Metabolism and Disposition of Vilanterol, a Long-Acting Beta$_2$ Adrenoceptor Agonist, for Inhalation use in Human.

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DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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Running Title:

Metabolism and disposition of vilanterol in human.

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Number of text pages: 30

Tables: 5

Figures: 5

References: 10

Word Counts:

Abstract: 221

Introduction: 724

Discussion: 1319

DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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Abbreviations:

AMS, accelerator mass spectrometry

$AUC_{(0\text{-}t)}$, area under the curve to last sample time where drug was measurable

COPD, chronic obstructive pulmonary disease

DMSO, dimethylsulfoxide.

DRM, drug-related material

GSK, GlaxoSmithKline

HMR, Hammersmith Medicines Research Centre

HPLC, high-performance liquid chromatography

HRS, human radiolabel study

LABA, long acting beta agonist

LSC, liquid scintillation counting

LLQ, lower limit of quantification

MS, mass spectrometry

NMR, nuclear magnetic resonance spectroscopy

NQ, non quantifiable

PRA, Pharmaceutical Research Associates

TR-FRET, Time Resolved Fluorescence Resonance Energy Transfer

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ABSTRACT

The metabolism and disposition of vilanterol, a novel long-acting $beta_2$ adrenoceptor agonist (LABA) for inhalation use, was investigated following oral administration to human. Single oral administrations of up to 500 μg vilanterol were shown to be safe and well tolerated in two clinical studies using healthy male subjects. In a human radiolabel study, six healthy male subjects received a single oral dose of 200 μg [^{14}C] vilanterol (74 kBq). Plasma, urine and faeces were collected up to 168 hours post dose and analysed for vilanterol, metabolites and radioactivity. At least 50% of the radioactive dose was orally absorbed. The primary route of excretion of drug-related material was via O-dealkylation to metabolites which were mainly excreted in urine. Vilanterol represented a very small percentage (<0.5%) of the total drug-related material in plasma - indicative of extensive first-pass metabolism. Circulating metabolites resulted mainly from O-dealkylation and exhibited negligible pharmacological activity. The therapeutic dose level for vilanterol is 25 μg by the inhalation route. At this low dose level, the likelihood of pharmacologically inactive metabolites causing unexpected toxicity is negligible. In addition to providing an assessment of the disposition of vilanterol in human, this work highlights a number of complexities associated with determining human ADME for inhaled molecules – mainly related to the low chemical doses and complications associated with the inhalation route of administration.

Introduction

Vilanterol, GW642444, or 4-[(1R)-2-[(6-{2-[(2,6-dichlorophenyl)methoxy]ethoxy}hexyl)amino]-1-hydroxyethyl]-2-(hydroxyl methyl) phenol, is a novel long acting beta$_2$ agonist (LABA) with inherent 24 h activity for once-daily clinical treatment of chronic obstructive pulmonary disease (COPD) and asthma in combination with the inhaled novel corticosteroid fluticasone furoate, also active for 24 h (Hanania, 2012; Lotvall, 2012). This publication describes in vitro and clinical studies conducted to determine the metabolism and disposition of vilanterol in human. This information is used to provide reassurance on vilanterol metabolite safety, to assess any metabolite contribution to the pharmacology and to define mechanisms of disposition and elimination which might be susceptible to drug interactions or relevant to special patient populations.

Traditionally the metabolism and disposition of a molecule is determined following administration of a radioactive drug analogue by the intended clinical route and includes aspects such as radioactive recovery in excreta, identification of metabolites and quantitative radio-metabolite profiles, usually in plasma and excreta. This study is commonly referred to as the human radiolabel study (HRS). There are significant challenges associated with the design and conduct of an HRS for low dose inhalation molecules, such as vilanterol, where the clinical dose is only 25 µg. These, described below, considerably impacted the approaches used to determine the metabolism and disposition of vilanterol.

Although precedent exists for dosing radiolabel by the inhalation route (Affrime et al., 2000), the inhalation route, for some molecules, is not necessarily the best or most feasible approach especially if the inhalation device and formulation are complex.

Recreating the commercial physical form of a radioactive drug analogue in its intended device is difficult and quantitative information following inhalation of a radioactive analogue is not usually, therefore, representative of the clinical situation. Exhalation of radioactive drug following inhalation administration not only creates safety and containment considerations but makes it very difficult to reliably quantify the administered radioactivity which is needed to fully interpret excretion and metabolism data. Other challenges arise due to the low chemical dose level of inhaled drugs which limit the radioactive dose and leads to biological samples containing very low concentrations of both metabolite chemical mass and radioactivity thereby impairing the ability to measure and identify metabolites. The inhalation route gives little opportunity to adjust chemical and radioactive doses to avoid these technical problems.

Further complications, specific to [^{14}C]vilanterol, included radiolysis of the [^{14}C] isotope which meant that it was unstable over the periods required to support manufacture and release of either intravenous or inhalation formulations, effectively ruling out these routes of administration. Radiolysis limited the [^{14}C]vilanterol specific activity to 370 kBq/mg, also restricting the amount of radioactivity that could be administered. The vilanterol HRS was, therefore, conducted by the oral route using a dose level of 200 µg containing only 74 kBq of radioactivity. The oral dose level of 200 µg [^{14}C]vilanterol was selected since it was higher than the inhaled clinical dose of 25 µg and allowed sufficient administered radioactivity to generate radioactive metabolite profiles for plasma, urine and faeces using Accelerator Mass Spectrometry (AMS) as a highly sensitive radioactivity counter. The chemical structure of [^{14}C]vilanterol showing the position of the [^{14}C] label is shown in Figure 1. A preceding clinical study (hereafter referred to as the tolerability study)

established safety and tolerability of oral administrations up to 500 µg of unlabelled vilanterol.

The emergence of AMS as a very low level radioactivity counter has enabled radioactive metabolite profiles to be generated in samples containing very low levels of radioactivity. The radioactive dose of 74 kBq permitted traditional liquid scintillation counting (LSC) to be used for some aspects of the study (e.g. radioactivity in urine and faeces) but AMS was essential to determine concentrations of radioactivity in plasma and for metabolite profile work. Whilst AMS is an excellent technique for low level radioactivity measurement, it does not directly provide structural information to assist with metabolite identification. The chemical dose level used here (200 µg), allowed limited application of high-performance liquid chromatography – mass spectrometry (HPLC-MS) to provide structural information on some metabolites. The majority of human metabolite structures were, however, assigned by chromatographic retention time comparison with metabolites whose full identification was achieved in other samples containing greater chemical mass e.g. in vitro/in situ experiments or animal studies. In vitro incubations conducted using mouse, rat, dog, rabbit and human hepatocytes which were the primary source of metabolite structures, are also, therefore, described.

Materials and Methods

Materials. Vilanterol, [^{14}C]vilanterol and four potential metabolites (GW630200, GSK167112, GW853734 and GSK932009), were supplied by Chemical Development, GlaxoSmithKline (GSK) R&D, Stevenage, UK. The specific activity of vilanterol was 370 kBq/mg. All other solvents and reagents were of analytical grade and were purchased from commercial suppliers.

Clinical Study Design, Study Centres and Subjects: The tolerability study was an ascending dose, single centre, open label study in nine healthy male subjects conducted at Hammersmith Medicines Research Centre (HMR), London, United Kingdom between 21 October 2008 and 17 December 2008 (Last Sample Last Visit). The HRS was a single centre open label study in six healthy male volunteers conducted at Pharmaceutical Research Associates (PRA), Zuidlaren, The Netherlands between 18 May 2010 and 9 July 2010. All subjects provided written informed consent before participation and the protocols were approved by the investigational centres' ethics committees. The subjects were in good health as shown by medical examination, clinical chemistry, haematology and urine analysis. They were non-smokers with no history of drug or alcohol abuse, were taking no other medication at the time of each study, and had taken no prescribed medication within 14 days of each study commencing. The target radioactive dose of 74 kBq corresponds to an effective radioactive dose of 20 μSv which is considerably lower than the upper limit for both the World Health Organisation (< 500 μSv) and International Commission on Radiological Protection (< 100 μSv) category I exposures which are considered variations in natural background radiation with a minimal risk. The effective radioactive dose was calculated by the United Kingdom Health Protection Agency according to the recommendations of the International Commission on Radiological Protection (ICRP, 1991).

