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Variation of Nephrotoxicity Biomarkers by Urinary Storage Condition in Rats

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Recently, there has been an increase in the use of several nephrotoxicity biomarkers in preclinical experiments. In addition, it has been indicated that the result may have been influenced by secondary factors, such as sample storage condition or storage period. In this study, we have assessed the variation in urinary nephrotoxicity biomarkers as a result of urine storage conditions and storage period of the urine. Urine was sampled from specific pathogen-free Sprague-Dawley rats (19 weeks old), which were housed individually in hanged stainless steel wire mesh cages. Urine was stored at 20°C, at 4°C, or at −70°C after sampling. The levels of the biomarkers such as beta-2 microglobulin (B2M), cystatin-C (Cys-C), *N*-acetyl-β-D-glucosaminidase (NAG), micro albumin (MA), micro protein (MP) were measured at 6, 24, 48 and 144 hr after sampling. The B2M level was significantly decreased at 6, 24, 48, and 144 hr compared to 0 hr at −70°C ($p < 0.05$, $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively) and 24 and 144 hr at 20°C ($p < 0.01$, $p < 0.01$, respectively). The Cys-C level was significantly decreased at 144 hr compared to 0 hr at 4°C ($p < 0.01$), at 20°C ($p < 0.05$) and at 70°C ($p < 0.01$). MP and MA levels were not different for 144 hr in all storage conditions. Taken together, B2M and Cys-C levels were modulated by storage temperature and period. For the enhancement of test accuracy, it is suggested that strict protocols be established for samples to minimize the effects of the storage conditions on the detected levels of biomarkers.

Key words: Nephrotoxicity, Biomarkers, Storage temperature, Storage period, Stability

INTRODUCTION

In drug development, kidney toxicity induced by drug candidates frequently results in the loss of considerable money and time. However, the current methods to detect nephrotoxicity, serum creatinine and blood urea nitrogen are insensitive and of poor diagnostic value (1,2). In addition, serum creatinine and blood urea nitrogen concentrations are influenced by several factors such as age and sex. Furthermore, serum creatinine and blood urea nitrogen do not reflect the current status of renal function after toxic injury (3,4). Therefore, the discovery and development of novel biomarkers that can diagnose kidney damage earlier and more accurately are needed for the effective prevention of drug-induced nephrotoxicity (5). Moreover, more sensitive and noninvasive approaches to monitor and manage nephrotoxicity in clinical situations where kidney damage cannot be avoided would considerably improve on current options for patient care.

To investigate novel biomarkers, superior in specificity and sensitivity for diagnosing acute kidney injury, the Food and Drug Administration and the European Medicines Agency through the Predictive Safety Testing Consortium's Nephrotoxicity Working Group, identified seven renal safety biomarkers that are qualified for use in nonclinical and clinical

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drug development to help guide safety assessments (6-8).

These urinary nephrotoxicity biomarkers facilitate the early estimation of kidney damage by testing urine, which can be collected through a non-invasive method. Indeed, urine is regarded as attractive and efficient sample because it can be obtained easily and non-invasively in considerable volume (5). Therefore, the identification of biomarkers can be detected in urine exposed to nephrotoxicity that is a promising approach (9). However, a rigorous evaluation of the stability of these biomarkers in urine is lacking. Although a published report has indicated that changes in of storage temperature and period can affect a novel biomarker the study focused on human urine (10-12). It has been recognized that the current understanding of the stability of novel biomarkers is insufficient. Therefore, this study assessed the effect of storage temperature and period on novel biomarkers by examined rat urine. We assessed cystatin-C (Cys-C), micro albumin (MA), micro protein (MP), and beta-2 microglobulin (B2M), which are recognized as a damage index of the glomerulus and proximal tubule, and the standard biomarker N-acetyl-β-D-glucosaminidase (NAG), which is sensitive in nephrotoxicity (13).

