METABOLISM OF [14C]OMAPATRILAT, A SULPHHYDRYL-CONTAINING VASOPEPTIDASE INHIBITOR IN HUMANS

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(Received April 19, 2000; accepted October 3, 2000)

ABSTRACT:

Omapatrilat, a potent vaso peptide inhibitor, is currently under development for the treatment of hypertension and congestive heart failure. This study describes the plasma profile along with isolation and identification of urinary metabolites of omapatrilat from subjects dosed orally with 50 mg of [14C]omapatrilat. Only a portion of the radioactivity in plasma was unextractable (40–43%). Prominent metabolites identified in plasma were S-methyl omapatrilat, acyl glucuronide of S-methyl omapatrilat, and S-methyl (S)-2-thio-3-phenylpropionic acid. Omapatrilat accounted for less than 3% of the radioactivity. However, after dithiothreitol reduction all of the radioactivity was extractable and was characterized to be omapatrilat and its hydrolysis product (S)-2-thio-3-phenylpropionic acid, both apparently bound to proteins via reversible disulfide bonds. Urinary profile of radioactivity showed no parent compound but the presence of several metabolites that can be grouped into three categories. 1) Three metabolites, accounting for 56% of the urinary radioactivity, resulted from the hydrolysis of the oxycyclic amide bond of omapatrilat. Two metabolites were diastereomers of S-methyl sulfoxide of (S)-2-thio-3-phenylpropionic acid, and the third was the acyl glucuronide of S-methyl (S)-2-thio-3-phenylpropionic acid. 2) One disulfide, identified as the L-cysteine mixed disulfide of omapatrilat, accounted for 8% of the radioactivity in the urine. 3) Five metabolites, derived from omapatrilat, accounted for 30% of the radioactivity in the urine. Two of these metabolites were mixtures of diastereomers of S-methyl sulfoxide of omapatrilat and the third was the S-methyl omapatrilat ring sulfoxide. The other two metabolites were S-methyl omapatrilat and its acyl glucuronide. These results indicate that omapatrilat undergoes extensive metabolism in humans.
metabolism of [14C]omapatrilat

Sample Analysis. HPLC analysis. Plasma samples were profiled with a Waters NovaPak C18 column (3.9 × 150 mm, 5 μm) at a flow rate of 1 ml/min on a Waters liquid chromatograph equipped with two 510 pumps, a 680 automated gradient controller, a 717+ WISP autosampler injection system, and a 486 tunable absorbance detector. Urine sample analysis, including profiling and metabolite isolation, were done on a Shimadzu Class VP system equipped with two pumps (model LC-10AT), an auto injector (SIL 10AD), and a diode array detector (SPD-M10A). Metabolite purification and profiling from urine was done on a semipreparative Zorbax RX C18 column (9.4 × 250 mm, 5 μm) at a flow rate of 3.8 ml/min. Chromatographic profiling of radioactivity in the pooled human urine samples, including samples generated after glucuronidase or DTT treatment, was done on a Zorbax RX C18 column (4.6 × 250 mm, 5 μm) at a flow rate of 1.0 ml/min. Liquid chromatography/mass spectrometry (LC/MS) and LC/MS/MS analysis of the isolated metabolite fractions from human urine was done with a Zorbax RX C18 column (2.1 × 150 mm, 5 μm) at a flow rate of 0.3 ml/min. The eluent from the HPLC was directed to the Finnigan (San Jose, CA) LCQ mass spectrometer through a divert valve set to divert the flow to waste from 0 to 7 min. From 7 min until the duration of the HPLC run, the eluent flow was directed to the mass spectrometer through a splitter, which split the flow in a 1:1 ratio. Half the eluent was directed to the mass spectrometer (flow rate, 150 μl/min), whereas the other half was allowed to go to waste.

All HPLC analyses were done by enclosing the column in an Eppendorf CH-30 column heater maintained at 35°C with an Eppendorf TC-45 temperature controller. Three different solvent systems, system I, II, and III, were used for HPLC analysis. Solvent system I consisted of solvent A, acetonitrile/water (5:95) containing 0.1% trifluoroacetic acid (pH 2.0) and solvent B, acetonitrile. System II consisted of solvent A, acetonitrile/water (5:95) containing 0.06% trifluoroacetic acid and 0.07% triethylamine (pH 2.5) and solvent B, acetonitrile/water (90:10) containing 0.06% trifluoroacetic acid and 0.07% triethylamine. System III consisted of solvent A, methanol/10 mM ammonium acetate (5:95, pH 4.9) and solvent B, methanol/10 mM ammonium acetate (90:10, pH 4.9). The HPLC fractions were collected with a Gilson (Middleton, WI) model 202 fraction collector and counted in a Packard (Middleton, WI) Tri-Carb 2550TR liquid scintillation counter or in a Packard Tri-Carb 2300 CA liquid scintillation counter. The dpm were determined from the sample count rates with a previously prepared quench curve. For each injection, the average dpm value for the first three fractions (column void volume) was subtracted from the dpm value of each subsequent fraction. Biotransformation profiles were prepared by plotting the resulting net dpm values against time-after-injection. Radioactive peaks in the biotransformation profiles are reported as a percentage of the total radioactivity collected during the entire HPLC run. The recovery of radioactivity from the column was quantitative.

