

Evaluation of factors that affect analytic variability of urine protein-to-creatinine ratio determination in dogs

Gabriele Rossi, DVM, PhD; Luca Giori, DVM; Simona Campagnola, DVM; Andrea Zatelli, DVM; Eric Zini, DVM, PhD; Saverio Paltrinieri, DVM, PhD

Objective—To determine whether preanalytic and analytic factors affect evaluation of the urinary protein-to-creatinine (UPC) ratio in dogs.

Sample—50 canine urine samples.

Procedures—The UPC ratio was measured to assess the intra-assay imprecision (20 measurements within a single session), the influence of predilution (1:10, 1:20, and 1:100) for urine creatinine concentration measurement, and the effect of storage at room temperature (approx 20°C), 4°C, and –20°C.

Results—The coefficient of variation at room temperature determined with the 1:20 predilution was < 10.0%, with the highest coefficients of variation found in samples with a low protein concentration or low urine specific gravity. This variability could result in misclassification of samples with UPC ratios close to the thresholds defined by the International Renal Interest Society to classify dogs as nonproteinuric (0.2), borderline proteinuric (0.21 to 0.50), or proteinuric (> 0.51). A proportional bias was found in samples prediluted 1:10, compared with samples prediluted 1:20 or 1:100. At room temperature, the UPC ratio did not significantly increase after 2 and 4 hours. After 12 hours at room temperature and at 4°C, the UPC ratio significantly increased. The UPC ratio did not significantly change during 3 months of storage at –20°C.

Conclusions and Clinical Relevance—The intra-assay precision of the UPC ratio was sufficiently low to avoid misclassification of samples, except for values close to 0.2 or 0.5. The optimal predilution ratio for urine creatinine concentration measurement was 1:20. A 1:100 predilution is recommended in samples with a urine specific gravity > 1.030. The UPC ratio must be measured as soon as samples are collected. Alternatively, samples should be immediately frozen to increase their stability and minimize the risk of misclassification of proteinuria. (*Am J Vet Res* 2012;73:779–788)

Quantification of proteinuria with the UPC ratio is a useful method for identification of renal disease in dogs.¹ Urinary loss of plasma proteins is one of the earliest functional defects recognized in association with glomerulonephritis.² Proteinuria is also a risk factor positively associated with development of uremic cri-

ABBREVIATIONS

CI	Confidence interval
CV	Coefficient of variation
UPC	Urine protein to creatinine
USG	Urine specific gravity

Received December 10, 2010.

Accepted May 9, 2011.

From the Department of Veterinary Pathology, Hygiene and Public Health, University of Milano, 20133 Milan, Italy (Rossi, Giori, Campagnola, Paltrinieri); Clinica Veterinaria Pirani, Via Majakovski 2/L,M,N, 42100 Reggio Emilia, Italy (Zatelli); the Department of Veterinary Clinical Sciences, University of Padua, 35020 Agripolis, Legnaro, Italy (Zini); Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, 8057 Zurich, Switzerland (Zini); and Istituto Veterinario di Novara, Strada Provinciale 9, Granozzo Con Monticello, NO 28060 Italy (Zini).

Presented in abstract form at the 20th European College of Veterinary Internal Medicine Companion Animals Congress and the 13th European Society of Veterinary Clinical Pathology Congress, Toulouse, France, September 2010.

The authors thank Dr. Giuliano Ravasio and Ugo Gandolfi for technical assistance.

Address correspondence to Dr. Paltrinieri (saverio.paltrinieri@unimi.it).

sis and renal-related death in dogs with chronic kidney disease.³ In addition, in a study² of dogs with glomerulonephritis, severity of proteinuria was associated with disease progression and outcome. Quantification of proteinuria has thus been included by the International Renal Interest Society in the staging system for chronic kidney disease.⁴ As recently reported,⁵ proteinuria can be routinely assessed via semiquantitative methods, such as the urine dipstick test. However, even if dipstick test results are reliable when interpreted together with USG, precise quantification of proteinuria remains mandatory to measure its magnitude, as defined by the International Renal Interest Society staging system. The gold standard to quantify urinary protein excretion is 24-hour urine collection, but this method is not practical in dogs. As an alternative, the UPC ratio is more fre-

quently used because it correlates well with daily protein excretion and can be assessed in 1 randomly collected urine sample.^{1,6-8} The reliability of the UPC ratio is based on the assumption that the test is precise and is not influenced by analytic or preanalytic variables, such as intra-assay repeatability of results and the influence of automated sample predilution on urine creatinine concentration. In addition, because urine samples intended for UPC ratio measurement are usually delivered to external laboratories by private practitioners, stability of urine protein or creatinine over time and at different storage temperatures represents a potentially critical factor. To the best of our knowledge, no studies have been reported regarding assessment of the analytic and preanalytic variability of UPC ratio measurement in dogs.

The purposes of the study reported here were to determine whether preanalytic and analytic factors affect evaluation of the UPC ratio in dogs and to estimate their clinical consequences. Specifically, the intra-assay repeatability of UPC ratio measurement and the effect of storage at various temperatures and times were evaluated. In addition, given that urine samples are routinely diluted to 1:20 before measuring the urine creatinine concentration, the influence of preanalytic dilution was also determined; in highly concentrated urine, a 1:20 dilution may not be sufficient to decrease the urine creatinine concentration into the linear range of the assay, leading to inaccurate results.

Materials and Methods

Animals and samples—The present study was performed with 50 urine samples collected, under informed consent of the owners, from 50 client-owned dogs following admission at the University of Milan for routine wellness visits or because of various diseases, in accordance with the guidelines of the Comitato Etico Tutela Animali, the Institutional Animal Care and Use Committee of the University of Milan. Because the investigation focused on the analytic performance and not on the diagnostic role of the UPC ratio, samples were collected irrespective of the dog's age, sex, breed, and underlying disease. Samples were collected via either ultrasonographically guided cystocentesis ($n = 36$) or free catch (14), according to established protocols.⁹ Eight to 10 mL of urine was collected from each dog, and all samples were immediately placed in 15-mL sterile conical tubes.

Urinalysis—From the collected urine samples, 5 mL was transferred into a fresh tube to perform sediment analysis; the remaining 3 to 5 mL was placed into another tube to obtain supernatants to determine USG and for UPC ratio measurement. Both tubes were centrifuged (10 minutes at $450 \times g$) within 30 minutes after collection. During the period between collection and centrifugation, samples were kept at room temperature (approx 20°C).

