PHARMACOKINETICS AND PHARMACODYNAMICS OF INTRAVENOUS AZOSEMIDE IN MUTANT NAGASE ANALBUMINEMIC RATS

EUN J. KIM, AE K. LEE, SO H. KIM, SANG G. KIM, AND MYUNG G. LEE

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, South Korea (E.J.K., A.K.L., S.G.K., M.G.L.); and Department of Pharmacology, College of Dentistry and Research Institute of Oral Science, Kangnung National University, Gangwon-Do, South Korea (S.H.K.)

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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 ml/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.

Azosemide [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 (Iinoue 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) (Iinoue 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 hypalbuminemic patients who were resistant to furosemide (Iinoue 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 32.6%, 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 (Krück et al., 1978) and, therefore, a similar binding independence 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-sulfamoyl)-tetrazole], were donated by Sam Jin Pharmaceuti- cal 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 polyclonal anti-rat CYP1A2, CYP2E1, and CYP3A23 antibodies were obtained from Dako (Glostrup, Denmark) and Bioclinica (Belgium). 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 (Tech Vacre, 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 treat- ment with azosemide) from control rats (n = 5) and NARs (n = 5) was obtained. After measuring the pH (Microprocessor pH/C Meter; Eutech Cybernetics, 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 from the abdominal area, and the hematocrit was measured (Readacarat Centrifuge; Clay Adams, Parsippany, NJ). Serum and heparin-treated 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 heparin-treated plasma for protein 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 immu- noblot 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 electrophotographed 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-indoylphosphate 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-TAGCCTGAGATGTGTT-3′) and the reverse primer (5′-TGTTGAAAA-GACGCGCATGTGTT-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 accord- ing to a reported method (Puissant and Hourdeine, 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% polyvinylpyrroli- dine and 0.1% bovine serum albumin (Pentex Fraction Ⅴ), 0.1% SDS, 200 μg/ml of sonicated salmon sperm DNA, and 5 × SSPE (1 × SSPE: 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 complement- ary to 18S rRNA to quantify the amount of RNA loaded onto the membranes.

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

Protein Binding Study. The ability of azosemide to plasma protein binding of control rats and NARs was determined using an equilibrium dialysis technique (Lee and Lee, 1995). One millilitre of plasma was dialyzed against 1 ml of isotonic Sörensen 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 (Guettner 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 concentra- tion of 1 μg/ml: 3.1% α-globulin, 1.8% β- plus 0.63% γ-globulin, 0.63% γ-globulin, 1.3% γ-globulin, and 3.1% α- plus 1.8% β- plus 1.3% γ-globulin (each rat globulin, dissolved in isotonic Sörensen phosphate buffer of pH 7.4 con- taining 3.0% dextran) was dialyzed 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 4.2% mg% human serum albumin was also measured.

Measurement of Vₘₐₓ, Kₐᵢᵣₑ, and CLᵢᵣₑ 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-injection volume was about 1 ml. Approximately 0.12-ml aliquot of blood clearance (CLint) was calculated by dividing linear regression and the method of least-squares. Intrinsic M1 formation was calculated using the Lineweaver-Burk plot (Lineweaver and Burk, 1934) by ice-cold 8% ZnSO4 and 0.1 ml of saturated Ba(OH)2. The precipitated proteins were removed by centrifugation at 15,000g for 10 min, and the supernatant was stored at -70°C until HPLC analysis of M1 (Lee and Lee, 1994). The kinetic constants (Km and Vmax) for the formation of M1 from azosemide were calculated using the Lineweaver-Burk plot (Lineweaver and Burk, 1934) by linear regression and the method of least-squares. Intrinsic M1 formation clearance (CLi) was calculated by dividing Vmax by Km.

**Intravenous Study.** In the early morning, the carotid artery and the jugular vein of each control rat and NAR were cannulated with polyethylene tubing (Clay Adams) while the animals were under light ether anesthesia. Both cannulas were exteriorized to the dorsal side of the neck and terminated with long silastic tubing (Dow Corning, Midland, MI). Both silastic tubings were inserted into a wire sheath to allow free movement of the rat. The exposed areas were surgically sutured. Each rat was housed individually in a rat metabolic cage (Daejong Scientific Company, Seoul, South Korea) and was allowed for 4 to 5 h to recover from the anesthesia before commencement of the experiment. They were not restrained during the study. Heparinized 0.9% NaCl-injectable solution (20 units/ml), 0.3 ml, was used to flush each cannula to prevent blood from clotting.

