PHARMACOKINETICS AND PHARMACODYNAMICS OF INTRAVENOUS AZOSEMIDE IN MUTANT NAGASE ANALBUMINEMIC RATS

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ABSTRACT:

This paper reports 1) the increase in expression of CYP1A2 in mutant Nagase analbuminemic rats (NARs), 2) the role of globulin binding of azosemide in circulating blood in its urinary excretion and hence its diuretic effects in NARs, and 3) the significantly faster renal (CLR) and nonrenal (CLNR) clearances of azosemide in NARs. Azosemide (mainly metabolized via CYP1A2 in rats), 10 mg/kg, was intravenously administered to control rats and NARs. Northern and Western blot analyses revealed that the expression of CYP1A2 increased ~3.5-fold in NARs as compared with control. The plasma protein binding of azosemide in control rats and NARs was 97.9 and 84.6%, respectively. In NARs, plasma protein binding (84.6%) was due to binding to α- (82.6%) and β- (68.9%) globulins. In NARs, the amount of unchanged azosemide excreted in 8-h urine was significantly greater (37.7 versus 21.0% of intravenous dose) than that in control rats due to an increase in intrinsic renal active secretion of azosemide. Accordingly, the 8-h urine output was significantly greater in NARs. The area under the plasma concentration-time curve of azosemide was significantly smaller (505 versus 2790 µg · min/ml) in NARs because of markedly faster CLR (7.36 versus 0.772 ml/min/kg, secondary to a significant increase in urinary excretion of azosemide and intrinsic renal active secretion). Additionally, CLNR was significantly faster (12.4 versus 3.05 µl/min/kg, because of ~3.5 fold increase in CYP1A2) in NARs compared with control. Based on in vitro hepatic microsomal studies, the intrinsic M1 [a metabolite of azosemide; 5-(2-amino-4-chloro-5-sulfamoylphenyl)-tetrazole] formation clearance was significantly faster (67.0% increase) in NARs than that in control rats, and this supports significantly faster CLNR in NARs. Renal sensitivity to azosemide was significantly greater in NARs than in control rats with respect to 8-h urine output (385 versus 221 ml/kg) and 8-h urinary excretions of sodium, potassium, and chloride. This study supports that in NARs, binding of azosemide to α- and β-globulins in circulating blood play an important role in its diuretic effects.

Azosemide1 [5-(4-chloro-5-sulfamoyl-2-thenylaminophenyl)-tetrazole] is a sulfonamide loop diuretic closely resembling furosemide in its diuretic action (Krück et al., 1978). Its main sites of action are both the cortical and medullary segments of the thick ascending limb of loop of Henle (Brater, 1979) where it inhibits water and solute reabsorption. It is used clinically in the treatment of edematous states and arterial hypertension; specific indications are cardiac and renal edema and ascites (Michel, 1992). Eleven metabolites of azosemide were found in rat urine and bile (Asano et al., 1984), but the diuretic effect of the drug does not require metabolism to an active metabolite (Greven, 1991).

After i.v. administration of furosemide to mutant Nagase analbuminemic rats (NARs), an animal model for human familial analbuminemia, the diuretic effects of the drug (urine output and urinary excretion of sodium) were essentially negligible, whereas the effects were rapid and marked in control Sprague-Dawley rats (Inoue et al., 1987). This was explained by the lower plasma protein binding of the diuretic in NARs (the plasma protein binding of furosemide was only 12% compared with 98.9% in control rats) and the reduced amount of unchanged furosemide excreted in the urine (7 versus 26% of a i.v. dose in 30 min) (Inoue et al., 1987). However, injected furosemide bound to albumin markedly promoted diuresis in NARs (the total amount of unchanged furosemide excreted in 30-min urine being 18% of an i.v. dose of the drug) and rapidly increased the urine volume in hypoalbuminemic patients who were resistant to furosemide (Inoue et al., 1987). This suggests that binding of furosemide to circulating plasma albumin is important for the delivery of furosemide to the thick ascending limb of loop of Henle, the site for its action, and hence its diuretic effects.
The importance of plasma protein binding of phenolsulfonphthalein (Inoue et al., 1985) and mercapturic acid (Okajima et al., 1985) for their urinary excretion has also been reported. The serum protein binding of phenolsulfonphthalein in control rats and NARs was 84.2 to 94.4 and 12.5 to 32.0%, respectively, and the percentage of an i.v. dose excreted in 6-h urine as unchanged phenolsulfonphthalein was 49.2 and 22.4% for control rats and NARs, respectively (Inoue et al., 1985). Plasma protein binding of mercapturic acid in control rats and NARs was 80.1 and 18.4%, respectively, and the percentage of an i.v. dose excreted in 20-min urine as unchanged mercapturic acid was 57.6 and 25.6% for control rats and NARs, respectively (Okajima et al., 1985).

