BIOTRANSFORMATION OF IRBESARTAN IN MAN

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ABSTRACT:
The metabolism of irbesartan, a highly selective and potent nonpeptide angiotensin II receptor antagonist, has been investigated in humans. An aliquot of pooled urine from healthy subjects given a 50-mg oral dose of [14C]irbesartan was added as a tracer to urine from healthy subjects that received multiple, 900-mg nonradioabeled doses of irbesartan. Urinary metabolites were isolated, and structures were elucidated by mass spectroscopy, proton NMR, and high-performance liquid chromatography (HPLC) retention times. Irbesartan and the following eight metabolites were identified in human urine: (1) a tetrazole N2-β-glucuronide conjugate of irbesartan, (2) a monohydroxylated metabolite resulting from ω-1 oxidation of the butyl side chain, (3, 4) two different monohydroxylated metabolites resulting from oxidation of the spirocyclopentane ring, (5) a diol resulting from ω-1 oxidation of the butyl side chain and oxidation of the spirocyclopentane ring, (6) a keto metabolite resulting from further oxidation of the ω-1 monohydroxyl metabolite, (7) a keto-alcohol resulting from further oxidation of the ω-1 hydroxy group of the diol, and (8) a carboxylic acid metabolite resulting from oxidation of the terminal methyl group of the butyl side chain. Biotransformation profiles of pooled urine, feces, and plasma samples from healthy male volunteers given doses of [14C]irbesartan were determined by HPLC. The predominant drug-related component in plasma was irbesartan (76–88% of the plasma radioactivity). None of the metabolites exceeded 9% of the plasma radioactivity. Radioactivity in urine accounted for about 20% of the radiolabeled dose. In urine, irbesartan and its glucuronide each accounted for about 5 to 10% of the urinary radioactivity. The predominant metabolite in urine was the ω-1 hydroxylated metabolite, which constituted about 25% of the urinary radioactivity. In feces, irbesartan was the predominant drug-related component (about 30% of the radioactivity), and the primary metabolites were monohydroxylated metabolites and the carboxylic acid metabolite. Irbesartan and these identified metabolites constituted 90% of the recovered urinary and fecal radioactivity from human subjects given oral doses of [14C]irbesartan.

Irbesartan (SR47436, BMS-186295), chemically designated as 2-butyl-3-[[2′-(1H-tetrazole-5-y1)[1,1′-biphenyl]-4-yl)methyl]-3,3-diazaspiro[4,4]non-1-en-4-one, is a potent, long-acting angiotensin II receptor component (about 30% of the radioactivity), and the primary metabolites were monohydroxylated metabolites and the carboxylic acid metabolite. Irbesartan and these identified metabolites constituted 90% of the recovered urinary and fecal radioactivity from human subjects given oral doses of [14C]irbesartan.

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Abbreviations used are: HPLC, high-performance liquid chromatography; AII, angiotensin II; COSY, 1H-1H correlated spectroscopy; FAB, fast atom bombardment; NOE, nuclear Overhauser effect; SR 49498, 1-[(1-oxopentyl)amino]-N-[2′-(1H-tetrazole-5-y1)[1,1′-biphenyl]-4-yl)methyl]cyclopentane-1-carboxamide; DMSO, dimethyl sulfoxide.

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Fig. 1. Structure of irbesartan.

The site labeled with 14C is indicated with an asterisk. Designations of protons used for the interpretation of the NMR spectra of irbesartan and its metabolites are also shown and are that of Perrier et al. (1994)

(Necciari et al., 1994; Vachharajani et al., 1995). Irbesartan was found to undergo N-glucuronidation on the tetrazole moiety during in vitro incubations with hepatic microsomes from rats, monkeys, and humans (Perrier et al., 1994). In addition to the glucuronide conjugate of irbesartan, a number of oxidized metabolites were identified by
LC/MS as metabolites of irbesartan in *Macaca fascicularis* monkeys (Tronquet et al., 1996). This report describes the isolation and identification of the metabolites of irbesartan from human urine and the quantification of some of these metabolites in plasma, urine, and feces after oral and iv administration of [14C]irbesartan to humans.

**Materials and Methods**

**Chemicals.** [14C]Irbesartan, with a specific radioactivity of 19.8 μCi/mg, was provided by Sanofi Recherche (Montpellier, France). The radiochemical purity of the [14C]irbesartan was 98.8%. Nonradioabeled irbesartan, SR 49498, chemically designated as 1-{[1-oxopentyl]amino}-N-[2-[(1H-tetrazol-5-yl)1,1'-biphenyl]-4-y]methyl)cyclopentanecarboxamide, and the tetrazole N-glucuronide of irbesartan were also obtained from Sanofi Recherche. Metabolites M1 through M8 were isolated from human urine and were used as reference standards. Ecolite liquid scintillation cocktail was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). For solid phase extractions, Bond Elut C18 6-cc cartridges were obtained from Analytech Incorporated (Harbor City, CA). HPLC-grade solvents were used throughout. Acetonitrile was

**Dosage and Sample Collection.** Plasma, urine, and/or fecal samples were obtained from three clinical studies as follows.

**Clinical study 1.** In an open label, balanced, randomized, 3-way crossover study, each of 12 healthy male volunteers received a single 50-mg dose of irbesartan administered as follows: [14C]irbesartan solution infused over 30 min, [14C]irbesartan solution orally or nonradioabeled irbesartan in a capsule. Blood, urine, and fecal samples were collected over 168 hr after the radioabeled doses (75 μCi per subject). HPLC biotransformation profiles were determined for pooled plasma (1 and 6 hr) and urine samples (0–48 hr) from subjects that received [14C]irbesartan in this study. There were numerous missing fecal samples; thus, biotransformation profiles were not determined for fecal samples from this study. This study was later repeated (clinical study 2).

