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Title

Investigation of Metabolism and Disposition of GSK1322322, a PDF Inhibitor, in Healthy Humans Using Entero-Test® For Biliary Sampling

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Running Title

GSK1322322 Disposition in Healthy Humans

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Nonstandard Abbreviations:

PDF	peptide deformylase
AMS	accelerator mass spectrometry
LSC	liquid scintillation counting
PK	pharmacokinetics
AUC	area under the curve
HPLC	high performance liquid chromatography

GSK	GlaxoSmithKline
MS	mass spectrometry
MS^n	tandem mass spectrometry
ACD	advanced chemistry development
NMR	nuclear magnetic resonance spectroscopy
HRS	human radiolabel study
ADME	absorption, distribution, metabolism and excretion
HPLC-MS	high performance liquid chromatography-mass spectrometry

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Abstract

GSK1322322 is an antibiotic in development by GlaxoSmithKline. In this study, we investigated metabolism and disposition of [^{14}C]GSK1322322 in healthy humans, and demonstrated the utility of Entero-Test® in a human radiolabel study. We successfully collected bile in five men with this easy-to-use device following single intravenous (1000 mg) and oral administration (1200 mg in solution) of [^{14}C]GSK1322322. GSK1322322 had low plasma clearance (23.6 L/h) with a terminal elimination half life of ~4 h following IV administration. Following oral administration, GSK1322322 was readily and almost completely absorbed (T_{max} of 0.5 h; bioavailability of 97%). GSK1322322 predominated in systemic circulation (> 64% of total plasma radioactivity). An O-glucuronide of GSK1322322 (M9) circulated at levels between 10-15% of plasma radioactivity, and was pharmacologically inactive. Humans eliminated the radioactive dose in urine and feces at equal proportions after both IV and oral doses (~ 45-48% each). Urine contained mostly unchanged GSK1322322, accounting for 30% of the dose. Bile contained mostly M9, indicating that glucuronidation was likely a major pathway in humans (up to 30% of total dose). In contrast, M9 was found in low amounts in feces, indicating its instability in the gastrointestinal tract. Therefore, without the Entero-test bile data, the contribution of glucuronidation would have been notably under-estimated. An unusual N-dehydroxylated metabolite (a secondary amine) of GSK1322322 was primarily observed in the feces, and was most likely formed by gut microbes.

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Introduction

GSK1322322 (*N*-((*R*)-2-(cyclopentylmethyl)-3-(2-(5-fluoro-6-((*S*)-hexahydropyrazino[2,1-*c*][1,4]oxazin-8(1*H*)-yl)-2-methylpyrimidin-4-yl)hydrazinyl)-3-oxopropyl)-*N*-hydroxy-formamide) is a novel, potent, and reversible inhibitor of bacterial peptide deformylase (PDF) that is in development at GlaxoSmithKline (GSK) for potential intravenous (IV) and oral treatment of hospitalized community-acquired bacterial pneumonia (CABP) and acute bacterial skin and skin structure infections (ABSSSIs). PDF is essential for bacterial protein maturation and a likely new target for antibiotic action (Giglione et al., 2001). Because of this novel mechanism, GSK1322322 has demonstrated activity against pathogens resistant to existing antibiotics (β-lactams, macrolides, and quinolones).

Plasma, urine, and feces are typically collected to understand the metabolism and elimination of a drug, however, bile collection is not common. Bile sampling can often provide crucial information about the disposition of the compound. Several methods have been attempted to collect human bile with varied success (Balani et al, 1997; Wang et al, 2006). Unfortunately, these methods tend to be invasive or difficult to use. The Entero-Test® capsule is a simple apparatus that was first developed over 30 years ago for the sampling of duodenal bile acids (Vonk et al., 1986). Recently, GSK has pioneered its use in metabolism studies. The device has proven useful in sampling bile from both dogs and human subjects in non-radiolabel metabolism studies (Guiney et al., 2011, Bloomer et al., 2012), but has never been tested in a conventional human radiolabel study where its application could be fully evaluated.

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In this study, we investigated the absorption, bioavailability, metabolism, and excretion of [^{14}C]GSK1322322 in healthy humans using the novel combination of Entero-Test® and accelerator mass spectrometry (AMS).

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Materials and Methods

Chemicals and Reagents. [^{14}C]GSK1322322 (human dosing: mesylate salt, specific activity of 0.04 μCi/mg, radiochemical purity of >99% as free base; in vitro experiments: free base, specific activity of 124.4 μCi/mg, radiochemical purity of 99.5%) was prepared by GSK (Stevenage, UK). As illustrated in Fig. 1, a single ^{14}C label was located at the pyrimidine ring of the molecule. Unlabeled GSK1322322 (chemical purity 99.2%) and a metabolite standard, GSK2733752 (M37, N-dehydroxylated GSK1322322; chemical purity >95%), were prepared at various sites of GSK. Two other metabolite standards, GSK2998310 (M6; deformylated glucuronide of GSK1322322; chemical purity >95%) and GSK2998314 (M9; a glucuronide of GSK1322322; chemical purity >90%), were synthesized by WuXi AppTec Co. (Shanghai, China).

Chemicals and solvents of reagent or HPLC grade were purchased from standard commercial sources. Scintillation cocktails Ultima Gold, Permafluor, Carbosorb CO_2 absorbent, and SafeScint 1:1 were obtained from PerkinElmer Life and Analytical Sciences (Waltam, MA) or LabLogic (Sheffield, UK). HPLC columns were purchased from Phenomenex (Torrance, CA). Sucrose (IAEA-C6) (certificated value = 1.506 times modern) was purchased from the International Atomic Energy Agency (Vienna, Austria) and used as the source of graphite (6.5 mg sugar for each graphite produced) in AMS instrument normalization and process control. Synthetic graphite 200 Mesh (99.9999%), from Alfa Aesar (Heysham, UK), was used as the AMS instrument background determination standard.

Dosing of Human Subjects. This open-label, single dose (IV and oral solution), cross-over study to investigate disposition of GSK1322322 in healthy humans was conducted at DaVita Clinical Research (Minneapolis, MN) under clinical study PDF112167 (registered as NCT01663389). Five healthy adult Caucasian male volunteers between the ages of 30 and 55 years (body weight ≤ 75 kg and body mass index between 21 and 24 kg/m^2) were enrolled. Subjects fasted at least 10 h before dosing for each dosing period. In Period 1, each subject received a single intravenous dose of 1000 mg (45.5 μCi) of [^{14}C]GSK1322322 as a 100 mg/mL solution in sterile water (10 mL) via an IV infusion over 60 min. Period 2 commenced after the total radioactivity in both urine and feces were <1% of the administered dose in two consecutive 24 h collections. In Period 2, each subject received a single oral dose of 1200 mg (54.5 μCi) of [^{14}C]GSK1322322 as a 100 mg/mL solution in sterile water (12 mL). The amount of [^{14}C]GSK1322322 ingested by each subject was determined by analyzing the dosing solution in each dosing bottle before and after dose administration.

Collection of Samples. In each subject, blood samples (8 mL) were drawn at predose, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h postdose for pharmacokinetic analysis of GSK1322322, M6, and M9. In addition, 10 mL blood samples were collected at both periods at predose, 0.5 h (period 2 only), 1 h (period 1 only), 2, 4, 8, 24, and 48 h for metabolic profiling. Plasma was harvested by centrifugation. Urine (10 mL) was aliquotted at predose, 0-6 h, 6-12 h, and 12-24 h during day 1 and in 24 h intervals up to 216 h postdose for Period 1 and 192 h for Period 2.

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Bile was collected non-invasively using the Entero-Test® capsule. The capsule was inserted at 3.5 h predose and removed 2 h post-IV infusion in Period 1. In Period 2, the capsule was inserted 2 h post oral dosing and removed 6.5 h post-dose. Food cues were used to stimulate emptying of the gall bladder at 0.5 h post-infusion start or 5.5 h post-oral dose. The strings were gently removed by pulling the line upward and out while the subject's head was tilted backwards.

Feces were collected at predose and then over 24 h collection intervals up to 216 h postdose in Period 1 and 192 h in Period 2. The steel weight from the Entero-test® was removed from the feces. The fecal samples were then mixed with water (5 times the sample weight) and homogenized using a probe-type homogenizer.

Analysis of Radioactivity.

Blood and feces. Duplicate weighed aliquots of each blood and fecal homogenate sample (0.2 g) were combusted and analyzed for radioactivity. The combustion was performed in a Packard model 307 sample oxidizer (Packard Instrument, Co., Meriden, CT). The resulting CO_2 was trapped in a Permafluor and Carbo-Sorb mixture, and the radioactivity was measured by LSC.

