Human mass balance, pharmacokinetics and metabolism of rovatirelin and identification of its metabolic enzymes in vitro

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ABSTRACT

1. The mass balance, pharmacokinetics and metabolism of rovatirelin were characterised in healthy male subjects after a single oral dose of [14C]rovatirelin. [14C]Rovatirelin was steadily absorbed, and the peak concentrations of radioactivity and rovatirelin were observed in plasma at 5–6 h after administration. The AUCinf of radioactivity was 4.9-fold greater than that of rovatirelin. Rovatirelin and its metabolite (thiazoylalanyl)methylpyrrolidine (TAMP) circulated in plasma as the major components. The total radioactivity recovered in urine and faeces was 89.0% of the administered dose. The principal route of elimination was excretion into faeces (50.1% of the dose), and urinary excretion was the secondary route (36.8%). Rovatirelin was extensively metabolised to 20 metabolites, and TAMP was identified as the major metabolite in plasma and excreta among its metabolites.

2. To identify the metabolic enzymes responsible for TAMP formation, the in vitro activity was determined in human liver microsomes. The enzymatic activity depended on NADPH, and it was inhibited by ketoconazole. Furthermore, recombinant human cytochrome P450 (CYP) 3A4 and CYP3A5 displayed enzymatic activity in the assay. Therefore, CYP3A4/5 are the most important enzymes responsible for TAMP formation.

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Introduction

Thyrotropin-releasing hormone (TRH) was originally isolated from the hypothalamus (Guillemin, 1978; Schally, 1978), and TRH is distributed throughout the central nervous system (Morley, 1979). TRH binds to the G protein-coupled TRH receptor (TRHR) (Yamada et al., 1993). The endocrine functions of TRH include controlling the levels of thyrotropin, thyroid-stimulating hormone (TSH) and prolactin. In addition to these endocrine functions, TRH also possesses neuropharmacological functions, including cerebral nerve activation (stimulation of motor function), effects on spinal function (stimulation of spinal motor neurons) and effects on the central nervous system (antineurodepressant activity) (Daimon et al., 2013; Khomane et al., 2011). Spinocerebellar degeneration (SCD) is a degenerative disease caused by the slow degeneration of the brainstem and cerebellum, and its major symptom is ataxia. TRH has been clinically investigated for the treatment of SCD (Sobue et al., 1983), and TRH agents, such as protirelin (synthetic TRH) and taltirelin (TRH analogue), are currently prescribed to patients with SCD in Japan. However, protirelin is administered via intravenous or intramuscular injection because of its short plasma half-life (4–5 min), poor metabolic stability and low bioavailability (Bassiri & Utiger, 1973; Griffiths, 1976; Khomane et al., 2011; Kinoshita et al., 1998). These disadvantages are not as severe for taltirelin, and the drug is approved for twice-daily oral administration (Kinoshita et al., 1998).

Rovatirelin (1-[N-(4S, 5S)-(5-methyl-2-oxooxazolidin-4-yl)carbonyl]-3-(thiazol-4-yl)-L-alanyl)- (2R)-2-methylpyrrolidine) hydrate has also been tested in clinical studies as an oral TRH agent for the treatment of SCD. In phase I studies in healthy adult males, rovatirelin exhibited a linear pharmacokinetics in a single-ascending dose (0.1 to 10 mg) and a benign safety profile supportive of once-daily oral administration (Kinoshita et al., 1998). From results of Phase II and III studies to evaluate the efficacy and safety of rovatirelin in SCD patients, a daily dose of 1.6 to 3.2 mg of rovatirelin have been considered to be dosage level intended for clinical use as once-daily oral administration (Shimizu et al., 2018). Rovatirelin exhibited greater affinity for TRHR (Kd = 702 nmol/L) than taltirelin (Kd = 3880 nmol/L) in receptor binding studies using the membrane fraction of human TRHR-expressing cells, and rovatirelin increased noradrenaline levels in the medial prefrontal cortex and locomotor activity in rats.

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compared with the effects of taltirelin (Ijiro et al., 2015).

Pre-clinical pharmacokinetic studies in rats and dogs indicated that rovatirelin was absorbed rapidly with absolute bioavailabilities of 7.3% in rats and 41.3% in dogs after oral administration, and orally administered [14C]rovatirelin was predominantly excreted in faeces (Kobayashi et al., 2019). In addition, the ability of rovatirelin to cross the blood-brain barrier was relatively high in rats. Furthermore, rovatirelin was stable in rat plasma and brain homogenates. In vitro metabolite profile studies illustrated that TAMP was the major metabolite of rovatirelin in human hepatocytes (Kobayashi et al., 2019).

The objectives of this study were to (1) characterise the pharmacokinetic profiles of rovatirelin after a single oral dose of [14C]rovatirelin in humans, (2) investigate the pharmacokinetic fate of rovatirelin in humans, (3) evaluate the amounts of predominant rovatirelin metabolites formed in humans and (4) identify the enzymes responsible for producing a major metabolite of rovatirelin.

Materials and methods

Chemicals

[14C]Rovatirelin was synthesised at Selcia Ltd. (Essex, UK) with a certificate of analysis of radiochemical purity (97.7%) and specific activity (5.52 MBq/mg) and stored at −80°C. Non-radiolabelled rovatirelin hydrate and authentic standards of rovatirelin hydrate and rovatirelin-d_{10} were supplied by Shionogi & Co., Ltd. (Osaka, Japan). Authentic metabolites of rovatirelin, namely rovatirelin aminopentanoic acid (rovatirelin-acid), rovatirelin pyrrolidine (4S)-hydroxy rovatirelin-OH) and KP051, were synthesised by Kissei Pharmaceutical Co., Ltd. Rovatirelin aminopentanoic acid (rovatirelin-ketone), (thiazolylalanyl)methylpyrrolidinone (TAMP) di-toluenesulphonate and 3-(4-thiazoyl)-L-alanine (TA) were supplied by Sumika Technoservice Corporation (Takarazuka, Japan), Nard Institute, Ltd. (Amagasaki, Japan) and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), respectively. The solubiliser Soluene-350, radioactive dioxide absorber Carbo-Sorb E, scintillation cocktail for high-performance liquid chromatography (HPLC) Ultima Flo-M and scintillation cocktail Emulsifier-Safe/Permafluor E+ were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). A pooled human liver S9 fraction (pool of 42 donors) and microsomes (HLM, pool of 24 donors) were purchased from BD Gentest (Woburn, MA, USA). Recombinant human cytochrome P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 supersomes) were also purchased from BD Gentest. Oxidised nicotinamide adenine dinucleotide phosphate (NADP^{+}), oxidised diphosphopyridine nucleotide (NADH), glucose-6-phosphate (G-6-P) disodium salt dehydrate and G-6-P dehydrogenase (G-6-P DH) from yeast were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used were of analytical grade and purchased commercially.