**Clinical study 2.** In an open label, balanced, randomized, 2-way crossover study, each of 6 healthy male volunteers received a single 150-mg oral dose (75 μCi) and a single 50-mg iv dose (75 μCi, 30-min infusion) of [14C]irbesartan on two separate occasions. Blood, urine, and fecal samples were collected over a period of 168 hr. HPLC biotransformation profiles were determined for representative pooled plasma (1 and 6 hr), urine (0–48 hr), and fecal samples (0–120 hr) from this study. These time intervals were chosen for pooling of the urine and fecal samples because most of the radioactivity (90% or greater) recovered in urine was recovered during the first 48 hr after dosing and most of the radioactivity in feces was recovered during the first 120 hr after dosing.

**Clinical study 3.** This study was a placebo-controlled, double blind within dose group, single and multiple dose study. Four groups of 9 volunteers each received a 150, 300, 600, or 900-mg dose of nonradioabeled irbesartan on day 1 followed by placebo for days 2, 3, and 4. The study subjects at each dose level then received a daily dose of irbesartan for 1 week (days 5–11).

For isolation and identification of the metabolites of irbesartan, 500 ml of pooled urine (4–8 hr) from subjects that had been given an oral 50-mg dose of radioalyzed irbesartan in clinical study 1 was added as a tracer to 1500 ml of pooled urine (4–8 hr) collected on day 11 from subjects that received the 900-mg nonradioabeled doses of irbesartan in clinical study 3.

**Biotransformation Profiles.** HPLC was performed on a Hewlett Packard model 1090 liquid chromatograph equipped with a photodiode array ultraviolet detector. Chromatography was performed on a Waters μBondapak C18 analytical column (3.9 × 300 mm, Part No. 27324, Millipore Corp., Milford, MA). A gradient mobile phase system was utilized for determination of biotransformation profiles. The mobile phase consisted initially of 20% acetonitrile and 80% water containing 0.1% diethylamine, adjusted to pH 5.2 with glacial acetic acid. The acetonitrile content of the mobile phase was increased in a linear gradient over a period of 15 min to a final composition of 40% acetonitrile. This composition was then maintained until the end of the run at 25 min. The mobile phase flow rate was 1.0 ml/min. All injections were performed using an automatic injector, which delivered 225 μl of sample. Nonradioabeled reference irbesartan and irbesartan glucuronide (the glucuronide was not added to all samples, as it was in very limited supply) were added to urine and extracts of plasma and feces before HPLC analysis to allow unambiguous identity of the radioactivity corresponding to these metabolites. HPLC fractions were collected at 0.5-min intervals using a Gibson model 202 fraction collector (Gilion Medical Electronics, Middleton, WI). Each fraction of column eluate was mixed with 15 ml of Ecolite and counted for radioactivity using a Packard Tri-Carb 1900CA liquid scintillation analyzer. The radioactive fractions corresponding to each radioactive peak were summed to determine the relative percentage distribution of radioactivity for each metabolite (or metabolites, as some metabolites were not completely resolved by the HPLC system utilized). The assignment of the isolated metabolites (the isolation and identification of the metabolites of irbesartan from urine is described below) to the proper radioactive peaks in the biotransformation profile was further accomplished by separate HPLC runs using the same conditions as described above, where the radioactive metabolites isolated and identified from human urine (M1–M8) were co-injected with the urine or with the extract of the fecal samples (this procedure could not be utilized for plasma because of the low amount of radioactivity in the plasma samples). For HPLC runs with co-injected radioactive metabolites, an increase in a radioactive peak indicated the added metabolite was contained within that peak. A number of co-injection runs had to be performed for each sample to allow unambiguous assignment of the identity of each radioactive peak. Some radioactive peaks contained more than one metabolite, as some metabolites were not completely resolved by the HPLC system utilized.

Specific details for the analysis of pooled human urine, plasma, and fecal samples were as follows.

**Human Urine.** A 360-μl aliquot of human urine was spiked with up to 20 μl of a methanol solution containing irbesartan as a reference standard. Following high speed centrifugation (10,000g) for 1 min, a 225-μl aliquot was injected into the HPLC.

Radioabeled metabolites of irbesartan that were isolated and identified from human urine (M1–M8, see below) were also available to be used as reference standards. Small aliquots (1–10 μl, corresponding to 300–600 dpm) of solutions of the identified metabolites of irbesartan (M1–M8) were added to the pooled urine sample (0–48 hr) obtained after an oral dose in clinical study 2 and analyzed as described above. These separate HPLC profiles were not used for quantification purposes but to establish the identity of the radioactive chromatographic peaks via co-chromatography.

**Human Plasma.** Plasma samples (1.0 ml) were deproteinized by adding 2.0 ml of acetonitrile while the sample was mixed on a Vortex mixer. After centrifugation of the acetonitrile/water mixture for 10 min at 700g, the supernatant fraction was removed and saved, and the protein precipitate was washed twice with 1.0 ml of acetonitrile/water (2:1,v/v). The combined supernatant fractions were evaporated to dryness under nitrogen and reconstituted in 430 μl of the HPLC mobile phase (initial conditions). The extraction recovery from plasma was 90% or greater in all cases. The reconstituted sample was spiked with approximately 20 μl of a methanol solution containing irbesartan and irbesartan glucuronide as a reference standard, and following high speed centrifugation for 1 min, a 225-μl aliquot was injected into the HPLC.