The pharmacological action of azosemide closely resembles that of furosemide (Krick et al., 1978) and, therefore, a similar binding dependence in its diuretic effects might be expected. Accordingly, renal effects of azosemide in NARs were compared with those in control rats.

### Materials and Methods

**Chemicals.** Azosemide and one of its metabolites, M1 [5-(2-amino-4-chloro-5-sulfamoylphenyl)-tetrazole], were donated by Sam Jin Pharmaceuticals Company (Seoul, South Korea) and Roche Diagnostics (Mannheim, Germany), respectively. [α-32P]dCTP (3000 mCi/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). Random prime-labeling kit was purchased from Promega (Madison, WI). Mouse polynuclear anti-rat CYP1A2, CYP2E1, and CYP3A23 antibodies were obtained from Detroit R&D Inc. (Detroit, MI). Biotinylated donkey anti-goat IgG, biotinylated goat anti-rabbit IgG, alkaline phosphatase-conjugated goat anti-mouse IgG and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD) and Bio-Rad (Hercules, CA). Rat globulins, G5140 (predominantly α-globulin), G4890 (57.5% β-globulin and 20.2% γ-globulin) and G2885 (98% γ-globulin), were supplied by Sigma-Aldrich (St. Louis, MO). Other chemicals were of reagent grade or high-performance liquid chromatographic (HPLC) grade and, therefore, were used without further purification.

**Rats.** Male Sprague-Dawley rats (control rats, weighing 310–345 g) and NARs (weighing 220–315 g) of 9 weeks of age were purchased from Japan SLC Inc. (Hamamatsu, Japan). All rats were maintained in a clean room (Animal Center for Pharmaceutical Research, College of Pharmacy, Seoul National University, Seoul, South Korea) at a temperature between 20 and 23°C with 12-h light and dark cycles and a relative humidity of 50%. Rats were individually housed in metabolic cages (Tecniplast, Varese, Italy) under a supply of filtered pathogen-free air, with food (Samyang Company, Seoul, South Korea) and water ad libitum. The protocol of animal study was approved by Animal Care and Use Committee of College of Pharmacy, Seoul National University.

**Preliminary Study.** A 0- to 24-h baseline urine collection (without treatment with azosemide) from control rats (n = 5) and NARs (n = 5) was obtained. After measuring the pH (Microprocessor pH/C Meter; Eutech Cybertronics, Singapore, Singapore) and volume, an aliquot was stored at −70°C for subsequent measurements of sodium, potassium, chloride, and creatinine. At the same time, as much blood as possible was collected via the abdominal artery, and the hematocrit was measured (Readacart Centrifuge; Clay Adams, Parsippany, NJ). Serum and hepatic protein and EDTA-treated plasma were also stored at −70°C for subsequent analysis; serum for total proteins, aspartate transaminase (AST), alanine transaminase (ALT), urea nitrogen and creatinine, EDTA-treated plasma for aldosterone and antiuretic hormone (ADH), and hepatic protein for plasma binding using an equilibrium dialysis technique (Lee and Lee, 1995). After sacrificing each rat by cervical dislocation, the abdomen was opened, and the liver was removed, rinsed with cold 0.9% NaCl-injectable solution, and blotted dry with tissue paper. Hepatic microsomal fractions of control rats and NARs were prepared by differential centrifugation, washed in pyrophosphate buffer (pH 7.4), and stored in 50 mM Tris-acetate buffer (pH 7.4) containing 1 mM sodium EDTA and 20% glycerol. Microsomal preparations were stored in a −70°C freezer until use. Protein (Lowry et al., 1951) and hepatic microsomal cytochrome P450 (P450) contents (Omura and Sato, 1964) were determined according to the reported methods.