Plasma and urine. Duplicate weighed aliquots (0.2 g) of each plasma and urine sample were transferred to scintillation vials and analyzed directly for radioactivity. Ultima Gold XR scintillation cocktail (5 mL) was added to each vial and counted in a scintillation counter for 5 min or 100,000 counts. The total plasma and urine radioactivity concentrations were calculated by converting the LSC data in disintegrations per min per

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milliliter (DPM) to concentrations (mass equivalents per gram) using the specific activity of [^{14}C]GSK1322322 (0.044 μCi/mg).

Plasma by AMS. The AMS analysis procedure was previously reported (Young et al., 2008). Plasma samples, especially those collected at later time points that had levels of radioactivity too low to be determined by LSC, were subject to AMS analysis. Aliquots of plasma (0.01 mL) from these samples were supplemented with an aliquot of control human plasma (0.05 mL) to yield the optimum carbon content equivalent to about 2.6 mg after preparation to graphite. The resulting graphite/cobalt mix was packed into an aluminum cathode and each sample was analyzed using a 250 kV single-stage accelerator mass spectrometer instrument (National Electrostatics Corp., Middleton, WI). A generic value of 4.14%, based on GSK historical data, for the carbon content of human plasma generated using a Costech Elemental Combustion System (Model 4010; Valencia, CA) CHNS-O Analyzer (Pelican Scientific Ltd., Chester, UK), was used for all samples analyzed. The AMS data, expressed as percent modern carbon, were used to calculate the disintegrations per min per milliliter of each sample, where 100% modern carbon equals 0.01356 DPM/mg carbon.

Quantitation of GSK1322322, M6, and M9 in Plasma.

Pharmacokinetic plasma samples were analyzed for the presence of GSK1322322 using a validated LC-MS/MS analytical method similarly described (Naderer et al, 2013A). PK plasma samples were also analyzed to determine the concentration of M6 and M9. GSK2298310 (M6) and GSK2298314 (M9) were extracted from human plasma by protein precipitation using acetonitrile containing [^{13}C $^{15}N_2$]GSK1322322 as an internal

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standard. Extracts were analyzed by UHPLC-MS/MS using a TurboIonspray™ interface with positive ion multiple reaction monitoring. The assay range was 20 to 20000 ng/mL and 5 to 5000 ng/mL for M6 and M9, respectively. Both metabolites were stable in plasma for at least 8 hours at ambient temperature and 76 days in storage at -80°C.

Pharmacokinetic Analysis of Radioactivity, GSK1322322, M6, and M9. Blood and plasma total radioactivity and plasma GSK1322322 PK parameters including area under the curve (AUC_{0-t}, and $AUC_{0-\infty}$), maximum concentration (C_{max}), time of C_{max} observed (T_{max}), terminal half-life ($T_{1/2}$), systemic clearance (CL) (IV only), and volume of distribution at steady-state (V_{ss}) (IV only) were determined for each subject by noncompartmental analysis using WinNonlin (version 6.2.1; Pharsight, Mountain View, CA). Plasma PK parameters for M6 and M9, including AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, T_{max}, and $T_{1/2}$ were also determined by noncompartmental analysis using Phoenix WinNonlin. The ratio of plasma GSK1322322 to plasma total radioactivity was calculated to assess the relative amount of radioactivity present as parent compound versus metabolites. The ratio of blood to plasma total radioactivity concentrations at each PK sampling timepoint was calculated to assess GSK1322322 partitioning into red blood cells. Concentration units for radioactivity were expressed as microgram equivalents of GSK1322322 per milliliter.

Preparation of Samples for Radio-profiling.

Plasma. Equal volume aliquots (0.05 mL) from each PK sampling time point up to and including 24 h were combined across subjects to create 22 discrete IV and oral dose pooled plasma samples. These samples were then pooled according to the method

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described by Hop et al. (1998) to provide two pools of plasma. Plasma samples at 48 h postdose were not examined due to the very low levels of radioactivity present. Each pooled plasma sample (50 µL) was diluted 20-fold with water and dimethylformamide (DMF) (1:18:1, v/v/v), which contained authentic non-radiolabeled reference standards of GSK1322322, M6 and M9 each at a concentration of 1666 µg/mL (83 µg/mL after dilution). The diluted pooled plasma samples were analyzed by HPLC with radiometric detection by off-line analysis by AMS.

Urine. The urine samples from each subject were pooled to produce a single representative sample for the subject on the basis of the total sample weight collected from each interval. Samples with less than 2% of the administered dose were not pooled. The pooled urine samples were centrifuged at 3000 g_{av} at 4°C for 5 min to remove any particulates before analysis by radio-HPLC. Triplicate aliquots of each pooled urine sample were analyzed before and after centrifugation to determine whether any notable loss of radioactivity occurred upon centrifugation.

Feces. Fecal samples were pooled to generate a representative sample for each subject. The pooled samples were extracted twice with a mixture of methanol and water (1:4, v/v) followed by extraction with methanol. Each extraction involved addition of 3 volumes of the solvent, followed by sonication for 0.5 min, mixing at room temperature for 1 h, and centrifugation (3000 g_{av} at 4°C for 5 min). The supernatants were combined and triplicate aliquots of the combined supernatant were analyzed by LSC to determine the radioactivity content. Each extract was then concentrated on wet ice under a stream of nitrogen gas. The concentrated sample was mixed at 10°C for 30 min and centrifuged

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(3000 g_{av} at 4°C for 5 min). Triplicate aliquots of the concentrate supernatant were analyzed by LSC to determine the total radioactivity prior to radio-HPLC analysis.

Bile. Radioactive material was extracted from each bile string with the addition of 3 mL of methanol and water (1:4, v/v). The string was gently agitated by inversion of the container. The extract was decanted into another container. The string was then rinsed twice with 3 mL of methanol and all washes were combined with the initial extract to produce 10 individual bile string extracts (five from each period). Equal volume aliquots (1 mL) from the individual bile string extracts were combined to produce two route specific pools. An aliquot (1 mL) was removed from each pool for analysis by LSC to determine the radioactivity content. The two pooled bile string extracts were then diluted 20- and 100-fold, respectively, with a mixture of water and DMF containing authentic non-radiolabeled reference standards of GSK1322322, M6 and M9 each at concentrations of 1666 μg/mL (83 μg/mL after dilution). The diluted bile samples were analyzed by HPLC with radiometric detection by off-line analysis by AMS.

In Vitro Incubation of [^{14}C]GSK1322322 with Human Fecal Contents.

Fecal incubation. The human fecal homogenate incubation procedure was modified from an established method by Fouda et al. (1997). A fresh feces sample pooled from three anonymous healthy volunteers was commercially obtained (Bioreclamation Co., Westbury, NY). Upon receipt, the feces sample was homogenized with 0.1 M sodium phosphate buffer pH 7.4 (previously gassed with nitrogen) under nitrogen to minimize atmospheric contact. Then, the sample was transferred to an anaerobic chamber purged with nitrogen. Approximately 10 mL of the feces homogenate was removed into a

culture bottle containing 20 mL of media (0.5% glucose, 0.5% yeast extract and 0.5% peptone in 0.1 M sodium phosphate buffer, pH 7.4). The culture bottle was sealed with Teflon tape, removed from the anaerobic chamber and placed in a 37°C water bath for approximately 1 h. After the pre-incubation, the fecal content suspension was returned to the anaerobic chamber.

In the chamber, 1 mL aliquot of incubation medium containing 52 µM [^{14}C]GSK1322322 was added to each of the culture tubes containing 1 mL of fecal homogenate suspension. The tubes were sealed with Teflon tape, removed from the anaerobic chamber, and incubated in a water bath (37°C) for 24 h protected from light. Following completion of the incubation, reactions were stopped by addition of 2 mL of methanol. Samples were vortex mixed, and centrifuged at approximately 3500 g_{av} for 5 min. The resulting supernatant was analyzed by HPLC-MS^n with offline radio-detection. Control incubations without GSK1322322 were conducted in parallel using a blank medium containing 25 µL of methanol. Control incubations without fecal homogenate were also conducted using the incubation medium containing the same amount of [^{14}C]GSK1322322.

Bacterial viability check. To confirm fecal bacterial activity, incubations were performed by incubating 1 mL of fecal homogenate suspension with 1 mL of 50 µg/mL methylene blue in media for 24 h at 37°C (LeBoffe and Pierce, 1996). Bacterial activities in the incubations were verified at 24 h by noting any color change in the samples (blue = no activity; absence of blue = activity).

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Heat/antibiotic treatment. To further verify the bacterial activity in the feces, approximately half of the original human fecal homogenate suspensions were heat-treated by placing the suspension tubes in a boiling water bath for 15 min. The samples were then allowed to cool to room temperature when a mixture of antibiotics (2.44 mL of 50 mg/mL bacitracin in water, 0.976 mL of 125 mg/mL neomycin in water, and 0.976 mL of 125 mg/mL streptomycin in water) (Kinouchi et al., 1993) was added to the samples and mixed. Incubations with heat- and antibiotics-treated samples were conducted similarly as discussed above.

