A Method for Determining Urinary Enzyme Activities as Nephrotoxic Indicators in Rats

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Accepted July 17, 1990

Abstract—A simple, useful method was developed for detecting drug nephrotoxicity in rats. Rat urine excreted by stimulation of the sacral part of the back was collected in a beaker, and enzymes in 0.2 ml of the urine were partially purified by centrifugal ultrafiltration using an Amicon MPS-1 kit. Activities of N-acetyl-β-D-glucosaminidase (NAG), alanine aminopeptidase (AAP), γ-glutamyltranspeptidase (γGTP) and lactate dehydrogenase (LDH), and the protein concentration of the enzyme preparation suspended in phosphate-buffered saline were represented as creatinine ratios. A marked increase in these enzyme activities and protein concentration were observed in rats with kidneys damaged by treatment with cephaloridine, HgCl2, cisplatin or gentamicin. The patterns of increase of these indicators differed with the drug, but in general, LDH showed the highest response and NAG, the longest lasting one. These data agreed with the results reported by other researchers using dialyzed 24-hr urine. Thus, we concluded that this method is an efficient one for determining drug nephrotoxicity in rats.

As many enzymes are released from kidney tissue into urine when the kidney is injured (1), urinary enzymes have recently been added to the list of markers of nephrotoxicity, which can also be determined from plasma urea nitrogen and creatinine levels and by urinalysis using test paper. Many reports have appeared on the changes in urinary enzyme activities induced by drug nephrotoxicity in animals, especially rats (1-5). However, despite the sensitivity of urinary enzymes as markers, using them for analysis is difficult because urine collection and partial purification of the urine, which must be done to determine urinary enzyme activities, are troublesome. Usually, 24-hr urine is collected in a metabolism cage with cooling equipment to maintain the stability of the enzymes in collected urine (2-6). Rough purification of urinary enzymes, using methods such as dialysis or gel filtration, is also required to remove enzyme inhibitors present in urine (2-5, 7-9).

To overcome the problems involved in using urinary enzymes as markers, we developed a simple and useful method for measuring urinary enzyme activities in rats. The gist of the method consists of using spot urine excreted by stimulation of the sacral part of the back as reported by Khosho et al. (10) and subjecting the sample to centrifugal ultrafiltration with an Amicon MPS-1 kit to remove enzyme inhibitors. Enzyme activities are represented as creatinine ratios. Changes in urinary enzyme activities and protein concentration following administration of cephaloridine, HgCl2, cisplatin and gentamicin are represented by the plasma levels of creatinine and urea nitrogen and the histological findings of the kidney.

Materials and Methods
Male Sprague-Dawley rats (Jcl:SD rats), 7-8 weeks old, were used. The rats were kept in a temperature-controlled room at 25±1°C with a light-dark cycle of 12/12 (light period=8 a.m.–8 p.m.) and 50-60% relative humidity, and they were supplied with food (CA-1, Clea Japan) and tap water ad libitum. Urine was collected by the modified
method of Khosho et al. (10). A rat was held under the forelegs with the first and third fingers and lifted lightly and stimulated tactiley on the sacral part of the back using the fingers of the opposite hand. The tail was held by the remaining fingers at the same time. The released urine was collected in a beaker. When the urine volume was less than 0.2 ml, the procedure was repeated one hour later. When an older male rat (over 10 weeks old) was used, strong stimulation was avoided, because it could cause excretion of semen, which is rich in N-acetyl-β-D-glucosaminidase (11). The collected urine was kept in ice water and centrifuged at 2000 rpm for 5 min. Next, 0.2 ml of the supernatant was applied to a tube of MPS-1 (micropartition system standard kit, membrane YMT, Amicon) and 0.8 ml of 50 mM KH₂PO₄-K₂HPO₄ (pH 7.4) in 0.9% saline (PBS) was applied over it. Ultrafiltration was done by centrifugation at 3000 rpm for 30 min at 5°C. Enzyme preparation was done by suspending protein on the membrane of the kits in 1 ml of PBS. The lower vessel of the kit containing the ultrafiltrate was removed, then 0.5 ml of PBS was added to the upper tube holding the protein on the membrane. A suspension of the protein was prepared by vortexing for about 5 sec using a Therm-mixer apparatus. After the suspension was transferred into a tube, the same procedure was repeated, and the wash was mixed with the suspension. Enzyme activities of the preparation were stable for 9 days at 5°C. The creatinine concentration of the filtrate was determined by the Jaffe reaction (12).

