In Vivo Analysis of Amantadine Renal Clearance in the Uninephrectomized Rat: Functional Significance of In Vitro Bicarbonate-Dependent Amantadine Renal Tubule Transport

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ABSTRACT

Amantadine transport into renal proximal and distal tubules is bicarbonate dependent. In the present study, we addressed the effects of bicarbonate on renal clearance and urinary excretion of amantadine. Renal clearance of kynurenic acid was also studied to determine whether bicarbonate effects are specific for organic base transport by the kidney. After a moderate diuresis was established, animals received i.v. [3H]amantadine or [3H]kynurenic acid followed by an acute dose of sodium bicarbonate or physiological saline. Urine and blood samples were analyzed for [3H]amantadine or [3H]kynurenic acid, blood gases, and pH. Amantadine and kynurenic acid were excreted by the kidneys, and both compounds underwent renal tubular secretion. Amantadine metabolism occurred, and one metabolite was detected in the urine. In the bicarbonate-treated rats, the total amount of amantadine excreted in the urine was decreased, whereas the amount of metabolite recovered was similar in both groups. Bicarbonate treatment caused a sustained increase in blood bicarbonate levels, a mild increase in blood pH, and a decrease in amantadine renal clearance and in the kynurenic acid/creatinine clearance ratio. Only a transient decrease in the renal clearance of kynurenic acid and the kynurenic acid/creatinine clearance ratio was observed. This study demonstrates that short-term changes in bicarbonate concentration may have significant effects on renal organic cation elimination. Coupled with our previous in vitro demonstration of bicarbonate-dependent organic cation transport, the present study suggests that bicarbonate inhibition of renal tubule organic cation secretion may explain the previous observation that bicarbonate dosing decreases amantadine excretion by the kidney.

Organic cation transport plays an important role in the renal tubule secretion and the elimination of many exogenous cationic compounds from the body (Rennick, 1981). Amantadine, an organic cation drug, is eliminated from the body by the kidneys, and renal tubule secretion is important in this process (Bleidner et al., 1965; Tilles, 1974; Aoki et al., 1979; Sitar et al., 1997). When human volunteers taking oral amantadine were given chronic oral bicarbonate, a decrease in amantadine excretion followed by an increase in plasma amantadine concentration was observed (Geuens and Stephens, 1967). From their study, it was inferred that bicarbonate decreased amantadine excretion by increasing urine pH and thus passive reabsorption of amantadine. In contrast, in vitro rat experiments have demonstrated that at constant pH, the energy-dependent uptake of the organic cation amantadine into proximal and distal tubules is primarily mediated by bicarbonate-dependent transport sites (Escobar et al., 1994; Escobar and Sitar, 1995).

In the study by Geuens and Stephens (1967), no attempts were made to address the possibility that the depression in renal elimination of amantadine might be mediated by bicarbonate driven changes in secretion or filtration in addition to or rather than pH-mediated passive reabsorption. The novelty of the present study is our extension of the importance of secretion and filtration components of amantadine clearance as a function of an acute exposure to bicarbonate. The objective of the current study was to determine whether the modulating effects of bicarbonate on the renal tubule amantadine (organic cation) transport system that is located in proximal and distal tubules (Wong et al., 1991, 1992; Escobar et al., 1994, 1995; Escobar and Sitar 1995, 1996) contribute to the...
previous observation that bicarbonate dosing decreases amantadine renal excretion. The $K_a$ value (about 22 mM) for bicarbonate at these transport sites is close to normal plasma bicarbonate concentrations (25 mM). Therefore, we proposed that increases in plasma bicarbonate above physiological levels (or above the transporter $K_a$ value for bicarbonate) would be expected to increase amantadine renal tubule uptake at the bicarbonate-dependent organic cation transport sites. The increased amantadine uptake might then be reflected as a measurable change in the amantadine renal clearance in vivo. Disorders in which plasma bicarbonate concentration rises above normal are quite common in humans and include metabolic alkalosis and metabolic compensation to respiratory acidosis (Dubose et al., 1996). These may represent potential conditions in which organic cation elimination by the kidney may be compromised.

It has also been suggested that organic cation and anion transport in the kidney may be facilitated by common transporters (Ullrich et al., 1993; Ullrich, 1994). Therefore, the effects of bicarbonate on renal elimination of kynurenic acid (an organic anion) were evaluated to determine whether bicarbonate effects on tubule transport are specific for organic cations. Kynurenic acid is a minor end product of tryptophan metabolism (Stone and Connick, 1985). In rats, kynurenic acid is rapidly eliminated predominantly unchanged by the kidneys (Turski and Schwarz, 1988). It therefore represents a good organic anion model substrate for these studies.

## Materials and Methods

### Experimental Design

The experimental protocol was approved by the Protocol Management and Review Committee of the University of Manitoba according to the guidelines of the Canadian Council on Animal Care. The use of the uninephrectomized rat model has been established and characterized previously (Intengan and Smyth, 1996). Rats were initially assigned to one of five groups. Control rats (group 1) received i.v. physiological saline only. Treatment groups received amantadine plus saline (group 2), amantadine plus bicarbonate (group 3), kynurenic acid plus saline (group 4), and kynurenic acid plus bicarbonate (group 5). At a later date, two additional groups were added to the experimental protocol to analyze blood gases and urine pH in animals receiving amantadine plus saline (group 6) and amantadine plus bicarbonate (group 7). The detailed methods that we used are presented below.