Radio-HPLC Analysis of Metabolites.

Plasma and Bile. The Agilent 1100 LC system (South Queensferry, Scotland, UK) consisted of a binary pump, autosampler, UV detector (λ 254 nm) and fraction collector. Aliquots (100 µL) of the diluted samples were injected onto a Phenomenex Synergi Polar-RP column (4.6 x 250 mm, 4 µm, 80Å) and eluted at 1 mL/min with solvents A (25 mM ammonium formate buffer, pH 3.78) and B (methanol). The following four step gradient was used: 0-5 min, 5% solvent B in solvent A; 5-80 min, 5 to 85% B in A; 80-80.2 min, 85 to 100% B in A; and 80.2-85 min, 100% B. After elution of GSK1322322 and its metabolites, the gradient was switched back to 5% B in A, and the column was washed for 5 min before the next injection. Each sample was individually fractionated at a frequency of 0.25 minutes over a time range of 15-70 min by HPLC. The fractionates were collected directly into quartz glass tubes (York Glassware Services, York, UK) containing carbon carrier (40 µl of aqueous sodium benzoate solution [625 mM]; a source of carbon for isotopic dilution equating to 2.1 mg; essentially containing no ^{14}C). Each HPLC fraction was evaporated to dryness in a centrifugal evaporator for 4 h, then carbon

in the sample converted to graphite and subsequently analyzed for radioactivity by AMS (Young et al., 2008). HPLC column recoveries were determined for the individual plasma and bile extract fractionated chromatograms using AMS through comparison of pre-column injectates with total radioactivity of all fractions. Chromatograms were reconstructed by importing the AMS data into Laura (LabLogic, Broomhill, Sheffield, UK).

Urine and Feces. Urine and fecal samples were analyzed using Agilent 1200 LC systems. Except for injection volume (200 μL), the system configuration and LC gradient were identical to above description. After injection, HPLC eluent was collected onto four 96-well scintillator-coated microtitre plates (PerkinElmer Life and Analytical Sciences) at 0.194 min/well. The solvents in the microtitre plates were vacuum centrifuged in the speedvac (Model 1450; ThermoFisherScientific, Waltham, MA) at ambient temperature. The dried plates were then sealed using TopSeal-A film (PerkinElmer Life and Analytical Sciences) and analyzed using a Trilux Microbeta (Model 1450; PerkinElmer Life and Analytical Sciences). Each well was counted for 15 min without background subtraction. The resulting Microbeta data were imported into the Laura software using the "LSC Import" function to reconstruct radio-HPLC chromatograms. Each radioactive peak was calculated as a percentage of the total counts detected and was expressed as the percentage of the dose recovered in feces (corrected for the combustion and extraction recovery) and in urine (corrected for centrifugation efficiency). The lower limit of quantitation was defined as two times the background area integrated in each chromatogram.

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Mass Spectroscopic Analysis of Metabolites. Metabolite structural characterization was performed by LC-MS^n analysis on a LTQ Orbitrap XL or LTQ XL ion trap mass spectrometers, with an electrospray ion source operating in positive ion-mode. The LC conditions were identical to those used for radio-profiling as described above using an Agilent 1260 LC system. Synthetic standards were used for comparison of chromatographic retention times as well as to provide characteristic MS^n fragmentation patterns. All instruments used a CTC PAL autosampler for sample injection. Data was acquired and processed using Xcalibur software (version 2.1; ThermoFisherScientific) and ACD (version 12; Toronto, Ontario).

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Results

Safety. Following the single IV and oral administration of [^{14}C]GSK1322322, the most reported adverse event (AE) was headache. Four subjects had headaches during Period 1, while two subjects had headaches and one had other AEs (diarrhea, nausea, oropharyngeal pain, throat irritation, and pain in extremity) during Period 2. No serious AEs were reported. No subject discontinued the study medication or was withdrawn prematurely from the study as a result of an AE.

Elimination and Mass Balance of Radioactivity. Five healthy male volunteers received a single 1050 mg IV dose of [^{14}C]GSK1322322 (46.1 μCi) during Period 1 and a separate single 1270 mg oral dose of [^{14}C]GSK1322322 (55.9 μCi) during Period 2. Period 2 commenced after the total radioactivity in urine and feces were <1% of the administered dose after two consecutive 24 h collections. T he mean (range) total recoveries of radioactivity from the IV and oral administration were high at 96% (92-98%) and 92% (66-100%), respectively. The radioactive dose was eliminated in urine and feces at approximately equal proportions after both routes of administration (~ 45-48% each) (Table 1). Urinary elimination of radioactivity was basically completed by 48 h and fecal elimination by six days post-dose.

Pharmacokinetics of Total Radioactivity, GSK1322322 and Metabolites. Mean concentration-time profiles for blood and plasma radioactivity and plasma GSK1322322, M6, and M9 were displayed in Fig. 2. The profiles were similar between the two routes of administration. Radioactivity and GSK1322322 concentrations quickly peaked post-dose (T_{max} between 0.5 and 1 h) followed by biexponential decline. A summary of blood

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and plasma total radioactivity and plasma GSK1322322, M6 and M9 PK parameters was presented in Table 2. Following IV administration, the mean blood radioactivity and plasma GSK1322322 clearance was similar at 24 L/h. Plasma radioactivity clearance was lower at 15 L/h. The mean volume of distribution at steady state ranged from 49 L for radioactivity in blood, to 53 L for GSK1322322 in plasma, and to 61 L for radioactivity in plasma. Blood radioactivity concentrations were 58 – 85% and 64 – 88 % of plasma radioactivity concentrations at corresponding time points after IV and oral administration, respectively (data not shown). Consistently, the mean blood:plasma radioactivity $AUC_{0-\infty}$ ratio (calculated based on data in Table 2) ranged from 0.63 to 0.75, indicating minimal association of GSK1322322 or its metabolites with blood cells. The plasma exposure to GSK1322322 accounted for 64 – 67% of the total plasma exposure to radioactivity for both dose routes, suggesting that the unchanged drug predominated in circulation (Table 2 and Fig. 2). Plasma exposure of M9 and M6 were 22 – 28% and 3 – 4% of the total plasma radioactivity exposure, respectively. Following IV or oral administration, the median half life ($T_{1/2}$) of GSK1322322 was 4.2 – 5.0 h. The median $T_{1/2}$ for M6 and M9 were approximately 2.5 h after dosing. Following oral administration, GSK1322322 was measurable in the first sampling occasion (0.25 h) with a T_{max} observed at 0.5 h post-dose.

Metabolite Profiles in Plasma. HPLC radiochromatograms of pooled plasma were depicted in Fig. 3. The relative amounts of GSK1322322 and metabolites in plasma after IV and oral administration of [^{14}C]GSK1322322 were shown in Table 3. Unchanged GSK1322322 was the predominant circulating component, accounting for 77 - 80% of total plasma radioactivity in the pooled IV and oral plasma samples. M9, a glucuronide

of GSK1322322, accounted for 11 and 15% of total radioactivity after IV and oral administration, respectively, while M6, a product of deformylation of M9, was a minor component (<2% of total radioactivity in either regimen).

Metabolite Profiles in Urine. A representative HPLC radiochromatogram of urine was depicted in Fig. 4. The individual and mean quantification of metabolites from the five subjects were shown in Table 4. The metabolite profiles were similar across subjects and between IV and oral administration profiles, with unchanged GSK1322322 and its co-eluting N-dehydroxylated metabolite (M37) being the major radiocomponents, accounting for 32% of the administered dose (23 – 46%). M9 accounted for approximately 5% (3 – 7%) of the dose after IV and oral administration. M1-M4 (products of mono-oxygenation of GSK1322322), along with their N-dehydroxylated forms (M38-41), and M32 (a product of oxidative deamination plus ketone formation) were all minor, each accounting for <1% of the administered dose.

Metabolite Profiles in Feces. A representative HPLC radiochromatogram of fecal homogenate extracts was shown in Fig. 5. The individual and mean percentages of GSK1322322 and metabolites excreted in feces were shown in Table 4. The metabolite profiles were similar across subjects and between IV and oral administration profiles. Unchanged GSK1322322 and its co-eluting reduced form (M37) were predominant radioactive components, accounting for approximately 10% (6 – 14%) of the dose. Metabolites M1-M4 and their N-dehydroxylated analogues (M38-M41) were also notable, together accounting for 17% of the dose. M6, M9, and M18 (a product of

deformylation plus N-dehydroxylation) were all minor, each accounting for <5% of the dose.

