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Metabolism and pharmacokinetics of indacaterol in humans

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Running title. Metab., pharmaco. and excret. of indacaterol in humans

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List of nonstandard abbreviations.: AUC, area under curve; ADME, absorption, distribution, metabolism and excretion; COPD, chronic obstructive pulmonary disease; HPLC, high performance liquid chromatography; LC, liquid chromatography; LOQ, limit of quantitation; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LSC, liquid scintillation counting; MRM, multiple reaction monitoring; NMR, nuclear magnetic resonance; PK, pharmacokinetics;
QCs, quality controls; SPE, solid phase extraction; UGT, uridinediphosphate-glucuronosyltransferase
Abstract

The metabolism, pharmacokinetics and excretion of [14C]-indacaterol was 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 administration of a radiolabeled compound via the inhalation route. The sub-milligram doses administered in this study made metabolite identification and structural elucidation by mass spectrometry especially challenging. In serum, the mean $t_{\text{max}}$, $C_{\text{max}}$ and $AUC_{0-\text{last}}$ 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 while 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 4-13%. In addition, the N-glucuronide (2-amino) conjugate (P37.7) and two metabolites (P38.2 and P39) which resulted from the cleavage about the amino-ethanol group linking the hydroxy-quinolinone and diethyl-indane 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 greater than 1.3%.
Introduction

Indacaterol (5-[(R)-2-(5,6-Diethyl-indan-2-ylamino)-1-hydroxy-ethyl]-8-hydroxy-1H-quinolin-2-one; QAB149) is a novel, long-acting inhaled β2-adreneregic receptor agonist intended for long-term, once daily, maintenance treatment in patients with chronic obstructive pulmonary disease (COPD) (Battram et al., 2006). Currently, it has received regulatory approval in the United States, Japan, the European Union, and in a number of other countries worldwide at once-daily doses ranging from 75 µg to 300 µg. Indacaterol’s once-daily treatment favorably differentiates it from other currently marketed long-acting inhaled β2-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 COPD, the most common reason given for treatment noncompliance was “frequency of administration” with 83.2% of respondents stating a preference for once-daily administration (Tamura and Ohta, 2007). Indacaterol has been shown to maintain its efficacy for up to one year with no evidence of tolerance or tachyphylaxis to the bronchodilator effect (Haney and Hancox, 2005 and Dahl et al., 2010). Indacaterol has also 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 one year’s duration. At doses of 75 µg and greater, indacaterol has demonstrated efficacy similar to or better than that of current standard bronchodilators (Cope et al., 2011 and Dahl et al., 2010).
The objectives of the current [¹⁴C]-indacaterol human 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 study participants gave written informed consent before 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 hours prior to 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 hours post-dose). Subjects fasted for at least 10 hours prior to 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, 168 hours post-dose. Urine was collected in discreet 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, 288-312 hours post-dose) while all fecal samples were collected individually up to 312 hours post-dose. All samples were analyzed for total radioactivity while select samples were also analyzed for their metabolite content. Additionally, the serum samples were analyzed by a validated 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 of indacaterol. The results predicted that the exposure in the organ expected to receive the highest radioactive dose, the colon, was 21-fold than the 3 rem limit set by the regulations. Hence, the radiation risk 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 institute 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 benzylic carbon of one of the two ethyl side chains of the diethyl-indane moiety as shown in Figure 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 in order to determine blood total radioactivity, serum total radioactivity, serum PK,
and serum metabolite characterization. All samples (blood, serum, urine and feces) were kept frozen at ≤ -20°C until the time of analysis.

