Metabolism and Pharmacokinetics of Indacaterol in Humans

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ABSTRACT:

The metabolism, pharmacokinetics, and excretion of [14C]indacaterol were investigated in healthy male subjects. Although indacaterol is administered to patients via inhalation, the dose in this study was administered orally. This was done to avoid the complications and concerns associated with the administration of a radiolabeled compound via the inhalation route. The submilligram doses administered in this study made metabolite identification and structural elucidation by mass spectrometry especially challenging. In serum, the mean C_{max}, t_{max}, and AUC_{0-last} values were 1.75 h, 0.47 ng/ml, and 1.81 ng·h/ml for indacaterol and 2.5 h, 1.4 ngEq/ml, and 27.2 ngEq·h/ml for total radioactivity. Unmodified indacaterol was the most abundant drug-related compound in the serum, contributing 30% to the total radioactivity in the AUC_{0-24h} pools, whereas monohydroxylated indacaterol (P26.9), the glucuronide conjugate of P26.9 (P19), and the 8-O-glucuronide conjugate of indacaterol (P37) were the most abundant metabolites, with each contributing 4 to 13%. In addition, the N-glucuronide (2-amino) conjugate (P37.7) and two metabolites (P38.2 and P39) that resulted from the cleavage about the aminomethyl group linking the hydroxquinolinone and diethylidene moieties had a combined contribution of 12.5%. For all four subjects in the study, ≥90% of the radioactivity dose was recovered in the excreta (85% in feces and 10% in urine, mean values). In feces, unmodified indacaterol and metabolite P26.9 were the most abundant drug-related compounds (54 and 17% of the dose, respectively). In urine, unmodified indacaterol accounted for ~0.3% of the dose, with no single metabolite accounting for >1.3%.

Introduction

Indacaterol (5-[(R)-2-(5,6-diethyl-indan-2-ylamino)-1-hydroxy-ethyl]-8-hydroxy-1Hquinolin-2-one; QBA149) is a novel, long-acting inhaled β_{2}-adrenergic receptor agonist intended for long-term, once-daily, maintenance treatment in patients with chronic obstructive pulmonary disease (COPD) (Batram et al., 2006). Currently, it has received regulatory approval in the United States, Japan, the European Union, and a number of other countries worldwide at once-daily doses ranging from 75 to 300 μg. Indacaterol’s once-daily treatment favorably differentiates it from other currently marketed long-acting inhaled β_{2}-adrenergic receptor agonists, such as formoterol and salmeterol, which require more frequent dosing for regular maintenance treatment of asthma or COPD (Cazzola and Matera, 2008). In an Internet-based questionnaire study involving 1470 patients with asthma and/or pulmonary disease (COPD) (Batram et al., 2006), it has been shown to have a wide therapeutic margin, with once-daily doses up to 600 μg being safe and well tolerated in studies of up to 1 year’s duration. At doses of 75 μg and greater, indacaterol has demonstrated efficacy similar to or better than that of current standard bronchodilators (Dahl et al., 2010; Cope et al., 2011).

The objectives of the current [14C]indacaterol human absorption, distribution, metabolism, and excretion (ADME) study were to identify and quantify indacaterol and its metabolites in serum, urine, and feces, to determine the rate and routes of indacaterol excretion, to characterize the pharmacokinetics of total radioactivity in blood and serum, and to evaluate the completeness of the dose recovery in excreta. This information was critical for verifying the suitability of the preclinical safety studies and for designing appropriate clinical drug-drug interaction studies. The current study was especially challenging from an analytical sensitivity perspective due to the low dose (800 μg) that was administered.

Materials and Methods

Clinical ADME Study Design. This was an open-label, single-dose study in which four healthy adult male subjects each received a single 800-μg (free base) oral dose of indacaterol maleate under fasted conditions. Before the study started, an institutional review committee approved the protocol and the informed consent document. All of the study participants gave written in-
formed consent before the initiation of the study. Each dose contained 50 μCi of [14C]indacaterol. On the day before dosing, the subjects reported to the study site at least 16 h before dosing to undergo safety evaluations and to confirm eligibility. Eligible subjects remained domiciled until adequate recovery of the radioactive dose was confirmed (up to 312 h postdose). Subjects fasted for at least 10 h before receiving their dose. The dose was administered with 240 ml of water. Adequate recovery was defined as ≥85% of the radioactivity dose, with ≤1% of the dose being collected in consecutive urine and/or feces samples. Serum was collected at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h postdose. Urine was collected in discrete intervals (0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144, 144–168, 168–192, 192–216, 216–240, 240–264, 264–288, and 288–312 h postdose), whereas all of the fecal samples were collected individually up to 312 h postdose. All of the samples were analyzed for total radioactivity, whereas select samples also were analyzed for their metabolite content. In addition, the serum samples were analyzed by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for indacaterol. No medications other than indacaterol were allowed from the start of screening until the end of the study, except for paracetamol and any other medications that were needed to treat adverse events (documented in the Case Report Forms).

**Radiation Safety of Subjects.** The expected radiation exposure of a subject receiving a 50-μCi oral dose of [14C]indacaterol was estimated prognostically according to regulatory guidelines (Code of Federal Regulations, 2004). The estimate was based on available human pharmacokinetic data and animal ADME data for indacaterol. The results predicted that the exposure in the organ expected to receive the highest radioactivity dose, the colon, was 21-fold higher than the 3 roentgen equivalent in man limit set by the regulations. Hence, the radiation risks in the current study were estimated to be low.