**Immunoblot Analysis.** SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to the published procedures (Kim and Cho, 1996; Kim et al., 1996). Microsomal proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose paper. The nitrocellulose paper was incubated with each polyclonal mouse anti-rat CYP1A2, CYP2E1, or CYP3A23 antibody, followed by incubation with biotinylated secondary antibody, and then developed using 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium (Kim et al., 1996).

**Preparation of cDNA Probes for Cytochrome P450s.** Specific cDNA probes for CYP1A2, CYP2E1, CYP3A23, and CYP3A23 genes were amplified by reverse transcriptase-polymerase chain reaction using the selective primers for each gene (Zhang et al., 1996) and cloned in the pGEM+T vector (Promega). The mRNA prepared from rat liver was reverse-transcribed and amplified by polymerase chain reaction using the forward primer (5′-CAGGG-TAGCTGAGATGGTT-3′) and the reverse primer (5′-TGTTGGAAAGACGGCCTGATGT-3′). The polymerase chain reaction product was cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA), and the sequence was confirmed by DNA sequencing (probe size = 343 base pairs).

**Northern Blot Hybridization.** Total RNA was isolated using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction according to a reported method (Puissant and Houdébine, 1990). Northern blot analysis was carried out as described previously (Kim et al., 1996). Briefly, total RNA isolated from rat livers was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nitrocellulose paper. The nitrocellulose paper was baked in a vacuum oven at 80°C for 2 h. The blot was incubated with hybridization buffer containing 50% deionized formaldehyde, 5 × Denhardt’s solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin (Pentex Fraction V)), 0.1% SDS, 200 μg/ml of sonicated salmon sperm DNA, and 5 × SSC (1 × SSC: 0.15 M NaCl, 10 mM NaH2PO4 and 1 mM sodium EDTA, pH 7.4) at 42°C for 1 h without probe. Hybridization was performed at 42°C for 18 h with a heat-denatured cDNA probe, which was random-prime-labeled with [α-32P]dCTP. Filters were washed twice in 2 × standard saline citrate and 0.1% SDS for 10 min at room temperature and once in 0.1 × standard saline citrate and 0.1% SDS for 60 min at 60°C. After quantitation of mRNA levels, the membranes were stripped and rehybridized with a labeled-specific DNA probe complementary to 18S rRNA to quantify the amount of RNA loaded onto the membranes.

**Scanning Densitometry.** Scanning densitometry was performed with a Image Scan and Analysis System (α-Inotech Corporation, San Leandro, CA).

**Protein Binding Studies.** The ability of azosemide to plasma protein of control rats and NARs was determined using an equilibrium dialysis technique (Lee and Lee, 1995). One milliliter of plasma was dialyzed against 1 ml of isotonic Sorense phosphate buffer (pH 7.4) containing 3.0% dextran (“the buffer”) to minimize volume shift, using a 1 ml dialysis cell (Fisher Scientific Co., Fair Lawn, NJ) and a Spectral/Por 4 membrane (molecular weight cutoff of 12,000–14,000; Spectrum Medical Industries Inc., Los Angeles, CA). Azosemide was added to the plasma side (Guentert and Oie, 1982) at a concentration of 10 μg/ml. The dialysis cell was incubated for 24 h in a water-bath shaker kept at 37°C and at a rate of 50 oscillations per min. Similar experiment was also performed with rat globulins at an azosemide concentration of 1 μg/ml: 3.1% α-globulin, 1.8% β-globulin plus 0.63% γ-globulin, 0.63% γ-globulin, 1.3% γ-globulin, and 3.1% α-, 1.8% β- plus 1.3% γ-globulin (each rat globulin, dissolved in isotonic Sorense phosphate buffer of pH 7.4 containing 0.5–2% dextran, was placed in the dialysis cell instead of plasma). Since globulin G4890 contained β-globulin with 0.63% γ-globulin as an impurity, the protein binding of azosemide to 0.63% γ-globulin was measured (n = 3, each). The binding of azosemide to 42 mg% human serum albumin was also measured.

