Pharmacokinetics and Metabolism of an αβ-Blocker, Amosulalol Hydrochloride, in Mice: Biliary Excretion of Carbamoyl Glucuronide

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The pharmacokinetics and metabolism of an αβ-blocker, amosulalol hydrochloride, were investigated in mice. After intravenous administration (10 mg/kg), the plasma concentration of the unchanged drug declined biphasically, with a terminal half-life of 1.1 h. The maximum plasma concentrations were reached at 0.25 h after oral administration, and then declined with apparent half-lives of 0.8—1.3 h. The systemic bioavailability of a 10-mg/kg dose was 38.7%. The area under the plasma concentration curve increased more than proportionally to the dose, which suggests metabolic saturation. After oral and intravenous administrations of 14C-labelled amosulalol hydrochloride, 64.7% and 81.0% of the radioactivity were recovered, respectively, in the urine within 48 h. HPLC-UV and LC/MS analyses demonstrated that the major urinary metabolite was the glucuronide of M-2 (desmethyl metabolite at the o-methoxyphenoxy group) followed by M-5, the M-3 glucuronide, and the M-4 glucuronide, in that order. In the bile sample, amosulalol carbamoyl glucuronide was found as a new metabolite of this drug.

Key words amosulalol; pharmacokinetics; carbamoyl glucuronide; metabolism; mouse

Amosulalol hydrochloride is a combined α- and β-blocker that was selected from a series of sulphonamide-substituted phenylethylamines. It blocks both postsynaptic α1- and β1-adrenoceptors to almost the same extent. When administered to conscious, spontaneously hypertensive rats, it reduces blood pressure via its α1-blocking activity without causing reflex tachycardia because of its β1-blocking action. This drug exhibited dose-dependent hypotensive effects in healthy volunteers, and is used for the treatment of essential hypertension. In contrast to a structurally related α1- and β-blocker, labetalol, amosulalol’s first-pass metabolism was almost negligible in humans. However, this drug was extensively metabolized in laboratory animals yielding an order of total body clearance as follows: rats>dogs>monkeys>humans. Amosulalol hydrochloride was administered intravenously as a solution in saline and orally as an aqueous solution. Mice were fasted overnight before oral administration, and fed 4 h after dosing. Blood samples were taken from the inferior vena cava with heparinized syringes under diethyl ether anesthesia, and each animal was sacrificed at each sampling point. For urine and fecal sample collection, animals were kept in glass metabolic cages for 24 or 48 h after dosing. For the metabolite identification, bile samples were obtained from bile duct-cannulated mice during hours 0 and 24 after dosing. This research was conducted in accordance with the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised 1985).

Determination of Radioactivity The urine collected was diluted 10-fold with distilled water. AQUAOL-2 (10 ml, New England Nuclear) was then added to a 0.1-ml aliquot of the diluted sample. In addition, about 100 mg of dried feces was combusted in a sample oxidizer (Packard, Model 306). The resulting 14CO2 was absorbed in 7 ml of Oxisorb-CO2 (New England Nuclear), after which it was mixed with 11 ml of Oxiprep-2 (New England Nuclear). The radioactivity was counted with a liquid-scintillation spectrometer (Packard, Model 3255), with quench corrected by the external standard.

Quantification of Amosulalol The concentration of the unchanged drug in plasma and urine was determined using HPLC with fluorescence detection and gas chromatography with nitrogen-sensitive detection, respectively.

Pharmacokinetic Analyses Four animals were used at each sampling point, and the mean plasma concentration–time curves obtained were used for pharmacokinetic analysis. The concentration curve obtained after intravenous administration was fitted to a two-compartment open model using the equation \( Cp = Ae^{-\alpha t} + Be^{-\beta t} \) (α>β), where A and B are ordinate axis intercepts, and α and β are the composite rate constants for the distribution and elimination phases. The area under the plasma concentration curve (AUCtot), elimination half-life (t1/2β), and total body clearance (CLtot) were calculated using the nonlinear least-square program.
NONLIN four according to the following equations:

\[ AUC_{v} = A + B / \beta, \quad t_{1/2} = 0.693 / \beta, \quad CL_{na} = \text{Dose} / AUC_{v} \]

The \( AUC \) after oral administration was estimated using the following equation:

\[ AUC_{p.o} = AUC_{p.o,0-\infty} + C_{p}(t) / \beta \]

where \( AUC_{p.o,0-\infty} \) is the \( AUC \) from zero to the last time \( t \) at which a measurable concentration was observed, by the trapezoidal method, and \( C_{p}(t) \) is the plasma concentration at time \( t \). Systemic availability \( (F) \) was calculated using the following equation:

\[ F = (AUC_{p.o} / AUC_{i.v.}) \times 100 \]

