Effect of Storage Time and Temperature on Canine Urine Enzymes N-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyl transpeptidase (GGT)

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Presented in part as a poster at the American College of Veterinary Internal Medicine Forum 2011, Denver, CO.

KEY WORDS: canine, nephrotoxicity, GGT, NAG, enzymuria, stability

ABSTRACT

Measurement of enzymes that have leaked from damaged renal tubular epithelial cells into the urine of dogs is a valuable diagnostic tool. However, storage of the urine sample prior to analysis may affect enzyme activity. The objective of this study was to determine if urine NAG and GGT activity is decreased in samples that have been refrigerated or frozen. Canine urine samples submitted to the Clinical Pathology Laboratory at Kansas State University were analyzed by complete urinalysis. Samples were excluded if the pH was ≥ 8, there were > 10 white blood cells/hpf, > 50 red blood cells/hpf, or bacteriuria or gross pigmenturia was present. Urine NAG and GGT activities were measured from the fresh urine supernatant and then aliquots of urine supernatant (n = 41) were refrigerated at 4° C for 5 days and frozen at -20° C for 5 and 30 days, at which time urine enzyme activity was compared with day 0 values. Additionally, aliquots of urine supernatant (n = 22) were evaluated as above, but with the additional time/temperatures of 4° C for 30 days and -70° C for 30 days added to the evaluation. The analytical uncertainty in quantifying NAG was non-trivial when the urine NAG level was low. For samples whose NAG concentrations were above 1 U/L at day 0, their urine NAG/creatinine ratios under all storage methods led to <0.01 false positive rate (FPR) based on a defined 50% decline over day 0. The GGT concentrations in >50% of samples

frozen at -20° C for either 5 days or 30 days dropped below the limit of detection. The urine GGT/creatinine ratios in samples frozen under -70° C for 30 days carried >0.10 FPR, whereas those refrigerated at 4° C for 5 days or 30 days have <0.01 FPR. Time and temperature storage conditions affect urine GGT activity. Urine GGT activity is most severely affected by freezing at -20° C.

INTRODUCTION

Acute kidney injury (AKI) in dogs can occur secondary to administration of potentially nephrotoxic drugs (eg, antibiotics, antifungals, chemotherapeutics, and NSAIDs) and has a high mortality rate.[1] Even in cases with reversible renal lesions, hospital-acquired AKI is often associated with prolonged hospitalization and expensive treatment. Early recognition of kidney injury, before changes in routine blood work are detected, is important, since continued administration of nephrotoxic medications may lead to more serious injury. In addition, when discontinuing potentially nephrotoxic therapeutics is not possible, dose adjustments may be implemented (eg, either increasing time between doses or dose reduction), and/or supportive treatments may be initiated (eg, fluid therapy).

Measurement of enzyme activity in urine may aid diagnosis of early nephrotoxicant-induced AKI. γ-glutamyl transpeptidase (GGT) and N-acetyl-β-D-glucosaminidase (NAG) are two of the more commonly measured urine enzymes. GGT originates from the proximal tubule brush border and NAG is present in the proximal tubule lysosomes.[2] Although both enzymes are present in the plasma, they are too large to be freely filtered by the normal glomerular capillary wall. In the absence of glomerulopathy and/or high magnitude hematuria, enzymuria occurs associated with tubular cell damage. Previous studies have shown that measurement of these enzymes in the urine facilitates detection of early renal tubular damage prior to changes in serum biochemical parameters and urine specific gravity.[3,4]

After acquisition of a urine sample, enzymuria determination may be delayed for sample batching and/or shipping to an outsourced laboratory. In addition, longitudinal evaluations may be performed on an individual patient. For example, baseline urine samples may be saved and run with post-treatment urine samples collected at a later time. Therefore, it is important to know how storage of urine samples affects enzyme activity.

Previous studies of GGT stability in the urine of healthy human beings found no changes in enzyme activity after short-term storage (24 hours) at room temperature or at 4° C, but found only partial preservation of enzyme activity in urine supernatant stored at – 70° C. Urine enzyme stability was not maintained even after a few days of freezing at – 20° C.[5] A recent study of urinary biomarkers in dogs with hereditary renal disease assessed urine NAG stability and reported decreased enzyme activity in two of four urine samples stored at room temperature for 12-24 hours.[6] In addition, there was increased urine enzyme activity in two of four samples stored at 4° C.[6] Freezing at – 20° C or – 80° C for up to 1 year did not affect urine NAG activity.[6] To the authors' knowledge, no previous studies have evaluated the stability of GGT in canine urine. The purpose of the present study was to determine how sample storage affects NAG and GGT activity in canine urine.