Mass spectral analyses of all standards and isolated metabolites were performed on a Finnigan LCQ mass spectrometer with an electrospray ionization (ESI) probe, in negative and positive ion modes. For samples introduced

(methylnalsulfonyl)benzenepropanoic acid, l-cysteine mixed disulfide adduct of omapatrilat ([45S,7S,10aS]-4-[2H]-2-amino-2-carboxyethyl [dithio]-1-oxo-3-phenylpropylaminooctahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid), diastereomeric sulfides of S-methyl omapatrilat ([45S,7S,10aS]-octahydro-4-[1-(methylsulfonyl)-1-oxo-3-phenylpropyl]aminooctahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid), diastereomeric ring sulfides of S-methyl omapatrilat ([45S,7S,10aS]-octahydro-4-[1-(methylsulfonyl)-1-oxo-3-phenylpropyl]aminooctahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid), diastereomeric ring sulfides of S-methyl omapatrilat ([45S,7S,10aS]-octahydro-4-[1-(methylsulfonyl)-1-oxo-3-phenylpropyl]aminooctahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid 1-oxide), (S)-2-thio-3-phenylpropionic acid ([S]-2-mercaptobenzenepropanoic acid), (S)-2-thiomethyl-3-phenylpropionic acid ([S]-2-(methylthio)benzenepropanoic acid), omapatrilat ([45S,[4a(R*)]-7S,10a(R*)]-octahydro-4-[2-mercapto-1-oxo-3-phenylpropyl]aminooctahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid), S-methyl omapatrilat ([45S,[4a(R*)]-7S,10a(R*)]-octahydro-4-[2-(methylthio)-1-oxo-3-phenylpropyl]aminooctahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid), and disulfide of omapatrilat ([45S,7S,10aS]-4,4′-dithiobilis[2S-1]-1-oxo-2-(phenylmethyl)-2,1-ethanediyldilinino)bis[octahydro-5-oxo-7H-pyrido[2,1-b][1,3]thiazepine-7-carboxylic acid] were supplied by the radiochemistry group. Phenolphthalene glucuronide, phenolphthalain solution in ethanol (1 mg/ml), β-glucuronidase (Helix pomatia type H-1 with sulfate activity; 420,800 units/g), glycine, and 1,4-saccharalactone were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) and triethylamine were grade or better. [1,3]Thiazepine-7-carboxylic acid} were supplied by the radiochemistry group.

FIG. 1. Structure of omapatrilat.

The site labeled with 14C is indicated with an asterisk.
by infusion, the flow rate was 3 to 5 \( \mu \)l/min and the solvent was either acetonitrile/water (1:1) or acetonitrile/10 mM ammonium acetate buffer, pH 4.9 (1:1). The capillary temperature used for analysis was 220 to 230°C. The nitrogen gas flow rate, spray current, and voltages were adjusted for each metabolite to give the maximum sensitivity.

**Biotransformation Profiles. Preparation of plasma samples for HPLC profiling.** Pooled plasma samples (1 and 6 h, 2 ml) were mixed with acetonitrile (6 ml), vortexed vigorously, and centrifuged. The precipitates were again extracted with acetonitrile (2\( \times \)3 ml) and the supernatants from previous extraction were combined. The pooled extracts were evaporated at room temperature under a stream of nitrogen and the residues were reconstituted in HPLC mobile phase. A portion of the sample was mixed with Ecolite, counted for radioactivity with a liquid scintillation counter, and the remaining sample used for HPLC profiling. Pooled 1-h plasma sample collected without methyl acrylate was treated with DTT, a disulfide reducing agent, as follows. Plasma (2 ml) was mixed with 0.3 M potassium phosphate buffer (0.2 ml, pH 7.4) and a solution of 0.1 M DTT (1.5 ml) in the same buffer. The mixture was allowed to stand at room temperature for 5 min to ensure complete reduction of any sulfhydryl compounds to the methyl acrylate adducts generated after DTT reduction.

**Preparation of plasma samples for HPLC profiling.** Portions of the plasma extracts were loaded onto a NovaPak C18 column (3.9 \( \times \)150 mm, 5 \( \mu \)m) and eluted with solvent system I where solvent B was maintained at 0% for 5 min and then increased in a step manner as follows: 20% (15 min), 20% (25 min), 25% (30 min), 25% (35 min), 45% (45 min), 45% (55 min), and 60% (60 min).

