Renal Disposition of a Furan Dicarboxylic Acid and Other Uremic Toxins in the Rat

YASUHIRO TSUTSUMI, TSUNE OEGUCHI, MIKIHISA TAKANO, AKIRA TAKDATE, W. EDWARD LINDUP, and MASAKI OTAGIRI

ABSTRACT

The aim of this study was to understand the mechanisms that underlie the renal elimination of albumin-bound uremic toxins, particularly the highly bound furan acid 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), that accumulate in chronic renal failure. These toxins inhibit the binding of acidic drugs and have various other untoward effects. The pharmacokinetics and tissue distribution of CMPF plus three other such toxins, indoxyl sulfate, indole acetic acid, and hippuric acid, have been examined in the anesthetized rat. The effects of p-aminohippuric acid (PAH) acid and tetraethylammonium on the uptake of CMPF by rat renal cortical slices in vitro were also investigated to characterize its mechanism of uptake. Plasma and tissue concentrations of the uremic toxins were determined by high-performance liquid chromatography. The rate of elimination of the toxins from plasma was indoxyl sulfate > hippuric acid > indole acetic acid > CMPF. Although the renal clearance of CMPF was low, its main elimination pathway was via urinary excretion with active tubular secretion. In renal cortical slice experiments, mutual inhibition between CMPF and PAH was observed. In addition, a-ketoglutarate stimulated the uptake of CMPF by renal cortical slices. The base tetraethylammonium did not inhibit slice uptake of CMPF. The pharmacokinetics of CMPF was characterized by slow plasma clearance and localization in the kidney. Furthermore, the evidence from experiments with renal cortical slices indicates that the uptake of CMPF is mediated by an anion/dicarboxylate exchanger, similar to that for PAH.

When kidney function is impaired, a variety of pathological changes occur that are collectively referred to as the uremic syndrome. A part of this syndrome involves elevated serum levels of a number of substances (Ringoir et al., 1987). In patients who have chronic renal failure, uremic toxins accumulate in the serum by a combination of the following four mechanisms: 1) a decrease in renal clearance (indoxyl sulfate), 2) an accumulation of abnormal metabolites (methylguanidine), 3) an increase in production (parathyroid hormone), and 4) a decreased rate of catabolism by the kidney (β2-microglobulin) (Niwa, 1996).

3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), a furan dicarboxylic acid derivative, was first detected in normal human urine in 1979 (Spiteller and Spiteller, 1979) and was subsequently isolated and identified in human blood (Pfordt et al., 1981). Since these initial reports, it has also been reported to accumulate in uremic plasma, reaching concentrations in the range of 60 to 370 μM (Niwa et al., 1988). In addition, a growing body of evidence suggests that it is a significant uremic toxin (Niwa, 1996).

CMPF has a high affinity for albumin (number of binding sites, n = 1; apparent association constant, K.a = 1.3 × 107 M−1) and inhibits the binding of other ligands, especially those that bind to site I (Lindup et al., 1986; Mabuchi and Nakahashi, 1988b; Henderson and Lindup, 1990; Sakai et al., 1995). It is believed to be a major factor in the decreased level of drug binding in uremic plasma because of its considerable affinity for albumin and also because of increased CMPF concentrations in uremic plasma. The concentration of CMPF can approach a 1:1 molar ratio with albumin, particularly when the production of albumin is reduced during chronic renal failure.

It has also been suggested that CMPF may play a role in a variety of pathological conditions, including the anemia that occurs during chronic renal failure (Niwa et al., 1990; Costigan et al., 1995), irregularities in thyroid function (Lim et al., 1993), and neurological symptoms that may be caused by the inhibition of organic anion transport at the blood-brain barrier (Costigan et al., 1996a). This furan dicarboxylic acid also inhibits phase I (O-demethylation) and phase II (glutathione

ABBREVIATIONS: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; IS, indoxyl sulfate; IA, indole acetic acid; HA, hippuric acid; PAH, p-aminohippuric acid; α-KG, α-ketoglutarate; TEA, tetraethylammonium; HPLC, high-performance liquid chromatography; CL, clearance; GFR, glomerular filtration rate; OAT, organic anion transporter.
conjugation and glucuronidation) pathways of drug metabolism in rabbit liver homogenates in vitro (Walters et al., 1995). In addition, there is evidence that CMPF inhibits active tubular secretion in the kidney (Henderson and Lindup, 1992). Thus, CMPF can be classified as a uremic toxin and, as a result, a compound of pharmacological interest.