**Human Feces.** Fecal homogenates (1.0 g) were extracted by addition of 2.0 ml of acetonitrile while the sample was mixed on a Vortex mixer. After centrifugation of the acetonitrile/water mixture for 10 min at 700g, the supernatant fraction was removed and saved, and the protein precipitate was washed twice with 2.0 ml of acetonitrile/water (2:1,v/v). The extraction recovery from feces was approximately 100%. A 1.5-ml aliquot of the combined supernatant fraction was evaporated to dryness under nitrogen and reconstituted in 430 μl of the HPLC mobile phase (initial conditions). The reconstituted sample was spiked with 20 μl of a methanol solution containing irbesartan as a reference standard, and following high speed centrifugation for 1 min, a 225-μl aliquot was injected into the HPLC.

To confirm the identity of chromatographic peaks from pooled fecal samples, fecal extracts were co-chromatographed with the isolated metabolites (see below). These separate HPLC analyses were not for quantification of the
ammonium acetate was adjusted to pH 5.2 with glacial acetic acid. Human urine was applied to a column of XAD–2 resin (3 cm). Ammonium acetate was adjusted to pH 5.2 with glacial acetic acid. The acetonitrile content of the mobile phase was increased in a linear gradient from 0 to 48% acetonitrile over 48 min. Incubations were performed using phenolphthalein glucuronide (0.45 mM) as a positive control and with the β-glucuronidase inhibitor, D-saccharic acid 1,4-lactone (24 mM), added to selected incubations to differentiate sulfate activity from β-glucuronidase activity.

Isolation of Metabolites from Human Urine. HPLC was performed on a Hewlett Packard model 1090 liquid chromatograph equipped with a photo-diode array ultraviolet detector. Chromatography was performed on a Whatman Partisil 10 ODS-3 Magnum 9 semi-preparative reverse phase column (25 cm × 4.6 mm, 5 μm). The mobile phase flow rate was 1 ml/min. Fractions of HPLC eluent were collected at 0.5- or 1-min intervals. HPLC runs were profiled for radioactivity by mixing eluent fractions with either 6 or 15 ml of Ecolite and counting using a Packard Tri-Carb 1900CA liquid scintillation analyzer.

Eight different mobile phase compositions, designated HPLC systems I to VIII, were used for isolation of the metabolites of irbesartan from human urine. HPLC system I was the only gradient system used during the isolation of irbesartan metabolites. It was used in the separation of radioactivity into eight fractions designated fractions 1, 2, 3, 4, 5a, 5b, 6, and 7 (Fractions 5a and 5b resulted from a single radioactive peak that appeared to be composed of two or more components). These eight designated fractions were then further purified using HPLC systems II to VIII to yield nine purified metabolites M1, M2, M3, M4, M5, M6, M7, M8, and M9. The mobile phase composition of the different HPLC systems used were as follows.

System I. Isocratic elution with 20% acetonitrile and 80% 0.05 M ammonium acetate was adjusted to pH 5.2 with glacial acetic acid. This composition was then maintained till the end of the run at 65 min.

System II. Isocratic elution with 20% acetonitrile and 80% 0.05 M ammonium acetate was adjusted to pH 5.2 with glacial acetic acid.

System III. Isocratic elution with 21% acetonitrile and 79% 0.05 M ammonium acetate was adjusted to pH 5.2 with glacial acetic acid.

System IV. Isocratic elution with 24% acetonitrile and 76% 0.05 M ammonium acetate was adjusted to pH 4.5 with glacial acetic acid.

System V. Isocratic elution with 24% acetonitrile and 76% 0.05 M ammonium acetate was adjusted to pH 5.2 with glacial acetic acid.

System VI. Isocratic elution with 28% acetonitrile and 72% 0.05 M ammonium acetate was adjusted to pH 4.5 with glacial acetic acid.

System VII. Isocratic elution with 25.5% acetonitrile and 74.5% 0.05 M ammonium acetate was adjusted to pH 5.2 with glacial acetic acid.

System VIII. Isocratic elution with 30% acetonitrile and 70% 0.05 M ammonium acetate was adjusted to pH 5.2 with glacial acetic acid.

The overall scheme used for the isolation of the metabolites of irbesartan from human urine is shown in fig. 2. Briefly, a total volume of 2 liters of human urine was applied to a column of XAD–2 resin (3 × 35 cm) and eluted slowly under gravity. The column was then washed with 1 liter of water using a Lab Pump Jr. (model RHSY, Fluid Metering Inc., Oyster Bay, NY) to maintain the column flow at approximately 2 ml/min. The column was then washed with 1 liter of acetonitrile followed by 1 liter of a mixture of 80% acetonitrile/20% water. All column eluate was collected in 500-ml aliquots and counted for radioactivity. The first 500 ml of acetonitrile eluate and the first 500 ml of 80% acetonitrile/20% water eluate were combined because together they contained 95% of the radioactivity. This combined eluate was evaporated to dryness in vacuo.

The residue from the combined eluate was then dissolved in 25 ml of 0.2 M KH$_2$PO$_4$, pH 2.5. The resulting solution was adjusted to pH 2.8 using dropwise addition of a mixture of concentrated phosphoric acid and water (1:10, v/v) while vortexing. The acidified solution was then extracted twice with 100 ml of water-saturated ethyl acetate in a separatory funnel. The pH of each extract was immediately adjusted to neutrality with dropwise addition of diethylamine. Both ethyl acetate extracts were combined and found to contain 95% of the radioactivity. The combined extracts were evaporated to dryness in vacuo, and the radioactivity redissolved in approximately 6 ml of a mixture of acetonitrile and water.