### Preparation of Amantadine and Kynurenic Acid Solutions

Solutions for amantadine HCl and kynurenic acid infusion were prepared on the day of the experiment from stock solutions. Amantadine HCl was dissolved in physiological saline. Kynurenic acid was dissolved in 1 M NaOH; the solution was back titrated with 0.1 M HCl to pH 7.4 and volume adjusted with physiological saline. [3H]Amantadine or [3H]Kynurenic acid was added to an aliquot of the stock amantadine and kynurenic acid solutions, respectively, such that the final molar ratio of $[^3H]$ label/nonlabel was consistently 1:10,000.

### Animal Preparation

Male Sprague-Dawley rats (200–225 g) were obtained from the University of Manitoba (Charles River breeding stock). The rats were housed in metal cages, at 22°C, with a 12-h light/dark cycle. They had free access to food (standard Purina rat chow) and tap water. The right kidney was removed under ether anesthesia via a flank incision, and a minimum 1-week recovery period was imposed. Renal clearance experiments were performed 7 to 14 days after the unilateral nephrectomy. On the day of the experiment, the rats (270–340 g) were anesthetized with sodium pentobarbital (150 mg/kg i.p.). Body temperature was monitored with a rectal thermometer and maintained at 37.5°C with a thermostatically controlled heating pad. A tracheotomy was performed, and the animals were allowed to breathe spontaneously. The left carotid artery was cannulated with PE-50 polyethylene tubing and connected to a Grass polygraph via a Statham pressure transducer (model P23Db) for monitoring blood pressure and heart rate. The left jugular vein was cannulated with PE-160 polyethylene tubing for administration of saline, bicarbonate, amantadine, or kynurenic acid. Additional anesthetic (3.0 mg) as required throughout the experiment was injected through a latex adapter into the main i.v. line. A left flank incision was made, the remaining kidney was exposed, and the ureter was cannulated with PE-50 polyethylene tubing to allow for urine collection.

### Amantadine and Kynurenic Acid Renal Clearance Experiments

Immediately after completion of the surgical preparation, rats were started on a continuous infusion of 5 IU heparin in isosmotic (300 mOsm/l) physiological saline (0.9% w/v) at 97 µl/min and were allowed to stabilize for 45 min. The heparin/saline infusion was maintained for the remainder of the procedure, except during amantadine, kynurenic acid, and bicarbonate infusion periods. Immediately after the stabilization period, 3 mg/kg [3H]amantadine (groups 2 and 3) or [3H]kynurenic acid (groups 4 and 5) were infused in a total volume of 200 µl at a rate of 97 µl/min. The control animals (group 1) received an equivalent volume of physiological saline over the same duration. Five minutes after the completion of drug administration, the first 20-min urine collection was started. At the start of the second urine collection period, groups 3 and 5 were infused with hypertonic (2000 mOsm/l) sodium bicarbonate (5 mmol/kg i.v.) at 111 µl/min, whereas the remaining groups (groups 1, 2, and 4) were maintained on the heparin/saline infusion. After the start of the bicarbonate administration, there were five successive 20-min urine collection periods. All urine samples were collected into preweighed vials, and the urine volume was determined gravimetrically. Blood sampling was done from the carotid artery cannula at the beginning of each urine collection period (7, 27, 47, 67, 87, and 107 min after the start of amantadine or kynurenic acid infusion). Blood samples (100 µl) were collected into microcentrifuge tubes that contained 1.0 IU of heparin sulfate. A final blood sample (2 ml) was taken at the end of the final urine collection period for determination of plasma creatinine levels, osmolality, and plasma ions. Blood was centrifuged immediately to separate the plasma. Two 20-µl aliquots from each plasma and urine sample were placed in plastic scintillation vials, suspended in 5 ml of Beckman Ready-Safe Scintillation Cocktail, vortexed for 30 s, and counted for radioactivity in a Beckman model LS5801 scintillation counter (Beckman Instruments, Fullerton, CA). To address the issues of blood gas and urine pH, we performed additional experiments to measure plasma pH, bicarbonate, pCO$_2$, and urine pH in rats treated with amantadine plus saline (group 6, $n = 4$) and rats given amantadine plus bicarbonate (group 7, $n = 4$). The same procedures were used for these experiments as described above, with the addition of the following minor changes. A slightly larger volume of blood was necessary for blood gas measurements than for measurements of plasma amantadine (140 versus 100 µl). Therefore, to keep the volume of blood withdrawal similar over the duration of the experiment, we reduced the number of blood collections from seven to five. The five blood samples were taken at 7, 27, 47, 87, and 127 min after amantadine infusion. To facilitate rapid and efficient withdrawal of blood samples, an arteriovenous loop was inserted connecting the carotid artery and the jugular vein (Xie et al., 1996). Samples were drawn directly from the arterial side of the loop into a capillary tube, sealed, and measured for HCO$_3$-, pCO$_2$, and pH immediately using an Instrumentation Laboratory System model 1302 Blood-Gas Analyzer. Saline, bicarbonate, and amantadine were all infused through the venous side of the loop. Urine pH was measured using a standard microelectrode. Plasma and urine osmolalities were determined with a Precision System Micro Osmometer. The plasma and urine Na$^+$ concentrations were determined using a Nova Electrolyte Analyzer.
Thin-Layer Chromatography (TLC). TLC was performed according to previously described methods to confirm the presence of unmetabolized [3H]amantadine and [3H]kynurenic acid in urine samples (Uchiyama and Shibuya, 1969; Turski and Schwarzrz, 1988). [3H]Amantadine and [3H]kynurenic acid standards were dissolved in saline control urine samples and run in parallel with the test urine samples on fluorescent silica gel plates (Analtech Inc., Newark, DE). The developing solvent for amantadine was a mixture of n-butanol/acetic acid/water (4:1:5), and that for kynurenic acid was a mixture of n-butanol/methanol/water/ammonium hydroxide (60:20:19:1). The developing time ranged between 50 and 60 min. Sections (0.5 cm) from the origin to the solvent front of the plate were scraped and counted for [3H]radioactivity. Analysis of plasma was not possible due to the small volume and low specific radioactivity of plasma samples. For calculations, it was assumed that all radioactivity counted in plasma was associated with the parent compounds.