From the first tube, 4.5 mL of supernatant was removed and the remaining 0.5 mL of urine was used to resuspend the urine sediment. A drop of the resuspended sediment was microscopically examined to count the

mean number of RBCs and WBCs per hpf. Bacteriuria, spermaturia, lipiduria, and the presence of epithelial cells, casts, and crystals were also evaluated according to a semiquantitative scale (rare, moderate, abundant, and very abundant). Sediments were classified as active on the basis of the presence of one or more of the following findings: bacteriuria, a moderate number of casts, and > 5 RBCs, WBCs, or epithelial cells/hpf. Samples with none of these findings were classified as inactive. From the second tube, supernatant was used to determine the UPC ratio; this was calculated by measuring the concentration of creatinine and protein with an automated spectrophotometer^a and by the following formula: UPC ratio = urine protein concentration/urine creatinine concentration. Urine protein concentrations were determined with the pyrogallol red method^b from undiluted urine, and urine creatinine concentrations were determined with the modified Jaffè method^c from urine samples that were prediluted to 1:20. Also from the second tube, a drop of supernatant was used to determine the USG with a handheld refractometer^d calibrated daily with distilled water.

Supernatants from the tubes were transferred into a third tube, and depending on the volume of available supernatant, one or more of the following analytic or preanalytic variables potentially affecting the UPC ratio measurement were investigated: intra-assay repeatability, effect of predilution of urine samples, and effect of storage duration and temperature.

Intra-assay repeatability—Twenty consecutive measurements of the UPC ratio were performed on 25 urine supernatants immediately after centrifugation. For each sample, the mean and SD of urine protein concentration, urine creatinine concentration, and UPC ratio were measured, and the CV was calculated as follows: $CV = SD/mean \times 100$.

To assess whether intra-assay test imprecision had an influence on urine categorization according to the International Renal Interest Society staging system, samples were defined as nonproteinuric if the mean UPC ratio of the 20 measurements was ≤ 0.20 , borderline proteinuric if the UPC ratio was from 0.21 to 0.50, and proteinuric if the UPC ratio was > 0.50 . The possible influence of intra-assay imprecision was assessed on samples with a mean UPC ratio close to the International Renal Interest Society cutoffs (0.2 or 0.5); the 20 replicate readings from these samples were examined to count the number of readings in the same International Renal Interest Society class of the mean value and the number of readings in a different International Renal Interest Society class.

Effect of predilution of urine samples—After centrifugation, 3 aliquots of 25 urine supernatants were diluted to 1:10, 1:20, and 1:100. The UPC ratio was measured in triplicate. The mean value of each triplicate measurement was used to estimate the actual value of each sample.

Effect of storage duration and temperature—The supernatant of 20 samples was used to prepare 3 aliquots of 300 μ L to assess possible changes of the UPC ratio that occurred at room temperature after short-

term storage (2 and 4 hours). The supernatant of 25 samples was divided into 5 aliquots of 300 μ L, which were stored at room temperature and then analyzed immediately and 12, 24, 48, and 72 hours after storage. In addition, 24 samples were used to prepare 6 aliquots of 300 μ L stored at 4°C and analyzed 12, 24, 48, and 72 hours after and 1 week after storage; 18 samples were used to prepare 10 aliquots of 300 μ L that were stored at -20°C and analyzed 24, 48, and 72 hours after storage; 1, 2, and 3 weeks after storage; and monthly for up to 3 months after storage. Before analysis, refrigerated and frozen samples were brought to room temperature and further centrifuged. Then, the UPC ratio was analyzed in triplicate, as described. Mean values of each triplicate measurement were used to estimate the actual value of each sample.

Statistical analysis—To identify possible correlations between the intra-assay CV of urine protein concentration, urine creatinine concentration, or UPC ratio and the actual values of each of these variables or the USG, the Spearman correlation test was used. Similarly, possible differences between intra-assay CV values recorded in samples with active or inactive sediments were assessed by use of the Mann-Whitney *U* test.

For tests focused on determining the effect of predilution of urine samples, the results obtained at dilutions of 1:10, 1:20, and 1:100 were compared with the Friedman test, followed by the Bonferroni correction; the agreement between results obtained with the 3 dilutions was assessed via the Passing-Bablok and Bland-Altman tests.

To assess the influence of storage on urine protein and creatinine concentrations and on the UPC ratio, results obtained at different times for each storage condition (ie, room temperature, 4° and -20°C) were compared with the Friedman test, followed by the Bonferroni correction, with the basal value used as a reference. These tests were performed on either the whole set of data or the data grouped according to the presence or absence of an active sediment or according to the type of sampling (cystocentesis vs free catch). Statistical

analysis was performed with software.⁶ Values of *P* < 0.05 were considered significant.

Results

Characteristics of the samples—Thirty-three samples had an inactive sediment, and 17 samples had an active sediment. The most common sediment abnormalities were the presence of leukocytes or epithelial cells. Less commonly, crystals, spermatozoa, or RBCs were observed. Bacteria or casts were not detected in any urine sediments.

In the set that included all samples, the concentration of protein ranged from 1.6 to 479.0 mg/dL (mean \pm SD, 68.9 \pm 103.8 mg/dL; median, 28.0 mg/dL), the creatinine concentration ranged from 10.6 to 418.1 mg/dL (mean, 137.8 \pm 103.4 mg/dL; median, 98.3 mg/dL), the UPC ratio ranged from 0.02 to 5.29 (mean, 0.57 \pm 0.83; median, 0.32), and the USG ranged from 1.008 to 1.072 (mean, 1.032 \pm 0.017; median, 1.030).

Intra-assay repeatability—Results of individual intra-assay repeatability revealed that the intra-assay precision was lower for the urine creatinine concentration than for urine protein concentration and, consequently, for the UPC ratio (Table 1). This variability led to misclassification of samples that had UPC ratios close to the threshold values that were used to separate the 3 International Renal Interest Society classes (UPC ratios, 0.2 and 0.5; Tables 2 and 3).

A significant negative correlation between mean values and intra-assay CVs was recorded for the urine protein concentration ($r = -0.62$; $P < 0.001$) and UPC ratio ($r = -0.59$; $P = 0.002$). A significant positive correlation between USG and urine creatinine concentration ($r = 0.56$; $P = 0.005$), but not between USG and urine protein concentration ($r = 0.13$; $P = 0.055$) or UPC ratio ($r = 0.32$; $P = 0.128$), was found. Similarly, the USG was not correlated with the intra-assay CV of urine creatinine concentration ($r = -0.19$; $P = 0.361$), urine protein concentration ($r = -0.04$; $P = 0.862$), or UPC ratio ($r = -0.11$; $P = 0.616$).