MATERIALS AND METHODS

Ninety-eight canine urine samples submitted to the Clinical Pathology Laboratory at Kansas State University were initially evaluated. Samples were collected by cystocentesis, urethral catheterization, and free-catch voiding from clinical patients at the Veterinary Health Center at Kansas State University that had a variety of medical or surgical disorders. In addition, samples were selected based on the hours of operation of Clinical Pathology Laboratory to ensure timely sample analysis and a minimum urine sample volume of 10 ml was required to allow aliquoting for storage and subsequent analysis.

A complete urinalysis, including physical appearance, specific gravity via refractometer, chemical assessment via dipstick[a] read by an analyzer,[b] and microscopic evaluation of the sediment was performed on each sample. Samples were excluded (n = 35) if the pH was ≥ 8, there were > 10 white blood cells/hpf, > 50 red blood cells/hpf, or bacteriuria or gross pigmenturia. Measurement of NAG, GGT, and creatinine from fresh urine supernatant was performed on an automated chemistry analyzer.[c] The NAG activity was assessed by colorimetric assay in which 3-Cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide is hydrolyzed by NAG resulting in the release of 3-cresolsulfonphthalein (also referred to as 3-cresol purple). The amount of 3-cresol produced was subjected to photometric analysis to determine the NAG activity in the sample and two repeated readings were collected (U/L). The GGT was measured once by a colorimetric assay in which GGT transfers the γ-glutamyl group of L-γ-glutamyl-3-carboxy-4-nitroanilide to glycylglycine and GGT activity in the sample is proportional to the amount of 5-amino-2-nitrobenzoate (measured photo metrically) produced in the sample (U/L).

For these methodologies for NAG and GGT, the amount of enzyme activity is determined by measuring the amount of product produced (ie, the amount of product produced is directly proportional to the concentration of active enzyme). Urine creatinine concentration was measured once using the Jaffe reaction in which creatinine reacts with picrate in an alkaline solution to produce a yellow-red complex.[c] The creatinine concentration in the sample is directly proportional to the color intensity of the yellow-red complex produced (mg/dl).

In the first phase of the study aliquots of urine supernatant were refrigerated at 4° C for 5 days and frozen at -20° C (in a non-freeze-thaw cycle freezer) for 5 and 30 days, at which time enzyme activity was reassessed (n = 41). In a second phase of the study, additional time and temperature points were added to the previous time points to include a 30-day refrigerated sample (4° C) (n = 20) and a 30-day frozen sample (-70° C) (n = 22).

The analysis of NAG/creatinine ratios was based on a linear mixed model.[d] It was observed that large analytical uncertainty occurs when the NAG concentrations were below 1 U/L. Statistical analysis was therefore conducted with and without those six urine samples whose NAG concentrations at day 0 were <1 U/L. Let Y_{ijk} be the NAG/creatinine ratio from sample i under storage condition j where the NAG level is recorded from the k^{th} repeated reading (i=1, …, 41; j=fresh, 4°C for 5 days, 4°C for 30 days, -20°C for 5 days, -20° C for 30 days, -70°C for 30 days; k=1, 2). To better fulfill the normality assumption required by the linear mixed model, data first underwent natural-log transformation and then were analyzed by the model given below.

$$\ln(Y_{ijk}) = Subject_i + \mu_j^{NAG} + Subject \times Storage_{ij} + Error_{ijk}$$

The effect of storage condition j, denoted by μ_j^{NAG}, is fixed. Parameter $\exp(\mu_j^{NAG})$, corresponds to the median of the marginal distribution of its NAG/creatinine ratios and $\exp(\mu_j^{NAG} - \mu_{Fresh}^{NAG})$ corresponds to the median of changes over fresh. Random effects due to subject, subject-by-storage interaction and error are independently normally distributed with zero means. Let $\sigma^2_{Subject \times Storage}$ and σ^2_E be the respective variance for subject-by-storage interaction and error. A two-fold increase over baseline is compatible with a diagnosis of AKI.[4] Let Y_i^* and Y_{fresh} be the NAG/creatinine ratio in two random aliquots of the same sample under storage condition j and at day 0, respectively. False positive diagnosis compatible with AKI was defined as a NAG/creatinine ratio from a stored aliquot that decreased to 50% of that in the fresh aliquot, ie, $Y_j^*/Y_{fresh} \leq 0.5$. Stability of storage condition j is then evaluated in terms of its false positive rate (FPR) given below.