**Identification and Quantification of Metabolites**

After oral administration of 100 mg/kg amosulalol hydrochloride, the urine and bile samples were collected and then pooled. A 0.5-ml portion of the sample was diluted with 100 mM ammonium acetate (1 ml for urine, 1.5 ml for bile) and applied to an Oasis HLB cartridge (1 cc/30 mg, Waters, MA, U.S.A.) that was preconditioned with 1 ml of methanol followed by 1 ml of water. The cartridge was washed with water and eluted with 2 ml of methanol containing 0.1% acetic acid. The eluate obtained was successively concentrated under reduced pressure. The residue was dissolved in methanol (0.5 ml) and 100 mM ammonium acetate (0.5 ml) and injected into a LC/MS for metabolite identification.

The basic metabolites, M-1, M-2, M-3, M-4, and M-6, and their conjugates in urine were quantified using HPLC-UV methods monitored at 271 nm both before and after enzymatic or acid hydrolysis. The acidic metabolite, M-5, was converted to its methyl ester using ethereal diazomethane, and analyzed using GC-MS.

**Purification of an Unknown Biliary Metabolite**

The bile sample (3.5 ml) was diluted with 100 mM ammonium acetate (14 ml) and loaded onto an Oasis HLB cartridge (20 cc/30 mg, Waters, MA, U.S.A.) that had been conditioned with 20 ml of methanol followed by 20 ml of water. The cartridge was washed with water and then eluted stepwise with 20-ml aliquots of solutions of ammonium acetate–methanol–water–formic acid with the following compositions and in the following order, A (100 : 250 : 650 : 1), B (100 : 500 : 500 : 1), C (100 : 750 : 250 : 1), and D (100 : 900 : 0 : 1). The B and C eluates were concentrated and applied to a preparative HPLC fitted with an Inertsil ODS-3 (250 mm×10 mm I.D. particle size 5-μm column (Shimadzu LC-10A systems, Kyoto, Japan) maintained at 40°C. The column was eluted with mixtures of ammonium acetate–methanol–water–formic acid with the following coupling constants were given in Hz.

**LC/MS**

LC/MS was performed using a HP1100-series HPLC system (Agilent Technologies, Inc., Waldbronn, Germany), equipped with a Surveyor photodiode array detector (ThermoElectron, CA, U.S.A.), ion trap mass spectrometer LCQ Deca equipped with an electro-spray interface (ESI) (ThermoElectron), and a Capcell Pak C18 UG120 column (250 mm×2.0 mm I.D. particle size 5-μm; Shiseido Fine Chemicals, Tokyo, Japan). The column was maintained at 40°C for all HPLC experiments. The mobile phases consisted of 10 mM ammonium acetate (pH 9.0)–methanol (19 : 1) (A) and 10 mM ammonium acetate (pH 9.0)–methanol–acetonitrile (2 : 19 : 19) (B). The linear gradient solvent program began with 0% B, which increased to 30% B at 10 min, 35% at 20 min, and 80% B at 30 min, with a constant flow rate of 0.21 ml/min. The metabolite was detected at 271 nm. All eluates were introduced to the ESI interface for ionization and scanned at m/z 150—1000 between 5 and 35 min in positive ion mode. The divert valve was set to “waste” for minutes 0 to 4, then to “source” for minutes 4 to 35. The heated capillary temperature was set at 300°C, and the spray voltage was set at 3.5 kV for all scans. Comprehensive characterization of the metabolites in the urine and bile samples was conducted by subtracting the raw mass data obtained from the respective blank samples using Metabolite ID® 2.0 (ThermoElectron).

**Mass Spectrometer**

The total ion scan and product ion scan of the purified unknown metabolite from mouse bile were obtained using TSQ7000 (ThermoElectron) with an ESI interface using flow injection analysis (FIA). Elution for FIA was subjected to a mixture of 0.1% formic acid–acetonitrile (7 : 3) as the mobile phase at the rate of 1.0 ml/min. The heated capillary temperature was set at 300°C, and the spray voltage was set at 4.5 kV. Nitrogen was used as the sheath gas (60 psi), and no auxiliary gas was employed. The total ion scan was conducted from m/z 200 to 900 in positive ion mode. The product ion scan was conducted as follows: parent ion at m/z 618.10 [M+NH₄]⁺, collision energy −15 eV, scan range from m/z 10 to 630, and argon gas for collision set at 1.5 kgf/cm².

**NMR Spectrometer**

The NMR spectra of amosulalol hydrochloride and its metabolite were recorded on a JNM-ALPHA500 (500 MHz; JEOL Ltd., Tokyo, Japan). The sample was dissolved in methanol-d₄, and chemical shifts downfield from tetramethylsilane were reported in ppm (δ). The coupling constants were given in Hz.