Metabolite Profiles in Entero-test Collected Bile. Representative HPLC radiochromatograms of pooled duodenal bile string extracts were depicted in Fig. 6, with relative abundance of GSK1322322 and metabolites shown in Table 5. The glucuronide conjugate of parent (M9) predominated in bile following IV and oral administration, accounting for 40% and 53% of total biliary radioactivity, respectively. Assuming the Entero-Test® snapshot represented the full biliary excretion (% of dose in bile should be equivalent to the dose eliminated in feces due to almost complete absorption of GSK1322322), glucuronidation (M6 and M9 together) would account for 20% (42% of 48% of fecal dose) and 25% of dose (56% of 45% of fecal dose), respectively, following IV and oral administration. Unchanged GSK1322322 and coeluting M37 accounted for 30% of biliary radioactivity, but were less abundant in the bile after the oral dose (9% of biliary radioactivity). M1-M4 and their N-dehydroxylated forms (M38-M41), M18, and M6 were minor components, each accounting for <7% of the bile radioactivity for both dose routes.

In Vitro Human Fecal Microbial Metabolism. The HPLC radiochromatograms and MS spectra of the fecal extracts following incubation with [^{14}C]GSK1322322 were depicted in Fig. 7. In the presence of viable gut microbes as evidenced by methylene blue assays, GSK1322322 readily degraded to M37 as indicated by LC/MS data of the co-eluting reduced analogue. However, in the absence of viable gut microbes, GSK1322322 was stable.

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Identification of Metabolites. Several metabolites (M1-M4, M6 and M9) have previously been identified in preclinical studies by HPLC-MS^n and NMR analysis (Supplemental Materials and Methods). In this study, the structures of these metabolites and a few others (e.g., M37) were confirmed or characterized by HPLC-MS^n analysis. The MS^n fragmentation of GSK1322322 was shown in Fig. 8 and was used as a reference in characterizing the metabolites. Summaries of HPLC retention times and MS^n data of observed metabolites were depicted in Table 6. When possible, relevant standards were also analyzed to assist in metabolite identification.

Metabolites M6 and M9. M6 was characterized as a N-deformation and O-glucuronidation metabolite of GSK1322322. The protonated pseudo-molecular ion along with the diagnostic fragment ion of m/z 452 (loss of 176 Da) supported this characterization. The fragmentation ions at *m/z* 283, 419 and 434 were consistent with the fragmentation of GSK1322322. M9 was characterized as an O-glucuronidation at the hydroxyl amine moiety. The pseudo-molecular ion of M9 at *m/z* 656 was 176 Da higher than GSK1322322, indicating glucuronidation. The fragment ion at *m/z* 267 is consistent with the MS^2 fragmentation of GSK1322322. *m/z* 480 is the loss of 176, which is typical of glucuronide conjugates, *m/z* 462 and others matched with the MS^n from initiative characterizations. The M6 and M9 were previously characterized by accurate MS^n and NMR earlier from preclinical studies with definite structures (Supplemental Tables 1-4).

Metabolites M1-M4. For metabolite M1- M3, the pseudo-molecular ion at m/z 496 was 16 Da higher than that of GSK1322322, indicating mono-oxygenation. The fragment

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ions at *m/z* 223, 283, were consistent with the fragmentation of GSK1322322, and together with the fragment ion at *m/z* 435 indicated oxidation of the ethyl-cyclopentane moiety as shown in Fig. 1. M4 was 14 Da higher than that of GSK1322322, indicating the metabolism pathway is ketone formation. Fragment ions indicate ketone formation on the ethyl-cyclopentane moiety as well as shown in the Fig 1. M1-M4 were initiatively characterized from previous preclinical studies (Supplemental Tables 1-4).

Metabolite M37. A synthetic standard (GSK2733752) was available to confirm structural assignment of this metabolite. The pseudo-molecular ion at *m/z* 464 was consistent with N-dehydroxylation. The MS^2 was 446, which was a loss of H_2O from *m/z* 464 by accurate mass confirmation. The fragmentation ions at *m/z* 223, 268, 281, 347, 419, and 428 matched with the MS^3 spectrum of GSK2733752. The chromatographic retention time of M37 matches with the standard too.

Other minor metabolites. Several other minor metabolites were characterized by HPLC/MS^n. However, their structural assignments were not described further here as they were present in minute quantities (M18, M32, and M38-M41).

Discussion

This paper described the results of an excretion and mass balance study in healthy human subjects that utilized Entero-Test® as a novel method for parent and metabolite characterization in bile. Single doses of GSK1322322, given either intravenously (1000 mg) or orally (1200 mg), were well tolerated in healthy men without any notable adverse events. Following the IV administration, GSK1322322 had a volume of distribution approximating total body water (~42 L for a 70 kg person), indicating the drug may be distributing into all body water, but not extensively into tissues. Following the oral dose, GSK1322322 was quickly absorbed, as evidenced by its short T_{max} (0.5 h). Dose-normalized $AUC_{0-\infty}$ from the oral dose was nearly identical to that following IV administration, suggesting close to complete absorption and bioavailability (97%) of the molecule. Systemic exposure (C_{max} of 22 µg/mL and $AUC_{0-\infty}$ of 49 µg.h/mL) following 1200 mg oral solution was similar, but higher than that in a previous study (C_{max} of 19 µg/mL and $AUC_{0-\infty}$ of 40 µg.h/mL) following oral administration of 1500 mg of a powder-in-bottle formulation, confirming that oral solution had better bioavailability than other oral formulation(s) (Naderer et al, 2013A). The terminal elimination half-life of GSK1322322 (5 h) following the oral administration was also similar to a previous observation (5.3-6.8 h after single dose) (Naderer et al, 2013A). Clearance and Vss following 1000 mg IV dose were in agreement with previous observations (Naderer et al, 2013B)

Pharmacokinetic comparison of total plasma radioactivity with GSK1322322 indicated that GSK1322322 was the predominant component in circulation following both IV and oral administration, a finding that was confirmed by plasma metabolite profiling.

Metabolite profiling and specific/selective LC-MS assays identified and quantified two glucuronides of GSK1322322 (M6 and M9) in circulation. Metabolite M9 circulated at a level slightly exceeding 10% of total circulating compound-related materials, thus suggesting a need for further safety testing (ICH M3(R2)). However, M9 had a short half-life (2.4 h) and was unlikely to accumulate to a higher exposure level. The other glucuronide (M6) was circulating only at minute quantities (1% total plasma radioactivity). Neither glucuronide metabolite has been shown to have antibiotic activity (unpublished data).

Following a single dose of GSK1322322 in healthy male subjects, the radioactivity excretion patterns were almost identical between IV and oral administration, with radioactivity approximately evenly split between urine and feces. This similarity in excretion patterns between the two routes was expected since GSK1322322 was almost completely absorbed and bioavailable following the oral dose. These patterns highlighted the equal importance of both biliary and renal elimination in GSK1322322 clearance and the potential effect of impairment of the liver and/or kidney on GSK1322322 disposition.

GSK1322322 was largely eliminated unchanged in the urine (30% of dose). The potential interaction of GSK1322322 with drugs that rely highly on renal elimination by transporters is possible and may be investigated in future studies.

Since glucuronidation was a predominant pathway in the preclinical species (unpublished data), it was predicted to be predominant in humans as well. Indeed, Entero-Test® collected bile sample analysis confirmed that the glucuronide of GSK1322322 (M9) was

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the major biliary radio-component in humans. Although the Entero-Test®, a non-invasive and easy-to-use device, has been used in human bile collections before (Vonk et al, 1986; Muraca et al, 1989; Bloomer et al, 2012), this is the first study, to our knowledge, that it has been successfully incorporated into a conventional human radiolabel study. Biliary metabolites were often crucial in evaluating drug disposition, especially if the metabolites were unstable following biliary secretion into the gut. Historically in human radiolabel studies, bile was frequently collected by duodenal intubation followed by suction or aspiration (Balani et al, 1997; Wang et al, 2006). These methods were complicated and invasive. The Entero-Test®, on the other hand, is much less invasive, cheaper, and simpler method to obtain this data. No subjects withdrew or suffered any adverse events related to this device in the study. Bile was successfully collected from all five subjects in both IV and oral cross-over sampling occasions. In addition, a food cue was all that was needed to induce sufficient bile flow in this study, unlike in other reported studies which involved an intravenous administration of cholecystokinin to stimulate gallbladder contraction (Balani et al, 1997; Wang et al, 2005). The successful implementation of Entero-test® in this study enabled an accurate assessment of GSK1322322 disposition in humans. Assuming the Entero-Test® snapshot represented the full biliary excretion (% of dose in bile should be equivalent to the dose eliminated in feces due to almost complete absorption of GSK1322322), glucuronidation alone could account for metabolism of up to 30% of the administered dose in humans (25% in bile and 5% in urine following oral administration). We have not investigated the enzymes responsible for the glucuronidation pathway. However, UGTs in general are high capacity enzymes with low substrate-specificity. UGT

inhibition has rarely resulted in significant clinical drug interactions (Williams et al, 2004).