Table 1—Intra-assay repeatability of tests (n = 25 samples tested 20 times) for creatinine concentration, protein concentration, and UPC ratio in canine urine samples.

Variable	Urine creatinine	Urine protein	UPC ratio
Mean \pm SD CV (%)	3.2 \pm 3.0	9.3 \pm 6.5	10.0 \pm 6.5
Median (range) CV (%)	2.0 (0.8–14.2)	8.3 (1.9–29.7)	8.6 (2.5–29.9)
No. of samples with CV < 5%	22	5	6
No. of samples with CV from 5% to 10%	2	14	9
No. of samples with CV from 10.1% to 20%	1	4	8
No. of samples with CV > 20.1%	0	2	2

Table 2—Frequency of misclassification of 5 canine urine samples tested 20 times and with mean UPC ratio close to the cutoff value of 0.2 that differentiates nonproteinuric versus borderline proteinuric patients.

Variable	Sample				
	1	2	3	4	5
Mean UPC ratio	0.17 (NP)	0.21 (BP)	0.22 (BP)	0.23 (BP)	0.23 (BP)
UPC ratio \leq 0.20 (NP)	20	9	9	2	0
UPC ratio > 0.20 (BP)	0	11	11	18	20

BP = Borderline proteinuric. NP = Nonproteinuric. P = Proteinuric.

Although the urine protein concentration ($P = 0.026$) and UPC ratio ($P = 0.041$) were significantly higher in the 10 samples with active sediments (70.04 ± 41.70 mg/dL and $0.96 \pm 1.47\%$, respectively), compared with the 15 samples with inactive sediments (38.01 ± 38.02 mg/dL and $0.43 \pm 0.60\%$, respectively), no significant differences were found between the intra-assay CVs recorded in samples with active or inactive sediments, with regard to the urine protein concentration ($8.13 \pm 4.74\%$ and $8.64 \pm 5.43\%$, respectively; $P = 0.649$) and UPC ratio ($8.40 \pm 5.08\%$ and $9.91 \pm 5.38\%$, respectively; $P = 0.531$). Similarly, no significant differences were found between the USG of samples with active sediments (1.032 ± 0.02) and inactive sediments (1.027 ± 0.012).

Effect of predilution of urine samples—Mean urine creatinine concentration and UPC ratio determined by means of the 1:10 dilution were significantly lower and higher, respectively, than those determined with the 1:20 dilution and 1:100 dilution (Table 4). The Bland-Altman test revealed a significant ($P = 0.003$) overestimation of urine creatinine concentration at the 1:100 dilution, compared with the 1:10 dilution, with a proportional bias detected by use of the Passing-Bablok test (intercept, -12.16 [95% CI, -22.36 to 7.53]; slope, 1.36 [95% CI, 1.09 to 1.48]). Nevertheless, with regard to the UPC ratio, no constant or proportional bias between the 1:10 and 1:100 dilutions was detected (intercept, -0.01 [95% CI, -0.10 to 0.05]; slope, 0.89 [95% CI, 0.73 to 1.18]) and the Bland Altman test did not reveal significant ($P = 0.318$) differences.

Significant differences and proportional or constant errors were not detected by comparison of the 1:10 and 1:20 ($P = 0.058$) dilutions for urine creatinine concentration (intercept, 2.89 ; [95% CI, 6.68 to 7.22]; slope, 1.04 [95% CI, 0.99 to 1.18]), whereas the UPC ratio had a slight proportional bias (intercept, 0.00 [95% CI, -0.01 to 0.01]; slope, 0.94 [95% CI, 0.87 to 0.97]) with a slight underestimation at increasing values.

Table 3—Frequency of misclassification of 3 canine urine samples tested 20 times and with mean UPC ratio close to the cutoff value of 0.5 that differentiates borderline proteinuric from proteinuric patients.

Variable	Sample		
	1	2	3
Mean UPC ratio	0.48 (BP)	0.50 (BP)	0.51 (P)
Measurements ≤ 0.50 (BP)	14	9	9
Measurements > 0.50 (P)	6	11	11

See Table 2 for key.

Table 4—Mean \pm SD (median) results of urine creatinine concentration measurements and UPC ratio with various predilution ratios for 25 canine urine samples.

Variable	Dilution ratio			Friedman test	Wilcoxon test
	1:10	1:20	1:100		
Urine creatinine (mg/dL)	127.1 ± 91.3 (89.9)	144.4 ± 105.2 (99.1)	156.5 ± 115.2 (107.7)	*	1:10 vs 1:20†; 1:10 vs 1:100†
UPC ratio	0.70 ± 1.14 (0.34)	0.53 ± 0.59 (0.32)	0.46 ± 0.42 (0.32)	*	1:10 vs 1:20*; 1:10 vs 1:100‡

*Values are significantly ($P < 0.001$) different. †Values are significantly ($P < 0.01$) different. ‡Values are significantly ($P < 0.05$) different.

Significant differences and proportional or constant errors were not detected between mean values recorded with the 1:20 and 1:100 dilutions for either the urine creatinine concentration (intercept, -8.08 [95% CI, -22.73 to 3.47]; slope, 1.12 [95% CI, 0.96 to 1.31]) or UPC ratio (intercept, -0.01 [95% CI, -0.08 to 0.05]; slope, 1.06 [95% CI, 0.78 to 1.28]).

Effect of storage time and temperature—During short-term storage at room temperature (Figure 1), mean values of the urine protein concentration, urine creatinine concentration, and UPC ratio did not significantly increase during the first 4 hours after sampling, either in the presence ($n = 6$) or in the absence (14) of an active sediment. Similarly, differences were not recorded in samples collected via cystocentesis ($n = 13$) or free catch (7; data not shown).

During long-term storage at room temperature (Figure 2), mean urine creatinine concentration did not significantly change for up to 72 hours. In all samples, a high individual variability was present. Urine protein concentration increased over time, with significant differences, compared with baseline, beginning after 48 hours of storage. Similarly, the UPC ratio had a significant progressive increase that was evident after 12 hours of storage, compared with baseline. The changes were detected either in the whole set of data or in samples grouped according to the presence of an active ($n = 11$) or inactive (14) sediment or to the type of sampling (cystocentesis, 14; free catch, 11; data not shown).

