

## **Renal Clearance of Endogenous Hippurate Correlates with Expression Levels of Renal Organic Anion Transporters in Uremic Rats**

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d) **Abbreviations:** HA, hippurate; CRF, chronic renal failure; 5/6 Nx rats, 5/6 nephrectomized rats;

OAT, organic anion transporter; PAH, *p*-aminohippuric acid; BBB, blood-brain barrier; BCSFB, blood-cerebrospinal fluid barrier; BUN, blood urea nitrogen; HPLC, high-performance liquid chromatography; GFR, glomerular filtration rate; TBS-T, tris-buffered saline containing tween 20; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; OCT, organic cation transporter.

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## Abstract

Hippurate (HA) is a harmful uremic toxin that accumulates during chronic renal failure and failure of the excretion system for uremic toxins is thought to be responsible. Recently, we reported that organic anion transporter 1 (rOat1) is the primary mediator of HA uptake in the kidney and so now we have studied the pharmacokinetics and tissue distribution of HA after a single i.v. dose of HA to normal and 5/6 nephrectomized rats (5/6Nx rats). In control rats, the renal and biliary clearances of HA were 18.1 and 0.1 mL/min/kg, respectively. Plasma clearance decreased as dosage increased from 0.1 to 5 mg/kg, which suggests that renal tubular secretion is the primary route for elimination of HA. The plasma clearance of HA was significantly decreased in 5/6 Nx rats, compared with normal rats. In 5/6 Nx rats, renal clearance of endogenous HA correlated more closely with clearance of *p*-aminohippurate (PAH) than with that of creatinine. Protein expression of rOat1 and rOat3, assessed by Western blot analysis, was decreased in 5/6 Nx rats. Furthermore, in 5/6 Nx rats, the renal secretory clearance of endogenous HA correlated closely with protein expression of renal rOats. Thus, HA is primarily eliminated from the plasma via the kidney by active tubular secretion. The renal clearance of endogenous HA appears to be a useful indicator of changes in renal secretion that accompany the reduced levels of OATs protein in chronic renal failure.

## Introduction

In chronic renal failure (CRF) patients, uremic toxins accumulate in the serum because of impaired renal clearance (Niwa, 1996). Serum levels of the uremic toxin hippurate (HA) are markedly elevated in patients with uremia (Vanholder et al., 2003). It has been suggested that HA plays a role in a variety of pathological conditions, including stimulation of ammoniogenesis (Dzurik et al., 2001), and inhibition of both plasma protein binding (Sakai et al., 1995) and organic anion secretion by the kidney (Boumendil-Podevin et al., 1975). HA also inhibits glucose utilization in muscles and so may be involved in development of muscular weakness in uremia (Spustova et al., 1987; Spustova et al., 1989). Serum and cerebrospinal fluid concentrations of HA correlate positively with neurophysiological indices (Schoots et al., 1989), which suggests that HA induces neurological symptoms, perhaps via inhibition of organic anion transport at the blood-brain barrier (BBB) (Ohtsuki et al., 2002) or blood-cerebrospinal fluid barrier (BCSFB) (Porter et al., 1975). In addition, HA accelerates the renal damage associated with CRF (Sato et al., 2003). Thus, HA can be classified as a uremic toxin and is consequently a compound of pharmacological interest.

Despite the important role of HA in the pathophysiology of uremia, little information is available regarding its pharmacokinetics in animals, and no studies of its tissue distribution have been reported. HA is the glycine conjugate of benzoate, which is formed primarily from aromatic amino acids by

gastrointestinal flora and is added to foods and beverages as a preservative (Niwa, 1996). Active tubular secretion is the primary route for elimination of HA from the plasma via the kidney, and functional failure of this system causes accumulation of HA in blood (Tsutsumi et al., 2002). Recently, we reported that rat organic anion transporter 1 (rOat1) plays a major role in the renal uptake of HA on the basolateral membrane of the proximal tubules (Deguchi et al., 2004). Also, there is evidence that HA inhibits OAT1- or OAT3-mediated transport in predialysis patients, leading to acceleration of serum accumulation of uremic toxins and reduction of plasma elimination of drugs via OAT1 and OAT3. The distribution and accumulation of HA in various tissues appears to be an important step in the development of uremic toxicity in renal failure. Therefore, it is important to clarify the changes in HA pharmacokinetics that occur in uremia.

To investigate the mechanisms of uremic symptoms and pharmacokinetics of HA, we conducted the present pharmacokinetic study, in which normal and 5/6 nephrectomized (5/6 Nx) rats received a single i.v. administration of HA. We also examined the renal and biliary excretion of HA after i.v. administration of HA to anaesthetized rats, and examined tissue distribution of endogenous HA. Additionally, we evaluated the suitability of HA clearance as a clinical marker of renal function.

## Materials and Methods

### Materials

[<sup>14</sup>C]HA (55.0 mCi/mmol), [<sup>14</sup>C]carboxyl-inulin (2.0 mCi/g) and [<sup>3</sup>H]inulin (1.03 mCi/g) were purchased from American Radiolabeled Chemicals (St.Louis, MO). [<sup>3</sup>H]*p*-Aminohippurate (PAH) (4.54 Ci/mmol) was purchased from PerkinElmer Life Science (Boston, MA). HA was obtained from Sigma Chemical Co. (St. Louis, MO). PAH was obtained from Nacalai Tesque (Kyoto, Japan). Polyclonal antibodies for rOat1 and rOat3 were purchased from Trans Genic Inc. (Kumamoto, Japan). Polyclonal antibody for Na<sup>+</sup>-K<sup>+</sup> ATPase was purchased from Upstate Biotechnology Inc (Lake Placid, NY). All chemicals were of analytical grade.