Nevertheless, the mechanism by which CMPF accumulates remains unclear. To understand the pathway for the accumulation of CMPF, we have examined the pharmacokinetic properties of CMPF after intravenous administration and compared them with those of other uremic toxins, namely, indoxyl sulfate (IS), indole acetic acid (IA), and hippuric acid (HA). The renal and biliary excretion of CMPF and its tissue distribution after intravenous administration to the anesthetized rat have also been studied. We have also investigated the uptake of CMPF by renal cortical slices in vitro to see whether an organic anion/dicarboxylate exchanger was involved in the renal excretion of CMPF.

**Materials and Methods**

**Materials.** CMPF was synthesized according to the method of Costigan et al. (1996b), which was based on that of Pfordt et al. (1981). IS, IA, HA, p-aminohippuric acid (PAH), glutarate, and succinate were obtained from Nacalai Tesque (Kyoto, Japan). α-Keto-glutarate (α-KG) was obtained from Sigma-Aldrich (St. Louis, MO). Tetraethylammonium (TEA) was obtained from Wako Pure Chemicals (Osaka, Japan). Malonate was purchased from Tokyo Kasei (Tokyo, Japan), and methylsuccinate was bought from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of analytical grade.

**Pharmacokinetics of Uremic Toxins in the Anesthetized Rat.** Male Wistar rats (250–290 g; bred and maintained in the departmental animal facility) were anesthetized with sodium pentobarbital (60 mg/kg) by intraperitoneal injection, and the left femoral vein and artery were then cannulated.

All uremic toxins (CMPF, IS, IA, and HA) were administered at a dose of 5 mg/kg (21 μmol/kg) by rapid infusion into the femoral vein. After each infusion the cannulae were flushed with a small volume of 0.9% NaCl/H2PO4, and an aliquot of a stock solution of fenbufen was added as an internal standard. After this, 5 ml of ethyl acetate was added, and the samples were gently shaken for 15 min. After centrifugation at 3000 × g for 10 min the supernatant (2 ml) was evaporated to dryness. The samples were then reconstituted in 0.2 ml of 0.2 M acetate buffer, pH 4.5/acetone/acidic acid (63:37:0.5, v/v/v), the mobile phase for the HPLC system. For the other uremic toxins (IS, IA, and HA), an aliquot of plasma sample (100 μl) was added directly to 200 μl of acetone/water (1:2, v/v).

**Determination of the Free (Unbound) Concentration of Uremic Toxins.** The serum-free concentrations of uremic toxins were estimated by ultrafiltration as described previously (Teutsumi et al., 1999). The free fractions of uremic toxins were determined according to the following equation:

\[ f = \left( \frac{C_f}{C_t} \right) \times 100 \, (\%) \]  

where \( f \) represents free fraction of uremic toxin, \( C_f \) represents the free concentration, and \( C_t \) represents the total concentration of uremic toxin, respectively.

**Urinary and Biliary Excretion.** The urinary excretion and biliary excretion of CMPF were measured to find the major excretory pathway of CMPF. Male Wistar rats (250–290 g; bred and maintained in the departmental animal facility) underwent a surgical procedure under light anesthesia with phenobarbital where cannulae were inserted into the femoral vein and artery, using polyethylene tubing (polyethylene-50; 0.58 mm i.d.; 0.9655 mm o.d.; BD Biosciences, Parsippany, NJ). The bile duct was also cannulated with polyethylene tubing (polyethylene-10; 0.28-mm i.d.; 0.61-mm o.d.), as was the bladder (polyethylene-3; 2.33-mm o.d.; Hibi Co., Tokyo, Japan). The body temperature of the rats was maintained by heat from a lamp. Thirty minutes before the i.v. injection of CMPF, control samples of bile and urine were collected. Bile and urine were collected at 0 to 60, 60 to 120, 120 to 180, 180 to 240, and 240 to 300 min.

**Tissue Distribution of CMPF.** Rats were weighed and cannulated as described above. CMPF was then administered via the femoral vein, and 1 h later the rats were sacrificed by decapitation. The brain, heart, lungs, liver, spleen, kidneys, and testes were removed and weighed. A sample (0.5 g) of each tissue was homogenized in 3 ml of 1 M KH2PO4, and the CMPF in each tissue was extracted using the procedure described for the preparation of plasma samples. The distribution of CMPF in each tissue is expressed as the concentration of CMPF per gram of each tissue divided by the concentration of CMPF in plasma (i.e., the \( C_t/C_f \) ratio).