**Measurement of indacaterol concentrations in serum.** 0.20 mL aliquots of serum samples, blanks, standards, and QC’s were transferred into the appropriate wells of a 2-mL, 96-well block. For the blanks, QC’s, 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 seconds. A 25 µL aliquot of the internal standard solution [M+4]13CD3-indacaterol (62.4 ng/mL) was added to each well except the blank samples, and the samples were vortexed again. A 1mL aliquot of tert-butyl methyl ether was added to each well. The block was then capped and vortexed for an additional 5 min. The 96-well block was centrifuged for 10 minutes at approximately 1000 x g. The organic layer was transferred into a clean 96-well block using a Tomtec work station, 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 (ESI) in the positive ion mode with multiple reaction monitoring. [12C]-indacaterol was monitored by the m/z 393.1 → m/z 116.8 transition and [M+4]13CD3-indacaterol was monitored by the m/z 397.4 → m/z 118.0 transition. Response factors for [12C]-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 $[^{12}\text{C}]$-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 x concentration data using a quadratic regression with $1/x^2$ weighting. The lower limit of quantitation (LLOQ) for $[^{12}\text{C}]$-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 $[^{12}\text{C}]$-indacaterol concentrations in order to obtain the total indacaterol concentrations ($^{12}\text{C} + ^{14}\text{C}$) 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 (Packard Tri-Carb 3170 TR/SL, PerkinElmer Life and Analytical Sciences, Boston, 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 the $[^{14}\text{C}]$-indacaterol (dpm/ng). The lower limit of quantitation (LLOQ) for total radioactivity was 0.070 ngEq/ml using 0.8 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. in Kansas City, Missouri. 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 prior to homogenization using a Tekmar lab blender. Fecal samples were pooled into 24 h intervals prior to homogenization. The volume of diluent added to each pooled fecal sample prior to 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®. The fecal homogenate samples were allowed to dry overnight in a fume hood. Fecal homogenate samples were combusted in a Packard sample Oxidizer Model 307. Approximately 10 mL of carbo-Sorb E and 10 mL of Permafluor E scintillant (Packard) was added to each combusted fecal homogenate sample. The total radioactivity in each urine and feces sample was counted for 5 minutes using a Packard Tricarb 3100TR liquid scintillation counter (LSC). The counting time of 5 minutes was verified to be sufficient to achieve %CV values that were ≤ 3% for control urine and feces samples spiked with [14C]-indacaterol at concentrations relevant to this study. For the urine, two aliquots were prepared from each sample. For the 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 ([14C]-indacaterol standard solution) into each matrix and comparing this amount with that determined from LSC counting. The recovery of radioactivity for three different concentrations of radioactivity was validated for each matrix. Matrix-specific pre-dose aliquots were counted to determine matrix-specific background radioactivity values. The limit of quantitation (LOQ) for a matrix was set at three times the matrix-specific background radioactivity value for that matrix. Sample radioactivity values below the
LOQ were recorded as being below the limit of quantitation (BLQ). The radioactivity measured for each sample of urine or feces was in units of dpm (disintegrations per minute). The measured radioactivity was converted to a % dose value using the weight of the aliquot, the total weight of the collected sample, the measured radioactivity of the dose, and the conversion factor: 1 µCi per 2.22x10^6 dpm.

**Determination of metabolite profiles and identification of metabolites.** Human serum samples at 2 h, 8 h, and an AUC pool were analyzed for metabolite profiles by pooling equal volumes of serum from each subject. The serum AUC pool was prepared using a previously published method (Hamilton et al., 1981).

Serum samples were prepared by solid phase extraction using Sep-Pak cartridges (C18, 12cc, 2g, from Waters, Milford, MA). Cartridges were first washed two times with 10 mL of methanol, followed by two washes with 10 mL of water. Serum samples were then loaded onto cartridges with very low or no vacuum. SPE columns were washed with 10 mL of water. Samples were eluted from the column with 10 mL of methanol under very low or no vacuum. The final eluant was dried under nitrogen 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 approximately 77%.

