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**Absorption, Metabolism and Excretion of [¹⁴C]Mirabegron (YM178), a Potent
and Selective β_3 -Adrenoceptor Agonist, after Oral Administration to Healthy
Male Volunteers**

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Running title page

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d) List of non-standard abbreviations used in this paper

AR: adrenoceptor

AUC: area under the concentration-time curve

C_{max}: maximum concentration

HPLC: high-performance liquid chromatography

IS: internal standard

LC: liquid chromatography

LSC: liquid scintillation counter

Mirabegron (YM178):

2-(2-amino-1,3-thiazol-4-yl)-*N*-[4-(2-[(2*R*)-2-hydroxy-2-phenylethyl]amino)ethyl]phenyl]acetamide

MS: mass spectrometry, mass spectrometer

MS/MS: tandem mass spectrometry

OAB: overactive bladder

RAD: radiochemical detector

ROE: rotational nuclear Overhauser effect

ROESY: rotational nuclear Overhauser effect spectroscopy

t_{max}: time to reach maximum concentration

TOCSY: total correlated spectroscopy

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Abstract

The mass balance and metabolite profiles of [¹⁴C]mirabegron [2-(2-amino-1,3-thiazol-4-yl)-*N*-[4-(2-{{(2*R*)-2-hydroxy-2-phenylethyl}amino)ethyl][U-¹⁴C]phenyl]acetamide], a β₃-adrenoceptor agonist for the treatment of overactive bladder, were characterized in four young, healthy, fasted male subjects after a single oral dose of [¹⁴C]mirabegron (160 mg, 1.85 MBq) in a solution. [¹⁴C]Mirabegron was rapidly absorbed with a plasma t_{max} for mirabegron and total radioactivity of 1.0 and 2.3 h post-dose, respectively. Unchanged mirabegron was the most abundant component of radioactivity, accounting for about 22% of circulating radioactivity in plasma. Mean recovery in urine and feces amounted to 55% and 34%, respectively. No radioactivity was detected in expired air. The main component of radioactivity in urine was unchanged mirabegron, which accounted for 45% of the excreted radioactivity. A total of 10 metabolites were found in urine. On the basis of the metabolites found in urine, major primary metabolic reactions of mirabegron were estimated to be amide hydrolysis (M5, M16 and M17), accounting for 48% of the identified metabolites in urine, followed by glucuronidation (M11, M12, M13 and M14) and *N*-dealkylation or oxidation of the secondary amine (M8, M9 and M15), accounting for 34% and 18% of the identified metabolites, respectively. In feces, the radioactivity was recovered almost entirely as the unchanged form. Eight of the metabolites characterized in urine were also observed in plasma. These findings indicate that mirabegron, administered as

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a solution, is rapidly absorbed after oral administration, circulates in plasma as the unchanged form and metabolites, and is recovered in urine and feces mainly as the unchanged form.

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Introduction

Mirabegron [YM178, 2-(2-amino-1,3-thiazol-4-yl)-*N*-[4-(2-[(2*R*)-2-hydroxy-2-phenylethyl]amino)ethyl]phenyl]acetamide] (Fig. 1), synthesized at Astellas Pharma Inc. (Ibaraki, Japan), is a potent and selective agonist for the human β_3 -adrenoceptor (AR) (Takasu et al., 2007), and is the first of a new class of compounds developed for the treatment of overactive bladder (OAB). Mirabegron activates β_3 -ARs on the detrusor muscle of the bladder to facilitate filling of the bladder and storage of urine, without inhibiting bladder voiding contractions (Yamaguchi and Chapple, 2007; Takasu et al., 2007). In a phase 2 dose-ranging study to evaluate the efficacy of mirabegron versus placebo in patients with OAB, mirabegron at doses of 50, 100 and 200 mg in extended release formulations once daily had superior efficacy results compared with placebo (Chapple et al., 2010). The results of two pivotal phase 3 clinical trials for mirabegron confirmed that mirabegron significantly improves key OAB symptoms – urinary incontinence and frequency of micturition (Khullar et al., 2011; Nitti et al., 2011). Overall, the incidence of treatment-emergent adverse events in mirabegron groups was low across studies, and mirabegron was generally well tolerated (Chapple et al., 2010; Khullar et al., 2011; Nitti et al., 2011). The therapeutic dose is 50 mg in extended release formulations once daily.