**Uptake by Rat Renal Cortical Slices.** The uptake of CMPF and PAH by rat renal cortical slices was investigated using the procedure described by Henderson and Lindup (1992). Rats were anesthetized and the kidneys promptly removed, decapsulated, and placed in an ice-cold oxygenated rinse medium, which consisted of 97 mM NaCl, 40 mM KCl, 0.74 mM CaCl2, 22 mM glucose, 7.5 mM sodium phosphate-chloride buffer, pH 7.4. Renal cortical slices (weight 10–20 mg/slice; about 0.5 mm in thickness) were cut from freshly killed and decapitated rats and were stored in the oxygenated rinse medium on ice for no longer than 15 min before the start of incubation. Two slices were placed in each flask and the medium (which consisted of rinse medium, 10 mM l-lactate, and 10 mM l-pyruvate) in each flask was thoroughly gassed with 100% oxygen for about 1 min both before and after the addition of the slices. A concentration of 20 μM was chosen for the uptake (CMPF) because this is the concentration normally found in healthy humans (Niwa et al., 1988). The slices were then tightly sealed with rubber stoppers and incubated in a shaking water bath at 37 °C for 60 min. After the incubation, the slices were placed on ice, the slices promptly removed from the flasks, gently blotted, and weighed. Tissue blanks were prepared by omission of CMPF from the incubation medium.

Another incubation medium (modified Cross and Taggart saline) was used to investigate the effect of α-KG on the uptake of CMPF and PAH by renal cortical slices and this contained 95 mM NaCl, 80 mM mannitol, 5 mM KCl, 0.74 mM CaCl2, and 9.5 mM Na2PO4, pH 7.4 (Pritchard, 1995).

For the renal cortical slice experiments, uptake of CMPF was expressed as the concentration of CMPF per gram of kidney tissue divided by the concentration of CMPF per milliliter of medium (i.e., the slice-to-medium ratio). Active uptake velocity was obtained by subtracting the uptake velocity in the presence of nitrogen (nonspecific uptake) from that in the presence of oxygen.

**HPLC Conditions.** The HPLC system consisted of an L-6200 intelligent pump (Hitachi, Tokyo, Japan) and either an F-1050 fluorescence spectrophotometer or L-4000 UV detector (Hitachi). A column of LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as a stationary phase. The mobile phase consisted of acetate buffer (20 M, pH 4.5/acetone (93:7, v/v), for HA; 65:35, v/v, for IA and IS), oxygenated with nitrogen gas. The HPLC system consisted of an L-6200 pump, an L-7410 autosampler, an L-7480 spectrophotometer detector (Hitachi, Tokyo, Japan), and an L-7410 integrator (Hitachi, Tokyo, Japan). The column used was a Shimadzu LC-10AD column (4.6 mm i.d., 15 cm). The mobile phase consisted of methanol/water (50:50, v/v) and the flow rate was 1 ml/min. The temperature was maintained at 4°C. The detection wavelength was set at 280 nm. For the other uremic toxins (IS, IA, and HA), an aliquot of plasma sample (100 μl) was added directly to 200 μl of acetone/water (1:2, v/v).
Pharmacokinetic parameters for uremic toxins after i.v. administration (5 mg/kg)

**TABLE 1**  
Different experiments. Each point represents the mean ± S.E. of four different experiments.

![Figure 1](plasma_concentration_time.png)  
**Fig. 1.** Plasma concentration-time profiles for a series of uremic toxins. All uremic toxins (CMPF, IA, HA, and IS) were administered at a dose of 5 mg/kg by rapid infusion into the femoral vein. Blood samples were taken from the femoral artery at 1, 3, 6, 15, 30, 60, 90, 120, 180, 240, and 300 min. Each point represents the mean ± S.E. of four different experiments.

Acetate buffer (0.2 M, pH 4.5)/acetone/nitrileacet acid (60:40:0.5, v/v, for CMPF), and methanol/acetonitrile/tetrahydrofuran/acetate buffer (70 mM, pH 4.0), which contained 3 mM tetra-n-butylammonium bromide (6.5:20:2:91.3, v/v, for PAH). The flow rate was 1.0 ml/min. HA, CMPF, and PAH were detected by UV at 240, 261, and 254 nm, respectively. IA and IS were detected by means of a fluorescence monitor. The excitation/emission wavelengths were 280/375 nm, respectively, for both IA and IS. The coefficients of variation of unbound CMPF were 14.3 ± 0.8 (for IS), 20.2 ± 0.9 (for IA), and 16.1 ± 0.8 (for HA) for each concentration level. The recovery of authentic CMPF, IS, IA, and HA from rat plasma was greater than 95% in each case. Normal rat plasma contained substances that have chromatographic characteristics that are identical to CMPF, IS, IA, and HA, and the mean (±S.E.) endogenous concentrations of these substances were 0.85 ± 0.06, 1.62 ± 0.17, 0.25 ± 0.04, and 1.25 ± 0.14 µg/ml, respectively (n = 5).