Dose Formulation and Administration. Vilanterol and [^{14}C]vilanterol were supplied to the study centres by GSK, Ware, UK, as sterile solutions. For the tolerability study, a solution (5% ethanol in water) containing 50 μg vilanterol/ml was supplied to HMR. Subjects received a single oral dose of either 4 ml or 10 ml of this solution containing 200 or 500 μg vilanterol respectively. For the HRS, a solution

DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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(80% ethanol in water) containing 200 µg [^{14}C]vilanterol/ml (74 kBq radioactivity /ml) was supplied to PRA. For each volunteer, the solution was diluted 1 in 20 with water by PRA. All subjects received a single 20 ml oral dose containing 200 µg [^{14}C]vilanterol (74 kBq of radioactivity) administered via syringe. After administration of each vilanterol solution, water (2 x 20 ml) was administered to the subject using the same syringe to ensure that the complete dose was given. A third volume of water (20 ml) was drawn up into the syringe (HRS only), dispensed into a suitable container and residual radioactivity was determined by liquid scintillation counting (LSC). Total residual radioactivity was deducted from the dispensed radioactivity in order to determine the overall administered radioactive dose.

Sample Collection. Blood samples (2 ml in the tolerability study; 6 ml in the HRS) were collected at pre-defined times up to 7 days after dosing via either an indwelling cannula or by direct venepuncture into EDTA-containing polypropylene tubes. These were used for plasma measurement of vilanterol (both studies) and radioactivity (HRS only). Additional blood (18 ml, HRS only) was collected at 0.5, 3.5 and 24 h after dosing and used for metabolic investigations. Blood was placed on crushed ice prior to centrifugation in a refrigerated centrifuge at approximately 1500 x g for 10 minutes to yield plasma which was then stored frozen at nominally –20°C. All urine and faeces (HRS only) were collected at 24 hour intervals up to at least 7 days post-dose. The faecal and urine samples were stored frozen at –20°C or less prior to analysis.

In vitro Incubations. [^{14}C]vilanterol was incubated at a concentration of 10 µM in the presence of mouse, rat, dog, rabbit and human cryopreserved hepatocytes (In vitro technologies INC, Baltimore, USA) using Williams Medium E incubation media supplemented with antibiotics, at 37°C for 24 hours. Following incubation, methanol (1.2 ml) was added to all hepatocyte incubations (0.6 ml), the precipitate was

centrifuged at 7500 g at ambient temperature for 5 minutes and the supernatant removed for further analysis.

Assay for Total Radioactivity (HRS only). After measurement of the volume of urine or weight of stool, the levels of radioactivity were determined by LSC (Beckman LS series, Bucks, UK or Perkin Elmer Life Sciences instruments, Bucks, UK) with quench correction performed by an automatic external standard ratio method, which was established using sealed ^{14}C standards. Aliquots of liquid samples (e.g. urine, dose dilutions) or extracts of samples were mixed with scintillation fluid. Faecal samples were homogenised with a minimal amount of water and reweighed. Aliquots of homogenised faecal material were combusted using a Packard model 307 oxidiser (Canberra Packard, Didcot, UK) prior to radioassay. In some urine and faeces samples the levels of radioactivity were too low to be determined by LSC. The levels of radioactivity in these samples were determined, following combustion to graphite, using AMS (a 5MV tandem accelerator mass spectrometer) at Xceleron Ltd, York, United Kingdom. Analysis of plasma was conducted at GSK using AMS (a 250kV single-stage accelerator mass spectrometer; Young et al., 2008). The lower limit of quantitation (LLQ) was previously established as 10% above endogenous background levels of total radioactivity in human plasma with greater than 95% confidence (data not shown). Based on the pre-dose plasma values for the six subjects in the human ADME study and the specific activity administered, this corresponded to 2.45 pg equivalents of vilanterol/ml.

Determination of Radiochemical Purity (HRS only). The radiochemical purity of [^{14}C]vilanterol solution was determined using the HPLC system described below for metabolite analysis. The radiochemical purity of the administered [^{14}C]vilanterol determined on the day of dosing was 99.4%.

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Quantification of Vilanterol in Plasma (Tolerability Study and HRS).

Concentrations of vilanterol in human plasma samples were determined using analytical methods validated to GSK worldwide Standard Operating Procedures which are based on guidelines set out by the US Food and Drug Administration Agency. The extraction method for vilanterol utilised solid phase extraction to achieve a Low Limit of Quantitation (LLQ) of 10 pg/ml from a 200 µl aliquot of plasma where [$^{2}H_{12}$]-vilanterol was added as an isotopically labelled internal standard. Extracts were analysed using HPLC (Waters™ Acquity System, Milford USA) and tandem mass spectrometry (HPLC-MS/MS). The mass spectrometer used was an API-4000 triple quadrupole using a TurboIonSpray™ interface (AB Sciex, Framingham, USA) in positive mode with multiple reaction monitoring. The mass transitions were 486 to 159 and 498 to 159 for vilanterol and [$^{2}H_{12}$]-vilanterol respectively.

Quantification and Profiling of Metabolites. An equal volume of plasma from each subject was pooled to produce a single discrete representative sample per time point. A single discrete pooled human urine sample was prepared by mixing proportional volumes of 0-24 h urine collected from each volunteer which individually represented >95% of the radioactivity excreted by this route. Representative faecal homogenates were obtained by pooling across sampling times on a total sample weight basis, to generate a discrete pool containing 80% or greater of the radioactivity excreted in faeces for each subject. These were subsequently pooled across individuals to obtain a single faeces homogenate sample representative of the whole study. Radioactive material was extracted from plasma and faeces samples by vortex-mixing successive aliquots of acetonitrile. The extracts were evaporated to near dryness under a stream of nitrogen before reconstitution in small

volumes of ammonium formate buffer (50 mM, pH 2.5) and acetonitrile. Plasma and faeces extracts and human urine were spiked with dimethylsulfoxide (DMSO) solutions containing authentic reference standards of vilanterol, GW630200 and GSK932009 as chromatographic UV retention time markers. The spiked plasma, urine and faeces samples were subsequently analysed by HPLC with UV detection and radiometric detection by off-line AMS analysis. The supernatants from hepatocyte incubations were analysed with no further treatment using HPLC with off-line radio detection by microtitre plate scintillation counting and HPLC-MS.

Chromatographic instrumentation consisted of an Agilent system (South Queensferry, Scotland, UK) consisting of a 1100 binary pump, column oven (25°C), UV detector (λ 275 nm) and auto-sampler which were linked to a fraction collector (Gilson, Luton, UK). A Luna Phenyl Hexyl RP column (250 x 4.6 mm, 5 micron particle size) was employed. The mobile phase consisted of 50 mM ammonium formate pH 2.5 (solvent A) and acetonitrile (solvent B, supplied by Fisher Scientific, Loughborough, Leicestershire, UK) at a flow rate of 1 ml/min. A linear gradient was used as follows: 10% to 37% B over 13 min; 37 to 52% B by 14 min; 52% B to 64% B by 35 min; 64% B to 90% B by 40 min; conditions being held at 90% B until 45 min. The column was re-equilibrated for a minimum of 6 minutes after each injection. Fractions from the HRS samples were collected into quartz glass tubes (York Glassware Services, York, UK) every 30 s for off-line radiodetection by AMS. For hepatocyte samples, HPLC fractions were collected into 96 deep-well microtitre plates containing yttrium silicate solid scintillant (Perkin Elmer, Bucks, UK) every 12 s. Each HPLC eluate was evaporated to dryness in a drying oven overnight and the dried plates were sealed using a Microplate Heat sealing film (Perkin Elmer).