In the preclinical pharmacokinetic studies, when single doses of mirabegron were

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orally administered to rats and dogs, mirabegron plasma concentrations reached C_{max} 0.1 to 4 h after administration. AUC increased more than dose-proportionally with dose in both rats and dogs. Absolute bioavailability was 23.0%, 48.4% and 75.7% at doses of 3, 10 and 30 mg/kg, respectively, in rats, and 41.8%, 64.6% and 77.1% at doses of 0.25, 0.5 and 1 mg/kg, respectively, in dogs (unpublished observations). In monkeys, when repeated doses of mirabegron at 3, 10 and 30 mg/kg/day were orally administered, AUC of mirabegron increased almost proportionately with increasing doses of mirabegron (unpublished observations). Orally administered [^{14}C]mirabegron was excreted as unchanged drug and metabolites in urine and feces in rats and monkeys. Total recovery in urine and feces was more than 94% of the administered dose (unpublished observations). Mirabegron was safe and well tolerated in healthy subjects after single and multiple dose administration up to 240 mg in immediate release solid dosage formulations daily in the phase 1 clinical trials of non-radiolabeled mirabegron (data on file). The objectives of the present study were: 1) to investigate the routes of elimination of mirabegron; 2) to quantify the levels of total radioactivity in blood and plasma, and mirabegron in plasma; 3) to examine the metabolite profiles of mirabegron; and 4) to demonstrate mass balance for [^{14}C]mirabegron in healthy human male subjects after a single oral dose.

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Materials and Methods

Radiolabeled Material and Other Materials

[¹⁴C]Mirabegron (Fig. 1) was synthesized at Amersham Biosciences (Buckinghamshire, UK), with a certificate of analysis of the radiochemical purity (98.5%) and specific activity (3.51 MBq/mg), and was stored at –20°C in the absence of moisture, light and air. Authentic standards of non-labeled mirabegron and its metabolites, YM-538852 (M5) hydrochloride, YM-538853 (M8) trifluoroacetate, YM-340790 (M9), YM-382984 (M11), YM-538858 (M12), YM-538859 (M13), YM-554028 (M14) formate, YM-9636324 (M15) and YM-208876 (M16) hydrochloride, were supplied by the Process Chemistry Labs or the Chemistry Research Labs of Astellas Pharma Inc. (Ibaraki, Japan). The internal standard (IS) for determination of unchanged mirabegron in plasma and urine, YM-88796 (Fig. 1), was also supplied by Astellas Pharma Inc. All other reagents were of high-performance liquid chromatography (HPLC) grade or analytical grade and were obtained from commercial sources.

Dose Preparation

The total dose of mirabegron was 160 mg per subject and it contained 1.85 MBq of [¹⁴C]mirabegron. Non-labeled mirabegron and [¹⁴C]mirabegron were supplied as dry powder and dissolved in 100 mL of a 20 mM sodium citrate buffer solution at pH 4.5. The radioactive dose of 1.85 MBq was well below the maximum allowed limits for

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radiation burden in clinical studies. The radiation exposure in this study, approximately 0.66 mSv, fell into category IIa studies (0.1–1 mSv) of the International Commission on Radiological Protection guidelines (1992). The radioactive dose was decided based on the minimum amount of radioactivity which was deemed necessary to determine the parameters set in the study objectives according to the principle of ALARA ('as low as reasonably achievable').

Study Design

This study was an open-label study involving four healthy male Caucasian subjects, who were aged 19 to 35 years with a height from 174 to 181 cm, a body weight between 65.6 and 85.7 kg and a body mass index between 21.7 and 26.7 kg/m². The clinical phase of this study was conducted at the clinical unit of PRA International B.V. (previously Pharma Bio-Research Group B.V.) (Zuidlaren, The Netherlands) in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. The clinical study protocol was approved by an independent ethics review committee, and all subjects provided written informed consent before the study.

All subjects were in good health based on screening results of routine safety laboratory tests, physical examinations, 12-lead electrocardiograms (ECGs) and vital signs. After an overnight fast in the clinical unit from the day before dosing, with only water allowed up to 2 h before drug administration, subjects received study drug at

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approximately 8:00 AM. Immediately after dose intake, the dosing container was rinsed with three subsequent portions of 50 mL water, which were also taken by the subjects. Subjects continued to refrain from food and drinks until 4 h after dosing, and thereafter were given standardized meals, non-alcoholic drinks, and decaffeinated beverages at normal hours. Smoking and consumption of caffeine, alcohol and grapefruit-containing products were not permitted during the study. Safety evaluations including 12-lead ECG, vital signs, and laboratory tests (hematology, biochemistry, and urinalysis) were performed throughout the admission period.

Sample Collection

Blood samples (20 mL) were collected by an indwelling catheter or by direct venipuncture into lithium heparin tubes at 0 (before administration), 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 36, 48, 72, 96, 120, 144 and 168 h after administration. One milliliter of the sample was used to assess total radioactivity in whole blood.