Azosemide (15 mg of azosemide powder was dissolved in 1 ml of 0.1 N NaOH solution, filtered through a 0.45-μm filter, and diluted with 0.9% NaCl-injectable solution before use, pH = 10), 10 mg/kg, was infused over 1 min via the jugular vein of control rats (n = 10) and NARs (n = 9). The injection volume was about 1 ml. Approximately 0.12-ml aliquot of blood sample was 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. Heparinized 0.9% NaCl-injectable solution, 0.3 ml, was used to flush the cannula immediately after each blood sampling. The blood samples were centrifuged immediately, and a 50-μl aliquot of each plasma sample was stored at -70°C until HPLC analysis of azosemide (Lee and Lee, 1994). The pharmacokinetic and pharmacodynamic parameters of i.v. azosemide have been reported to be dependent on the rate and composition of fluid replacement (Park et al., 1997). Therefore, the loss of fluid and electrolytes in urine induced by azosemide was immediately replaced volume-for-volume with lactated Ringer’s solution via the jugular vein for up to 8 h after dosing. Urine samples were collected between 0 and 30, 30 and 60, 60 and 90, 90 and 120, and 120 and 480 min. At 480 min, the metabolic cage was rinsed with 15 ml of distilled water, and the rinsings were combined with 120 to 480 min of urine sample. After measuring the exact volume of urine output and combined urine (for 120 to 480 min sample), aliquots of each urine sample were stored at -70°C until HPLC analysis of azosemide and M1 (Lee and Lee, 1994), sodium, potassium, and chloride. At the end of experiment (8 h), the entire gastrointestinal tract (including its contents and feces) was removed, transferred into a beaker containing 50 ml of 0.01 NaOH (to facilitate the extraction of azosemide), and cut into small pieces using scissors. After shaking manually and stirring with a glass rod for 10 min, two 100-μl aliquots of the supernatant were collected from each beaker and stored at -70°C until HPLC analysis of azosemide and M1 (Lee and Lee, 1994).

**Analysis of Sodium, Potassium, Chloride, Creatinine, Total Proteins, AST, ALT, Urea Nitrogen, Aldosterone, and Antidiuretic Hormone.** The sodium, potassium, chloride, and creatinine levels in urine and total proteins, AST, ALT, urea nitrogen, and aldosterone, and antidiuretic hormone plasma levels were measured using a Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan). The aldosterone and ADH levels in plasma were measured using a radioimmunoassay (Green Cross Reference Lab., Seoul, South Korea).

**Pharmacokinetic Analysis.** The following pharmacokinetic parameters were estimated (Gibaldi and Perrier, 1982): area under the plasma concentration-time curve from time 0 to time infinity (AUC), time-averaged total body clearance (CL), area under the first moment of plasma concentration-time curve, mean residence time (MRT), apparent volume of distribution (Vd), half-life (t1/2), terminal half-life (t1/2), clearance (CL), and terminal half-life (t1/2) assuming that kidney function was stable during the experimental period. CLcr was estimated by dividing the total amount of unchanged creatinine excreted in 8-h urine by AUC0-8, 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 humans in bratch (Brater et al., 1983) appear to be sigmoidal. The Emax and EC50 were calculated with the WinNonlin program (Scientific Consulting Inc., Cary, NC) using the following Hill equation:

\[ E = E_{max} \times \frac{C}{C + EC_{50}} \]

where \( E_{max} \), C, and EC50 represent the intensity of diuretic effect (urine flow rate or urine 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 urine excretions of sodium, potassium, and chloride and CLcr 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 %