**Test Article.** The high specific activity batch of radiolabeled indacaterol was prepared by the Isotope Laboratory within the Department of Drug Metabolism and Pharmacokinetics at the Novartis Institutes for Biomedical Research (East Hanover, NJ). The high specific activity [14C]indacaterol was diluted with unlabeled indacaterol to provide capsules containing nominally 25 μCi of radioactivity in 400 μg of indacaterol. The position of the 14C label was at the benzyl carbon of one of the two ethyl side chains of the diethylindane moiety, as shown in Fig. 1. The chemical and radiochemical purity of the final drug substance was verified to be ≥98% from the time of manufacture to dose administration.

**Collection of Biological Samples.** Blood samples were taken by either direct venipuncture or an indwelling cannula inserted into a forearm vein, at the times specified above. At each of these time points, blood was collected into four separate tubes to determine blood total radioactivity, serum total radioactivity, serum pharmacokinetics, and serum metabolite characterization. All of the samples (blood, serum, urine, and feces) were kept frozen at or below −20°C until the time of analysis.

**Measurement of Indacaterol Concentrations in Serum.** The 0.20-ml aliquots of serum samples, blanks, standards, and quality controls were transferred into the appropriate wells of a 2-ml, 96-well block. For the blanks, quality controls, and standards, blank human serum was transferred. Calibration standards were prepared daily in duplicate by adding 20 μl of the appropriate spiking solution to the 0.2 ml of blank human serum and mixing thoroughly for 60 s. A 25-μl aliquot of the internal standard solution [M + 4] [14CD3]indacaterol (62.4 ng/ml) was added to each well except the blank samples, and the samples were vortexed again. A 1-ml aliquot of methyl tert-butyl ether was added to each well. The block then was capped and vortexed for an additional 5 min. The 96-well block was centrifuged for 10 min at approximately 1000g. The organic layer was transferred into a clean 96-well block using a workstation (Tomtec, Hamden, CT) and evaporated to dryness under a nitrogen flow at 45°C. After complete evaporation, the samples were reconstituted with 150 μl of reconstitution solution (methanol/water, 35:65, v/v containing 0.1% formic acid). A 10-μl aliquot of the extract was injected into the LC-MS/MS system.

The samples were analyzed by LC-MS/MS using electrospray ionization in the positive ion mode with multiple reaction monitoring (MRM). [12C]Indacaterol was monitored by the m/z 393.1 → m/z 116.8 transition, and [M + 4] [14CD3]indacaterol was monitored by the m/z 397.4 → m/z 118.0 transition. Response factors for [14C]indacaterol were calculated by dividing the intensity of its mass spectrometric response by the intensity of the mass spectrometric response for the internal standard. Calibration curves for [14C]indacaterol were constructed by plotting the response factors of the standard solutions versus their known concentrations. The calibration curves were fit to the response factor × concentration data using a quadratic regression with 1/x2 weighting. The lower limit of quantitation (LLOQ) for [14C]indacaterol was 0.050 ng/ml using 0.2 ml of human serum. Concentrations below the LLOQ were reported as 0 ng/ml. A multiplicative factor of 1.58 was applied to the measured [14C]indacaterol concentrations to obtain the total indacaterol concentrations (1°C + 1°C) reported in this publication.

**Measurements of Total Radioactivity in Serum.** Total radioactivity in serum was determined by analyzing a serum aliquot from each time point using a liquid scintillation counter (Tri-Carb 3170 TR/SL, PerkinElmer Life and Analytical Sciences, Waltham, MA). Serum concentrations of total radioactivity (ngEq/ml) in human subjects were determined by dividing the radioactivity measurement (dpm/ml) by the specific activity of [14C]indacaterol (dpm/ng). The LLOQ for total radioactivity was 0.070 ngEq/ml using 8.0 ml of human serum.

**Measurements of Total Radioactivity in Urine and Feces.** Analysis of total radioactivity in feces and urine was performed by Aptuit, Inc. (Kansas City, MO). For the urine samples, 0.5-ml aliquots were transferred directly into liquid scintillation vials along with approximately 10 ml of Ultima Gold scintillant. The fecal samples were handled frozen at approximately −20°C before homogenization using a lab blender (Tekmar-Dohrmann, Mason, OH). Fecal samples were pooled into 24-h intervals before homogenization. The volume of diluent added to each pooled fecal sample before homogenization was determined by multiplying the calculated weight of the pooled fecal sample by three. The diluent used was 60:40 water/methanol. For the feces homogenate samples, aliquots of −0.3 g were pipetted into Combusto-Cones containing Combusto-Pads (PerkinElmer Life and Analytical Sciences). The fecal homogenate samples were allowed to dry overnight in a fume hood. Fecal homogenate samples were combusted in a sample oxidizer (model 307, PerkinElmer Life and Analytical Sciences). Approximately 10 ml of CarboSorb E and 10 ml of Permafluor E scintillant (PerkinElmer Life and Analytical Sciences) were added to each combusted fecal homogenate sample. The total radioactivity in each urine and feces sample was counted for 5 min using a liquid scintillation counter (Tri-Carb 3100TR; PerkinElmer Life and Analytical Sciences). The counting time of 5 min was verified to be sufficient to achieve percentage of coefficient of variation values that were ≤3% for control urine and feces samples spiked with [14C]indacaterol at concentrations relevant to this study. For urine, two aliquots were prepared from each sample. For feces, three aliquots were prepared from each sample.

