Determination of and correlation between urine protein excretion and urine protein-to-creatinine ratio values during a 24-hour period in healthy horses and ponies

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Objective—To determine whether urine protein-to-creatinine (UP:C) ratio assessment provides an estimate of urine protein excretion (UPE) over a 24-hour period in horses and ponies, establish a preliminary UP:C ratio reference range, and determine UP:C ratio variation over time in healthy equids.

Animals—11 female horses and 6 female ponies.

Procedures—Urine was collected from all equids at 4-hour intervals for 24 hours. Total 24-hour UPE (mg of protein/kg of body weight) and UP:C ratio were determined; these variables were also assessed in aliquots of urine collected at 4-hour intervals. On 2 additional days, urine samples were also obtained from 6 horses (1 sample/horse/d) to determine day-to-day variation in UP:C ratio. Correlation between 4-hour or 24-hour UPE and UP:C ratio values was assessed. Reference ranges for 24-hour UPE, 24-hour UP:C ratio, and 4-hour UP:C ratios were calculated as central 95th percentiles of observed values.

Results—Mean 24-hour UPE (4.28 ± 2.99 mg/kg) and 24-hour UP:C ratio (0.0 to 0.37) had excellent correlation ($\rho = 0.826$; $P < 0.001$) in both horses and ponies; analysis of 4-hour data also revealed good correlation ($\rho = 0.782$; $P < 0.001$) with these variables. Calculated UPE and UP:C ratio reference ranges were similar to established ranges in other species. Day-to-day variability in UP:C ratio was minimal, and all results were within the reference range calculated by use of the 24-hour urine samples.

Conclusions and Clinical Relevance—Assessment of the UP:C ratio appears to be a reliable method for estimating 24-hour UPE in horses and ponies. (*Am J Vet Res* 2009;70:1551–1556)

Protein in urine may be prerenal, renal, or postrenal in origin, and renal protein loss is subclassified as glomerular or tubular proteinuria.[1] Retention of macromolecules and cells during formation of the glomerular ultrafiltrate requires maintenance of a maximum glomerular endothelial pore size, negative electrostatic charge of the glomerular basement membrane, and an intact podocyte slit diaphragm. In various disease states, this filtration system is disrupted, which results in excess protein loss in urine. This loss of glomerular integrity may develop either with primary glomerular diseases or with systemic inflammatory conditions and presumptive secondary deposition of immune complexes within the glomeruli. Regardless of the cause, severity of proteinuria is significantly associated with the rate of progression of kidney disease in dogs and humans with naturally occurring diseases.[2–5] Determination of UP concentration is therefore a useful ancillary test for the early diagnosis of inflammatory diseases in dogs, cats, and people because detection of excess UP may be the first indication that kidney, vascular, or systemic inflammatory disease is present.[4–9]

The gold standard for quantification of UPE involves 24-hour collection of all excreted urine and measurement of total protein concentration in a representative urine sample of known volume.[10,11] Unfortunately, this procedure is particularly cumbersome in animals and limits its clinical application. However, measurement

Abbreviations

UC	Urine creatinine
UP	Urine protein
UP:C	Urine protein-to-creatinine
UPE	Urine protein excretion

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of the UP:C ratio in dogs, cats, and humans is a considerably less labor intensive, but still accurate, means of estimating UPE because the UP:C ratio has excellent correlation with 24-hour UPE in these species.[11–15] Standard laboratory assays are available for measurement of UP and UC concentrations and require only a single arbitrarily collected urine sample. Values of UP:C ratio > 0.5 in dogs and > 0.4 in cats are considered abnormal; when such abnormal values are detected in these species, further diagnostic testing is recommended if glomerular proteinuria is suspected.[1]

Primary renal disease is rare in horses; however, subclinical renal damage develops secondary to many systemic diseases.[16] Metabolic derangements such as insulin resistance may result in microvascular disease, and proteinuria of glomerular origin may result from this vascular damage.[17] Urine protein excretion in horses has not been thoroughly investigated, to our knowledge. Although transient postexercise proteinuria in horses has been reported,[18] a reference range for 24-hour UPE has not been established and correlation of a random sample UP:C ratio with 24-hour UPE is unknown. Therefore, the primary objective of the study of this report was to determine whether UP:C ratio assessment provides an estimate of UPE over a 24-hour period in horses and ponies, establish a preliminary UP:C ratio reference range, and determine UP:C ratio variation over time in healthy equids. We hypothesized that horses and ponies have UP:C ratios that are comparable to those in other species and that UP:C ratio does not vary significantly over a 24-hour period or from day to day.