### Animals

Adult male Wistar rats were housed in an air-conditioned room with free access to commercial feed and water, and fasted for 16 h before experiments. All animal experiments were conducted according to the guidelines of Kumamoto University for the care and use of laboratory animals.

### Induction of CRF by Surgical Reduction of Renal Mass

Experimental CRF was induced by 5/6 Nx (Deguchi et al., 2003). Male Wistar rats (130 to 150 g)

were anesthetized with sodium pentobarbital (60 mg/kg) by intraperitoneal injection. During surgery, the body temperature of the rats was maintained using a warming lamp. The left kidney was exposed via a left flank incision, and was gently dissected free from the adrenal gland, followed by excision of the upper and lower poles. One week later, the rats were again anesthetized with sodium pentobarbital, and the right kidney was exposed via a right flank incision, dissected free from the adrenal gland, and completely removed. Rats were maintained in metabolic cages for 24 hours before the experiment *in vivo* to measure normal urine output and urinary levels of creatinine, protein and HA. Metabolic and pharmacokinetic studies were performed 4 weeks after nephrectomy. Correlations of HA clearance with the clearance of either creatinine, PAH or protein expression of rOats were examined between weeks 1 and 6 after nephrectomy. The blood urea nitrogen (BUN) was determined with the urease/indophenol method (Mizuno et al., 1997) and creatinine in serum and urine was determined by the Jaffé reaction. Measurements were performed using assay kits from Wako Pure Chemical Industries (Osaka, Japan). The concentration of endogenous HA in serum and urine was measured by high-performance liquid chromatography (HPLC) (Tsutsumi et al., 2002). Clearance of endogenous HA and creatinine was calculated by dividing the rate of urinary excretion by the serum concentration. The renal secretory clearance of endogenous HA was calculated by subtracting creatinine clearance multiplying the unbound fraction of HA, as the glomerular filtration rate (GFR), from renal clearance of HA. Urine



protein levels were determined using the Bradford assay (Bradford, 1976).

### **Tissue Distribution of HA**

After normal and 5/6 Nx rats were sacrificed by decapitation, their brain, heart, lungs, liver, kidneys, spleen, testes and skeletal muscles were removed and weighed. A sample (0.5 g) of each tissue was homogenized in 5 mL of 1 M  $\text{KH}_2\text{PO}_4$ . A 50- $\mu\text{L}$  aliquot of this solution was added directly to 100  $\mu\text{L}$  of acetonitrile. After centrifugation at 3000 x g for 10 min, the supernatant was assayed by HPLC. The distribution of HA in each tissue is expressed as the  $K_p$  value (concentration of HA per gram of each tissue, divided by the concentration of HA in serum).

### **Pharmacokinetics of HA in Anaesthetized Rats**

Under light anesthesia with phenobarbital (60 mg/kg), normal Wistar rats (250 to 290 g) and 5/6 Nx rats underwent a surgical procedure in which cannulae were inserted into the femoral vein and artery using polyethylene tubing (polyethylene-50: i.d., 0.58 mm; o.d., 0.9655 mm; Becton Dickson & Co., Parsippany, NJ) (Deguchi et al., 2003). The bile duct was also cannulated with polyethylene tubing (polyethylene-10: i.d., 0.28 mm; o.d., 0.61 mm), as was the bladder (polyethylene-8: o.d., 2.33 mm; Hibiki Co., Tokyo, Japan). Body temperature of the rats was maintained using a warming lamp. Tracer

amounts of [ $^{14}\text{C}$ ]HA (3  $\mu\text{Ci/kg}$ ) or [ $^3\text{H}$ ]PAH (2  $\mu\text{Ci/kg}$ ) were administered with radiolabeled inulin ([ $^3\text{H}$ ]inulin (10  $\mu\text{Ci/kg}$ ) or [ $^{14}\text{C}$ ]carboxyl-inulin (0.15  $\mu\text{Ci/kg}$ )) as a rapid infusion into the femoral vein. GFR was assumed to be equal to the renal clearance of inulin. After each infusion, the cannulae were flushed with a small volume of heparinized saline in order to ensure the complete administration of each dose and to prevent clot formation. Blood samples (200  $\mu\text{L}$ ) were taken from the femoral artery at a designated time. To avoid an effect on the pharmacokinetics, only 4 blood samples were taken from each rat. Blood was placed in graduated microcentrifuge tubes containing a drop of heparinized saline, which served as an anticoagulant. Blood samples were centrifuged (3000 x g for 10 min) and plasma was removed. Bile and urine were collected at 0 to 30, 30 to 60, 60 to 90, 90 to 120, 120 to 150, 150 to 180 and 180 to 240 min post-injection. Hionic-fluor (10 mL; PerkinElmer Life Sciences, Boston, MA) was added to aliquots (50  $\mu\text{L}$ ) of plasma, bile and urine, followed by measurement of double-isotope radioactivity with a liquid scintillation counter. The radioactivity of an aliquot of the solution used for the injection was measured simultaneously.