Dose preparation

[14C]Rovatirelin oral solution was manufactured from non-radiolabelled rovatirelin hydrate and an ethanolic solution of [14C]rovatirelin and assembled in unit doses. An equivalent amount of rovatirelin (non-radiolabelled plus radiolabelled) was formulated in sterile water/ethanol (90:10, v/v) and the actual concentrations were 0.063 mg/mL and 43.29 KBq/mL. Each unit dose contained 3.2 mg of rovatirelin and [14C]rovatirelin (2.16 MBq) as the anhydrate form. The dosing formulation was prepared at the clinical site, and all doses were administered within 72 h of manufacture. The radiation exposure in this study, approximately 0.48 mSv, fell into category Ila studies (0.1–1 mSv) in the International Commission Radiological Protection guidelines (1992). The radioactive dose was set according to human dosimetry calculations established by the Centre for Radiation, Chemical and Environmental Hazards Health Protection Agency (Oxfordshire, UK). The radioactive dose of [14C]rovatirelin of 3.2 mg/2.16 MBq was considered adequate to define the total recovery of radioactivity (mass balance), routes of excretion of radioactivity and pharmacokinetics of total radioactivity in blood, plasma and excreta. In addition, this dose level of rovatirelin was also considered to be in the therapeutic dose range and was high enough to fully characterise the single oral dose pharmacokinetics of rovatirelin and the major metabolite, TAMP.

Study design

This study (Kissei protocol number: KPS1105) was an open-label, single-oral-dose study involving six healthy Caucasian male subjects aged 36–53 years (mean, 45 years) with body weights of 65.2–93.1 kg (mean, 78.53 kg) and body mass indices (BMI) of 23.1–27.9 kg/m² (mean, 25.53 kg/m²). The clinical phase of this study was conducted at Covance Clinical Research Unit (CCRU, Leeds, UK) in accordance with good clinical practice guidelines and ethical principles originating from the Declaration of Helsinki, International Conference on Harmonisation (ICH) guidelines and applicable laws and regulations. Before starting the study, the protocol and consent form were reviewed and approved by the Research Ethics Committee (REC). The study commenced after receiving a Clinical Trials Authorisation from the Medicines and Healthcare products Regulatory Agency and REC approval. Permission to perform the study was also granted by the Administration of Radioactive Substances Advisory Committee.

For this study, healthy Caucasian men, between 35 and 55 years of age and a BMI between 18.5 and 30 kg/m², were eligible for enrolment and the key inclusion and exclusion criteria are described in the Supplementary Table 1. Eligible subjects were in good health as determined by a medical history review, physical examination, 12-lead electrocardiogram (ECG) and clinical laboratory evaluations.

The subjects were administered [14C]rovatirelin oral solution (50 mL) in the fasted state on the morning of day 1. The dosing vessel was then rinsed twice (2 × 100 mL), and both
rinses were ingested by the subject, resulting in a total fluid intake at dosing of 250 mL. Each subject participated in only one study period and remained resident in the CCRU from day −1 (the day before dosing) until at least day 11 (24 h after administration), with the possibility of extension to in-house visits on days 11–15 (336 h after administration) and outpatient visits on days 21 (±1 day) and 28 (±1 day) at the CCRU. On day 11, subjects were discharged from the CCRU if no radioactivity was detected in two consecutive plasma samples, <1% of the total radioactive dose was recovered in excreta (urine and faeces) in two consecutive 24-h collection periods and there were no medical reasons for a longer stay. All subjects completed the study, but one subject vomited approximately 50% of his dose within 6 h after administration. His data have been excluded from the calculation of safety assessment.

Safety was assessed throughout the study via clinical laboratory evaluations, body weight measurements, physical examinations, 12-lead ECG, vital sign assessments and monitoring for adverse events.

Sample collection
Blood samples for blood and plasma radioactivity (6 mL) and plasma analyte pharmacokinetic analyses (4 mL) were collected into lithium heparin/sodium heparin tubes before dosing and 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h after administration. For one subject who did not meet the exit criteria at 240 h after administration and who was required to remain in the clinical unit, further blood and plasma samples were collected at 264, 288 and 312 h after administration. Blood samples for metabolite profiling/identification (10 mL) were collected into lithium heparin tubes before dosing and 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 36, 48, 72, 120 and 168 h after administration. Blood samples (2 mL) were refrigerated and used to assess blood radioactivity for assessments of plasma radioactivity, plasma analyte pharmacokinetics and metabolic profiles, the blood samples were centrifuged at 1500 × g for 10 min at 4 °C. The separated plasma was stored at 4 °C for plasma radioactivity and −70 °C for plasma analyte pharmacokinetics and metabolic profiling/identification.

Urine samples were collected at the following intervals: pre-dose (−12–0 h) and 0–6, 6–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144, 144–168, 168–192, 192–216 and 216–240 h after administration. For one subject, additional 24-h collections continued up to 312 h after administration. During the collection interval, urine was stored in a refrigerator. Three aliquots were subsequently taken for radioactivity measurements (20 mL), urine analyte analyses (4 mL) and metabolite profiling (20 mL), and then the samples were stored at 4, −70 and −70 °C, respectively.

Faecal samples were collected at the following intervals: pre-dose (−24–0 h), 0–24, 24–48, 48–72, 72–96, 96–120, 120–144, 144–168, 168–192, 192–216 and 216–240 h after administration. For one subject, additional 24-h collections continued up to 312 h after administration. Immediately after voiding, faecal samples were stored at −20 °C until analysis of total radioactivity and metabolite profiling. Each pooled collection was homogenised in an appropriate volume of deionised water avoiding excessive dilution and sub-samples were removed for analyses of total radioactivity and metabolite profiling and stored at −20 and −70 °C, respectively.