For urine, a 0-t h pool was prepared for each subject by combining equal volume percents from each time period. Each urine pool was then concentrated approximately 10-fold, centrifuged, and analyzed directly. For feces, a 0-t h pool was prepared for each subject by combining equal weight percents of fecal homogenates from each
collection. Each fecal pool was extracted three times with methanol followed by centrifugation and collection of the supernatant. The supernatant from each extraction was then 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 approximately 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 HPLC with off-line radioactivity detection. The analytical system consisted of a Waters Alliance 2695 (Waters, Milford, MA) HPLC system, equipped with a Kromasil C18 column (3 x 150 mm, 3.5 µm, Peeke Scientific), preceded by a Security guard cartridge 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 employed.

The HPLC effluent from the serum sample analyses were fraction collected into 7 mL liquid scintillation vials using a fraction collector (FC 204, Gilson Inc., Middleton, WI) at a collection rate of 0.5 min per vial. The vials were each filled with 5 mL Flo-Scint II cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA), capped, mixed well, and counted on a Packard Tri-Carb 3170 TR/SL liquid scintillation analyzer. Data files generated by the liquid scintillation analyzer were imported into Excel (V. 2002 xr-2), reformatted and converted to comma-delimited files (.csv), and then imported into
Winflow radio chromatography software (β-ram V. 1.4a (116), Lablogic-IN/US Systems, Tampa, FL) for creation of radiochromatograms and integration of peaks. In urine and feces sample analyses, the HPLC effluent was fraction collected into 96-well Lumaplate (Packard Instrument Co, Downers Grove, IL) microplates using the same fraction collector as described above, at a collection rate of 0.13 min per well. The Lumaplates were dried, sealed, and counted on a Packard TopCount microplate scintillation counter. Data generated by the microplate scintillation counter was processed in the same way as described previously.

Radioactive peaks were visually selected from the radiochromatograms. The radioactivity in the region encompassing the beginning and ending of the peak was summed. All further calculations were based on radioactivity. The fraction of radioactivity (FRA) 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 the integrated peaks. The concentration or amount of each component was calculated by multiplying its FRA by the total compound-related plasma radioactivity concentration (ngEq/mL), or the percent of dose in the excreta. There was no correction for extraction recovery.

Indacaterol and its metabolites were characterized by LC/MS. The HPLC system consisted of two Shimadzu Liquid Chromatographs (model LC-10AD, Shimadzu, Kyoto, Japan), a Shimadzu System Controller (model LC-10Avp), and a PAL autosampler (Leap Technologies, Carrboro, NC). The analytical column, mobile phase, and gradient program were the same as used for the metabolite profiling. The HPLC effluent was split (3:1, v/v) to deliver 0.075 mL/min to the mass spectrometer (Finnigan TSQ
Quantum Ultra, ThermoFisher Scientific Corporation, San Jose, CA). 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 acquisition of a high-quality MS product ion spectrum for each peak (unpublished data). Three diagnostic products 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 multiple reaction monitoring (MRM) mode. Metabolite structures were proposed based on 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.

**Pharmacokinetic data analysis.** PK parameters for total radioactivity and indacaterol in serum were determined using non-compartmental methods via WinNonlin Professional (Version 4.1). Serum concentrations were expressed in mass per volume units. As described previously, the concentration of total radioactivity in serum was determined by liquid scintillation counting (LSC) while the concentration of indacaterol in serum was determined using a validated LC-MS/MS bioanalytical method. Descriptive statistics included mean and SD. Additional pharmacokinetic determinations for indacaterol and selected metabolites in serum based on radioactivity measurements
were also performed. The maximum serum 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 (Ver.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 graphically in Figure 2 while the mean values of $C_{\text{max}}$, $t_{\text{max}}$ and $AUC_{0-\text{last}}$ for indacaterol and total radioactivity are listed in Table 1. 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 dependant 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 $AUC_{0-\text{inf}}$. Using the last timepoints with measurable concentrations (listed in Table 2), 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 stereochemical 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 graphically in Figure 3. For all four subjects, ≥ 85% of the administered dose radioactivity was recovered by 168 hours post dose and ≥ 90% was recovered by study completion. The radioactivity was primarily excreted 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 in the *Methods* section, 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 hepatocytes incubation study based on chromatographic retention time matching, the association was furthered confirmed by comparing the MRM intensity patterns from the two studies. These results are displayed in the *Supplemental Data* section (Supplemental Figure 1 – Supplemental Figure 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 human is displayed in Figure 4.