N-Acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.30) was measured by a modification of the method of Maruhn (9). A 0.1-ml sample of the enzyme preparation and 0.5 ml of the substrate solution (5 mM p-nitrophenol-N-acetyl-β-D-glucosamine (Sigma) in 50 mM citric acid-K₂HPO₄-KOH, pH 4.2) were incubated for 30 min at 37°C. The reaction was stopped by addition of 1 ml of 0.1 M borate buffer (H₃BO₃-KOH), pH 10.5. The amount of p-nitrophenol was measured spectrophotometrically at 405 nm. The activity was represented as nmol of p-nitrophenol formed per min.

Alanine aminopeptidase (AAP, EC 3.4. 11.2) was measured by the method of Jung and Scholz (13) with a slight modification. A 0.1-ml sample of the enzyme preparation and 1 ml of the substrate solution (2 mM L-alanine-4-nitroanilide (Merck) in 50 mM Tris-HCl buffer, pH 7.6) were incubated for 10 min at 37°C. The reaction was stopped by addition of 0.1 ml of 20% sodium laurylsulfate (SDS). The amount of p-nitroaniline was measured at 405 nm within one hour. The activity was represented as nmol of p-nitroaniline formed per min.

γ-Glutamyl-transpeptidase (γ-GTP, EC 2.3.2.2.) was measured by a modification of the method of Szasz (14). A 0.05-ml sample of the enzyme preparation and 2 ml of the substrate solution (4.2 mM L-glutamyl-p-nitroanilide (Sigma), 263 mM glycylglycine, 10.5 mM MgCl₂ in 50 mM Tris-HCl buffer, pH 9.3) were incubated for 10 min at 37°C, and the reaction was stopped by addition of 0.2 ml of 20% SDS.

Lactate dehydrogenase (LDH, EC 1.1.1.27) was measured by a modification of the method of Bergmeyer and Bernt. (15). A 0.5-ml sample of the enzyme preparation and 0.3 ml of 0.1 M KH₂PO₄-K₂HPO₄ buffer, pH 7.4 (phosphate buffer), containing 0.2% Triton X-100, 0.1 ml of 10 mM sodium pyruvate in phosphate buffer, and 0.1 ml of 1 mg/ml NADH (Boehringer), were incubated at 30°C, and the amount of decrease in NADH was determined at 340 nm by rate assay (for 2 min). The activity was represented as nmol of decreased NADH per min. The protein content of the enzyme preparation was determined by the method of Lowry et al. (16) using bovine serum albumin as the standard. Urinary enzyme activities and protein concentration were represented as creatinine ratios. Plasma creatinine and urea nitrogen were determined by the Technicon C9100 system.

For histological observation, the kidney was fixed in 10% neutral formalin and then stained with hematoxylin and eosin.

Cephaloridine (CER), mercuric chloride (HgCl₂) and cisplatin (CDDP) were dissolved in 0.9% saline, and 5 ml/kg body weight of the solution was administered intravenously once. Gentamicin sulfate (GM) was administered subcutaneously once a day for 4 days. Significant differences were determined by
Student’s t-test (equal variance) or the Cochran-Cox test (unequal variance). Before the test, the values of enzyme activities and protein concentration were converted to common logarithm values.

Results

Studies on assay method

An example of the individual urine volume obtained by sacral stimulation is shown in Fig. 1. Over 0.2 ml of urine, enough for the assay, was obtained from 9/10 rats from one session in the morning. As for the remaining rat, enough urine for the assay was obtained at the second session. With repeated urine collection in the afternoon with the same rats, all animals excreted more than 0.2 ml of urine.