### **Determination of the Unbound Concentration**

Plasma concentrations of unbound HA were estimated by ultrafiltration as previously described (Tsutsumi et al., 1999). Free fractions of HA were calculated according to the following equation:

$$f_u = \frac{C_f}{C_t} \times 100 \quad (\%) \quad (1)$$

where  $f_u$  represents the free fraction of HA,  $C_f$  represents the free concentration of HA, and  $C_t$  represents the total concentration of HA.

### Western blot analysis

The rat kidney plasma membrane fraction was prepared using the standard procedure (Nakajima et al., 2000). Rat kidney plasma membrane proteins (40  $\mu$ g) were electrophoresed on 10% SDS-polyacrylamide gel with a 4.4% stacking gel. Separated proteins were transferred to a polyvinylidene difluoride membrane using a blotter at 15 V for 1 h. The membrane was blocked with Tris-buffered saline (137 mM NaCl, 20 mM Tris, pH 7.5) containing 0.1% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature. After washing 3 times with TBS-T for 5 min, the membrane was incubated over night at 4°C with primary antibody specific for rOat1 (1:2000 dilution), rOat3 (1:2000 dilution) or Na<sup>+</sup>-K<sup>+</sup> ATPase (1:10000 dilution). After washing, the membrane was incubated with a horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Biosciences UK, Ltd.) diluted 1:2500 in TBS-T for 1 h at room temperature, and labeling was detected using ECL plus (Amersham Biosciences UK, Ltd.).

## Data and Statistical Analysis

Plasma concentration profiles were analyzed by fitting the following biexponential equation using the nonlinear least-squares method (MULTI) (Yamaoka et al., 1981):

$$C_p = A \cdot \exp^{(-\alpha \cdot t)} + B \cdot \exp^{(-\beta \cdot t)} \quad (2)$$

Pharmacokinetic parameters were calculated using the following equations:

$$AUC_{0 \rightarrow \infty} = \frac{A}{\alpha} + \frac{B}{\beta} \quad (3)$$

$$CL_{tot} = \frac{\text{Dose}}{AUC_{0 \rightarrow \infty}} \quad (4)$$

$$t_{1/2\beta} = \frac{0.693}{\beta} \quad (5)$$

$$CL_{renal} = CL_{tot} \times f_{urine} \quad (6)$$

$$CL_{biliary} = CL_{tot} \times f_{bile} \quad (7)$$

where  $AUC_{0 \rightarrow \infty}$ ,  $CL_{tot}$ ,  $t_{1/2\beta}$ ,  $CL_{renal}$ ,  $f_{urine}$ ,  $CL_{bile}$  and  $f_{bile}$ , represent the AUC from zero to infinity, total body clearance,  $\beta$ -phase half-life, renal clearance, fraction of test compound recovered in the urine, biliary clearance and fraction of test compound recovered in the bile, respectively.

Unless otherwise indicated, all data represent the mean  $\pm$  SE, and n refers to the number of animals used in each experiment. An unpaired, two-tailed Student's *t* test was used to determine the significance of differences between means of two groups. Fitting was performed using the nonlinear least-squares method with the MULTI program and the Damping Gauss Newton Method algorithm

(Yamaoka et al., 1981).

## Results

### Renal function in normal and 5/6 Nx rats

Four weeks after nephrectomy, 5/6 Nx rats exhibited significant increases in urine volume, BUN, serum creatinine, proteinuria and serum concentration of endogenous HA (Table 1). In addition, there was a decrease in body weight and creatinine clearance, indicating that renal function was significantly impaired in 5/6 Nx rats.

### Pharmacokinetics of HA in 5/6 Nx rats

Table 2 shows the tissue distribution of endogenous HA in control and 5/6 Nx rats. These results indicate that the highest concentration of HA occurred in the kidney. The HA tissue-to-serum concentration ratio ( $K_p$ ) was markedly reduced in 5/6 Nx rat kidneys, compared with the normal rat kidneys. In contrast, the  $K_p$  value of the brain was significantly increased in 5/6 Nx rats, compared with control rats.

In order to roughly delineate the major route for elimination of HA from plasma, we examined urinary and biliary excretion in normal and 5/6 Nx rats (Fig. 1 and Table 3). Most of the HA was excreted in an intact form (data not shown), and the main route was via the urine (Fig. 1B). The plasma

clearance of HA was significantly decreased in 5/6 Nx rats. The biological half-life of HA was longer in 5/6 Nx rats (156 min) than in the control rats (22 min). These values appear to reflect plasma and renal clearance, suggesting that plasma clearance of HA was reduced by impairment of renal function. The unbound fraction of HA was increased in 5/6 Nx rats, compared with control rats (Table 3), indicating that plasma protein binding of HA was inhibited by other strongly protein-bound uremic retention compounds, such as 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and indoxyl sulfate. In normal rats, the renal clearance of unbound HA was about 16 times greater than the GFR, which suggests that active tubular secretion is involved in the urinary excretion of HA. The excretion ratio of 5/6 Nx rats was 50% of that of control rats, an indication that the contribution of secretion to the renal elimination of HA was reduced in 5/6 Nx rats.

### **Dose-dependent pharmacokinetics of HA in normal rats**

We examined the dose-dependency of pharmacokinetic parameters after iv. administration of HA (Fig. 2A). Plasma clearance decreased as the dosage increased from 0.1 to 5 mg/kg (Table 4), indicating that renal tubular secretion is the main route for elimination of HA. In normal rats, renal clearance of HA was comparable to that of PAH (Table 4), which suggests that the excretion rate of HA (like that of PAH) was determined by the renal plasma flow rate.