**Measurement of Vmax, Km, and CLint for the Formation of M1 from Azosemide in Hepatic Microsomal Fractions.** The livers of control rats (n = 5) and NARs (n = 5) were homogenized in an ice-cold buffer of 0.154 M KCl/50 mM Tris-HCl in 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 9700g for 30 min, and the supernatant fraction was further centrifuged at 27,000g for 90 min. The microsomal pellet was resuspended in the buffer of...
centrifuged immediately, and a 50-cannula immediately after each blood sampling. The blood samples were collected via the carotid artery at 0, 1 (at the end of the infusion), 5, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min after i.v. administration. The content and composition of fluid replacement have been reported to be dependent on the rate and composition of fluid replacement (Brater et al., 1983) in rats (Greven and Heidenreich, 1981) as well as urinary excretion rate of sodium (Brater et al., 1983) in humans (Brater et al., 1983) appear to be sigmoidal. The measured values of each clearance (Chiou, 1980, 1980, Chiou, 1979) and terminal half-life (Eatman et al., 1977) were estimated by the harmonic mean method.

The glomerular filtration rate was estimated by measuring creatinine clearance (Cl_{GFR}) assuming that kidney function was stable during the experimental period. Cl_{GFR} was estimated by dividing the total amount of unchanged creatinine excreted in 8-h urine by AUC_{0-8} of creatinine in plasma.

**Pharmacodynamic Analysis.** The relationships between log urinary excretion rate of azosemide and urine flow rate (Greven and Heidenreich, 1981) as well as urinary excretion rate of sodium (Brater et al., 1983) in rats (Greven and Heidenreich, 1981) and in humans (Brater et al., 1983) appear to be sigmoidal. The E_{max} and EC_{50} were calculated with the WinNonlin program (Scientific Consulting Inc., Cary, NC) using the following Hill equation:

\[
E = E_{max} \times C/(C + EC_{50})
\]

where \(E_{max}, C,\) and \(EC_{50}\) represent the intensity of diuretic effect (urine flow rate or urinary excretion rate of sodium, potassium, or chloride), maximal diuretic effects, urinary excretion rate of azosemide, and urinary excretion rate of azosemide required to produce 50% maximal diuretic effects, respectively.

**Statistical Analysis.** For the molecular biology study, one-way analysis of variance was used to assess significant effect of the treatment, the Newman-Keuls test for comparison of multiple group means, and the Student’s t test to determine whether two population means differed significantly. For the pharmacokinetic/pharmacodynamic studies, the t test between the two means for unpaired data were employed. A p value of less than 0.05 was considered to be statistically significant. All results are expressed as mean ± S.D.