Plasma, urinary and faecal samples for metabolite profiling were selected after confirming the total radioactivity in plasma, urine and faeces, respectively.

Analysis of total radioactivity in samples
Aliquots of plasma (up to 800 µL) and urine (up to 1 mL, weighed aliquots) were added directly to Emulsifier-Safe scintillation fluid prior to liquid scintillation counting. Aliquots of blood (400 µL) samples and faecal homogenate samples (0.2 to 0.5 g) were combusted using a Packard sample oxidiser System 307 (PerkinElmer Life and Analytical Sciences). The resulting 14CO2 was trapped in Carbo-sorb E and then mixed with Permafluor E+. The efficiency of oxidation was determined via combustion of quality control standards. All of the samples in the scintillation fluid were counted using a TriCarb scintillation counter (2100TR, 2900TR or 3100TR, PerkinElmer Life and Analytical Sciences).

Determination of rovatirelin and TAMP in plasma and urine
Rovatirelin and TAMP in plasma (100 µL) or urine (50 µL) were extracted using an Oasis HLB µElution Plate (Waters Corporation, Milford, MA, USA). The concentrations of rovatirelin and TAMP were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS) with electrospray ionisation in the positive ion mode, which consisted of API5000 (for plasma) or API4000 (for urine) (AB Sciex Pte. Ltd., Framingham, MA, USA) and Acquity HPLC systems (Waters Corporation). Chromatographic separation was achieved using a Hypersil GOLD PFP column (100 mm × 3 mm i.d., Thermo Fisher Scientific Inc., Waltham, MA, USA) maintained at 50 (for plasma) or 40 °C (for urine) by employing two solvents as the mobile phase, namely solvent A (10 mmol/L ammonium formate containing 0.2% formic acid) and solvent B (acetonitrile containing 0.2% formic acid).
The gradient and flow rate for plasma samples were set as follows: 30% B (0 min, 0.25 mL/min) → 30% B (4.5 min, 0.25 mL/min) → 90% B (4.55 min, 0.6 mL/min) → 90% B (5.80 min, 0.6 mL/min) → 30% B (5.85 min, 0.6 mL/min) → 30% B (6.80 min, 0.6 mL/min) → 30% B (6.85 min, 0.25 mL/min) → 30% B (7.20 min, 0.25 mL/min). For urinary samples, the mobile phase consisted of 70% solvent A and 30% solvent B delivered isocratically at various flow rates as follows: 0 min, 0.3 mL/min; 30 min, 0.3 mL/min; 3.1 min, 0.6 mL/min; 3.5 min, 0.6 mL/min; 3.6 min, 0.3 mL/min; and 4.0 min, 0.3 mL/min. The multiple reaction monitoring transition was monitored for rovatirelin, TAMP and the internal standard (rovatirelin-d10) at m/z 367 → 254, m/z 240 → 127 and m/z 377 → 254, respectively. The concentrations in plasma and urine were represented as those of rovatirelin and TAMP. Calibration curves for both of rovatirelin and TAMP ranged from 0.02 to 40 ng/mL for plasma and from 0.5 to 1000 ng/mL for urine. The assay methods were validated for selectivity, linearity, accuracy, precision and stability prior to the analysis. Furthermore, incurred sample reproducibility was evaluated, and these data were acceptable in plasma and urine analyses.

Pharmacokinetic analysis
Pharmacokinetic parameters were calculated from the individual subject data by non-compartmental methods using WinNonlin Enterprise Version 5.2 (Pharsight Corporation, Mountain View, CA, USA). The area under the concentration-time curve (AUC) was calculated by the linear trapezoidal method, and AUC from time zero to infinity (AUC∞) was estimated by via extrapolation of the terminal phase. The elimination half-life (t1/2) was calculated from the slope of the linear regression of the terminal portion of the log-transformed plasma concentration-time curve (1/z). The maximum concentration (Cmax) and time at the maximum concentration (tmax) were used as observed values. Oral clearance (CL/F) was calculated by dividing the dose by AUCinf. The volume of distribution during the terminal phase (Vz/F) was calculated by dividing CL/F by 1/z.

The concentrations of total radioactivity in excreta (urine and faeces) were multiplied by the respective weights to obtain the amounts recovered in each collected interval, which are expressed in total terms (Ae) and as percentages of the administered radioactivity dose. The amounts recovered in each collection interval of faeces and urine were separately added to obtain the cumulative recovery in each of these excreta. Renal clearance (CLR) was calculated by dividing urinary AE by AUC.

Sample preparation for metabolite profiling
Plasma samples at 3, 4, 5, 6, 8 and 12 h from the five subjects were pooled for each time point. In addition, plasma samples were pooled by subject using an established AUC pooling technique (Hamilton et al., 1981). The time-points included in the AUC pools for each subject were dependent on the level of radioactivity present. AUC pooled samples were prepared from plasma samples at 0.5–12, 2–12 or 3–72 h. The samples were mixed with 4 volumes of acetonitrile and centrifuged at 4°C for 10 min to separate the supernatant. The residue was further extracted with 4 volumes of acetonitrile/methanol (1:1, v/v) in the same manner as the first extraction. The extracts were evaporated to near-dryness under a stream of nitrogen gas at room temperature. The residue was reconstituted in 10 mmol/L ammonium acetate pH 9.0/acetonitrile (97:3, v/v) (800 μL) and centrifuged for HPLC analysis. The final extract was analysed by liquid scintillation counting to calculate the recovery of radioactivity. The extraction recoveries ranged from 66.3 to 79.0% (mean, 74.2%).

Urine samples from each subject were pooled across time-points so that the pool included >90% of the total radioactivity excreted via urine. The pooled samples were prepared by combining equal sample weights of each constituent. A portion (2 mL) of each urine pool was centrifuged for HPLC analysis. To quantify recovery from urine samples, the radioactivity of the supernatant was measured by liquid scintillation counting. The extraction recoveries ranged from 96.9 to 102.3% (mean, 100.6%).