**Preparation of urine samples for HPLC profiling.** Pooled 0- to 24-h and 0- to 168-h human urine samples were concentrated on an Oasis HLB 3.0-ml cartridges. The cartridge was loaded with pooled urine (3 ml) and washed sequentially with water containing 0.1% acetic acid (2 \( \times \)3 ml, pH 3.2) and methanol (2 \( \times \)3 ml). Liquid scintillation counting of the water and the methanol extracts showed quantitative recovery of radioactivity in the methanol fractions. The methanol extracts were combined, evaporated to dryness under a stream of nitrogen, and dissolved in HPLC mobile phase (300 \( \mu \)l) consisting of acetonitrile/water (20:80) containing 0.06% trifluoroacetic acid and 0.07% triethylamine. A portion of the extract was loaded onto a Zorbax RX C18 column (4.6 \( \times \)250 mm, 5 \( \mu \)m) and eluted with solvent system II where solvent B was maintained at 0% for 5 min and then increased in a step manner as follows: 20% (15 min), 25% (25 min), 25% (30 min), 25% (35 min), 45% (45 min), 45% (55 min), and 60% (60 min).

**Glucuronidase and DTT Treatment of Human Urine.** A solution of \( \beta \)-glucuronidase (0.2 ml, 2104 units) in water was added to a solution of 0- to 24-h pooled human urine (1.0 ml) in 0.2 M sodium acetate buffer (1.0 ml, pH 4.8). The mixture was incubated at 37°C in a water bath for 24 h. Three control incubations were done: negative control in the absence of the enzyme, positive control in the presence of phenolphthalein glucuronide (1 mg), and incubation in the presence of the \( \beta \)-glucuronidase inhibitor 1,4-saccharolactone (10 mg). A 0.2 M glycine buffer (8.0 ml, pH 10.5) was added at the end of the incubation to the positive control. The amount of phenolphthalein generated was quantified against a phenolphthalein standard curve with a Perkin Elmer (Norwalk, CT) Lambda 2 UV-visible spectrophotometer operating at 550 nm. All other samples were centrifuged for 5 min in a bench top microcentrifuge and stored at \(-20°C\) until further analysis.

A 0.2 M solution of DTT (1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) was added to 0- to 24-h pooled human urine (1.0 ml), and the solution was incubated at 37°C in a water bath for 45 min. A control incubation was done in the absence of DTT at 37°C for 45 min. The samples were centrifuged for 5 min in a bench top microcentrifuge and stored at \(-20°C\) until further analysis. The samples were analyzed by HPLC under the same conditions as described above for the analysis of 0- to 24- and 0- to 168-h pooled urine samples.

**Isolation and Identification of Metabolites in Human Urine.** The overall scheme used for isolation of the metabolites of omapatrilat from human urine is shown in Fig. 2. A 1.5-liter portion of 0- to 24-h pooled human urine was applied to a XAD-2 resin column (3 \( \times \)35 cm). The column was washed sequentially with water (500 ml), acetonitrile/water (500 ml, 8:2), and acetonitrile (500 ml). The acetonitrile/water and the acetonitrile eluates were combined and evaporated to dryness in vacuo. The residue was dissolved in water, acidified to pH 2.2 with 6 N hydrochloric acid, and extracted with ethyl acetate (5 \( \times \)150 ml). The organic extracts were combined and evaporated to dryness in vacuo. The residue was dissolved in 1:1 mixture of acetonitrile/water (8.0 ml) and subjected to semipreparative HPLC in 0.8-ml portions with solvent system II. The gradient used was the same as described above for analysis of 0- to 24-h pooled urine samples. The column eluate was collected as 1-min fractions for 60 min. A 10-\( \mu \)l sample from each fraction was added to Ecolite (4.5 ml) and counted for 10 min each in a liquid scintillation counter. Radioactive fractions from each preparative run were combined into five fractions designated as fractions I, II, III, IV, and V (Fig. 2). Each fraction was evaporated to dryness in vacuo, dissolved in mobile phase, and subjected to second semipreparative HPLC as described below.

**Purification of fraction I.** Fraction I from the first HPLC run was subjected to a second semipreparative HPLC with solvent system II. The column eluate was collected as 1-min fractions for 60 min. Isocratic elution with 5% solvent B gave two radioactive peaks, M1 and M2. Metabolites M1 and M2 were further purified by preparative TLC with methanol/ethylene acetate/acetic acid (6:4:0.01) as the solvent. The TLC plates were imaged and the silica gel showing the radioactive band was removed with a razor blade. The silica gel
was extracted with a mixture of methanol/water (8:2, 5 × 50 ml) containing 0.1% acetic acid. The mixture was filtered and the solvent was evaporated to dryness in vacuo. The sample was suspended in water containing 0.1% acetic acid (pH 3.2) and loaded onto Sep-Pak Vac 20.0-ml (C18, 5.0-g) cartridges. The cartridge was washed with water containing 0.1% acetic acid (2 × 10 ml, pH 3.2) and the metabolite was eluted with acetonitrile. The solvent was removed in vacuo and the residue was dissolved in the mobile phase. Metabolites M1 and M2 were further purified by semipreparative HPLC with solvent system III. Solvent B was maintained at 0% B for 5 min and then increased linearly to 25% B in 20 min. The column eluate was collected as 30-s fractions for 20 min. The fractions with radioactivity were combined, evaporated to dryness, and dissolved in water containing 0.1% acetic acid (pH 3.2). The solution was loaded onto a Waters Oasis HLB 3-ml cartridge for desalting. The cartridge was washed with water containing 0.1% acetic acid (3 × 3 ml, pH 3.2) and the metabolite was eluted with acetonitrile. After HPLC analysis to determine the purity, both metabolites M1 and M2 were analyzed by NMR spectroscopy and mass spectrometry.