Figure 2 show the enzyme recovery from the urine. A quantitative relationship was found between the urinary volumes used and the enzyme activities obtained, with the line passing through the zero point. LDH was slightly more variable than other enzymes because of its weaker activity. For the determination of LDH activity, Triton X-100 was added to the reaction medium. The LDH activity increased about 2 times with 0.02-0.5% Triton X-100 (Table 1). The other enzymes, however, were not influenced by these treatments. Only 0.2 ml of urine was sufficient for the assay of these enzymes.

Fig. 1. Individual urine volume obtained by stimulation of the sacral part of the back of male rats.

Fig. 2. Relationship between sampling volume of urine and optical density in assay of enzyme activities. Specimens of 0.1 ml to 0.5 ml of pooled normal rat urine were ultrafiltrated with Amicon MPS-1 kits. The enzyme assay method is described in the text.
Table 1. Effect of Triton X-100 added to the assay medium on assay of urinary LDH activity

| Concentration (%) of Triton X-100 | LDH activity (decreased O.D. at 340 nm/min) |
|----------------------------------|--------------------------------------------|
| 0                                | 0.043                                      |
| 0.02                             | 0.104                                      |
| 0.05                             | 0.107                                      |
| 0.10                             | 0.106                                      |
| 0.20                             | 0.111                                      |
| 0.50                             | 0.104                                      |

Urine was obtained from normal rats, and the urine sample was dialyzed overnight against phosphate-buffered saline at 4°C.

Table 2. Accuracy of the urinary enzyme assay

| Enzyme | No. of samples | Activitya            | Cephaloridine         |
|--------|----------------|----------------------|-----------------------|
|        |                | Normal               | Cephaloridine         |
| NAG    | 8              | 18.3±0.3 (4.6)b      | 76.1±0.8 (3.0)        |
| AAP    | 8              | 83.9±1.5 (5.1)       | 184.1±2.1 (3.2)       |
| γGTP   | 8              | 1355±18 (3.8)        | 1689±11 (1.8)         |
| LDH    | 8              | 36±2 (15.7)          | 2344±22 (2.6)         |

Spot urine samples were collected 24 hr after i.v.-administration of 1 g/kg of cephaloridine. aU/mg creatinine. bMean±S.E.M. (coefficient of variation).

Fig. 3 Daily variation of urinary enzyme activities and protein concentration in normal rats. At 9:00–10:00 a.m. and 3:00–4:00 p.m. everyday for 5 days, the sacral part of the back was tickled and urine was collected. Each point represents the mean±S.E.M. of 6 rats.
The accuracy of these methods for enzyme activities are shown in Table 2. The values for the coefficient of variation (CV) of the activities in normal rat urine were below 5% except the CV of LDH, which was 15.7%. The CV of the enzyme activities including LDH decreased to about 3% in the urine of cephaloridine (1 g/kg, i.v.)-treated rats, in which enzyme activities markedly increased.

Normal rat urine was collected twice a day in the morning (9:00–10:00 a.m.) and afternoon (3:00–4:00 p.m.) for 5 days. As shown in Fig. 3, urinary enzyme activities and protein concentration were higher in the afternoon. Fluctuations of urinary activities of NAG and LDH were small at both times, but the activities of AAP and rGTP were slightly more variable in the afternoon.

Effect of drugs

Cephaloridine (CER): As shown in Fig. 4, increased in urinary enzymes except for NAG and protein were observed even 6 hr after administration of 1000 mg/kg of CER, and the levels of all of these components reached maxima 24 hr later. LDH showed the highest response among these components. Recovery of AAP and rGTP to the control levels was observed at 48 hr, when increases in NAG, LDH and protein were lasting. CER at 500 mg/kg caused no increase in these urinary components.

Table 3 shows the plasma levels of creatinine and urea nitrogen and the pathological findings of the kidney in this experiment.
Increases in creatinine, urea nitrogen (not significant) and kidney weight were observed 2 day after 1000 mg/kg of CER. Proximal tubular necrosis was also observed at a moderate to severe degree. No change was observed after treatment with 500 mg/kg of CER.