At 4°C , the urine creatinine concentration did change significantly for up to 1 week of storage, whereas the urine protein concentration was significantly higher, compared with baseline values, after 1 week of storage (Figure 3). The UPC ratio was significantly increased after 12 hours of storage. The comparison of results collected in the whole set of data with those obtained from samples with active ($n = 11$) and inactive (13) sediments or from samples collected via cystocentesis (14) and free catch (10) provided the same results for both the urine protein and creatinine concentrations (data not shown).

At -20°C (Figure 4), the urine creatinine concentration was stable. The concentration of urine protein was significantly higher in samples analyzed 1 week and 2 months after collection, and the UPC ratio was significantly higher in samples analyzed 1 week after collection. Most of these differences were not significant when results were analyzed on the basis of the presence ($n = 9$) or absence (9) of an active sediment or the type of sampling (cystocentesis, 9; free catch, 9; data not shown).

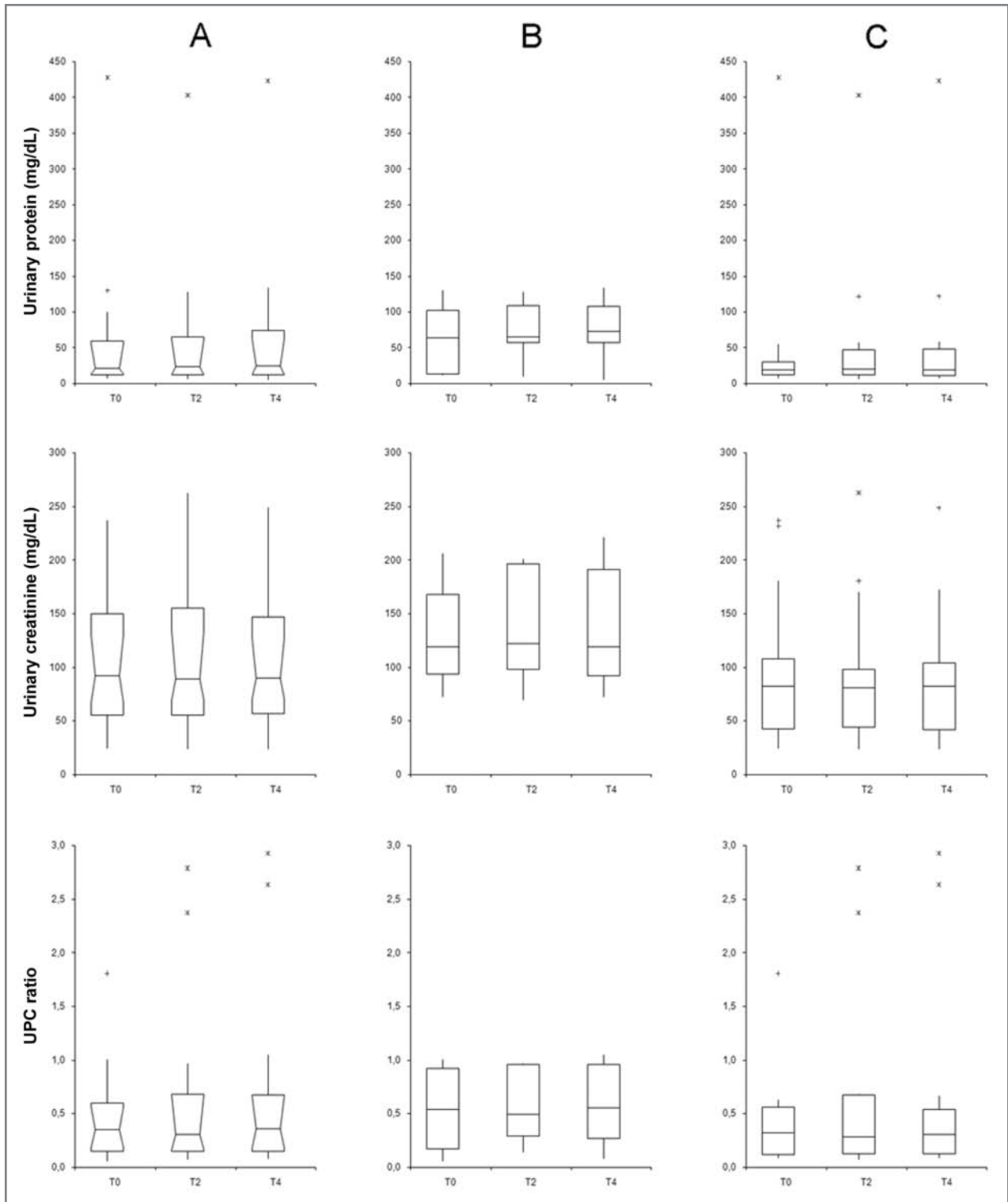


Figure 1—Distribution of the time of sample collection urine protein concentration, urine creatinine concentration, and UPC ratio recorded at the time of sample collection (T0) and after 2 and 4 hours (T2 and T4, respectively) of storage at room temperature (approx 20°C) urine samples from dogs. A—All samples (n = 20). B—Samples with active sediment (n = 6). C—Samples with inactive sediment (n = 14). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value of each group. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile. Crosses indicate outliers $> 1.5 \times$ IQR; asterisks indicate outliers $> 3.0 \times$ IQR.

Discussion

Measuring proteinuria is essential for the detection of renal damage and for establishing prognosis and

monitoring progression of renal disease in dogs. However, to provide useful clinical information, knowledge of the analytic and preanalytic factors that affect the methods used to determine the UPC ratio is a prerequi-