Purification of fraction III. Fraction III from the first HPLC run was subjected to a second semipreparative HPLC with solvent system II. The column eluate was collected as 30-s intervals for 60 min. Isocratic elution with 25% solvent B gave three radioactive peaks, M4, a mixture of M5 and M6, and M7. The metabolites M4, mixture of M5 and M6, and M7 were further purified and desalted in a manner similar to purification of metabolites M1 and M2. The preparative TLC mobile phase used was the same as for M1 and M2 but the percentage of composition of solvent B in semipreparative HPLC (system III) varied for each metabolite. For metabolite M4, solvent B was varied from 20% at 0 min to 80% at 30 min. For metabolite mixtures M5 and M6, solvent B was varied from 10% at 0 min to 55% at 30 min, whereas for metabolite M7, solvent B was varied from 10% at 0 min to 65% at 30 min. After desalting, M4, mixture of M5 and M6, and M7 were analyzed by NMR spectroscopy and mass spectrometry.

Purification of fractions IV and V. Fractions IV and V from the first HPLC run were subjected to a second semipreparative HPLC with solvent system II. The column eluate was collected as 1-min fractions for 60 min. Isocratic elution with 25% solvent B gave single radioactive peaks for fraction IV (metabolite M8) and for fraction V (metabolite M9). Metabolites M8 and M9 were further purified and desalted in a manner similar to purification of metabolites M1 and M2. The preparative TLC solvent used for M8 and M9 was methanol/ethyl acetate/acetic acid (4:6:0.01) and ethyl acetate/hexane (1:1) containing 0.1% acetic acid, respectively. The percentage of composition of solvent B in semipreparative HPLC (system III) varied for each metabolite. For metabolite M8, solvent B was varied from 0% at 2 min to 80% at 30 min, whereas for metabolite M9, solvent B was varied from 10% at 0 min to 80% at 30 min. After desalting, M8 and M9 were analyzed by NMR spectroscopy and mass spectrometry.

LC/MS Analysis of Fraction II and Metabolite Mixture MS5 and M6. Fraction II was directly analyzed by LC/MS and LC/MS/MS (LC/MS²) without further purification by HPLC. The HPLC analysis was done with solvent system III on Zorbax RX C18 column (2.1 × 150 mm, 5 μm) where solvent B was varied from 10% at 0 min to 60% at 40 min. For fraction II, LC/MS was done both in the negative ion mode and in the positive ion mode, whereas LC/MS² and LC/MS/MS/MS (LC/MS³) was done only in the positive ion mode. Analysis of metabolite mixture M5 and M6 was done by LC/MS and LC/MS² in the positive ion mode and by LC/MS/MS in the negative ion mode. The HPLC was done with solvent system III where solvent B was varied from 10% at 0 min to 60% at 30 min. Partial separation of metabolites M5 and M6 was achieved in this solvent system.

Results

Radioactivity Elimination Profile. The recovery of radioactivity in urine and feces collected over 168 h post dose was 64% and 8%, respectively. Most of the recovered radioactivity was collected within the first 72 h post dose. The radioactivity recovered in the urine and feces collected after 72 h post dose was extremely low (less than 1%). The plasma radioactivity and omapatrilat concentration versus time is plotted on Fig. 3. The radioactivity was substantially higher than the concentration of unchanged omapatrilat over the time of blood collection.

Biotransformation Profiles. Plasma profile. Distribution of radioactivity among HPLC fractions from pooled human 1-h plasma samples is shown in Table 1. In the 1-h plasma samples collected in the absence and presence of methyl acrylate, unextractable radioactivity accounted for 40.6 and 43.2%, respectively. However, after DTT reduction, all the radioactivity presumably bound to the protein through disulfide bond was extractable (Table 1, footnote). Even though omapatrilat accounted for less than 3% of the radioactivity in 1-h plasma sample, collected in the presence of methyl acrylate, it accounted for nearly 21% of the radioactivity in DTT-treated plasma sample. The remaining unextractable radioactivity (20%) was accounted for by (S)-2-thio-3-phenylpropionic acid, the hydrolysis product of omapatrilat. The profiles of the 6-h plasma samples were qualitatively similar to that of the 1-h plasma sample (data not shown).