Data Analysis. The radioactivity in each plasma and urine sample was recorded as disintegrations per minute. Background radioactivity, consistently about 20 dpm, was subtracted to obtain the specific value. Plasma and urine amantadine and kynurenic acid concentrations were determined from the respective dpm values. TLC demonstrated that for amantadine-treated rats, amantadine and an unidentified metabolite were present in the urine. Therefore, all urine dpm values for amantadine-treated rats were corrected for the percentage of the [3H]metabolite in the urine as follows: amantadine dpm (urine) = total dpm (urine) × area under the plasma concentration-versus-time curve (AUC) (amantadine peak on TLC)/AUC (amantadine peak plus metabolite peak on TLC). A colorimetric assay based on the Jaffé reaction (Diagnostic Kit, procedure 555; Sigma Chemical Co., St. Louis, MO) was used to quantify urine and plasma creatinine. The renal clearance values of amantadine (groups 2 and 3), kynurenic acid (groups 4 and 5), and creatinine (all groups) were calculated for each of the six collection intervals using the AUC method (Gibaldi and Perrier, 1982). Renal clearance equals the amount of the drug eliminated in the urine during each time interval divided by the AUC plasma drug concentration for each time interval. AUC was calculated with the plasma concentration data at the beginning and end of each time interval.

Renal clearance ratios of amantadine and kynurenic acid to creatinine were calculated to evaluate the effect of bicarbonate on their renal tubule secretion. Additional pharmacokinetic analysis was performed by fitting the plasma concentrations of amantadine and kynurenic acid to the two-compartment open model of drug disposition with zero order iv infusion using the nonlinear regression program WinNonlin version 1.1 (Pharsight Corporation, Palo Alto, CA). The parameters determined were AUC, half-life of initial drug disposition (α1/2) and terminal disposition (β1/2), plasma drug clearance (Clp), and the apparent volume of distribution at steady state (Vd). All data are presented as mean ± S.E.M. of a least four experiments. Data were analyzed for treatment and time effects by mixed model repeated measures (for time) ANOVA using Systat for Windows, version 6.01 (Statistical Solutions Inc., Boston, MA). Significant differences between means were determined with Tukey’s honest significant difference (HSD) test. Differences between mean values with a value of p ≤ .05 were considered to be significant.

Chemicals. [3H]Amantadine HCl (28 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). Amantadine was obtained from Dupont Canada, Inc. (Mississauga, Ontario, Canada). [3H]Kynurenic acid (14 Ci/mmol) was obtained from Amersham Canada (Oakville, Ontario, Canada). Kynurenic acid and creatinine assay kits (procedure 555) were obtained from Sigma Chemical Co. Physiological saline (0.9% w/v) was obtained from Baxter Corporation (Toronto, Ontario, Canada). NaHCO3 for i.v. injection (8.4%) was obtained from Abbott Laboratories Limited (Montreal, Quebec, Canada). All other chemicals were of the highest grade available from commercial suppliers.

Results

Metabolism of Amantadine and Kynurenic Acid. Urine samples from amantadine-treated rats consistently revealed two peaks of radioactivity, indicating metabolism of the parent compound (data not shown). The retention factor (r) value for the parent compound and the metabolite were consistently 0.67 and 0.53, respectively. Considering the significant amantadine metabolism observed in our experiments, we attempted to identify the metabolite but were unsuccessful. By gas chromatographic analysis of urine samples, we confirmed that the metabolite was not acetylamantadine. Urine samples from the kynurenic acid-treated rats displayed one peak that was consistent with that of the standard (r = 0.80), indicating that the radioactivity recovered in the urine was associated with the parent compound.

Renal Clearance Measurements. Creatinine clearance was used as a general marker for renal glomerular filtration (Figs. 1a and 2a). For clarity of interpretation of creatinine clearance, the same saline control data are shown separately in each figure. However, groups 1 to 5 were compared simultaneously for statistical analysis. Increases in creatinine clearance were observed in amantadine-treated rats that received bicarbonate and in kynurenic acid-treated rats independent of bicarbonate treatment (p < .05). Amantadine treatment alone did not alter the creatinine clearance. Creatinine clearance for the saline controls did not decrease with time, indicating maintenance of renal filtration function over the duration of our experiments.