Materials and Methods

Experimental animals—The study was reviewed and approved by the Purdue University Animal Care and Use Committee. Eleven mares (weight range, 492 to 591 kg) and 6 female ponies (weight range, 144 to 360 kg) of varying adult ages and mixed breeds were selected from the Purdue University Veterinary Teaching Hospital teaching herd for inclusion in the study. Physical examination of each equid prior to enrollment in the study did not reveal any abnormalities. Serum and urine samples were analyzed at the time of enrollment, and all variables (including BUN and serum creatinine concentrations) were within reference ranges for all equids. The horses and ponies were each housed in individual box stalls with free-choice hay and water.

During the experimental period, health status of each equid was monitored via physical examinations performed every 12 hours and via assessment of activity level and appetite performed every 4 hours. A 24-hour urine collection procedure was performed for each of the 17 equids. From among the 11 mares, a subset of 6 horses (weight range, 500 to 591 kg) was established. Two urine samples were collected via urinary bladder catheterization on 2 nonconsecutive additional days over a period of 4 months (during spring and summer) from each of the subset horses (1 sample/horse/d). Thus, 3 urine samples (one 24-hour collection and 2 random samples) were obtained from each of the 6 subset mares; one 24-hour collection was performed for the other 5 mares and 6 ponies.

Urethral catheterization and urine collection protocol—Indwelling urethral catheters were placed in all equids for 24-hour urine collections as previously described.[16] In brief, each horse's or pony's tail was bandaged and the perineal area was aseptically cleansed. A 28-F, 30-mL balloon catheter[a] was placed transurethrally into the bladder, urine in the bladder was evacuated, and a closed urine collection system was attached to the catheter. Each system emptied via passive flow into a sterile 5-L plastic collection bag that was attached to the tail. When necessary because of temperament, longer tubing was used and the bag was attached instead to the horse's or pony's mane. Equids were unrestrained within their stalls throughout the period of urine collection. Because some study animals were visibly stressed during urethral catheter placement, an interval of 2 hours was allowed to elapse before commencement of urine collection from all equids to minimize possible effects of stress-associated proteinuria or proteinuria associated with microtrauma during catheterization. After this 2-hour period, urine bags were emptied and the collected urine was discarded; this time point was designated as 0 hours. Urine was then removed from the collection bags at 4-hour intervals for 24 hours (at 4, 8, 12, 16, 20, and 24 hours); at each time point, urine volume was measured by use of a graduated cylinder and recorded. Ten percent of each 4-hour collection volume was removed to contribute to a representative pooled 24-hour sample for each equid. An additional 5 mL of urine from each 4-hour collection volume was removed and stored at –20°C for measurement of UP and UC concentrations. All indwelling catheters were removed after the 24-hour collection period, and equids were returned to housing on pasture within the Purdue University Veterinary Teaching Hospital teaching herd; animals were monitored every 12 hours for 24 hours for adverse effects or complications of catheterization.

The 6 horses in the subset were housed within the teaching herd and fed grass hay and free-choice water during a period of 4 months (during spring and summer). To collect a random urine sample from each subset horse on each of 2 days, the perineal region was aseptically prepared and the urinary bladder was catheterized by use of a rigid urinary catheter and a standard clinical technique. On each of the 2 additional days, 5 to 10 mL of urine was collected and frozen at –20°C until analysis.

