URINALYSIS FOR DETECTION OF CHEMICALLY INDUCED RENAL DAMAGE (2)

Changes in urinary excretions of enzymes and various components caused by p-aminophenol, puromycin aminonucleoside and hexadimethrine

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Abstract In order to establish sensitive methods of detecting minor renal damage, changes of enzymes, protein, tubular cell counts, and creatinine in the urine were investigated in rats to which nephrotoxic chemicals had been administered.

Daily administration of p-aminophenol (PAP) dose-dependently increased urinary excretions of lactate dehydrogenase (LDH) and its isoenzymes (LDH5 > LDH4 > LDH3 > LDH2 > LDH1), aspartate aminotransferase (GOT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), leucine aminopeptidase (LAP), lysozyme (LZM), N-acetylmuramidase (NAG) and acid protease together with increased counts of tubular cells in the urine. Tubular cell counts, LDH and GOT were more sensitive indicators in the PAP tubulonephritis.

Single i.v. injection of puromycin aminonucleoside (PM) dose-dependently increased urinary excretions of LDH and its isoenzymes (LDH1 = LDH5 > LDH2 > LDH4 > LDH3), GOT, NAG, acid protease and protein but degree of the increases in these enzymes was lower than those in the rats treated with PAP. PM increased excretions of high molecular weight proteins but did not increase ALP, γ-GTP, LAP, LZM and tubular cell excretions.

Single i.v. injection of hexadimethrine increased urinary excretion of LDH and its isoenzymes (LDH1 = LDH5 > LDH2 > LDH3 = LDH4), GOT, LZM, NAG and acid protease together with increased counts of tubular cells in the urine but did not increase ALP, γ-GTP and LAP excretions.
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It is concluded that tubular cell counts, LDH isoenzymes and battery of these enzymes in urine are useful markers for detecting the severity and the site of renal damage in addition that urinary protein is a useful marker for detecting glomerular damage.

**Key words**: Urinalysis, renal damage, urinary enzyme excretion, \( p \)-aminophenol, puromycin aminonucleoside, hexadimethrine

**INTRODUCTION**

The chemically induced renal damage has been verified by physiological function tests, blood analysis and urinalysis (Diezi and Biollaz, 1979; Berndt, 1981). Recently much interest has been shown in the use of urinary enzymes for detecting renal damage in an early phase (Raab, 1972; Piperno, 1981; Price, 1982; Takahashi and Ohata, 1982). In the previous report we have shown that the severity and/or the site of renal damage can be presumed by periodic determination of urinary tubular cell counts, lactate dehydrogenase isoenzymes and some enzyme excretions in rats with tubulonephritis caused by mercuric chloride and by gentamicin (Ohata et al., 1987). However, usefulness of this urinalysis for detecting renal damage caused by other nephrotoxic chemicals is uncertain.

In this study, in order to establish methods of detecting renal damage in an early phase and estimating the damaged site, changes in some enzymes, LDH isoenzyme patterns, tubular cell counts of urine were investigated in rats treated with well known nephrotoxic chemicals, \( p \)-aminophenol causing tubulonephritis (Calder et al., 1971; Davis et al., 1981), puromycin aminonucleoside causing glomerulonephritis (Gang and Mautner, 1972; Price and Ellis, 1976) and hexadimethrine causing nephritis (Horvath and Kovacs, 1968; Hunsicher et al., 1981).

**MATERIALS AND METHODS**

**Materials**

\( p \)-Aminophenol (PAP) was purchased from Tokyo Kasei Co., and puromycin aminonucleoside (PM) and hexadimethrine (HD) were purchased from Sigma Chemical Co. All other chemicals were of commercially available analytical grade.

**Animals**

Male Wistar rats (9–10 weeks of age) were used throughout this study. The animals were housed in a temperature (23–25°C), humidity (50–70%), and light-cycle (12 h light, 12 h dark) controlled room with free access to standard rat chow and water.

**Treatments**

The chemicals were dissolved in saline and injected (2 ml/kg body weight) as follows: PAP was injected s. c. for 7 days in doses of 0.25 mmole (27.5 mg)/kg/day
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(PAP-L), 0.5 mmole (55 mg)/kg/day (PAP-M), or 1.0 mmole (110 mg)/kg/day (PAP-H). PM was administered as a single i. v. injection in doses of 37.5 mg/kg (PM-L) or 75 mg/kg (PM-H). HD was administered as a single i. v. injection in dose of 15 mg/kg. Control animals were injected s. c. or i. v. with saline (2 ml/kg/day) in each experiment.