## **Correlation between renal clearance of endogenous HA and creatinine clearance, PAH clearance and expression levels of renal organic anion transporters**

To evaluate the renal clearance of endogenous HA corrected by the unbound fraction as a clinical marker, we examined the linear regression of the renal clearance of unbound HA against creatinine clearance (Fig. 3A) and PAH clearance corrected by its unbound fraction (Fig. 3B). In normal and 5/6 Nx rats, the renal clearance of unbound HA correlated more closely with clearance of unbound PAH ( $r = 0.846$ ,  $p < 0.01$ ) than with the clearance of creatinine ( $r = 0.571$ ,  $p < 0.01$ ).

We used Western blotting to assess the expression levels of renal rOats. We observed primary bands for rOat1, rOat3 and  $\text{Na}^+\text{-K}^+$  ATPase with sizes of 62, 71 and 125 kDa, respectively (Fig. 4A). Compared to normal rats, the kidneys of 5/6 Nx rats had markedly decreased protein expression levels of rOat1 (42.0 % of normal) and rOat3 (49.3% of normal) (Fig. 4B), whereas there was no significant difference in expression of  $\text{Na}^+\text{-K}^+$  ATPase between the normal and 5/6 Nx rats (Fig. 4A). Figure 4C shows the correlation between the renal secretory clearance of endogenous HA and expression levels of rOats in 5/6 Nx rats. The renal secretory clearance of HA significantly correlated with the levels of rOat1 ( $r = 0.786$ ,  $p < 0.01$ ) and rOat3 ( $r = 0.653$ ,  $p < 0.01$ ).



## Discussion

Endogenous HA was mainly localized in the kidney in control and 5/6 Nx rats (Table 2), and the renal concentration of HA in 5/6 Nx rats was approximately 3 times greater than that of normal rats. It has been suggested that the uptake mechanism of HA plays a key role in the induction of HA nephrotoxicity (Sato et al., 2003). Interestingly, the  $K_p$  value in the brain was significantly greater for 5/6 Nx rats than for control rats. HA is markedly elevated in the serum and cerebrospinal fluid of uremic patients (Porter et al., 1975). In addition, the serum concentration of HA in hemodialysis patients correlates positively with neurophysiological indices (Schoots et al., 1989), suggesting that HA is related to neurological symptoms in uremia. Likewise, a relationship was observed in patients between the increasing severity of abnormalities attributable to the uremic state and higher plasma concentrations of CMPF (Costigan et al., 1996). Evidence obtained by Ohtsuki et al (2002) suggests that uremic toxins (e.g., indoxyl sulfate and HA) inhibit rOat3-mediated brain-to-blood transport in uremic patients, leading to accumulation of neurotransmitter metabolites and drugs in the brain (Ohtsuki et al., 2002). These findings highlight the importance of carrier-mediated transport of uremic toxins such as HA across the blood-brain barrier and the mechanism of neurological symptoms of uremic syndrome in CRF patients.

In the present study, we examined the distribution of the uremic toxin HA in normal and 5/6 Nx

rats following i.v. administration of HA. The total clearance of HA was equal to the renal clearance, and nearly all excreted HA was eliminated via the kidney (Table 2) in a largely unchanged form. Additionally, the plasma and renal clearance of HA was significantly decreased in 5/6 Nx rats (Fig. 1), indicating that HA is mainly excreted via the kidney. In normal rats, the renal clearance of unbound HA was about 16 times greater than the GFR, which suggests that active tubular secretion is involved in the urinary excretion of HA. Furthermore, our observations indicated that the plasma clearance of HA was dose-dependent (Fig. 2 and Table 4). HA significantly inhibited PAH transport in the kidney (Boumendil-Podevin et al., 1975), suggesting that both compounds are transported by the organic anion transport system. Recently, we demonstrated that rOat1 and human OAT1 (hOAT1) play an important role in renal uptake of HA (Deguchi et al., 2004). Together, these findings indicate that organic anion transporters play an important role in renal transport of HA, and that functional failure of the system responsible for excretion of HA causes accumulation of HA in blood. The renal clearance of HA has been reported to be 590 mL/min in healthy people (Ilic et al., 2000), suggesting that HA is also rapidly and efficiently cleared by the kidney in rats. In the normal rats of the present study, the renal clearance of HA was comparable to that of PAH (Table 4), indicating that the excretion rate of HA (like that of PAH) is dependent on the renal plasma flow rate.

Residual nephrons make a significant contribution to the removal of uremic waste products in

patients on chronic dialysis treatment (van Olden et al., 1998). HA is one of the most abundant waste products in uremic serum, accumulating to concentrations as high as 2.5 mM (Vanholder et al., 2003), and HA suppresses PAH transport by the kidney (Boumendil-Podevin et al., 1975). These findings indicate that accumulation of uremic toxins including HA inhibit both their own renal elimination and that of other organic anions by inhibiting transport via rOats/hOATs (Deguchi et al., 2004). Therefore, the inhibition of rOats by HA and other uremic toxins accumulated in serum may be partly involved in the decrease of HA clearance in 5/6 Nx rats (Table 3). These observations also suggest that serum HA is a useful indicator of interactions between uremic toxins and drugs in patients with uremia. In addition, plasma and urine specimens from healthy subjects contain small amounts of uremic toxins (Sakai et al., 1996), allowing quantification of renal clearance *in vivo* and prediction of renal anion secretion. In the 5/6 Nx rats of the present study, the renal clearance of unbound HA correlated more closely with the clearance of unbound PAH than with the clearance of creatinine (Fig. 3), which implies that renal clearance of endogenous HA reflects the renal secretion of organic anions. It is known that a small fraction of creatinine is cleared through active tubular secretion, partially by human organic cation transporter 2 (hOCT2; SLC22A)-mediated transport (Urakami et al., 2004). In CRF, the urinary excretion of cationic substrates is reduced, partly because of the reduced expression of OCT2 (Ji et al., 2002), which suggests that both renal secretion of creatinine and glomerular filtration may be attenuated.