Measurements of UP and UC concentrations—Urine total protein and UC concentrations were determined at the Purdue University Veterinary Teaching Hospital Clinical Pathology Laboratory. Urine samples were centrifuged for 8 minutes at 3,500 × *g* prior to analysis, and UP and UC concentrations were then measured by use of standard methods.[b,c] The UPE values were calculated as the amount of UP (in mg) per kg of body weight. Urine protein-to-creatinine ratio was calculated by normalizing the UP concentration against the UC concentration to generate a unitless ratio.

Statistical analysis—The distributions of 24-hour UPE, UPE determined at 4-hour intervals, 24-hour UP:C ratios, and UP:C ratios determined at 4-hour intervals for horses, ponies, or all equids as a single group were

analyzed by means of Kolmogorov-Smirnov goodness-of-fit tests. All variables were nonnormally distributed. By use of the Wilcoxon rank sum test, median 24-hour UPE in horses versus 24-hour UPE in ponies, UPE determined at 4-hour intervals in horses versus UPE determined at 4-hour intervals in ponies, 24-hour UP:C ratios in horses versus 24-hour UP:C ratios in ponies, and UP:C ratios determined at 4-hour intervals in horses versus UP:C ratios determined at 4-hour intervals in ponies were compared. The UPE and UP:C ratio results from each 4-hour interval collection for the 11 horses, 6 ponies, or all 17 equids as a single group and UP:C ratio results for the subset of 6 horses on the 3 different study days were compared with each other by use of the Kruskal-Wallis equality-of-populations rank test. Correlation was determined by use of the Spearman rank correlation coefficient. Significance was designated at a value of $P < 0.05$ for all analyses. Reference ranges for 24-hour UPE, 24-hour UP:C ratio, and 4-hour UP:C ratio were constructed by use of the central 95th percentile of values (obtained from all 17 equids) following transformation into parametric distribution. All analyses were conducted by use of a statistical software program.[d]

Results

All equids remained healthy throughout the study (determined on the basis of results of physical examinations performed every 12 hours and observation of activity level and appetite performed every 4 hours). No adverse effects or complications occurred during or following the 24-hour urine collections in any equids or during or following the intermittent catheterizations in the subset of 6 horses.

The 24-hour UPE (ie, amount of UP [in mg]/kg of body weight) in horses and ponies ranged from 1.02 to 11.98 mg/kg (median, 3.29 mg/kg; mean ± SD, 4.28 ± 2.99 mg/kg). The UP:C ratio for 24-hour collection periods (determined from pooled aliquots from urine volumes obtained at each 4-hour collection time point) ranged from 0.06 to 0.41 (median, 0.11; mean ± SD, 0.14 ± 0.10). The pooled 24-hour UP:C ratio correlated well with 24-hour UPE ($\rho = 0.826$) and was significant ($P < 0.001$; **Figure 1**). The median 24-hour UPE in ponies was not significantly ($P = 0.315$) different from that in horses (4.07 vs 3.19 mg/kg, respectively); however, the median 24-hour UP:C ratio for ponies (median, 0.13; range, 0.10 to 0.41) was significantly ($P = 0.035$) greater than that determined for horses (median, 0.09; range, 0.06 to 0.21).

Both UPE values and UP:C ratios at the 4-hour time points varied moderately over the 24-hour collection period, but were well correlated ($\rho = 0.782$; $P < 0.001$ [data not shown]). The UP:C ratio of 1 of the 4-hour samples (n = 102 samples) obtained from the 17 equids could not be measured because the sample was too viscous to allow assessment of creatinine concentration; therefore, this sample was excluded from the analyses. The UP:C ratio determined at 4-hour collection time points ranged from 0.03 to 0.93 (median, 0.11; mean ± SD, 0.15 ± 0.14). The 4-hour UPE values did not differ significantly ($P = 0.395$) among time points. The 4-hour UP:C ratios did not differ significantly ($P = 0.323$) among time points, although the ratios in ponies did increase at the later time points (**Figure 2**).

The UP:C ratios obtained in the random urine samples collected via urinary bladder catheterization from each of the 6 subset horses on 2 additional days did not vary widely from the 24-hour pooled UP:C value determined for the same subset horse; among the 6 subset horses, maximum variation from the 24-hour UP:C ratio was 0.11 (**Figure 3**). For the subset horses, there were no significant differences in UP:C ratios among dates of experiment ($P = 0.960$) or among horses ($P = 0.781$). All UP:C ratios for subset horses remained within the reference range calculated by use of the 24-hour UP:C ratio derived from the entire group of equids.

