Effect of twice-daily oral administration of a chondroitin sulfate–containing supplement on urine chondroitin sulfate concentrations in dogs

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OBJECTIVE
To quantify the magnitude and duration of changes in urine chondroitin sulfate concentration (uCS) as a result of oral administration of a chondroitin sulfate–containing supplement in dogs.

ANIMALS
8 healthy privately owned dogs.

PROCEDURES
A urine sample was collected from each dog via cystocentesis on day 1; free-catch midstream urine samples were collected once daily on days 2 through 5. Pretreatment uCS was established from those samples. Each dog then received a chondroitin sulfate–containing supplement (20 to 30 mg/kg, PO, q 12 h) for 8 days (on days 7 through 14). Urine samples were collected on days 8 through 12 and day 15. For each sample, uCS was quantified by liquid chromatography–tandem mass spectrometry. Variable urine concentration was accounted for by dividing the uCS by urine creatinine concentration (uCrea) to determine the uCS:uCrea ratio. Pretreatment uCS:uCrea ratios were compared with treatment uCS:uCrea ratios to calculate the fold change in uCS after supplement administration.

RESULTS
Among the study dogs, oral administration of the chondroitin sulfate–containing supplement resulted in a 1.9-fold increase in the median uCS:uCrea ratio. Data obtained on days 8 through 12 and day 15 indicated that the daily increase in uCS remained consistent and was not additive.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that oral administration of supplemental chondroitin sulfate to dogs modestly increased uCS within 24 hours; however, subsequent supplement administration did not have an additive effect. A potential therapeutic benefit of persistently increased uCS in preventing recurrent urinary tract infections in dogs warrants investigation. (*Am J Vet Res* 2019;80:799–805)

In healthy humans and other animals, GAGs (including chondroitin sulfate) in the urine protect the urothelium from exposure to urine contents.[1–3] In cases of UTI with *Escherichia coli*, virulence factors produced by bacteria can injure the GAG barrier, thereby allowing increased urothelial bacterial adherence and permeability.[4,5] The subsequent leakage of bacterial components, potassium, and ammonia into the suburothelium initiates an inflammatory cascade that culminates in the local production of antiproliferative factor and decreased urothelial GAG concentrations and predisposes the affected individual to additional inflammation and further urothelial injury.[6–9] The importance of GAG barrier replenishment and repair to reduce the clinical signs and inflammation associated with interstitial cystitis in humans and with idiopathic cystitis in cats has been well described.[10] However, more recently, ineffective urinary GAG production and barrier repair has been associated with the pathogeneses of other chronic inflammatory lower urinary tract disorders including detrusor overactivity, toxin-induced cystitis, and UTIs.[11–17]

Administration of GAGs to manage recurrent UTI in people through repair of the urothelial barrier is not a novel concept. Results of numerous human clinical studies performed over the last decade have indicated that weekly followed by monthly intravesicular instillations of chondroitin sulfate or hyaluronic acid (or both) can reduce reinfection rates by as many as 3.41 infections/y, increase the mean time to UTI recurrence by 187.35 days, and increase patients' quality of life scores.[18–23] Overall, this results in a reduced need for antimicrobial treatment of UTIs.

ABBREVIATIONS

GAG	Glycosaminoglycan
IQR	Interquartile (25th to 75th percentile) range
uCrea	Urine creatinine concentration
uCS	Urine chondroitin sulfate concentration
UTI	Urinary tract infection

Although intravesicular GAG instillations have the potential to augment urine GAG concentrations and prevent recurrent UTIs in people, there are considerations that may limit their usefulness in veterinary medicine. The use of equivalent intravesicular treatment protocols would include weekly to monthly instillations requiring regular sedation or anesthesia to pass urinary catheters in female dogs and a means to prevent urination after GAG administration (to extend contact time with the urothelium to 2 hours) in all treated dogs.[18,23] These obstacles make intravesicular instillations in dogs both expensive and impractical; hence, an alternative method for providing urinary GAG supplementation in canine patients is necessary.

For dogs, a potential alternative treatment to intravesicular instillations is the provision of a GAG, such as chondroitin sulfate, in an orally administered formulation. Chondroitin sulfate has the advantage that the compound remains largely unchanged as it passes through the stomach and small intestine, where it is absorbed via endocytosis or pinocytosis and excreted from the body through the urinary tract.[24–27] In dogs, oral bioavailability of chondroitin sulfate ranges from 4.8% to 5% for single doses; with multiple-day dosing, a cumulative effect has been reported.[28] In veterinary medicine, chondroitin sulfate combined with glucosamine is commonly orally administered to dogs as a chondroprotective agent in the management of osteoarthritis. Given that chondroitin sulfate can safely be orally administered daily, this compound may be ideal for maintaining a constant excretion of GAGs in the urine of dogs.