The liquid scintillation counter efficiency and the oxidizer recovery were verified periodically during the study. The recovery of radioactivity for the assay was validated for the urine and feces sample matrices by spiking a known amount of radioactivity (1°C indacaterol standard solution) into each matrix and comparing this amount with that determined from liquid scintillation counting (LSC). The recovery of radioactivity for three different concentrations of radioactivity was validated for each matrix. Matrix-specific predose aliquots were counted to determine matrix-specific background radioactivity values. The limit of quantitation for a matrix was set at three times the matrix-specific background radioactivity value for that matrix. Sample radioactivity values below the limit of quantitation were recorded as being below the limit of quantitation. The radioactivity measured for each sample of urine or feces was in units of disintegrations per minute (dpm). The measured radioactivity was converted to a percentage dose value using the weight of the

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**Fig. 1.** Chemical structure of indacaterol. The asterisk designates the position of the 14C label at one of the two benzyl carbons.
incurred approximately 10-fold, centrifuged, and analyzed directly. For feces, a 0-h pool was prepared for each subject by combining equal weight percent-ages of fecal homogenates from each collection. Each fecal pool was extracted threentimes with methanol, followed by the centrifugation and collection of the supernatant. The supernatant from each extraction then was combined, evaporated to near-dryness, and reconstituted in acetonitrile/ammonium acetate (20 mM, pH 4.3) (10:90, v/v). The recovery of radioactivity from this sample preparation procedure was ~78%.

For urine, a 0-h pool was prepared for each subject by combining equalvolume percentages from each time period. Each urine pool then was concen-trated approximately 10-fold, centrifuged, and analyzed directly. For feces, a 0-h pool was prepared for each subject by combining equal weight percentages of fecal homogenates from each collection. Each fecal pool was extracted three times with methanol, followed by the centrifugation and collection of the supernatant. The supernatant from each extraction then was combined, evaporated to near-dryness, and reconstituted in acetonitrile/ammonium acetate (20 mM, pH 4.3) (10:90, v/v). The average recovery of radioactivity from this extraction procedure was ~78% for the fecal samples. The overall time periods for the urine and feces time pools were chosen to capture ~90% of the total radioactivity measured for that matrix.

Radiochromatograms for indacaterol and its metabolites in serum, urine, and feces were acquired using high-performance liquid chromatography (HPLC) with off-line radioactivity detection. The analytical system consisted of an HPLC system (Alliance 2695; Waters), equipped with a Kromasil C18 column (3 × 150 mm, 3.5 μm; Peeke Scientific, Redwood City, CA), preceded by a SecurityGuard cartridge (Phenomenex, Torrance, CA) of the same type; the column and guard column were maintained at 30°C. The mobile phase consisted of 20 mM ammonium acetate containing 0.2% acetic acid (pH 4.3) (solvent A) and acetonitrile containing 0.2% acetic acid (solvent B). A linear gradient elution program was used.

The HPLC effluent from the serum sample analyses were fraction collected into 7-ml liquid scintillation vials using a fraction collector (FC 204; Gilson, Middleton, WI) at a collection rate of 0.5 min per vial. The vials each were filled with 5 ml of Flo-Scint II cocktail (PerkinElmer Life and Analytical Sciences), capped, mixed well, and counted on a liquid scintillation analyzer (Tri-Carb 3170TR/SL; PerkinElmer Life and Analytical Sciences). Data files generated by the liquid scintillation analyzer were imported into Excel (version 2002 xR-2; Microsoft, Redmond, WA), reformatted, and converted to comma-delimited files, and then imported into WinFlow radiochromatography software [β-ram, version 1.4a (116); Lablogic-INUS Systems, Tampa, FL] for the creation of radiochromatograms and integration of peaks. In urine and feces sample analyses, the HPLC effluent was fraction collected into 96-well Lum- maPlate (PerkinElmer Life and Analytical Sciences) microplates using the same fraction collector as was used for the serum samples, at a collection rate of 0.13 min per well. The LummaPlates were dried, sealed, and counted on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Data generated by the microplate scintillation counter were pro-cessed in the same way as described previously.

Radioactive peaks were selected visually from the radiochromatograms. The radioactivity in the region encompassing the beginning and ending of the peak was summed. All of the further calculations were based on radioactivity. The fraction of radioactivity in a particular peak, Z, was calculated by dividing the dpm value associated with that peak by the dpm value associated with the sum of all of the integrated peaks. The concentration or amount of each component was calculated by multiplying its fraction of radioactivity by the total compound-related plasma radioactivity concentration (ngEq/ml) or the percentage of the dose in excreta. There was no correction for extraction recovery.

Indacaterol and its metabolites were characterized by LC/MS. The HPLC system consisted of two liquid chromatographs (model LC-10AD; Shimadzu, Kyoto, Japan), a system controller (model LC-10Avp; Shimadzu), and a PAL autosampler (LEAP Technologies, Carboro, NC). The analytical column, mobile phase, and gradient program were the same as those used for the metabolite profiling. The HPLC effluent was split (3:1, v/v) to deliver 0.075 ml/min to the mass spectrometer (Finigan TSQ Quantum Ultra; Thermo Fisher Scientific, Waltham, MA). Samples were analyzed by electrospray ionization in the positive ion mode. Due to the small dose of indacaterol administered in this study (800 μg), the analyte concentrations were too low to analyze by mass spectrometry in the full-scan product ion mode. Instead, radiochromatogram peaks from the current study were matched by retention time with radiochromatogram peaks from a human hepatocyte incubation performed with indacaterol concentrations high enough to allow for the acquisi-tion of a high-quality MS product ion spectrum for each peak (Novartis Pharmaceuticals Corporation, unpublished data). Three diagnostic product ions from each of these spectra were selected for side-by-side MS analysis of the samples from the current study with the human hepatocyte incubation sample using the more sensitive MRM mode. Metabolite structures were proposed based on the interpretation of product ion spectra, NMR spectra (when available), and comparison with synthetic standards (when available). The MRM ion transitions selected for the metabolites of indacaterol are listed in Table 2.