The sample was subjected to preparative HPLC (system I) in 0.7-ml portions. Each 0.7-ml portion of this sample was evaporated to dryness under a stream of nitrogen and redissolved in 1.8 ml of mobile phase. The samples were each centrifuged at about 10,000g in an Eppendorf model 5415 microcentrifuge (Brinkman Instrument Co., Westbury, NY), and the supernatant was injected into the HPLC. The HPLC eluent was collected in 1-min fractions, and a 25-μl aliquot of each fraction was counted in 6 ml of Ecolite. Radioactive fractions from each preparative run were combined in eight fractions designated fractions 1, 2, 3, 4, 5a, 5b, 6, and 7. Each fraction was evaporated to dryness in vacuo and redissolved in 2.0 ml of acetonitrile.

Fraction 5 was divided into two fractions because UV analysis indicated at least two components were present (5a had a retention time of 40 min and 5b had a retention time of 41 min). Fraction 5a was further purified into two metabolites by chromatography using system VI.

Purification of Fractions 1, 2, 3, 4, 5b, 6, and 7. Each fraction was further purified to yield a single metabolite by use of preparative HPLC utilizing the mobile phase systems indicated in fig. 2. This was accomplished by evaporating each fraction to dryness, redissolving the residue in mobile phase, and making multiple injections of 1.8 ml each until the entire fraction was processed. Eluate fractions from all runs that corresponded to a single peak were combined and evaporated to dryness in vacuo. The metabolites were each redissolved in approximately 3 ml of an acetonitrile/water mixture. To remove salt remaining from the mobile phase, each metabolite solution was submitted to solid phase extraction. The resulting solutions were each evaporated to dryness under nitrogen, and the residues were each redissolved in 25 ml of water. These aqueous samples were each loaded onto two 6-cc C$_8$ solid phase cartridges, which had been mounted one on top of the other. The cartridges were each washed with 25 ml of water, 25 ml of acetonitrile, and 25 ml of acetonitrile/water (50:50). The final two washes, which contained the metabolites, were combined and evaporated to dryness in vacuo and redissolved in 1 or 2 ml of methanol. Following HPLC analysis, which indicated each metabolite was pure, each of the metabolites (M1, M2, M3, M4, M7, M8, and M9) were submitted for NMR and mass spectral analysis.

Purification of Fraction 5a. Fraction 5a contained two metabolites (M5 and M6), which were purified by preparative HPLC system VI. The column eluate corresponding to M5 was combined and evaporated to dryness under nitrogen and submitted to solid phase extraction as previously described. M5 was redissolved in 2 ml of methanol. HPLC analysis indicated that M5 was pure. The column eluate corresponding to M6 was combined and evaporated to dryness under nitrogen and submitted to solid phase extraction as previously described. HPLC analysis indicated that M6 still contained some M5. The sample was chromatographed in HPLC system VI. Column eluate that corresponded to M6 was combined and evaporated to dryness in vacuo. M6 was submitted to solid phase extraction as previously described and was redissolved in 2 ml of methanol. HPLC analysis indicated that M6 was pure.

Spectroscopy. Proton NMR spectroscopy was performed on a Varian Unity Plus-400. All compounds were dissolved in DMSO-d$_6$. All chemical shifts (δ$_H$) are reported in ppm relative to tetramethylsilane as an internal standard. One-dimensional spectra were collected using both the 5P2UL and PRESAT pulse sequences, whereas GCOSY and CYCLENOE pulse sequences were used for acquiring two-dimensional correlation spectra and one-dimensional NOE data, respectively.

Mass spectral analyses of all standards and isolated metabolites were performed on both a Sciex API-III and a JEOL HX-110 mass spectrometer. Ionization techniques employed included FAB and ion spray. FAB was performed on the JEOL HX-110 to yield exact mass and high resolution results. The FAB matrix utilized consisted of thioglycolic acid (50:50). All MS/MS mass spectrometry was performed on a Sciex API-III triple quadrupole. Samples were infused at a flow rate of 3 μl/min in a mobile phase of 20 mM ammonium acetate, pH 5, and methanol (90:10). The MS/MS collision gas was argon, and the collision energy was 50 eV.
Biotransformation profiles of pooled plasma (6 hr), urine (0–48 hr), and fecal samples (0–120 hr) from six healthy male volunteers given a single 150-mg oral dose of [14C]irbesartan are shown in fig. 3. Reference standards or previously isolated radioactive metabolites of irbesartan (M1–M8, isolation and identification of these metabolites from urine are discussed below) were co-injected with the pooled urine and fecal samples for determination of their retention times in this HPLC system. Co-injection of samples with isolated radioactive metabolites was accomplished in separate HPLC runs. The retention times of these metabolites are indicated in fig. 3, and the relative distribution of each metabolite in pooled plasma (1 and 6 hr), urine (0–48 hr), and feces (0–120 hr) is summarized in table 1. Biotransformation profiles of pooled plasma (1 and 6 hr) and urine (0–48 hr) from a separate clinical study where 12 healthy male volunteers received single 50-mg oral and iv doses of [14C]irbesartan (clinical study 1) gave similar results.

Biotransformation profiles of the plasma samples also were similar after either oral or iv administration of [14C]irbesartan. At 1 hr after drug administration, the amount of unchanged irbesartan ranged from about 81 to 88% of the plasma radioactivity, and the amount of glucuronide (M8) was about 7% of the plasma radioactivity; none of the other metabolites constituted greater than 4% of the plasma radioactivity. At 6 hr, the amount of unchanged irbesartan ranged from about 76 to 83% of the plasma radioactivity, and the amount of the glucuronide conjugate (M8) was about 5% of the plasma radioactivity. Other metabolites were present to a greater extent at 6 hr than at 1 hr but still constituted a minor portion of the plasma radioactivity, with the carboxylic acid metabolite (M3) accounting for about 9% and other metabolites less than 5% of the plasma radioactivity. These minor metabolites were estimated solely based upon retention times, as the peaks were barely above background noise and were too small to be properly co-chromatographed with isolated metabolites.