Biliary glucuronides of GSK1322322 were not stable once entering the gastrointestinal (GI) tract, as evidenced by the observed low levels of these metabolites in the feces in comparison with the bile samples. Without the Entero-Test®, the contribution of glucuronidation to GSK1322322 metabolism would have been notably under-estimated. These glucuronides were likely hydrolyzed back to the aglycones by the microbes in the large intestine. Glycoside/glucuronide hydrolysis is a common metabolic pathway by gut microbes (Sousa et al., 2008). The resulting aglycone could then be reabsorbed and complete the cycle of entero-hepatic recirculation. Although not evident in this study, entero-hepatic recirculation might be responsible for the appearance of a second peak at 4 hour post-dose in the GSK1322322 plasma concentration-time profiles in a phase I study with GSK1322322 (Naderer et al, 2013C).

Human feces contained high levels of an N-dehydroxylated product of GSK1322322 (M37). N-dehydroxylation has been observed in xenobiotic metabolism, including reduction of N-hydroxyformamide, the same moiety as in GSK1322322 (Sugihara et al, 2000; Stokvis et al, 2004). The mechanism of this reduction has been extensively studied. Cytochrome b5 and its reductase and several molybdenum-containing enzymes such as aldehyde oxidase were all capable of carrying out the reduction (Vonk et al, 1986; Saulter et al, 2005; Havemeyer et al, 2010). Sugihara et al (2000) also reported non-enzymatic N-dehydroxylation by hemoglobin under anaerobic conditions. In this study, we have demonstrated that gut microbes likely dehydroxylated GSK1322322.

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Gut microbes contained high levels of reductases (Sousa et al., 2008). Weisburger et al (1970) demonstrated gut microbial N-dehydroxylation of a carcinogen (N-hydroxy-N-2-fluorenylacetamide). Although GSK1322322 had antibacterial activity, it was only active against selected bacterial strains (Naderer et al, 2013A). We speculated that the N-hydroxyformamide moiety of GSK1322322 and its non-glucuronidated metabolites (e.g., M1-M4), derived from biliary secretion or following microbial degradation, were susceptible to this gut microbial reduction. Boiling fecal contents followed by treatment with broad-spectrum antibiotics prevented the reduction of GSK1322322 *in vitro*. The reduced product was devoid of antibiotic activity (unpublished data). However, human reductase-catalyzed and/or non-enzymatic formation of M37 via hemoglobin-assisted reduction could not be ruled out, since the reduction product was also detected in human blood, urine and bile, albeit at low concentrations.

In summary, we report the results of a human metabolism and excretion study with successful bile collection using Entero-Test® on an experimental antibiotic, GSK1322322. GSK1322322 was quickly absorbed with near complete (97%) bioavailability following single oral administration of 1200 mg in solution. Unchanged parent predominated in circulation following oral or intravenous administration (1200 or 1000 mg). A glucuronide metabolite of GSK1322322 (M9) also circulated at levels slightly >10% of total plasma radioactivity. A notable portion of GSK1322322 was excreted unchanged, mainly in urine (30% of total dose). Glucuronidation was likely the major pathway in metabolism (up to 30% of dose), with products excreted mostly via the bile. Oxidation played a minor role (<20% of dose) in elimination. Comparison of the

biliary and fecal profiles indicate that the glucuronides are likely labile to the gut lumen. Gut microbes were capable of reducing GSK1322322 prior to fecal elimination.

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Authorship Contribution

Participated in research design: Mamaril-Fishman, Zhu, Lin, Jones, Stump, Naderer, Dumont, Patel, Chen, Deng

Conducted experiments: Mamaril-Fishman, Lin, Felgate, Jones, Stump, Bowen

Contributed new reagents or analytical tools: NA

Performed data analysis: Mamaril-Fishman, Zhu, Lin, Felgate, Jones, Stump, Pierre, Gorycki, Wen, Deng

Wrote or contributed to the writing of the manuscript: Mamaril-Fishman, Zhu, Lin, Felgate, Jones, Stump, Pierre, Bowen, Naderer, Dumont, Patel, Gorycki, Wen, Chen, Deng

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Footnotes

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Legends for Figures

Figure 1 Metabolic pathways of [^{14}C]GSK1322322 in humans.

Figure 2 Mean concentration-time profiles of plasma [^{14}C]GSK1322322 and metabolites M6 and M9, and blood and plasma total radioactivity after IV and Oral administration.

Figure 3 Reconstructed AMS radiochromatograms of human plasma 0-24 h proportional pool after IV (1000 mg, 45.5μCi) and oral (1200 mg, 54.5μCi) administration of [^{14}C]GSK1322322.

Figure 4 Representative HPLC radiochromatogram of pooled human urine (0-48 h) after IV administration of [^{14}C]GSK1322322 (1000 mg, 45.5μCi). The urine samples pooled from subject 2 was subjected to LC/MS analysis with radiometric detection.

Figure 5 Representative HPLC radiochromatogram of pooled human feces sample (24-96 h) after oral administration of [^{14}C]GSK1322322 (1200 mg, 54.5μCi). Pooled fecal samples from each of the five human subjects were individually analyzed by solvent extraction followed by LC/MS analysis with radiometric detection.

Figure 6 Reconstructed AMS radiochromatogram of human bile 0-3 h after IV administration of [^{14}C]GSK1322322 (1000 mg, 45.5uCi) and 0-6.5 h after oral administration (1200 mg, 54.5uCi). Bile samples from each of the five human subjects per dose were subsequently pooled together and analyzed by solvent extraction followed by LC/MS analysis.

Figure 7 HPLC radiochromatogram and MS chromatogram of anerobic incubation with human fecal homogenate with 50uM of [^{14}C]GSK1322322 for 24 h without antibiotics (A) and with antibiotics (B). The fecal sample was analyzed by solvent extraction followed by LC/MS analysis with radiometric detection. HPLC radiochromatogram of anerobic incubation with human fecal homogenate with 50uM of [^{14}C]GSK1322322 for

24 hours. The fecal sample was analyzed by solvent extraction followed by LC/MS analysis with radiometric detection.

Figure 8 Mass fragmentation pattern of GSK1322322.

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Table 1 **Excretion of radioactivity in human urine and feces following a single intravenous and oral dose of [^{14}C]GSK1322322**

Collection Interval (h)	% of Mean Administered Dose			
	Mean for IV dose	SD	Mean for oral dose	SD
Urine				
0-6	36.5	3.8	33.0	10.9
6-12	5.2	0.4	8.0	2.0
12-24	2.6	0.5	3.0	0.6
24-48	2.3	0.8	2.2	0.4
48-72	0.7	0.3	0.6	0.1
72-96	0.3	0.2	0.1	0.1
96-120	0.1	0.1	0.03	0.1
120-144	NS	NS	0.02	0.05
144-168	NS	NS	0.02	0.05
Subtotal	47.5	4.1	47.0	10.0
Feces				
0-24	0.79	0.7	4.9	6.78
24-48	21.5	16.9	21.6	10.8
48-72	16.6	10.0	13.4	6.2
72-96	5.3	7.0	3.9	3.2
96-120	3.6	5.8	0.2	0.3
120-144	0.2	0.2	0.4	0.3
144-168	0.3	0.3	0.1	0.04
168-192	0.1	NA	0.2	0.09
192-216	0.1	NA	NS	NS
subtotal	48.3	2.9	44.7	10.2
Total[a]	95.8	2.4	91.7	16.1

NS = no sample obtained.
NA = not applicable. Subject discharged from clinical unit and excluded from calculation of mean and SD.
a. The total recovery included the radioactivity recovered from toilet tissues (less than 0.1% of dose per subject).