The objective of the study reported here was to quantify the magnitude and duration of changes in uCS as a result of oral administration of a chondroitin sulfate–containing supplement to healthy dogs. Our hypothesis was that orally administered chondroitin sulfate would increase uCS in healthy dogs. To test this hypothesis, the uCS in a group of dogs was determined before and after 8 days of daily oral administration of a chondroitin sulfate–containing supplement. The intent was to provide baseline data that could be used to compare uCS following administration of supplemental chondroitin sulfate by intravesicular instillation and the oral route.

Materials and Methods

Animals

For this prospective study, 16 dogs owned by community members (faculty, students, and staff) at the School of Veterinary Medicine, University of Wisconsin-Madison were recruited. Prior to study admission, the dog owners were asked to sign an informed consent form and agreed to administer the chondroitin sulfate supplement as recommended. Within the study, each dog served as its own control. For each dog, the diet was standardized in the pretreatment and treatment periods; owners were required to consistently maintain each dog's diet and administer medications and supplements throughout the study. Historical information and a list of current medications were also collected for each dog. Dogs with a history of polyuria and polydipsia, pollakiuria, stranguria, or UTIs as well as those receiving chondroitin sulfate in food or supplements, corticosteroids, or anticoagulants including heparin, aspirin, or clopidogrel were excluded from the study. Other medications were permitted provided that they were administered consistently throughout the study.

Enrolled dogs underwent a physical examination, cystocentesis, and venipuncture. Urine and serum samples were submitted to the university's Veterinary Care Clinical Pathology Laboratory for urinalysis and biochemical analysis, respectively. Urine samples were also cultured on a blood agar plate at 37°C for 5 days to assess for bacterial growth. Dogs were excluded from the study if high serum liver enzyme activity (alkaline phosphatase, alanine aminotransferase, or γ-glutamyltransferase) or high concentrations of azotemia markers (creatinine or BUN), evidence of urinary tract inflammation, or bacteriuria was detected. Urinary tract inflammation was defined as the presence of proteinuria or hematuria detected by urine dipstick evaluation or the presence of WBCs or RBCs detected by microscopic evaluation of urine sediment. The University of Wisconsin-Madison Institutional Animal Care and Use Committee approved the study design and execution.

Pretreatment urine samples

For each dog, initial urine samples were collected via cystocentesis on day 1. Free-catch midstream urine samples were collected and stored in urine specimen cups by the owners at home once daily on days 2 through 5. Each owner was instructed to collect the single urine sample at the same time each day, although this timing was not a requirement for retention of the dog in the study. Samples were stored at 4°C until submission for analysis; at the laboratory, urine samples were maintained at 4°C and processed within 24 hours after collection. One aliquot of each sample underwent urine creatinine quantification with an automated biochemical analyzer.[a] The remainder of each urine sample was centrifuged at 1,000 × *g* for 5 minutes prior to being divided into 1-mL aliquots for storage at –80°C.

Treatment

On day 7, oral administration of a supplement[b] containing chondroitin sulfate, glucosamine, and manganese was commenced for each dog. The supplement was provided twice daily according to the manufacturer's dosing recommendation for the next 7 days. The mean elimination half-life of orally administered chondroitin sulfate is 9.35 to 12.1 hours in dogs,[28] and the 8-day treatment period (days 7 through 14) allowed for a steady-state plasma concentration to be achieved. Among the 16 dogs, the chondroitin sulfate dose ranged from 20 to 30 mg/kg.

Treatment urine samples

During the 8-day period of supplement administration, free-catch midstream urine samples were collected on days 8 through 12 and day 15. The dogs did not receive the chondroitin sulfate–containing supplement prior to urine sample collection on day 15. The treatment urine samples were processed as described for pretreatment samples collected on days 2 through 5.