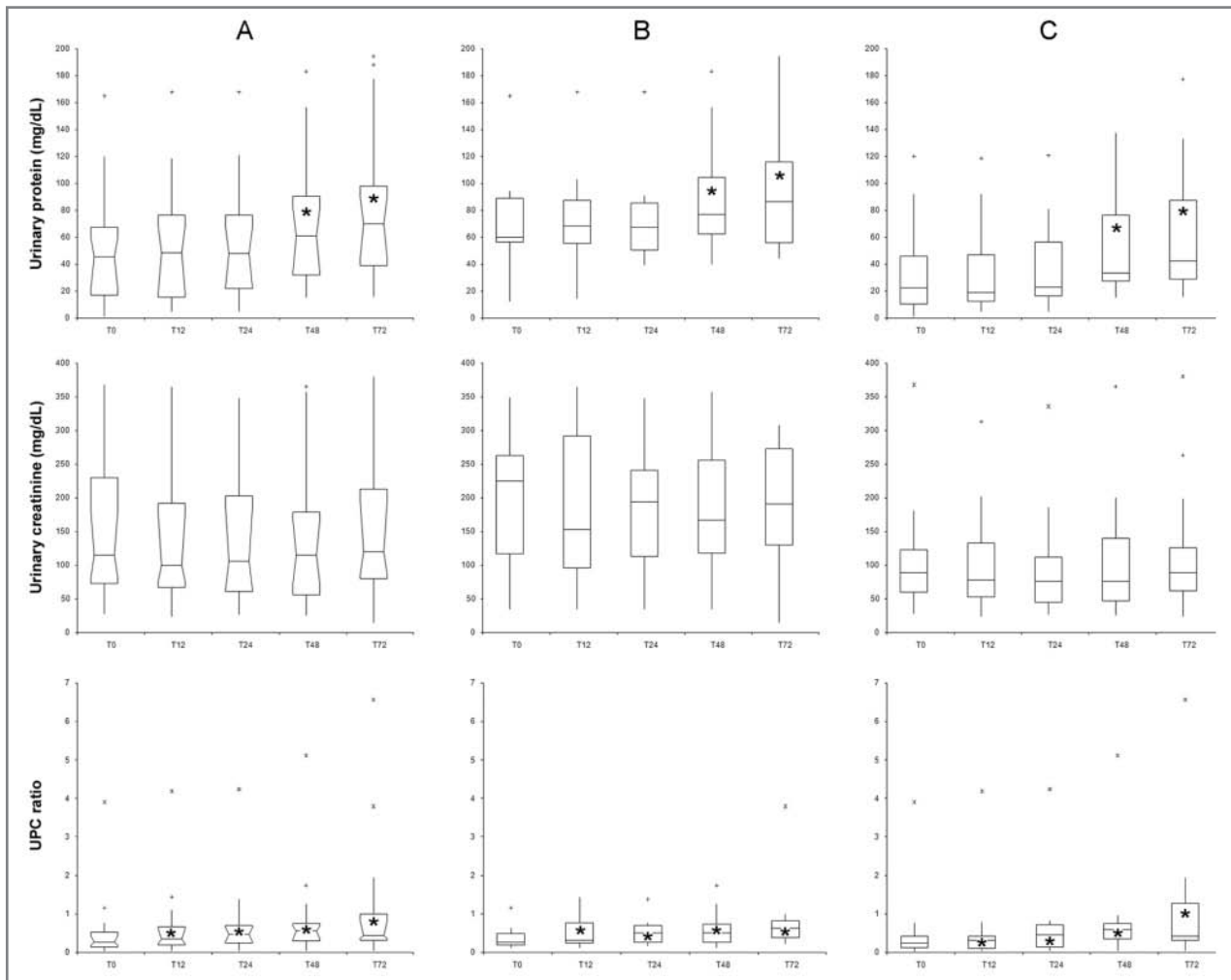


Figure 2—Distribution of urine protein concentration, urine creatinine concentration, and UPC ratio recorded at the time of sample collection (T0) and after 12, 24, 48, and 72 hours (T0 to T72) of storage at room temperature for urine samples from dogs. A— All samples (n = 25). B— Samples with active sediment (n = 11). C— Samples with inactive sediment (n = 14). Asterisks within boxes indicate a significant ($P < 0.05$) difference, compared with T0. See Figure 1 for remainder of key.

site. Data regarding reliability of methods used to quantify proteinuria in dogs have not been reported to date.

In the present study, median intra-assay precisions for urine creatinine concentration, urine protein concentration, and UPC ratio were 2%, 8.3%, and 8.6%, respectively. The analytic variability was higher at low urine protein and creatinine concentrations, which is similar to many analytes¹⁰ at values close to the lower limit of detection (ie, the smallest amount of analyte that can be measured with sufficient precision and accuracy by use of a particular method). Thus, because the urine creatinine concentration was directly correlated to the USG, it may have major analytic variability at a low USG. For this reason, the USG should always be taken into account to confidently interpret UPC ratio results, similar to other methods used to quantify proteinuria in dogs, such as the urine dipstick test.⁵ In contrast, the urine protein concentration in the present study did not depend on USG. As recently reported, urine protein concentration is minimally affected by the method of collection,⁹ unless the sediment is active because of urinary tract inflammation. In clinical practice, the UPC ratio should not be determined in

dogs with pyuria, hematuria, or bacteriuria because the chance of albuminuria increases.¹¹ In the study reported here, samples with active sediments and various degrees of proteinuria were included to explore analytic variability under various clinical conditions. However, comparison of intra-assay CVs recorded in dogs with active and inactive sediments revealed that the presence of an active sediment does not influence UPC ratio variability. Although it is theoretically possible that this difference would be significant in a larger sample population or in a population with a different proportion of samples with an active sediment, this is unlikely, considering that mean values were close to each other and P values were largely far from significant; hence, the potential bias attributable to inclusion of different causes of proteinuria should not have affected the reliability of the results.

From a practical standpoint, these results suggest that in samples that are highly diluted or have low urine protein concentrations, the magnitude of analytic variability increases, possibly leading to an incorrect categorization according to the International Renal Interest Society staging system. This was the case