**Metabolite P26.9.** A protonated molecular ion at *m/z* 409 which was 16 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 diethyl-indanylamine portion of the molecule. A separate NMR analysis of the purified P26.9 metabolite (unpublished data) indicated that hydroxylation had occurred at one of the two benzylic carbons in the diethyl-indane moiety. Hydroxylation at the benzylic carbon in the diethyl-indane 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 time and mass spectra of P26.9 matched closely with that of the synthetic standard designated by the code: QBA089.

**Metabolite P30.3.** A comparison of its mass spectrum and chromatographic retention time with that of P26.9 and the diastereomeric standard (described above) designated by the code: QBA088 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 were also consistent with the glucuronide assignment. Chromatographic retention time matching with a synthetic standard confirmed that P37 was the glucuronide conjugate of indacaterol (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. Based on its chromatographic retention time with respect to P37 and the results of previous ADME studies in mouse, rat, and dog (internal reports), P37.7 was tentatively assigned as the glucuronide conjugate of indacaterol where the glucuronidation has occurred at the indanyl-amine 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 diethyl-indane 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 cleavage of the carbon-carbon bond connecting the hydroxy-quinolinone substructure to the diethyl-indanylamino-ethanol substructure. Two possible biotransformation pathways for formation of this unusual metabolite will be proposed in the Discussion section that follows.

Metabolite P39. A protonated molecular ion at $m/z$ 190 which was 203 amu less than that of indacaterol indicated that P38.2 was the result of a cleavage mechanism. The MS/MS fragment ions at $m/z$ 91, 117, and 129 were consistent with the assignment of P39 as the N-diethyl-indanylamine 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 a 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 UGT1A1 produced a glucuronide with the same chromatographic retention time and mass spectrum as P19. UGT1A1 had previously been shown to be the primary UGT isoform responsible for the glucuronidation of indacaterol to form P37 (unpublished data). Therefore, P19 was
assigned as the glucuronide conjugate of P26.9 with glucuronidation occurring on the phenolic oxygen of the hydroxy-quinolinone functionality. The assignment of the site of glucuronidation was further supported by the similarity of the relative retention times of P19 versus its aglycone, P26.9, compared to 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 employed.

**Metabolite profiles of indacaterol in serum.** The radioactivity profiles labeled with the contributing metabolites for the 2 hour, 8 hour and $AUC_{0-24h}$ serum pools are displayed in Figure 5. Quantitative amounts for the individual metabolites in the 2 hour and $AUC_{0-24h}$ serum pools are listed in Table 3. The $C_{\text{max}}$ of total radioactivity in serum occurred at 2 hours post-dose in three of the four subjects in the study. In both the $C_{\text{max}}$ and the $AUC_{0-24h}$ pools, unmodified indacaterol was the most abundant circulating component ($\approx 30\%$ 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 indacaterol (P37) were the most abundant circulating metabolites of indacaterol (4-13% of total radioactivity) that could be chromatographically resolved. Additionally, the N-glucuronide conjugate of indacaterol (P37.7) and the two metabolites resulting from cleavage (P38.2 and P39) had a combined contribution of $\approx 12.5\%$ to the circulating radioactivity. P30.3, the diastereomer of P26.9, was also 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 more rapidly cleared 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 hour 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 the urine and feces of one of the four subjects is displayed in Figure 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 while 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

$[^{14}\text{C}]$-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 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 was also justified based on several literature reports indicating that 80-90% of a dose administered via a dry powder or metered-dose inhaler is swallowed orally (Taburet and Schmit, 1994 and Roland et al., 2004).