uCS quantification by mass spectrometry

The chondroitin sulfate quantification method used in the study was adapted from a previously described technique for measuring GAG concentrations in samples of urine or CFS from humans.[29,30] Briefly, stored urine samples were diluted 1:1 with ultrapure water (resistivity, 18MΩ•cm) from which a volume of 25 µL was dried under a nitrogen stream. Chondroitin sulfate within the urine samples was derivatized and chemically cleaved by methanolysis to produce disaccharides. After methanolysis, samples were resolubilized in 200 µL internal standard–containing solution, dried again under a nitrogen stream, and resolubilized in 200 µL of 10mM ammonium acetate in 90% acetonitrile and 10% water (high-performance liquid chromatography solvent B). The internal standard solution was created by taking 300 µg of a 70% chondroitin sulfate A powder[c] and adding 2M ^{2}HCl in methanol-d_4.[d] Calibration curve standards were prepared from pooled urine of healthy dogs with fixed amounts of chondroitin sulfate A prior to derivatization. Standards were prepared at 0, 10, 25, 50, 100, 250, and 500 µg of chondroitin sulfate A/mL. Derivatization of calibration curve standards was performed exactly as described for the experimental samples.

Data were collected by use of a liquid chromatography–tandem mass spectrometry multiple-reaction method with hydrophilic interaction liquid chromatography. High-performance liquid chromatography was performed in normal mode.[e] Mass spectrometry was performed in multiple-reaction method mode.[f] Solvents of 10mM ammonium acetate in 10% acetonitrile (solvent A) and 10mM ammonium acetate in 90% acetonitrile (solvent B) were used in hydrophilic interaction liquid chromatography with a 1.7 µm X 2.1 mm X 50-mm column.[g] Analytes were gradient eluted at 300 µL/min, starting with 100% solvent B and ramping to 88% solvent B over a 10-minute period, then to 70% solvent B over a 5-minute period, and returning to 100% solvent B over a 5-minute period, and equilibrated for 10 minutes. The column was held at 40°C while the autosampler was kept at 4°C. The multiple-reaction method was performed in positive ion mode with the turbo ion spray source. Three transitions were monitored: 2 for derivatized chondroitin sulfate A and 1 for the isotopically labeled derivatized chondroitin sulfate A internal standard. For chondroitin sulfate A transitions, Q1 was 426.4 with Q3 of 236.2 (quantifier) at a collision energy of 10 and 50-millisecond dwell time or with Q3 of 204.2 (qualifier) at a collision energy of 15 and 50-millisecond dwell time. For internal standards, Q1 was set to 432.4 with Q3 of 239.2 at a collision energy of 10 and 50-millisecond dwell time. The injection volume was 10 µL for all standards and samples.

Acquisition and quantitation were performed with commercially available software.[h,i] Chromatographic peak areas were derived by integration and normalized to the internal standard peak area. A best-fit line was determined, and correction for endogenous chondroitin sulfate A was made by not forcing the y-intercept through the origin. Upper-level detection limits were calculated daily and were between 100 and 200 µg/mL. Correlation coefficients were > 0.999 for all calibration curves acquired. For every 8 samples prepared and analyzed, a quality control spike was created from the eighth sample by spiking it with 50 µg of chondroitin sulfate/mL. This helped to ensure reliable quantitation by demonstrating an appropriate increase in the measured chondroitin sulfate concentration. The mean ± SD spike recovery was 80.2% ± 12.91% of the expected level. Samples were run in singlets.

Statistical analysis

To normalize uCS in the face of variable urine production or concentration, a ratio of uCS (µg/mL) to uCrea (mg/dL) was calculated as previously described.[31] The collection of data in the pretreatment and treatment periods allowed each dog to be its own control. Median uCS:uCrea ratios for the pretreatment (days 1 through 5) and treatment (days 8 through 12 and day 15) periods were obtained for each dog. The median individual dog uCS:uCrea ratios for the pretreatment period were compared, as were the indi-

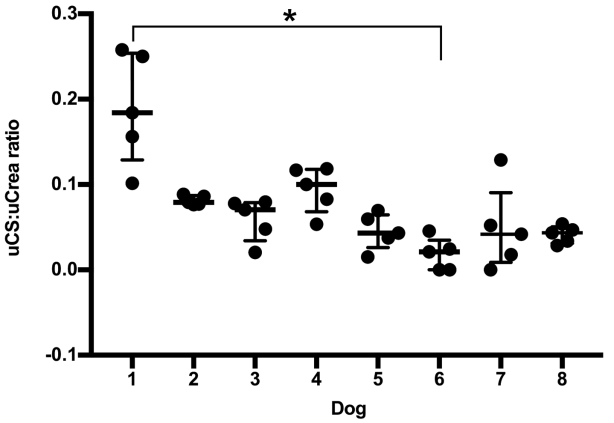

Figure 1—Plots of pretreatment ratios of uCS to uCrea for 8 dogs enrolled in a study to quantify the magnitude and duration of changes in uCS as a result of oral administration of a chondroitin sulfate-containing supplement. For each dog prior to treatment, a urine sample was collected via cystocentesis on day 1; a free-catch midstream urine sample was collected once daily on days 2 through 5. For each dog, data points represent the ratio calculated for each of those 5 pretreatment days; the middle horizontal bar represents the dog's median uCS:uCrea ratio, and the whiskers represent the IQR. *The median pretreatment uCS:uCrea ratios for dog 1 and dog 6 are significantly (P = 0.001) different.