Urinary profile. The radioactivity in the 0- to 24-h pooled urine sample represents 92% of the radioactivity excreted in the urine over the entire 168 h of collection and 59% of the administered radioactivity. The radiochromatographic profile of 0- to 168-h pooled human urine (data not shown) was similar both qualitatively and quantitatively to that of the 0- to 24-h pooled human urine. Hence, the 0- to 24-h pooled urine sample was considered to represent the entire 0- to 168-h collection. Radiochromatographic profiles of 0- to 24-h pooled human urine before and after glucuronidase and DTT treatment are shown in Fig. 4 and the relative distribution of radioactivity among various peaks in the profiles is shown in Table 2. The profiles of control samples in the absence of β-glucuronidase/sulfatase and DTT showed no change from the original profile (data not shown), indicating that the metabolites were stable for the duration of the incubation.

Profile A in Fig. 4 shows the distribution of radioactivity in untreated urine. The peaks for the identified metabolites are labeled M1 to M9 and they accounted for 94% of the radioactivity excreted in the 0- to 24-h pooled urine. The profile of untreated urine showed no radioactivity peak corresponding to the parent compound. The major route for formation of metabolites found in human urine was due to hydrolysis of the exocyclic bond of the parent compound. These metabolites included M1 (8%), M2 (44%), and M4 (4%), which together accounted for 56% of the radioactivity excreted in 0- to 24-h pooled urine (Table 2). The remaining metabolites identified were formed from modification of omapatrilat. Metabolites M3 and M5 to M9 accounted for 38% of the radioactivity (Table 2).

Omapatrilat and its metabolites have carboxylic acid groups that
Retention time of peak M3 matched with that of L-cysteine mixed (9%) appeared in the radiochromatogram (Table 2, footnote). The reduction any disulfide conjugates of omapatrilat and its metabolites.

Chromatogram of 0- to 24-h pooled human urine treated with DTT to potentially form disulfides in vivo with protein thiols and soluble M8 were further confirmed by LC/MS and NMR as described below.

Omapatrilat and its metabolites with a free sulfhydryl group can potentially form acyl glucuronides in vivo. Figure 4B and Table 2 show the distribution of radioactivity in β-glucuronidase-treated 0- to 24-h pooled urine. Comparison of profiles A and B showed that M4 and M8 were hydrolyzed to give a new peak at 47 min and an increase in the radioactivity of M9 from 13 to 23%, respectively. Incubation in the presence of 1,4-saccharolactone, a β-glucuronidase inhibitor, prevented the hydrolysis of M4 and M8 (data not shown). The peak at 47 min (Fig. 4B) had retention time similar to that of (S)-2-thiomethyl-3-phenylpropionic acid. Peak M9 retention time was similar to that of S-methyl omapatrilat. Metabolites M4 and M8 were tentatively identified as the acyl glucuronides of (S)-2-thiomethyl-3-phenylpropionic acid and S-methyl omapatrilat, respectively. The structure of M4 and M8 were further confirmed by LC/MS and NMR as described below.

Omapatrilat and its metabolites with a free sulfhydryl group can potentially form disulfides in vivo with protein thiols and soluble thiols such as cysteine and glutathione. Figure 4C shows the radiochromatogram of 0- to 24-h pooled human urine treated with DTT to reduce any disulfide conjugates of omapatrilat and its metabolites. Comparison of profiles A and C showed that peak M3 (8%) was reduced and a peak corresponding to the retention time of omapatrilat (9%) appeared in the radiochromatogram (Table 2, footnote). The retention time of peak M3 matched with that of the L-cysteine mixed disulfide adduct of omapatrilat. Another peak was observed at 37 min in profile C, which accounted for 3% of the radioactivity. The origin of this peak is not known and this peak was not further characterized.

Isolation and identification of metabolites. The purification scheme for isolation of metabolites of omapatrilat from 0- to 24-h pooled urine is shown in Fig. 2. The profile of the ethyl acetate extract was similar to that of the starting pooled urine except that peak M3 was recovered to a smaller extent. Preparative HPLC of ethyl acetate extract yielded five major radioactive fractions (fractions I, II, III, IV, and V). Further purification of fractions I, III, IV, and V yielded eight metabolites (M1, M2, M4, M5, M6, M7, M8, and M9) (Fig. 2). Metabolites M5 and M6 were isolated as a mixture. Fraction II was not further purified but was directly analyzed by LC/MS, LC/MS2, and LC/MS3. Metabolite M3 was identified in fraction II by LC/MS.

Structural elucidation of the isolated metabolites was based upon mass spectrometry, 1H NMR, COSY analysis, and by comparison of the HPLC retention times with those of the reference standards. Mass spectral data for molecular ions and the fragments generated are shown in Table 3. 1H NMR and COSY spectra were obtained for M1, M2, M4, mixture of M5 and M6, M8, and M9, and the chemical shifts for characteristic protons are shown in Table 4. M3 was not analyzed by NMR because it was not isolated and purified from fraction II. The proton NMR of M7 is not reported because the spectrum showed peaks of endogenous impurity, which made the assignment of protons difficult. The evidence and the rationale for the structures assigned to these nine metabolites isolated from human urine follows.