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Radioactivity in each well was subsequently counted using a TopCount NXT counter (Perkin Elmer).

Structural Identification of Metabolites. Structural characterisation was performed on selected HRS samples by HPLC-MS using an Accela autosampler and HPLC system coupled to an Orbitrap XL mass spectrometer with Xcalibur software (ThermoFisher Scientific, Hemel Hempstead, UK). The majority of human metabolites were identified through chromatographic comparisons to metabolites identified in other samples such as hepatocyte incubations. Metabolites in the hepatocyte incubations were characterised using an Agilent HP1100 autosampler and HPLC system coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer with MassLynx software (Waters MS Technologies, Manchester, UK) or a ThermoFinnigan LTQ linear trap mass spectrometer with Xcalibur software (ThermoFisher Scientific, Hemel Hempstead, UK). All mass spectrometric analyses employed electrospray ionisation in the positive and negative ion modes. The flow was split 1:2 (Orbitrap) or 1:20 (Quattro and LCT) between the mass spectrometers and fraction collector (CTC Analytics HTX PAL). Metabolites in human hepatocyte supernatant were also separated by preparative HPLC using an Agilent series 1100 Preparative-LC system (Waldbronn, Germany) for characterisation by nuclear magnetic resonance spectroscopy (NMR). Separations were carried out on a Luna Phenyl Hexyl HPLC column (250 x 10 mm i.d. 5 micron particle size) at ambient temperature with a mobile phase of 50 mM ammonium formate (pH 2.5, solvent A) and acetonitrile:methanol (20:80 v/v, solvent B) at a constant flow rate of 4 ml/min with an initial gradient of 10% B increasing linearly to 37% B at 13 min, then to 52% B at 14 min, 64% B at 25 min and 90% B at 40 min where it was held for a further 5 min. HPLC eluent was collected into fractions, in a time-slice mode, into two 96-

deep-well plates using a frequency of 15 s per fraction. This resulted in 180 fractions, each containing 1 ml of column eluent. The flow was split 100:1 into a Micromass ZQ mass spectrometer (Waters, Manchester, UK) fitted with an electrospray source operated in the positive ionization mode. System control was mediated through MassLynx™ and FractionLynx™ (Waters, Milford, USA). The fractions were taken to dryness under nitrogen at 37°C within the 96-deep-well plates using a Micro DS96 dry down station (Porvair Scientific Ltd, Shepperton, UK) and then reconstituted in approximately 0.6 ml of deuterium oxide : acetonitrile (1:1) before being transferred to 5 mm NMR tubes. 1D proton NMR experiments were performed on all 180 fractions using a Bruker 600 MHz spectrometer equipped with an inverse 5 mm TXI Cryo- Probe™ (1H/13C/15N) operating at 600.13 MHz under the control of TopSpin software (Bruker, Rheinstetten, Germany).

Accelerator Mass Spectrometry. The ^{14}C content of human plasma, faecal homogenates, urine, plasma extracts and HPLC fractions were measured by AMS, which measures the radiocarbon content in a sample through separation of the isotopes of carbon present by their different mass-to-charge ratios. Before analysis by AMS, the samples were graphitised via a two-step process of oxidation and reduction (Young et al., 2008). The graphite, containing a cobalt catalyst, was packed into an aluminium cathode and loaded into a sample wheel that was then placed into the ion source of the AMS instrument (either NEC 5MV tandem or 250KV single-stage AMS system, WI, USA). A generic value of 4.14%, based on GSK historical data, for the carbon content of plasma was used for any neat plasma samples analysed, and values were adjusted appropriately to allow for any dilution. For faecal homogenates the carbon content for each sample was measured independently using an elemental analyser (NA2100 Brewanalyser, CE Instruments, UK). The carbon content for the

HPLC fractions and for urine was deemed to be insignificant and only the carbon content of the carbon carrier was used in calculations to determine the ^{14}C content based on the determined ^{14}C to ^{12}C ratio in the samples. The AMS data, which are expressed as percent modern carbon were used to calculate the dpm/ml of sample, where 100 percent modern carbon equals 0.01356 dpm/mg carbon.

Pharmacokinetic Calculations. Pharmacokinetic analyses of plasma vilanterol concentration-time data (tolerability study only) and ^{14}C-radioactivity concentration-time data (HRS only) were conducted using non-compartmental Model 200 (for extra vascular administration) of WinNonLin Professional Edition version 5.2 (Pharsight Corporation, CA, USA). Values for the following pharmacokinetic parameters were estimated; the maximum observed plasma concentration (Cmax), the first time to reach Cmax (tmax), concentration at last measurable time point (Ct) and the time of the last observed plasma concentration (tlast). Where possible, the terminal plasma elimination rate-constant (λz) was estimated from log-linear regression analysis of the terminal phase of the plasma concentration time profile. The area under the plasma concentration-time curve from time zero to the last quantifiable time point (AUC(0–t)) and extrapolated to infinity (AUC(0–∞)) were calculated by a combination of linear and logarithmic trapezoidal methods.

In vitro Pharmacological Activity of Metabolites. The $beta_1$ and $beta_2$ activities of vilanterol, GW630200 (M29), GSK932009 (M33) GSK167112 (M20) and GW853734 (a potential metabolite of vilanterol undetected in animals or human) were determined in cAMP TR-FRET LANCE™ (Time Resolved Fluorescence Energy Transfer; Perkin Elmer) agonist assays. These were either metabolites or potential metabolites of vilanterol, selected to represent routes of human metabolism. Compounds were dissolved in DMSO at a concentration of 10 mM and serially

DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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diluted in DMSO using a 1 in 4 dilution step to provide 11-point concentration–response curves (CRCs). Chinese Hamster Ovary cells stably expressing either recombinant $beta_1$ or $beta_2$ receptor were thawed at 37°C, diluted in phosphate buffered saline, and centrifuged at 1500x g for 5 minutes. Cells were re-suspended (2 million cells/ml) in stimulation buffer (Hanks Balanced Salt Solution containing 0.01% Bovine Serum Albumin, 500 μM 3-isobutyl-1-methylxanthine, 5 mmol/L HEPES final assay concentration, pH 7.4 with potassium hydroxide).

The in vitro activity against either $beta_1$ and $beta_2$ was measured by adding CRCs of compound or controls, 100 nl/well to a white Greiner polypropylene low volume 384-well plate to which the relevant cell suspension (10,000 cells/well at 5μl addition), and antibody solution (stimulation buffer containing Alexia fluor 647at 5 μl/well) were added. Plates were incubated for 30 minutes at room temperature then detection mixture was added (kit components; detection buffer, europium W8044-labelled streptavidin and biotin cAMP at 10 μl/well). Plates were covered and incubated at room temperature for 4 hours before being analysed on a Viewlux™ Microplate Imager (Perkin Elmer, Bucks, UK).

Results

Demographic, Safety, and Tolerability Data. Nine and six healthy male subjects were enrolled into the tolerability and HRS studies, respectively. Demographic data from the tolerability study and HRS are shown in Table 1. Vilanterol was safe and well tolerated following oral administrations up to 500 μg, with no drug-related adverse events or clinically significant changes in vital signs (including heart rate and electrocardiogram interval) observed. No concomitant medication was reported for any subject during the studies.