**Results**

**Preliminary Study.** The hematocrit, serum levels of total proteins, AST, ALT, urea nitrogen and creatinine, plasma level of aldosterone, urine pH, 24-h urine output, 24-h urinary excretions of sodium, potassium, and chloride and Cl_{GFR} were not significantly different between control rats and NARs (Table 1). This data indicated that kidney and liver functions in NARs were essentially the same as those in control rats. Similar results have also been reported previously (Nagase et al., 1979). However, in NARs, kidney size based on %

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Rats (n = 5)</th>
<th>NARs (n = 5)</th>
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<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
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<tr>
<td>Hematocrit (%)</td>
<td>46.0 ± 1.00</td>
<td>45.5 ± 0.70</td>
</tr>
<tr>
<td>Serum</td>
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<tr>
<td>Total proteins (g/dl)</td>
<td>5.86 ± 0.313</td>
<td>5.85 ± 0.212</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>126 ± 17.3</td>
<td>114 ± 14.8</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>42.6 ± 4.98</td>
<td>39.8 ± 6.13</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>20.2 ± 1.74</td>
<td>18.7 ± 1.68</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.571 ± 0.00488</td>
<td>0.557 ± 0.00535</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
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<tr>
<td>Plasma (heparin-treated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein binding (%)</td>
<td>97.9 ± 2.10</td>
<td>84.6 ± 0.768a</td>
</tr>
<tr>
<td>Plasma (EDTA-treated)</td>
<td></td>
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<tr>
<td>Aldosterone (ng/dl)</td>
<td>34.1 ± 19.0</td>
<td>47.4 ± 18.5</td>
</tr>
<tr>
<td>ADH (pg/ml)</td>
<td>15.5 ± 8.40</td>
<td>29.8 ± 1.37b</td>
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<tr>
<td>pH</td>
<td>8.61 ± 0.265</td>
<td>8.43 ± 0.310</td>
</tr>
<tr>
<td>Urine output (ml/kg)</td>
<td>62.2 ± 13.5</td>
<td>71.3 ± 16.1</td>
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<tr>
<td>Na+ excretion (mmol/kg)</td>
<td>5.04 ± 1.16</td>
<td>5.00 ± 1.71</td>
</tr>
<tr>
<td>Cl- excretion (mmol/kg)</td>
<td>12.6 ± 3.14</td>
<td>12.9 ± 3.51</td>
</tr>
<tr>
<td>K+ excretion (mmol/kg)</td>
<td>4.36 ± 2.00</td>
<td>5.22 ± 1.63</td>
</tr>
<tr>
<td>Cl_{GFR} (ml/min/kg)</td>
<td>4.35 ± 0.455</td>
<td>4.38 ± 0.391</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>13.7 ± 0.402</td>
<td>15.1 ± 0.297a</td>
</tr>
<tr>
<td>Kidney weight (% body weight)</td>
<td>0.647 ± 0.0366</td>
<td>0.604 ± 0.0357b</td>
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</table>

\* p < 0.001, b p < 0.01, a p < 0.05.
body weight was significantly smaller (6.65% decrease), and liver was significantly larger (10.2% increase) than the values in control rats (Table 1). Also, plasma protein binding of azosemide was significantly smaller (13.6% decrease), and plasma ADH level was significantly higher (92.3% increase) than those in control rats (Table 1). The mean plasma ADH level in male Sprague-Dawley rats of 8 weeks of age has been reported to be 1.4 ± 0.3 pg/ml (Terashima et al., 1998), high ADH levels in both strains are due to acute hemodynamic effects of anesthesia and hemorrhage.

**Albumin mRNA Expression.** The plasma albumin levels in control rats and NARS were reported to be 3.84 and 0.0042%, respectively (Nagase et al., 1980). Northern blot analysis confirmed that the expression of albumin mRNA was blocked in livers of NARS, whereas the mRNA level was highly expressed in livers of control rats (Fig. 1). Equal loading of the mRNA in each lane was confirmed by hybridization of the blots with a radio-labeled probe for 18S rRNA (Fig. 1). This result is in good agreement with the low albumin concentration in plasma of NARS (Nagase et al., 1980).