An 800 µg dose of indacaterol was chosen for this study to insure sufficient analytical sensitivity for metabolite detection, although the approved therapeutic doses are 75 µg, 150 µg and 300 µg. At this supra-pharmacological dose, the maximum serum concentration of indacaterol was ≈ 1 nM while the $K_m$ values for the drug metabolizing enzymes responsible for the formation of the major metabolites of indacaterol in human were in the low µM range (internal report). Thus, it is highly unlikely that there was any saturation of enzyme activities at the dose employed in this study. The high volumes of distribution for indacaterol that were determined from intravenous administration to various preclinical species (5-34 L/kg) (internal reports) were 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 hepatocytes incubations as described in the Methods section. 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 hours using radiometric profiling (0.42 ng/mL). This observation provides further confidence in the accuracy of the serum concentrations determined for the metabolites since the same radiometric method was used.

Unmodified indacaterol was the most abundant drug-related component in the serum, contributing 32.5% to the $AUC_{0-24h}$ pool radioactivity. The monohydroxylated indacaterol metabolite, P26.9, and its glucuronide conjugate, P19, were also relatively abundant, contributing 12.4% and 5.8%, respectively. Metabolite P37 contributed 4.2% while 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 CYP450 and UGT enzymes (internal reports). 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 was also little indication from the in vitro data that P37.7, P38.2 or P39 would be detected at significant concentrations. This disconnect between the human in vitro and in vivo data is currently unexplained.

The unexpectedly high concentrations of several of the human serum metabolites was a source of additional interest since 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 (internal reports). This apparent species difference was eventually reconciled when more targeted MS analyses in remaining serum samples from high dose toxicology studies (internal report) confirmed the presence of the human metabolites
(P19, P26.9, P30.3 and P38.2) at concentrations high enough to satisfy “MIST” requirements (CDER, 2008 and ICH, 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 - 3.5 that was determined from other clinical studies (Moen MD, 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 (Figure 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 diethyl-indanyl-aminoethanol moieties. The closest analogue 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 diethyl-indanyl-aminoethanol moiety. All of these analogues 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 (Rechner et al., 2004 and Miyake et al., 2000), the anthracycline anti-cancer drug, doxorubicin (Reszka et al., 2005), and the metabolic intermediate of bacterial nicotine degradation, 2,6-dihydroxy-*pseudo*-oxynicotine (DHPON) (Schleberger et al., 2007). The respective
authors of these publications proposed a hydrolytic mechanism for the formation of DHPON, 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 Figure 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 can then 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 alkoxy radical. The alkoxy radical can then undergo simultaneous carbon-carbon bond cleavage and formation of a carbonyl group by a well-established beta-scission pathway (Carey and Sundberg, 1990 and Lowry and Richardson, 1987) which is driven by the favorable energetics associated with replacement of a carbon-carbon bond with an additional carbon-oxygen bond. Although the energetics associated with the hydrogen atom abstraction is 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 is slightly favorable. Assuming these two steps occur in a concerted rather than sequential fashion, the overall proposed mechanism is plausible. The aldehyde that is initially formed 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 Figure 7) involved an initial hydroxylation on the hydroxyquinolinone moiety at the carbon atom adjacent to the diethyl-indanylamino-
ethanol group followed by a retro-Aldol reaction. The resulting aldehyde (same as in Scheme 1) would again be further oxidized 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, Pohl et al., 1982; Taskinen et al., 1991 and Sipes 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 does not allow for distinguishing between the two proposed biotransformation pathways. However, future experiments utilizing dual radiolabels are being planned that may help in this endeavor.

On average, approximately 10% of the administered radioactivity was recovered in the urine while approximately 85% was recovered in the feces. Less than 0.5% of the dose was eliminated in the 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 the urine was distributed between a number of different metabolites. Approximately 55% of the dose was recovered in the 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 (internal report), is that a significant portion of the indacaterol detected in the 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
diasteriomerically dihydroxylated metabolites, P26.9 and P30.3, were the only other abundant drug-related compounds found in the 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 (internal report). Not unexpectedly, a clinical drug-drug interaction study with the potent CYP3A4 inhibitor, ketoconazole, resulted in an almost two-fold increase in the serum indacaterol $AUC$ (internal report).