**Pharmacokinetics of Uremic Toxins.** Fig. 1 shows the plasma-time concentration profiles for the uremic toxins after administration of doses of 5 mg/kg to the rats. The plasma clearance of CMPF was the lowest of all the uremic toxins tested in this experiment (Table 1). The biological half-life (t1/2) was 356 ± 18 min (Table 1), and this value seems to reflect its high affinity for albumin (the percentage bound was about 98%); Table 1). IS, however, which had a similar unbound fraction (2%) to CMPF in rat plasma under the conditions of this experiment, was eliminated from plasma much more rapidly than CMPF. Other uremic toxins, including IA and HA, had a moderate plasma clearance and a lower affinity for albumin than either CMPF or IS.

The urinary and biliary excretion of CMPF were also examined. The renal (CLr) and biliary (CLb) clearances of unbound CMPF were 14.3 ± 0.6 and 0.09 ± 0.01 ml/min/kg, respectively. Most of the CMPF was excreted unchanged and the main route was via the urine (Table 2). It is noteworthy in this respect that the main elimination pathway of the other uremic toxins was also via renal excretion (Table 3). When the renal clearance of unbound CMPF (CLr) is compared with the glomerular filtration rate (GFR), the results suggest that active tubular secretion is involved in the urinary excretion of CMPF (Table 2). These results are consistent with the finding that CMPF is mainly distributed in the kidney. The concentration of CMPF in the kidney after 1 h (at the 5-mg/kg dose) was 34.3 µg/g, after intravenous administration (Fig. 2). The low renal clearance of CMPF may be due to the saturation of tubular secretion at a 5-mg/kg dose in the rat. Because of this, the dose dependence of CMPF was examined. Plasma clearance decreased with increasing the dose from 1 to 5 mg/kg (Table 4), suggesting that at 5 mg/kg, tubular secretion, which is responsible for the plasma clearance of CMPF, was saturated.

**Results**

All data are presented as the mean ± S.E., and n refers to the number of animals used in each experiment. Student’s t test was used to analyze differences between two groups. Analysis of variance was used to analyze differences among more than two groups, and the significance of difference between two means in these groups was evaluated using the modified Fisher’s least-squares difference method.

**Table 1:** Pharmacokinetic parameters for uremic toxins after i.v. administration (5 mg/kg)  
Each value represents the mean ± S.E. (n = 4).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Uremic Toxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMPF</td>
</tr>
<tr>
<td>AUC (µg/mL · min)</td>
<td>17,377 ± 101</td>
</tr>
<tr>
<td>CLtot (mL/min/kg)</td>
<td>0.288 ± 0.002</td>
</tr>
<tr>
<td>t1/2 (min)</td>
<td>356 ± 18</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>f (%)</td>
<td>2</td>
</tr>
</tbody>
</table>
Mechanism of Uptake of CMPF by Renal Cortical Slices. The uptake of CMPF by renal cortical slices was examined next. In the presence of oxygen, the slice-to-medium ratio was 4.6 ± 0.5 (n = 3), whereas under anaerobic conditions (in the presence of nitrogen), this value was 1.1 ± 0.2 (n = 3). These results suggest that active transport is involved in the uptake of CMPF by renal cortical slices and provides further support for the above-mentioned in vivo study. The active uptake of CMPF by renal cortical slices was linear for at least 20 min (data not shown). To estimate the $K_m$ and $V_{\text{max}}$ values for the active uptake of CMPF from the initial rate, the uptake velocity of CMPF over a range of concentrations (20–320 μM) was measured after incubation for 20 min at 25°C in three separate experiments. Nonspe-

cific uptake measured in the absence of oxygen was subtracted from the uptake in the presence of oxygen. The mean $K_m$ and $V_{\text{max}}$ values for the active uptake of CMPF were 98.1 ± 15.2 μM and 36.7 ± 10.2 nmol/min/g kidney, respectively. These values were smaller than those reported by Henderson and Lindup (1992) ($K_m = 194$ μM; $V_{\text{max}} = 55$ nmol/min/g kidney) who used a longer incubation time of 90 min. In the present study, $K_m$ and $V_{\text{max}}$ values were calculated from the initial rate and therefore the affinity of the renal uptake system for CMPF may be higher than reported previously.