**Table 1**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; ngEq/ml</th>
<th>t&lt;sub&gt;max&lt;/sub&gt; h</th>
<th>AUC&lt;sub&gt;0-last&lt;/sub&gt; ngEq-h/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indacaterol</td>
<td>0.47 (46.8)</td>
<td>1.75 (54.7)</td>
<td>1.81 (77.9)</td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>1.4 (52.8)</td>
<td>2.5 (40.0)</td>
<td>27.2 (49.1)</td>
</tr>
</tbody>
</table>

* For indacaterol, the last time points included in the AUC calculation were 3, 6, 8, and 12 h for the four subjects. For total radioactivity, the last time points included in the AUC calculation were 48, 144 (two subjects), and 168 h.

† There were too few later time points with measurable analyte concentrations to estimate t<sub>1/2</sub> or AUC<sub>C<sub>max</sub></sub> values for indacaterol. For total radioactivity, the terminal phase was defined insufficiently to allow estimation of t<sub>1/2</sub> or AUC<sub>C<sub>max</sub></sub> values.
concentrations ($C_{\text{max}}$) of total radioactivity and indacaterol along with the times at which they occurred ($t_{\text{max}}$) were recorded. The total radioactivity and indacaterol concentrations were plotted in a log-linear fashion and analyzed by noncompartmental analysis using WinNonlin (version 4.1). The area under each concentration-time curve from 0 to the last time point analyzed ($\text{AUC}_{0-\text{last}}$) was calculated using the linear trapezoidal rule.

**Results**

**Indacaterol and Total Radioactivity Concentrations in Serum.**

The concentrations of indacaterol and total radioactivity in serum are displayed in Fig. 2, whereas the mean values of $C_{\text{max}}$, $t_{\text{max}}$, and $\text{AUC}_{0-\text{last}}$ for indacaterol and total radioactivity are listed in Table 1.

### Table 2

<table>
<thead>
<tr>
<th>Metabolite Name</th>
<th>Proposed Structure with Assigned MS/MS Product Ions</th>
<th>Precursor Ion</th>
<th>Diagnostic MRM Product Ions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19</td>
<td></td>
<td>567†</td>
<td>171, 203, 373††</td>
</tr>
<tr>
<td>P26.9</td>
<td></td>
<td>409</td>
<td>143, 171, 188‡,¶,√</td>
</tr>
<tr>
<td>P30.3</td>
<td></td>
<td>409</td>
<td>143, 171, 188§</td>
</tr>
<tr>
<td>P37</td>
<td></td>
<td>569</td>
<td>173, 188, 375†</td>
</tr>
<tr>
<td>P37.7</td>
<td></td>
<td>569</td>
<td>173, 188, 375</td>
</tr>
<tr>
<td>P38.2</td>
<td></td>
<td>248</td>
<td>117, 145, 173†</td>
</tr>
</tbody>
</table>
The mean $t_{\text{max}}$ and $C_{\text{max}}$ values were 1.75 h and 0.47 ng/ml for indacaterol and 2.5 h and 1.4 ngEq/ml for total radioactivity. For indacaterol, the LC-MS/MS assay was not sensitive enough to capture the terminal portion of the serum concentration-time profile. This prevented a terminal half-life and other dependent parameters such as AUC$_{0-\text{inf}}$, CL/$F$, and $V/F$ from being estimated. For total radioactivity, the inability to clearly define a terminal slope for the serum concentration-time profile also prevented the estimation of $t_{1/2}$ and AUC$_{0-\text{inf}}$. Through the use of the last time points with measurable concentrations (listed in Table 1), mean AUC$_{0-\text{last}}$ values were calculated for indacaterol and total radioactivity. They were 1.81 ng·h/ml and 27.2 ngEq·h/ml, respectively. Analysis of urine obtained from humans (internal report) provided evidence that stereoisomeric conversion of indacaterol (the pure R-enantiomer) to the S-enantiomer in vivo does not occur to any significant extent.

**Excretion and Dose Recovery in Urine and Feces.** The excretion of radioactivity from all four subjects was measured over a period of up to 13 days after dosing and is presented in Fig. 3. For all four subjects, $\geq 85\%$ of the administered dose radioactivity was recovered by 168 h postdose, and $\geq 90\%$ was recovered by study completion. The radioactivity was excreted primarily via the fecal route (mean, 85.3% of dose). The renal excretion route was minor by comparison (mean, 9.7% of dose).

**Elucidation of Metabolite Structures.** As described under *Materials and Methods*, the MS product ion spectra used for metabolite structural elucidation were acquired from human hepatocyte incubations rather than the samples from the clinical study. The assignment of these product ions to the proposed metabolite structures are shown in Table 2. From each of these full-scan product ion spectra, three diagnostic MS/MS transitions were selected for monitoring by the more sensitive MRM MS technique. For each metabolite in the clinical study that was associated with a metabolite from the hepatocyte incubation study based on chromatographic retention time matching, the association was confirmed further by comparing the MRM intensity patterns from the two studies. These results are displayed in Supplemental Figs. 1 to 6. For all of the metabolites in Table 2, the match of the MRM intensity patterns between the two studies was excellent. An overall metabolic scheme for indacaterol in humans is displayed in Fig. 4.