In summary, $[^{14}C]$-indacaterol administered orally to healthy male subjects was fairly rapidly absorbed with $C_{\text{max}}$ occurring at 1.75 hours. Unmodified indacaterol was the major circulating drug-related component with the dihydroxylated 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 the urine accounted for approximately 10% and 0.55% of the dose respectively. Excretion of radioactivity, indacaterol and (P26.9 + P30.3) into the feces accounted for approximately 85%, 55% and 24% of the dose respectively.
Acknowledgments

We thank scientists from multiple departments at Novartis Institutes for Biomedical Research and Novartis Pharmaceuticals Corporation (East Hanover, NJ; Basel Switzerland; Horsham, England) for the synthesis of [14C]-indacaterol and metabolite standards of P26.9, P30.3, P37, P38.2, and P39, the NMR analysis of metabolite P26.9, and the analysis of indacaterol concentrations in human serum. We also thank the contract research organization, Quintiles (Lenexa, KS), for conduct of the clinical portion of this study, collection of biological samples, and analysis of total radioactivity in urine and feces.
Authorship Contributions

Participated in research design: Dain and Reynolds

Conducted experiments: Peng and Kagan

Contributed new reagents or analytic tools: n.a.

Performed data analysis: Kagan, Dain, Peng, and Reynolds

Wrote or contributed to the writing of the manuscript: Kagan
References


Code of Federal Regulations (Revised as of April 1, 2004) Radioactive drugs for certain research uses. Title 21 Part 361 Section 361.1: 320-325


Katchen B and Buxbaum S (1975) Disposition of a new, nonsteroid, antiandrogen, \(\alpha,\alpha,\alpha\)-trifluoro-2-methyl-4'-nitro-m-propionotoluidide (flutamide), in men following an single oral 200 mg dose. *JCE & M* **41**: 373-379


Footnotes

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Legends for figures

1. Chemical structure of indacaterol. The asterisk designates the position of the [14C] label at one of the two benzylic carbons.

2. Time course of indacaterol and total radioactivity concentrations in human serum. The indacaterol concentrations (lower plot) are expressed in units of ng/mL and were determined by a validated LC-MS/MS assay. The total radioactivity concentrations (upper plot) are expressed in units of ngEq/mL and were determined by liquid scintillation counting.

3. Cumulative recovery of total radioactivity in excreta expressed as percentage of dose. The upper trace represents the feces data while the lower trace represents the urine data.

4. Proposed metabolic pathways of indacaterol in humans

5. Metabolite profiles of indacaterol and its metabolites in human serum. The radiochromatograms were reconstructed from off-line liquid scintillation counting of sequentially collected HPLC fractions. The top panel displays the data from a 2 h time pool, the middle panel displays the data from an 8 h time pool. The bottom panel displays the data from an AUC pool. The preparation of these pools is described in the Methods section.

6. Representative metabolite profiles of indacaterol and its metabolites from one of four study subjects in human urine (top panel) and feces (bottom panel).
radiochromatograms were reconstructed from off-line counting of sequentially collected HPLC fractions with a microplate scintillation counter.

7. Proposed biotransformation mechanisms for formation of metabolite P38.2
Tables

### TABLE 1

*Serum pharmacokinetic parameter estimates for indacaterol and total radioactivity*

Values are means (percentage coefficient of variation of the mean); \( n = 4 \)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>( C_{\text{max}} ) ( \text{ng(Eq)/mL} )</th>
<th>( t_{\text{max}} ) ( \text{h} )</th>
<th>( AUC_{0-\text{last}}^{a,b} ) ( \text{ng(Eq) \cdot 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 point included in the \( AUC \) calculation was 3 h, 6 h, 8 h and 12 h for the 4 subjects. For total radioactivity, the last time point included in the AUC calculation was 48 h, 144 h (2 subjects) and 168 h.