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Pharmacokinetics of Vilanterol and Radioactivity. Pharmacokinetic data for vilanterol and ^{14}C radioactivity in both studies are summarised in Table 2. Plasma concentrations of vilanterol following oral administration of 200 or 500 μg vilanterol in the tolerability study were low (≤ 24 and 65 pg/ml, respectively) and could only be quantified up to 0.8 h (median T_{last}) after administration of 200 μg and up to 2.8 h (median T_{last}) following the 500 μg dose. In the HRS, vilanterol was only quantified in plasma (LLQ 10 pg/ml) in a single individual (n=1/6; subject 102) at 0.5 h (14 pg/ml) and 3.0 h (15 pg/ml) post-dose; at all other sampling times vilanterol concentrations were non-quantifiable (NQ). Since two NQ values occurred in succession after the quantifiable concentration at 0.5 h, the profile was deemed to have terminated at the first NQ value and the subsequent quantifiable concentration at 3.0 h was omitted from pharmacokinetic calculations. In contrast, plasma concentrations of ^{14}C-radioactivity (total drug-related material or DRM, representing vilanterol and its metabolites) were substantially higher than vilanterol concentrations (Table 2 and Figure 2). For subject 102 (Table 2), the vilanterol Cmax (14 pg/ml) represented a very small percentage (<0.5%) of the drug-related material (DRM) where Cmax was 3148 pg vilanterol equivalents/ml (data not shown). For other subjects, where vilanterol concentrations were not quantifiable, vilanterol Cmax was <10 pg/ml (the limit of quantitation for the assay) which also corresponded to <0.5% of the DRM Cmax (2060 pg equivalents/ml), indicating that vilanterol represented a small proportion (<0.5%) of the total drug-related material in plasma.

Excretion of [^{14}C]Vilanterol Drug-Related Material. Cumulative percentage recovery of administered radioactivity in urine and faeces is depicted in Figure 3. DRM was mainly excreted in urine (50% administered radioactivity or 70% of the recovered radioactivity). Faecal excretion accounted for a further 21% of the

administered radioactivity (or 30% of the recovered radioactivity). Absorption of vilanterol was at least 50% based on urinary recovery. Total recoveries of radioactivity were relatively low (72% of the administered radioactivity). Excretion of radioactivity, however, was essentially complete by the end of Day 4 (>99% of recovered radioactivity) with <0.3% of administered radioactivity being recovered, in total, between Days 5 and 7 after dosing.

Metabolite Profiles. Radio-chromatograms of separated metabolites were generated from HPLC eluate fractions using AMS as a highly sensitive off-line radio-detector. Representative radio-chromatograms for human plasma, urine and faeces are shown in Figure 4 with each radioactive peak given an alphabetical assignment (A-M). Solvent extraction efficiencies were 64% for the 0.5 h plasma (where metabolites were quantified) and 75% from faeces homogenates. Solvent extraction efficiency from the 24 hour plasma sample was very low, being <10%. Low chemical mass precluded the generation of interpretable MS spectra for all but a few metabolites. The majority of metabolites were, therefore, assigned by comparison of chromatographic retention times to either authentic reference material or metabolites identified in other non-clinical studies (Table 3) where higher amounts of metabolite facilitated more detailed identifications. Characterised metabolites were given a numerical designation preceded by the letter M (e.g., M1), each number representing a distinct metabolite structure. Metabolite structures assigned to each radioactive peak, along with supporting evidence from other studies, are shown in Table 3.

***Plasma Metabolites*.** HPLC radiochromatograms of human plasma extracts from samples taken at 0.5 and 3.5 h after dosing are shown in Figure 4. Radioactive peaks from the 0.5 h sample time (Cmax of vilanterol in plasma) are quantified in Table 4. The largest radioactive peaks (F and G) were assigned as a mixture of O-dealkylated

metabolites (including M33/GSK932009 and M29/GW630200). These metabolites represented approximately 33% of plasma radioactivity in the 0.5 hour plasma extract. Peak J2 (assigned as M26, a C-dealkylated metabolite) and Peak B (M1, an O-dealkylated glucuronide conjugate) represented 18 and 13% of plasma radioactivity in the 0.5 hour sample. No other radioactive peak represented greater than 10% plasma drug-related material. All systemically circulating metabolites, with the exception of Peak K (M16, an oxidative metabolite), were the products of O- or C-dealkylation.

***Urine and Faeces Metabolites*.** HPLC radiochromatograms of pooled human urine and faeces extracts are shown in Figure 4 and are quantified in Table 4. The largest radioactive peaks in human urine and faeces, as in plasma, were Peaks F and G, assigned as a mixture of O-dealkylated metabolites. Peaks F and G accounted for 50% of urinary radioactivity, 35% of faecal radioactivity and a combined 51% of the recovered dose. Peaks B, D and E (assigned as M1, M3, M4 and M30) were also associated with O-dealkylation pathways and represented a combined total of 32% urinary radioactivity, 14% faecal radioactivity or a combined 27% recovered radioactive dose. M26 was a minor urinary component. Unchanged vilanterol was not detected in human urine but represented about 15% faecal radioactivity (5% of the recovered dose).

***In vitro Metabolites*.** HPLC radiochromatograms of mouse, rat, dog, rabbit and human hepatocyte incubations are shown in Figure 5. The main route of metabolism of vilanterol in human hepatocyte incubations was O-dealkylation to yield M29 (GW630200) with subsequent oxidation to M33 (GSK932009), M30 and M39. O-dealkylation was also a notable route of metabolism in all animal species except for the mouse where direct glucuronidation of vilanterol to yield M12 was the major pathway. O-glucuronidation with O-dealkylation to M3 was a major pathway in the

rat (M3). C-dealkylation of the salicyl alcohol moiety to M26 was a major pathway in dog and rabbit hepatocytes.

Pharmacological Activity of Metabolites. The pharmacological activity of potential metabolites using human $beta_1$ and $beta_2$ cAMP LANCE assays are shown in Table 5 along with their chemical structures. Tested metabolites were representative of the main routes of human metabolism. M29 (GW630200) and M33 (GSK932009) were major in vitro pathways representing O-dealkylation. M20 (GSK1676112) and GW853734 were investigated to understand the effect of N-dealkylation (both halves of the molecule) on its pharmacological activity. All cleaved structures tested were ≥ 2500 times less potent compared to vilanterol on the $beta_2$ receptor indicating that metabolic cleavage largely removes the $beta_2$ agonist pharmacology associated with vilanterol. Similarly the metabolites and cleaved analogues of vilanterol were either inactive against $beta_1$ or were of such low potency that beta agonist related effects would be unlikely in human.

Discussion

The results described provide an understanding of the metabolism and disposition of vilanterol in human. The oral route of administration represents the swallowed portion of an inhaled dose and, based on urinary recovery of radioactivity (Figure 3), at least 50% of the vilanterol oral dose solution was absorbed. Metabolite profiles, however, indicated that <5% of the administered radioactivity was associated with unchanged vilanterol in faeces potentially reflecting the percentage of unabsorbed drug (Table 4). Oral absorption of vilanterol is probably higher, therefore, than the estimate based on urinary excretion alone and is likely virtually complete. Systemic exposure to vilanterol represented a very small proportion (<0.5%) of DRM in plasma (Figure 2) and together with the presence of several circulating metabolites, was indicative of extensive first-pass metabolism of vilanterol. Considered together, systemic vilanterol concentrations measured following inhalation administration are therefore due to absorption through the lung with little or no contribution from the swallowed portion of the dose which is well absorbed but undergoes extensive first pass metabolism.

The total recovery of radioactivity was relatively low (71.6% of the administered radioactivity) and the precise cause is unknown but is most likely due to technical reasons related to the low radioactive dose (74 kBq; 25 to 50- fold lower than that used in more traditionally designed studies) and the low vilanterol chemical mass administered (200ug). This results in excreta containing very low mass concentrations where the measurement of radioactivity would be sensitive to very low levels of non-specific binding to any apparatus used. The mixed methods used for radioactivity measurement may also have contributed to the low radioactive recovery.

Any concerns about the slow release of drug-related material (DRM) from a depot within the body or loss due to exhalation of radioactivity were not supported by the data. By 4 days after dosing, elimination of radioactivity was essentially complete (>99% of the recovered radioactivity detected in urine or faeces collections) with only <0.3% of radioactivity recovered within the next 3 days and there was little evidence for substantial metabolism associated with atoms close to the [^{14}C] isotope to easily explain exhalation of radioactivity as carbon dioxide. A retrospective analysis of mass balance data (Roffey et al) suggests total radioactive recovery in human, using routine methodology, should normally be 80% or greater. The authors conclude that a low mass balance is more likely due to technical limitations which often confound the generation of a full mass balance and should not be regarded as tissue sequestration per se.