**Urine collection**

The rats were housed singly in metabolic cages (Nippon Clea) with free access to standard rat chow and water. The urine was collected in tubes cooled by Coolnit (CML-III, Taiyo Scientific Industrial Co.) to 4–8°C. In PAP-L, PAP-M, PAP-H and HD groups, the urine samples were collected during the periods of 0–10 and 10–24 h after the first injection, and then 24 h urine samples were collected on days 2, 3, 5 and 7. In the PM treated groups, 24 h urine samples were collected on days 1, 3, 5, 7, 9 and 14.

**Urinalysis**

Tubular cell counts, concentration of creatinine, activities of lactate dehydrogenase (LDH, EC 1.1.1.27), aspartate aminotransferase (GOT, EC 2.6.1.2), alkaline phosphatase (ALP, EC 3.1.3.1), γ-glutamyl transpeptidase (γ-GTP, EC 2.3.2.2), leucine aminopeptidase (LAP, EC 3.4.11.1), lysozyme (LZM, EC 3.2.1.17), N-acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.30) and acid protease (EC 3.4.23--), and profile of LDH isoenzymes were determined as described in the previous report (Ohata et al, 1987). Urinary excretion of each parameter was expressed as activity or as amount per h per kg of body weight.

Urinary protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of urinary proteins was performed in 7.5% acrylamide gel according to the method of Fairbanks et al. (1971).

**RESULTS**

1. Changes in urinary parameters from PAP treated rats

1-1) Urine volume, creatinine, protein and tubular cell counts

Urine volume was not increased in the PAP-L group and was increased only on day 2 in the PAP-M group. In the PAP-H group, urine volume increased to 2–3 times the control value from the 10–24 h period to day 3, and then declined on day 5. However, the value on day 5 was significantly higher than the control value (Fig. 1-A). Creatinine excretion was increased only to 1.3 and 1.6 times the control value in the PAP-L and the PAP-H groups, respectively, on day 2 (Fig. 1-B). Urinary protein excretion was increased to 1.4 times the control value only on day 5 in the PAP-L group and to 1.7 times the control value during the 0–10 h period and day 2 in the PAP-M group. In the PAP-H group, urinary protein excretion was increased to 6.5 times the control value, and then declined. However, the value on day 7 was still

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Fig. 1. Effects of p-aminophenol on various parameters in rat urine. Each point is mean ± SE of five animals. ○ — ○ : control, □ — □ : 0.25 mmole/kg/day s. c. for 7 days (PAP-L), ● — ● : 0.5 mmole/kg/day s. c. for 7 days (PAP-M), ■ — ■ : 1.0 mmole/kg/day s. c. for 7 days (PAP-H), A : urine volume, B : creatinine, C : protein, D : tubular cells, *, ** : significantly different (* : p<0.05, ** : p<0.001) from the control group.

significantly higher than the control value (Fig. 1-C). In case of tubular cell counts, this value was increased even in the PAP-L group to 18 times the control value on day 2 and the increase persisted until day 7. In the PAP-M and the PAP-H groups, tubular cell counts were increased to 94 and 250 times the control value, respectively, during the 10–24 h period, and maintained the higher levels until day 7 (Fig. 1-D).

1-2) Urinary enzymes

Changes in excretions of LDH, GOT, ALP, \( \gamma \)-GTP, LAP, LZM and acid protease in the urine from PAP treated rats are shown in Fig. 2. All the enzyme excretions were dose-dependently increased. In the PAP-L group, excretions of LDH and GOT were increased to 2–4 times the control values but excretions of the other enzymes were not increased. Excretions of these enzymes except LZM in the PAP-M group and excretions of all the enzymes in the PAP-H group were increased.
Fig. 2. Effects of p-aminophenol on various enzymes in rat urine. Each point is mean ± SE of five animals. ○ ○ : control, □ □ : 0.25 mmole/kg/day s. c. for 7 days (PAP-L), ● ● : 0.5 mmole/kg/day s. c. for 7 days (PAP-M), ■ ■ : 1.0 mmole/kg/day s. c. for 7 days (PAP-H), A : LDH, B : GOT, C : ALP, D : γ-GTP, E : LAP, F : LZM, G : acid protease, * * : significantly different (* : p < 0.05, ** : p < 0.001) from the control group.
Excretions of LDH, GOT, ALP, $\gamma$-GTP and LAP reached to the peak values during the 0–10 h period in the PAP-M and the PAP-H groups, and then declined. The peak values of LDH, GOT, ALP, $\gamma$-GTP and LAP were 14, 24, 7.9, 9.5, and 7.0 times the control values, respectively, in the PAP-M group and were 53, 49, 20, 16 and 23 times the control values, respectively, in the PAP-H group. Excretions of LDH, GOT and LAP on day 7 were significantly higher (2–6 times) than the control values. LZW excretion was significantly increased only in the PAP-H group and the peak value was 13.5±5.9 $\mu$g/kg/h during the 10–24 h period, but, after day 3, the values were not significantly different from the control values (Fig. 2-F). Acid protease excretion was maximally increased to 1.6 and 4.6 times the control value in the PAP-M and the PAP-H groups, respectively, during the 10–24 h period. Then it declined, although the values on days 7 was still significantly higher than the control value (Fig. 2-G).