Although it is difficult to estimate the contributions of GFR and secretion in CRF, the present results suggest that the renal clearance of HA would not reflect glomerular filtration and OCTs-mediated secretion.

In renal tubules, membrane transport systems mediate the tubular secretion of endogenous and exogenous organic anions, including various drugs, toxins and endogenous metabolites. rOat1 (*Slc22a6*), a typical substrate of which is PAH, is expressed predominantly in the kidney and is localized on the basolateral membrane of the middle proximal tubules (S2) (Kusuhara and Sugiyama, 2002; Miyazaki et al., 2004). rOat1 has broad substrate specificity and transports a variety of organic anions. Other isoforms in rodents, referred to as Oat2 (*Slc22a7*), Oat3 (*Slc22a8*) and Oat5 (*Slc22a19*), are expressed in the kidney (Kusuhara and Sugiyama, 2002; Youngblood and Sweet, 2004). Immunolocalization studies have revealed that Oat2 is apical in rat kidney, but basolateral in human kidney (Enomoto et al., 2002; Kojima et al., 2002). Additionally, the localization of Oat5 in the kidney has not been identified yet and so the role of Oat2 and Oat5 in the renal transport of organic anions is unclear. In human kidney, the renal brush border membrane possesses an influx/efflux transport system for organic anions, such as hOAT4 (*SLC22A11*), and it has been suggested that hOAT4 is partly involved in the apical efflux of uremic toxins in human proximal tubules (Enomoto et al., 2003). rOat3 expressed in the kidney is located on the basolateral membrane of all segments (S1, S2 and S3) of the

proximal tubules (Kusuhara and Sugiyama, 2002; Miyazaki et al., 2004). Functional characterization shows that substrates of rOat3 include organic anions and the organic cation cimetidine (Kusuhara and Sugiyama, 2002; Hasegawa et al., 2003). The contribution of rOat1 and rOat3 to renal uptake of organic anions has been evaluated with kidney slices and the results suggest that rOat1 is the primary mediator of the renal uptake of small hydrophilic molecules, whereas rOat3 mediates renal uptake of more bulky organic anions (Hasegawa et al., 2003). It has also been reported that uptake of taurocholate, estrone sulfate and PAH by kidney slices is markedly reduced in mOat3 knockout mice (Sweet et al., 2002). hOAT1 and hOAT3 are predominantly expressed in the kidney, and are coexpressed on the basolateral membrane in some parts of the proximal tubules (Hosoyamada et al., 1999; Cha et al., 2001; Motohashi et al., 2002). Given the results of transport studies in rats and mice, it is thought that hOAT1 and hOAT3 play a predominant role in the transport of organic anions across the basolateral membrane of human proximal tubules.

In patients with renal failure, pathophysiological changes may affect the activity of transporters. As mentioned above, uremic toxins, especially HA, appear to inhibit OAT1- and OAT3-mediated transport *in vivo* in cases of CRF (Deguchi et al., 2004), leading to an acceleration of the accumulation of uremic toxins in serum. On the other hand, the expression levels of some transporters are changed in 5/6 Nx rats and patients with renal disease (Laouari et al., 2001; Ji et al., 2002; Sakurai et al., 2004),

which suggests that expression levels of drug transporters are related to changes in renal anion secretion. The present Western blot analysis demonstrated that the protein expression levels of rOat1 and rOat3 were markedly decreased in the kidneys of 5/6 Nx rats, compared to normal rats (Fig. 4B), reflecting the fact that 5/6 Nx rats have a decreased  $K_p$  value of endogenous HA in the kidneys (Table 2) and a decreased excretion ratio of HA (Table 3). This result agrees with previous work using cDNA array and quantitative RT-PCR analysis (Aoyama et al., 2003). Furthermore, the renal secretory clearance of endogenous HA correlated significantly with the levels of rOat1 and rOat3 (Fig. 4C). This suggests that the changes in expression levels of rOat1 and rOat3 may affect the secretion of renal anions such as HA in uremia. The present results indicate that HA is suitable as a reference compound for estimation of renal clearance of organic anions and protein expression of OATs. Such information could be used to prevent excessive accumulation of drugs in the body prior to treatment. In the previous report, rOat1 mainly accounted for HA uptake in the kidney (Deguchi et al., 2004). However, protein expression of rOat3 correlated with renal clearance of HA. There are two possibilities that may account for this. Firstly, HA is partly taken up by rOat3. Our kinetic experiments suggested that rOat1 accounted for about 70% of the renal uptake of HA, and the remaining fraction was accounted for by a pravastatin- or benzylpenicillin-sensitive transporter, which may be rOat3 (Deguchi et al., 2004). Secondly, rOat1 and rOat3 are co-regulated in 5/6 Nx rats. Previous determinations of chromosomal locations noted that

OAT1 and OAT3 genes are tightly linked in the mouse and human genomes (Eraly et al., 2003), which suggests that the pairing might exist to facilitate the co-regulation of their genes. Protein expression of rOat3 could therefore be correlated indirectly with the renal clearance of HA. On the other hand, it has been reported that normal expression levels of rOat1 and rOat3 protein are maintained in rats two weeks after 5/6 nephrectomy (Ji et al., 2002). However, in the present study, 5/6 Nx rats were used for experiments more than 4 weeks after surgery. Thus, there may be a progressive reduction of the renal expression of rOat1 and rOat3 after 5/6 nephrectomy.