As shown in Fig. 3, the uptake of CMPF by renal cortical slices was inhibited by PAH, a typical substrate for an organic anion transporter in the kidney. CMPF uptake by renal cortical slices was concentration dependent over the range 20 μM to 1 mM. PAH (1 mM) produced a significant reduction in CMPF uptake (7.76 ± 2.93% of the uptake in the control study). The apparent inhibitory constant ($K_i$) of PAH to inhibit the CMPF uptake, determined from a Dixon plot, was 97.4 μM. CMPF uptake was not inhibited by TEA, a typical substrate for an organic cation transporter in the kidney.
These results suggest that organic anion transporters, which recognize PAH as a substrate, are also involved in the active uptake of CMPF by renal cortical slices.

To estimate the inhibitory effect of CMPF on the organic anion transport system, we investigated the effect of CMPF on the uptake of PAH by renal cortical slices. As shown in Fig. 4, CMPF inhibited the uptake of PAH in a concentration-dependent manner. At 400 \text{ \mu M}, CMPF inhibited the uptake of PAH by 81%. The \( K_I \) value of CMPF for inhibition of PAH uptake was 91 \text{ \mu M}. The mutual inhibition suggests that PAH and CMPF shared the transport system.

Characterization of the CMPF Transport System in Renal Cortical Slices. It is well known that the active transport of PAH across the basolateral membrane occurs as the result of the coupling of two transport processes: \( Na^+ \)-dicarboxylate symport and PAH-dicarboxylate exchange (Pritchard, 1995; Orlov Yu, 1997). Because of the mutual inhibition between PAH and CMPF in the renal cortical slice experiments, the same mechanism seems to be involved in the uptake of CMPF. To confirm whether the uptake of CMPF might be mediated by the same transport system, we investigated the effect of \( \alpha-KG \) on the uptake of CMPF by rat renal cortical slices.

As has been shown previously for PAH (Pritchard, 1990, 1995), the external addition of \( \alpha-KG \) resulted in a biphasic and lithium-sensitive effect on the uptake of 20 \text{ \mu M} CMPF by rat renal cortical slices (Fig. 5). Peak stimulation was observed at an \( \alpha-KG \) concentration of 50 \text{ \mu M}, and uptake was decreased at higher concentrations. Furthermore, 5 \text{ mM} lithium completely blocked the stimulatory effect of 50 \text{ \mu M} \( \alpha-KG \). Based on these observations, we propose that an organic anion/dicarboxylate exchanger is involved in the uptake of CMPF by rat kidney slices, in the same manner as for PAH.

The \( Na^+ \)-dicarboxylate transport system has \( Na^+ \) and \( Li^+ \) dependence (Pritchard, 1990; Pajor, 1999). To obtain further evidence about whether the organic anion/dicarboxylate exchanger was responsible for the uptake of CMPF in the kidney, the ionic dependence of CMPF uptake was investigated by measuring CMPF uptake in the presence of various inorganic salts (Table 5). Control uptake was measured in the presence of NaCl. Replacement of Na\(^+\) with an equimolar concentration of cations such as K\(^+\) and choline inhibited the uptake of CMPF by 38.9 ± 2.3 and 43.5 ± 7.2\%, respectively. This indicates that the renal uptake of CMPF was diminished by inhibition of Na\(^+\)-dicarboxylate symport, which links with the organic anion/dicarboxylate exchanger. Replacement of Na\(^+\) with \( Li^+\) decreased the uptake by 31.2 ± 8.7\%, but its inhibitory effect was weaker than that of K\(^+\) and choline. Moreover, in the presence of NaCl and 5 \text{ mM} LiCl, the uptake of CMPF was reduced by 26.4 ± 8.6\%. These results indicate that transport of CMPF was sensitive to \( Li^+\), and so the organic anion/dicarboxylate exchanger could be involved in the transport of CMPF in the kidney.

**Table 5**

Influence of monovalent cations on the uptake of CMPF by renal cortical slices

Renal cortical slices were incubated with 20 \text{ \mu M} CMPF at 25°C for 1 h, either in the control buffer (25 mM Heps/Tria, pH 7.5, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 5 mM glucose, and 140 mM NaCl) or in buffer in which NaCl was replaced with 140 mM of various inorganic salts. The effect of lithium (5 mM) on the uptake of CMPF by renal cortical slices is also shown. Each value represents the mean ± S.E. of six different experiments.