**RESULTS**

**Pharmacokinetics**

After intravenous administration of 10 mg/kg amosulalol hydrochloride, the plasma concentration of the unchanged drug declined in a biphasic manner, and the values for \( t_{1/2} \), \( AUC_{v} \), and \( CL_{na} \) were 1.1 h, 0.93 μg·h/ml, and 10.7/h/kg, respectively (Fig. 1). After oral administration (10—100 mg/kg), the maximum plasma concentrations were obtained at 0.25 h, after which they declined with apparent half-lives of 0.8—1.3 h. The \( AUC_{p.o} \) increased at a rate more than proportional to the dose (0.36, 1.54 and 7.94 μg·h/ml at 10, 30 and 100 mg/kg, respectively). The maximum plasma concentration obtained after 10-mg/kg dosing (0.31 μg/ml) was similar to that found in humans after a therapeutic dose of this drug (12.5 mg/man). The systemic bioavailability at 10 mg/kg was 38.7%.

**Excretion of Radioactivity into Urine and Feces**

Within 48 h after oral administration of the 14C-labelled compound, 64.7±2.0% of the radioactivity was excreted in the

August 2007
urine, and 29.3 ± 0.6% in the feces. The respective recoveries after intravenous administration reached 81.0 ± 3.0% and 17.8 ± 0.2% within 48 h, most of which was excreted within the first 24 h.

**Excretion of Amosulalol and Its Metabolites into Urine and Bile** After intravenous administration of amosulalol hydrochloride, urinary excretion of the unchanged drug was 16.6 ± 3.1% (0—24 h). Renal clearance ($CL_{\text{renal}}$) was calculated to be 1.8 l/h/kg, which was much less than the $CL_{\text{tot}}$ of 10.7 l/h/kg. Following oral administration of the drug, 7.6 ± 1.6% of the dose was recovered as the unchanged form.

Figure 2 shows the HPLC-UV chromatograms of the urine and bile samples. Several peaks corresponding to metabolites previously reported to be present in rats, dogs, and monkeys were found. Quantification of the metabolites revealed that M-2 glucuronide was the major metabolite in the urine, accounting for 22.1% of the dose (Table 1). The second major metabolite was M-5, followed by M-3 glucuronide and M-4 glucuronide. The total of the quantified urinary metabolites (53.8%) was about 10% less than that measured by radioactive counting (64.7%), which may suggest the presence of other unknown metabolites in the urine. The LC/MS analyses of the bile with reconstruction of the total ion mass chromatograms from m/z 350 to 650 revealed that one large unknown peak was present at the retention time of approximately 21 min (data not shown).

**Identification of an Unknown Biliary Metabolite** The MS spectrum of the unknown peak was present at m/z 618 and 623, which corresponded to the adduct ions of [M+
and Na/H11002, respectively (Fig. 3A). The molecular ion of the unknown metabolite was considered to be m/z 600, which was 44 u (CO₂) and 176 u (glucuronosyl moiety) greater than that of the parent drug. Product ions observed at m/z 381 (M-H-H₁1001H₁1002220) as aglycone and m/z 425 (M-H-H₁1001H₁1002176) as N-carboxy amosulalol in the product ion scan at m/z 618 supported the assertion that an additional functional group was present (Fig. 3B). A fragment ion at m/z 407 caused by the elimination of glucuronic acid was also observed. Subsequent 1H-NMR and TOCSY experiments were conducted on the metabolite in order to elucidate its structure by comparing it with that of the unchanged drug. The NMR data are listed in Table 2. A couple of anomeric proton doublet signals were observed in the spectrum at δ 5.43 and 5.45 [0.5H (J = 7.8 Hz), each]. The anomeric proton signal values exhibited a significant downfield chemical shift compared to those of the typical carbinol glucuronides. This shift seemed to result from the deshielding of the glucuronosyl anomeric proton by the carbonyl group. The proton signals of the aglycone moiety exhibited almost the same chemical shift values as those of amosulalol, except for those adjacent to the nitrogen atom. This suggests that the carbonyl group was attached at the secondary amino moiety. The large coupling constant value of the anomeric proton signal indicated that the glucuronide was a β-anomer.¹²) The anomeric proton signal split suggested the presence of two conformational isomers of the carbamoyl glucuronide in methanol-d₄. This is probably due to the slow rotation of the carbonyl glucuronic acid moiety at the C–N bond of the carbamate.¹³) An overview of the postulated metabolic pathways of this drug in mice is shown in Fig. 4.