**Metabolite P26.9.** A protonated molecular ion at $m/z$ 409, which was 16 atomic mass units (amu) greater than that of unmodified indacaterol, allowed P26.9 to be assigned as monooxygenated indacaterol. The MS/MS fragment ion at $m/z$ 188 was unchanged relative to the corresponding fragment ion for indacaterol, indicating that the oxygenation had occurred in the diethylindanylamine portion of the molecule. A separate NMR analysis of the purified P26.9 metabolite (Novartis Pharmaceuticals Corporation, unpublished data) indicated that hydroxylation had occurred at one of the two benzylic carbons in the diethylindane moiety. Hydroxylation at the benzylic carbon in the diethylindane moiety results in four possible diastereomeric structures. Authentic standards for all four of these diastereomers were synthesized, but the stereochemistry of each about their two chiral centers was not determined. Therefore, the exact stereochemistry of metabolite P26.9 was not assigned. However, the retention

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**TABLE 2—Continued**

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</tr>
</thead>
<tbody>
<tr>
<td>P39</td>
<td><img src="attachment.png" alt="Proposed Structure" /></td>
<td>190</td>
<td>91,117,129‡</td>
</tr>
</tbody>
</table>

* Due to the low metabolite concentrations in this study, the mass spectrometric analysis of the samples was performed in the MRM mode rather than the full-scan product ion mode to insure sufficient sensitivity. Additional details can be found in the *Materials and Methods*.

† Although the actual protonated molecular weight of M19 is 585 Da, $m/z$ 567 was chosen as the precursor ion, because M19 was found to readily lose a molecule of water in the mass spectrometer ion source.

‡ The standard for M19 was generated via incubation with UGT1A1 of a synthetic standard for P26.9.

§ Comparison of NMR spectrum with that of a synthetic standard provided additional structural confirmation.

¶ Four individual diastereomeric standards were synthesized.

The mean $t_{\text{max}}$ and $C_{\text{max}}$ values were 1.75 h and 0.47 ng/ml for indacaterol and 2.5 h and 1.4 ngEq/ml for total radioactivity. For indacaterol, the LC-MS/MS assay was not sensitive enough to capture the terminal portion of the serum concentration-time profile. This prevented a terminal half-life and other dependent parameters such as AUC$_{0-\text{inf}}$, CL/$F$, and $V/F$ from being estimated. For total radioactivity, the inability to clearly define a terminal slope for the serum concentration-time profile also prevented the estimation of $t_{1/2}$ and AUC$_{0-\text{inf}}$. Through the use of the last time points with measurable concentrations (listed in Table 1), mean AUC$_{0-\text{last}}$ values were calculated for indacaterol and total radioactivity. They were 1.81 ng·h/ml and 27.2 ngEq·h/ml, respectively. Analysis of urine obtained from humans (internal report) provided evidence that stereoisomeric conversion of indacaterol (the pure R-enantiomer) to the S-enantiomer in vivo does not occur to any significant extent.

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**Fig. 3.** Cumulative recovery of total radioactivity in excreta expressed as percentage of dose. The upper trace represents the feces data, whereas the lower trace represents the urine data.
time and mass spectra of P26.9 matched closely with that of the synthetic standard designated by the code QBA089 (5-{(R)-2-[5-ethyl-6-(1-hydroxy-ethyl)-indan-2-ylamino]-1-hydroxy-ethyl}-8-hydroxy-1H-quinolin-2-one).

**Metabolite P30.3.** A comparison of its mass spectrum and chromatographic retention time with those of P26.9 and the diastereomeric standard (described above) designated by the code QBA088 (5-{(R)-2-[5-ethyl-6-(1-hydroxy-ethyl)-indan-2-ylamino]-1-hydroxy-ethyl}-8-hydroxy-1H-quinolin-2-one) allowed P30.3 to be assigned as a diastereomer of P26.9.

**Metabolite P37.** A protonated molecular ion at m/z 569, which was 176 amu greater than that of indacaterol, allowed P37 to be assigned as a glucuronide of indacaterol. The MS/MS fragment ions at m/z 188 and 375 along with the more complete product ion spectra obtained from the human hepatocyte sample also were consistent with the glucuronide assignment. Chromatographic retention time matching with a synthetic standard confirmed that P37 was the glucuronide conjugate of indacaterol (Novartis Pharmaceuticals Corporation, unpublished data) where glucuronidation had occurred at the hydroxyl group in the quinolinone moiety.

**Metabolite P37.7.** Although P37.7 was not detected in human hepatocytes, it was detected in human serum using the same mass spectrometric filter as was used to detect P37. On the basis of its chromatographic retention time with respect to P37 and the results of previous ADME studies in mouse, rat, and dog (Novartis Pharmaceuticals Corporation, unpublished data), P37.7 was assigned tentatively as the glucuronide conjugate of indacaterol where the glucuronidation has occurred at the indanylamine nitrogen.

**Metabolite P38.2.** A protonated molecular ion at m/z 248, which was 145 amu less than that of indacaterol, indicated that P38.2 was the result of a cleavage mechanism. The MS/MS fragment ions at m/z 117, 145, and 173 indicated that the diethylindane portion of the molecule was still intact. Chromatographic retention time and mass spectral matching with a synthetic standard confirmed that P38.2 was the carboxylic acid resulting from the cleavage of the carbon-carbon bond connecting the hydroxyquinolinone substructure to the diethylindanylaminoethanol substructure. Two possible biotransformation pathways for the formation of this unusual metabolite will be proposed under Discussion.