The plasma half-life of radioactivity was very long (see Figure 2) and the low extraction efficiencies (<10% extracted at 24 hours after dosing) were consistent with tight or covalent binding of DRM to plasma protein rather than slow release of DRM from a depot. This binding phenomenon, which occurs in many metabolism studies, is accentuated when using the very high sensitivity conferred by AMS when compared to traditional LSC counting.

The human metabolic profiles in human plasma, urine and faeces (Figure 4) were broadly similar to that observed using human hepatocytes (Figure 5). Elimination of vilanterol was mainly by metabolism (Table 4, Figure 4) followed by excretion of metabolites in urine (70% of recovered radioactivity) and faeces (30% of recovered radioactivity). Direct secretion or direct conjugation of vilanterol were not major routes of elimination with less than 5% of the administered dose associated with

unchanged vilanterol. The major route of metabolism (Figure 1) was via O-dealkylation with up to 78% of the recovered dose (in all human excreta) eliminated as O-dealkylated metabolites (Tables 3 and 4). N- dealkylation and C-dealkylation were minor pathways representing a combined 5% of the recovered dose. Although C-dealkylation is uncommon, it has been described elsewhere as a route of metabolism for another beta agonist, indacaterol (Kagan et al., 2012) which shares the same (amino-hydroxyethyl) phenol moiety with vilanterol. The authors tentatively proposed two mechanisms for this carbon- carbon cleavage: one initiated by a one electron oxidation of the oxygen in the phenol moiety followed by a beta-scission reaction; the other initiated by phenolic hydroxylation, subsequent keto/enol tautomerism followed by a retro-Aldol reaction. Both mechanisms are also feasible for the C-dealkylation of vilanterol.

The proposed commercial dose for vilanterol is 25 μg, once daily, by the inhalation route. All identified routes of metabolism for vilanterol are associated with innocuous (as opposed to reactive) chemical mechanisms. The maximum daily body burden to any metabolite does not exceed the vilanterol dose level of 25 μg/day which is negligible when compared with the 10 mg daily threshold for clinical adverse drug reactions mediated by reactive metabolite mechanisms proposed by a number of key opinion leaders (Smith, 2005; Uetrecht, 1999). Also, at this low dose level, the likelihood of a metabolite causing new unexpected toxicity, unrelated to its beta-agonist pharmacology, is negligible. As a result, a major focus of the metabolism work for vilanterol was to establish whether any metabolites possessed beta-agonist pharmacological activity that could result in unwanted systemic effects. Firstly, following oral administration of vilanterol at up to 500 μg, plasma concentrations of metabolites were significantly higher than unchanged vilanterol as well as being

DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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significantly greater than metabolite concentrations produced after an inhaled administration at the lower therapeutic dose of 25 μg. Despite the higher metabolite concentrations there were no changes in measured vital signs or heart rate on either clinical study, indicating a lack of metabolite beta adrenergic activity. Secondly, synthesized metabolites, representative of the major human metabolic routes, were ≥ 2500 times less potent against $beta_2$ receptors compared to vilanterol (Table 5). The synthesised metabolites were either inactive against $beta_1$ receptors or of such low potency that no $beta_1$-agonist related effects would be seen in human after the human daily dose. The metabolites tested represented both halves of the molecule following cleavage metabolism and demonstrate how O- or N- dealkylation considerably reduces potency against both $beta_1$ and $beta_2$ receptors. The lack of potency associated with GW853734 demonstrates that N-dealkylation to metabolites where the [^{14}C] radioisotope label might be lost will also dramatically reduce pharmacological activity. These data represent an extension to previously published data showing how $beta_2$-agonist pharmacology is lost following metabolism of vilanterol in human microsome incubations to M29 (GW630200), [Ford et al, 2010].

The metabolites detected in human matrices were also present in the species used for toxicology assessment (data not shown) demonstrating their relevance to the assessment of safety for vilanterol in human. Additionally, all major routes of metabolism in human hepatocytes were also shown in mouse, rat, dog and rabbit hepatocyte incubations. The largest radioactive chromatographic peaks in human plasma, following oral administration were Peak G (M33/GSK932009), Peak F (several O-dealkylated and oxidised metabolites including GW630200), Peak J2 (M26) and Peak B (M1) - see Table 4 and Figure 4. These radioactive peaks each represented >10% plasma DRM (the threshold for further investigation as

recommended by ICH M3[R2]), 0.5 h after an oral administration of vilanterol. It should be noted, however, that the proposed commercial dose of vilanterol (25 μg) is considerably lower than the 10 mg threshold proposed in ICH M3(R2) guidance, below which a more lenient threshold than 10% plasma DRM is deemed appropriate. Concentrations of M33 (GSK932009) and M29 (GW630200) were measured in plasma from asthma and COPD patients dosed up to 50 μg vilanterol by the inhalation route and were generally lower than the quantification limit (180 and 90 pg/ml, respectively, data not shown). Both metabolites circulated at higher concentrations in mice, rats and dogs compared with human, when measured as part of the inhalation toxicology studies.

In summary, oral vilanterol is well absorbed in human, and subject to extensive first pass metabolism to metabolites with negligible beta agonist pharmacological activity. The metabolism of vilanterol is mainly by O-dealkylation and metabolites are excreted both via faeces and urine. To a large extent the low dose levels often associated with inhalation molecules (25 μg in this case) mitigate any metabolite or metabolism safety concerns.

DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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Acknowledgements

We thank Dr Geoff Badman (Chemical Development, GSK R&D, Stevenage, UK) for the synthesis and supply of [^{14}C]vilanterol and the metabolite standards. We thank Dr Michael Butler and his staff at Xceleron Ltd, York, UK for the accelerator mass spectrometry analysis of faecal homogenates. We thank Dr Gordon Dear for the metabolite identification work from hepatocyte incubations and Steve Corless for AMS analysis of GSK graphites.

Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Harrell, Beaumont, Siederer, Young, Roberts, Kempsford.

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Footnotes

This work was funded by GlaxoSmithKline R&D, UK as part of the development programme for vilanterol as a novel therapeutic agent. Data in this manuscript corresponds with clinical study numbers B2C106180 (GSK company report GM2009/00020/00) and B2C106181 (GSK company report YM2010/00088/00) with metabolite identifications reported in study number 10DMW020 (GSK company report 2011N115614). Hepatocyte incubations were reported in GSK company report WD2006/02574/00. Pharmacological activities of metabolites were reported in GSK company report HR2008/00016/00. All authors were employees of GlaxoSmithKline Research and Development Ltd. at the time of conducting the work.

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DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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Figure Legends

Figure 1

Metabolic scheme for vilanterol in human

Chemical structures are shown for vilanterol and important human metabolites. ^{14}C indicates position of labelled carbon.

Figure 2

Mean concentrations of vilanterol and radioactivity in plasma following oral administration of [^{14}C]vilanterol to humans

The difference in mean plasma concentrations of radioactivity (pg equivalents DRM /ml) and concentrations of unchanged vilanterol (pg/ml) are highlighted on this concentration/time semi-log plot generated using data collected following a single oral administration of 200 μg [^{14}C]vilanterol containing 74 kBq radioactivity.

Figure 3

Mean percentage cumulative recovery of radioactivity in urine and faeces (expressed as % administered radioactivity) following oral administration of [^{14}C]vilanterol (74 kBq) to humans

Cumulative recovery of radioactivity is shown in urine and faeces collected for up to 7 days following a single oral administration of 200 μg [^{14}C]vilanterol containing 74 kBq radioactivity. Mean data from 6 subjects is presented.

DMD Fast Forward. Published on October 4, 2012 as DOI: 10.1124/dmd.112.048603
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Figure 4

HPLC radio-chromatograms of plasma, urine and faeces following oral administration of [^{14}C]vilanterol to humans.