The effect of bicarbonate on the interval renal clearances of amantadine and kynurenic acid is shown in Figs. 1b and 2b, respectively. It was important to compare the interval renal clearances rather than just the overall renal clearances so that the persistence of any effects of bicarbonate treatment could be identified. The interval amantadine renal clearance (Fig. 1b) was similar in both amantadine-treated groups before bicarbonate administration (collection period 1). After bicarbonate administration (collection periods 2–6), the interval amantadine renal clearances (Fig. 1b) were 30 to 60% lower than the respective controls (p < .05). The interval amantadine renal clearance decreased with time regardless of treatment (p < .01). The overall mean renal clearance of amantadine was lower in the bicarbonate-treated group (0.76 ± 0.04 ml/min/100 g) versus the amantadine plus saline-treated rats (1.16 ± 0.04 ml/min/100 g; p < .01). In the kynurenic acid-treated rats, the interval kynurenic acid renal clearance was similar in both groups before and after bicarbonate infusion. The mean interval kynurenic acid renal clearance decreased with time (p < .01). There was no effect of bicarbonate treatment on overall mean kynurenic acid clearance (1.11 ± 0.05 ml/min/100 g) versus the kynurenic acid plus saline-treated rats (1.19 ± 0.05 ml/min/100 g).

Renal clearance data were normalized to creatinine clearance for amantadine (Fig. 1c) and kynurenic acid (Fig. 2c). Initially, all amantadine/creatinine and kynurenic acid/creatinine clearance ratios were substantially greater than 1, indicating renal tubule secretion of both amantadine and kynurenic acid. In the two amantadine treatment groups, the mean amantadine/creatinine clearance ratios were similar before bicarbonate treatment and were 55 to 70% lower than the respective time controls after bicarbonate treatment (p < .01). The overall amantadine/creatinine clearance ratio was...
reduced in the bicarbonate-treated rats (1.62 ± 0.12) compared with the amantadine plus saline-treated rats (2.97 ± 0.12) (p < .01). Similar to amantadine clearance, the amantadine/creatinine clearance ratio decreased with time after amantadine infusion (p < .001). The kynurenic acid/creatinine clearance ratio decreased with time after dosing (p < .05).
.001) and was lower only during collection interval 2 for the bicarbonate-treated group compared with the control (p < .01). There was no effect of bicarbonate treatment on the overall mean kynurenic acid/creatinine clearance ratio for bicarbonate-treated (2.26 ± 0.09) versus for the saline-treated (2.31 ± 0.09) rats.

For the current experiments, a direct comparison of amantadine clearance with the clearance of its major metabolite was not possible because we were unable to determine metabolite concentrations in the plasma. However, it was possible to compare the rates of excretion and total excretion of amantadine versus the amantadine metabolite (Table 1).

Before bicarbonate administration, the rate of urinary amantadine excretion was similar in both groups and was reduced after bicarbonate administration (collection periods 2 and 3; p < .01), which is consistent with the observed decrease in amantadine clearance. Total amantadine excretion was lower in the bicarbonate-treated rats (p < .01). The rate of amantadine metabolite excretion for all urine collection periods and the total amount of amantadine metabolite excreted in the urine were similar in the amantadine plus saline- and the amantadine plus bicarbonate-treated rats. In contrast to amantadine, there were no appreciable changes in the rate of kynurenic acid excretion, or total kynurenic acid recovered in the urine, for the bicarbonate-treated rats compared with controls.

**Correlation between Urine Flow Rate and Amantadine/Creatinine and Kynurenic Acid/Creatinine Clearance Ratios.** Urine flow rates (µl/min) for each urine collection period and group of rats are shown in Table 2. Urine flow rates for all groups increased with time (p < .001) and reached a plateau between 60 and 100 µl/min. Initially (collection period 1), the urine flow rates were similar in all groups. After bicarbonate treatment, the urine flow rate was greater during urine collection periods 2 and 3 compared with the groups that did not receive bicarbonate (p < .05).

The more rapid diuresis in the bicarbonate-treated rats is likely due to the greater Na⁺ load from the hypertonic NaHCO₃ infusion compared with the isotonic heparin saline infusion. After the third urine collection period, the urine flow rates in the bicarbonate-treated rats decreased toward values similar to those of the groups that did not receive bicarbonate treatment. Amantadine/creatinine and kynurenic acid/creatinine clearance ratios versus urine flow rates are presented in Fig. 3. The amantadine/creatinine clearance ratio was not correlated with a change in urine flow rate in the amantadine plus saline-treated rats (r² = 0.092) and in the amantadine plus bicarbonate-treated rats after the start of bicarbonate administration (r² = 0.022). Conversely, the kynurenic acid/creatinine clearance ratio in kynurenic acid plus saline-treated rats was moderately correlated with urine flow rate (r² = 0.421). However, no effect of urine flow rate on the kynurenic acid/creatinine clearance in the bicarbonate-treated group was demonstrated (r² = 0.057).

**Comparison of Blood Gas and Urine pH.** Blood gas and urine pH values for group 6 (amantadine plus saline treated) and group 7 (amantadine plus bicarbonate treated) rats are shown in Table 3. The blood gas and urine pH measurements were not performed in the kynurenic acid-treated rats because there was no major effect of bicarbonate on kynurenic acid renal clearance. In the amantadine plus saline-treated rats, blood bicarbonate, pCO₂, and pH and urine pH were similar over the duration of the experiment. Before bicarbonate infusion, bicarbonate, pCO₂, and pH and urine pH were similar between the two groups. After the acute bicarbonate dose, blood bicarbonate increased to a maximum level of 34.6 ± 0.41 mM, which was approximately 8 mM higher than for the amantadine plus saline-treated rats (26.9 ± 0.67 mM) at the same time point (p < .001). The plasma bicarbonate levels dropped in the last two collection intervals but remained greater than the respective controls (p < .001). In contrast to the large increase in blood bicarbonate, the blood pH remained only slightly elevated (7.45–7.48) compared with control measurements (7.40–7.41) at the same time points (p < .01). Blood pCO₂ increased slightly but was not significant. Urine pH in the amantadine plus saline-treated rats remained constant and slightly acidic (6.6–6.8), whereas the urine pH became alkaline immediately after bicarbonate infusion and remained elevated at (pH 8) in the amantadine plus bicarbonate-treated rats (p < .001).