vidual dog uCS:uCrea ratios for the treatment period. For each period, an overall median uCS:uCrea ratio was calculated for comparison. Statistical analyses were performed by use of commercially available software.[j] Data were tested for normality. Parametric data were analyzed with a Student *t* test, and values were reported as the mean ± SD. Nonparametric data were analyzed with a Wilcoxon matched-pairs signed rank test when paired comparisons of pretreatment and treatment period samples were performed. The Mann-Whitney *U* test was used for unpaired data, whereas the Friedman test of repeated measures with the Dunn multiple comparisons correction was used for repeated measures. Data were paired when comparing pretreatment and treatment period values as well as when comparing day-to-day variations in the uCS and uCS:uCrea ratio. Nonparametric data were reported as the median and IQR. Significance was set at a value of $P \leq 0.05$. A post hoc Wilcoxon matched-pairs signed rank power analysis was performed with commercially available software.[k]

Results

Sixteen dogs were considered for the study. Five dogs were excluded because the initial urine culture yielded bacterial growth, and a sixth dog was excluded because of high serum alkaline phosphatase activity. Ten dogs were enrolled in the study. During the study, 2 dogs were withdrawn; one dog ingested chocolate (day 5), and the urine sample for the other dog was improperly stored prior to submission for analysis (day 10). Eight dogs successfully completed the study. Despite the small number of dogs, the study maintained a power of 0.8 for comparison of pretreatment and treatment data.

Of the 8 study dogs, 4 were spayed females, 1 was a sexually intact male, and 3 were castrated males. The dogs' median age was 7 years (range, 2 to 14 years). Breeds represented included Alaskan Malamute, American Staffordshire Terrier, Australian Shepherd, Rough Collie, and German Shorthaired Pointer (1 each); there were 3 mixed-breed dogs. The 8 dogs' median weight was 28.8 kg (range, 17.8 to 40.9 kg). Mean ± SD uCrea in the pretreatment period (220.7 ± 80.14 mg/dL for days 1 through 5) was not significantly ($P = 0.82$) different from that in the treatment period (216.9 ± 74.27 mg/dL for days 8 through 12 and day 15). No adverse effects were reported during the entire study period.

During the pretreatment period (days 1 through 5), the median uCS:uCrea ratio for all dogs was 0.057 (IQR, 0.035 to 0.088) with an absolute median uCS of 12.60 µg/mL (IQR, 5.85 to 27.18 µg/mL). To assess how normal daily variations in chondroitin sulfate excretion may have confounded the data, the median pretreatment uCS:uCrea ratios were calculated for each dog for comparison. Among dogs, only the median pretreatment uCS:uCrea ratios for dogs 1 and 6 differed significantly ($P = 0.001$; **Figure 1**). On examination of each dog's pretreatment data, the uCS:uCrea ratios obtained on days 1 through 5 did not differ significantly. Across all dogs, there was also no difference ($P = 0.855$) detected between the median uCS:uCrea ratio for urine samples collected via cystocentesis on day 1 (0.067) and the median ratio for