Faecal samples from each subject were pooled across time-points so that the pool included >95% of the total radioactivity excreted faecally. The pooled samples were prepared by combining equal weights of the aqueous homogenates of each constituent. A sample (2 g) of each faecal homogenate pool was extracted twice with 4 volumes of acetonitrile/methanol (1:1 v/v) in the preparation, and samples for HPLC analysis were prepared as described previously for the plasma samples. To quantify recovery from faecal samples, the radioactivity of the final extract was measured by liquid scintillation counting. The extraction recoveries ranged from 93.2 to 108.1% (mean, 100.7%).

HPLC analysis of metabolites in plasma, urine and faeces
Chromatography was performed using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using a Luna C18 column (250 mm × 4.6 mm i.d., 5 μm, Phenomenex, Belmont, CA, USA) maintained at 40°C by employing two solvents as the mobile phase: solvent A (10 mmol/L ammonium acetate, pH 9.0) and solvent B (acetonitrile). A linear gradient at a flow rate of 1.0 mL/min was set as follows: 3% B (0 min) → 3% B (5 min) → 18% B (35 min) → 70% B (45 min) → 70% B (50 min) → 3% B (50.1 min) → 3% B (60 min). The column eluate was introduced to the UV detector set at a wavelength of 210 nm and fractionated every 10 s into 96-well microplates (Deep-Well LumaPlate, PerkinElmer Life and Analytical Sciences) using a HTC PAL fraction collector (BCLA124, CTC Analytics AG, Zwingen, Switzerland). After evaporation of HPLC solvents from the microplates, the radioactivity of residues was determined using a microplate scintillation and luminescence counter (TopCount NXT, PerkinElmer Life and Analytical Sciences). Data were analysed using Laura software v3.4 (Lablogic Systems Ltd., Sheffield, UK). The metabolites were...
identified by comparing retention times between the radioactive peaks of samples and UV peaks of authentic standards on the HPLC connected to the UV detector. Identification of the metabolites was further conducted by comparing retention times between the ion peaks of samples and those of authentic standards on an LC-MS system equipped with a multiple-stage mass spectrometer (MSn).

The column recoveries of radioactivity were 89.7% for plasma, 85.8% for urine and 100% for faeces. Therefore, no notable losses of radioactivity were observed across the analytical system.

The radiolabelled components in each chromatogram (greater than 3-fold background) were evaluated to determine retention times and percentage peak area values as percentages of the total radioactivity in the chromatogram. The peaks not associated with the metabolite reference standards were assigned the prefix HM (human metabolite) followed by a number based on the retention time. The amounts of rovatirelin and its metabolites in urine and faeces were expressed as percentages of the dose administered.

Identification of rovatirelin and its metabolites using LC-MS (MSn)

Analytical samples prepared from plasma, urine and faeces were analysed using an LC-MS (MSn), as they contained the peaks of interest. Chromatography was performed using an Accela HPLC system (Thermo Fisher Scientific). The HPLC conditions were the same as described in a previous section. The HPLC eluent was split post-column in a 1:10 ratio between a mass spectrometer and a Beta-RAM radioactivity detector (model 4, Lablogic Systems Ltd.). MS analysis was performed using an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific). The analytical conditions of the mass spectrometer were as follows: atmospheric pressure ionisation interface, electrospray ionisation; acquisition, full scans in the positive ion mode; scan range, 100–750 m/z (MSn data dependant); source voltage, 4.2 kV; and capillary temperature, 310 °C. Metabolites in the analytical samples were identified by comparing the retention time, protonated molecule and fragment ions with those of the authentic standards. Compound-related molecular ions were identified by comparing the retention times on the radiochromatogram. In all cases, the 12C ion was the major species in the molecular ion cluster. For accurate mass confirmation of a suspected metabolite, the measured accurate mass had to be within 5 ppm of the theoretical value to confirm the empirical formula. The fragmentation given was based on the 12C ion unless stated otherwise.

Assay for TAMP formation from rovatirelin in human liver preparations and recombinant human CYPs

A typical incubation mixture (100 μL) contained 100 mmol/L potassium phosphate buffer, pH 7.4, 3.3 mmol/L MgCl₂, 1.3 mmol/L NADP⁺, 3.3 mmol/L G-6-P, 0.4 units/mL G-6-P DH, the enzyme fraction (4.0 mg/mL for human liver S9, 2.0 mg/mL for HLM, and 100 pmol/mL for recombinant human CYPs) and 200 μmol/L rovatirelin. In the case of inhibition studies, the reaction mixture contained a CYP or aldehyde dehydrogenase (ALDH) inhibitor, namely 500 μmol/L 1-aminobenzotriazole (broad-spectrum CYP inhibitor), 10 μmol/L furafylline (CYP1A2 inhibitor), 2 μmol/L trans-2-phenylcyclopropylamine (CYP2A6 inhibitor), 1 μmol/L ticlopidine (CYP2B6 inhibitor), 0.1 μmol/L montelukast (CYP2C8 inhibitor), 10 μmol/L sulfaphenazole (CYP2C9 inhibitor), 1 μmol/L (S)-(+)-N-3-benzylindanoxan (CYP2C19 inhibitor), 1 μmol/L quinidine (CYP2D6 inhibitor), 50 μmol/L diethyldithiocarbamate (CYP2E1/ALDH inhibitor), 1 μmol/L ketoconazole (CYP3A4/5 inhibitor) or 100 μmol/L disulphiram (ALDH inhibitor). Rovatirelin hydrate was dissolved in distilled water, and all inhibitors were dissolved in acetonitrile/distilled water (1:1, v/v). The final concentration of acetonitrile in the reaction mixtures was 0.5% (v/v). The reaction mixture was typically incubated at 37 °C for 120 min, and the reaction was terminated with acetonitrile (100 μL). After removal of the protein by centrifugation at 20,800 × g for 10 min, the supernatant was evaporated under a gentle stream of nitrogen. The residue was reconstituted in distilled water (100 μL), and then the solution was filtered by centrifugation at 10,000 × g for 2 min using Ultrafree-MC (pore size 0.22 μm, Merck KGaA, Darmstadt, Germany).