Biotransformation profiles of urine samples were also similar after oral or iv administration of [14C]irbesartan. Recovery of radioactivity in urine accounted for about 20% of the radiolabeled dose. In pooled human urine (0–48 hr), irbesartan and its glucuronide conjugate (M8) each accounted for about 10% or less of the urinary radioactivity. The predominant metabolite excreted in urine was M4, the α-1 monohydroxylated metabolite, which constituted approximately 25% of the urinary radioactivity. The carboxylic acid metabolite (M3) constituted approximately 15% of the urinary radioactivity. Irbesartan and the
eight metabolites identified accounted for about 90% of the urinary radioactivity (table 1).

Table 1: Biotransformation profiles of irbesartan in representative pooled plasma, urine, and fecal samples, as determined by HPLC, after a single 50-mg iv and 150-mg oral dose of [14C]irbesartan to six healthy subjects

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Percentage Distribution of Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma (1 hr)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>iv</td>
</tr>
<tr>
<td>M1 + M2</td>
<td>0.4</td>
</tr>
<tr>
<td>M3</td>
<td>0.5</td>
</tr>
<tr>
<td>M4</td>
<td>0.5</td>
</tr>
<tr>
<td>M5 + M6 + M7</td>
<td>2.5</td>
</tr>
<tr>
<td>M8</td>
<td>7.2</td>
</tr>
<tr>
<td>M9 (Irbesartan)</td>
<td>87.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentrations (ng-eq of irbesartan per ml) of total radioactivity were 2980 (po) and 1280 (iv) in the pooled 1-hr plasma samples and 495 (po) and 231 (iv) in the pooled 6-hr plasma samples.

<sup>b</sup> Recoveries of the radioactive doses were 17% (po) and 19% (iv) in urine over a 48-hr collection period and 54% (po) and 50% (iv) in feces over a 120-hr collection period.

About 30% of the fecal radioactivity corresponded to irbesartan; the remainder corresponded primarily to the metabolites identified in urine (table 1 and fig. 3). About 3.6% of the fecal radioactivity corresponded to SR 49498 after either the oral or iv dose of [14C]irbesartan. SR49498 is the dihydroimidazole ring-opened product of irbesartan (structure shown in fig. 4) and was present as a minor impurity in the radiolabeled dose.

For metabolite isolation, an aliquot of the urine collected from subjects given an oral 50-mg dose of radiolabeled irbesartan in clinical study 1 was added as a tracer to urine collected on day 11 from
subjects that received the 900-mg nonradiolabeled doses of irbesartan in clinical study 3. Urinary metabolites were concentrated on an XAD-2 column, extracted with ethyl acetate, and purified by extensive preparative HPLC to give metabolites M1 through M9.

Structural elucidation of the isolated metabolites was based upon mass spectrometry, proton NMR, COSY analysis, and chromatographic retention time using HPLC. Mass spectral analyses were performed using two different ionization techniques. Ion spray MS/MS produced ions resulting from cleavage of the methylene bridge between the butyl diazospirononenone and the biphenyl tetrazole moieties of irbesartan and its metabolites. This fragmentation of the molecule into two readily interpretable product ions allowed metabolic changes to be assigned to one or the other of these two ring systems. Fast atom bombardment (FAB) on the JEOL HX–110 mass spectrometer gave mass spectra of high resolution, allowing the determination of the exact mass of the (M+H) ion of the isolated metabolites. A fragmentation scheme and a summary of both the ion spray MS/MS and FAB exact mass determinations are shown in table 2.

NMR spectral data are summarized in table 3. Proton NMR and interpretable COSY spectra were obtained on each metabolite. Initial attempts to obtain interpretable proton NMR spectra of the metabolites in MeOH-d4 failed due to the gradual exchange of the 1b protons of the butyl side chain with deuterium. Thus, the proton NMR of all metabolites was determined in DMSO–d6. The aromatic protons of the two phenyl rings present in irbesartan are clearly visible downfield in the NMR spectra between about δ 6.8 and δ 7.7. The methyl protons of the butyl side chain demonstrate a distinct triplet at about δ 0.8, and the methylene protons of this same butyl side chain are differentiated from each other at chemical shift values between δ 1.2 and δ 2.4. The benzylic methylene protons produced a sharp singlet at about δ 4.66.

A scheme for the biotransformation of irbesartan in humans is presented in fig. 4. The evidence and the rationale for the structures assigned to these nine metabolites isolated from human urine follow.

**Identification of M1.** FAB mass spectrometry of M1 produced a protonated molecular ion (M+H) of m/z 461. Exact mass determination of the (M+H) ion resulted in an experimental value of m/z 461.2301 (m/z 461.2301 calculated for C25H29O3N6), which was consistent with dihydroxylation of irbesartan. MS/MS of the m/z 461 parent ion using ion spray resulted in product ions at m/z 227 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 227 indicated two additional oxygen atoms were added to the butyl diazospirononenone ring of irbesartan. The proton NMR of this metabolite suggested that ω-1 oxidation of the butyl side chain had occurred. Consistent with this interpretation is the presence of a midfield proton at δ 3.58, the downfield shift of the 4b protons from δ 0.8 to δ 1.0 when compared with the respective protons of irbesartan and the conversion of these same 4b protons from a triplet to a doublet. The COSY analysis of M1 was consistent with these observations.
with this assignment of the butyl side chain protons. The proton NMR of this metabolite also indicated that hydroxylation of the spirocyclopentane ring had occurred. An oxygen-bearing methine proton with a chemical shift of δ 4.37 was observed, and the COSY spectrum demonstrated pronounced cross-peaks between this proton and other protons on the spirocyclopentane ring. The number and nature of the cross-peaks in the COSY spectrum indicated a pronounced cross-peak between proton 2b and the spiro carbon atom (designated H$_2$ or H$_3$ in the NMR numbering scheme). NOE analysis was not able to differentiate between the two possible sites of hydroxylation because of the spiro nature of the pentane ring. M1 was identified as a dihydroxy metabolite of irbesartan, with hydroxylation occurring at the ω-1 position of the butyl side chain and at one of the positions β to the spiro carbon atom.