**Metabolite P39.** A protonated molecular ion at m/z 190, which was 203 amu less than that of indacaterol, indicated that P39 was the result of a cleavage mechanism. The MS/MS fragment ions at m/z 91, 117, and 129 were consistent with the assignment of P39 as the N-diethylindanylamine resulting from N-alkyl cleavage. Chromatographic retention time and mass spectral matching with a synthetic standard confirmed the proposed structure for P39.

**Metabolite P19.** The protonated molecular ion at m/z 567 and the MS/MS fragment ions at m/z 373 and m/z 203 initially supported the structural assignment of P19 as a glucuronide conjugate of an M – 2H metabolite of indacaterol. However, incubation of the P26.9 synthetic standard, QBA089, with uridinediphosphate-glucuronosyltransferase (UGT) 1A1 produced a glucuronide with the same chromatographic retention time and mass spectrum as P19. UGT1A1 had been shown previously to be the primary UGT isoform responsible for the glucuronidation of indacaterol to form P37 (Novartis Pharmaceuticals Corporation, unpublished data). Therefore, P19 was assigned as the
The glucuronide conjugate of P26.9 with glucuronidation occurring on the phenolic oxygen of the hydroxyquinolinone functionality. The assignment of the site of glucuronidation was supported further by the similarity of the relative retention times of P19 versus its aglycone, P26.9, compared with the relative retention times of P37 versus its aglycone, indacaterol. Apparently, P19 readily loses a molecule of water in the mass spectrometer ion source under the experimental conditions used.

**Metabolite Profiles of Indacaterol in Serum.** The radioactivity profiles labeled with the contributing metabolites for the 2 and 8 h and AUC₀–₂₄₉ serum pools are displayed in Fig. 5. Quantitative amounts for the individual metabolites in the 2 h and AUC₀–₂₄₉ serum pools are listed in Table 3. The Cₘₐₓ of total radioactivity in serum occurred at 2 h postdose in three of the four subjects in the study. In both the Cₘₐₓ and the AUC₀–₂₄₉ pools, unmodified indacaterol was the most abundant circulating component (~30% of the total radioactivity). Monohydroxylated (hydroxylation at the 5-(1-ethyl) position) indacaterol (P26.9), P19 (glucuronide conjugate of P26.9), and the 8-O-glucuronide conjugate of P37.7, P38.2, and P39 were the most abundant circulating metabolites of indacaterol (4–13% of total radioactivity) that could be resolved chromatographically. In addition, the N-glucuronide conjugate of indacaterol (P37.7) and the two metabolites resulting from cleavage (P38.2 and P39) had a combined contribution of ~12.5% to the circulating radioactivity. P30.3, the diastereomer of...
P26.9, also was observed as a minor circulating metabolite. The abundances of P19, P26.9, and P37 relative to indacaterol were greater in the 2 h pool than in the 8 h pool, suggesting that these metabolites were cleared more rapidly than indacaterol. Indacaterol and its well characterized metabolites (P19, P26.9, P30.3, P37, P37.7, P38.2, and P39) accounted for 67.8% of the radioactivity in the serum AUC0–24h pool. The serum radioactivity associated with the 24 h time period represented by the AUC pool accounted for 51.8% of the serum radioactivity measured out to the last detectable time point for each subject.

**Metabolite Profiles of Indacaterol in Excreta.** The radioactivity profiles labeled with the contributing metabolites for urine and feces of one of the four subjects is displayed in Fig. 6. Quantitative amounts for the individual metabolites are listed in Table 4. In feces, unmodified indacaterol accounted for ~54% of the total dose. Metabolite P26.9 was the most abundant metabolite (~17%), and metabolites P30.3, P31.6, and P39 each accounted for 6% or less of the dose. The chemical structure of P31.6 was not determined in this study. In urine, unmodified indacaterol accounted for ~0.3% of the total dose, whereas the metabolites P19, P26.0, P26.9, P30.3, P37, P38.2, and P39 each accounted for ~1.3% of the dose, respectively. The chemical structure of P26.0 was not determined in this study.

**Discussion**

[14C]Indacaterol was administered orally in this study, even though its intended route of administration to patients is via inhalation. This was done to avoid the complications and concerns associated with the administration of a radiolabeled compound via the inhalation route in a mass balance study (e.g., radioactivity containment and ensuring that subjects receive the entire dose). The use of oral dosing as a surrogate for inhalation dosing also was justified based on several...
literature reports indicating that 80 to 90% of a dose administered via a dry powder or metered-dose inhaler is swallowed orally (Taburet and Schmit, 1994; Roland et al., 2004).

An 800-μg dose of indacaterol was chosen for this study to ensure sufficient analytical sensitivity for metabolite detection, although the approved therapeutic doses are 75, 150, and 300 μg. At this suprapharmacological dose, the maximum serum concentration of indacaterol was ~1 nM, whereas the \( K_{\text{m}} \) values for the drug-metabolizing enzymes responsible for the formation of the major metabolites of indacaterol in human were in the low micromolar range (Novartis Pharmaceuticals Corporation, unpublished data). Thus, it is highly unlikely that there was any saturation of enzyme activities at the dose used in this study. The high volumes of distribution for indacaterol that were determined from intravenous administration to various preclinical species (5–34 l/kg) (Novartis Pharmaceuticals Corporation, unpublished data) was another likely contributor to the low serum concentrations observed in the current study. For the reasons just stated, the circulating metabolites of indacaterol had to be identified indirectly by comparison with human hepatocyte incubations as described under Materials and Methods. Despite these challenges, there was close agreement between the mean serum concentrations of indacaterol determined at \( C_{\text{max}} \) by a validated LC-MS/MS method (0.47 ng/ml) and at 2 h using radiometric profiling (0.42 ng/ml). This observation provides further confidence in the accuracy of the serum concentrations determined for the metabolites, because the same radiometric method was used.