Remaining blood samples were centrifuged at 4 °C for 10 minutes, and approximately 1.5 mL of the separated plasma samples was used to assess total radioactivity in the plasma. The plasma for metabolite profiling (approximately 5 mL) was transferred to pre-cooled polypropylene tubes containing 50 µL of a dichlorvos solution (0.1% (w/v) in saline) to protect mirabegron from degradation by esterases. The plasma for metabolite profiling and the remaining plasma for analysis of mirabegron

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(approximately 3 mL) were stored at approximately -70°C .

Urine samples were collected at $t = 0$ h (before administration), between 0 to 6, 6 to 12,

12 to 24 h after administration and at subsequent 24 h intervals until 17 days (408 h)

after administration. During the collection interval, the urine was stored in a

refrigerator at 4°C . At the end of each interval, the samples were mixed and then the

total volume was recorded, and the samples were divided into three parts: for

radioactivity counting (12 mL), mirabegron analysis (6 mL) and metabolite profiling

(100 mL). The urine for metabolite profiling was transferred to a polypropylene tube,

containing 1 mL of a dichlorvos solution (0.1% (w/v) in saline). The samples for

analysis of mirabegron and metabolite profiling were stored at approximately -70°C .

Fecal samples were collected at $t = 0$ h (before administration) and 24 h intervals after

administration until 17 days (408 h) after administration. For each collection interval,

the weight of the feces was recorded and the whole samples were homogenized using

water. The volume of water added was recorded to quantify the dilution of the samples.

Portions of the homogenized feces were used for radioactivity counting. For metabolite

profiling, a 20-mL aliquot of the homogenized feces was transferred into a

polypropylene tube containing 0.2 mL of a dichlorvos solution (0.1% (w/v) in saline),

stirred well, and then stored at -70°C .

Expired air was sampled for assessment of $^{14}\text{CO}_2$ expiration before dosing and at 1, 2,

3, 4, 6, 8, 12, 24, 48, 72 and 96 h post-dose. Expired air was blown through a mixture

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of 2 mL hyamine hydroxide (approximately 1.0 N) and 2 mL ethanol, containing an indicator (thymolphthalein), until the color changed from clear blue to colorless, indicating that 2 mmol of CO₂ had been trapped.

Analysis of Total Radioactivities in Samples

An aliquot of plasma (0.25 mL) and urine (1 mL) samples was dissolved in liquid scintillation fluid, Ultima Gold™ (PerkinElmer Inc., Waltham, MA, USA). An aliquot of blood (0.5 mL) samples was added with tissue solubilizer, Solvable™ (PerkinElmer), and the samples were incubated for 60 min at 60 °C to be solubilized. After cooling, 0.1 mL of 0.1 M ethylenediaminetetraacetic acid was added and the samples were decolorized by adding four times a volume of 0.1 mL aliquot of 30% hydrogen peroxide. The mixture was heated again for 20 min at 45 °C, followed by 40 min at 60 °C. After cooling, the mixture was dissolved in Ultima Gold™. Fecal homogenate samples (approximately 0.5 g) were combusted using a Packard 307 sample oxidizer (PerkinElmer). The ¹⁴CO₂ generated was collected in the absorbing fluid, CarboSorb®-E (PerkinElmer) and scintillation fluid, Permafluor® E⁺ (PerkinElmer). The expired air sample was mixed with liquid scintillation fluid, Emulsifier Safe® (PerkinElmer) by vortex-mixing.

All of the samples in the scintillation fluid were counted in the scintillation counter, Packard Tri-Carb 3100TR (PerkinElmer), until a statistical error of 0.5% was obtained,

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with a maximum counting time of 10 min. The lower limit of quantification (LLOQ) was defined as 30 dpm/mL, 50 dpm/mL, 10 dpm/mL, 20 dpm/g and 20 dpm in plasma, whole blood, urine, feces and expired air, respectively.

Determination of Unchanged Mirabegron in Plasma and Urine

Concentrations of unchanged mirabegron in plasma and urine were determined using validated liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods (Astellas internal report). The plasma assay method consisted of a single liquid-liquid extraction of mirabegron and internal standard YM-88796 (structure analogue) using hexane:ethylacetate (1:1 v/v). The urine assay method consisted of dilution of urine with methanol and ammonium acetate buffer. Separation of mirabegron and YM-88796 from matrix constituents was achieved using a reversed phase Symmetry[®] C18 column (Waters Corporation, Milford, MA, USA), 100 mm × 2.1 mm i.d., $d_p = 3.5 \mu\text{m}$ or 150 mm × 3.9 mm i.d., $d_p = 5 \mu\text{m}$, coupled to Thermo TSQ7000 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using Atmospheric Pressure Chemical Ionization interface in positive ion mode. The mobile phase consisted of 20 mM ammonium acetate and acetonitrile (30:70, v/v). The reaction monitoring transitions selected (all m/z masses are $[\text{M}+\text{H}]^+$) were m/z 397 to 260 for unchanged mirabegron and m/z 376 to 358 for the IS.