**Identification of M2.** FAB mass spectrometry of M2 produced a protonated molecular ion (M+H)$^+$ of m/z 459. Exact mass determination of the (M+H)$^+$ ion resulted in an experimental value of m/z 459.2133 (m/z 459.2145 calculated for C$_{25}$H$_{27}$O$_3$N$_6$+$^+$), which was consistent with dihydroxylation and further oxidation of irbesartan. MS/MS analysis of the m/z 459 parent ion using ion spray resulted in product ions at m/z 235, 207, and 192 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 225 indicated two additional oxygen atoms were present on the butyl diazospironenone ring and that, in addition to dihydroxylation, further oxidation had occurred on this part of the molecule.

The proton NMR of M2 indicated that hydroxylation of the spirocyclopentane had occurred. A midfield proton at δ 4.34 was observed, and the COSY spectrum of this metabolite demonstrated pronounced cross-peaks between this proton and other protons on the spirocyclopentane ring. The number and nature of the cross-peaks in the COSY spectrum of this metabolite and the complexity of the proton at δ 4.34, a multiplet instead of a triplet, indicated the site of hydroxylation was β to the spiro carbon atom. Analysis of the protons of the butyl side chain of this metabolite indicated a keto group at the ω-1 position. The terminal methyl protons (4b) had a chemical shift of δ 0.3, a multiplet instead of a triplet, indicated the site of hydroxylation was δ to the spiro carbon atom. Analysis of the protons of the butyl side chain were obscured by a large solvent peak, but the COSY spectrum indicated a pronounced cross-peak between proton 2b and protons at the chemical shift of the solvent peak, confirming this assignment. M2 was identified as the further oxidized product of M1 with the oxidation occurring at the ω-1 hydroxyl group.

**Identification of M3.** FAB mass spectrometry of M3 produced a protonated molecular ion (M+H)$^+$ of m/z 459. Exact mass determination of the (M+H)$^+$ ion resulted in an experimental value of m/z 459.2148 (m/z 459.2144 calculated for C$_{25}$H$_{27}$O$_3$N$_6$+$^+$), which was consistent with dihydroxylation and further oxidation of irbesartan. MS/MS analysis of the m/z 459 parent ion using ion spray resulted in product ions at m/z 235, 207, 192, and 180 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 225 indicated two additional oxygen atoms were present on the butyl diazospironenone ring and that, in addition to dihydroxylation, further oxidation also occurred on this part of the molecule.

The proton NMR of M3 indicated no change in the spirocyclopentane ring as compared with irbesartan and that only the butyl side chain had undergone metabolism. The protons of the terminal methyl group of the butyl side chain were absent, and the 3b protons had shifted downfield to δ 2.23. Based on these data, M3 was identified as the 4b-carboxylic acid derivative of irbesartan.
### TABLE 3

Proton NMR data of irbesartan and its metabolites determined in DMSO-d$_6$.

Values are chemical shifts (in ppm). b, broad; s, singlet; d, doublet; t, triplet; q, quartet; quin, quintuplet; m, multiplet.

<table>
<thead>
<tr>
<th>1H</th>
<th>Irbesartan</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
<th>M9</th>
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<td>8'</td>
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<td>7.63 (1H,d)</td>
<td>7.64 (1H,d)</td>
<td>7.62 (1H,d)</td>
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<td>7.62 (1H,d)</td>
<td>7.8 (1H,d)</td>
<td>7.56 (1H,d)</td>
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<td>7.6 (1H,s)</td>
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<td>7.54 (1H,d)</td>
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<td>7.62 (1H,d)</td>
<td>7.44 (1H,d)</td>
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<td>7.52 (1H,d)</td>
<td>7.53 (1H,d)</td>
<td>7.48 (1H,d)</td>
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<td>7.48 (1H,d)</td>
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<td>7.45 (1H,d)</td>
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<td>7.34 (1H,d)</td>
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<tr>
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<td>7.08 (2H,d)</td>
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<td>7.07 (4H,d)</td>
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<td>7.08 (2H,d)</td>
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<td>7.04 (2H,d)</td>
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<td>4.65 (2H,s)</td>
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<td>4b-Me</td>
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<td>3.58 (1H,m)</td>
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<td>2.23 (2H,t)</td>
<td>3.59 (1H,m)</td>
<td>1.24 (2H,m)</td>
<td>—</td>
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<tr>
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<td>2.07 (3H,s)</td>
<td>—</td>
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<tr>
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<td>1.66 (2H,b)</td>
<td>1.65 (2H,b)</td>
<td>1.87 (1H,d)</td>
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<td>1.53 (1H,m)</td>
<td>1.63 (2H,b)</td>
</tr>
</tbody>
</table>

* Proton was hidden under DMSO-d$_6$ peak. COSY analysis indicated a strong correlation between 2b and the solvent peak.
* Proton was hidden under water peak from solvent.
* The glucuronic acid protons.

