AM, Titmarsh H, et al. Cats with Inflammatory Bowel Disease and Intestinal Small Cell Lymphoma Have Low Serum Concentrations of 25-Hydroxyvitamin D. *Journal of Veterinary Internal Medicine*. 2014; 28:351–5. [PubMed: 24433362]
5. Lalor SM, Mellanby RJ, Friend EJ, et al. Domesticated Cats with Active Mycobacteria Infections have Low Serum Vitamin D (25(OH)D) Concentrations. *Transboundary and Emerging Diseases*. 2012; 59:279–81. [PubMed: 21999899]
6. Titmarsh HF, Cartwright JA, Kilpatrick S, et al. Relationship between vitamin D status and leukocytes in hospitalised cats. *Journal of Feline Medicine and Surgery*. 2016
7. Morris JG. Cats discriminate between cholecalciferol and ergocalciferol. *Journal of Animal Physiology and Animal Nutrition*. 2002; 86:229–38. [PubMed: 15379909]
8. Roberts MD, Toedebusch RG, Wells KD, et al. Nucleus accumbens neuronal maturation differences in young rats bred for low versus high voluntary running behaviour. *The Journal of Physiology*. 2014; 592:2119–35. [PubMed: 24665095]
9. Association of American Feed Control Officials. 2016 Official publication. Champaign, IL: 2016.

10. Huang M, LaLuzerne P, Winters D, et al. Measurement of vitamin D in foods and nutritional supplements by liquid chromatography/tandem mass spectrometry. *J AOAC Int.* 2009; 92:1327–35. [PubMed: 19916369]
11. Lensmeyer GL, Wiebe DA, Binkley N, et al. HPLC method for 25-hydroxyvitamin D measurement: comparison with contemporary assays. *Clin Chem.* 2006; 52:1120–6. [PubMed: 16574756]
12. Singh RJ, Taylor RL, Reddy GS, et al. C-3 Epimers Can Account for a Significant Proportion of Total Circulating 25-Hydroxyvitamin D in Infants, Complicating Accurate Measurement and Interpretation of Vitamin D Status. *The Journal of Clinical Endocrinology & Metabolism.* 2006; 91:3055–61. [PubMed: 16720650]
13. Horst RL, Littledike ET, Riley JL, et al. Quantitation of vitamin D and its metabolites and their plasma concentrations in five species of animals. *Analytical Biochemistry.* 1981; 116:189–203. [PubMed: 6272611]
14. Lensmeyer G, Poquette M, Wiebe D, et al. The C-3 Epimer of 25-Hydroxyvitamin D3 Is Present in Adult Serum. *The Journal of Clinical Endocrinology & Metabolism.* 2012; 97:163–8. [PubMed: 22013102]
15. Bianchini C, Lavery P, Agellon S, et al. The Generation of C-3 α Epimer of 25-Hydroxyvitamin D and Its Biological Effects on Bone Mineral Density in Adult Rodents. *Calcified Tissue International.* 2015; 96:453–64. [PubMed: 25712257]
16. Bailey D, Veljkovic K, Yazdanpanah M, et al. Analytical measurement and clinical relevance of vitamin D3 C3-epimer. *Clinical Biochemistry.* 2013; 46:190–6. [PubMed: 23153571]
17. Masuda S, Kamao M, Schroeder NJ, et al. Characterization of 3-epi-1 α ,25-dihydroxyvitamin D3 involved in 1 α ,25-dihydroxyvitamin D3 metabolic pathway in cultured cell lines. *Biol Pharm Bull.* 2000; 23:133–9. [PubMed: 10706373]
18. Rehan VK, Torday JS, Peleg S, et al. 1 α ,25-Dihydroxy-3-epi-vitamin D3, a natural metabolite of 1 α ,25-dihydroxy vitamin D3: production and biological activity studies in pulmonary alveolar type II cells. *Molecular Genetics and Metabolism.* 2002; 76:46–56. [PubMed: 12175780]
19. Kamao M, Tatematsu S, Hatakeyama S, et al. C-3 epimerization of vitamin D3 metabolites and further metabolism of C-3 epimers: 25-hydroxyvitamin D3 is metabolized to 3-epi-25-hydroxyvitamin D3 and subsequently metabolized through C-1 α or C-24 hydroxylation. *J Biol Chem.* 2004; 279:15897–907. [PubMed: 14757768]
20. Young LR, Backus RC. Oral vitamin D supplementation at five times the recommended allowance marginally affects serum 25-hydroxyvitamin D concentrations in dogs. *J Nutr Sci.* 2016; 5:e31. [PubMed: 27547394]
21. National Research Council. Nutrient requirements of the cat and dog. Washington, DC: National Academy Press; 2006.
22. Sih TR, Morris JG, Hickman MA. Chronic ingestion of high concentrations of cholecalciferol in cats. *Am J Vet Res.* 2001; 62:1500–6. [PubMed: 11560284]