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**Table 1**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Rats (n = 5)</th>
<th>NARs (n = 5)</th>
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</thead>
<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>
<td></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>12.6 ± 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 ± 0.0488</td>
<td>0.557 ± 0.0535</td>
</tr>
<tr>
<td>Plasma (heparin-treated)</td>
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</tr>
<tr>
<td>Protein binding (%)</td>
<td>97.9 ± 2.10</td>
<td>84.6 ± 0.768</td>
</tr>
<tr>
<td>Plasma (EDTA-treated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>34.1 ± 19.0</td>
<td>47.8 ± 18.5</td>
</tr>
<tr>
<td>ADH (pg/ml)</td>
<td>15.5 ± 8.40</td>
<td>29.8 ± 13.7</td>
</tr>
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**Note:** p < 0.001, bp < 0.05, *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 the probe for 18S rRNA. Arrow indicates the band of CYP3A23.

**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 increased approximately 3.5-fold 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_{E_{azos}}, 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_{e} (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_{E_{Na}^+}}, 93.9% increase), potassium (A_{E_{K}^-}}, 35.0% increase) and chloride (A_{E_{Cl}^-}, 79.5% increase) were signi-

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**Fig. 1.** The mRNA levels of hepatic albumin in control rats and mutant NARs.

Northern blot analysis was performed to determine the mRNA levels for albumin in total RNA fractions (20 µg each). Equal loading of the mRNA was confirmed by rehybridization of the blots with the probe for 18S rRNA.

**Fig. 2.** Hepatic P450 expression in control rats and mutant NARs.

A, immunoblot analyses of CYP1A2, CYP2E1, and CYP3A23 were carried out with rat hepatic microsomal proteins prepared from control rats and NARs. Each lane was loaded with 10 µg of microsomal proteins. B, Northern blot analyses were performed to determine the mRNA levels for CYP1A2, CYP2E1, CYP3A23, and CYP3A2 in the total RNA fractions (20 µg each). Equal loading of the mRNA was confirmed by rehybridization of the blots with the probe for 18S rRNA. Arrow indicates the band of CYP3A23.
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 azosamide and urine flow rate as well as urinary excretion rates of sodium and chloride, the renal sensitivity of the kidney to azosamide was significantly greater in NARs than that in control rats (Fig. 5). In NARs, the $E_{\text{max}}$ values of urinary excretion rates of sodium (48.4% increase) and chloride (35.8% increase) were significantly greater, whereas $EC_{50}$ 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 azosamide 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 azosamide to α- and β-globulins whereas binding to albumin is more important for furosemide. Like azosamide, 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 azosamide 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 $CL_{NR}$. Considerating the plasma protein binding (Table 1) and $CL_{R}$ of azosamide (Table 3), the estimated $CL_{R}$ values for free (unbound in plasma) fraction of azosamide 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 azosamide is actively secreted in both groups of rats. Active renal secretion of azosamide was also found in other rat studies (Lee and Lee, 1996). Considering the $CL_{R}$ of azosamide (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 ($CL_{R}$/renal plasma flow) of azosamide were 3.63 and 36.7% for control rats and NARs, respectively. This data indicated that azosamide was poorly 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 azosamide in NARs could be due to considerable increase in intrinsic renal active secretion.

The $CL_{NR}$ of azosamide 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 azosamide 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 azosamide was approximately 20% in rats, indicating that azosamide is a low hepatic extraction ratio drug. Hence, hepatic clearance of azosamide 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 azosamide in NARs. The 6.3-fold increase in free (unbound in plasma) fraction of azosamide in NARs could also contribute to the faster $CL_{NR}$ of azosamide in NARs. The faster CL of azosamide in NARs resulted in significantly shorter terminal half-life and MRT than those in control rats (Table 3).