On the basis of the data obtained, preliminary reference ranges for 24-hour UPE, 24-hour pooled UP:C ratio, and 4-hour UP:C ratio in female horses and female

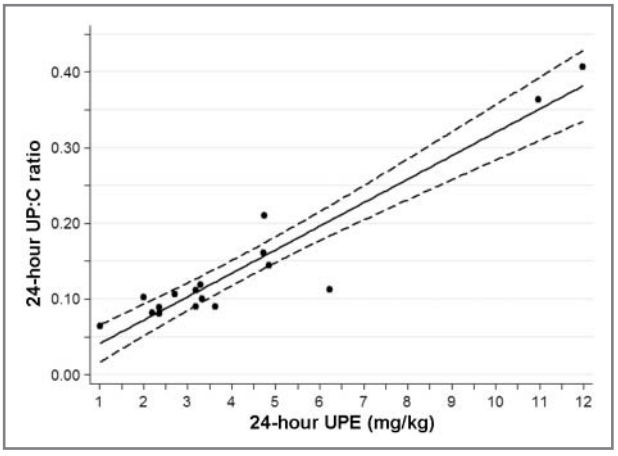

Figure 1—Correlation between 24-hour total UPE and UP:C ratio in pooled representative samples from urine volumes obtained at 4-hour intervals during a 24-hour collection period in 11 female horses and 6 female ponies. Dotted lines indicate 95% confidence intervals. There is good correlation between these values ($\rho = 0.826$; $P < 0.001$).

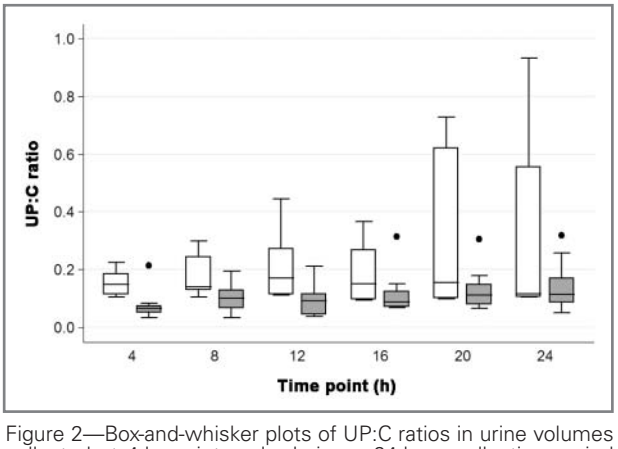

Figure 2—Box-and-whisker plots of UP:C ratios in urine volumes collected at 4-hour intervals during a 24-hour collection period from 11 female horses (gray boxes) and 6 female ponies (white boxes). For each box, the horizontal line represents the median value and the upper and lower boundaries represent the 75th and 25th percentiles, respectively. Whiskers represent the maximum and minimum values (range) of observed values. Outlier results are indicated by dots. Values in horses and ponies do not differ ($P = 0.323$) among time points, despite an increase in the SDs of the values over time in ponies.

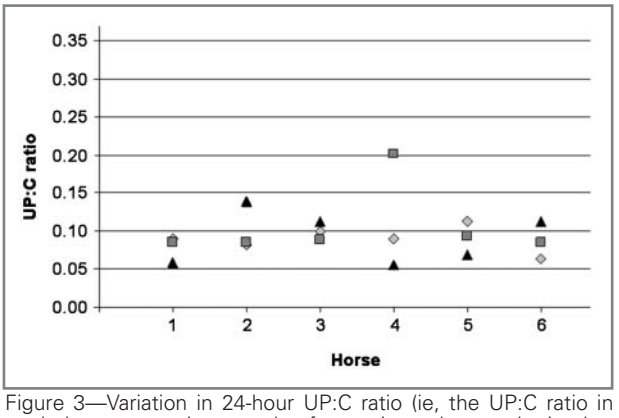

Figure 3—Variation in 24-hour UP:C ratio (ie, the UP:C ratio in pooled representative samples from urine volumes obtained at 4-hour intervals during a 24-hour collection period [diamonds]) versus UP:C ratios in 2 random samples of urine collected via urinary bladder catheterization on each of 2 additional days (1 sample/d [squares and triangles, respectively]) during a 4-month period following 24-hour UP:C ratio assessment in 6 female horses. No significant differences in UP:C ratio were detected across dates or among horses.