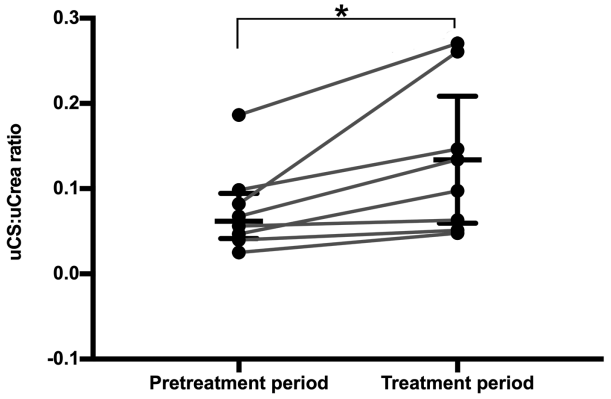

Figure 2—Median ratios of uCS to uCrea for each of the 8 dogs in Figure 1 determined by analysis of urine samples collected in the pretreatment period (days 1 through 5) and in the treatment period (days 8 through 12 and day 15) of the study. For each dog, a urine sample was collected via cystocentesis on day 1; a free-catch midstream urine sample was collected once daily on days 2 through 5, days 8 through 12, and day 15. Beginning on day 7, dogs were administered a chondroitin sulfate-containing supplement (20 to 30 mg/kg) orally twice daily for 8 days. Individual data points represent the median uCS:uCrea ratio for each individual dog during the pretreatment and treatment periods. The middle horizontal black bar in each grouping represents the overall median uCS:uCrea ratio for that period; the whiskers represent the IQR. Gray lines are drawn to connect the pretreatment and treatment period uCS:uCrea ratios for each individual dog. *The median overall uCS:uCrea ratios for the pretreatment and treatment periods are significantly ($P = 0.008$) different.

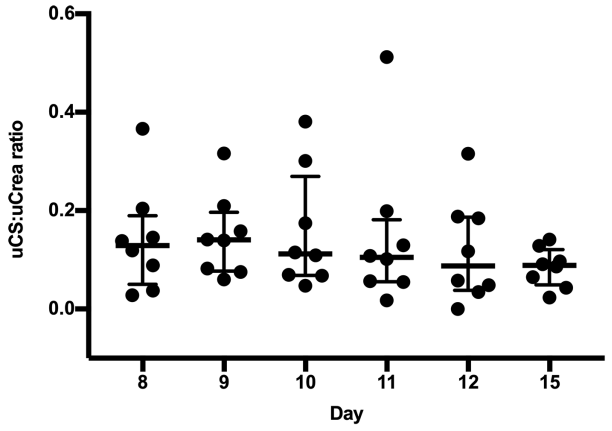

Figure 3—Plots of treatment ratios of uCS to uCrea for each of the 8 dogs in Figures 1 and 2. The dogs were treated with the chondroitin sulfate–containing supplement on days 7 through 14. Data points represent the uCS:uCrea ratio for individual dogs on each day of urine sample collection in the treatment period. The middle horizontal black bar in each grouping represents the overall median uCS:uCrea ratio for that day; the whiskers represent the IQR. The overall median uCS:uCrea ratio did not differ significantly among days.

all urine samples collected by free catch on days 2 through 5 (0.057). Oral administration of supplemental chondroitin sulfate (on days 7 through 14) to the dogs significantly (P = 0.008) increased the median uCS:uCrea ratio for all urine samples collected on days 8 through 12 and day 15 to 0.109 (IQR, 0.059 to 0.171) with an absolute median uCS of 19.90 µg/mL (IQR, 12.35 to 35.78 µg/mL; **Figure 2**). These data indicated that administration of an oral supplement containing chondroitin sulfate to the dogs resulted in a 1.91-fold increase in the median uCS:uCrea ratio, compared with the pretreatment period value. The observed increase in the median uCS:uCrea ratio from the pretreatment period value was detectable after the first day of supplement administration (P = 0.016) and remained consistent for each subsequent day of supplement administration. However, a progressive increase in the median uCS:uCrea ratio was not observed with continued chondroitin sulfate administration on days 8 through 12 and day 15 (P = 0.075; **Figure 3**).

Discussion

Results of the present study indicated that daily oral administration of a supplement containing chondroitin sulfate and glucosamine increased uCS in dogs. In urine samples collected during the treatment period, there was a nearly 2-fold increase in uCS after the first day, which persisted with continued supplement administration. A previous study[28] revealed that chondroitin sulfate disaccharide accumulation was appreciable in the plasma of dogs when measured after 7 days of oral administration of a supplement. A cumulative effect of multiple-day dosing was not observed in the urine from dogs of the present study. A possible explanation for this difference in plasma and urine accumulations is that chondroitin sulfate in plasma is distributed throughout the body and not just excreted in the urine. However, other possibilities include the facts that the sample size used in the present study was limited and the dosage of chondroitin sulfate administered to dogs in the aforementioned plasma study exceeded (by 4 to 8 times) the recommended dosage that was administered to dogs in the present study.[32] Whether higher dosages of chondroitin sulfate administered to dogs would result in progressive increases in uCS over time is unknown.