This study was performed in order to test and establish the optimal storage period and temperature of the early nephrotoxicity biomarkers for nephrotoxicity assessments. The ability to reliably measure these biomarkers in serum and urine samples is critically dependent on appropriate sample processing, which can significantly affect findings. In this study, we examined how to the storage temperature and period of novel and existing biomarkers that might be affected the measurement of urine biomarkers.

MATERIALS AND METHODS

Animals. Five 19-weeks-old male specific pathogen-free Sprague-Dawley rats, strain Crl:CD (SD) were purchased from Orientbio Inc. (Gapyeong, Republic of Korea). The animals had been housed individually in hanging stainless steel wire mesh cages in an animal room under the following conditions: temperature, 19~25°C; relative humidity 30~70%; air ventilation 10~15 times/hr; and illumination, 12 hr (0700~1900 hr). They had been fed a commercial diet (Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C). The animals were housed in a facility (Biotoxtech Co. Ltd., Cheongju-si, Chungcheongbuk-do, Republic of Korea) where approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Urine collection and storage methods. Urine was collected from rats fasted for 24 hr in standard metabolic cages. However, drinking water was provided ad libitum. Aliquots of collected urine specimens were stored at 20°C, at 4°C, and at −70°C, individually, and assessed 6, 24, 48, and 144 hr.

Measurement of urinary biomarkers. Urine specimens were analyzed for Cys-C, NAG, MA and MP using an automatic analyzer (7180; Hitachi, Tokyo, Japan). B2M levels were measured with an immunological analyzer (IMMULITE 1000, Siemens Healthcare Diagnostics, Tarrytown, NY, USA).

Statistical analysis. Statistical analysis was performed using SAS Program (version 9.3, SAS Institute Inc., Cary, NC). Data were analyzed utilizing Bartlett's test for homogeneity of variance. One-way analysis of variance was employed on homogeneous data, and if significant, Dunnett's test was then applied for multiple comparisons (significance level: $p < 0.05$ and $p < 0.01$, two-tailed). The Kruskal-Wallis was employed on heterogeneous data, and then, if significant, Steel's test was then applied for multiple comparisons (significance level: $p < 0.05$ and $p < 0.01$, two-tailed).

RESULTS

The B2M level was significantly decreased after 6, 24, 48 and 144 hr at −70°C compared to 0 hr ($p < 0.05$, $p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively) and after 24 and 144 hr at 20°C ($p < 0.01$ and, $p < 0.01$, respectively). However, the B2M level was not different after 144 hr of refrigeration when compared to 0 hr (Table 1).

The Cys-C level was not different after 6, 24, or 48 hr at any of the tested storages temperature when compared to 0 hr. However, it was significantly decreased after 144 hr under refrigeration ($p < 0.01$), at 20°C ($p < 0.05$), or at −70°C ($p < 0.01$) (Table 2) compared to 0 hr.

NAG was present at a significantly decreased level after

Table 1. Changes of β2-microglobulin by storage time and temperature

	Storage period (ng/ml)				
	0 hr	6 hr	24 hr	48 hr	144 hr
Refrigerated	3.36 ± 0.11[a]	3.35 ± 0.07	3.28 ± 0.07	3.26 ± 0.05	3.26 ± 0.04
Room temperature	3.36 ± 0.11	3.33 ± 0.05	3.19 ± 0.05**	3.25 ± 0.04	3.18 ± 0.06**
Deep freezer	3.36 ± 0.11	3.18 ± 0.13*	3.15 ± 0.07**	3.19 ± 0.07*	3.20 ± 0.05*

*, ** Significantly different from control ($p < 0.05$, $p < 0.01$, respectively).
[a]Data represent mean ± S.D.

Table 2. Changes of Cystatin C by storage time and temperature

	Storage period (mg/L)				
	0 hr	6 hr	24 hr	48 hr	144 hr
Refrigerated	0.37 ± 0.01[a]	0.35 ± 0.02	0.36 ± 0.01	0.36 ± 0.02	0.16 ± 0.03**
Room temperature	0.37 ± 0.01	0.37 ± 0.02	0.37 ± 0.02	0.38 ± 0.02	0.17 ± 0.07*
Deep freezer	0.37 ± 0.01	0.36 ± 0.02	0.36 ± 0.01	0.37 ± 0.01	0.18 ± 0.03**

*, ** Significantly different from control ($p < 0.05$, $p < 0.01$, respectively).
[a]Data represent mean ± S.D.