ponies were 1.086 to 11.700 mg/kg, 0.063 to 0.473, and 0.056 to 0.200, respectively.

Discussion

In the present study, values of 24-hour total UPE (ie, amount of UP [in mg]/kg of body weight) in healthy horses and ponies were determined, and the reliability of the UP:C ratio as a clinically reasonable approximation of this gold standard was established. Total 24-hour UPE in the 17 study equids (4.28 ± 2.99 mg/kg) was lower than a previously reported value determined in mares (12.0 ± 7.0 mg/kg)[16]; however, this finding was similar to 24-hour UPE values in dogs (4.76 mg/kg) and cats (4.93 ± 1.34 mg/kg).[1,11,15,19] The present study revealed an excellent correlation ($\rho = 0.826$) between 24-hour UPE and the UP:C ratio in horses and ponies. The UP:C ratio reference ranges generated for the study population of equids from urine collections at 4-hour intervals or from pooled samples of those urine collections during a 24-hour period were all lower than those considered normal in healthy dogs (≤ 0.5) and cats (≤ 0.4).[1] Finally, UP:C ratio in the study horses and ponies remained relatively constant and within the ranges reported for other species over an extended period of time.

The strong correlation between 24-hour UPE and the UP:C ratio was weakened by the large amount of variability associated with the regression line (Figure 1). Also, although most of the UPE values were clustered at the lower end of the range, the range was extended by 2 high 24-hour UPE values. This suggests that some apparently healthy horses and ponies may have markedly greater proteinuria than most other equids. The small experimental population in the present study makes it difficult to extrapolate to a larger population; assessment of a larger population of equids is required to definitively establish a reference range for UPE in horses and ponies.

Although not significant, UP:C ratios determined at the 4-hour time points did increase during the collection period in some ponies. This may have been attributable to sterile lower urinary tract inflammation that developed secondary to urethral catheterization rather than reflecting a true daily variation in UP concentration. Alternatively, the apparent difference in UPE between horses and ponies may reflect physiologic differences in glomerular protein handling or the volume of urinary tract secretions. In humans, UP concentration fluctuates over a 24-hour period and values are typically highest in the evening.[20] This end-of-day increase in UP concentration is thought to be a result of orthostatic hypertension; increased blood pressure while standing or sitting increases glomerular hydrostatic pressure, thereby increasing release of protein into the urine.[20,21] Because equids do not undergo the same wide variations in body position throughout the day, we considered this less likely as the cause of the changes in UP:C ratio in ponies over time. Regardless of the biologic cause for this observation, this change was evident because of an increase in UP concentration in only 2 of the 6 ponies; despite the apparent increase in UP:C ratio, most values remained well within the preliminary reference range for 24-hour UP:C in equids (derived from data obtained from all horses and ponies) and within reference ranges in other species. Finally, the higher UP:C ratios determined in those 2 ponies were nevertheless considered low; if similar values were detected in dogs or cats, it is likely that small animal clinicians would consider them clinically insignificant or worthy of reevaluation at a later time point without immediate further diagnostic testing or intervention. The comparison of UP:C ratios between different species has not been validated, but values in healthy domestic animals are < 1.0. Determination of reference ranges for a larger population of healthy equids would help to refine the reference range and better determine the importance of the apparent increase in UP:C ratio over 24 hours in some ponies.