Unmodified indacaterol was the most abundant drug-related component in the serum, contributing 32.5% to the AUC0–24h pool radioactivity. The monohydroxylated indacaterol metabolite, P26.9, and its glucuronide conjugate, P19, also were relatively abundant, contributing 12.4 and 5.8%, respectively. Metabolite P37 contributed 4.2%, whereas P37.7, P38.2, and P39 together contributed 12.9%. These results were unexpected based upon what had been observed previously from incubations of indacaterol with human liver microsomes, hepatocytes, and liver slices and recombinant human cytochrome P450 and UGT enzymes (Novartis Pharmaceuticals Corporation, unpublished data). From these studies, it was predicted that P37 would be the major circulating metabolite and that P26.9, if present, would be at much lower levels. There also was little indication from the in vitro data that P37.7, P38.2, or P39 would be detected at significant concentrations. This disconnect between human in vitro and in vivo data is unexplained currently.

The unexpectedly high concentrations of several of the human serum metabolites was a source of additional interest, because P37 and P37.7 were the only metabolites detected in the serum of the two toxicology species, rat and dog, in the initial radiolabeled ADME studies (Novartis Pharmaceuticals Corporation, unpublished data). This apparent species difference was reconciled eventually when more targeted MS analyses in remaining serum samples from high-dose toxicology studies (Novartis Pharmaceuticals Corporation, unpublished data) confirmed the presence of the human metabolites (P19, P26.9, P30.3, and P38.2) at concentrations high enough to satisfy “MIST” requirements (Center for Drug Evaluation and Research, 2008; International Committee on Harmonization, 2009).

Although the metabolite exposure values determined from this study were from a single dose, the values determined at steady state are not expected to be significantly higher. This assertion is based on the accumulation ratio for indacaterol of 2.9 to 3.5 that was determined from other clinical studies (Moen, 2010) and the observation from the current study that the major human serum metabolites (P19, P26.9, and P37) appeared to be cleared faster than indacaterol (Fig. 5).

The biotransformation pathway leading to the formation of metabolite P38.2 was unusual in that it involved the cleavage of the carbon-carbon bond linking the hydroxyquinolinone and diethylindanylaminoethanol moieties. The closest analog to this pathway that could be found in the literature was for substrates in which the hydroxyl group on the carbon adjacent to the hydroxyquinolinone moiety was replaced by a carbonyl group and the NHR group in the dihydroquinone imine substructure was replaced by a second hydroxyl group to form a dihydroquinone. In all of these, one of the hydroxyl groups in the dihydroquinone substructure was ortho to the diethylindanylaminoethanol moiety. All of these analogs were shown to produce metabolites in which the carbon-carbon bond linking the dihydroquinone substructure to the carbinol group was cleaved. Examples included the flavonoids, eriocitrin, and naringin (Miyake et al., 2000; Rechner et al., 2004), the anthracycline anticancer drug doxorubicin (Reszka et al., 2005), and the metabolic intermediate of bacterial nicotine degradation 2,6-dihydroxy-pseudo-oxynicotine (Schleberger et al., 2007). The respective authors of these publications proposed a hydrolytic mechanism for the formation of 2,6-dihydroxy-pseudo-oxynicotine, a peroxidase-mediated one-electron oxidation mechanism for the formation of doxorubicin and colonic metabolism for the formation of eriocitrin and naringin.

For the formation of metabolite P38.2, we tentatively propose two possible formation mechanisms. The first mechanism (Scheme 1 in Fig. 7) begins with a one-electron oxidation of the oxygen in the hydroxyquinolinone moiety, leading to a semiquinone imine radical intermediate. The site of the free radical then can migrate from the oxygen to the ring carbon adjacent to the carbinol group. Hydrogen atom abstraction from the carbinol oxygen results in the formation of an alkoxo radical. The alkoxy radical then can undergo simultaneous carbon-carbon bond cleavage and formation of a carbonyl group by a well established \( \beta \)-scission pathway (Lowry and Richardson, 1987; Carey and Sundberg, 1990), which is driven by the favorable energetics associated with the replacement of a carbon-carbon bond with an additional carbon-oxygen bond. Although the energetics associated with hydrogen atom abstraction are unfavorable due to the greater stability of an O-H bond relative to a C-H bond, the overall energetics of the hydrogen atom abstraction/\( \beta \)-scission reaction sequence are slightly favorable. Assuming these two steps occur in a concerted rather than sequential fashion, the overall proposed mechanism is plausible. The aldehyde that is formed initially should be readily oxidized further to the observed carboxylic acid metabolite P38.2.