Table 2 **Summary of PK parameters from plasma [^{14}C]GSK1322322, and blood total radioactivity, plasma M6 and M9 following intravenous and oral administration of [^{14}C]GSK1322322**

Dose	Analyte	Matrix	AUC(0-∞) (µg.h/mL)	Cmax (µg/mL)	Tmax (h)	T1/2 (h)	CL (L/h)	Vss (L)	F (%)
IV	GSK1322322[a]	Plasma	42.5 (14.6)	19.7 (17.8)	1.0 (1.0-1.1)	4.2 (44.6)	23.6 (14.6)	52.5 (13.1)	NA
	Total radioactivity[a]	Plasma	66.3 (31.7)	22.8 (19.9)	1.0 (1.0-1.1)	4.0 (115)	15.1 (31.7)	61.2 (56.4)	
	Total radioactivity[a]	Whole Blood	41.8 (13.9)	18.5 (20.5)	1.0 (1.0-1.1)	1.7 (15.6)	23.9 (13.9)	48.6 (20.7)	
	M6 (GSK2998310)[b]	Plasma	1.8 [1.2-2.6]	0.4 [0.3-0.6]	1.5 [1.50]	2.5 [2.1-2.9]	NA	NA	
	M9 (GSK2998314)[b]	Plasma	14.5 [10.1-19.2]	4.3 [3.0-6.1]	1.5 [1.0-2.0]	2.5 [1.8-2.9]	NA	NA	
Oral	GSK1322322[a]	Plasma	49.3 (12.0)[c]	21.9 (18.1)	0.50 (0.3-0.5)	5.0 (52.5)[c]	NA	NA	97
	Total radioactivity[a]	Plasma	73.5 (17.4)	24.6 (15.2)	0.50 (0.3-0.5)	2.8 (38.3)	NA	NA	
	Total radioactivity[a]	Whole Blood	54.8 (14.3)	20.2 (20.1)	0.50 (0.3-0.5)	2.3 (8.5)	NA	NA	
	M6 (GSK2998310)[b]	Plasma	2.9 [2.0-4.0]	0.5 [0.4-0.7]	1.0 [0.5-1.0]	2.4 [2.0-2.8]	NA	NA	
	M9 (GSK2998314)[b]	Plasma	20.3 [14.3-28.6]	6.2 [4.0-9.3]	1.0 [0.5-1.0]	2.4 [1.8-3.0]	NA	NA	

N=5; NA = not applicable; CVb% = between subject coefficient of variation.

a. Data are presented as geometric mean and (CVb%), except for T_{max}, which is presented as median (range).

b. Data are presented as geometric mean and [range], except for T_{max}, which is presented as median [range].

c. N=4

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Table 3 **Percent of circulating GSK1322322 and metabolites following a single 1000 mg intravenous administration and a single 1200 mg oral administration of [^{14}C]GSK1322322**

Metabolite ID[a]	% Plasma Radioactivity[a]	
	IV	Oral
M6	1.3	1.0
M9	10.7	14.6
GSK1322322	76.5	79.5[b]

M6 = GSK2998310; M9 = GSK2998314.
a. Structural identification based on co-chromatography with standards of GSK1322322, GSK2998314, and GSK2998310.
b. M37 which co-elutes with parent was determined to be <1% of GSK1322322 from mass spectrum response compared to synthetic standards in pooled Human plasma – Oral dose at 0.5 hour.

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Table 4 **Mean percentages of GSK1322322 related materials in human excreta following a single 1000 mg intravenous and single 1200 mg oral administration [^{14}C]GSK1322322**

Metabolite ID	% Administered Dose (IV/Oral)
	Mean
Urine[a]	
M9	5.4/5.2
GSK1322322, M37[b]	32.4/31.4
Feces	
M1,M38	6.9/6.1
M2,M39	5.0/4.9
M3,M40	3.2/3.4
M4,M32,M41	2.0/2.0
M6	NA/0.3
M6,M18[c]	1.6/1.3
M9	1.0/1.0
M18	NA/0.4
GSK1322322, M37[d]	10.4/9.6

LLQ = lower limit of quantitation; ND = not detected; NA = not applicable; M6 = GSK2998310; M9 = GSK2998314; M37 = GSK2733752.
a. M1-M4, M2, and M38-M41 were <2% of dose.
b. M37 was determined to be 12.6% of GSK1322322 from mass spectrum response compared to synthetic standards in human subject 2 urine - oral dose.
c. M6 and M18 coeluted in all but one subject.
d. M37 was determined to be 56.3% of GSK1322322 in human subject 2 feces - oral dose.

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Table 5 **Percent of GSK1322322 and metabolites in human duodenal bile following a single 1000 mg intravenous administration and a single 1200 mg oral administration of [^{14}C]GSK1322322**

Metabolite ID	% Bile Radioactivity[a]	
	IV dose 0-2 hr pooled duodenal bile	Oral dose 2-6.5 hr pooled duodenal bile
M1,M38	3.4	5.4
M2,M39	2.7	5.2
M3,M40	5.1	4.5
M4,M41	5.0	6.6
M18	2.0	3.4
M6	2.0	2.3
M9	39.9	53.4
GSK1322322, M37	29.8	9.1[b]

ND = not detected; M6 = GSK2998310; M9 = GSK2998314; M37 = GSK2733752.
a. LC/MS_n work was completed on the bile extracts (pooled across subjects per period) to identify the metabolites radioprofiled using AMS.
b. M37 was determined to be 4.3% of GSK1322322 from mass spectrum response compared to synthetic standards in pooled human bile – oral dose.

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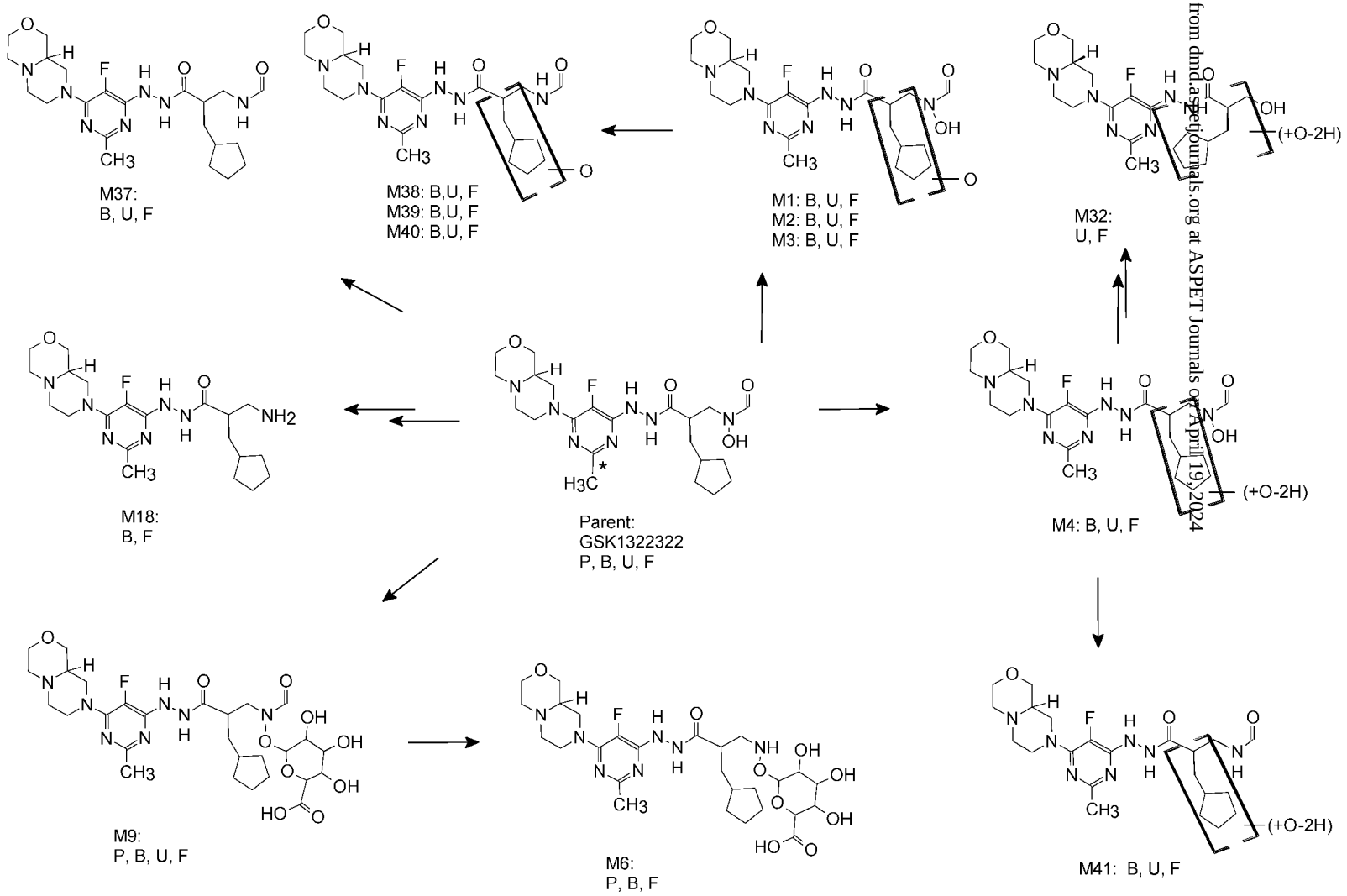
Table 6 **Selected HPLC retention times and MS^n data for GSK1322322 and its metabolites**