Identification of M1 and M2. Mass spectral analysis of M1, both in the negative ion ESI1 mode and in the positive ion ESI1 mode, produced a loss of 63 due to -CH3SO (Table 3). 1H NMR showed protons for the aromatic region, α-CH, benzylic -CH2, and the singlet for -CH3SO (Table 4). COSY spectra showed cross peaks for α-CH and benzylic -CH3 protons. Both mass spectral data, loss of 63 due to -CH3SO, and 1H NMR data, chemical shift of the methyl group of -CH3SO, indicate the presence of a sulfoxide in the molecule. These data are consistent with M1 being one of diastereomeric sulfoxide of (S)-2-thiomethyl-3-phenylpropionic acid. The mass spectral data of M2 are similar to that of M1 (Table 3). 1H NMR showed protons for the aromatic region, α-CH, benzylic -CH2 and the singlet for -CH3SO (Table 4).

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative distribution of radioactivity (%) among various peaks in the radiochromatographic profiles of 1-h pooled human plasma collected in the absence of methyl acrylate and in the presence of methyl acrylate</td>
</tr>
<tr>
<td>Radioactive peaks are reported as a percentage of the total radioactivity eluted from the column after background subtraction. The recovery of the radioactivity from the column was quantitative</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>1-h Pooled Human Plasma</th>
<th>(Distribution)</th>
</tr>
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<tr>
<td>Omapatrilat-L-cysteine disulfide + S-methyl (S)-2-thio-3-phenylpropionic acid sulfoxide</td>
<td>5.7%</td>
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<td>S-Methyl omapatrilat acyl glucuronide</td>
<td>11.8</td>
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<tr>
<td>S-Methyl (S)-2-thio-3-phenylpropionic acid</td>
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<td>S-Methyl omapatrilat</td>
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<tr>
<td>Omapatrilat</td>
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<tr>
<td>Unextractable</td>
<td>43.2</td>
</tr>
<tr>
<td>Total</td>
<td>82.8</td>
</tr>
</tbody>
</table>

a Percentage due to two metabolites is included since baseline resolution was not achieved in the radiochromatogram.

b Omapatrilat was detected as its methyl acrylate adduct.

c Unextractable radioactivity was bound to the protein via disulfide bonds. After DTT reduction of pooled 1-h plasma, collected in the absence of methyl acrylate, >99% of the unextractable radioactivity was extracted and was distributed between (S)-2-thio-3-phenylpropionic acid (20%) and omapatrilat (21%).

d Total is less than 100% because remainder of the radioactivity is distributed in small amounts (≤0.8%) in various fractions throughout the chromatogram.
TABLE 2

Relative distribution of radioactivity (%) among various peaks (M1–M9) in the radiochromatographic profiles of untreated, glucuronidase-treated, and DTT-treated 0- to 24-h pooled human urine

Radioactive peaks are reported as a percentage of the total radioactivity eluted from the column after background subtraction. The recovery of the radioactivity from the column was quantitative.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>No Treatment</th>
<th>Glucuronidase</th>
<th>DTT</th>
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<tbody>
<tr>
<td>Retention time range in min</td>
<td>% Distribution</td>
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<td></td>
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<tr>
<td>M1 (17–18)</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>M2 (19–20)</td>
<td>44</td>
<td>44</td>
<td>42</td>
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<tr>
<td>M3 (27–28)</td>
<td>8</td>
<td>8</td>
<td>—</td>
</tr>
<tr>
<td>M4 (29–30)</td>
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<td>3</td>
</tr>
<tr>
<td>M5 (31–32)</td>
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<td>3</td>
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<tr>
<td>M6 (33–34)</td>
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<tr>
<td>M7 (34–35)</td>
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<td>3</td>
</tr>
<tr>
<td>M8 (38–40)</td>
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<td>10</td>
</tr>
<tr>
<td>M9 (52–55)</td>
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<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>98</td>
<td>99</td>
</tr>
</tbody>
</table>

*Percentages less than 1% are not reported.

These data are consistent with M2 being the other diastereomeric sulfoxide of (S)-2-thiomethyl-3-phenylpropionic acid. In addition, the fragmentation pattern seen in the negative ion ESI mode and in the positive ion ESI mode for M1 and M2 were similar to the fragmentation of the synthetic standard. Moreover, the retention times of M1 and M2 in chromatographic system II were identical to the diastereomeric sulfoxides of (S)-2-thiomethyl-3-phenylpropionic acid.

Identification of M3. Metabolite M3 was identified from fraction II by LC/MS and LC/MS2 analysis. The fragmentation pattern seen in positive ion ESI was similar to the fragmentation of the synthetic standard, the L-cysteine mixed disulfide adduct of omapatrilat (Table 3). Based on the mass spectral data, metabolite M3 was identified as the L-cysteine mixed disulfide of omapatrilat. Moreover, M3 showed identical retention time on HPLC to that of the synthetic standard.