### DISCUSSION

Labetalol, an α and β-blocker that is structurally related to amosulalol hydrochloride, possesses a phenolic hydroxy group, the glucuronidation of which is one of the main elimination routes.⁴) Labetalol exhibited non-linear pharmacokinetics in humans, which lead to considerable variations in bioavailability in clinical practice.¹⁴,¹⁵) In contrast to labetalol, amosulalol hydrochloride does not possess any phenolic hydroxy moiety, and thus exhibited linear pharmacokinetics in humans²) as well as rats, dogs, and monkeys.⁶) The effect of first-pass metabolism is almost negligible in humans. However, the systemic bioavailability of this drug in rats, dogs, and monkeys was 22—31%, 51—59%, and 57—66%, respectively.⁶) Since ¹⁴C-amosulalol was almost com-

<table>
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<th>Position</th>
<th>Amosulalol hydrochloride</th>
<th>Amosulaloy carbamoyl glucuronide</th>
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<tr>
<td>1,2,5-Trisubstituted benzene ring</td>
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<td>7.34</td>
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<tr>
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<td>7.56</td>
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<td>Glucuronic acid</td>
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<tr>
<td>2”—5”</td>
<td>—</td>
<td>3.4—3.7</td>
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The position of the structure is tentatively numbered as shown in Fig. 3.
pletely absorbed in the gastrointestinal tract of rats and dogs, the reduced bioavailability for these animals could possibly be due to first-pass metabolism. The mice in this study showed a systemic bioavailability of 38.7% at the dose of 10 mg/kg, and the urinary excretion of radioactivity did not differ greatly between intravenous and oral administration. These findings suggest that this drug is well-absorbed in the gastrointestinal tract and undergoes first-pass metabolism in mice in a manner similar to that reported for rats. Unlike the rats or any of the other species tested, the mice exhibited non-linear pharmacokinetics after oral administration.

The \( CL_{renal} \) value obtained after intravenous administration to mice was relatively small compared to the \( CL_{tot} \) value, which also suggested that the main route of elimination was metabolism. After oral administration, 7.6% of the dose was found in the urine as the unchanged drug, together with a large number of metabolites. The primary urinary metabolites in rats, dogs, monkeys, and humans were reported to be M-6 sulphate, M-5, M-5, and M-3 sulphate, respectively. Unlike these species, 22.1% of the dose in mice was excreted into the urine as M-2 glucuronide. This metabolite, characteristic to mice, was derived through demethylation at the \( o \)-methoxyphenoxy group, and subsequent conjugation with glucuronide. The second major metabolite was M-5, which is derived through oxidative C–N cleavage, followed by M-3 glucuronide and M-4 glucuronide. M-3 sulfate was the only metabolite quantified in human urine (12.7% of the dose). Urinary excretion of this metabolite in mice, however, remained at 1.4%.

Amosulalol has a secondary aliphatic amine in its molecule. Some secondary amines, as well as some primary and tertiary amines, can be metabolized by direct conjugation with glucuronic acid. The conjugated form of amosulalol found in mouse bile, however, yielded a molecular ion 44 u greater than that of amosulalol plus the glucuronosyl moiety. The extreme downfield chemical shift of the anomeric proton signal observed in NMR analysis implies that the parent drug was conjugated with carbonyl glucuronic acid. The chemical shift of the protons adjacent to the nitrogen atom of the secondary aliphatic amine group suggested that this metabolite was amosulalol carbamoyl glucuronide.

The antiarrythmic agent, tocainide, was first reported to form a carbamoyl glucuronide in humans. Subsequently, reports on the formation of various carbamoyl glucuronides by primary and secondary amines were published (recently reviewed by Schaefer). It has been proposed that the mechanism that generates these glucuronides starts by forming carbamic acid as an intermediate under physiological conditions in the liver through a reversible reaction with CO2 dissolved in the environmental fluid. UDP-glucuronyl transferase (UGT) then causes the immediate conjugation of this carbamic acid with glucuronic acid by utilizing UDP-glucuronic acid as a cofactor. Recent studies using recombinant human enzymes suggest that UGT2B7, UGT1A3, UGT2B4, and UGT1A6 may be the UGT isozymes responsible for the formation of sertraline carbamoyl glucuronide, and UGT2B7 for the formation of varenicline carbamoyl glucuronide. The mechanism of amosulalol carbamoyl glucuronide formation is not known. However, this metabolite found in the bile may also be derived through the series of reactions described above in the mouse liver.

In conclusion, amosulalol hydrochloride was extensively metabolized with non-linear pharmacokinetics in mice. The major metabolite in urine was M-2 glucuronite. Carbamoyl glucuronide of amosulalol was isolated and characterized from bile samples.

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REFERENCES