**Pharmacokinetic Determinations.** Mean amantadine and kynurenic acid plasma concentrations versus time are shown in Fig. 4. The plasma amantadine and kynurenic acid concentration-versus-time profiles were similar in the bicarbonate-treated and control rats. The amantadine and kynurenic acid plasma concentrations from the individual experiments were fit to the two-compartment model of disposition to determine the pharmacokinetic parameters.

### TABLE 1
Amantadine, metabolite, and kynurenic acid excretion rate in the urine and total excretion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urine Collection Period</th>
<th>1 (nmol/min)</th>
<th>2 (µmol)</th>
<th>3 (µmol)</th>
<th>4 (µmol)</th>
<th>5 (µmol)</th>
<th>6 (µmol)</th>
<th>Total (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine excretion</td>
<td>Amantadine + saline</td>
<td>22.1 ± 1.8</td>
<td>11.6 ± 0.7</td>
<td>7.8 ± 0.6</td>
<td>6.1 ± 0.5</td>
<td>4.4 ± 0.4</td>
<td>3.9 ± 0.3</td>
<td>1.12 ± 0.07</td>
</tr>
<tr>
<td>Amantadine + bicarbonate</td>
<td>21.3 ± 1.4</td>
<td>6.4 ± 0.4</td>
<td>3.3 ± 0.5</td>
<td>3.3 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>2.5 ± 0.6</td>
<td>0.79 ± 0.05b</td>
<td></td>
</tr>
<tr>
<td>Amantadine metabolite excretion</td>
<td>Amantadine + saline</td>
<td>13.3 ± 0.7</td>
<td>13.2 ± 0.5</td>
<td>11.9 ± 0.8</td>
<td>9.9 ± 0.3</td>
<td>8.7 ± 0.1</td>
<td>7.2 ± 0.5</td>
<td>1.29 ± 0.04</td>
</tr>
<tr>
<td>Amantadine + bicarbonate</td>
<td>13.2 ± 1.0</td>
<td>11.6 ± 1.5</td>
<td>11.4 ± 1.0</td>
<td>10.6 ± 0.7</td>
<td>9.0 ± 0.6</td>
<td>8.8 ± 0.6</td>
<td>1.29 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Kynurenic acid excretion</td>
<td>Kynurenic acid + saline</td>
<td>33.4 ± 3</td>
<td>37.1 ± 2.7</td>
<td>11.4 ± 1.7</td>
<td>4.36 ± 0.65</td>
<td>1.99 ± 0.35</td>
<td>1.16 ± 0.13</td>
<td>3.79 ± 0.17</td>
</tr>
<tr>
<td>Kynurenic acid + bicarbonate</td>
<td>131 ± 11</td>
<td>23.6 ± 5.6</td>
<td>9.62 ± 1.3</td>
<td>3.61 ± 0.56</td>
<td>1.76 ± 0.26</td>
<td>0.97 ± 0.17</td>
<td>3.42 ± 0.24</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. of five separate determinations.

* p < .05 compared with the amantadine + saline-treated group, ANOVA followed by Tukey’s HSD.

* p < .01 compared with the amantadine + saline-treated rats, unpaired t test.

The total amount of metabolite eliminated was calculated assuming that there was a 1:1 conversion ratio for specific activity between the parent amantadine compound and the metabolite.
shown in Table 4. The amantadine pharmacokinetic parameters are provisional and highly variable because the experiment must span two \( t_{1/2} \) periods for a more reliable determination. In the bicarbonate-treated rats, \( V_d \) for amantadine was increased compared with the amantadine plus saline-treated rats (\( p < .05 \)). For kynurenic acid, the kinetics of disposition were similar in control versus bicarbonate-treated rats.

### Discussion

This study addressed the potential in vivo functional importance of a previously identified in vitro bicarbonate-dependent renal tubule amantadine (organic cation) transport mechanism (Escobar et al., 1994; Escobar and Sitar, 1995). The major finding of the present study was that acute bicarbonate administration decreased amantadine renal clearance, most likely by modulation of renal tubule secretion. Our present in vivo observations in rats support a previous report that chronic administration of bicarbonate reduced the renal excretion amantadine in humans (Geuens and Stephens, 1967). Similar effects of bicarbonate loading on decreasing the renal clearance of other organic bases in rats and dogs have also been reported (Torretti et al., 1962; Weiner and Roth, 1980). Our data demonstrated for the first time that a chronic alteration in circulating bicarbonate is not necessary to result in decreased renal clearance of amantadine.