Metabolite	t_R(min)	*m/z*	
		$[M+H]^+$	Typical MS^n fragments
GSK1322322	58.2	480	MS^2: 223, 267, 283, 311, 419, 434, 452, 462
M1	36.4	496	MS^2: 223, 267, 283, 311, 435, 468, 478
M2	37.7	496	MS^2: 223, 267, 283, 311, 435, 468, 478
M3	38.6	496	MS^2: 233. 267. 283. 311. 435. 468. 478
M4	39.8	494	MS^2: 223, 267, 283, 311, 433, 466, 476
M6	45.8	628	MS^2: 268, 283, 419, 434, 452, 610
M9	48.1	656	MS^2: 267, 462, 480, 638
M18	44.4	436	MS^2: 263, 283, 407, 419
M32	39.9	451	MS^2: 263, 283, 352, 403, 421, 433
M37	58.6	464	MS^2: 283, 446 MS^3: 223, 268, 281, 347, 419, 428
M38	36.5	480	MS^2: 283, 462 MS^3: 181, 283, 310, 426, 444
M39	37.6	480	MS^2: 283, 462 MS^3: 268, 281, 283, 310, 363, 426, 435, 444
M40	38.5	480	MS^2: 283, 462 MS^3: 268, 281, 363, 426, 435, 444

M41	40.0	478	MS^2: 283, 460 MS^3: 223, 268, 281, 283, 310, 361, 433, 442

Fig. 1

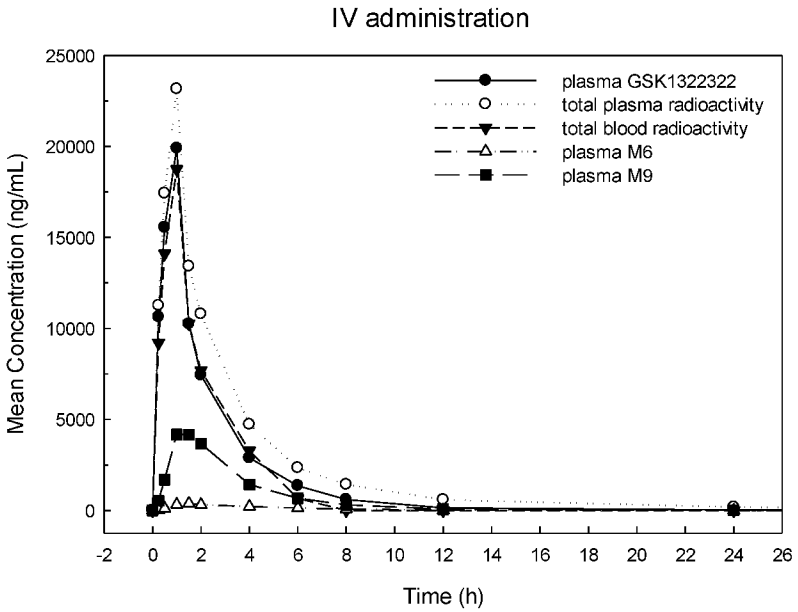


P=Plasma, B=Bile, U=Urine, F=Feces

* denotes the position of the ^{14}C label.

Note: Double arrow means metabolites might be formed by muiti-step reactions.