Analysis of random urine samples obtained from the subset of 6 horses over a 4-month period following the 24-hour experimental period revealed minor variations in UP:C ratios over time. Although the method of urine collection for this intrahorse comparison differed from the urine collection method used to establish 24-hour UPE values, all results were still within the 95% confidence intervals generated from data obtained from all of the equids in the study. Unfortunately, the small number of subset horses does not account for within-day or between-day variations in UP:C ratios. Nevertheless, the results of the present study have provided preliminary evidence that UPE likely varies minimally in healthy mares, and that collection and analysis of random samples of urine may provide results equivalent to those obtained from analysis of 24-hour catheter-assisted urine collections.

The median UP:C value from the 17 equids in our total study population was initially also compared with the median UP:C value from urine samples randomly collected from 25 flat racing Thoroughbreds.[e] Median Thoroughbred UP:C ratio in urine samples collected immediately after racing was significantly greater than the median 24-hour UP:C ratio reported in the present study, although the Thoroughbreds' median and individual animal UP:C values were still within the refer-

ence ranges reported for UP:C ratio in other species.[1,14] We elected not to examine the Thoroughbred data in the present study because preexercise urine samples from the Thoroughbreds were not available for comparison, and an increase in UP:C ratio after intense exercise has been reported previously in horses and humans[18,20,22]; however, postexercise increase in urine albumin concentration was not detected in dogs in another study.[23] On the basis of contradictory data obtained from various species, assessment of UP:C ratio before and after exercise in horses warrants further investigation.

Because of the large amount of mucoprotein that is typically in the urine of equids,[24] further research is required to establish whether 24-hour total UPE, 24-hour UP:C ratio, or UP:C ratios determined from randomly collected urine samples have sufficient specificity for diagnosis and monitoring of glomerular-origin protein in the urine of equids. In laboratories, urine samples are routinely centrifuged prior to measurement of protein concentration in the supernatant to minimize the confounding effects of the largest mucoproteins and protein aggregates; nevertheless, smaller mucoproteins likely still remain in solution. In humans, despite the ease of measurement of UP:C ratio, assessment of urine albumin concentration is more sensitive than determination of total UPE or UP:C ratio for early detection of increased glomerular permeability and is better correlated with severity of glomerular damage.[6] Microalbuminuria (urine albumin concentration greater than the upper reference limit but not sufficiently high to result in a positive urine dipstick result) is highly correlated with later development of renal failure and progression of established kidney disease and, for some diseases (including breast and lung cancers and myocardial infarction), is a predictor of disease severity.[5,6] Microalbuminuria also appears to be an early indicator of renal disease in dogs and cats, and results of several studies[1,5,8,9] suggest that assessment of urine albumin-to-creatinine ratio in dogs and cats may eventually be used to determine disease severity and predict progression as it is used in humans. The typically low concentration of albumin in urine in humans, dogs, and cats requires species-specific albumin ELISAs for assessment rather than the standard colorimetric assays performed in most laboratories[7,25]; to our knowledge, such an assay has not been developed yet for use in horses. Future research may establish that assessment of UP:C ratio or urine albumin-to-creatinine ratio in equids may also prove useful in assessing vascular damage in addition to primary renal diseases. In particular, vascular damage may be caused by dysregulation of insulin metabolism and has been proposed to play a role in the development of laminitis.[26,27]

In the present study, the reliability of UP:C ratio assessments in equids was established and a preliminary reference range for UP:C ratio in healthy animals was generated. However, because of the limited experimental population in the study, a reference range from a larger population should be determined for use in clinical settings. Furthermore, investigations are needed to evaluate changes in renal protein excretion in equids with various systemic inflammatory diseases.

a. Foley catheter Silkolatex Rusch Gold, Teleflex Medical, Bannockburn, Ill.
b. Vitros Crea quantitative color test, VITROS Chemistry, Ortho-Clinical Diagnostics, Rochester, NY.
c. Bio-Rad Microprotein dye-binding test, Bio-Rad Laboratories, Hercules, Calif.
d. STATA, version 10.1, Stata Corp, College Station, Tex.
e. Uberti B, Eberle DB, Moore GE, et al. Determination of 24-hour urine protein excretion and correlation with random-sample urine protein:creatinine ratio in equids (abstr). *J Vet Intern Med* 2008;22:709.

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