In the present study, the importance of secretion and filtration components of amantadine clearance as a function of an acute exposure to bicarbonate was determined. The relative contribution of secretion of amantadine and kynurenic acid to their overall renal clearance was determined by the amantadine or kynurenic acid/creatinine clearance ratio, respectively. Initially, amantadine and kynurenic acid underwent significant renal tubule secretion as indicated by an amantadine/creatinine and kynurenic acid/creatinine clearance ratio of more than 1. Based on the decrease in the observed amantadine/creatinine clearance ratio in face of a relatively constant creatinine clearance, it appears that bicarbonate dosing is decreasing amantadine clearance through effects on secretion and not filtration. Conversely, kynurenic acid secretion appears to be only transiently decreased during the

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**TABLE 2**

**Urine flow rates**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urine Collection Period</th>
<th>( \mu l/min )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1. Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Amantadine + saline</td>
<td>18 ± 5</td>
<td>39 ± 10</td>
</tr>
<tr>
<td>3. Kynurenic acid + saline</td>
<td>17 ± 4</td>
<td>33 ± 11</td>
</tr>
<tr>
<td>4. Amantadine + bicarbonate</td>
<td>31 ± 9</td>
<td>157 ± 27(^b)</td>
</tr>
<tr>
<td>5. Kynurenic acid + bicarbonate</td>
<td>21 ± 9</td>
<td>128 ± 13(^a)</td>
</tr>
</tbody>
</table>

Data are expressed as mean \( \pm \) S.E.M. of four or five separate determinations.

\(^a\) \( p < .05 \), \(^b\) \( p < .01 \), \(^c\) \( p < .001 \) compared with the nonbicarbonate-treated rats of the same urine collection period, ANOVA followed by Tukey's HSD.

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**Fig. 3.** Correlation between urine flow rate and the amantadine/creatinine or kynurenic acid/creatinine clearance ratio. The figures show the combined clearance versus urine flow rate data for all rats in each group. The solid line represents the least-squares regression for the correlation. a, amantadine plus saline-treated rats. b, amantadine plus bicarbonate-treated rats. c, kynurenic acid plus saline-treated rats. d, kynurenic acid plus bicarbonate-treated rats. b, the amantadine/creatinine clearance ratio before bicarbonate administration (\( \square \)) and after bicarbonate administration (\( \Box \)) are identified to emphasize the change in the amantadine/creatinine clearance ratio after bicarbonate administration. For this group, the regression fit was performed only on data points after the start of bicarbonate infusion.
TABLE 3
Blood HCO₃⁻, pCO₂, and pH and urine pH in rats treated with amantadine plus saline or amantadine plus bicarbonate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Blood Sample</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>1. HCO₃⁻</td>
<td>Control</td>
<td>27.5 ± 0.8</td>
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<tr>
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<td>Treatment</td>
<td>28.4 ± 0.5</td>
</tr>
<tr>
<td>2. pH</td>
<td>Control</td>
<td>7.41 ± 0.01</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>7.41 ± 0.01</td>
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<tr>
<td>3. pCO₂</td>
<td>Control</td>
<td>43.4 ± 2.2</td>
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<td>Treatment</td>
<td>44.6 ± 0.9</td>
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Urine Collection Period

<table>
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<tr>
<th>Parameter</th>
<th>Group</th>
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<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. Urine pH</td>
<td>Control</td>
<td>6.68 ± 0.22</td>
<td>6.73 ± 0.20</td>
<td>6.71 ± 0.13</td>
<td>6.81 ± 0.10</td>
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<td>Treatment</td>
<td>6.45 ± 0.27</td>
<td>8.03 ± 0.08*</td>
<td>8.24 ± 0.03*</td>
<td>8.00 ± 0.11*</td>
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</table>

Data are represented as mean ± S.E.M. of four separate experiments.
Control, amantadine plus saline-treated rats; treatment, amantadine plus bicarbonate-treated rats. HCO₃⁻ is expressed in units of mmol/L, and pCO₂ is expressed in units of mm Hg.

* p < .01 compared with the control group, ANOVA followed by Tukey’s HSD.

Fig. 4. Plasma concentration versus time profiles for amantadine (a) and kynurenic acid (b). Amantadine and kynurenic acid (3 mg/kg) were infused starting at time zero, and the duration of the infusion was 2 min. Plasma concentrations were measured at the beginning and end of each urine collection. The bicarbonate-treated rats (■) received 5 mmol/kg bicarbonate i.v.; at the beginning of the second urine collection (27 min), and the controls (□) received an equivalent volume of saline (0.9% i.v.). Amantadine or kynurenic acid concentrations (µg/ml) are represented as the mean ± S.E.M. of five separate experiments. Error bars are not visible for all points due to small S.E.M.

period of bicarbonate infusion. This observation may reflect temporary changes in renal function during the time of the infusion. With reference to our chosen organic cation and anion substrates, the effect of bicarbonate infusion on renal tubule transport appears to be specific for the organic base as opposed to a general phenomenon affecting both organic acid and base secretion.