$$FPR_{NAG}^{j} = \Pr\left(\frac{Y_j^*}{Y_{Fresh}} \leq 0.5\right) = \Pr\left(\ln(Y_j^*) - \ln(Y_{Fresh}) \leq \ln(0.5)\right) = \Phi\left(\frac{\ln(0.5) - (\mu_j^{NAG} - \mu_{Fresh}^{NAG})}{\sqrt{Var(\ln(Y_j^*/Y_{Fresh}))}}\right),$$

$$Var(\ln(Y_j^*/Y_{Fresh})) = \begin{cases} 2\sigma_E^2, & j = fresh, \\ 2\sigma^2_{Subject \times Storage} + 2\sigma_E^2, & j \neq fresh. \end{cases}$$

Table 1. *Summary statistics.*

Storage	N	NAG Median (Range)	GGT Median (Range)	Creatinine Median (Range)
Fresh	41	3.25 (0.2-43.9)	40.00 (4.0-364.0)	96.70 (16.0- 391.6)
5 day Refrigerator 4°C	41	3.00 (0.1-46.4)	40.00 (4.0-349.0)	97.20 (16.5-393.3)
30 day Refrigerator 4°C	20	1.57 (0.2-11.1)	35.00 (4.0- 286.0)	104.45 (17.4-288.4)
5 day Freezer -20°C	41	2.70 (0.1-46.6)	<3.0 (<3.0-195.0)	95.00 (16.5-396.8)
30 day Freezer -20°C	41	3.35 (0.2-46.6)	<3.0 (<3.0-49.0)	98.30 (16.5-396.4)
30 day Freezer -70°C	22	1.55 (0.1-10.2)	34.50 (<3.0-292.0)	103.30 (16.2-301.0)

Function Φ is the cumulative distribution function of a standard normal distribution. Note that FDR^{j}_{NAG} increases as $\sigma^2_{SubjectxStorage}$ and σ^2_E increases; it decreases as μ_j^{NAG} declines further from μ^{NAG}_{Fresh}; when μ_j^{NAG}, false positive diagnosis is still possible. The calculation of the confidence interval (CI) for FDR^{j}_{NAG} involved complex statistical methodology and is beyond the scope of this present work. Two-sided 95% CIs are provided in Table 2 for $\exp(\mu_j^{NAG})$ and $\exp(\mu_j^{NAG} - \mu^{NAG}_{Fresh})$ only.

The analysis of GGT/creatinine ratios also entailed a two-step process. The first step evaluated the numbers of GGT readings that dropped below the detection limit. The second step of the process, ie, mixed model analysis, focused on evaluating refrigeration (4° C) for 5 and 30 days and freezing (-70° C) for 30 days. Let Z_y be the GGT/creatinine ratio from sample i under storage condition j. The log-transformed data were modeled according to

$$\ln(Z_{ij}) = Subject + \mu_i^{GGT} + Error_{ij}.$$

The effect of storage condition is treated as fixed. For a given storage condition j, μ_i^{GGT} is the median of the marginal distribution of its GGT/creatinine ratios and $\exp(\mu_j^{GGT} - \mu^{GGT}_{Fresh})$ is the median of changes over fresh. Random effects due to subject and error are indepen-

Table 2. *Analysis results of urine NAG/creatinine ratio (U/L/mg/dl).*

Storage	Median (95% Confidence Interval)	Compared with Fresh (95% Confidence Interval)	False Positive Rate
Including samples with fresh [NAG] < 1 U/L			
Fresh	0.0302 (0.0203, 0.0448)	1 (--, --)	0.02458
5 day Refrigerator 4°C	0.0267 (0.0180, 0.0396)	0.884 (0.791, 0.989)	0.0978
30 day Refrigerator 4°C	0.0297 (0.0198, 0.0446)	0.986 (0.854, 1.139)	0.0618
5 day Freezer -20°C	0.0236 (0.0159, 0.0350)	0.781 (0.689, 0.875)	0.1558
30 day Freezer -20°C	0.0266 (0.0179, 0.0395)	0.882 (0.789, 0.987)	0.0987
30 day Freezer -70°C	0.0286 (0.0191, 0.0428)	0.948 (0.825, 1.090)	0.0732
Excluding samples with fresh [NAG] < 1 U/L			
Fresh	0.0379 (0.0258, 0.0558)	1 (--, --)	<0.00001
5 day Refrigerator 4°C	0.0346 (0.0236, 0.0509)	0.913 (0.851, 0.981)	0.00316
30 day Refrigerator 4°C	0.0364 (0.0247, 0.0538)	0.961 (0.875, 1.056)	0.00154
5 day Freezer -20°C	0.0318 (0.0216, 0.0467)	0.838 (0.780, 0.900)	0.00963
30 day Freezer -20°C	0.0331 (0.0225, 0.0487)	0.873 (0.813, 0.938)	0.00575
30 day Freezer -70°C	0.0353 (0.0239, 0.0521)	0.930 (0.849, 1.020)	0.00245