Representative HPLC radiochromatograms are presented following analysis of human plasma (0.5 and 3.5 h, panels A and B) extracts, human urine (C) and human faeces (D) extracts obtained following a single oral administration of 200 μg [^{14}C]vilanterol containing 74 kBq radioactivity. Structures assigned to peaks are depicted in Table 3.

Figure 5

HPLC radio-chromatograms of mouse, rat, dog, rabbit, human and control hepatocyte incubations with [^{14}C]vilanterol

Representative HPLC radiochromatograms are presented following analysis of incubations of [^{14}C]vilanterol with mouse (A), rat (B), dog (C), rabbit (D) and human (E) cryopreserved hepatocytes. A control incubation without hepatocytes (drug-only) is included for comparison (F).

Table 1

Summary of Subject Disposition and Demographic characteristics following oral administrations of 200 or 500 µg of vilanterol or 200 µg of [^{14}C] vilanterol to male subjects.

Subject disposition and demographic characteristics are summarised from two clinical studies. In the first tolerability study subjects received 200 or 500 µg vilanterol. In the subsequent human radiolabel study subjects received 200 µg [^{14}C] vilanterol containing 74 kBq radioactivity.

	Tolerability study (GSK study B2C106180)	Human radiolabel study (GSK study B2C106181)
Number of subjects completed n, (%)	9	6
Oral administration	200 or 500 µg vilanterol	200 µg [^{14}C] vilanterol
Age, years: Mean (range)	33.1 (19-47)	43.3 (35-53)
Race n, (%) White/Caucasian/European Heritage Asian Heritage African/American Heritage	 7 (78) 1 (11) 1 (11)	6 (100)
Ethnicity n, (%) Not Hispanic or Latino	9 (100)	6 (100)
Body Mass Index, kg/m^2: Mean (range)	24.8 (21.3 – 29.0)	26.2 (21.8 - 28.8)
Height, cm: Mean (range)	178 (166-186)	179 (163 - 189)
Weight, kg: Mean (range)	78.5 (66.9 – 90.8)	83.9 (57.8 – 102)

Table 2

Pharmacokinetic parameters in human plasma for vilanterol and [^{14}C] radioactivity following oral administration of 200 or 500 µg of vilanterol and 200 µg of [^{14}C] vilanterol.

Plasma $AUC_{(0\text{-}t)}$, C_{max} and T_{max} of vilanterol and ^{14}C radioactivity in plasma are shown following single oral administrations to healthy male subjects in two clinical studies. For the tolerability study parameters are presented for two dose levels following specific assay analysis for vilanterol. For the human radiolabel study parameters are presented from a single dose level and calculated from: mean specific assay data for vilanterol in all subjects; specific assay data for vilanterol in subject 102 who was the only subject with measurable concentrations; mean radioactivity measurements (DRM) in all subjects presented as pg equivalents of vilanterol per ml.

	Tolerability Study (200 µg or 500 µg vilanterol)						Human Radiolabel Study (200 µg [^{14}C] vilanterol)						
Parameter; units	**200 µg**	**n**	**n[1]**	**500 µg**	**n**	**n[1]**	**Vilanterol**	**Vilanterol [a]**	**n**	**n[1]**	**Radioactivity**	**n**	**n[1]**
$AUC_{(0\text{-}t)}$ (95% confidence interval); pg.h/ml	21.8 (NC)	3	1	62.6 (25, 156)	6	0	NC	NC	0	6	66000 [b] (52000, 84000)	6	0
C_{max} (95% confidence interval; pg/ml	18.5 (10, 33)	3	0	44.5 (32, 62)	6	0	NC	14	1	5	2060 [b] (1580, 2700)	6	0

T_{max} median [range]; h	0.5 [0.25 – 0.5]	3	0	0.5 [0.25 – 1.0]	6	0	NC	0.5	1	5	3.0 [2.5 – 4.0]	6	0
Tlast median [range]; h	0.8 [0.5 -3.0]	3	0	2.8 [1.0 -8.0]	6	0	NC	0.5	1	5	168 [168-168]	6	0

a Data is presented separately for subject 102 where there was a single quantifiable concentration of 14 pg/ml at 0.5 hours after dosing

b Radioactivity concentrations are expressed in terms of pg equivalents of vilanterol/ml

NC: Not calculable due to insufficient data or concentrations below lower limit of quantification (10 pg/ml).

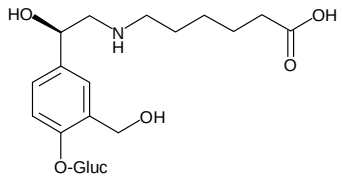
n : number of subjects with non-missing observations, including inputed NC values for AUC and Cmax.

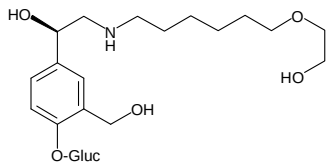
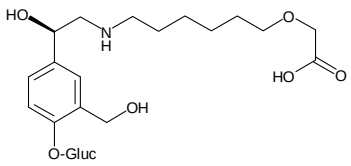
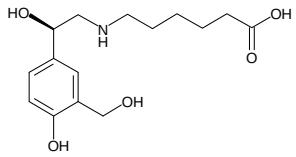
n^1 : number of subjects for whom parameters cannot be derived because of NQ concentrations.

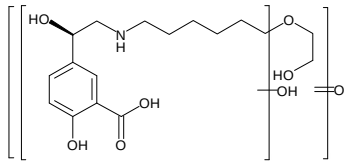
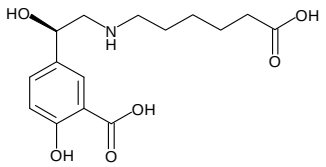
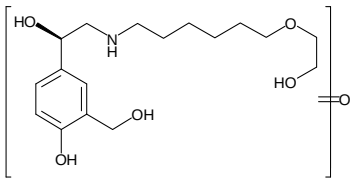
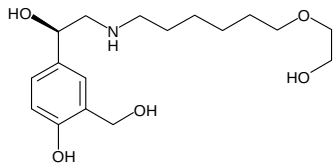
Table 3

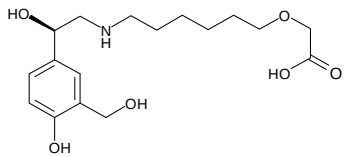
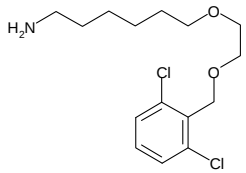
A summary of the metabolite structures assigned to radio-chromatographic peaks in human urine, faeces and plasma following a single oral administration of 200 µg [^{14}C]vilanterol.

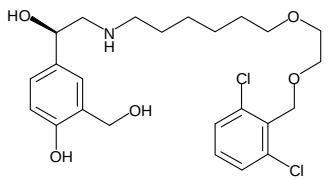
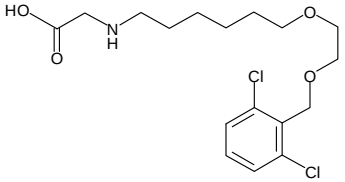
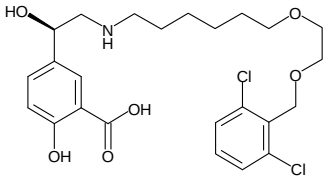
Chemical structures and supporting evidence from in vitro samples for metabolites assigned to radio-chromatographic peaks detected in human urine, faeces and plasma obtained following a single oral administration of 200 µg [^{14}C] vilanterol containing 74 kBq of radioactivity.