1–3) LDH isoenzymes

In normal rat urine, LDH1 and LDH5 predominated and each of them accounted for about 35% of the total LDH excretion (LDH1 =LDH5 >LDH2 >LDH4 > LDH3). In the PAP-L group, LDH isoenzymes during the 0–10 h period could not be determined because total LDH excretion during this period was too low to analyze the LDH isoenzymes. Excretions of LDH4 and LDH5 were increased to 2–3 times during the 10–24 h period and day 3 in the PAP-L group. In the PAP-M and the PAP-H groups, excretions of all the isoenzymes began to increase during the 0–10 h period and LDH 4 and LDH5 predominated. The isoenzyme profiles were not changed on day 3 when LDH total excretion was decreased to 10% of the peak value (Fig. 3).

2. Changes in urinary parameters from PM treated rats

2-1) Urine volume, creatinine and protein

Urine volume and creatinine excretion were not increased (Fig. 4-A, B). Urinary protein excretion reached to twice the control value on day 9 in the PM-L group, but the value was not significantly different from the control value. In the PM-H group, urinary protein excretion began to increase on day 3 and reached to the peak value of 13 times the control value on day 9. Then it declined to 4.8 times the control value on day 14 but the value was still significantly higher than the control value (Fig. 4-C). SDS-PAGE of the urinary proteins indicated that PM markedly increased excretion of proteins with molecular weight of over 60,000 (data not shown).

2-2) Urinary enzyme activities

Changes in excretions of LDH, GOT, ALP, $\gamma$-GTP, LAP, LZW, NAG and acid protease in urine from PM treated rats are shown in Fig. 5. Excretions of all the enzymes were not increased in the PM-L group. LDH and GOT excretions reached to the peak values of 18 times the control values on day 9 in the PM-H group, and then declined to 4.5 and 8.7 times the control values, respectively, on day 14 (Fig. 5-A, B). ALP, $\gamma$-GTP and LAP excretions were slightly increased to less than
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Fig. 3. Effects of p-aminophenol on pattern of LDH isoenzymes in rat urine.

Fig. 4. Effects of puromycin aminonucleoside on various parameters in rat urine. Each point is mean ± SE of five animals. ○ — ○: control, □ — □: 37.5 mg/kg single i.v. injection (PM-L), ● — ●: 75 mg/kg single i.v. injection (PM-H). A: urine volume, B: creatinine, C: protein, * , **: significantly different (*: p < 0.05, **: p < 0.001) from the control group.

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Fig. 5. Effects of puromycin aminonucleoside on various enzymes in rat urine. Each point is mean ± SE of five animals. ○——○: control, □——□: 37.5 mg/kg single i. v. injection (PM-L), ●——●: 75 mg/kg single i. v. injection (PM-H), A: LDH, B: GOT, C: ALP, D: γ-GTP, E: LAP, F: LZM, G: NAG, H: acid protease, *, **: significantly different (*: p<0.05, **: p<0.001) from the control group.
twice the control values in the PM-H group (Fig. 5-C, D, E). LZM excretion reached to 1.53 ± 0.81 μg/kg/h on day 9 in the PM-H group, but the value was not significantly different from the control value (Fig. 5-F). NAG and acid protease excretions reached to the peak values of 3.7 and 4.6 times the control values, respectively, on day 9 and declined to 1.7 and 2.1 times the control values, respectively, on day 14. However, the values on day 14 were significantly higher than the control values (Fig. 5-G, H).

2-3) LDH isoenzymes
LDH1 and LDH2 predominated on day 5, and then LDH1 and LDH5 became predominated on days 7 and 9 in the PM-H group (Fig. 6).

2-4) Tubular cell counts
In the urine from the rats treated with PM of 75 mg/kg single i. v., tubular cell counts reached to 10 times the value before the treatment, on days 7 and 9, but the values were not significantly different from the value before the treatment (Fig. 7).