**Cytochrome P450 Expression.** Since changes in P450 isoforms in NARS have not been previously reported, the levels of representative P450 isoforms were measured by Western blot analysis with the hepatic microsomes prepared from control rats and NARS (Fig. 2A). The expression of CYP1A2 was increased approximately 3.5-fold in NARS as compared with control (Fig. 2A). In contrast, the expression of CYP2E1 and CYP3A23 was not changed in NARS (Fig. 2A). The mRNA levels for the cytochrome P450s were also determined by Northern blot analysis in the livers of control rats and NARS (Fig. 2B). The mRNA level of CYP1A2 was 3.5- to 4-fold increased in NARS, whereas those of CYP2E1, CYP3A2, and CYP3A23 were not altered (Fig. 2B). These results (Fig. 2B) are in good agreement with those of Western blot analysis (Fig. 2A).

**Binding of Azosemide to Rat Globulins Using an Equilibrium Dialysis Technique.** Plasma levels of albumin, and α-, β- and γ-globulins in NARS were found to be approximately 0.0042, 3.1, 1.8, and 1.3%, respectively (Nagase et al., 1980). The extent of azosemide binding to plasma of NARS was 84.6% (Table 1). However, the value to 0.0042% human serum albumin was only 10.2%. Hence, the difference, approximately 74%, could be due to binding of azosemide to other plasma proteins in NARS. The binding values of azosemide to rat globulins at azosemide concentration of 1 μg/ml were 82.6, 68.9, 6.85, 16.9, and 83.1% for 3.1% α-globulin, 1.8% β- plus 0.63% γ-globulin, 0.63% γ-globulin, 1.3% γ-globulin, and 3.1% α-, 1.8% β- plus 1.3% γ-globulin, respectively. The value of 83.1% (at the same plasma levels of α- β- plus γ-globulins in NARS) was very close to 84.6% in plasma of NARS (Table 1). This data indicated that azosemide was considerably bound to α- and β-globulins in plasma of NARS. The mean plasma concentration of azosemide was close to 1 μg/ml at 60 min after i.v. administration of the drug to NARS (Fig. 3). Hence, azosemide concentration of 1 μg/ml was arbitrarily chosen in globulin binding studies.

**Measurement of V_{max}, K_m, and CL_{int} for the Formation of M1 from Azosemide in Hepatic Microsomal Fraction.** In NARS, V_{max} for the formation of M1 from azosemide in hepatic microsomal fraction was significantly faster (64.6% increase) than that in control rats (Table 2). However, K_m values were not significantly different between two groups of rats suggesting that the affinity of azosemide to the enzymes was not changed in NARS. Therefore, CL_{int} was significantly faster (67.0% increase) in NARS (Table 2) suggesting that formation of M1 would be faster in NARS.

**Intravenous Study.** After i.v. administration of azosemide, the plasma levels of the diuretic declined in a multieponential fashion for both groups of rats with lower levels in NARS (Fig. 3). NARS showed a smaller AUC (81.9% decrease), shorter terminal half-life (50.9% decrease) and MRT (64.1% decrease), faster CL (454% increase), CL_{int} (853% increase) and CL_{NR} (307% increase), greater amount (60.4% increase) and percentage (79.5% increase, expressed in terms of i.v. dose of azosemide) of unchanged azosemide excreted in 8-h urine (A_{0–8 h}), greater percentage of M1 recovered from gastrointestinal tract at 8 h (G_{M1, 0–8 h}, 144% increase, expressed in terms of the dose of azosemide) and larger V_{	ext{ss}} (104% increase) than those in control rats (Table 3). These differences were all statistically significant.

In NARS, the 8-h urine output (74.2% increase) and 8-h urinary excretions of sodium (A_{Na, 0–8 h}, 93.9% increase), potassium (A_{K, 0–8 h}, 35.0% increase) and chloride (A_{Cl, 0–8 h}, 79.5% increase) were signifi-
Fig. 3. Arterial plasma concentration-time profiles of azosemide after 1-min i.v. infusion of the drug, 10 mg/kg, to control rats (n = 10, ●) and mutant Nagase analbuminemic rats (n = 9, ○).

All data are mean ± S.D.