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IRBESARTAN BIOTRANSFORMATION

[Downloaded from dans.mephys.org on October 19, 2017]
Identification of M4. FAB mass spectrometry of M4 produced a protonated molecular ion (M+H)^+ of m/z 445. Exact mass determination of the (M+H)^+ ion resulted in an experimental value of m/z 445.2372 (m/z 445.2352 calculated for C_{25}H_{27}O_{2}N_{6}^+), which was consistent with monohydroxylation of irbesartan. MS/MS of the m/z 445 parent ion using ion spray resulted in product ions at m/z 235, 207, 192, and 180 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 211 indicated one additional oxygen atom was present on the butyl diazisopropionenone ring.

The proton NMR of this isolated metabolite suggested that ω-1 oxidation of the butyl side chain had occurred. Consistent with this interpretation was the presence of an additional midfield methine proton at δ 3.59, the downfield shift of the methyl protons from δ 0.8 to δ 1.0 when compared with the respective protons of irbesartan and the conversion of these same methyl protons from a triplet to a doublet. The COSY analysis of M4 was consistent with this assignment of the butyl side chain protons. No changes were noted with the protons of the spiropencyclopentane ring as compared with irbesartan. M4 was identified as the ω-1 hydroxylated derivative of irbesartan.

Identification of M5. FAB mass spectrometry of M5 produced a protonated molecular ion (M+H)^+ of m/z 445. Exact mass determination of the (M+H)^+ ion resulted in an experimental value of m/z 445.2350 (m/z 445.2352 calculated for C_{25}H_{29}O_{2}N_{6}^+), which was consistent with hydroxylation of irbesartan. MS/MS of the m/z 445 parent ion using ion spray resulted in product ions at m/z 235, 207, 192, and 180 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 211 indicated one additional oxygen atom was present on the butyl diazisopropionenone ring.

The proton NMR of this metabolite showed an oxygen-bearing methine proton with a chemical shift of δ 4.37, and the COSY spectrum of this metabolite demonstrated pronounced cross-peaks between this proton and other protons on the spiropencyclopentane ring. The number and nature of the cross-peaks in the COSY spectrum of this metabolite and the complexity of the proton at δ 4.37, a multiplet instead of a triplet, indicated the site of hydroxylation was ω-1 to the spiro carbon atom. NOE analysis was not able to differentiate between the possible sites of hydroxylation due to the spiro nature of the pentane ring. M5 was identified as a monohydroxylated metabolite of irbesartan, with hydroxylation occurring ω-1 to the spiro carbon of the spiropencyclopentane ring.

Identification of M6. FAB mass spectrometry of M6 produced a protonated molecular ion (M+H)^+ of m/z 443. Exact mass determination of the (M+H)^+ ion resulted in an experimental value of m/z 443.2183 (m/z 443.2195 calculated for C_{25}H_{29}O_{2}N_{6}^+), which was consistent with hydroxylation and further oxidation of irbesartan. MS/MS of the m/z 443 parent ion using ion spray resulted in product ions at m/z 235, 207, 192, and 180 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 209 indicated one additional oxygen atom was present on the butyl diazisopropionenone ring and that, in addition to hydroxylation, further oxidation also occurred on this part of the molecule.

No oxygen-bearing methine protons were observed in the proton NMR spectrum. The terminal methyl protons (4b) at δ 2.07 were observed as a singlet, which was consistent with being adjacent to a keto group. Other protons (1b and 2b) of the butyl side chain were shifted downfield when compared with the respective protons in irbesartan, also consistent with an adjacent keto group. The 1b protons of the side chain were partially obscured by the large solvent peak at δ 2.5, but the COSY spectrum indicated a pronounced cross-peak between proton 2b and the protons at the chemical shift of the solvent peak, confirming this assignment. M6 was identified as the further oxidized product of M4 and was assigned the structure of the ω-1 keto derivative of irbesartan.

Identification of M7. FAB mass spectrometry of M7 produced a protonated molecular ion (M+H)^+ of m/z 445. Exact mass determination of the (M+H)^+ ion resulted in an experimental value of m/z 445.2341 (m/z 445.2352 calculated for C_{25}H_{29}O_{2}N_{6}^+), which was consistent with monohydroxylation of irbesartan. MS/MS of the m/z 445 parent ion using ion spray resulted in product ions at m/z 235, 207, 192, and 180 indicative of an unchanged biphenyl tetrazole ring system. A product ion at m/z 211 indicated one additional oxygen atom was present on the butyl diazisopropionenone ring.

The proton NMR of this metabolite was similar to M5 and suggested that hydroxylation of the spiropencyclopentane ring had occurred. An oxygen-bearing methine proton with a chemical shift of δ 4.32 was observed, and the COSY spectrum of this metabolite demonstrated pronounced cross-peaks between this proton and other protons on the spiropencyclopentane ring. The number and nature of the cross-peaks in the COSY spectrum of this metabolite and the complexity of the proton at δ 4.32, a multiplet instead of a triplet, indicated the site of hydroxylation was ω-1 to the spiro carbon atom. M7 was identified as a monohydroxylated metabolite of irbesartan, with hydroxylation occurring ω-1 to the spiro carbon of the spiropencyclopentane ring.