Calibration ranged from 1 to 500 ng/mL for plasma and from 2 to 1000 ng/mL for

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urine. Accuracy and precision at all concentrations, including the LLOQ, were -8.2% to 8.8% and 4.0% to 14.2%, respectively, for plasma and 0.5% to 12.2% and 3.0% to 17.4%, respectively, for urine in the validation studies.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated from the individual subject data by non-compartmental methods using Parmplus version 7.0 (in-house SAS program developed at PRA International B.V.). The pharmacokinetic parameters included area under the drug concentration–time curve extrapolated to infinity (AUC_{inf}), maximum concentration (C_{max}), time to reach maximum concentration (t_{max}), and terminal half-life ($t_{1/2} = 0.693/k_{el}$) of radioactivity in whole blood and plasma, whole-blood-to-plasma ratio of radioactivity, AUC_{inf} , C_{max} , t_{max} , $t_{1/2}$, oral clearance ($CL/F = \text{dose}/AUC_{inf}$) and apparent volume of distribution based on the terminal phase ($V_z/F = (CL/F)/k_{el}$) of unchanged mirabegron in plasma, cumulative excretion of radioactivity in urine, feces and expired air, $t_{1/2}$ and cumulative excretion of unchanged mirabegron in urine, and renal clearance (CL_R). AUC values were estimated by the linear-log trapezoidal rule. The k_{el} , the apparent terminal elimination rate constant, was determined by linear regression of log-transformed concentration data over the terminal elimination phase, which was determined by visual inspection.

The total amounts of radioactivity excreted between $t = 0$ to the last quantifiable

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sample in the urine, feces and expired air and that of mirabegron in the urine were calculated and are expressed as percentages of the administered radioactivity (percentage of dose).

Metabolite Profiling

Pretreatment of urine, feces and plasma

The frozen urine samples (0–6, 6–12, 12–24 and 24–48 h) obtained from each subject were used individually. The urine sample (2 mL) was thawed and mixed with a 2-fold volume of 100 mM ammonium acetate/formic acid (100:1, v/v), and then loaded onto a solid phase extraction cartridge, Oasis[®] HLB (Waters). The cartridge was then washed with water and eluted with an acetic acid/methanol (0.1:100, v/v) mixture. The eluate was evaporated to dryness under reduced pressure, and the residue was reconstituted with 100 mM ammonium acetate/water/methanol (1:4:5, v/v/v). The mean extraction recovery was 91.9% (from 81.5% to 97.5%).

The frozen fecal homogenates (0–24, 24–48, 48–72 and 72–96 h) obtained from each subject were individually used. Five milliliters of concentrated hydrochloric acid/methanol (5:95, v/v) was added to the fecal homogenate samples (approximately 1 g). After vigorous shaking and centrifugation for 5 min at 4°C, the supernatant was transferred into a polypropylene tube. The same volume of extraction solvent was again added to the residue, which was then shaken vigorously and centrifuged. The

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resulting supernatant was then transferred into the above-mentioned polypropylene tube. The combined supernatants were evaporated to dryness under reduced pressure, then the residues were reconstituted with 100 mM ammonium acetate/water/methanol (1:4:5, v/v/v) and filtered through a Ultrafree C3-HV membrane filter (Millipore Corporation, Billerica, MA, USA). The mean extraction recovery was 70.9% (from 59.6% to 97.5%).

Plasma samples at 2 h (approximately t_{\max}) and at 4, 8 and 12 h (distribution/elimination phase) obtained from the four subjects were pooled for each time point. The pooled plasma (8 mL) was treated in the same way as the urine samples described above. The extraction recoveries were 70.6%, 72.0%, 61.1% and 53.3% for 2, 4, 8 and 12-h plasma, respectively.

An aliquot of the reconstituted solution was analyzed under the conditions described below.

HPLC analysis of metabolites in samples

Relative amounts of metabolites in the urine, feces and plasma were determined by HPLC connected to a radiochemical detector (RAD) or by a liquid scintillation counter (LSC) after collection of HPLC eluates. The metabolites were identified by comparison of retention times between radioactive peaks of samples and ultraviolet (UV) peaks of authentic standards on the HPLC connected to the UV detector.

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Identification of the metabolites was further conducted by comparison of retention times between ion peaks of urine or plasma samples and those of authentic standards on a HPLC equipped with a mass spectrometry (MS) system.