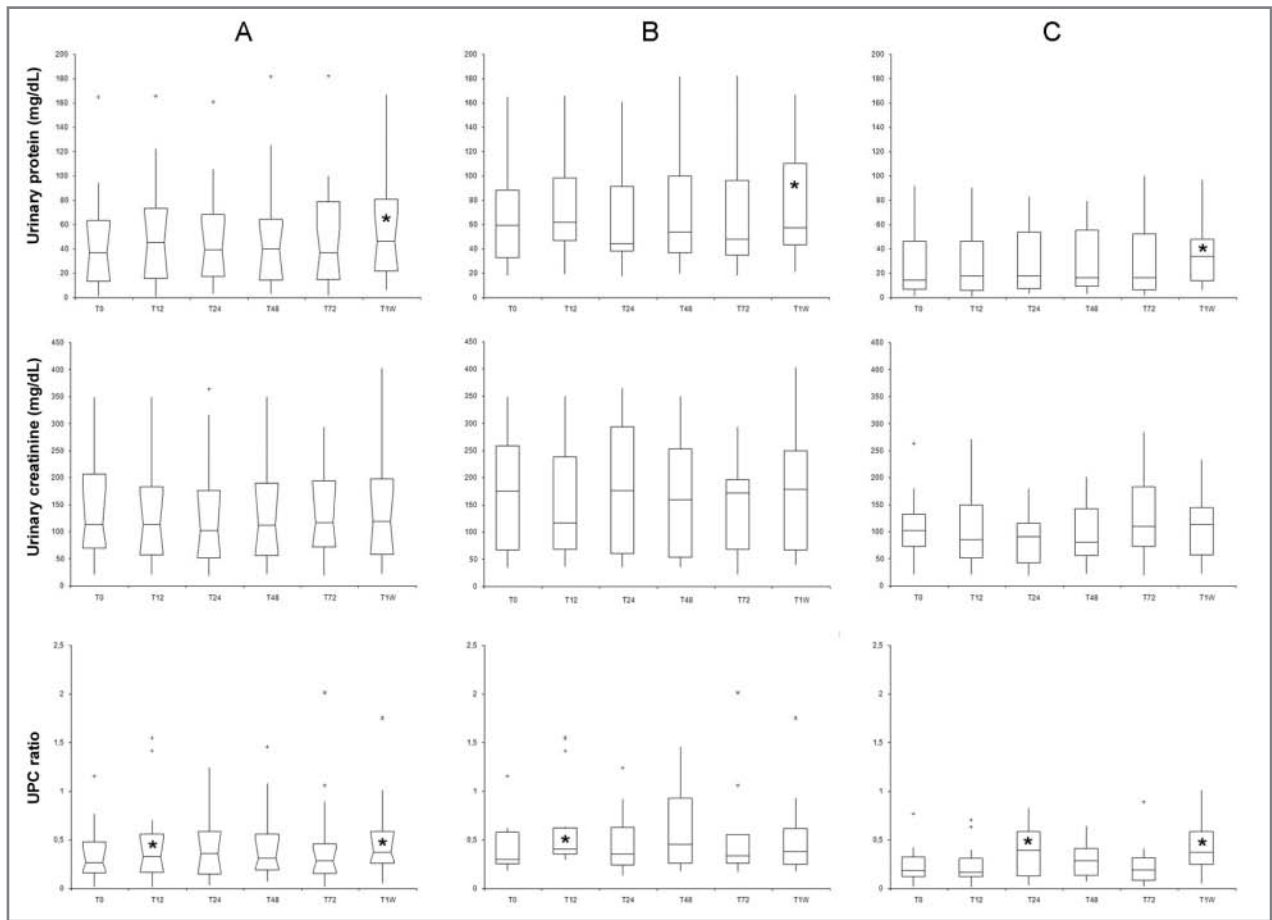


Figure 3—Distribution of urine protein concentration, urine creatinine concentration, and UPC ratio recorded at the time of sample collection (T0) and after 12, 24, 48, and 72 hours (T0 to T72) and 1 week (T1W) of storage at 4°C for urine samples from dogs. A—All samples (n = 24). B—Samples with active sediment (n = 11). C—Samples with inactive sediment (n = 13). See Figures 1 and 2 for remainder of key.

for urine samples with a UPC ratio close to the cutoff values between consecutive International Renal Interest Society stages. Because there is also some biological variability of the UPC ratio over time in individual dogs,^{1,12,13} both of these potential sources of erroneous interpretation (ie, analytic and biological variability) should be kept in mind. As suggested by LeVine et al,¹³ to reduce individual variability of the UPC ratio, pooling several urine samples from the same dog may be considered. Our results support this recommendation, although the analysis of pooled urine samples can be affected by storage artifacts.

The tests performed with different dilutions revealed that the 1:10 dilution was too low to reduce the urine creatinine concentration to the linear range of the assay, affecting its correct quantification and, in turn, the results obtained for the UPC ratio. On the other hand, there was no difference between samples diluted to 1:20 and 1:100, but analysis of individual results (data not shown) yielded large discrepancies in some instances, mostly characterized by high urine creatinine concentration. Because the USG correlates with urine creatinine concentration if the USG is high (> 1.030 in the present study), a 1:100 dilution is advisable so that the urine creatinine concentration will be in the linear range. However, in urine samples with a low USG, a 1:100 dilution might be excessive, leading

to measurement of urine creatinine concentration close to or less than the lower limit of detection and thereby increasing imprecision or inaccuracy; thus, with these samples, a 1:20 dilution would be recommended.

Assessing the influence of storage time and temperature was performed to mimic common conditions that occur in routine practice (eg, shipping samples to external laboratories for analysis within 3 days after collection and analysis of refrigerated samples within 1 week after collection) or in retrospective research studies (eg, analysis of frozen samples several months after collection). It was found that the storage of urine at room temperature leads to a preanalytic increase of urine protein concentration and, consequently, an artifactual increase of the UPC ratio. In the first 4 hours after sampling, this increase was mild and did not induce a significant difference, compared with baseline values, but it became significant at 12 hours. Urine protein concentration was more stable when samples were stored at 4°C, although the UPC ratio transiently increased at 12 hours (possibly because of slow cooling) and after 1 week. The frozen samples were only minimally affected by preanalytic variability during storage. Even though significant differences were occasionally recorded for frozen samples over time, they were more important from a statistical perspective than from a biological or diagnostic standpoint, given that the mean