**TABLE 2**

Mean (± S.D.) $V_{max}$, $K_m$, and $CL_{int}$ for the formation of M1 from azosemide in hepatic microsomes of control rats and NARs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Rats (n = 5)</th>
<th>NARs (n = 5)</th>
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<tbody>
<tr>
<td>$V_{max}$ (pmol/mg/min)</td>
<td>288 ± 89.9</td>
<td>474 ± 71.1 $^a$</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>107 ± 33.5</td>
<td>105 ± 13.6</td>
</tr>
<tr>
<td>$CL_{int}$ (10$^{-3}$ ml/min)</td>
<td>2.70 ± 0.185</td>
<td>4.51 ± 0.378 $^b$</td>
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$^a$ p < 0.05, $^b$ p < 0.001.

significantly greater than those in control rats (Table 3). In NARs, the timed-interval urine flow rate as well as urinary excretion rates of sodium, potassium, and chloride were significantly faster for up to 120-min urine collection than those in control rats (Fig. 4). In the relationship between log urinary excretion rate of azosemide and urine flow rate as well as urinary excretion rates of sodium and chloride, the renal sensitivity of the kidney to azosemide was significantly greater in NARs than that in control rats (Fig. 5). In NARs, the $E_{max}$ values of urinary excretion rates of sodium (48.4% increase) and chloride (35.8% increase) were significantly greater, whereas EC50 values of urine flow rate (64.9% decrease) and urinary excretion rates of sodium (49.4% decrease), potassium (47.9% decrease), and chloride (66.8% decrease) were significantly smaller than those in control rats based on Fig. 5 (Table 4).

**Discussion**

The extent of plasma protein binding of azosemide in NARs (84.6%) was significantly smaller than that in control rats (97.9%) (Table 1). This is a much smaller difference than with furosemide (98.9 versus 12%) (Inoue et al., 1987), and probably reflects the extensive binding of azosemide to α- and β-globulins whereas binding to albumin is more important for furosemide. Like azosemide, the binding of bumetanide (approximately 30–40%) to rat α- and β-globulins (Kim and Lee, 2001) and cisplatin (approximately 50%) to 1% rat γ-globulin (Takada et al., 1999) has also been reported.

After i.v. administration of azosemide to NARs, the AUC was significantly smaller due to significantly faster CL than those in control rats (Table 3). In NARs, significantly faster CL was due to significantly faster both $CL_R$ and $CL_{NR}$. Considering the plasma protein binding (Table 1) and $CL_R$ of azosemide (Table 3), the estimated $CL_R$ values for free (unbound in plasma) fraction of azosemide were 34.4 and 47.8 ml/min/kg for control rats and NARs, respectively; these values were considerably greater than the glomerular filtration rates measured by $CL_{CR}$ (4.35 and 4.38 ml/min/kg for control rats and NARs, respectively (Table 1). This indicates that azosemide is actively secreted in both groups of rats. Active renal secretion of azosemide was also found in other rat studies (Lee and Lee, 1996). Considering the $CL_R$ of azosemide (Table 3) and reported kidney blood flow in rats (36.8 ml/min/kg) (Davies and Morris, 1993) and hematocrit (Table 1), the estimated renal extraction ratios ($CL_{RP}$/renal plasma flow) of azosemide were 3.63 and 36.7% for control rats and NARs, respectively. This data indicated that azosemide was only extracted by the kidney in control rats. However, the value in NARs was 10 times greater than that in control rats. Therefore, the faster $CL_R$ of azosemide in NARs could be due to considerable increase in intrinsic renal active secretion.