The second possible mechanism that was proposed for the formation of metabolite P38.2 (Scheme 2 in Fig. 7) involved an initial

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Urine</th>
<th>Feces</th>
<th>Urine + Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19</td>
<td>0.83 (0.33)</td>
<td>0.00 (0.00)</td>
<td>0.83 (0.33)</td>
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<tr>
<td>P26.0</td>
<td>1.09 (0.36)</td>
<td>0.00 (0.00)</td>
<td>1.09 (0.36)</td>
</tr>
<tr>
<td>P26.9</td>
<td>1.24 (0.67)</td>
<td>17.44 (8.73)</td>
<td>18.68 (8.92)</td>
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<tr>
<td>P30.3</td>
<td>0.31 (0.14)</td>
<td>6.33 (2.74)</td>
<td>6.64 (2.85)</td>
</tr>
<tr>
<td>P31.6</td>
<td>0.00 (0.00)</td>
<td>2.82 (1.19)</td>
<td>2.82 (1.19)</td>
</tr>
<tr>
<td>P37</td>
<td>0.53 (0.31)</td>
<td>0.00 (0.00)</td>
<td>0.53 (0.31)</td>
</tr>
<tr>
<td>P38.2</td>
<td>0.97 (0.24)</td>
<td>0.00 (0.00)</td>
<td>0.97 (0.24)</td>
</tr>
<tr>
<td>P39</td>
<td>0.00 (0.00)</td>
<td>1.70 (1.17)</td>
<td>1.70 (1.17)</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>0.34 (0.37)</td>
<td>54.42 (20.95)</td>
<td>54.76 (20.69)</td>
</tr>
<tr>
<td>Indacaterol + Identifed</td>
<td>5.31 (2.22)</td>
<td>79.90 (11.17)</td>
<td>85.21 (9.36)</td>
</tr>
<tr>
<td>Metabolites*</td>
<td>9.7 (3.7)</td>
<td>85.3 (7.6)</td>
<td>95.0 (5.1)</td>
</tr>
</tbody>
</table>

* Metabolite P31.6 was not included, because its chemical structure was not determined.
hydroxylation on the hydroxyquinolinone moiety at the carbon atom adjacent to the diethylindanylaminoethanol group followed by a retro-aldol reaction. The resulting aldehyde (same as in Scheme 1) again would be oxidized further to the observed carboxylic acid metabolite P38.2. Although there were several examples in the literature of this biotransformation pathway occurring on aromatic side chains (Katchen and Buxbaum, 1975; Morris et al., 1982; Taskinen et al., 1991; Sauer et al., 1997), there were none that were analogous to our proposal for metabolite P38.2 in which the carbon-carbon bond cleavage occurred between the aromatic moiety and the carbon atom alpha to it. The currently existing data for metabolite P38.2 do not allow for distinguishing between the two proposed biotransformation pathways. However, future experiments using dual radiolabels are being planned that may help in this endeavor.

On average, ~10% of the administered radioactivity was recovered in urine, whereas ~85% was recovered in feces. Less than 0.5% of the dose was eliminated in urine as unmodified indacaterol, indicating that altered renal clearance in susceptible populations should not be of clinical concern. The remainder of the dose excreted in urine was distributed among a number of different metabolites. Approximately 55% of the dose was recovered in feces as unmodified indacaterol, suggesting that either oral absorption might be no greater than 45% or that biliary excretion of indacaterol is a major clearance mechanism. A third possibility, supported by the results of a rat ADME study performed with bile duct-cannulated animals (Novartis Pharmaceuticals Corporation, unpublished data), is that a significant portion of the indacaterol detected in human feces was actually excreted as the direct glucuronide, P37, that is subsequently hydrolyzed back to the aglycone by gut bacteria. Other than unmodified indacaterol, the diastereomeric monohydroxylated metabolites P26.9 and P30.3 were the only other abundant drug-related compounds found in feces, together accounting for 24% of the excreted dose on average. Phenotyping data indicated that CYP3A4 was the primary drug-metabolizing enzyme responsible for their formation (Novartis Pharmaceuticals Corporation, unpublished data). Not unexpectedly, a clinical drug-drug interaction study with the potent CYP3A4 inhibitor ketoconazole resulted in an almost 2-fold increase in the serum indacaterol AUC (Novartis Pharmaceuticals Corporation, unpublished data).

In summary, [14C]indacaterol administered orally to healthy male subjects was absorbed fairly rapidly with $C_{\text{max}}$ occurring at 1.75 h. Unmodified indacaterol was the major circulating drug-related component, with the monohydroxylated metabolite, P26.9, its glucuronide, P19, and the 8-O-glucuronide of indacaterol, P37, also contributing significantly to the serum profile. Excretion of radioactivity and indacaterol into urine accounted for ~10 and 0.55% of the dose, respectively. Excretion of radioactivity, indacaterol, and P26.9 + P30.3 into feces accounted for ~85, 55, and 24% of the dose, respectively.

Acknowledgments
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Authorship Contributions
Participated in research design: Dain and Reynolds.
Conducted experiments: Kagan and Peng.
Performed data analysis: Kagan, Dain, Peng, and Reynolds.
Wrote or contributed to the writing of the manuscript: Kagan.

References


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Positive multiple reaction monitoring (MRM) mass spectra of metabolite P19 (precursor mass = 567) in human serum AUC pool (A) and in human hepatocytes (B)
Positive multiple reaction monitoring (MRM) mass spectra of metabolite P26.9 (precursor mass = 409) in human serum AUC pool (A) and in human hepatocytes (B)
Positive multiple reaction monitoring (MRM) mass spectra of P30.3 (precursor mass = 409) in human serum AUC pool (A) and in human liver hepatocytes (B)
Positive multiple reaction monitoring (MRM) mass spectra of P37 (precursor mass = 569) in human serum AUC pool (A) and in human liver hepatocytes (B)
Positive multiple reaction monitoring (MRM) mass spectra of P38.2 (precursor mass = 248) in human serum AUC pool (A) and in human liver hepatocytes (B)
Positive multiple reaction monitoring (MRM) mass spectra of P39 (precursor mass = 190) in human serum AUC pool (A) and in human liver hepatocytes (B)