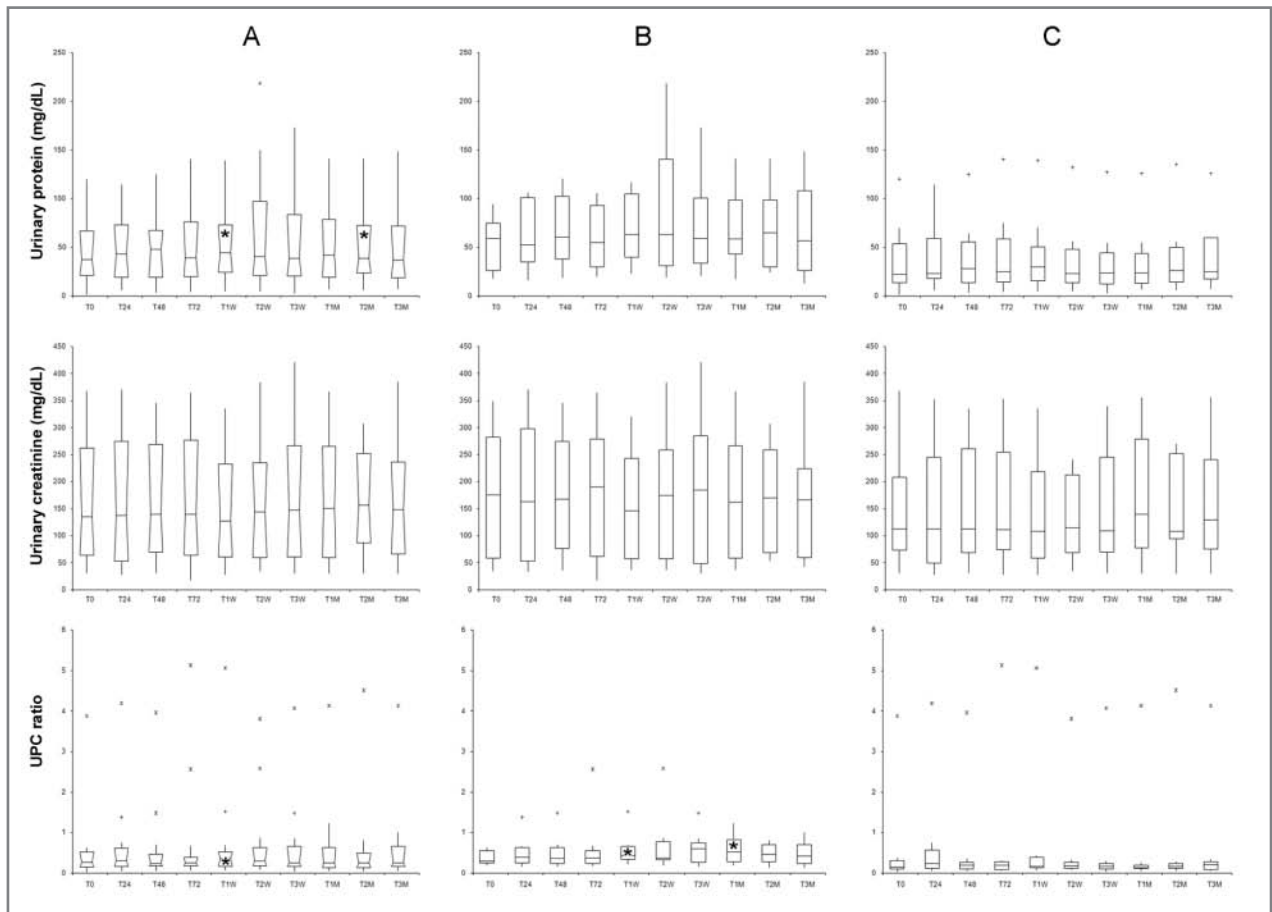


Figure 4—Distribution of urine protein concentration, urine creatinine concentration, and UPC ratio recorded at the time of sample collection (T0) and after 24, 48, and 72 hours (T0 to T72); 1, 2, and 3 weeks (T1W to T3W); and 1, 2, and 3 months (T1M to T3M) of storage at -20°C for urine samples from dogs. A—All samples ($n = 18$). B—Samples with active sediment ($n = 9$). C—Samples with inactive sediment ($n = 9$). See Figures 1 and 2 for remainder of key.

values recorded from paired samples at different times were similar.

It is difficult to explain the increase in urine protein concentration because denaturation of proteins associated with storage at room temperature would have induced a decrease, rather than an increase, in urine protein concentration. However, dehydration of the sample during storage, if it occurred, would have increased the urine creatinine concentration, thereby minimizing the effect on the UPC ratio. Bacteriologic culture of urine was not performed because it is not included in routine urinalysis unless clinical or cytologic suspicion of bacterial infection is present. Therefore, it is possible that a low number of bacteria might be present even in the absence of cytologic evidence of bacteriuria and that the increase in urine protein concentration occurred because of bacterial proteins. This is unlikely, because only detectable pyuria induces an increase of UPC ratio.¹¹ The lack of influence of bacteria potentially present in urine is supported by the detection of similar important findings regarding the UPC ratio in samples grouped according to the type of sampling (bacteria are more likely to be present in samples collected by free catch than in samples collected by cystocentesis) as well as in samples grouped according to the presence or absence of an active sediment. This latter comparison also allowed us to exclude the possibility that the increased urine protein concentration recorded over time was at-

tributable to leakage of proteins from cells (if these were not completely removed in the initial centrifugation steps, although this is also unlikely because samples were centrifuged again after thawing).

Contrary to what was expected, the present study revealed basically the same significant differences in samples with active and inactive sediments. Qualitative analysis of proteinuria (eg, by sodium dodecylsulphate electrophoresis or by western blotting) would be extremely useful to fully understand which proteins are responsible for the changes detected in stored samples. However, this information would not add much from a practical point of view because, independent of the mechanisms responsible for this artifact, an increase of the UPC ratio in stored samples can lead to a misclassification of proteinuria according to the International Renal Interest Society staging system. Therefore, to avoid storage artifacts, urine samples should be kept frozen or maintained at 4°C and analyzed within 3 days after collection. At room temperature, we detected storage artifacts 12 hours after collection but not in the first 4 hours after collection. Further studies on a larger number of samples are needed to investigate whether shorter time periods induce storage artifacts.

In the present study, measurement of the UPC ratio via the pyrogallol method to determine protein concentration revealed a moderate degree of analytic vari-

ability. Additional sources of variation in typical diagnostic laboratories include the instrument used, batch of reagent, and skill of technical staff. To minimize the possible influence of these factors, all the tests were performed by the same operators (GR and SC) and the same batch of reagent was used to process all the samples in the present study. The instrument used in our laboratory is one of the most popular spectrophotometers used in veterinary laboratories and is known to be precise and accurate for several analytes.^{14,15} At least for some analytes evaluated with this instrument, it seems that the manufacturer of the reagents was not associated with the quality of the results.¹⁵ In humans, it has been found that measurement of urinary proteins with this instrument and the pyrogallol method is precise,¹⁶ with intra-assay CVs similar to those recorded in the present study. However, the precision of the pyrogallol method, widely used in veterinary medicine, is highly independent of the instrument,¹⁷ and with the use of a specific calibrator for urinary proteins, as in the present study, performance of this method is similar to that with other reagents.¹⁸ Also, the Jaffé method used to measure urine creatinine concentration is considered precise and has analytic performance similar to that of other methods, including the enzymatic rate assay or the high-performance liquid chromatography method,¹⁹ which is considered the gold standard for urine creatinine concentration measurement.²⁰ The enzymatic method is recommended in plasma because it is less prone to analytic interference²¹ and provides results similar to those of high-performance liquid chromatography.²⁰ However, measurement of urine creatinine concentration by the Jaffé method is still recommended, considering that because of the low amount of interfering substances in urine, it has been reported not to result in overestimation.²¹