Peak	Numerical Assignment	Proposed Structure	Parent Ion $[M+H]^+$ *MS/MS Product Ions* (m/z)[1]	^{1}H NMR Chemical Shifts (ppm)	Evidence supporting structural assignment
A	Void Volume	Not applicable	Not applicable	Not applicable	Not applicable
B	M1 (O-dealkylation, oxidation & O-glucuronidation)		476 *440, 300, 282, 264, 246*	No NMR from in vitro samples[2]	Peak B assigned as M1 based on chromatographic retention time comparison to mouse and rat hepatocyte incubations

Peak	Numerical Assignment	Proposed Structure	Parent Ion [M+H]$^+$ *MS/MS Product Ions* (m/z)[1]	^{1}H NMR Chemical Shifts (ppm)	Evidence supporting structural assignment
D&E	M3 (O-dealkylation & O-glucuronidation)		506[2]	No NMR from in vitro samples[2]	Peaks D and E assigned as a mixture of M3, M4 and M30 based on chromatographic retention time comparisons to mouse, rat, rabbit, dog and human hepatocyte incubations.
	M4 (O-dealkylation, oxidation & O-glucuronidation)		520 *484, 344, 326, 308, 250, 232*	No NMR from in vitro samples[2]	
	M30 (O-dealkylation & oxidation)		330 *282, 264, 246, 228*	δ ppm 7.26 (d, J=1.6, 1H), 7.16 (dd, J=8,3,1.6, 1H), 6.84 (d, J=8.3, 1H), 4.86 (t, J=6.5, 1H), 4.59 (s, 2H), 3.14 (d, J=6.5, 2H), 3.00 (t. J=7.9, 2H), 1.49-1.67 (m , 6H)	

Peak	Numerical Assignment	Proposed Structure	Parent Ion $[M+H]^+$ *MS/MS Product Ions* (m/z)[1]	^{1}H NMR Chemical Shifts (ppm)	Evidence supporting structural assignment
F	M6 (O-dealkylation & oxidation)		No MS^2	No NMR	Peak F assigned as a mixture of O-dealkylated metabolites based on chromatographic retention time comparisons to metabolites indentified in other studies.
	M7 (O-dealkylation & oxidation)		314 *296, 278*	δ ppm 7.76 (d, J=2.3, 1H), 7.36 (dd, J=8.5,2.3, 1H), 6.87 (d, J=8.5, 1H), 4.89 (t, J=7.1, 1H), 3.03 (t, J=7.9, 2H), 2.27 (m, 2H)1.64-1.70 (m, 4H), 1.33 (m, 2H)	M6 and M19 assigned based on chromatographic comparisons to rat bile (data not shown). M7 assigned based on chromatographic comparison to human hepatocyte incubations.
	M19 and M39 (O-dealkylation & oxidation)		M19: No MS^2 M39: 344 *308,250, 232*	No NMR	M39, a metabolite identified in human hepatocyte incubations (Figure 4) was detected by HPLC-MS in human urine.
	M29 (GW630200, O-dealkylation)		330 *294, 250, 232*	δ ppm 7.26 (d, J=1.8, 1H), 7.15 (dd, J=8.1, 1.8, 1H), 6.84 (d, J=8.1, 1H), 4.86 (t, J=6.8, 1H), 4.59 (s, 2H), 3.61 (t, J=5.1, 2H), 3.48 (t, J=5.1, 2H), 3.45 (t, J=6.7, 2H), 3.13 (d, J=6.5, 2H), 2.99 (t, J=8.3, 2H), 1.64 (m, 2H), 1.53 (m, 4H) , 1.32 (m, 2H)	M29 was a major component in incubations with human hepatocytes (Figure 4) and human liver microsomes (data not shown). Despite the availability of reference standard (GW630200), the presence of this metabolite could not be confirmed by HPLC-MS in human urine.

Peak	Numerical Assignment	Proposed Structure	Parent Ion $[M+H]^+$ *MS/MS Product Ions* (m/z)[1]	^{1}H NMR Chemical Shifts (ppm)	Evidence supporting structural assignment
G	M33 (GSK932009, O-dealkylation & oxidation)		344 *308, 250, 232*	δ ppm 7.26 (d, J=2.2, 1H) 7.15 (dd, J=8.3,2.2, 1H), 6.84 (d, J=8.3, 1H), 4.86 (t, J=6.7, 1H), 4.59 (s, 2H), 3.79 (s, 2H), 3.43 (t, J=6.6, 2H), 3.14 (d, J=6.7, 2H), 3.00 (t, J=7.9, 2H), 1.64 (m, 4H), 1.54 (m, 2H), 1.33 (m, 2H	Peak G assigned as M33 based on chromatographic retention time comparisons to standard GSK932009. M33 was detected by HPLC-MS in human urine.
I	M20 (GSK1676112, N-dealkylation)		322 *159*	No NMR from in vitro samples[2]	Peak I assigned as M20 based on chromatographic retention time comparison to standard GSK1676112.

Peak	Numerical Assignment	Proposed Structure	Parent Ion $[M+H]^+$ *MS/MS Product Ions* (m/z)[1]	^{1}H NMR Chemical Shifts (ppm)	Evidence supporting structural assignment
J	Vilanterol		488 470, 452, 250, 234, 232, 159	δ ppm 7.40 (d, J=8.2, 2H), 7.30 (t, J=8.2, 1H), 4.59 (s, 2H), 7.25 (d, J=2.3, 1H), 7.13 (dd, J=8.2,2.3), 6.82 (d, J=8.2, ,1H), 4.79 (dd, J=9.1,4.4, 1H), 4.77 (2, 2H), 3.66 (m, 2H), 3.56 (m, 2H), 3.42 (t, J=6.6, 2H), 3.00 (m, 2H), 2.84 (d, J=7.6, 2H), 1.56 (m, 2H), 1.49 (m, 2H), 1.28 (m, 4H),	Peak J assigned as a mixture of unchanged vilanterol and M26 based on chromatographic retention time comparisons to vilanterol reference material and M26 in rabbit and dog hepatocyte incubations. M26 was detected by HPLC-MS in human urine. Using a second HPLC system (data not shown) and specific assays developed for vilanterol, M26 was assigned as the major component of Peak J in human urine and plasma whilst vilanterol was assigned as the major component of Peak J in human faecal extracts.
	M26 (C-dealkylation: oxidative cleavage of the salicyl alcohol moiety)		380 *334, 234, 204, 176, 159*	δ ppm 7.42 (d, J=8.0, 2H), 7.31 (t, J=8.0, 1H), 3.66 (m, 2H), 3.56 (m, 2H), 3.42 (t, J=6.7, 2H), 3.45 (s, 2H), 2.92 (m, 2H), 4.77 (s, 2H)	
K	M16 (oxidation)		502 *466, 264, 246*	δ ppm 7.75 (d, J=2.3, 1H), 7.38 (dd, J=8.6,2.3, 1H), 7.35 (d, J=8.1, 2H), 7.24 (t, J=8.1, 1H), 6.90 (d, J=8.6, 1H), 4.91 (t, J=6.8, 1H), 4.83 (s, 2H), 3.73 (m, 2H), 3.61 (m, 2H), 3.46 (t, J=6.8, 2H), 3.18 (d, J=6.8, 2H), 2.99 (t, J=7.9, 2H)	Peak K assigned as M16 based on chromatographic retention time comparison to rabbit and dog hepatocyte incubations.

Peak	Numerical Assignment	Proposed Structure	Parent Ion $[M+H]^+$ *MS/MS Product Ions* (m/z)[1]	^{1}H NMR Chemical Shifts (ppm)	Evidence supporting structural assignment
C,H	Unassigned	Not applicable	No MS	No NMR	No metabolite structures could be assigned to these peaks.

1 m/z values presented for ^{14}C isotope (major isotope for in vitro incubations)

2 Additional NMR and/or MS data available from *in situ* or *in vivo* rat study to allow structural characterisation of metabolite – data not shown

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Table 4

Summary of vilanterol metabolites in human plasma, urine and faeces following oral administration of 200 µg vilanterol.