<table>
<thead>
<tr>
<th>Inorganic Salt</th>
<th>CMPF Uptake (ml/h/g kidney)</th>
<th>Percentage of Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (NaCl)</td>
<td>3.00 ± 0.27</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>LiCl</td>
<td>2.06 ± 0.26</td>
<td>68.8 ± 8.7(^a)</td>
</tr>
<tr>
<td>KCl</td>
<td>1.83 ± 0.07</td>
<td>61.1 ± 2.3(^b)</td>
</tr>
<tr>
<td>Choline-CI</td>
<td>1.69 ± 0.22</td>
<td>56.5 ± 7.2(^b)</td>
</tr>
<tr>
<td>140 mM NaCl + 5 mM LiCl</td>
<td>2.21 ± 0.26</td>
<td>73.6 ± 8.6(^a)</td>
</tr>
</tbody>
</table>

\(^a P < 0.05, ^b P < 0.01, \) significantly different from control.

---

**Fig. 4.** Effect of CMPF on the uptake of PAH by renal cortical slices. The values are expressed as a percentage of 20 \text{ \mu M} PAH uptake by renal cortical slices at 25°C for 1 h in the absence of CMPF. Each column represents the mean ± S.E. of three different experiments. \( P < 0.05 \) (a), \( P < 0.01 \) (b), significantly different from control.

**Fig. 5.** Stimulation of CMPF uptake in renal cortical slices by external \( \alpha-KG \). CMPF uptake was assessed after a 30-min incubation with 20 \text{ \mu M} CMPF and 0 to 400 \text{ \mu M} \( \alpha-KG \) at 25°C in the modified Cross and Taggart saline. Lithium (5 mM) completely blocked the stimulation produced by 50 \text{ \mu M} \( \alpha-KG \). Each column represents the mean ± S.E. of four to five different experiments. a, \( P < 0.01 \) versus control (no added \( \alpha-KG \)); b, \( P < 0.01 \) versus 50 \text{ \mu M} \( \alpha-KG \).
TABLE 6
Effect of dicarboxylate on the uptake of CMPF by the renal cortical slice

<table>
<thead>
<tr>
<th>Dicarboxylate</th>
<th>CMPF Uptake (ml/h/g kidney)</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.94 ± 0.36</td>
<td>100 ± 9 (6)</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.45 ± 0.16</td>
<td>11.5 ± 4.1 (6)</td>
</tr>
<tr>
<td>Glutarate</td>
<td>1.40 ± 0.25</td>
<td>35.6 ± 6.3 (6)</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.40 ± 0.29</td>
<td>61.0 ± 7.3 (6)</td>
</tr>
<tr>
<td>Methylsuccinate</td>
<td>2.06 ± 0.33</td>
<td>52.3 ± 8.5 (6)</td>
</tr>
<tr>
<td>Malonate</td>
<td>3.80 ± 0.20</td>
<td>96.6 ± 5.0 (3)</td>
</tr>
</tbody>
</table>

* P < 0.01, significantly different from control.

We also examined the inhibitory effect of various dicarboxylates on CMPF uptake by renal cortical slices (Table 6). α-KG and glutarate, substrates and/or inhibitors of the organic anion transport system and Na\(^+\)-dicarboxylate symport (Sekine et al., 1997; Uwai et al., 1998; Kekuda et al., 1999), inhibited CMPF uptake by 88.5 ± 4.1 and 64.4 ± 6.3%, respectively. Succinate and methylsuccinate, which are substrates of Na\(^+\)-dicarboxylate symport but not inhibitors of the organic anion/dicarboxylate exchanger, also reduced the uptake by 39.0 ± 7.3 and 47.7 ± 8.5%, respectively. Malonate, which is not an inhibitor of either the Na\(^+\)-dicarboxylate symport or the organic anion/dicarboxylate exchanger, did not inhibit (Table 6). These observations suggest that the organic anion transport system plays an important role in the uptake process of CMPF in the kidney.

Discussion

The half-life of CMPF was longer than that of the other uremic toxins investigated in this study, in which IS was eliminated the most rapidly. CMPF and IS were bound to nearly the same extent in rat plasma in these experiments (Table 1), and the data in Table 3 show that the elimination of CMPF and IS from plasma is a function of urinary excretion. We conclude that this difference in rate of elimination is based on differences in affinity for the renal active transport system.