It is unlikely that technical or methodological factors influenced the analytic variability in the present study. Nevertheless, it would be advisable that each veterinary laboratory establishes its own intra-assay CV to be taken into account for correct interpretation of results. Moreover, the intra-assay imprecision is higher in samples with low urine protein concentration or low USG. In the latter situation, the intra-assay imprecision may be attributable to the lower urine creatinine concentration, compared with more concentrated urine. In contrast, highly concentrated urine can be associated with moderate inaccuracy attributable to a urine creatinine concentration beyond the linear range of the detection method. In this situation, the use of a predilution factor greater than that routinely used by most instruments (eg, 1:100 instead of 1:20) can improve the reliability of the method. Because of these sources of variability, UPC ratios close to the threshold limit should be carefully interpreted to avoid misclassification of samples and serial analysis should be considered. Furthermore, results of the present study confirmed the importance of determining the UPC ratio as soon as samples are collected to avoid an artifactual increase in protein concentration. If this is not possible, samples should be refrigerated and analyzed within 4 days after collection or, even better, immediately frozen to increase their stability and minimize the risk of

misclassification of proteinuria, especially when UPC ratios are close to the International Renal Interest Society cutoff points.

-
- a. Cobas Mira, Roche Diagnostics, Basel, Switzerland.
 - b. Fluitest USP, Analyticon Biotechnologies AG, Lichtenfels, Germany.
 - c. Creatinina, Real-Time Diagnostics, Viterbo, Italy.
 - d. Clinical Refractometer, model 105, Sper Scientific, Scottsdale, Ariz.
 - e. Analyse-it, Analyse-it Software Ltd, Leeds, West Yorkshire, England.
-

References

1. Lees GE, Brown SA, Elliott J, et al. Assessment and management of proteinuria in dogs and cats: 2004 ACVIM forum consensus statement (small animal). *J Vet Intern Med* 2005;19:377–385.
2. Grauer GF, Greco DS, Getzy DM, et al. Effects of enalapril versus placebo as a treatment for canine idiopathic glomerulonephritis. *J Vet Intern Med* 2000;14:526–533.
3. Jacob F, Polzin J, Osborne CA, et al. Evaluation of the association between initial proteinuria and morbidity rate or death in dogs with naturally occurring chronic renal failure. *J Am Vet Med Assoc* 2005;226:393–400.
4. Elliott J, Watson ADJ. Chronic kidney disease: staging and management. In: Bonagura JD, Twedt DC, eds. *Kirk's current veterinary therapy*. 14th ed. St Louis: Saunders, 2008;883–892.
5. Zatelli A, Paltrinieri S, Nizi F, et al. Evaluation of a urine dipstick test for confirmation or exclusion of proteinuria in dogs. *Am J Vet Res* 2010;71:235–240.
6. DiBartola SP, Chew DJ, Jacobs G. Quantitative urinalysis including 24-hour protein excretion in the dog. *J Am Anim Hosp Assoc* 1980;16:537–546.
7. Center SA, Wilkinson E, Smith CA, et al. 24-hour urine protein/creatinine ratio in dogs with protein-losing nephropathies. *J Am Vet Med Assoc* 1985;187:820–824.
8. Moore FM, Brum SL, Brown L. Urine protein determination in dogs and cats: comparison of dipstick and sulfosalicylic acid procedures. *Vet Clin Pathol* 1991;20:95–97.
9. Beatrice L, Nizi F, Callegari D, et al. Comparison of urine protein-to-creatinine ratio in urine samples collected by cystocentesis versus free catch in dogs. *J Am Vet Med Assoc* 2010;236:1221–1224.
10. Westgard JO. Basic method validation. 2nd ed. Madison, Wis: Westgard QC Inc, 2003.
11. Vaden SL, Pressler BM, Lappin MR, et al. The effects of urinary tract inflammation and sample blood contamination on urine albumin and total protein concentrations. *Vet Clin Pathol* 2004;33:14–19.
12. Nabity MB, Boggess MM, Kashtan CE, et al. Day-to-day variation of the urine protein:creatinine ratio in female dogs with stable glomerular proteinuria caused by X-linked hereditary nephropathy. *J Vet Intern Med* 2007;21:425–430.
13. LeVine DN, Zhang D, Harris T, et al. The use of pooled vs serial urine samples to measure urine protein:creatinine ratios. *Vet Clin Pathol* 2010;39:53–56.
14. Kanluan T, Intaramanee S, Tangvorasittichai S, et al. Laboratory evaluation of the Cobas Mira S random access analyser. *J Autom Chem* 1991;13:97–100.
15. Morandi PA, Deom A, Kessler D, et al. Retrospective analysis of 88,429 serum and urine glucose EQA results obtained from professional laboratories and medical offices participating in surveys organized by three European EQA centers between 1996 and 2007. *Clin Chem Lab Med* 2010;48:1255–1262.
16. Antunes VV, Veronese FJ, Morales JV. Diagnostic accuracy of the protein/creatinine ratio in urine samples to estimate 24-h proteinuria in patients with primary glomerulopathies: a longitudinal study. *Nephrol Dial Transplant* 2008;23:2242–2246.
17. Watanabe N, Kamel S, Ohkubo A. Urinary proteins measured with a pyrogallol red-molybdate complex, manually and in a Hitachi 726 automated analyzer. *Clin Chem* 1986;32:1551–1554.

18. Marshall T, Williams KM. Total protein determination in urine: elimination of a differential response between the Coomassie blue and pyrogallol red protein dye-binding assays. *Clin Chem* 2000;46:392–398.
19. Campins Falcò P, Tortajada Genaro LA, Meseger Lloret S, et al. Creatinine determination in urine samples by batchwise kinetic procedure and flow injection analysis using the Jaffè reaction: chemometric study. *Talanta* 2001;55:1079–1089.
20. Palm M, Lundblad A. Creatinine concentration in plasma from dog, rat, and mouse: a comparison of 3 different methods. *Vet Clin Pathol* 2005;34:232–236.
21. Braun JP, Lefebvre HP, Watson ADJ. Creatinine in the dog: a review. *Vet Clin Pathol* 2003;32:162–179.