**Mercuric chloride (HgCl₂): Changes in**

| Cephaloridine (mg/kg) | No. of rats | Kidney wt. (g/100 g BW) | Plasma |
|-----------------------|-------------|--------------------------|--------|
|                       |             |                          | Creatinine (mg/100 ml) | Urea-N (mg/100 ml) | Proximal tubular necrosis |
| 0                     | 5           | 0.78±0.01*                | 0.43±0.01          | 22±1               | -                         |
| 500                   | 4           | 0.80±0.03**               | 0.41±0.00          | 20±1               | -                         |
| 1000                  | 4           | 0.92±0.02**               | 0.75±0.07*         | 36±5               | + or ++                   |

Rats were sacrificed 2 days after i.v. administration of cephaloridine. *Mean±S.E.M. **: Significantly different from the control group (P<0.05, P<0.01). †Arbitrary grade: -, no change; +, moderate; ++, severe.

**Fig. 5. Effect of mercuric chloride on urinary enzyme activities and protein concentration.** HgCl₂ was administered i.v. Each point represents the mean±S.E.M. of 5 or 10 rats. *, **: Significantly different from the control group at P<0.05, P<0.01.
urinary enzymes and protein are shown in Fig. 5. Marked increases in these components were observed following administration of 0.8 mg/kg of HgCl₂, and the maximal responses were observed 1 day after dosing. All of these components, except for NAG, increased rapidly, and the levels were 2- to 5-fold higher than each control level 6 hr after drug administration. LDH showed the highest increase, and the maximal level was about 45 times higher than the control level. The activity, however, decreased to near the control level after 4 days. Similar fluctuation was seen in urinary protein, but the response was poor. Activities of AAP and γGTP decreased to normal levels after 2 days and fell below the control 4 days after drug administration. On the other hand, NAG showed a more continuous increase than the other components, and its level was higher than the control even 4 days after dosing. HgCl₂ at 0.4 mg/kg caused only a slight increase of LDH 1 day after dosing, and the level recovered 2 days after dosing.

Table 4 shows the plasma levels of creatinine and urea nitrogen, and the pathological findings of the kidney in this experiment. HgCl₂ at 0.8 mg/kg caused renal damage as shown by the marked increase in plasma levels of creatinine and urea nitrogen and severe proximal tubular necrosis. Increase in kidney weight was also observed. Recovery of creatinine and urea nitrogen levels and regeneration of tubules in the necrotic parts were observed 7 days after dosing. HgCl₂ at 0.4 mg/kg caused a slight increase in kidney weight and the plasma level of urea nitrogen. No changes were observed histopathologically.

**Table 4. Nephrotoxicity of mercuric chloride**

| HgCl₂ (mg/kg) | No. of rats | Days after dosing | Kidney wt. (g/100 g BW) | Plasma Creatinine (mg/100 ml) | Urea-N (mg/100 ml) | Proximal tubular necrosis |
|---------------|-------------|-------------------|-------------------------|-----------------------------|-----------------|-------------------------|
| 0             | 5           | 7                 | 0.77±0.02a               | 0.43±0.01                   | 22±1            | –                       |
| 0.4           | 5           | 2                 | 0.89±0.01**              | 0.53±0.07                   | 27±2*           | –                       |
| 0.8           | 5           | 2                 | 1.08±0.03**              | 1.61±0.17**                 | 111±17*         | ++                      |
| 0.8           | 5           | 7                 | 1.21±0.08**              | 0.55±0.08                   | 25±1            | regeneration |

Rats were sacrificed 2 days or 7 days after i.v. administration of HgCl₂. aMean±S.E.M. **,***: Significantly different from the control (P<0.05, P<0.01). bArbitrary grade: –, no change; ++, severe.