Metabolites of vilanterol are quantified in pooled plasma, urine and faeces obtained following a single oral administration of 200 µg [^{14}C]vilanterol

Peak ID	% Radioactivity 0.5 hour Human Plasma	% Radioactivity in Urine and Faecal extracts (% recovered dose)	
		Pooled Urine	Pooled Faeces
A	8.7	ND	2.8 (0.8)
B (M1)	12.8	9.4 (6.6)	ND
D (M3)	ND	13.5 (9.5)	ND
E (M30, M4)	7.3	9.0 (6.3)	13.8 (4.1)
F (M6, M7, M19, M29, M39)	32.5	31.0 (21.7)	21.5 (6.4)
G (M33)		19.3 (13.5)	33.9 (10.2)
H	ND	2.0 (1.4)	ND
I (M20)	< 1	ND	1.3 (0.4)
J1 GW642444 (P)	ND	ND	15.4 (4.6)
J2 (M26)	17.7	6.5 (4.6)	ND
K (M16)	6.0	ND	ND

ND Not Detected.
Peak J was assigned as unchanged vilanterol in faeces (J1) and M26 in plasma and urine (J2).

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Table 5

Pharmacological activity of selected human metabolites of vilanterol tested using human beta$_1$ and beta$_2$ cAMP LANCE assays.

Pharmacological activity is shown in terms of mean pEC_{50} for vilanterol, four human metabolites and one potential metabolite.

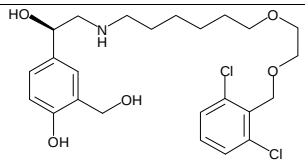
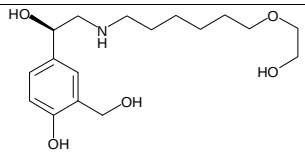
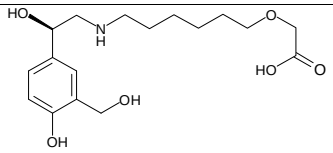
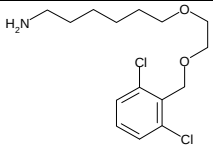
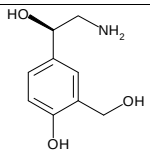
	Mean pEC_{50} (n = 7-24)	
	Beta$_1$	**Beta$_2$**
 Vilanterol	**7.0**	**10.4**
 GW630200 (M29)	**5.7**	**7.0**
 GSK932009 (M33)	**5.0**	**6.9**
 GSK1676112 (M20)	**< 4.5**	**5.0**
 GW853734 (potential metabolite – not detected in animals or human)	**5.3**	**5.4**

Figure 1

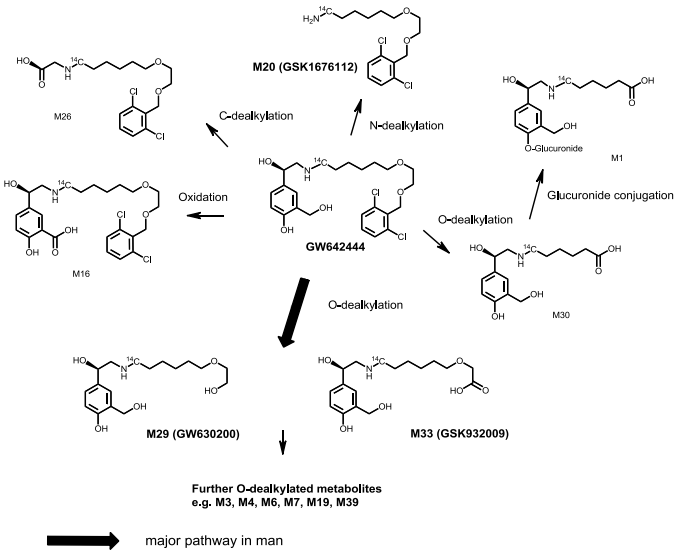


Figure 2

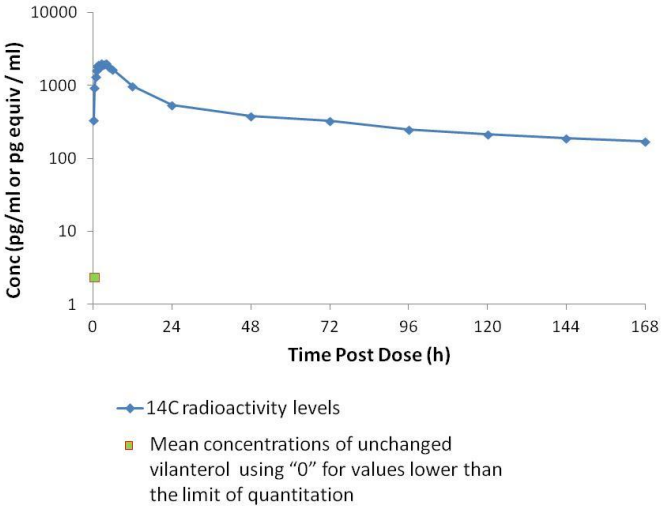


Figure 3

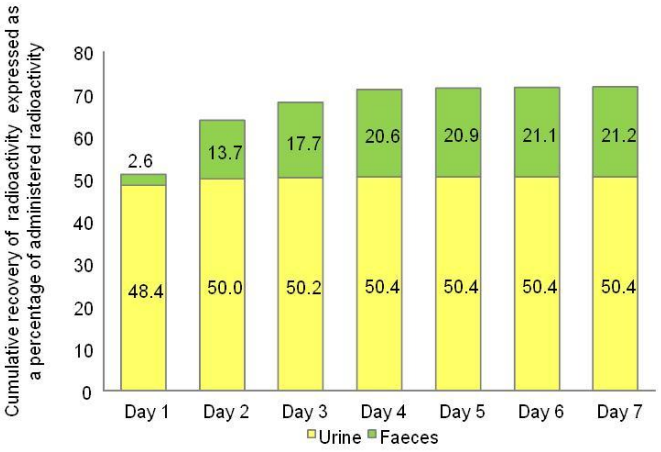


Figure 4

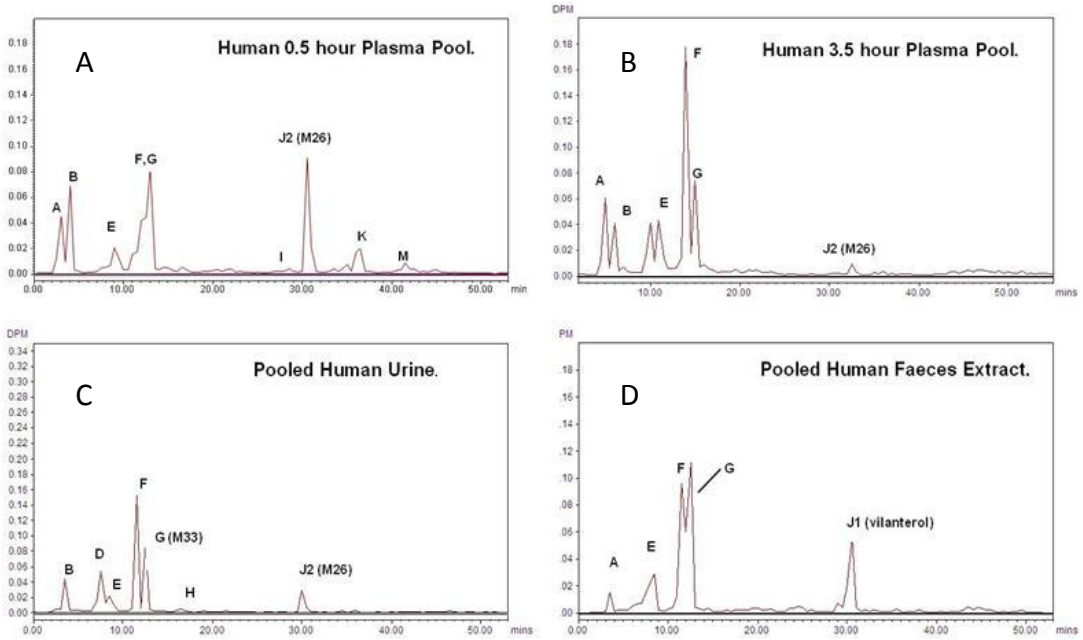


Figure 5