In conclusion, the results of the present experiments *in vivo* with 5/6 Nx rats indicate that the primary route for elimination of HA from the plasma is via the kidney by active tubular secretion, and that renal clearance of endogenous HA is a useful indicator of the changes in renal secretion that accompany reduction of OATs protein expression in CRF.

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## **Footnotes**

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## Legends for Figures

**Fig. 1 Disposition profiles after i.v. administration of [ $^{14}$ C]HA in normal and 5/6 Nx rats 4 weeks after nephrectomy.**

(A) HA was administered at a dose of 0.1 mg/kg by rapid infusion into the femoral vein in normal (○) and 5/6 Nx rats (●). (B) Bile and urine (normal rats, □; 5/6 Nx rats, ■) were collected at 180 min post-injection. See *Materials and Methods* for experimental details. Each point represents the mean  $\pm$  SE of 4 or 5 experiments.

**Fig. 2 Dose-dependent pharmacokinetics of HA after i.v. administration**

(A) HA was administered at a dose of 0.1 mg/kg (○) or 5 mg/kg (●) by rapid infusion into the femoral vein in normal rats. (B) Bile and urine (0.1 mg/kg, □; 5 mg/kg, ■) were collected at 180 min post-injection. See *Materials and Methods* for experimental details. Each point represents the mean  $\pm$  SE of 4 experiments.



**Fig. 3 Linear regression of endogenous HA clearance against endogenous creatinine clearance**

**(A) and [<sup>3</sup>H]PAH clearance (B) in normal and 5/6 Nx rats.**

The serum and urinary concentration of HA were measured by HPLC, and endogenous HA clearance was calculated.

**Fig. 4 Protein expression of rOat1 and rOat3 in normal and 5/6 Nx rats, and correlation between**

**rOats expression and the renal secretory clearance of endogenous HA.**

The protein expression levels of rOat1, rOat3 and Na<sup>+</sup>-K<sup>+</sup> ATPase were determined by Western blotting as outlined in the **Methods** section. (A) The image shows a blot of the crude plasma membranes isolated from normal or 5/6 Nx rats 4 weeks after nephrectomy. We observed a 62-kDa band for rOat1, a 71-kDa band for rOat3 and a 125-kDa band for Na<sup>+</sup>-K<sup>+</sup> ATPase. (B) The average mass of each protein band is expressed as units of the densitometry ratio of rOats to Na<sup>+</sup>-K<sup>+</sup> ATPase, for normal (open column) and 5/6 Nx rats (closed column). The values for normal rats were arbitrarily defined as 100%. Each column represents the mean  $\pm$  SE (7 - 10). \*p < 0.01, significantly different from normal rats. (C) The image shows the correlation between the expression levels of renal rOats and renal secretory clearance of endogenous HA in 5/6 Nx rats.

Table 1. Physiological parameters of normal and 5/6 Nx rats 4 weeks after nephrectomy

Parameters	Normal rats	5/6 Nx rats
Body weight (g)	339 ± 5	307 ± 6*
Urine volume (mL/day)	8.89 ± 1.04	19.6 ± 0.9*
BUN (mg/mL)	15.4 ± 0.5	43.1 ± 1.3*
Serum creatinine (mg/dL)	0.758 ± 0.024	1.89 ± 0.10*
Creatinine clearance (mL/min/kg)	2.83 ± 0.09	1.58 ± 0.06*
Proteinuria (μg/min/kg)	18.3 ± 2.2	152 ± 31*
Endogenous serum HA (μM)	12.3 ± 2.4	135 ± 12*

Each value represents the mean ± SE of 15 to 20 experiments.

\*p < 0.01, significantly different from the corresponding parameter in normal rats.

Table 2. Tissue distribution of endogenous HA in normal and 5/6 Nx rats 4 weeks after nephrectomy

	Normal rats		5/6 Nx rats	
	Concentration	K <sub>p</sub>	Concentration	K <sub>p</sub>
	(nmol/mg of tissue)	(mL/g of tissue)	(nmol/mg of tissue)	(mL/g of tissue)
Brain	1.62 ± 0.17	0.132 ± 0.015	72.8 ± 9.1	0.549 ± 0.044*
Heart	6.28 ± 0.23	0.515 ± 0.028	64.9 ± 6.1	0.492 ± 0.025
Lung	12.3 ± 3.9	0.987 ± 0.267	142 ± 10	1.08 ± 0.02
Liver	18.2 ± 2.7	1.51 ± 0.29	180 ± 14	1.36 ± 0.04
Kidney	258 ± 19	21.1 ± 1.4	723 ± 14	5.55 ± 0.44*
Spleen	7.72 ± 2.52	0.607 ± 0.183	84.6 ± 3.1	0.648 ± 0.045
Testis	6.60 ± 1.85	0.519 ± 0.119	64.5 ± 4.8	0.471 ± 0.053
Skeletal muscle	2.47 ± 0.56	0.197 ± 0.036	24.1 ± 4.1	0.180 ± 0.018

Endogenous HA was measured in various tissues of normal and 5/6 Nx rats by HPLC. Distribution of HA in each tissue is expressed as K<sub>p</sub>; i.e., the concentration of HA per gram of each tissue divided by concentration of HA in serum (normal rats, 12.3 ± 1.0 μM; 5/6 Nx rats, 132 ± 9 μM). Each value represents the mean ± SE of 3 experiments. \*p < 0.01, significantly different from the corresponding parameter in normal rats.