Fig. 2



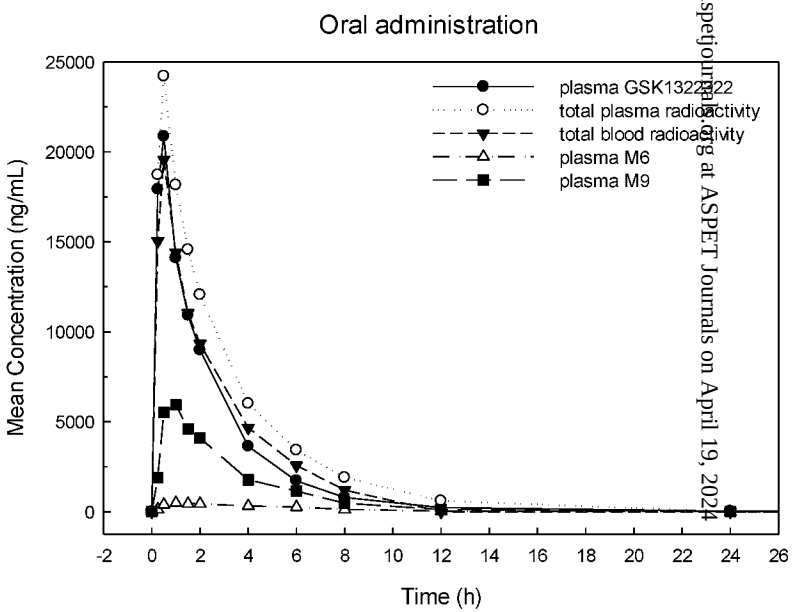


Fig. 3

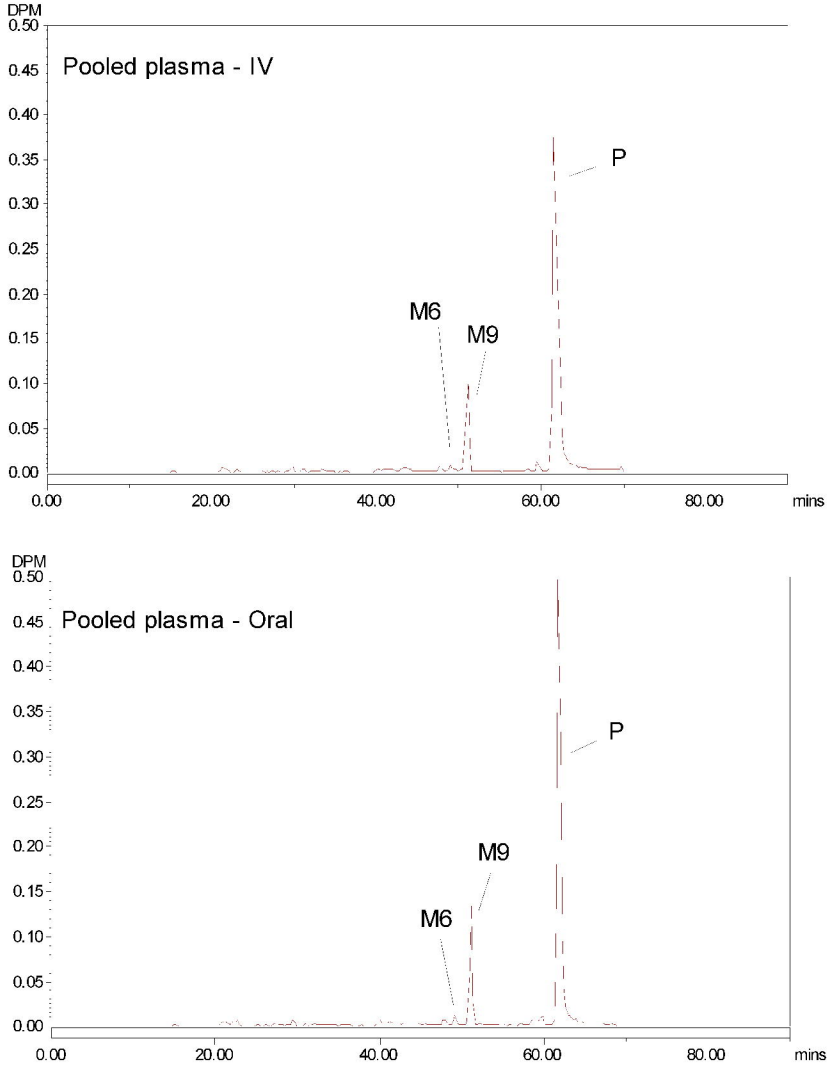


Fig. 4

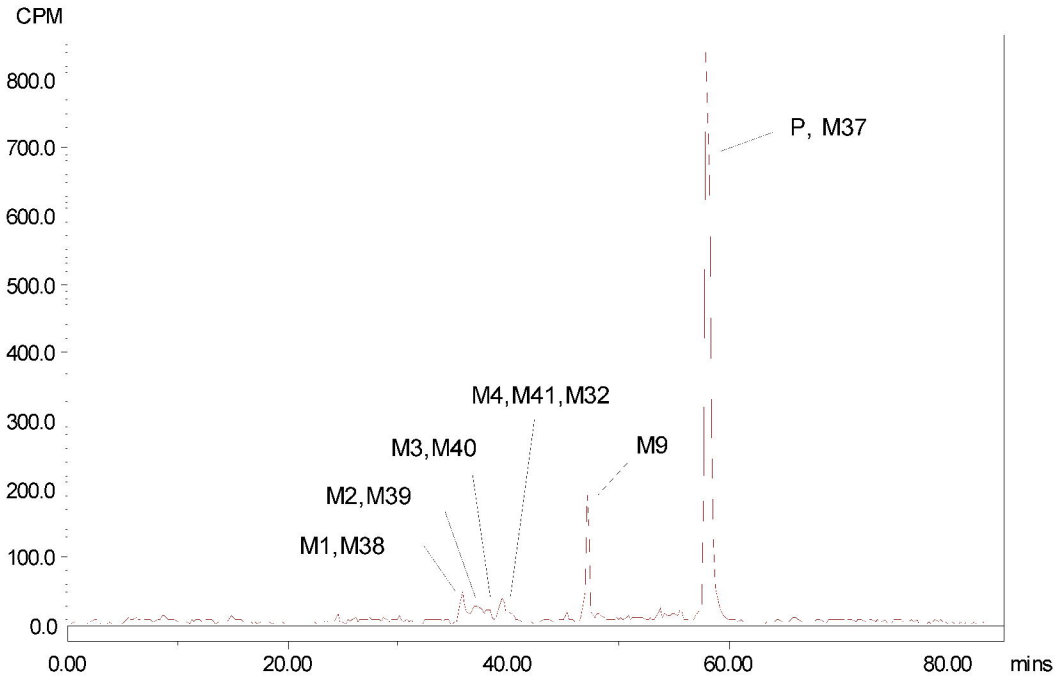


Fig. 5

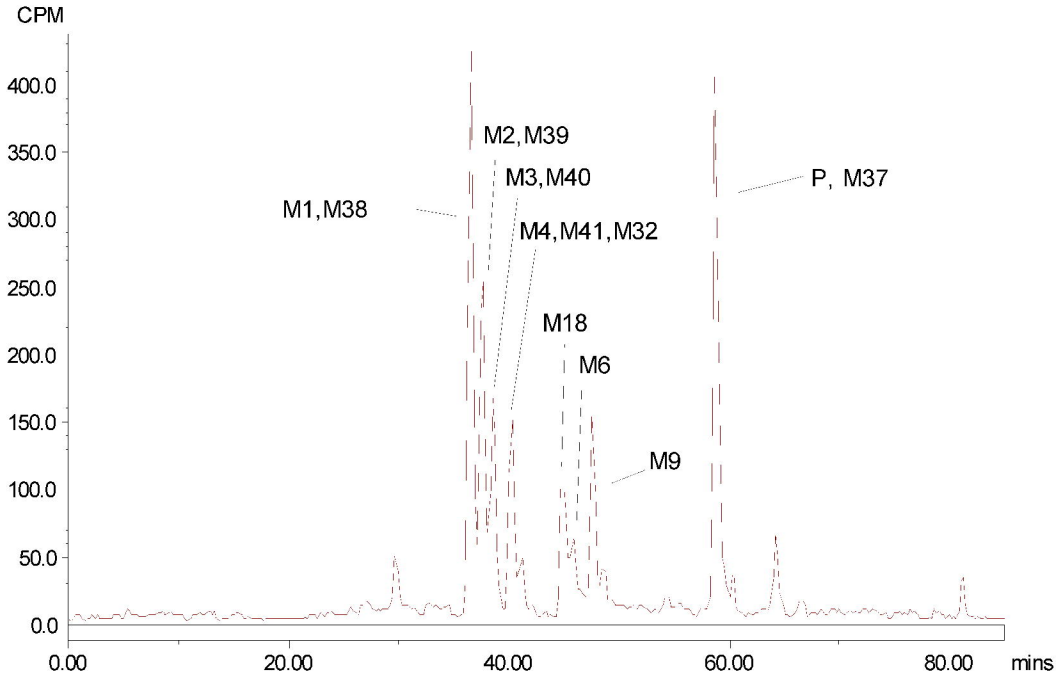


Fig. 6

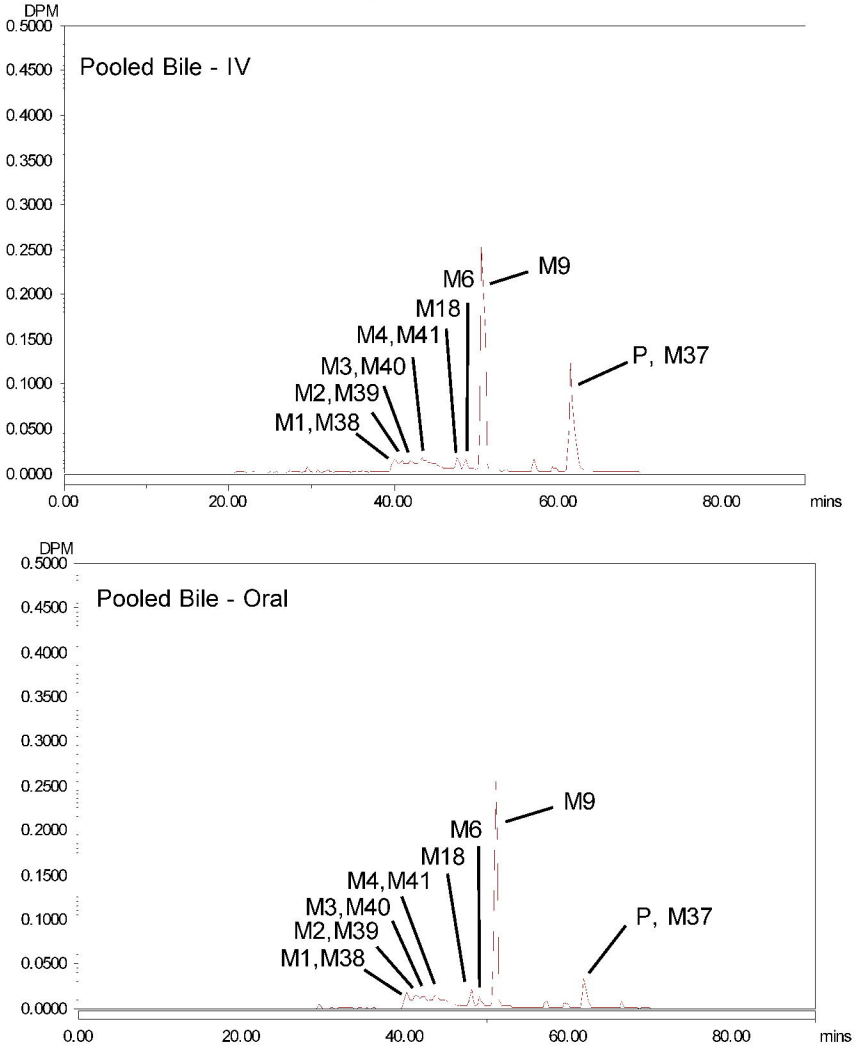
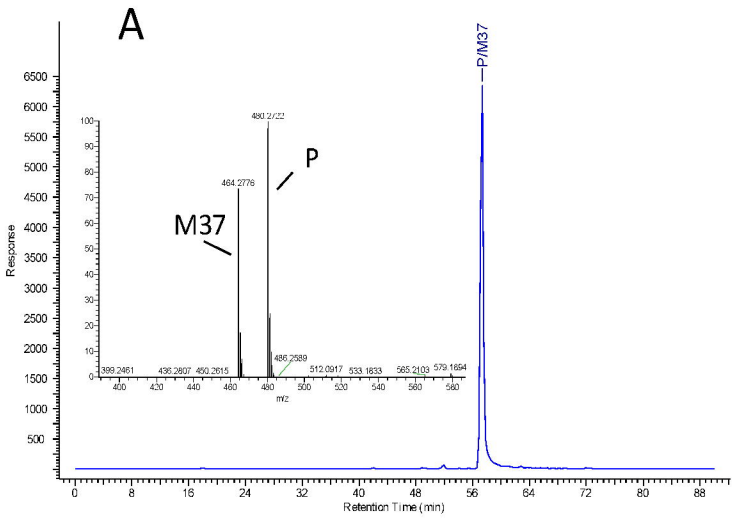


Fig. 7



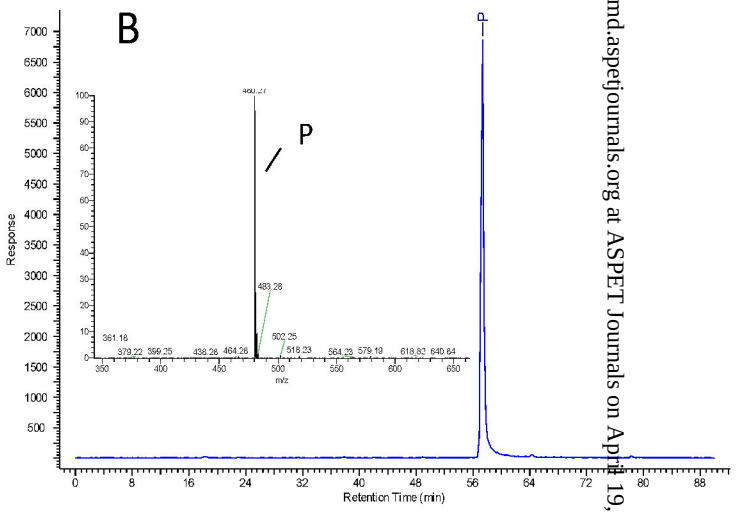


Fig. 8