The concentration of TAMP in the filtrate was determined using an HPLC system with UV detection, which consisted of a Hitachi L-7000 series (Hitachi High-Technologies Corporation, Tokyo, Japan). Chromatographic separation was achieved using an L-column ODS column (250 mm × 4.6 mm i.d., 5 μm, Chemicals Evaluation and Research Institute Japan, Tokyo, Japan) maintained at 40 °C by employing two solvents as the mobile phase, namely solvent A (20 mmol/L potassium phosphate pH 7.5) and solvent B (acetonitrile). A linear gradient at a flow rate of 1.0 mL/min was set as follows: 10% B (0 min) → 10% B (5 min) → 30% B (20 min) → 60% B (20.1 min) → 60% B (25 min) → 10% B (25.1 min) → 10% B (35 min). The wavelength of UV detection was set at 220 nm. The calibration curve of TAMP was linear in the concentration range 0.5–50 μmol/L. The assay method was validated for selectivity, linearity, accuracy, precision and stability after extraction prior to the analysis.

Results

Safety assessment

A single oral dose of [14C]rovatirelin (3.2 mg) was considered to be moderately well tolerated in the healthy male subjects. All adverse events were mild or moderate in severity and transient in nature. No severe or serious adverse events were reported, and no subject was withdrawn from the study because of adverse events. There were no safety concerns based on clinical laboratory results, vital signs, 12-lead ECG, body weight changes or physical examinations.

Pharmacokinetics of radioactivity, rovatirelin and TAMP

The concentration-time profiles and pharmacokinetic parameters of radioactivity, rovatirelin and TAMP are presented in
The plasma concentration of rovatirelin reached $C_{\text{max}}$ (7.92 ng/mL) at a $t_{\text{max}}$ of 5.02 h. After reaching $C_{\text{max}}$, the plasma concentrations appeared to decline in a biphasic manner with a terminal $t_{1/2}$ of 14.9 h. The percentages of $C_{\text{max}}, AUC_{0-8}$ h and $AUC_{0-36}$ h of rovatirelin relative to radioactivity were 73.0, 72.7 and 43.8%, respectively. The plasma concentration of TAMP slowly reached $C_{\text{max}}$ (0.976 ng/mL) at a $t_{\text{max}}$ of 8.00 h compared with radioactivity and rovatirelin. After reaching $C_{\text{max}}$, the plasma concentrations of TAMP declined in a multiphasic manner with a secondary peak at 48 h after administration. The percentages of $C_{\text{max}}, AUC_{0-8}$ h and $AUC_{0-36}$ h of TAMP relative to radioactivity were 9.0, 6.3 and 10.6%, respectively. The maximum levels of radioactivity in plasma and blood were reached at a similar time as that for rovatirelin, with a $t_{\text{max}}$ of 6.00 h after administration. A similar multiphasic decline as noted for TAMP was also observed in the profiles for plasma and blood radioactivity, with a secondary peak at 48 h after administration. The concentrations of radioactivity were lower in blood than in plasma (blood/plasma ratio: 0.713–0.771 between 3 and 12 h after administration).

### Excretion and recovery of radioactivity, rovatirelin and TAMP

The cumulative excretions of radioactivity in urine and faeces up to 240 h after administration were 36.8 and 50.1% of the administered dose, respectively (Table 2 and Figure 2). The sum of urinary and faecal excretion was 89.0% of the administered dose. The mean total amounts of substances excreted in urine represented 15.6 and 15.9% of the administered dose for rovatirelin and TAMP, respectively, and CL was calculated as 5.80 L/h for rovatirelin and 9.45 L/h for TAMP. At early times up to 12 h after administration, the urinary excretion of rovatirelin was abundant (71% relative to total radioactivity in urine).

### Metabolite profiles in plasma, urine and faeces

The representative chromatographic profiles of radiolabelled components in plasma, urine and faeces are shown in Figure 3, and the quantification of these components are summarised in Table 3.

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**Figure 1.** Concentrations of radioactivity in plasma and blood, and concentrations of rovatirelin and TAMP in plasma after a single oral dose of $^{14}$Crovatirelin (3.2 mg) to healthy male subjects. Each point and vertical bar represents the mean and standard deviation of five subjects, respectively. TAMP: (thiazolylalanyl)methylpyrrolidine.

**Table 1.** Pharmacokinetic parameters of radioactivity, rovatirelin and TAMP after a single oral dose of $^{14}$Crovatirelin (3.2 mg) to healthy male subjects.

| Pharmacokinetic parameters | Radioactivity ($n = 5$) | Rovatirelin ($n = 5$) | TAMP ($n = 5$) |
|----------------------------|------------------------|----------------------|----------------|
|                            | Plasma                 | Blood                | Plasma        | Plasma        |
| $C_{\text{max}}$ (ng eq./mL or ng/mL) | 10.8 (15.3)            | 8.09 (13.2)          | 7.92 (23.1)   | 0.976 (38.3)  |
| $t_{\text{max}}$ (h)        | 6.00 (5.00–8.00)       | 6.00 (5.00–8.00)     | 5.02 (5.00–6.00) | 8.00 (5.02–48.00) |
| $AUC_{0-8}$ h (ng eq./h/mL or ng·h/mL) | 48.8 (24.9)            | 36.0 (23.2)          | 35.5 (27.0)   | 3.09 (39.4)   |
| $AUC_{0-36}$ h (ng eq./h/mL or ng·h/mL) | 185 (16.7)             | NA                   | 81.1 (17.7)   | 19.7 (24.0)   |
| $AUC_{\text{last}}$ (ng eq./h/mL or ng·h/mL) | 278 (59.9)             | 122 (78.2)           | 85.9 (15.1)   | 35.1 (46.9)   |
| $AUC_{\infty}$ (ng eq./h/mL or ng·h/mL) | 425 (91.8)             | NC$^b$               | 87.2 (14.8)   | NC$^b$        |
| $t_{1/2}$ (h)               | 32.1 (167.1)           | NC$^b$               | 14.9 (19.8)   | NC$^b$        |
| $CL/F$ (L/h)                | NA                     | NA                   | 36.7 (14.8)   | NA            |
| $V_z/F$ (L)                 | NA                     | NA                   | 787 (30.7)    | NA            |

Geometric mean (CV%) data are presented.  
TAMP: (thiazolylalanyl)methylpyrrolidine; $C_{\text{max}}$: maximum concentration; $t_{\text{max}}$: time of $C_{\text{max}}$; $AUC$: area under the time-concentration curve; $t_{1/2}$: half-life; $CL/F$: oral clearance; $V_z/F$: volume of distribution during the terminal phase; NA: not applicable; NC: not calculated.