Table 3. *Counts of GGT reading that are above the limit of detection.*

Storage	[GGT] < 3 U/L	[GGT] ≥ 3 U/L	Total
Fresh	0	41	41
5 day Refrigerator 4°C	0	41	41
30 day Refrigerator 4°C	0	20	20
5 day Freezer -20°C	26	15	41
30 day Freezer -20°C	33	8	41
30 day Freezer -70°C	1	21	22

dently normally distributed with zero means. According to the summary statistics of log-transformed data (results not shown), heterogeneous error variance was employed to account for the variability difference between -70° C freezing for 30 days (denoted by σ^2_{E1}) and the other storage conditions (denoted by σ^2_{E2}). Again it was assumed that a two-fold increase over baseline is compatible with a diagnosis of AKI. Let Z_j^* and Z_{fresh} be the GGT/creatinine ratio in two random aliquots of the same sample under storage condition j and at day 0, respectively. The FPR under storage condition j is computed as

$$FPR^j_{GGT} = \Pr\left(\frac{Z_j^*}{Z_{fresh}} \le 0.5\right) = \Phi\left(\frac{\ln(0.5) - (\mu_j^{GGT} - \mu_{fresh}^{GGT})}{\sqrt{Var(\ln(Z_j^*) - \ln(Z_{fresh}))}}\right),$$

$$Var(\ln(Z_j^*) - \ln(Z_{fresh})) = \begin{cases} \sigma^2_{E1} + \sigma^2_{E2}, & j = -70^\circ\text{C for 30 days,} \\ 2\sigma^2_{E2}, & j = \text{fresh, 4°C for 5 days, 4°C for 30 days.} \end{cases}$$

Note that FPR^j_{GGT} increases as σ^2_{E1} and σ^2_{E2} increases; it decreases as μ_j^{GGT} decays further from μ_{Fresh}^{GGT}; false positive diagnosis is still possible even if $\mu_j^{GGT} = \mu_{Fresh}^{GGT}$.

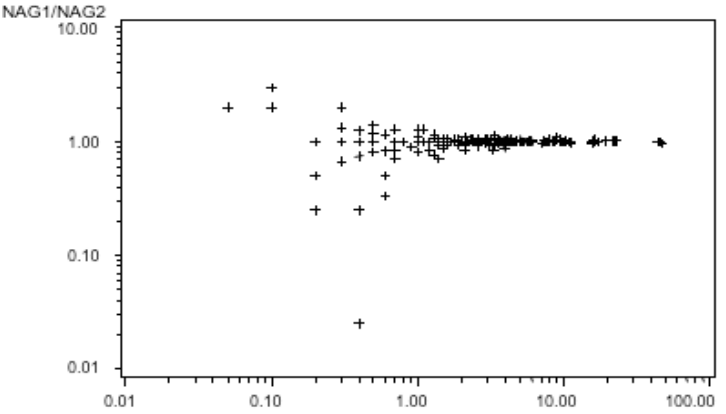
RESULTS

Table 1 presents the summary statistics, including median and range, of the urine NAG, GGT, and creatinine data. Table 2 shows the median urine NAG/creatinine ratio values for the various storage conditions along with the FPR. Inclusion of urine NAG activities < 1 U/L increased the FPR. Figure 1 plots the NAG1/NAG2 ratios between two repeated readings of urine sample aliquots and demonstrates the analytical variability when NAG activities are < 1 U/L. Figure 2 presents the schematic boxplots of the observed urine NAG/creatinine ratios at each storage condition. As seen in Table 3, the GGT concentration in all fresh samples was above the quantification limit. There were no below- detection limit readings after storing sample aliquots at 4° C. In contrast, 26 out of 41 sample aliquots upon 5-day freezing at -20° C had their GGT concentration dropped below the limit; 33 out of 41 sample aliquots upon 30-day freezing at -20° C had their GGT concentration dropped below the limit; freezing sample aliquots at -70° C for 30 days also generated one GGT reading that is below the limit. Substituting those non-quantifiable values with half the detection limit, Figure 3, presents the schematic boxplots of the observed urine GGT/creatinine ratios for each storage condition. Both Table 3 and Figure 3 show that freezing urine sample aliquots at -20° C for either