### Table 2

**Mean (± S.D.) Vmax, Kmax and CLmax for the formation of M1 from azosamide 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>
</tr>
</thead>
<tbody>
<tr>
<td>Vmax (pmol/mg/min)</td>
<td>288 ± 89.9</td>
<td>474 ± 71.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kmax (μM)</td>
<td>107 ± 33.5</td>
<td>105 ± 13.6</td>
</tr>
<tr>
<td>CLmax (10&lt;sup&gt;-3&lt;/sup&gt; ml/min)</td>
<td>2.70 ± 0.185</td>
<td>4.51 ± 0.378&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05, <sup>b</sup> p < 0.001

### Table 3

**Mean (± S.D.) pharmacokinetic and pharmacodynamic parameters of azosamide after 1-min i.v. infusion of the drug, 10 mg/kg, to control rats and mutant Nagase analbuminemic rats (NARs)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Rats (n = 10)</th>
<th>NARs (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>319 ± 11.7</td>
<td>287 ± 28.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Terminal half-life (min)</td>
<td>76.8 ± 46.0</td>
<td>44.4 ± 8.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC (μg·min/ml)</td>
<td>2790 ± 519</td>
<td>505 ± 158&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>69.6 ± 25.1</td>
<td>25.9 ± 5.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>3.59 ± 0.574</td>
<td>19.8 ± 6.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{NR}$ (ml/min/kg)</td>
<td>0.772 ± 0.233</td>
<td>7.36 ± 2.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{NR}$ (ml/min/kg)</td>
<td>3.05 ± 0.357</td>
<td>12.4 ± 3.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$V_{e}$ (ml/kg)</td>
<td>234 ± 57.9</td>
<td>496 ± 165&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$A_{E_{max}}$ (μg)</td>
<td>675 ± 195</td>
<td>1.080 ± 133&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$A_{E_{max}}$ (% of the dose)</td>
<td>21.0 ± 5.24</td>
<td>37.7 ± 2.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$A_{E_{max}}$ (% of the dose)</td>
<td>72.2 ± 27.0</td>
<td>90.0 ± 10.7</td>
</tr>
<tr>
<td>$A_{E_{max}}$ (% of the dose)</td>
<td>72.2 ± 27.0</td>
<td>90.0 ± 10.7</td>
</tr>
<tr>
<td>$C_{max}$ (mmol/kg)</td>
<td>8.83</td>
<td>11.7</td>
</tr>
<tr>
<td>$A_{C_{max}}$ (mmol/kg)</td>
<td>3.34 ± 1.24</td>
<td>4.51 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed in terms of azosamide. <sup>b</sup> p < 0.1, <sup>c</sup> p < 0.001, <sup>d</sup> p < 0.05.
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

![Fig. 4. Urine flow rate as well as urinary excretion rates of azosemide, sodium, potassium, and chloride as a function of time after 1-min i.v. infusion of azosemide, 10 mg/kg, to control rats (n = 10, •) and mutant Nagase analbuminemic rats (n = 9, ○). All data are mean ± S.D. * p < 0.05; ** p < 0.01; *** p < 0.001.](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 $\alpha$- and $\beta$-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

![Table 4](image)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Rats</th>
<th>NARs</th>
<th>Control Rats</th>
<th>NARs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$</td>
<td>EC$_{50}$</td>
<td>$E_{\text{max}}$</td>
<td>EC$_{50}$</td>
</tr>
<tr>
<td>Urine flow rate</td>
<td>2.42 ± 0.922</td>
<td>3.00 ± 0.736</td>
<td>8.83 ± 6.87</td>
<td>3.45 ± 2.01$^*$</td>
</tr>
<tr>
<td>Urinary excretion rate of sodium</td>
<td>0.283 ± 0.100</td>
<td>0.420 ± 0.106$^*$</td>
<td>8.02 ± 4.28</td>
<td>4.06 ± 1.97$^*$</td>
</tr>
<tr>
<td>Urinary excretion rate of chloride</td>
<td>0.352 ± 0.111</td>
<td>0.478 ± 0.101$^*$</td>
<td>9.59 ± 4.96</td>
<td>5.00 ± 2.40$^*$</td>
</tr>
<tr>
<td>Urinary excretion rate of potassium</td>
<td>0.0493 ± 0.0279</td>
<td>0.0367 ± 0.00834</td>
<td>26.6 ± 15.3</td>
<td>8.83 ± 8.40$^*$</td>
</tr>
</tbody>
</table>

$^*$ $p < 0.05$. 

The straight line and dotted line represent computer generated lines for control rats and NARs, respectively.

![Fig. 5](image)
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