Identification of M4. The fragmentation pattern for metabolite M4 in the negative ion mode and in the positive ion mode is shown in Table 3. Both negative ion ESI and positive ion ESI generated daughter ions resulting from the loss of a glucuronide. 1H NMR data of M4 are reported in Table 4. The chemical shifts of the methyl group at 2.4 and 2.6 ppm also suggested that M5 and M6 were diastereomeric sulfoxides with oxidation of the exocyclic sulfur group. Finally, the fragmentation pattern and the retention time of M5 and M6 on HPLC were identical to those of the synthetic diastereomeric S-methyl side chain sulfoxides of omapatrilat.

Identification of metabolite M7. The fragmentation pattern for metabolite M7 in the negative ion mode and in the positive ion mode is shown in Table 3. Although M7 generated the same molecular ion as M5 and M6, in both the negative ion mode and the positive ion mode, the fragmentation pattern was very different. No loss of 63 due to methyl sulfoxide, -CH3 SO, was observed in MS analysis of M7 (Fig. 5B). The 1H NMR data of M7 was not very clean but there were no singlets for -CH3 SO that would be observed at 2.6 or 2.4 ppm (data not shown). These data suggest that M7 was the sulfoxidation product of S-methyl omapatrilat at the ring sulfur group. Finally, the fragmentation pattern and the retention time of M7 on HPLC were identical to that of the synthetic ring sulfoxide of S-methyl omapatrilat.

Identification of metabolite M8. The fragmentation pattern for metabolite M8 in the negative ion mode and in the positive ion mode is shown in Table 3. Both negative ion ESI and positive ion ESI generated daughter ions resulting from the loss of a glucuronide. 1H NMR data of M8 are shown in Table 4. The protons of the glucuronide were assigned from the cross peaks observed in the COSY spectra. Based on mass spectral data and 1H NMR, M8 was identified as the acyl glucuronide of S-methyl omapatrilat. Incubation of 0- to 24-h pooled human urine with glucuronidase led to the hydrolysis of M8 (Fig. 4B), which indicated that M8 was a β-glucuronide adduct. The hydrolysis product had the retention time identical to that of the aglycone, S-methyl omapatrilat, in HPLC system II.

Identification of metabolite M9. The fragmentation pattern for metabolite M9, in the negative ion mode and in the positive ion mode, is shown in Table 3. 1H NMR data are shown in Table 4. Based on mass spectral data and 1H NMR, M9 was identified as the S-methylation product of omapatrilat. The fragmentation pattern and the retention time of M9 on HPLC were identical to that of the synthetic reference standard.

Discussion

Excretory profiles of radioactivity in the urine and feces of omapatrilat and its metabolites that retained the radiolabel suggest that the major route of elimination was the kidney. However, the recovery of radioactivity in urine and feces did not completely account for the administered dose of [14C]omapatrilat. Although the reason for incomplete recovery of radioactivity is not entirely clear, possible noncompliance of subjects in complete collection of the excreta may in part be responsible. On the day of discharge after the first dose, the recovery of radioactivity in urine, the major route of elimination, was less than 0.5% of the dose. The other possibility is the binding of omapatrilat to plasma and tissue proteins via reversible disulfide bonds. Plasma profiles of radioactivity showed that nearly 40% of the radioactivity was bound covalently to the protein that was completely released when the sample was treated with DTT. Omapatrilat accounted for nearly half of the released radioactivity in 1-h plasma,
To which the proton is attached. The number corresponds to numbering of the atoms in Fig. 5 of metabolite M8. The glucuronide carbons are numbered from C-1 to C-19.

The main objective of the studies done here was to completely characterize the human urinary metabolites of omapatrilat that retain the $^{13}$C label and in the process identify the major pathways responsible for the metabolism of omapatrilat. The proposed scheme for biotransformation of omapatrilat, based on the isolation and identification of human urinary metabolites, is shown in Fig. 6. Except for the osteocytic disulfide adduct of omapatrilat, M3, all other metabolites identified were methylated at the sulphydryl group. The metabolic pathways identified for omapatrilat include methylation and disulfide conjugation at the free thiol group, glucuronide conjugation of the C-terminal dipeptide mimetic portion necessary for the compound to bind tightly to the active site of the enzyme (Delaney et al., 1994).