Table 4. *Analysis results of urine GGT/creatinine ratio.*

Storage	Median (95% Confidence Interval)	Compared with Fresh (95% Confidence Interval)	False Positive Rate
Fresh	0.385 (0.298,0.497)	1 (--, --)	0.0003
5 day Refrigerator 4°C	0.376 (0.291,0.485)	0.976 (0.916, 1.040)	0.0005
30 day Refrigerator 4°C	0.327 (0.252,0.425)	0.849 (0.781, 0.924)	0.0044
30 day Freezer -70°C	0.288 (0.193,0.428)	0.747 (0.541, 1.031)	0.2915

Figure 1. *Plot of ratios of two repeated NAG measurements from urine sample aliquots.*



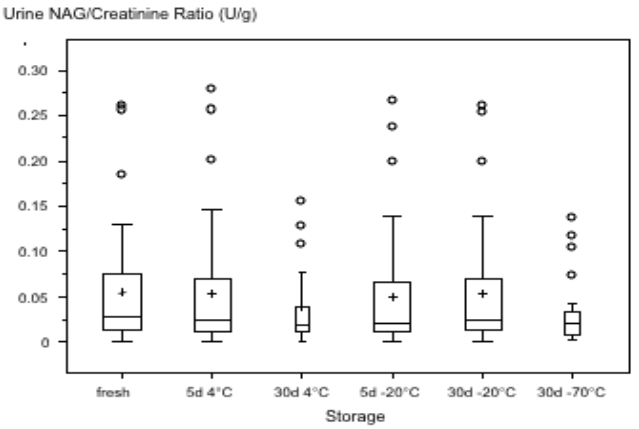
5 or 30 days caused a large decrease in urine GGT/creatinine ratios. Table 4 shows the median urine GGT/creatinine ratio values for the various storage conditions. The ratios from the 5 and 30 day -20° C frozen samples are not included due to the large number of samples below the detection limit.

DISCUSSION

Use of spot urine samples and urine enzyme/creatinine ratios (vs timed urine collections) facilitates monitoring enzymuria in the clinical setting and correlates with 24-hour urinary enzyme excretion.[8] Previous studies/recommendations have suggested that a 2-fold increase in urine GGT and NAG activity compared with baseline values is indicative of renal tubular cell damage.[4,9] The defined delta of 50% of the fresh urine enzyme/creatinine ratio was chosen based on these previous clinical recommendations. False positive results for renal tubular damage may occur when a stored baseline sample loses 50% enzyme activity. The present study suggests that time and temperature do not effect urine creatinine concentrations, have minimal effect on urine NAG activity, but that storage temperature does affect urine GGT activity.

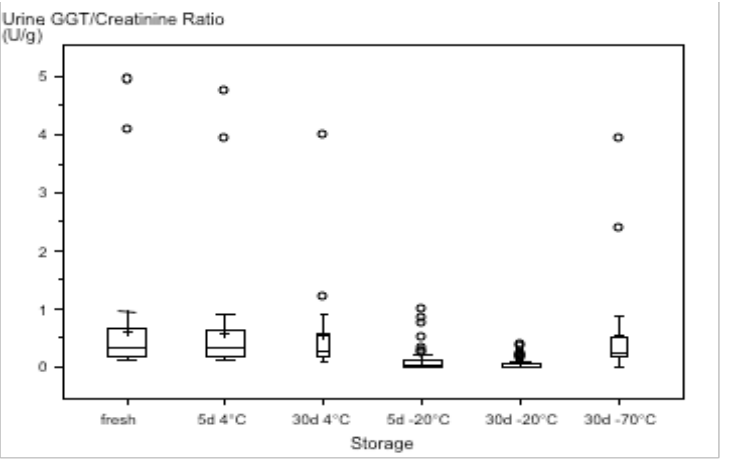
Previously published canine reference intervals for normal dogs for urine NAG/creatinine ratios (0.02 to 3.63 U/g) and for urine GGT/creatinine ratios (1.93 to 28.57 U/g) demonstrate wide variability.[10] Although we made no comparison with respect to gender, previous studies in age matched healthy beagle dogs found NAG activity to be double in males versus females.[11] Another study in healthy dogs found that GGT did not differ between sexes. However, there was a significant difference between male and female dogs and urine NAG.[10] Maddens, et al found significant differences in mean NAG/creatinine ratio between healthy dogs (2.3 U/g) and dogs with pyometra (14.3 U/g). However, significant overlap existed between the two groups (range 1.4-3.9 U/g in healthy dogs and 0.8-14.3 U/g in pyometra dogs).[12] This variability and

Figure 2. *Schematic boxplots of urine NAG/creatinine ratios for each storage condition. The box width is proportional to the sample size.*



the wide reference intervals underscore the importance of comparing pre- and post-treatment urine enzyme activity from the same patient rather than comparing an individual post-treatment value with a published normal value.