Table 3. Pharmacokinetic parameters of [ $^{14}\text{C}$ ]HA after i.v. administration to normal and 5/6 Nx rats 4 weeks after nephrectomy (0.1 mg/kg)

Parameters	Normal rats	5/6 Nx rats
AUC (% of dose/mL · min )	25.1 ± 1.1	165 ± 49*
$t_{1/2\beta}$ (min)	21.9 ± 1.3	156 ± 64*
$V_{d,ss}$ (mL/kg)	440 ± 22	401 ± 65
$CL_{tot}$ (mL/min/kg)	19.3 ± 0.7	3.55 ± 1.57**
$CL_{renal}$ (mL/min/kg)	18.1 ± 0.7	2.68 ± 1.19**
$CL_{biliary}$ (mL/min/kg)	0.092 ± 0.010	0.10 ± 0.01
GFR (mL/min/kg)	2.43 ± 0.18	0.59 ± 0.13**
$f_u$ (%)	47.4 ± 1.7	60.3 ± 0.8**
Excretion ratio	15.7	7.56

GFR was assumed to be equal to the renal clearance of inulin in normal or 5/6 Nx rats. Excretion ratio was calculated by dividing the renal clearance by the unbound fraction and GFR. Each value represents the mean ± SE of 4 or 5 experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , significantly different from the corresponding parameter in normal rats.

Table 4. Pharmacokinetic parameters of PAH and dose-dependent pharmacokinetics of HA after iv.  
administration to normal rats

Parameters	PAH	HA	
	0.1 mg/kg	0.1 mg/kg	5 mg/kg
AUC (% of dose/mL · min )	24.3 ± 1.5	25.1 ± 1.1	115 ± 15*
t <sub>1/2β</sub> (min)	24.5 ± 2.2	21.9 ± 1.3	73.9 ± 4.4*
V <sub>d,ss</sub> (mL/kg)	612 ± 37	440 ± 22	433 ± 41
CL <sub>tot</sub> (mL/min/kg)	21.8 ± 1.5	19.3 ± 0.7	4.57 ± 0.68*
CL <sub>renal</sub> (mL/min/kg)	20.4 ± 1.5	18.1 ± 0.7	4.24 ± 0.64*
f <sub>u</sub> (%)	91.0 ± 1.6	47.4 ± 1.7	46.0 ± 1.1
Excretion ratio	9.22	15.7	3.80

GFR was assumed to be equal to the renal clearance of inulin in normal rats ( $2.43 \pm 0.18$  mL/min/kg, n = 4). Excretion ratio was calculated by dividing the renal clearance by the unbound fraction and GFR. Each value represents the mean ± SE of 4 experiments. \*P < 0.01, significantly different from the corresponding parameter of HA (0.1 mg/kg).