The elimination of IS was more rapid than that of IA, which, like IS, is an indole derivative. IS contains a sulfate group, unlike IA. Sulfate conjugates, such as estrone sulfate and dehydroepiandrosterone sulfate, are high-affinity substrates for the various organic anion transporters (Kusuhara et al., 1999; Cha et al., 2000; Van Aubel et al., 2000). This points to the importance of the sulfate group in the recognition of compounds and their excretion by the transport system. The total clearance of IS was the same as renal clearance, and the renal clearance of IS divided by the free fraction (f = 0.02), which was much higher than that of the other uremic toxins, was about 41 times greater than the GFR (7.32 ml/min/kg; Trumper et al., 1998). This suggests that transport mechanisms for IS or the affinity of IS for the organic anion transporters may differ from that for carboxylate derivatives (IA, HA, and CMPF).

The elimination of CMPF, a dicarboxylic acid, was slower than that of IA and HA, which are monocarboxylic acid derivatives. Among these carboxylic acid derivatives, the rank order of total renal clearance was the same as that of the free fraction, and therefore renal clearance of the free fraction of these carboxylic acid derivatives was the same (Tables 1 and 3). This suggests that plasma protein binding affects the rate of glomerular filtration and also the concentration of the carboxylic acid derivative that can access the transporter at the basolateral membrane, i.e., the higher the protein binding, the lower the glomerular filtration rate and total renal clearance. The present data indicated that CMPF is almost totally excreted via the kidney (Table 2), mostly unchanged. CMPF can inhibit phase I (O-demethylation) and phase II (glutathione conjugation and glucuronidation) pathways of drug metabolism in the liver (Mabuchi and Nakahashi, 1988a; Walters et al., 1995). It seems that CMPF may act as an inhibitor of drug metabolism but not be metabolized itself. The renal transport system may therefore play an important role in the elimination of CMPF.

Because CMPF has a high affinity for albumin and is effectively excreted in urine, it is likely that an active transport system, such as an organic anion transporter is involved in its elimination from the kidney. It is reasonable to assume that CMPF is mainly eliminated by this mechanism because CMPF is an organic acid that structurally possesses the required hydrophobic area and two negative partial charges (Ullrich and Rumrich, 1988; Ullrich, 1997). Costigan and Lindup (1996) reported that the plasma clearance of CMPF is decreased as a result of the coadministration of PAH and probenecid. In addition, mutual inhibition between PAH and CMPF was observed in slice uptake experiments (Figs. 3 and 4), suggesting that the same transport system is shared with both PAH and CMPF.

The first step in active secretion is the extraction of organic anions from the peritubular blood plasma by the proximal tubule cells through the basolateral membrane. This basolateral uptake of organic anions has been extensively investigated with PAH as the test substrate. According to the proposed model (Pritchard, 1995; Orlov Yu, 1997), the first stage is the formation of a Na\(^+\) gradient as the result of the hydrolysis of ATP by Na\(^+\)-K\(^+\)-ATPase. This Na\(^+\) gradient is a driving force for the Na\(^+\)-dicarboxylate symport, which is sensitive to lithium (Pritchard, 1990; Pujor, 1999). As a result, an anion gradient (dicarboxylate) develops, which is directed from the cell. During the last step, the dicarboxylate anion is exchanged for PAH by the anion exchange transporter. Our observations also suggest that CMPF is recognized by an organic anion/dicarboxylate exchanger because the renal uptake of CMPF was markedly stimulated by 50 μM α-KG, and this stimulation was prevented by not only the presence of 400 μM α-KG but also the addition of 5 mM lithium (Fig. 5). Based on these observations, we propose that an organic anion/dicarboxylate exchanger is involved in the uptake of CMPF, in the same manner as for PAH.

To obtain further evidence about whether the organic anion/dicarboxylate exchanger was responsible for the uptake of CMPF in the kidney, the ionic dependence of CMPF uptake was investigated (Table 5). Replacement of Na\(^+\) with an equimolar concentration of cations such as K\(^+\), choline, and lithium inhibited the uptake of CMPF by 38.9 ± 2.3, 43.5 ± 7.2, and 31.2 ± 8.7%, respectively. These results support the view that the organic anion/dicarboxylate exchanger could be involved in the transport of CMPF in the kidney, because the organic anion/dicarboxylate exchanger is coupled with Na\(^+\)-

Renal Disposition of Uremic Toxins 885

at ASPET Journals on April 13, 2017 jpet.aspetjournals.org Downloaded from
dicarboxylate symport. Furthermore, CMFP uptake by renal cortical slices was strongly inhibited by α-KG and glutarate, which are substrates and/or inhibitors of the organic anion transport system and Na+-dicarboxylate symport (Sekine et al., 1997; Uwai et al., 1998; Kekuda et al., 1999) (Table 6). These results suggest that the organic anion transport system could play an important part in the uptake process for CMFP in the kidney.