In the 8 dogs of the present study, the median uCS achieved after oral administration of a supplement containing chondroitin sulfate was 19.90 µg/mL. This concentration is 1 one thousandth of the concentration of chondroitin sulfate in an intravesicular product[l] (1 g/50 mL) used in human clinical studies, which resulted in reduced rates of recurrent UTIs.[20,33] Hence, the usefulness of oral chondroitin sulfate administration for the prevention of recurrent UTIs in dogs is questionable. In a recent clinical study[34] of the efficacy of an orally administered combination of hyaluronic acid, chondroitin sulfate, curcumin, and quercetin for the prevention of recurrent UTIs in postmenopausal women, treatment for 1 year resulted in a reduced UTI rate, compared with findings prior to treatment.[34] That study used an oral product containing a combination of compounds, but the results provide some justification for a future study to examine whether long-term oral administration of chondroitin sulfate may have a beneficial effect in reducing UTI rates in dogs.

To our knowledge, a GAG dose-effect study for preventing UTIs in humans or other animals has yet to be performed, and the current GAG dosage recommendations used in human intravesicular clinical studies were adopted from interstitial cystitis research.[18,19,23,33,35] It remains possible that the chondroitin sulfate concentration at the urothelium-urine interface is more important than uCS with regard to UTI prevention. In rats and mice that have undergone acid injury procedures during which the urinary bladder GAG layer is disrupted, it has been shown that infused chondroitin sulfate preferentially binds to damaged urothelial tissue, thereby recreating a barrier that prevents bacterial adherence and increasing the impermeability of the bladder wall surface.[17,36,37] These GAGs also directly affect the urothelium by reducing nuclear factor κB–mediated inflammation and the rate of mast cell degranulation.[38,39] Hence, measurement of uCS does not necessarily quantify the protective effects of chondroitin sulfate on the bladder mucosa, and continuous excretion of low concentrations of chondroitin sulfate may be able to exert a beneficial effect. Because a specific relationship between urine GAG concentrations and mucosal GAG concentrations has not been established in dogs or people, evaluation of UTI recurrence in dogs receiving an oral supplement of chondroitin sulfate is necessary before this practice can be recommended by veterinarians to pet owners.

Limitations of the present study were that mass spectrometry is unable to identify the size of chondroitin sulfate molecules that are excreted into the urinary bladder after oral administration. Chondroitin sulfate exists as large molecules of alternating D-glucuronic acid and *N*-acetyl-D-galactosamine. Traditional methods for measuring uCS, such as the dimethylmethylene blue technique, indirectly measure chondroitin sulfate concentration by quantifying the amount of sulfated GAGs in the urine sample and are sensitive but not specific assays for uCS assessment. In comparison, the mass spectrometry technique used in the present report is more sensitive and specific for chondroitin sulfate quantification; however, this procedure cleaves the chondroitin sulfate molecules before measuring GAG fragments. Hence, the technique introduces some uncertainty as to whether the measured chondroitin sulfate concentration represents large molecules or fragments of molecules. The size of chondroitin sulfate fragments required to achieve a protective effect is not known.

In the dogs of the present study, daily oral administration of a chondroitin sulfate–containing supplement for 8 days consistently but modestly increased uCS. Whether long-term oral administration of chondroitin sulfate to dogs results in increases in uCS that are clinically relevant is unknown. A large-scale clinical study to compare UTI recurrence in dogs before and after oral administration of a chondroitin sulfate–containing supplement is warranted.

Acknowledgments

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The authors declare that there were no conflicts of interest.

Footnotes

a. Vitros 5.1 FS, Ortho Clinical Diagnostics, Raritan, NJ.
b. Cosequin DS, Nutramax Laboratories Veterinary Sciences Inc, Lancaster, SC.
c. ThermoFisher Scientific, Waltham, Mass.
d. Sigma-Aldrich Corp, St Louis, Mo.
e. Agilent 1100 HPLC Agilent, Santa Clara, Calif.
f. Sciex 3200 QTRAP mass spectrometer, Sciex, Framingham, Mass
g. Waters Acquity BEH Amide, 1.7 µm, 2.1 X 50 mm column, Waters Corp, Milford, Mass.
h. Analyst, version 1.4.2, Sciex, Framingham, Mass.
i. Excel, Microsoft Corp, Redmond, Wash.
j. GraphPad Prism 7, GraphPad Software, La Jolla, Calif.
k. G*Power 3.1, Heinrich Heine University of Düsseldorf, Düsseldorf, Germany.
l. iAluRil, Aspire Pharma Ltd, Petersfield, England.

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