Table 3. Changes of N-acetyl-β-D-glucosaminidase by storage time and temperature

	Storage period (U/L)				
	0 hr	6 hr	24 hr	48 hr	144 hr
Refrigerated	17.56 ± 4.07[a]	17.56 ± 4.42	17.98 ± 4.21	17.71 ± 4.67	18.29 ± 4.19
Room temperature	17.56 ± 4.07	17.92 ± 4.02	18.28 ± 3.94	17.15 ± 4.25	12.01 ± 6.18
Deep freezer	17.56 ± 4.07	17.90 ± 4.26	17.79 ± 3.94	17.81 ± 3.96	17.81 ± 4.08

*, ** Significantly different from control ($p < 0.05$, $p < 0.01$, respectively).
[a]Data represent mean ± S.D.

Table 4. Changes of Micro protein by storage time and temperature

	Storage period (μg/ml)				
	0 hr	6 hr	24 hr	48 hr	144 hr
Refrigerated	10.28 ± 3.79[a]	10.31 ± 3.83	10.30 ± 3.94	10.62 ± 3.90	10.68 ± 3.97
Room temperature	10.28 ± 3.79	10.32 ± 3.76	10.27 ± 3.63	10.71 ± 3.69	11.53 ± 3.85
Deep freezer	10.28 ± 3.79	10.31 ± 3.79	10.35 ± 3.79	10.74 ± 3.77	10.81 ± 3.89

*, ** Significantly different from control ($p < 0.05$, $p < 0.01$, respectively).
[a]Data represent mean ± S.D.

Table 5. Changes of Micro albumin by storage time and temperature

	Storage period (μg/ml)				
	0 hr	6 hr	24 hr	48 hr	144 hr
Refrigerated	23.26 ± 12.86[a]	22.93 ± 12.14	23.88 ± 12.59	23.13 ± 12.62	23.10 ± 12.85
Room temperature	23.26 ± 12.86	23.33 ± 12.05	24.71 ± 12.86	24.41 ± 12.12	25.36 ± 12.53
Deep freezer	23.26 ± 12.86	23.40 ± 12.26	23.92 ± 12.18	23.22 ± 12.43	22.95 ± 12.30

*, ** Significantly different from control ($p < 0.05$, $p < 0.01$, respectively).
[a]Data represent mean ± S.D.

storage at room temperature for 144 hr compared to 0 hr. However, the NAG level was not different after 144 hr of refrigerated or deep freezing (Table 3).

The MP level was significantly increased tendency after 144 hr at 20°C compared to 0 hr. However, the MP level was not different after storage for 144 hr at 4 °C or −70°C (Table 4).

After 144 hr at 20°C, the MA level was significantly increased tendency compared to 0 hr. However, the MA level was not different after 144 hr under refrigeration or at −70°C (Table 5).

DISSCUSSION

This study was performed in order to establish the optimal storage period and temperature for the preservation of the early nephrotoxicity biomarkers that are used for nephrotoxicity assessments.

The B2M level was significantly decreased after 6, 24, 48 and 144 hr at −70°C and after 24 and 144 hr storage at 20°C, compared to 0 hr. However, under refrigeration, the B2M level was not different after 144 hr when compared to 0 hr. Therefore, it has been recognized that the B2M level showed storage temperature dependent variation, and was the most stable under refrigeration. B2M is a 12-kDa polypeptide chain that is constantly synthesized throughout the body. It is filtered by the glomeruli and nearly completely reabsorbed and catabolized in the tubules so that only 0.3% of the filtered B2M is found in the urine (1). Hence, it is

used as early biomarkers for the detection of proximal tubule dysfunction, since its urine concentration is increased by an increase in its excretion due to absorption dysfunction caused by proximal tubule dysfunction (14,15). Ideally, B2M concentration should be measured as soon as possible after collecting the urine to obtain accurate results. However, if the analysis of B2M levels in the urine is delayed, the specimen must be preserved by refrigeration and measurement must be performed within 144 hr of storage in order to enable accurate results.