On the other hand, it is possible that CMFP is transported by Na+-dicarboxylate symport because CMFP is a dicarboxylic acid. Transport systems for dicarboxylates have been studied in the vesicles from brush-border membranes and basolateral membranes (Wright, 1985; Wright and Wunz, 1987), mostly with succinate as a test substrate. Succinate is a high-affinity substrate for the Na+-dependent dicarboxylate transporter (NaDC3; $K_i$ of succinate = 2 μM), which is present in the rat kidney (Kekuda et al., 1999). However, succinate exhibited only a moderate inhibitory effect on the uptake of CMFP by renal slices (Table 6). Furthermore, no inhibitory effect of 1 mM CMFP on the uptake of [14C]succinate by renal slices was observed (data not shown). These data suggest that CMFP and succinate would be transported by different carriers, and the contribution of Na+-dicarboxylate symport to the uptake of CMFP in the kidney would be very low. Considering the relatively little inhibition produced either by the replacement of Na+ (Table 5) or by succinate (Table 6), the effects on the uptake of CMFP may be secondary to the inhibition of Na+-dicarboxylate symport. This points to the importance of the organic anion transport system for CMFP excretion in the kidney. However, further work is needed to identify the CMFP transporter, and experiments with expression systems are needed to assess the contribution of individual transporters.

Recently, Sekine et al. (1997) reported the functional expression cloning of an organic anion/dicarboxylate exchanger (OAT1) and its characteristics as a multispecific organic anion transporter. They demonstrated that OAT1 plays an essential role in the elimination of numerous organic anions and that an outwardly directed dicarboxylate gradient is essential for expressing transport activity (Sekine et al., 2000). The necessity of the dicarboxylate gradient for the transport of CMFP suggests that OAT1 plays an important role in the elimination of CMFP. Moreover, other isoforms, named OAT2 and OAT3, are also expressed in the kidney and are sensitive to PAH (Sekine et al., 1998; Kusuhara et al., 1999; Kojima et al., 2002). In particular, CMFP may be a substrate of OAT3, because CMFP is a potent inhibitor of OAT3 expressed in oocytes (IC50 of CMFP = 4 μM) (Deguchi et al., 2002). These transporters may be responsible for the renal uptake of CMFP, because there was mutual inhibition of slice uptake between CMFP and PAH in the uptake experiments (Figs. 3 and 4; Henderson and Lindup, 1992).

In uremia, the accumulation of CMFP has various effects, i.e., inhibition of protein binding (Sakai et al., 1995), inhibition of drug metabolism (Walters et al., 1995), and inhibition of renal excretion (Henderson and Lindup, 1992; Walters et al., 1995). CMFP may therefore cause other uremic metabolites and/or drugs to remain in circulation and elevate their concentrations to toxic levels. Interestingly, however, the free fraction of CMFP was low, but its concentration in the kidney was almost the same as that of plasma (Fig. 2). Based upon this observation, CMFP may affect pharmacokinetics of drugs by inhibition of metabolism and renal excretion. This leads us to the suggestion that organic anion transport systems play an important role not only the elimination of CMFP via the kidney but also in mediating the uremic toxicity of CMFP. Furthermore, it has been reported that an organic anion/dicarboxylate exchanger also exists in the brain (Adkison and Shen, 1996; Sekine et al., 2000) as well as the kidney. These transporters also recognize drugs and endogenous compounds, and so a study of the effects of CMFP on such transporters will lead to a better understanding of not only drug-uremic toxin interactions but also the relationship between the uremic syndrome and uremic toxins.

In conclusion, we have found that CMFP is cleared more slowly from rat plasma than indoxyl sulfate, hippuric acid, or indole acetic acid and that the highest tissue concentration was found in the kidney. Furthermore, using renal cortical slices, we conclude that the uptake of CMFP would be mediated by an anion/dicarboxylate exchanger that is similar to that for PAH.

References
Renal Disposition of Uremic Toxins

Address correspondence to: Dr. Masaki Otagiri, Professor, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: otagirim@gpo.kumamoto-u.ac.jp