$^{a}$The estimation of a reliable $AUC_{\infty}$ and $t_{1/2}$ was impossible.
In time-point plasma samples, unchanged rovatirelin was the predominant component in the profiles at each time point (>70% of the total peak area), and the major plasma metabolite was TAMP (>6% of the total peak area). Two other minor components of radioactivity (HM2 and TA) were detected in the chromatograms of the time-point pooled samples. Regarding the AUC plasma samples, unchanged rovatirelin was the major component (74.21%). TAMP was the most abundant metabolite, accounting for 17.20% of the total drug-related exposure. HM2, which accounted for 2.92% of the total peak area, was detected in the AUC samples of all but one subject. The metabolite profile of the AUC pooled sample from one subject also exhibited two further components of interest (TA and HM10), and TA and HM10

Table 2. Cumulative excretion of radioactivity in urine and faeces and cumulative excretion of rovatirelin and TAMP in urine after a single oral dose of [14C]rovatirelin (3.2 mg) to healthy male subjects.

| Parameter          | Radioactivity (n = 5) | Rovatirelin (n = 5) | TAMP (n = 5) |
|--------------------|-----------------------|--------------------|--------------|
|                    | Urine | Faeces | Total | Urine | Faeces | Total |
| \( f_e \) (% of dose) | 36.8 (25.1) | 50.1 (24.9) | 89.0 (0.7) | 15.6 (16.1) | 15.9 (51.8) |
| \( \text{CLr} \) (L/h) | NA | NA | NA | 5.80 (19.1) | 9.45 (21.8) |

The periods of excretion of radioactivity, rovatirelin and TAMP were ≤240, 96 and 120 h after administration, respectively. Geometric mean (CV%) data are presented.

TAMP: (thiazolylalanyl)methylpyrrolidine; \( f_e \): percentage of the administered dose; \( \text{CLr} \): renal clearance; NA: not applicable.

In time-point plasma samples, unchanged rovatirelin was the predominant component in the profiles at each time point (>70% of the total peak area), and the major plasma metabolite was TAMP (>6% of the total peak area). Two other minor components of radioactivity (HM2 and TA) were detected in the chromatograms of the time-point pooled samples. Regarding the AUC plasma samples, unchanged rovatirelin was the major component (74.21%). TAMP was the most abundant metabolite, accounting for 17.20% of the total drug-related exposure. HM2, which accounted for 2.92% of the total peak area, was detected in the AUC samples of all but one subject. The metabolite profile of the AUC pooled sample from one subject also exhibited two further components of interest (TA and HM10), and TA and HM10

Figure 2. Cumulative recovery of radioactivity in urine and faeces, and cumulative recovery of rovatirelin and TAMP in urine after a single oral dose of [14C]rovatirelin (3.2 mg) to healthy male subjects. Each point and vertical bar represent the mean and standard deviation of five subjects, respectively. TAMP: (thiazolylalanyl)methylpyrrolidine.

Figure 3. Representative high-performance lipid chromatography radiochromatogram of plasma (12 h time-point pool), urine (0–72 h) and faeces (24–96 h) after a single oral dose of [14C]rovatirelin (3.2 mg) to healthy male subjects. HM: human metabolite (unknown); TA: 3-(4-thiazoyl)-L-alanine; TAMP: (thiazolylalanyl)methylpyrrolidine; rovatirelin-acid: rovatirelin aminopentanoic acid; rovatirelin-OH: rovatirelin pyrrolidine (4S)-hydroxy; rovatirelin-ketone: rovatirelin aminopentanone.
presented in Figure 5. Because of the low levels of radioactivity in the samples, only unchanged rovatirelin and TAMP could be confirmed in the samples from each matrix by LC-MS/MS analysis. The response of all other components was too low to permit the detection of rovatirelin-related ions, which would have enabled structural elucidation.

**Identification of enzymes for TAMP and unknown metabolite formation in vitro**

Regarding the requirement of NAD and NADPH via an NADPH-regenerating system for incubation with human S9 and HLM, respectively, TAMP was only formed from rovatirelin in the presence of NADPH and HLM. In addition, an unknown metabolite was also detected in the reaction mixture (Figure 7). Conversely, TAMP was not formed in human S9 fortified with NAD.

To identify the metabolic enzymes responsible for formation of TAMP and the unknown metabolite, chemical inhibitors of CYP and ALDH were added to the reaction mixtures of HLM fortified with an NADPH-regenerating system (Figures 6 and 7). TAMP formation was inhibited by 1-amino-2-benzothiazole (broad-spectrum CYP inhibitor), and formation of the unknown metabolite was also inhibited by the compound (data not shown). In addition, diethyldithiocarbamate (CYP2E1/ALDH inhibitor), ketoconazole (CYP3A4/5 inhibitor) and disulphiram (ALDH inhibitor) inhibited TAMP formation in HLM. Formation of the unknown metabolite was also inhibited by ketoconazole, but not by diethyldithiocarbamate and disulphiram (Figure 7).

In the assay with recombinant human CYPs, TAMP formation was only observed in the presence of CYP3A4 and CYP3A5, and its formation in the presence of CYP3A4 was 3-fold higher than that in the presence of CYP3A5 (Figure 8). The formation of the unknown metabolite was also detected in the presence of CYP3A4 and CYP3A5, and the ratio of the unknown metabolite to TAMP was greater than that in HLM (Figure 7).