Figure 1

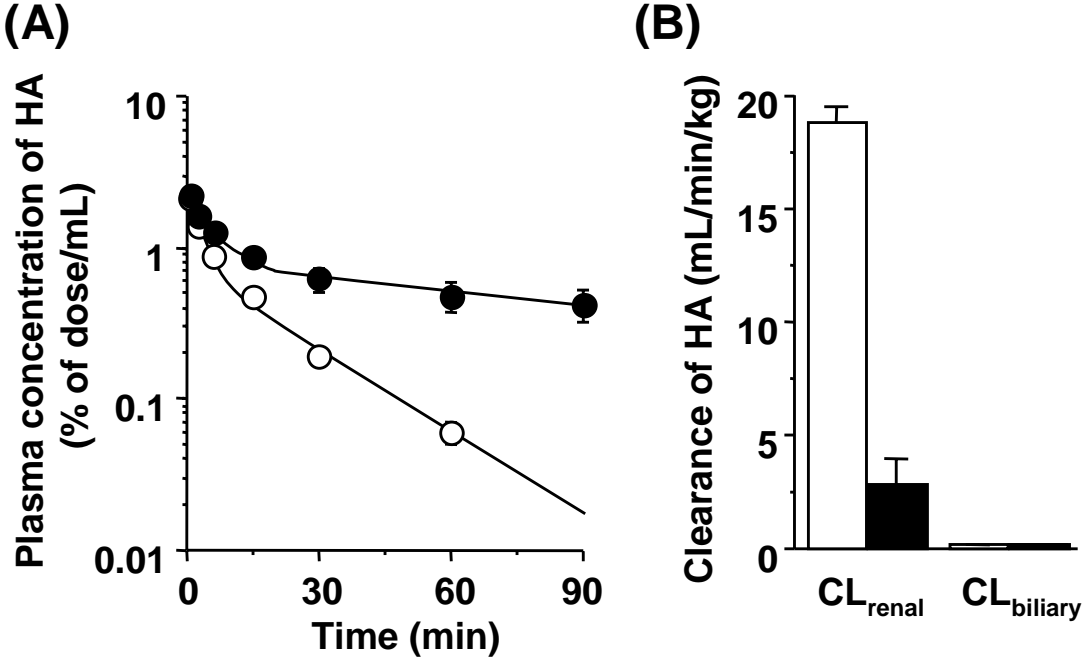


Figure 2

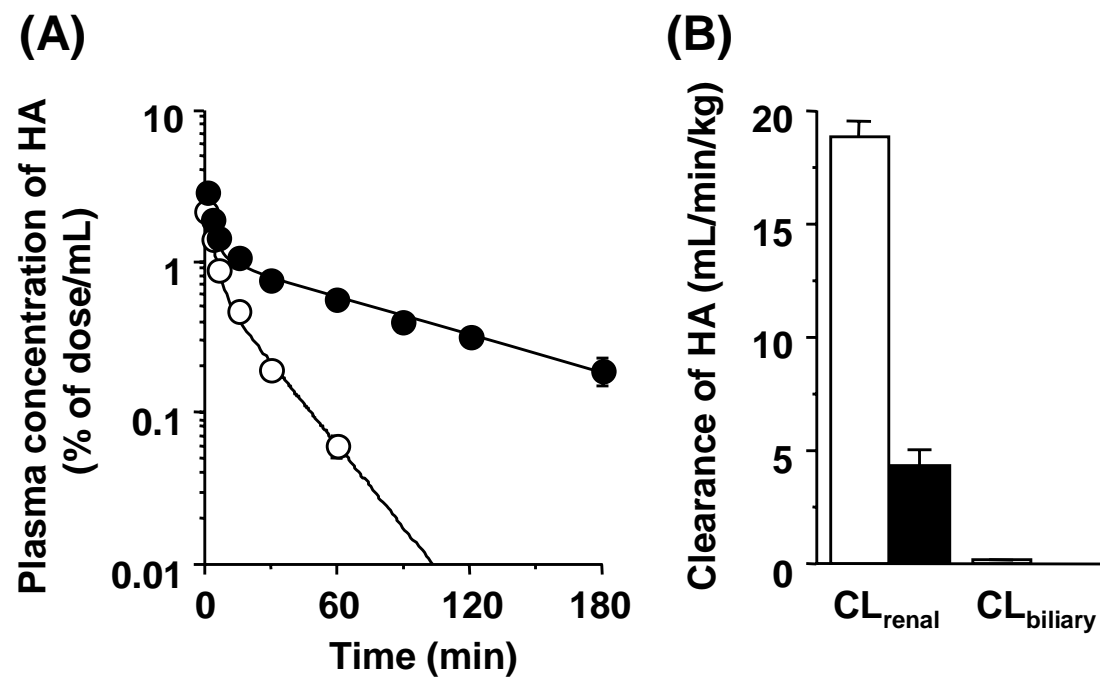


Figure 3

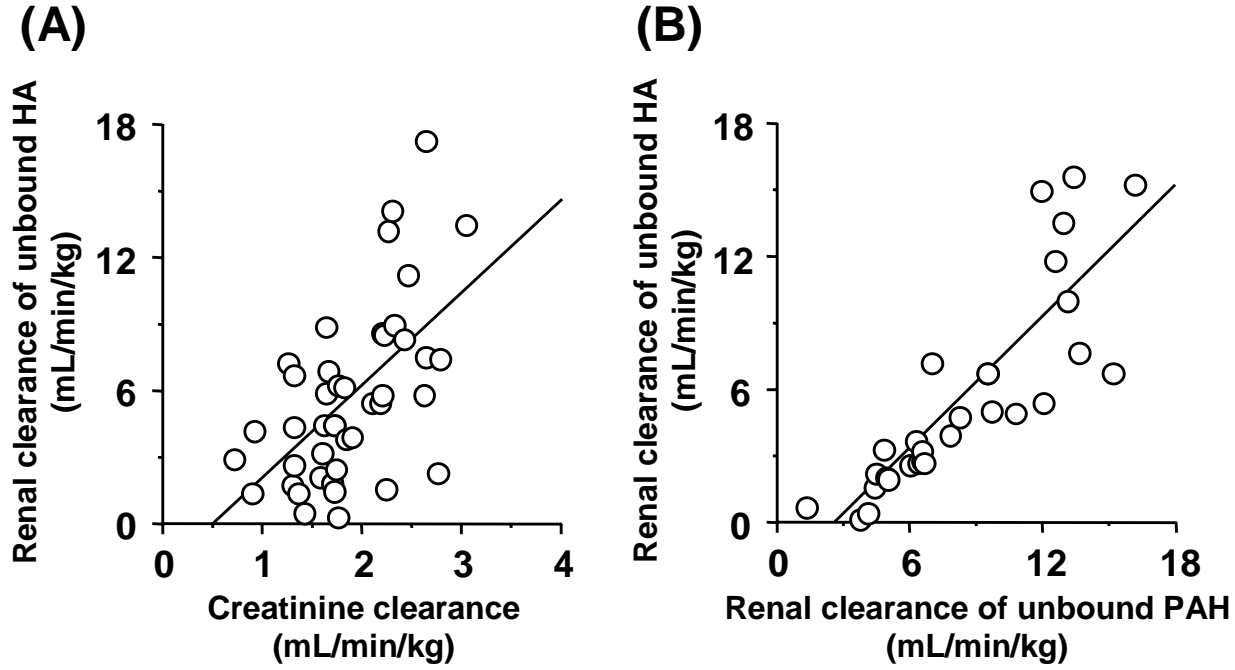




Figure 4