**Cisplatin (CDDP):** As shown in Fig. 6, urinary enzymes and protein increased following administration of 6 mg/kg of CDDP. The levels of these components reached their maxima 4 days after dosing. LDH showed the highest response, and its activity was higher than the control even after 6 hr and remained at a higher level 7 days after dosing. NAG also remained at a higher level than the control after 7 days, but the increase in the activity was delayed, and the activity showed no change until 24 hr after dosing. A similar delay was observed in urinary protein. Increases in AAP and γGTP started from 24 hr after dosing, and the levels recovered after 7 days.

Table 5 shows plasma levels of creatinine and urea nitrogen, and the pathological findings of the kidney in this experiment. Although slight to moderate necrosis of the proximal tubules was observed histologically, only a slight elevation of urea nitrogen was seen in the plasma 2 days after administration of 6 mg/kg of CDDP. At 7 days after dosing, increases in kidney weight and in plasma levels of creatinine and urea nitrogen (statistically not significant) were observed. Histological findings showed severe necrosis of the proximal tubules with partial regenerated tubules.

**Gentamicin (GM):** Rats were administered 25, 50 and 100 mg/kg of GM once a day for 4 days, and daily collection of urine was done before each drug administration. The rats were sacrificed after urine collection on the day after the last administration. As shown in Fig. 7, LDH increased in a manner dependent on dosage and administration frequency. Increases in NAG, AAP and protein were ob-
served mainly after dosing four times, but the responses were poor and not dose-related. No increase was observed in \( \gamma \)GTP. Table 6 shows the plasma levels of crea-

![Graphs of enzyme activities](image)

**Fig. 6.** Effect of cisplatin on urinary enzyme activities and protein concentration. Cisplatin was administered i.v. Each point represents the mean±S.E.M. of 4–8 rats. *: **: Significantly different from the control group at \( P<0.05 \), \( P<0.01 \).

| Cisplatin (mg/kg) | No. of rats | Days after dosing | Kidney wt. (g/100 g BW) | Plasma Creatinine (mg/100 ml) | Urea-N (mg/100 ml) | Proximal tubular necrosis* |
|------------------|-------------|-------------------|--------------------------|-----------------------------|-------------------|---------------------------|
| 0                | 6           | 7                 | 0.75±0.03*a              | 0.48±0.01                   | 21±0              | -                         |
| 3                | 4           | 2                 | 0.80±0.03                | 0.44±0.00*                  | 19±1*             | ~±                        |
| 6                | 4           | 2                 | 0.81±0.02                | 0.52±0.02                   | 26±1***           | ±~                         |
| 6                | 4           | 7                 | 1.19±0.09*               | 0.84±0.13                   | 66±20             | ++                         |

Rats were sacrificed 2 days or 7 days after i.v. administration of cisplatin. aMean±S.E.M. **: Significantly different from the control (\( P<0.05 \), \( P<0.01 \)). bArbitrary grade: -, no change; ±, slight; ++, severe.
tinine and urea nitrogen and the pathological findings of the kidney at sacrifice. GM caused a slight elevation of creatinine and urea nitrogen of 100 mg/kg/day and a slight

![Graph](image)

Fig. 7. Effect of gentamicin on urinary enzyme activities and protein concentration. Gentamicin sulfate was administered s.c. once a day for 4 days, and urine was collected just before the daily dosing. Each point represents the mean±S.E.M. of 5 rats. *, **: Significantly different from the control group at P<0.05, P<0.01.

| Gentamicin (mg/kg) | No. of rats | Kidney wt. (g/100 g BW) | Plasma |
|-------------------|-------------|--------------------------|--------|
|                   |             |                          | Creatinine (mg/100 ml) | Urea-N (mg/100 ml) | Hyaline droplet degeneration of proximal tubules |
| 0                 | 5           | 0.79±0.03*               | 0.36±0.01               | 19±1               | -            |
| 25                | 5           | 0.82±0.02                | 0.36±0.01               | 20±1               | - ~ ±        |
| 50                | 5           | 0.82±0.02                | 0.43±0.02*              | 21±5               | ± ~ +        |
| 100               | 5           | 0.87±0.02                | 0.52±0.02**             | 25±2*              | + ~ ++       |

Gentamicin was administered s.c. once a day for 4 days, and the rats were sacrificed 24 hours after the final administration. *Mean±S.E.M. **: Significantly different from the control (P<0.05, P<0.01).