Figure 1.
Representative chromatograms from three cats and two standards showing peaks eluting to 25(OH)D3, 25(OH)D2, and 3-epi-25(OH)D3

Figure 2.

Correlations between cat serum vitamin D metabolites determined with two analytical methods: HPLC and LC-MS. Best-fit regression lines for HPLC and LC-MS observations of 25(OH)D3 (plotted squares) and 3-epi-25(OH)D3 (plotted circles) serum concentrations (ng/mL) gave the equations $Y = 0.65x + 6.14$, $R^2 = 0.86$, $p < 0.01$ and $Y = 0.53x + 8.40$, $R^2 = 0.47$, $p = 0.06$, respectively.

Figure 3.
HPLC Analysis of serum metabolite concentrations in species observed

Table 1

Serum concentrations of 25(OH)D₃ and 3-epi-25(OH)D₃ of cats, rats, and dogs as analyzed by both HPLC and LC-MS

Species	Metabolite	HPLC (mean ± SEM)	LC-MS (mean ± SEM)
Cats Diet A (n = 8)	25(OH)D ₃ (ng/mL)	45.6 ± 10.3	35.7 ± 7.2
	3-Epi-25(OH)D ₃ (ng/mL)	23.5 ± 3.8	20.9 ± 3.0
	% Epimer	54.3 ± 16.5	56.1 ± 12.7
Cats Diet B (n = 6)	25(OH)D ₃ (ng/mL)	35.2 ± 20.3	—
	3-Epi-25(OH)D ₃ (ng/mL)	26.9 ± 12.2	—
	% Epimer	82.6 ± 13.4	—
Rats	25(OH)D ₃ (ng/mL)	8.8 ± 5.3	—
	3-Epi-25(OH)D ₃ (ng/mL)	1.3 ± 0.5	—
	% Epimer	21.5 ± 17.8	—
Dogs	25(OH)D ₃ (ng/mL)	23.0 ± 4.1	—
	3-Epi-25(OH)D ₃ (ng/mL)	0	—

Table 2Feline serum and plasma results of HPLC analyses for concentrations of 25(OH)D₃ and 3-epi-25(OH)D₃

Species	Metabolite	Serum (mean ± SEM)	Plasma (mean ± SEM)
Cats Diet B (n = 6)	25(OH)D ₃ (ng/mL)	41.0 ± 15.0	42.2 ± 10.7
	3-Epi-25(OH)D ₃ (ng/mL)	35.9 ± 5.2	38.0 ± 4.8

Table 3

Proximate, energy, and vitamin D contents of the experimental diets

	Cat Diet A	Cat Diet B	Rat	Dog
Proximate Analysis *				
Crude Protein (%)	31	26	23.6	25.5
Crude Fat (ether extract) (%)	14	9	6.7	8.5
Crude Fiber (%)	2	5.5	3.3	2.8
Ash (%)	6.2	6.2	6.1	7.2
Moisture (%)	10	10	≤12	≤12
NFE (%)			50.3	≤45
Metabolizable Energy *				
Protein (%)	29.2	28.7	26.5	27.8
Fat (%)	35.0	24.2	16.97	23.3
Carbohydrate (%)	35.8	47.1	56.5	49.0
Energy Density (kcal/g)	4.37	3.58	3.23	3.54
Vitamin D3 Content (IU/g)	1.36	5.62	3.43	2.44
IU cholecalciferol/day/kg (body weight)	15.3	66.7	198	—

* Reported by the manufacturer