**Discussion**

The pharmacokinetic properties of rovatirelin in humans were investigated in healthy male subjects after a single oral

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**Table 3. Metabolite profile of rovatirelin in plasma, urine and faeces after a single oral dose of [14C]rovatirelin (3.2 mg) to healthy male subjects.**

| Metabolite               | % Total Peak Area | % of Dose |
|--------------------------|------------------|-----------|
|                           | 3 h  | 4 h  | 5 h  | 6 h  | 8 h  | 12 h | AUC pool (0–12 h) | Urine | Faeces |
| Rovatirelin              | 88.23| 85.80| 86.48| 86.14| 84.95| 73.23| 74.21           | 18.07| 43.29 |
| TAMPe                    | 6.66 | 8.19 | 9.99 | 10.44| 12.12| 19.60| 17.20           | 16.68| 3.20  |
| TAe                      | ND   | ND   | ND   | ND   | ND   | 2.52 | 3.58            | 0.15 | 0.07  |
| Rovatirelin-acide        | ND   | ND   | ND   | ND   | ND   | ND   | ND             | 0.50 | 0.11  |
| Rovatirelin-ketonee      | ND   | ND   | ND   | ND   | ND   | ND   | ND             | ND   | 0.21  |
| KP051e                   | ND   | ND   | ND   | ND   | ND   | 2.92 | 2.92           | 0.23 | ND    |
| HM2                      | 2.79 | 5.62 | 2.86 | 3.09 | 2.75 | 4.29 | 2.92           | 0.21 | 0.46  |
| HM10                     | ND   | ND   | ND   | ND   | ND   | 1.19 | 0.21           | 0.95 | ND    |
| Other HMs                | ND   | ND   | ND   | ND   | ND   | 1.19 | 0.21           | 1.65 | ND    |
| Total                    | 97.85| 99.60| 99.33| 99.67| 99.82| 99.64| 99.10          | 36.12| 50.36 |

ND: Not detected (a value of zero was used for to calculate means); TAMPe: Thiapental/methylpyrrolidine; TAe: 3-[(4-thiazoyl)-l]-alanine; Rovatirelin-acide, rovatirelin aminopentanone; Rovatirelin-ketone: rovatirelin aminopentanoic acid; Rovatirelin-OH: rovatirelin pyrrolidine (4S)-hydroxy; Rovatirelin-ketone: rovatirelin aminopentanone; HM: human metabolite (unknown).

Values obtained from a time-point pooling sample from five subjects or a mean of five AUC pooling samples.

Values are the means of five subjects. Plotted samples were prepared from plasma samples at 0.5–12, 2–12 or 3–72 h. Values are the means of five subjects.

Values are the means of five subjects. Urinary pooling samples were prepared from each subject samples at 0–48, 0–72 or 0–96 h.

Values are the means of five subjects. Pooled faecal samples were prepared from each subject samples at 24–72 or 24–96 h.

Chemical structures are shown in Figure 9.

Total of HMs (HM1 alone) excreted in urine.

Total of HMs (HM3–9 and HM11–14) excreted in faeces.
Figure 4. Representative mass spectra of full MS (A), MS² of [MH]⁺ ion at m/z 367 (B) and MS³ of its fragment at m/z 282 (C) from rovatirelin reference standard and representative mass spectra of full MS (D), MS² of [MH]⁺ ion at m/z 367 (E) and MS³ of its fragment at m/z 282 (F) from rovatirelin in faeces (24–96 h pool).

Figure 5. Representative mass spectra of full MS (A), MS² of [MH]⁺ ion at m/z 240 (B) and MS³ of its fragment at m/z 127 (C) from the TAMP reference standard and representative mass spectra of full MS (D), MS² of [MH]⁺ ion at m/z 240 (E) and MS³ of its fragment at m/z 127 (F) from TAMP in faeces (24–96 h pool). TAMP: (thiazolylalanyl)methylpyrrolidine.
dose of $[^{14}C]$rovatirelin (3.2 mg), and the major metabolic enzyme of rovatirelin in humans was identified by in vitro studies. These studies are generally useful in understanding the fate of drug candidates in humans, drug-drug interaction (DDI) potential and the relevance of the animal species used for preclinical toxicity and pharmacodynamics studies (Isin et al., 2012; Penner et al., 2009, 2012; Walker et al., 2009).

After a single oral dose of $[^{14}C]$rovatirelin, radioactivity was steadily absorbed, reaching $C_{\text{max}}$ at 6.00 h after administration and declining thereafter with a $t_{1/2}$ of 32.1 h. Radioactivity was mainly recovered in faeces (50.1% of the dose) and to a smaller extent in urine (36.8% of the dose). The recovery of total radioactivity (89.0% of the dose) was considered satisfactory compared with the findings in the human mass balance studies conducted to date (Bruderer et al., 2012; Nijenhuis et al., 2016; Roffey et al., 2007). The principal route of elimination was faecal excretion, in line with the findings in non-clinical studies (Kobayashi et al., 2019). Urinary excretion was considered moderate, suggesting that no less than 36.8% of the dose of rovatirelin would be absorbed from the gastrointestinal tract. In addition, the metabolites in faeces accounted for 7.07% of the dose; thus, $>43\%$ of the dose (urinary excretion + metabolites in faeces) may be absorbed in total. Thus, the extent of absorption in humans is considered larger than that in rats (27.3%), and the bioavailability in humans may be higher than that in rats (7.3%) (Kobayashi et al., 2019).

The highest concentration of rovatirelin in plasma was observed at 5.02 h after administration, which was almost the same as that of total radioactivity in plasma, and the concentration decreased with a $t_{1/2}$ of 14.9 h (Table 1 and Figure 1). Conversely, rovatirelin was rapidly absorbed after oral administration to rats and dogs ($t_{\text{max}}$: 0.4–0.6 h after administration in rats, 1.0 h after administration in dogs), and $t_{1/2}$ was 3.3–7.7 h in rats and 2.7 h in dogs (Kobayashi et al., 2019). These findings illustrate that rovatirelin is likely to be steadily absorbed and slowly eliminated from plasma in humans. Similar to the blood-to-plasma ratio ($R_b$: 0.815–0.828) in an in vitro study (Kobayashi et al., 2019), $R_b$ in this mass balance study was as low as 0.713–0.771, exhibiting a limited degree of partitioning of drug-related material into blood cells in vivo. The urinary excretion of rovatirelin up to 12 h after administration was 71% relative to the total radioactivity in urine, revealing that rovatirelin was mainly
excreted in urine at early times after administration (Figure 2).