Identification of M8. The isolated M8 co-chromatographed with authentic tetrazole N^2-glucuronide of irbesartan obtained from Sanofi. FAB mass spectrometry of M8 produced a protonated molecular ion (M+H)^+ of m/z 605. Exact mass determination of the (M+H)^+ ion resulted in an experimental value of m/z 605.2744 (m/z 605.2724 calculated for C_{31}H_{37}O_{7}N_{6}^+), which was consistent with conjugation of irbesartan with glucuronic acid. MS/MS of the m/z 605 parent ion using ion spray resulted in a product ion at m/z 429, indicating the loss of glucuronic acid to give irbesartan. The product ion at m/z 383 (207 + 176), resulting from cleavage of the bond between the biphenyl tetrazole ring system and the methylene bridge carbon, confirmed the position of glucuronidation as the tetrazole ring.

The proton NMR of this metabolite was similar to the proton NMR of the tetrazole N^2-glucuronide of irbesartan isolated from an in vitro incubation of irbesartan with hepatic microsomal fraction from Cynomolgus monkeys (Perrier et al., 1994). Based on these results, M8 was identified as the tetrazole N^2-glucuronide of irbesartan.

Identification of M9. The isolated M9 co-chromatographed with irbesartan. FAB mass spectrometry of M9 produced a protonated molecular ion (M+H)^+ of m/z 429. Exact mass determination of the (M+H)^+ ion resulted in an experimental value of m/z 429.2399 (m/z 429.2403 calculated for C_{23}H_{29}O_{3}N_{6}^+), which was consistent with M9 being irbesartan. MS/MS of the m/z 429 parent ion using ion spray gave the same product ion spectrum as authentic irbesartan.

The proton NMR of M9 was also similar to the proton NMR of the reference standard. M9 was identified as irbesartan based on these results.

Discussion

The primary route of metabolism of irbesartan in humans was oxidative leading to mono- and dihydroxylated metabolites or metabolites resulting from further oxidation of these hydroxylated metabolites (i.e. keto, hydroxy-keto, and carboxyl metabolites). Hydroxylation occurs primarily at the ω-1 carbon of the butyl side chain or on the spiropencyclopentane ring. Hydroxylation of the spiropencyclopentane ring occurs on the carbon atom ω-1 to the spiro carbon atom. As two stereochemical orientations are possible at each of these carbon atoms, there are four possible isomers. Two of the three monohy-
droxylated metabolites identified were isomers resulting from hydroxylolation of the spiropentyl ring β to the spiro carbon atom. Attempts to distinguish between these isomers by use of NOE techniques were unsuccessful. Glucuronidation of the N2 nitrogen of the tetrazole ring was also observed in humans. This tetrazole-N2-β-glucuronide was previously identified from in vitro incubations of irbesartan with microsomes from rat, monkey, and humans (Perrier et al., 1994). A tetrazole-N2-β-glucuronide conjugate has been identified as a metabolite of losartan (Stearns et al., 1991, 1992) and other biphenyl tetrazole AII receptor antagonists (Huskey et al., 1993; Kondo et al., 1996).

Irbesartan was the largest drug-related component in feces (about 30% of recovered radioactivity). Any irbesartan glucuronide conjugate excreted in the bile would be hydrolyzed by intestinal microflora to irbesartan. Thus, the irbesartan found in feces may represent excretion of both intact irbesartan and its glucuronide conjugate. The polar M1 seems to present to a lesser extent in the feces; however, the other urinary metabolites are also present in feces. A minor amount of SR 49498 was observed in the biotransformation profiles, but the amount was only slightly more than the amount of SR 49498 present as an impurity in the radiolabeled dose of irbesartan. In fact, fecal homogenates spiked with [14C]irbesartan showed additional minor amounts (1–2%) of SR 49498 apparently formed during storage at −20°C. Incubation of fecal homogenate containing [14C]irbesartan for 18 hr at 37°C indicated little, if any, metabolism of irbesartan by the microflora present in the fecal samples. This suggests the SR 49498 observed in the biotransformation profiles resulted from the minor amount of impurity in the irbesartan dose with a trace amount of degradation of irbesartan that occurred during storage. However, the production of minor amounts of SR 49498 as a result of systemic metabolism cannot be excluded.

N-Dealkylation of irbesartan does not seem to be a major metabolic pathway in humans. N-Dealkylation of losartan was observed in incubations with liver slice preparations from rats and humans (Stearns et al., 1992). This cleavage was postulated to occur via oxidation of the benzylic carbon atom of losartan, followed by decomposition of the resulting hemiaminal metabolite. An analogous N-dealkylation is possible with irbesartan. If this cleavage were to occur with the [14C]irbesartan used for the clinical studies, the label would be retained in the resulting dihydromidazole moiety. This N-dealkylation of irbesartan has been observed in monkey (Tronquet et al., 1996). Three monohydroxylated metabolites of the butyl spiropentyl dihydroimidazole moiety were identified in the urine of monkeys given a 10 mg/kg oral dose of irbesartan. These metabolites would be expected to elute at or near the void volume in the HPLC system used for this study (fig. 3). The unidentified radioactivity in urine that eluted as polar metabolites at or near the void volume (fig. 3) could be metabolites resulting from N-dealkylation of irbesartan. However, this radioactivity corresponds to less than 10% of the urinary radioactivity and thus must be a minor metabolic pathway.

None of the identified human metabolites of irbesartan (M1–M8) are of greater potency than irbesartan (J. Gougat, unpublished results). M3, the major circulating metabolite in plasma (about 9% of the plasma radioactivity at 6 hr), was found to be more than 1000-fold less potent than irbesartan as an inhibitor of the AII receptor. These results suggest that irbesartan, and not its metabolites, is responsible for inhibition of the AII-mediated pressor response in humans.

Acknowledgments. We thank Dr. Maria Marino of the Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ) for the urine samples from healthy subjects given multiple 900-mg doses of irbesartan.

References


We thank Dr. Maria Marino of the Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ) for the urine samples from healthy subjects given multiple 900-mg doses of irbesartan.