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Omapatrilat accounted for 56% of the urinary radioactivity. Because the \(^{14}\)C label was on the carbonyl carbon of \((S)-2\)-thio-3-phenylpropionic acid part of the molecule (Fig. 1) no metabolites derived from the amine side after hydrolysis were identified. However, studies done with \(^{14}\)C label on both sides of the exocyclic amide bond show that the only metabolite derived from the amine side of the molecule after hydrolysis was the intact amine (R. A. Iyer, J. Mitroka, B. Malhotra, S. Bonacorsi, Jr., S. C. Waller, J. K. Rinehart, and K. Kripalani, in preparation). The proposed pathways for the formation of M1 and M2 in vivo from omapatrilat are shown in Fig. 6. M1 and M2, the diastereomeric sulfoxides of \((S)-2\)-thiomethyl-3-phenylpropionic acid, could be generated by three possible pathways. The first pathway could be hydrolysis of omapatrilat to give \((S)-2\)-thio-3-phenylpropionic acid followed by S-methylation to give \((S)-2\)-thiomethyl-3-phenylpropionic acid. \((S)-2\)-Thio-3-phenylpropionic acid and its S-methyl analog \((S)-2\)-thiomethyl-3-phenylpropionic acid were identified as metabolites in pooled human plasma (Table 1, footnote). Sulfoxidation of the S-methyl group could give M1 and M2. The second pathway could involve hydrolysis of S-methyl omapatrilat followed by oxidation of S-methyl group, and the third pathway could be hydrolysis of M5 and M6. It is not known to what extent each pathway contributes to the generation of M1 and M2. Metabolite M2 was the most prominent metabolite after hydrolysis of the exocyclic amide bond. When the ratio of the diastereomeric sulfoxides M1 and M2 was compared, metabolite M2 was formed at a much higher diastereomeric excess (69%) than M1. These data seem to indicate that the stereoselective S-oxygenation of \((S)-2\)-thiomethyl-3-phenylpropionic acid was probably enzymatic because chemically synthesized M1 and M2 were a 1:1 diastereomeric mixture of sulfoxides (data not shown). Enzyme-mediated stereoselective S-oxygenation of a number of sulfur-containing compounds such as cysteine S-conjugates of cis- and trans-1,3-dichloropropene, farnesylcysteine methyl ester, and \(p\)-tolyl methyl sulfide are known (Park et al., 1992, 1994; Sadeque et al., 1992). The third metabolite resulting from hydrolysis was the acyl glucuronide conjugate of \((S)-2\)-thiomethyl-3-phenylpropionic acid, M4, which was probably formed by glucuronidation of the corresponding aglycone.

Other metabolites identified in the urine were formed by modifi-
cation of omapatrilat. No intact omapatrilat was found, suggesting that in vivo omapatrilat is extensively metabolized. The L-cysteine mixed disulfide adduct of omapatrilat, M3, was the only disulfide adduct identified in the human urine. No disulfide adduct of glutathione or N-acetylcysteine was detected in the urine. This result is similar to metabolism of captopril in humans. The only disulfide adduct with endogenous thiols of captopril detected in human urine was the L-cysteine mixed disulfide. However, for captopril this was one of the most prominent metabolite and accounted for 45% of the radioactivity (Migdalof et al., 1984). Whereas for omapatrilat, the L-cysteine mixed disulfide adduct accounted for only 8% of the radioactivity. Also, in human urine no symmetrical disulfide of omapatrilat was detected, whereas in the urine of human subjects treated with 14C-labeled captopril, the symmetrical disulfide accounted for about 6% of the urinary radioactivity (Migdalof et al., 1984). Similarly, administration of D-[35S]penicillamine, a sulfhydril-containing compound, to human subjects led to the appearance of penicillamine-cysteine disulfide and penicillamine disulfide as metabolites in urine (Waring and Mitchell, 1988). These data indicate that disulfide adduct formation is a common metabolic pathway for sulfhydril-containing compounds in vivo even though the amount of various disulfides formed was different. Because disulfide conjugation is reversible in vivo (Gilbert, 1995), circulating cysteine disulfide adduct could be a reservoir for unchanged omapatrilat. This phenomenon has been observed in rats administered captopril-cysteine disulfide where unchanged captopril was identified as a metabolite in plasma (Kripalani et al., 1983).

The remaining metabolites of omapatrilat identified in human urine were derived from S-methyl omapatrilat. Glucuronidation of the carboxyl group gave the acyl glucuronide of S-methyl omapatrilat, whereas sulfoxidation of the S-methyl group or the bicyclic ring sulfur gave the corresponding regioisomeric sulfoxides. The regioisomeric sulfoxides were minor metabolites and accounted for less than 10% of the total radioactivity in human urine.

In summary, omapatrilat was extensively metabolized, and the identified metabolites of omapatrilat accounted for 94% of the radioactivity excreted in the urine. Except for the L-cysteine mixed disulfide adduct, because of its potential to revert to omapatrilat in vivo, all other metabolites identified are expected to be inactive against ACE and NEP.

**Acknowledgments.** We thank the Department of Clinical Pharmacology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, for providing the human plasma and urine samples from their study with radiolabeled drug. We also thank the Department of Analytical Sciences, Discovery Chemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, for providing NMR support for the isolated metabolites.

**References**


Park SB, Howald WN and Cashman JR (1994) S-Oxidative cleavage of farnesylcysteine and