TAMP has been assumed to be the major metabolite in humans based on in vitro study using human hepatocytes (Kobayashi et al., 2019). Thus, the TAMP concentration in plasma and urine was determined in this human mass balance study. The plasma concentration of TAMP peaked at 8.00 h after administration, and the cumulative excretion of TAMP in urine approached its maximum at approximately 72 h after administration (Figure 2), suggesting that the formation of TAMP is slow in humans. A secondary peak was observed in the plasma concentration time-curve of TAMP. Although the secondary peak was observed in the majority of the pharmacokinetics in the individual subjects, there was a large variability in the plasma concentration of TAMP from 24 to 48 h after administration. In the other clinical pharmacokinetics of TAMP, the secondary peak was not observed (Kissei Pharmaceutical Co. Ltd., data on file). These results show that the secondary peak is more likely due to variability in the pharmacokinetic samples. CLr of TAMP (9.45 L/h) was larger than that of rovatirelin (5.80 L/h), illustrating that TAMP tends to be more readily excreted in urine than rovatirelin.

In the metabolite profiling of rovatirelin, the major components of radioactivity in plasma, urine and faeces were unchanged rovatirelin and TAMP. Although unchanged rovatirelin was the predominant component in plasma, TAMP was identified as a major circulating metabolite according to the criteria in the ICH guideline M3(R2) (ICH, 2009). In the ICH guideline, a metabolite is considered major if its exposure in human plasma is >10% of the total drug-related material and further preclinical assessments are required. The exposure of TAMP was 17.20% of the total drug-related material, but TAMP was found to display no affinity for human TRHR and extremely low affinity for other pharmacological receptors and ion channels (Kissei Pharmaceutical Co. Ltd., data on file). The absence of pharmacological potencies of TAMP and its lower exposure relative to that of rovatirelin suggest that TAMP should not be expected to contribute to the pharmacological effects of rovatirelin in vivo. In addition, TAMP was also present in animals and adequately qualified in non-clinical safety studies (Kissei Pharmaceutical Co. Ltd., data on file). As minor metabolites, TA, HM2 and HM10 were detected in plasma and excreta. Although the chemical structures of HM2 and HM10 could not be assumed by LC-MS analyses, considering the retention time in HPLC analysis between this study and the non-clinical study in rats, HM2 and HM10 are considered to correspond to metabolites (Kobayashi et al., 2019). Thus, the minor metabolites found in human plasma (TA, HM2 and HM10) appear to be present in rat plasma, and it is considered that no human-specific metabolite exists among the circulating metabolites. Among the metabolites, TAMP was the most notable circulating metabolite and the major metabolite in excreta (approximately 20% of the dose), demonstrated that the major metabolic pathway of rovatirelin in vivo is the formation of TAMP from rovatirelin (Figure 9). Thus, we conducted in vitro studies to identify the major metabolic enzymes responsible for TAMP formation in humans. Although amount of TAMP was moderate in excreta in this human mass balance study, in vitro studies required high protein and substrate concentration for TAMP formation, which is thought to be due to a low turnover rate in vitro.

From the results of in vitro studies using human liver S9 and microsomal fractions, TAMP was not expected to be directly formed from rovatirelin by the enzymes present in these fractions, such as ALDH, aminopeptidase and esterase in the liver. Furthermore, rovatirelin was resistant to pyroglutamate aminopeptidase in the rat brain homogenate and rat plasma (Kobayashi et al., 2019). These findings indicated that TAMP is not formed from rovatirelin by hydrolysis as the first metabolic reaction. In HLM, formation of TAMP and the unknown metabolite was simultaneously observed depending on the presence of NADPH, and the formation of both compounds was inhibited by a typical CYP3A4/5 inhibitor (ketoconazole). An ALDH inhibitor (disulphiram) and its active metabolite (diethyldithiocarbamate) also inhibited the formation of TAMP in HLM, but not that of the unknown metabolite. In addition, TAMP was formed by recombinant human CYP3A4 and 3A5, but not by recombinant human CYP2E1, and only recombinant human CYP3A4 and 3A5 significantly formed the unknown metabolite. These results indicated that formation of TAMP and the unknown metabolite depends on CYP3A4/5 activity. Furthermore, it is reasonable to consider that TAMP may be generated depending on the formation of the unknown metabolite and that CYP3A4/5 activity could play an important role in the formation of the unknown metabolite from rovatirelin in addition to the possible direct formation of TAMP. Additionally, ALDH inhibitors affected the formation of TAMP from the unknown metabolite, suggesting that ALDH activity contributes to the formation of TAMP from the unknown metabolite. As ALDH is known to have NAD-independent esterase activity and is widely distributed in liver cell fractions (Marchitti et al., 2008; Sidhu & Blair, 1975; Yoshida et al., 1998), there is a possibility of hydrolysing the amide bond that the unknown metabolite may possess.

The metabolism of rovatirelin in humans is summarised in Figure 9. Although there are some metabolic routes, the major metabolic pathway of rovatirelin is conversion to TAMP. CYP3A4/5 activity is believed to be greatly involved as a rate-determining factor for TAMP formation based on the
results of in vitro studies, and the mean fractional metabolism is estimated to be 46% (urinary and faecal excretion of TAMP/total excretion) from the metabolite profiling in this human mass balance study. The DDI guidelines recommend that if the enzyme is responsible for >25% of the drug’s elimination based on the in vitro phenotyping studies and human pharmacokinetic data, clinical DDI studies should be conducted (EMEA guideline, 2012; FDA draft guidance, 2017; MHLW guideline, 2018). Thus, it is necessary to evaluate DDIs for TAMP formation in vivo.

Conclusion

After a single oral dose of [14C]rovatirelin in healthy male subjects, rovatirelin was steadily absorbed, and rovatirelin and TAMP circulated in plasma as the major components. The total radioactivity recovered in urine and faeces was 89.0% of the administered dose. The principal route of elimination was faecal excretion (50.1% of the dose), and urinary excretion was moderate (36.8%). Urinary excretion of rovatirelin and TAMP accounted for 15.6 and 15.9% of the total dose administered, respectively. Although rovatirelin was extensively metabolised in healthy subjects, with 20 metabolites observed across plasma, urine and faeces, TAMP was identified as the major metabolite of rovatirelin. The major metabolic pathway of rovatirelin is considered the conversion of rovatirelin to TAMP, and it appears that several metabolic enzymes are involved in this pathway. The most important enzymes responsible for the pathway are believed to be CYP3A4/5.

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