In this study, an acute dose of bicarbonate was sufficient to impair the renal clearance of amantadine for an extended period of time. The chosen dose of bicarbonate (5 mmol/kg) for these studies was based on its apparent volume of distribution (0.4–0.5 liters/kg) in dogs and was expected to increase peak plasma bicarbonate levels by 10 mM (Adrogue et al., 1983). The peak blood bicarbonate concentration observed in our experiments, approximately 5 min after the bicarbonate infusion was stopped, was about 8 mM higher than the respective control blood bicarbonate levels and remained elevated thereafter. Because blood bicarbonate levels remain elevated, it is suggestive that the increase in bicarbonate ion concentration may be responsible for decreasing amantadine clearance. We cannot rule out that the increased Na⁺ load in the NaHCO₃-treated rats contributes to the observed alteration in amantadine renal clearance. However, for the following reasons, we believe that increased Na⁺ had little effect on renal clearance of amantadine. In terminal plasma samples, Na⁺ levels were the same in the bicarbonate-treated (147 ± 2 mM) and control (145 ± 1 mM) rats; yet bicarbonate remained elevated and amantadine clearance remained depressed. In addition, in vitro studies have suggested that amantadine transport into isolated renal proximal and distal tubules is independent of Na⁺ concentration in the incubation medium (Escobar and Sitar, 1996).

We believe passive reabsorption is likely to have only minor importance in explaining the decrease in amantadine clearance for the following reasons. Due to the high pKₐ of amantadine (pKₐ, 10.1), only a limited gradient for passive reabsorption of amantadine from the tubule lumen to the peritubular capillaries would be established by the increase in urine pH. Second, with increasing urine flow rates with time in our animals, there would be a predicted increase in net renal drug excretion and increased clearance due to less contact time for passive reabsorption of the drug into the peritubular capillaries. This effect was not apparent, because a large increase in urine flow rate in the bicarbonate-treated rats was not correlated with a substantial increase in the amantadine/creatinine clearance ratio. Furthermore, drug
clearance ratio (Clr/Clp) for amantadine (0.65 in controls and 0.95 in amantadine in these species. The median renal/plasma Sitar et al., 1997), indicating similar renal secretory capacity reported in humans and in dogs (Tilles, 1974; Aoki et al., 1979; Holohan and Ross, 1980). These organic cation transporters may mediate the passage of organic cations from the tubule cell into the tubule lumen (Kinsella et al., 1979; Takano et al., 1984; Rafizadeh et al., 1987). Therefore, the exact identity of the metabolite is not critical for the understanding of the present findings.

The finding that the bicarbonate-dependent increase in net renal secretion of amantadine remains elusive at this time. We may speculate that to satisfy in vitro data of increased amantadine renal tubule accumulation in the presence of bicarbonate and in vivo data of decreased secretion, bicarbonate may be able to decrease the luminal efflux of amantadine in addition to its stimulatory effect on amantadine uptake at basolateral membrane. Renal tubule luminal organic cation transporters may mediate the passage of organic cations from the tubule cell into the tubule lumen (Kinsella et al., 1979; Holohan and Ross, 1980). These organic cation transporters may represent sites for the observed bicarbonate effect. Evidence for bicarbonate modulation of luminal proximal tubule organic cation transporters as opposed to increased nonionic diffusion has already been demonstrated for the organic base procainamide (McKinney, 1984). However, aside from the McKinney report, bicarbonate modulation on luminal membrane organic cation transporters has not been studied. Alternatively, evidence exists that indicates secretion of some organic cations across the brush border membrane of proximal tubules is coupled to an inwardly directed proton gradient that is driven by the Na+/H+ exchanger located in the brush border membrane (Holohan and Ross, 1981; Takano et al., 1984; Rafizadeh et al., 1987). Therefore, it is possible that the alkalization of the tubule fluid that occurs after bicarbonate administration may cause a decrease in the driving force for H+/organic cation exchange across the brush border membrane of proximal tubules and thus a decrease in amantadine clearance.

Certain organic cationic drugs, such as aminoglycoside antibiotics, are highly toxic to the kidney (Bennett, 1989). The finding that the bicarbonate-dependent increase in amantadine uptake in vitro is not linked to increased renal
disposition studies in humans showed amantadine renal clearance was not dependent on urine pH (Aoki et al., 1979). The slight increase in blood pH (<0.1) resulting from the bicarbonate infusion will not significantly change the degree of ionization of amantadine in the plasma; thus, there should be little effect of pH changes on whole body distribution of amantadine.

While studying bicarbonate effects on amantadine and kynurenic acid disposition, we were able to determine pharmacokinetic data for amantadine and kynurenic acid in the rat. These parameters have not been published previously. The plasma concentration data do not reflect the dramatic effect of bicarbonate on renal clearance of amantadine, as do the more robust measurements of interval renal clearance. With the exception of amantadine Vdss (greater in bicarbonate-treated rats than in controls), the large variability in the amantadine plasma pharmacokinetic disposition parameters in the presence of bicarbonate precluded our ability to definitively interpret other pharmacokinetic comparisons between the two groups. It is apparent from these data that a longer sampling time (two $\beta_{1/2}$ intervals) for amantadine plasma concentrations is necessary to verify our initially determined kinetic parameters. Although there are species differences in amantadine metabolism and elimination (Bleidner et al., 1965), the distribution characteristics for amantadine in the control rats (Vdss = 6.35 ± 0.66 liters/kg) is similar to that reported in adult male humans (Vdss = 6.59 ± 1.49 liters/kg) after i.v. amantadine infusion (Aoki and Sitar, 1988). Our reported control amantadine/creatinine clearance ratio is also similar to amantadine/creatinine ratios previously reported in humans and in dogs (Tilles, 1974; Aoki et al., 1979; Sitar et al., 1997), indicating similar renal secretory capacity for amantadine in these species. The median renal/plasma clearance ratio (Clr/Clp) for amantadine (0.65 in controls and 0.78 in the bicarbonate-treated rats) is consistent with routes of amantadine elimination in addition to renal excretion. The observed Clr/Clp ratio for kynurenic acid was about 0.75. This may indicate that kynurenic acid is being metabolized to a small extent, but the presence of any metabolite was undetectable by TLC.