* There were too few later time points with measurable analyte concentrations to estimate \( t_{1/2} \) or \( AUC_{0-\text{inf}} \) values for indacaterol. For total radioactivity, the terminal phase was insufficiently defined to allow estimation of \( t_{1/2} \) or \( AUC_{0-\text{inf}} \) values.
### TABLE 2

*Diagnostic MS/MS product ions used to propose metabolite structures*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proposed structure with assigned MS/MS product ions</th>
<th>Precursor ion</th>
<th>Diagnostic MRM product ionsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19</td>
<td>![Diagram of P19 metabolite]</td>
<td>567(^b)</td>
<td>171, 203, 373(^{c,d})</td>
</tr>
<tr>
<td>P26.9</td>
<td>![Diagram of P26.9 metabolite]</td>
<td>409</td>
<td>143, 171, 188(^{c,e,f})</td>
</tr>
</tbody>
</table>

\(^a\) *Product ions that are diagnostic for the metabolite.*
Due to the low metabolite concentrations in this study, the mass spectrometric analysis of the samples was performed in the multiple reaction monitoring (MRM) mode rather than the full-scan product ion mode to insure sufficient sensitivity. Additional details can be found in the Methods section.

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

Mass spectrometric and chromatographic retention time matching with a synthetic standard
provided additional structural confirmation

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

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

f Four individual diastereomeric standards were synthesized.
### TABLE 3

*Metabolite concentrations in serum*

<table>
<thead>
<tr>
<th>Sample</th>
<th>P19</th>
<th>P26.9</th>
<th>P37</th>
<th>P37.7/P38.2/P39(^a)</th>
<th>Indacaterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ngEq/mL</td>
<td>ngEq·h/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h pool(^b)</td>
<td>0.12</td>
<td>0.18</td>
<td>0.10</td>
<td>0.16</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>(9.31)</td>
<td>(13.2)</td>
<td>(7.42)</td>
<td>(11.7)</td>
<td>(31.4)</td>
</tr>
<tr>
<td>(AUC_{0-24h}) pool(^b)</td>
<td>0.82</td>
<td>1.8</td>
<td>0.60</td>
<td>1.82</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td>(5.78)</td>
<td>(12.4)</td>
<td>(4.22)</td>
<td>(12.9)</td>
<td>(32.5)</td>
</tr>
</tbody>
</table>

\(^a\) P37.7, P38.2 and P39 were not chromatographically resolved. Therefore, only their combined contribution is reported.

\(^b\) Sample pools were prepared as described in the *Methods* section.
### TABLE 4

Recovery of indacaterol, metabolites and total radioactivity in excreta

Values are mean (standard deviation of the mean); \( n = 4 \)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Urine</th>
<th>Feces</th>
<th>Urine + Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of dose</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P19</td>
<td>0.83 (0.33)</td>
<td>0.00 (0.00)</td>
<td>0.83 (0.33)</td>
</tr>
<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>
</tr>
<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 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identified</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></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total radioactivity</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 since its chemical structure was not determined
Figure 1

Chemical structure of a chiral molecule.
Figure 2

Indacaterol equivalents (ng/mL)

- indacaterol
- radioactivity

Time (h)

0 24 48 72 96 120 144 168

0.01 0.1 1 2
Figure 3

A graph showing the percentage of dose over time for feces and urine. The graph indicates a rapid increase in the percentage of dose in feces up to 80h, followed by a slower increase. Urine shows a steady increase from 0 to 120h, with no significant change thereafter.
Figure 4

* P26.9 and P30.3 are diastereomers of each other
Figure 5

- **2 h**
- **8 h**

Graphs showing radioactivity (CPM) over time (min) for different time points (2 h and 8 h) with peaks labeled P19, P26.9, P30.3, P37, P38.2, P39, and QAB149.