3. Changes in urinary parameters from HD treated rats
3-1) Urine volume, creatinine, protein and tubular cell counts
Urine volume was increased to 6.2 times the control value during the 10–24 h period and did not decline on days 2–7 (Fig. 8-A). Excretion of creatinine was slightly increased on days 5 and 7 (Fig. 8-B). Excretion of protein was increased to about 3 times the control value during the 0–24 h period and then declined (Fig.
Fig. 7. Effects of puromycin aminonucleoside (75 mg/kg single i. v. injection) on tubular cells in rat urine. Each point is mean ± SE of five animals.

8-C). SDS-PAGE of the urinary proteins indicated that HD increased excretion of proteins with molecular weight of over 60,000 (data not shown). Tubular cell counts began to increase during the 0–10 h period and was maximally increased to 49 times the control value on day 2 (Fig. 8-D). In the rats treated with HD of 10 mg/kg single i. v., urine volume and excretions of creatinine and protein were not increased. In the 5 rats treated with HD of 20 mg/kg single i. v., 3 rats died within 3 days after the injection (data not shown).

3-2) Urinary enzymes
Changes in excretions of LDH, GOT, ALP, 7-GTP, LAP, LzM, NAG and acid protease in the urine from HD treated rats are shown in Fig. 9. LDH and GOT excretions began to increase during the 0–10 h period and maintained the higher levels (20–40 times the control values) on days 1–3 (Fig. 9-A, B). ALP, 7-GTP and LAP excretions were not significantly higher than the control values except 7-GTP excretion on day 7 (Fig. 9-C, D, E). LzM excretion began to increase during the 0–10 h period and was maximally increased to 30.5 ± 4.8 g/kg/h on day 2 and then declined (Fig. 9-F). NAG excretion was slightly increased on days 1 and 2 (Fig. 9-G). Acid protease excretion was began to increase during the 0–10 h period and was maximally increased to 9.5 time the control value on day 5 (Fig. 9-H).
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Fig. 8. Effects of hexadimethrine (HD) on various parameters in rat urine. Each point is mean ± SE of five animals. ○——○: control, □——□: HD 15 mg/kg single i. v. injection, A: urine volume, B: creatinine, C: protein, D: tubular cells, *, **: significantly different (*: p<0.05, **: p<0.001) from the control group.

In the rats treated with HD of 10 mg/kg single i. v., excretions of LDH, GOT, LZM and acid protease were increased to 2.5, 1.9, 13 and 2.8 times the control values, respectively, on day 1 but the other enzyme excretions were not increased (data not shown).

3-3) LDH isoenzymes

LDH1 and LDH5 predominated during 0–24 h period, and then LDH1 and LDH2 became predominated on days 2 (Fig. 10).

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Fig. 9. Effects of hexadimethrine (HD) on various enzymes in rat urine. Each point is mean ± SE of five animals. ○——○: control, □——□: HD 15 mg/kg single i. v. injection, A: LDH, B: GOT, C: ALP, D: γ-GTP, E: LAP, F: LZM, G: NAG, H: acid protease, *: significantly different (*: p<0.05, **: p<0.001) from the control group.
DISCUSSION

We have reported that determinations of urinary tubular cell counts and some enzymes are very useful for detecting renal tubular damage caused by mercuric chloride (HgCl₂) and gentamicin (GM) (Ohata et al. 1987). In the present report, changes in the same parameters in renal damage caused by other three typical nephrotoxic compounds which damage different sites were investigated to establish sensitive methods of detecting minor renal damage.

Daily administration of p-aminophenol (PAP) dose-dependently increased tubular cell counts in urine. In particular, they were significantly increased also in the PAP-L group. On the contrary, other parameters measured simultaneously with tubular cell counts were not increased or were only slightly increased in the PAP-L group. Further, in the PAP-M and the PAP-H groups, tubular cell counts outlasted some urinary enzymes and maintained the higher level until day 7. Increased tubular cell counts in PAP groups were renal origin from their morphology. These results resemble those of tubular damage caused by HgCl₂ (Ohata et al., 1987). Hexadimethrine (HD) increased tubular cell counts in the urine. Tubular cell counts in HD group may include also extrarenal epithelial cell counts because other type of epithelial cells was observed in urine of HD group. However, most of
increased tubular cell counts were probably renal origin from their morphology. These results suggest that HD damages also renal tubules, in addition to the thick ascending of Henle's loop (Horvath and Kovacs, 1968). On the other hand, puromycin aminonucleoside (PM) did not significantly increased tubular cell counts in the urine. From these results, it is concluded that tubular cell count is a sensitive marker in the renal tubular damage.