In humans, a variety of amantadine metabolites have been identified by mass spectrometry, with the predominant metabolite of amantadine being N-acetylamantadine (Köppel and Tenczer, 1985). Hypothesizing that the metabolite profile for amantadine in rat is similar to that in humans, we performed gas chromatography analysis for acetylamantadine according to previously published methods (Bras et al., 1998). We were unable to detect acetylamantadine in our rat urine samples and thus can exclude acetylamantadine as the major metabolite of amantadine formed in our experiments. The fact that the total excretion of amantadine but not the metabolite changes in our experiments suggests that 1) amantadine and the metabolite do not interact at an identical point in the renal excretion pathway, and 2) it is likely that bicarbonate is reducing amantadine clearance solely by modulating renal tubule transport. Because it is likely that amantadine and the metabolite do not interact at a common tubule secretory pathway, the exact identity of the metabolite is not critical for the understanding of the present findings.

The exact mechanism of bicarbonate reduction in net renal secretion of amantadine remains elusive at this time. We may speculate that to satisfy in vitro data of increased amantadine renal tubule accumulation in the presence of bicarbonate and in vivo data of decreased secretion, bicarbonate may be able to decrease the luminal efflux of amantadine in addition to its stimulatory effect on amantadine uptake at basolateral membrane. Renal tubule luminal organic cation transporters may mediate the passage of organic cations from the tubule cell into the tubule lumen (Kinsella et al., 1979; Holohan and Ross, 1980). These organic cation transporters may represent sites for the observed bicarbonate effect. Evidence for bicarbonate modulation of luminal proximal tubule organic cation transporters as opposed to increased nonionic diffusion has already been demonstrated for the organic base procainamide (McKinney, 1984). However, aside from the McKinney report, bicarbonate modulation on luminal membrane organic cation transporters has not been studied. Alternatively, evidence exists that indicates secretion of some organic cations across the brush border membrane of proximal tubules is coupled to an inwardly directed proton gradient that is driven by the Na+/H+ exchanger located in the brush border membrane (Holohan and Ross, 1981; Takano et al., 1984; Rafizadeh et al., 1987). Therefore, it is possible that the alkalization of the tubule fluid that occurs after bicarbonate administration may cause a decrease in the driving force for H+/organic cation exchange across the brush border membrane of proximal tubules and thus a decrease in amantadine clearance.

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### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC (mg/min/L)</th>
<th>$\sigma_{\alpha}$ (min)</th>
<th>$R_{\alpha}$ (min)</th>
<th>Vdss (L/kg)</th>
<th>Clr (ml/min/100 g)</th>
<th>Clr/Clpa</th>
<th>Clr (ml/min/100 g)b</th>
<th>Clr/Clpb</th>
</tr>
</thead>
<tbody>
<tr>
<td>.+ Saline</td>
<td>253 ± 144</td>
<td>19.7 ± 2.1</td>
<td>518 ± 307</td>
<td>6.35 ± 0.66</td>
<td>1.9 ± 0.4</td>
<td>0.95</td>
<td>1.16 ± 0.04</td>
<td>0.95</td>
</tr>
<tr>
<td>.+ Bicarbonate</td>
<td>1279 ± 938</td>
<td>23.8 ± 1.9</td>
<td>3480 ± 2840</td>
<td>9.0 ± 1.0</td>
<td>1.0 ± 0.3</td>
<td>1.0</td>
<td>0.76 ± 0.04**</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>.+ Saline</td>
<td>150 ± 7</td>
<td>8.55 ± 0.42</td>
<td>0.300 ± 0.006</td>
<td>1.53 ± 0.05</td>
<td>1.11 ± 0.05</td>
<td>0.70</td>
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<tr>
<td>.+ Bicarbonate</td>
<td>189 ± 19</td>
<td>8.01 ± 0.50</td>
<td>0.354 ± 0.060</td>
<td>1.66 ± 0.20</td>
<td>1.19 ± 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. of four or five separate determinations. Median values for amantadine Clr/Clp are shown in parentheses.

*Model fit was only possible for four of five animals for this group.

a Clr, mean renal clearance over the duration of the experiment.

bt $p < .05$, ** $p < .01$ compared with amantadine plus saline-treated rats, unpaired t test.

All pharmacokinetic parameters for the two kynurenic acid-treated groups were similar, unpaired t test.
excretion of amantadine in vivo raises the issue of pharmacological consequences of increased renal or serum accumulation of amantadine or potential nephrotoxic organic cations such as aminoglycosides. Our data suggest that acute changes in acid/base status that result in increased plasma bicarbonate levels may compromise renal elimination of amantadine and possibly other organic cation drugs that are specifically handled by bicarbonate-dependent organic cation transporters in the kidney.

The exact mechanism of the bicarbonate-mediated decrease in amantadine clearance was not determined. However, the present study, coupled with our previous in vitro demonstration of bicarbonate-dependent organic cation transport, further suggests that bicarbonate modulation of certain renal tubule organic cation transporters is a complex process and contributes to impaired secretion as a mechanism by which bicarbonate dosing decreases amantadine renal clearance.

Acknowledgments
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References