Figure 3. *Schematic boxplots of urine GGT/creatinine ratios for each storage condition. The box width is proportional to the sample size.*



Exclusion criteria for the urine samples in this study were selected based on knowledge that certain conditions in the urine (pH ≥ 8, and the presence of gross pigmenturia, bacteria and/or pyuria) may affect accurate measurement of enzyme activity. Under alkaline conditions (pH of about 8) NAG isoenzyme A, which is the major measured enzyme in normal urine, can become inactivated. There were no changes in urine NAG activity when measured in acidic urine.[13] It has, therefore, been suggested to evaluate isoenzyme B if measurement should be performed on alkaline urine. Another study found that a pH ≥ 8 may (Author, please confirm this change) be associated with significant decreases in total NAG activity, and that more accurate results were obtained by determining urine NAG after ultrafiltration, dialysis, and chromatographic separation on DEAE cellulose to isolate separate isoenzymes.[14] Inasmuch as these additional techniques were not used in the current study, samples containing alkaline urine were excluded.

Samples containing gross pigmenturia were also excluded. The current study used a colorimetric assay to evaluate enzyme activity of both NAG and GGT. Therefore, any sample that had pigmenturia could potentially lead to inaccurate results. In addition to pigmenturia, samples with hematuria (defined in this study as > 50 RBC/hpf) were also excluded. The intention of measuring NAG and GGT in urine is to determine if there is increased release of enzymes as a result of renal tubular epithelial cell injury. However, since these enzymes are also present in the blood, any sample that contained > 50 RBC/hpf was excluded.

Samples that contained any bacteriuria or pyuria (pyuria was defined as > 10 WBC/hpf in this study) were also excluded. A study that evaluated NAG and GGT activity in healthy adult dogs found concentrations within reference intervals for patients with positive urine cultures.[10] However, since other studies found the enzyme to creatinine ratio may be affected by the presence of pyuria or infection,[2,8] samples containing bacteriuria or pyuria were excluded in the present study.

This study has several limitations. No attempt was made to standardize or compare pH at different storage conditions. Since pH has been previously shown to affect enzymuria measurement, it is possible that changes in pH could affect protein stability. Comparing pH between individual samples, or standardization of pH prior to storage could be considered for future studies. It is unclear if the same decrease in enzyme activity would occur if serum/

plasma samples were similarly analyzed, or if a compound(s) present in the urine contributes to the enzyme activity instability at -20°C. Finally, based on the assay performed, we were not able to determine if the concentration of the enzyme or the activity of the enzyme is responsible for the decline in measured activity. Future studies to evaluate the structure of the protein before and after storage in combination with measured results could provide more insight into reasons for the difference seen between freezing temperatures.

In summary, measurement of urine NAG and GGT activity may aid early detection of AKI. To ensure accurate and precise results, it is important for the clinician to be aware of factors that affect urine enzyme activity measurement, including appropriate storage techniques. The interpretation of urine enzyme activity should always include a complete urinalysis as well as a creatinine concentration for determination of the enzyme/creatinine ratio. If possible, a pretreatment (baseline sample) sample should be collected prior to potential nephrotoxic treatment for comparison to urine samples collected after initiation of the treatment. Time and temperature storage conditions affect urine GGT activity. Urine GGT activity is severely affected by freezing.

FOOTNOTES

a. Seimens Multisix® Reagent Strips, Siemans Healthcare Diagnostics, Inc., Tarrytown, NY 10591

b. Bayer Clinitek® 100 Urine Chemistry Analyzer, Bayer Diagnostics Division, Elkhart, IN 46515

c. Cobas c501, Roche Diagnostics, Indianapolis, IN, 46250.

d. Proc MIXED, SAS/STAT, version 9.3, SAS Institute Inc., Cary, NC.

ACKNOWLEDGEMENTS

Kansas State University Clinical Pathology Laboratory for technical support.

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