The Cys-C level was not different following 6, 24, or 48 hr at any storage temperature tested compared to 0 hr. However, following 144 hr at any storage temperature, its levels were significantly decreased compared to 0 hr. Hence, measuring Cys-C within 48 hr of sampling the urine is likely to yield acceptable results. In addition, even though Cys-C was stable at all temperature, it should be measured within 48 hr after urine collection. Cys-C is a 13-kDa, nonglycosylated basic protein, produced at a constant rate by all nucleated cells (16). Cys-C is extremely sensitive to very small changes in glomerular filtration rate, and therefore facilitates the detection of early stage kidney dysfunction (17). In contrast to B2M, infection, inflammation and malignancy do not increase the urinary concentration of Cys-C as Cys-C is produced and filtered at a constant rate. These advantages may promote the widespread use of urinary Cys-C as a marker of tubular injury in pre-clinical experiments. Cys-C, the biomarker of damage to the glomerulus and proximal tubules, was unaffected after 48h storage at any of the temperatures tested. However, in cases of delays in measurement, it is recommended that measurement be performed within 48 hr of sample collection in order to enable the acquisition of accurate results, unaffected by storage temperature or duration.

The NAG level was a significantly decreased tendency after 144 hr at 20°C compared to 0 hr. However, it was not different after 144 hr at 4°C or −70°C. Therefore, NAG levels are affected by storage temperature, and we found it to be most stable at 4°C or −70°C. These results are similar to previous studies of urinary enzymes (18). NAG is a type of lysosomal enzyme that mainly exists in the liver, spleen, brain and kidney. An especially high concentration of NAG is present in the epithelial cells of the proximal tubules (19,20). It is utilized as a marker for the early detection of proximal tubule dysfunction, since the majority of it is derived from proximal tubules. Therefore, the measurement of NAG enzymatic activity in urine is widely used to assess of renal tubular damage resulting from various renal diseases, drug nephrotoxicity and rejection after renal transplantation (18). Ideally, NAG concentration should be measured as soon as possible after urine collection to obtained results. However, if the analysis of NAG levels in the urine is delayed, we have found that the sample can be stably stored for 144 hr at 4°C or −70°C. No effect on biomarker levels was seen within 48 hr of storage at 20°C, but a rapid decreasing tendency in concentration was found when measurements were taken 144 hr after urine collection. Thus, 20°C storage is concluded to be unacceptable storage condition for samples in which NAG will be measured.

MP and MA levels in urine were found to be highly stable, as the concentration detected were not different after storage for 144 hr, at all temperature tested. Although the stability of specimens MP and MA was consistent for up to 144 hr after urine collection, further experiments were required to address their stability after longer storage periods.

The purpose of this study was to provide information for the assessment of influences on urine sample examination. We have established the optimal storage conditions of urine for the preservation of proteins utilized as early biomarkers for the detection of nephrotoxicity. Overall, the highest stability of all urine biomarkers types was achieved under refrigeration; however, there were differences among the tested biomarkers. Therefore, urine specimens should be tested as soon as possible after collection in order for clinical laboratory to accurately determine the concentration of novel biomarkers. These data will facilitate the measurement of the nephrotoxicity of test substances in nephrotoxicity and efficacy studies.

Taken together, the ability to reliably measure biomarkers in urine samples is critically dependent on appropriate sample processing. Thus, it is desirable to measure biomarker levels as soon as possible after the collection of urine. When performing measurements after sample preservation, it should be determined that storage of the urine for up to 48 h under refrigeration. And we suggest that strict protocols be established for the storage of urine samples, minimize variations in biomarker detection as a results of these procedures.

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