A Capcell Pak C₁₈ UG120 column (4.6 × 250 mm, 5 μm; Shiseido Co. Ltd., Tokyo, Japan) was used as the analytical HPLC column. As the mobile phase, the mixture of 5 mM ammonium acetate/0.029% aqueous ammonia/methanol (475:475:50, v/v/v) (A) and 5 mM ammonium acetate/0.029% aqueous ammonia/methanol (25:25:950, v/v/v) (B) was flowed at 1 mL/min in the following linear gradient mode: starting with 0% of B composition, increasing to 30% in 0 to 60 min, increasing to 70% in 60 to 80 min, maintaining 70% in 80 to 85 min, decreasing to 0% in 85 to 85.1 min and finally maintaining 0% in 85.1 to 110 min. The column was maintained at 40 °C. The column eluate was introduced to the UV detector (Waters 2487; Waters) set at a wavelength of 250 nm and, for urine and feces, then the RAD (Radiomatic FSA150TR; PerkinElmer). The UV and digitalized RAD signal was sent to the host computer running Millennium Chromatography Manager (Waters). The elution pattern of metabolites in urine and feces was determined using the RAD with 6-s integration. As scintillation fluid for feces, Ultima FloTM-M (PerkinElmer) was delivered to the HPLC eluate at a 3-fold flow rate of the mobile phase. The HPLC eluates were simultaneously collected every 30 s for urine and plasma, and dissolved in liquid scintillation fluid, Pico-Fluor (PerkinElmer), to quantify radioactivity (unchanged mirabegron and metabolites) by

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the LSC. For the sensitive detection of radioactivity in plasma, the radioactivity was counted on the low-level counting mode of the LSC (Tri-Carb 3100TR; PerkinElmer) for 20 min. For urine, the radioactivity was counted on the LSC (Tri-Carb 2700TR; PerkinElmer) for 5 min. The counting efficiency was corrected by an external standard radiation source. Detection limits of radioactivity for quantification of metabolite peaks in the LSC assays were defined as two times the background values.

On average, 97.4% and 95.5% of the injected radioactivity from urine and fecal extracts were recovered from the HPLC column. HPLC column recovery experiments were not conducted for plasma, because the radioactivity counts in 8 and 12-h plasma samples were very low, only 10 and 6-times the background value, respectively and multiple metabolites were present in the plasma.

Data processing

The ratio of counts of each radioactive peak to the total radioactivity in the HPLC eluent (percentage on HPLC chromatogram) was obtained to determine the compositions of metabolites to the radioactivities in the urine (percentage in sample).

The radioactivities of the metabolites excreted in the urine were calculated from the individual values of the total radioactivities excreted and are expressed as a percentage of the radioactivity administered (% of dose). Since mean recovery rates upon solid-phase extraction and HPLC analysis were as high as 91.9% and 97.4%, no

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corrections were made to account for the extraction recovery and HPLC column recovery. Quantitative analysis of metabolites in feces and plasma was not conducted because insufficient recoveries were observed for these samples upon extraction. For plasma, the ratio of counts of each radioactive peak to the total radioactivity in the HPLC injection sample (percentage of the radioactivity injected into HPLC, % profiled radioactivity) was calculated to estimate the relative abundance of the metabolites in the plasma.

Identification of Mirabegron, M5, M8, M9, M11, M12, M13, M14, M15 and M16 using LC-MS

Preparation of urine and plasma samples for LC-MS analysis

Urine samples collected for pre-dose, 0–6 and 6–12 h were pooled for each period. Plasma samples collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h were pooled. The pooled urine (4 mL) and plasma samples (16 mL) were diluted with a 2-fold volume of 100 mmol/L ammonium acetate/formic acid (100:1, v/v), and then loaded onto a solid-phase extraction cartridges, Oasis[®] HLB. The cartridges were then washed with water and eluted with a formic acid/methanol (0.1:100, v/v). The eluates were evaporated to dryness under reduced pressure, and the residues were reconstituted with 50% (v/v) methanol/water to prepare analytical samples.

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Comparison of metabolites in urine and plasma samples with authentic standards using LC-MS

Metabolites in urine and plasma samples were identified by comparing the retention time, molecular ion and fragment ion peaks with the authentic standards achieved by separation on LC with ion trap-MS. LC-MS conditions were as follows: mass spectrometer: LCQ^{Deca} XP Plus (Thermo Fisher Scientific, Waltham, MA, USA); atmospheric pressure ionization interface: electrospray ionization (ESI); acquisition: full scans in both positive and negative ion modes; column: Capcell Pak C₁₈ UG120 (3.0 × 250 mm, 5 μm); column temperature: 40°C; mobile phase and gradient conditions were identical with the HPLC analysis of metabolites in samples; flow rate: 0.42 mL/min; spray voltage: 5 kV; capillary voltage: 15 V; sheath gas (N₂): 80 units; auxiliary gas (N₂): 0 units; capillary temperature: 275°C; scan range: *m/z* 150–1000. Product ion scans including the target parent ion and scan range (between brackets), were set at *m/z* 299 (from *m/z* 80 to 309) for M5, at *m/z* 292 (from *m/z* 80 to 302) for M8, at *m/z* 194 (from *m/z* 50 to 204) for M9, at *m/z* 573 (from *m/z* 155 to 583) for M11 and M14, at *m/z* 615 (from *m/z* 165 to 625) for M12, at *m/z* 617 (from *m/z* 165 to 627) for M13, at *m/z* 589 (from *m/z* 160 to 599) for M15, and at *m/z* 257 (from *m/z* 70 to 267) for M16. Normalized collision energy for each product ion scan was set at 30%.

