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 crisis 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...
and centrifugation, samples were kept at room temper-

cally guided cystocentesis (n = 36) or free catch (14),

 Samples were collected via either ultrasonographi-

analytic performance and not on the diagnostic role

of Milan. Because the investigation focused on the

ous diseases, in accordance with the guidelines of

Milan for routine wellness visits or because of vari-

owned dogs following admission at the University of

ed consent of the owners, from 50 client-

performed with 50 urine samples collected, under

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 cre-
atinine 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 vari-
ous 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 ultrasonographi-
cally guided cystocentesis (n = 36) or free catch (14),
according to established protocols. 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 sedi-
ment 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 X g) within 30 minutes
after collection. During the period between collection and
centrifugation, samples were kept at room temper-
ature (approx 20°C).

From the first tube, 4.5 mL of supernatant was re-
moved and the remaining 0.5 mL of urine was used to
resuspend the urine sediment. A drop of the resuspend-
ed 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 ac-
tive 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 meas-
uring the concentration of creatinine and protein with an
automated spectrophotometer and by the following
formula: UPC ratio = urine protein concentration/urine
creatinine concentration. Urine protein concentrations
were determined with the pyrogallol red method from
undiluted urine, and urine creatinine concentrations
were determined with the modified Jaffe method from
urine samples that were prediluted to 1:2.0. Also from
the second tube, a drop of supernatant was used to de-
terminate the USG with a handheld refractometer cali-
brated 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 repeatabil-
ity, 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 centrifuga-
tion. For each sample, the mean and SD of urine pro-
tein concentration, urine creatinine concentration, and
UPC ratio were measured, and the CV was calculated as
follows: CV = SD/mean × 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, border-
line proteinuric if the UPC ratio was from 0.21 to 0.50,
and proteinuric if the UPC ratio was > 0.50. The pos-
sible influence of intra-assay imprecision was assessed
on samples with a mean UPC ratio close to the Interna-
tional 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 cen-
trifugation, 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 tripli-
cate 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 ali-
quots of 300 μL 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 µL, which were stored at room temperature and then analyzed immediately and 1, 2, 4, 8, and 72 hours after storage. In addition, 24 samples were used to prepare 6 aliquots of 300 µL stored at 4°C and analyzed 1, 2, 4, 8, and 72 hours after and 1 week after storage; 18 samples were used to prepare 10 aliquots of 300 µL 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.

In the set that included all samples, the concentration of protein ranged from 1.6 to 479.0 mg/dL (mean ± SD, 68.9 ± 103.8 mg/dL; median, 28.0 mg/dL), the creatinine concentration ranged from 10.6 to 418.1 mg/dL (mean, 137.8 ± 103.4 mg/dL; median, 98.3 mg/dL), the UPC ratio ranged from 0.02 to 5.29 (mean, 0.57 ± 0.83; median, 0.32), and the USG ranged from 1.008 to 1.072 (mean, 1.032 ± 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 ($r = 0.13; P = 0.053$) 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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Urine creatinine</th>
<th>Urine protein</th>
<th>UPC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD CV (%)</td>
<td>3.2 ± 3.0</td>
<td>9.3 ± 6.5</td>
<td>10.0 ± 6.5</td>
</tr>
<tr>
<td>Median (range) CV (%)</td>
<td>2.0 (0.8–14.2)</td>
<td>8.3 (1.9–29.7)</td>
<td>8.6 (2.5–29.9)</td>
</tr>
<tr>
<td>No. of samples with CV &lt; 5%</td>
<td>22/5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>No. of samples with CV from 5% to 10%</td>
<td>2/14</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>No. of samples with CV from 10% to 20%</td>
<td>1/4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>No. of samples with CV &gt; 20%</td>
<td>0/2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

### 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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean UPC ratio</td>
<td>1</td>
</tr>
<tr>
<td>UPC ratio ≤ 0.20 (BP)</td>
<td>0.17 (NP)</td>
</tr>
<tr>
<td>UPC ratio &gt; 0.20 (BP)</td>
<td>20</td>
</tr>
</tbody>
</table>

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 ± 1.47%, respectively), compared with the 15 samples with inactive sediments (38.01 ± 38.02 mg/dL and 0.43 ± 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 ± 4.74% and 8.64 ± 5.43%, respectively; P = 0.649) and UPC ratio (8.40 ± 5.08% and 9.91 ± 5.38%, respectively; P = 0.531). Similarly, no significant differences were found between the USG of samples with active sediments (1.027 ± 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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean UPC ratio</td>
<td>0.48 (BP)</td>
<td>0.50 (BP)</td>
<td>0.51 (P)</td>
<td></td>
</tr>
<tr>
<td>Measurements ≤ 0.50 (BP)</td>
<td>14</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Measurements &gt; 0.50 (P)</td>
<td>6</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>1:10</th>
<th>1:20</th>
<th>1:100</th>
<th>Friedman test</th>
<th>Wilcoxon test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine creatinine (mg/dL)</td>
<td>127.1 ± 91.3 (89.9)</td>
<td>144.4 ± 105.2 (99.1)</td>
<td>156.5 ± 115.2 (107.7)</td>
<td>*</td>
<td>1:10 vs 1:20; 1:10 vs 1:100</td>
</tr>
<tr>
<td>UPC ratio</td>
<td>0.70 ± 1.14 (0.34)</td>
<td>0.53 ± 0.59 (0.32)</td>
<td>0.46 ± 0.42 (0.32)</td>
<td>*</td>
<td>1:10 vs 1:20; 1:10 vs 1:100</td>
</tr>
</tbody>
</table>

*Values are significantly (P < 0.001) different. †Values are significantly (P < 0.01) different. ‡Values are significantly (P < 0.05) different.
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-
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 analytes 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. 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, 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. 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...
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, both of these potential sources of erroneous interpretation (ie, analytic and biological variability) should be kept in mind. As suggested by LeVine et al, 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 protein 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
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. 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 attributable 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,11 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 Jaffe 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.20 However, measurement of urine creatinine concentration by the Jaffe 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 artificial increase in protein concentration. If this is not possible, samples should be refrigerated and analyzed within 24 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.

References