In the PAP-M and the PAP-H groups, excretions of LDH, GOT, ALP, LAP and $\gamma$ -GTP declined after reaching to the peak values during the 0–10 h period, while the increase in tubular cell counts maintained the higher level until day 7. The pattern and the degree of these enzyme excretions resemble those of tubular damage caused by HgCl$_2$ (Ohata et al., 1987). It has been reported that PAP damaged distal third of the proximal convoluted tubules (Calder et al, 1971 ; Davis et al., 1983). Kojima and Suzuki (1984) reported that HgCl$_2$ damaged proximal tubular epithelial cells in the renal inner cortex. Koseki et al. (1980) reported that $\gamma$ -GTP, ALP and LAP were localized only in the proximal tubule in rat and that increased urinary excretions of the enzymes after administration of mercuric chloride probably originate from the kidney, particularly proximal tubule. Therefore, it seems that the site damaged by PAP is close to that by HgCl$_2$ and that it is mainly the proximal tubular brush border membrane where $\gamma$ -GTP, ALP and LAP are localized. PM and HD increased excretions of LDH and GOT but did not increase excretions of ALP, $\gamma$ -GTP and LAP. These results suggest that PM and HD do not damage the proximal tubular cells. However, HD might reduce reabsorption ability in renal tubules, because LZM excretion was markedly increased by HD. These results resemble those of the changes in urinary enzymes excretions by GM (Ohata et al., 1987). In a histological study, Kirino et al. (unpublished data) has observed that PM damaged mainly glomeruli and that HD damaged renal tubules, particularly distal tubules including ascending part of Henle's loop. Therefore, we suppose that the site of renal damage can be estimated by simultaneous determination of battery of these urinary enzymes.

PAP markedly increased excretions of LDH4 and LDH5. Profiles of the LDH isoenzymes resemble those on HgCl$_2$ tubulonephritis (Ohata et al., 1987). It has been reported that the distribution of LDH isoenzymes in the rat kidney is not homogeneous, and LDH1 and LDH2 predominate in the renal cortex whereas LDH4 and LDH5 predominate in the renal inner medulla (Ringoir, 1970 ; Cestonaro et al., 1979). Therefore, these results also suggest that the site damaged by PAP in the renal tubules is close to that by HgCl$_2$. In case of PM, excretions of LDH1 and LDH2 which do not predominate in serum were increased on day 5. Since LDH also exists in glomeruli (Guder and Ross, 1984), LDH which was increased by PM on day 5 might be derived from the renal tissue. After day 7, PM increased excretion of LDH5 which predominate in normal serum. This result suggests that glomerular damage caused by PM further progressed after day 7. We supposed that the increase in excretion of LDH5 by HD during the 0–24 h period might be also caused by the
glomerular damage. This can be also confirmed by the facts that HD increased excretion of high molecular weight proteins as described above and that heavy proteinuria caused by HD resulted from neutralization of glomerular polyanions (Hunsicker et al., 1981). However, HD increased excretions of LDH1 and LDH2 on day 2. Results of the urinalysis suggest that HD damages both glomeruli and tubules. Therefore, it is concluded that LDH isoenzyme is one of the most sensitive indicators of renal damage and that the site of damage can be presumed if the distribution of LDH isoenzymes in the nephron is ascertained.

Acid protease excretion was increased by PM as well as NAG excretion, although the increases in excretions of γ-GTP, ALP and LAP caused by PM were slight. The increase in acid protease excretion caused by PM was in agreement with those of Baricos and Shah (1984). In case of HD, acid protease excretion maintained the higher level until day 7 whereas the increases in LDH, GOT, LZM and NAG excretions were transient. In the histological study by Kirino et al. (unpublished data), severe necrosis of distal tubules caused by HD has been observed on day 5 after the injection. Therefore, we suppose that the change in acid protease excretion reflected severity of renal histological change more precisely than the other enzymes.

Urinary protein excretion was markedly increased by PM. This result is in agreement with those of Price and Ellis (1976). In particular, PM increased urinary proteins with molecular weight of over 60,000. These results indicate the characteristics of the glomerular damage caused by PM unlike the tubular damage.

From these results, it is concluded that tubular cell counts, LDH isoenzymes and a battery of these enzymes in urine are useful markers for detecting the severity and the site of renal damage in addition that urinary protein is a useful marker for detecting glomerular damage.

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