The $CL_{NR}$ of azosemide was also significantly faster in NARs than in control rats (Table 3). This could be due to 3.5- to 4-fold increase in CYP1A2 in NARs (Fig. 2B) since azosemide was mainly metabolized by CYP1A2 in rats (Lee and Lee, 1997). It has been reported (Kim et al., 1997) that the hepatic first-pass effect of azosemide was approximately 20% in rats, indicating that azosemide is a low hepatic extraction ratio drug. Hence, hepatic clearance of azosemide could be dependent on $CL_{int}$ and unbound fraction in plasma rather than hepatic blood flow (Wilkinson and Shand, 1975). Based on in vitro hepatic microsomal studies, the $CL_{int}$ in NARs was significantly faster than that in control rats (Table 2). This resulted in significantly faster $CL_{NR}$ of azosemide in NARs. The 6.3-fold increase in free (unbound in plasma) fraction of azosemide in NARs could also contribute to the faster $CL_{NR}$ of azosemide in NARs. The faster CL of azosemide in NARs resulted in significantly shorter terminal half-life and MRT than those in control rats (Table 3).
The $V_{ss}$ was significantly larger in NARs (Table 3), which could be due to higher concentrations of tissue globulins in NARs (Renkin et al., 1993) and 6.3-fold increase in free fraction of azosemide in plasma (Table 1). The larger $V_{ss}$ of azosemide due to increased free fraction in plasma has also been reported in Sprague-Dawley rats (Lee and Lee, 1996). The $V_{ss}$ of furosemide was also significantly greater in NARs than that in control rats (Inoue et al., 1987).

The 8-h urine output was significantly larger in NARs than that in control rats (Table 3). This could be due to significantly greater urinary excretion of unchanged azosemide because of considerable increase in intrinsic renal active secretion of azosemide (Table 3, Fig. 4) and greater renal sensitivity of the kidney to azosemide in the rats (Table 4, Fig. 5). However, the effect of the increase (633%) in free (unbound in plasma) fraction of azosemide in NARs did not appear to be considerable. Smith and Benet (1982) found that the ability to excrete furosemide in the urine was independent of plasma protein binding because furosemide is highly bound (i.e., > 96% to plasma protein), and it appears that the majority of furosemide available for excretion in the urine is delivered by active secretion rather than passive filtration (Ponto and Schoenwald, 1990). This could also be applied to bumetanide (Odlind et al., 1983) and azosemide (Lee and Lee, 1996). In NARs, the contribution of passive filtration to total excretion of unchanged azosemide was 9.16%. In NARs, the 8-h urine output and 8-h urinary excretions of sodium and chloride

![Figure 4](image-url)
were also significantly greater than those in control rats (Table 3 and Fig. 4). This could be due to significantly greater renal sensitivity of the kidney to azosemide in NARs (Table 4, Fig. 5). However, the renal sensitivity of the kidney to furosemide (Inoue et al., 1987), torasemide (Kim and Lee, in press), and bumetanide (Kim and Lee, 2001) was identical between two groups of rats. This difference only in azosemide could not be explained by the differences in the rate and composition of fluid replacement. Although fluid replacement was not mentioned in the furosemide study (Inoue et al., 1987), lactated Ringer’s solution was immediately replaced volume-for-volume in the bumetanide (Kim and Lee, 2001), torasemide (Kim and Lee, in press), and the present azosemide studies. However, this could at least partly be explained by the differences in the mechanisms of action among the loop diuretics, furosemide, bumetanide, and azosemide (Brater et al., 1979).

Diuretic effects of azosemide in NARs were greater than those in control rats due to considerable protein binding of azosemide to $\beta_1$- and $\beta_2$-globulins and greater renal sensitivity of the kidney to azosemide. It has been reported (de Sain-van der Velden et al., 1998) that plasma $\alpha_2$-macroglobulin increased significantly in nephrotic patients ($3.13 \pm 0.33, n = 7$ versus $1.64 \pm 0.15 \text{g/l, } n = 4, p = 0.012$). Therefore, it could be possible that the diuretic effects could be expected from i.v. azosemide in the nephrotic patients without using...
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i.v. furosemide-albumin complex. More studies are required in the patients to prove this hypothesis. Although the binding of azosemide to plasma globulins is an important component of its renal excretion and hence diuretic effects of azosemide in NARs, the renal excretion of azosemide in the rats appears to be more complex than that which can be attributed to binding to globulins alone.

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References