Arbitrary grade: -, no change; ±, slight; +, moderate; ++, severe.
elevation of only creatinine of 50 mg/kg/day. No increase in kidney weight was observed in all treated groups. In the histological observation, the appearance of hyaline droplet degeneration in the epithelium of proximal tubules was dose-related. Scattering necrosis of the tubular epithelium was also found in the 100 mg/kg/day group.

Discussion
Urinary enzyme determinations provide sensitive noninvasive indicators of renal damage (1), but the procedures for collection and partial purification of urine before enzyme assay are troublesome. Usually, rats are individually housed in metabolic cages to collect urine for 18 or 24 hr without contamination by feces, and the urinary enzymes are rapidly purified by dialysis (2–5) or gel filtration (9) because urine contains enzyme inhibitors (7–9). In contrast, the method developed in this study uses spot urine excreted by stimulation of the sacral part of the back. For enzyme assays, 0.2 ml of urine is needed and over 0.2 ml of urine could be obtained from about 80% of the rats in one session. For the remaining rats, urine could be obtained by a second session 1 hr later. This collection procedure is very easy. For 18- or 24-hr urine collection, a cooling apparatus is needed because some enzymes are unstable in urine at room temperature (2–4). However, our method eliminates the need for such an apparatus. For partial purification of urinary enzymes, our method uses centrifugal ultrafiltration with an MPS-1 kit. YMT membranes used in the MPS-1 kits retain more than 99.9% of serum protein and less than 5% of L-thyroxine (Instructions for ultrafiltration membrane type YMT for MPS-1, Amicon). This purification method is simple and quantitative, and the values of the enzyme activities obtained were equal to values obtained by a dialysis method.

The enzyme activities found in this study are given as the ratio against urinary creatinine. As endogenous creatinine filtrated by the renal glomeruli appears in the urine without any modification by the tubules, the urinary creatinine level is related to the glomerular filtration rate. Therefore, the enzyme activity/creatinine (E/C) ratio must be more constant than the concentration of enzyme activity in spot urine. Indeed, enzyme activity indicated by the E/C ratio of spot urine obtained at the same time of day was fairly constant day by day in normal rats, although it was higher in the afternoon than in the morning for all tested enzymes. This variation in the levels of urinary enzymes may be due to differences in the time elapsed after intake of food containing creatine or its precursor and/or to diurnal variation in renal function (17, 18).

Renal damage was caused by treatment of rats with CER, HgCl₂, CDDP or GM as shown by the histological and plasma biochemical findings. Although the renal damage appeared mainly in the proximal tubules, the type of damage was different among the four drugs: proximal tubular necrosis by CER, necrosis in the straight part of the proximal tubules by HgCl₂, necrosis and dilatation in renal tubules by CDDP, and accumulation of hyaline droplets in the proximal tubular epithelium by GM as reported previously (19–21). Urinary enzymes also increased with administration of these drugs, and the patterns of change in enzyme levels also differed. These observations agreed with results reported by other researchers using 24-hr urine (2, 4, 22). AAP and rGTP, localized in the brush border, increased in an early stage after administration of CER, HgCl₂, or CDDP. However, the activities decreased to levels lower than the control ones at 4 and 7 days after HgCl₂ or CDDP administration. NAG, localized in the lysosomes, increased more slowly than other enzymes, and the responses of NAG and LDH were lasting. The response of LDH was highest among the tested enzymes and protein, and the response to GM was dose-dependent. LDH also showed a temporal increase without any histological change after administration of 0.4 mg/kg of HgCl₂. Changes in urinary enzymes were more sensitive than histological changes 2 days after administration of 3 mg/kg CDDP. These observations show that renal damage can occasionally be detected more sensitively by changes of enzyme activities in a spot urine than by light microscopical observation. In addition to this sensitivity, changes within a short time after drugs administration can be
observed by this method. Thus, the method introduced here is a simple and efficient one for determining drug nephrotoxicity in rats.

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