Structural Characterization of M17 in Urine Samples using LC-MS and NMR

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Characterization of unidentified metabolite M17 in urine samples

An unidentified radioactive peak, M17, was observed at a retention time of 44.7–45.9 min in urine samples collected between 0 and 6 h after oral administration. A comparison of LC-MS analysis between post- and pre-dose urine samples was conducted and molecular weight of M17 was found to be 448. Product ion scans of the presumed molecular ion were also performed to characterize the metabolite.

Isolation and purification of M17 from urine samples

M17 was isolated and purified from pooled human urine (approx. 1 L) collected between 0 and 12 h after oral administration of non-labeled mirabegron at doses of 60 to 200 mg q.d. in other clinical studies. The pooled urine was loaded on an absorbent, LC-SORB SP-B-ODS (200 g, Chemco Scientific Co., Ltd., Osaka, Japan). The absorbent was washed with water and methanol/water/formic acid (1:9:0.01, v/v/v) and then eluted with a mixture of methanol containing 0.1% formic acid. The eluate was concentrated under reduced pressure, and subsequently applied to low pressure column chromatography as follows: a glass column, 20 × 300 mm, packed with Wakosil 40C18 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), flow rate: 10 mL/min, mobile phases: (A) 100 mmol/L ammonium acetate/water/methanol/formic acid (1:8:1:0.01, v/v/v/v), (B) 100 mmol/L ammonium acetate/water/methanol/formic acid (1:1:8:0.01, v/v/v/v), solvent gradient program: 0 min (B: 0%) to 100 min (B: 100%) in linear

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mode, column temperature: ambient. The M17 fraction was concentrated under reduced pressure and re-chromatographed using the same Wakosil column using the following conditions: flow rate: 10 mL/min, mobile phases: (A) 100 mmol/L ammonium acetate/water/methanol (1:8:1, v/v/v), (B) 100 mmol/L ammonium acetate/water/methanol/formic acid (1:1:8, v/v/v), solvent gradient program: 0 min (B: 0%) to 70 min (B: 70%), column temperature: ambient. M17 was obtained from a fraction containing M17 by successive three-step preparations using a Shimadzu HPLC 10A system (Shimadzu Co., Kyoto, Japan) with three different reversed phase columns. As a result, less than 0.01 mg of purified M17 was yielded from human urine.

Structural elucidation of M17

Purified M17 was dissolved in methanol- d_4 containing tetramethylsilane (TMS) as an internal reference. ^1H NMR spectroscopic data of M17 were recorded on the Varian Inova 600 MHz spectrometer (Agilent Technologies, Palo Alto, CA, USA) at 25 °C. Chemical shift values were reported on the δ scale (ppm) downfield from TMS signal set at 0 ppm. Structural elucidation of M17 by NMR was based on the data of ^1H NMR spectrum, total correlated spectroscopy (TOCSY) and rotational nuclear Overhauser effect spectroscopy (ROESY).

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Results

Safety Assessment

A single oral dose of 160 mg [^{14}C]mirabegron was well tolerated in the four subjects tested, with a single event of somnolence and headache reported as treatment-related adverse events. There were no clinically important changes in clinical laboratory values, vital signs, ECG parameters and physical examination data during the study.

Pharmacokinetics of Unchanged Mirabegron and Total Radioactivity

Time profiles of the concentrations of radioactivity in the whole blood and plasma as well as unchanged mirabegron in the plasma after a single oral dose of 160 mg of [^{14}C]mirabegron are illustrated in Fig. 2. The time profiles of mirabegron for all four individual subjects had two peaks, the first at 0.5 or 1 h and the second at 2 or 4 h. The key pharmacokinetic parameters are summarized in Table 1. AUC_{inf} of mirabegron accounted for 22% of that of total radioactivity in plasma. The whole-blood-to-plasma ratio of radioactivity increased from the range of 0.8 to 1.0 shortly after dosing (0.5 to 6 h after dosing) to approximately 2 after 36 h. The whole-blood-to-plasma ratio of radioactivity for C_{max} and AUC_{inf} was 0.88 and 1.4, respectively.

Excretion and Recovery of Unchanged Mirabegron and Total Radioactivity

The mean cumulative excretion in urine by 96 h post-dose was 48.7% of the dose

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administered; in feces this was 29.3% (Fig. 3). The excretion gradually continued afterwards, and the mean cumulative excretion of radioactivity by 408 h after dosing was 55.0% in urine, 34.2% in feces and 89.2% in total (Table 2). For one subject, urine and fecal samples were collected until 18 days (432 h) after administration because excretion of radioactivity in urine and feces continued. The major route of excretion of radioactivity was via the urine. No radioactivity was detected in expired air. The mean total amount of unchanged mirabegron excreted in urine accounted for 45% of the excreted radioactivity and for 25% of the administered dose, while the remainder of the radioactivity excreted in urine represented one or more metabolites of mirabegron.

Quantitative Metabolite Profiles and Identification of Metabolites in Urine

Representative radiochromatograms of urine are shown in Fig. 4. Ten peaks were present in these chromatograms (Table 3). Assignment of the radioactive peaks to mirabegron and its metabolites were done by comparison of retention times and mass spectra including product ion scans with ten authentic reference compounds (Table 4). The peak at 74.4–74.5 min was assigned to unchanged mirabegron because the retention time and the product ion spectra corresponded with those of mirabegron in subsequently conducted identification experiments (Fig. 5; Table 4). Similarly, the metabolite peaks at 5.8–6.5, 13.1–14.9, 60.2–62.8, 66.8–67.4, 70.2–70.3 and 72.8–73.0 min were identified as M9 (YM-340790), M8 (YM-538853), M11 (YM-382984), M15

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(YM-9636324), M16 (YM-208876) and M5 (YM-538852), respectively (Fig. 6; Table 4). The peak at 57.4–61.8 min corresponded to a mixture of metabolites M12 (YM-538858) and M13 (YM-538859). A structural isomer of metabolite M11 was found between the mixture peak of M12/M13 and the M11 peak on the chromatogram, and was called M14 (YM-554028). M14 could not be specifically assigned to either of the mixture peak of M12/M13 or the M11 peak because they were so close. The peak at 3.1–3.2 min could not be identified. The structure of the metabolite with a peak at 44.7–45.9 min was newly elucidated using LC-MS and NMR, and was named M17. The urinary excretion of radioactivity for each peak fraction detected is listed in Table 3. Between 0 and 48 h post-dose, the mean urinary excretion of unidentified metabolite at 3.1–3.2 min, M9, M8, M17, a mixture of M12/M13 (and M14), M11 (and M14), M15, M16, M5 and unchanged [¹⁴C]mirabegron amounted to 1.1%, 0.6%, 1.3%, 2.0%, 1.4%, 3.2%, 0.6%, 1.7%, 2.9% and 18.4% of the dose, respectively. Mirabegron represented the largest component.

Metabolite Profiles in Feces

In the radiochromatograms of extracts of fecal homogenates, the peak corresponding to unchanged mirabegron was detected (Fig. 7). No clear metabolite peaks were detected in the feces samples, suggesting that almost all radioactivity was unchanged mirabegron.

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Metabolite Profiles and Identification of Metabolites in Plasma

Radiochromatograms of plasma extracts are shown in Fig. 8. Eight peaks were present in these chromatograms. The peak at 74.5 min was assigned to unchanged mirabegron. The peak at 3.0 min could not be identified. Peaks at 15.5, 62.0–63.0, 66.5, 70.0 and 73.0 min were identified as metabolites M8, M11, M15, M16 and M5, respectively. As seen in the urine, the peak at 59.5–60.5 min corresponded to a mixture of M12 and M13, and M14 was also present between the mixture peak of M12/M13 and the M11 peak. The ratio of radioactivity for each peak fraction detected in plasma is listed in Table 3. The ratio of unidentified metabolite at 3.0 min, M8, a mixture of M12/M13 (and M14), M11 (and M14), M15, M16, M5 and unchanged mirabegron to the total profiled radioactivity (% of profiled radioactivity) was 2.4%, 2.2%, 14.0%, 13.3%, 6.9%, 3.3%, 3.7% and 46.4%, respectively, at 2 h, which was the t_{\max} of plasma radioactivity, and 3.1%, ND (not detected, below detection limit), 13.4%, 11.2%, ND, 7.6%, 13.0% and 31.0%, respectively, at 12 h post-dose. Mirabegron represented the largest component at all time points. As for metabolites, a mixture of M12 and M13 (and M14), and M11 (and M14) each might account for more than 10% of the total profiled radioactivity. The ratio of M5 and M16 increased as time passed after administration.

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Structure Elucidation of M17

Purified M17 showed a protonated molecule $[M+H]^+$ of m/z 449, and its product ions were observed at m/z 431 $[M+H-18]^+$, 273 $[M+H-176]^+$, and 255 $[M+H-194]^+$, suggesting that M17 in urine was an *O*-glucuronide of M16 (MW254) (Fig. 9). M17 was further characterized by NMR (Table 5). Proton signals of M17 were elucidated by analysis of 1H - 1H relayed correlations obtained by TOCSY experiment. As a result, partial structures, M16 and *O*-glucuronosyl moieties, were confirmed. Connection of glucuronosyl moiety to M16 via an oxygen was elucidated by ROESY. ROE correlations were observed from 10-H to 8-H, 7-H and 1'-H, respectively (Fig. 9). In addition, 14-H in 4-aminobenzene ring was also correlated to 8-H in ROESY, indicating that 11-H of M16 had undergone replacement with an oxygen. Glucuronidation occurred at the additional oxygen of M16.

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Discussion

In the present study, the absorption and elimination kinetics and metabolite profiles of mirabegron were investigated in four healthy male subjects after a single oral administration of 160 mg of [¹⁴C]mirabegron as a solution. Metabolites found in urine and plasma were identified by LC-MS/MS and NMR analyses.

[¹⁴C]Mirabegron was rapidly absorbed with a plasma t_{\max} for mirabegron and total radioactivity of 1.0 and 2.3 h post-dose, respectively. These findings are similar to the results in the preclinical ADME studies in rats and monkeys (unpublished observations), in which the total radioactivity in the plasma peaked within 3.0 h after oral administration of [¹⁴C]mirabegron as a solution. Furthermore, 55.0% of the administered dose of radioactivity was excreted in the urine, showing that at least a 55.0% dose of mirabegron was absorbed from the gastrointestinal tract. All individual concentration–time profiles of mirabegron in plasma showed distinct peaks at approximately 0.5 to 1 h and 2 to 4 h after administration. Individual plasma radioactivity concentration-time profiles also generated double peaks, but they were plateau-like and not distinct. A similar double-peak phenomenon in the plasma mirabegron concentration–time profiles was seen in rats, showing the first peak at 0.25 h and the second peak at 3.0 h after administration (unpublished observations). For humans, the first peak tended to be the highest, whereas for rats the second peak was the larger peak. Several structurally diverse drugs with adequate lipid solubility, such

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as celiprolol, pafenolol, acebutolol, cimetidine, danazol, veralipride and talinolol, generate double or multiple peaks or even plateau-like plasma concentration–time profiles (Voinchet et al., 1981; Plusquellec et al., 1987; Lin, 1991; Charman et al., 1993; Lennernäs and Regardh, 1993; Lipka et al., 1995; Mostafavi and Foster, 2003; Weitschies et al., 2005). The following mechanisms can cause erratic absorption: enterohepatic circulation, fractionated gastric emptying, and separated “absorption windows” along the intestinal tract (Roberts et al., 2002; Oberle and Amidon, 1987; Gramatté et al., 1994). However, enterohepatic recycling is not likely associated with mirabegron absorption, as there were no fluctuations in the plasma mirabegron concentration–time profiles after intravenous administration (unpublished observations). Also, extended release formulations of mirabegron generally do not show double peaks but only a single peak with a t_{\max} window of 2 to 4 h after administration in the plasma mirabegron concentration-time profiles (unpublished observations). Therefore, two separated “absorption windows” along the small intestine, but not fractionated gastric emptying, are hypothesized to cause this irregular absorption profile; in particular, low absorption from the jejunum, compared with the absorption from the duodenum and ileum. To elucidate these hypotheses of possible absorption mechanisms of mirabegron, additional investigations will be necessary. After the rapid increase in mirabegron and radioactivity plasma concentrations, an initial steep decline was observed, followed by a much slower terminal elimination

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phase with a $t_{1/2}$ of 47.9 and 28.2 h for mirabegron and radioactivity, respectively (Fig. 2, Table 1). Plasma concentrations could be measured up until 144 and 36 h post-dose for mirabegron and radioactivity, respectively. The difference in $t_{1/2}$ for mirabegron and radioactivity can be explained by the difference in the time interval over which they could be measured. The estimate of terminal $t_{1/2}$ of mirabegron and radioactivity was as long as 72.9 and 84.5 h when based on urinary excretion data, which could be both measured up to 396 h (384–408 h interval) post-dose. On the metabolite profiling, the largest component of radioactivity in plasma and urine was unchanged mirabegron at all time points. The ratio of M5 and M16 to the total radioactivity appeared to increase with time and, therefore, they might contribute to the somewhat longer $t_{1/2}$ of radioactivity.

Approximately 55.0% and 34.2% of the administered dose of [^{14}C]mirabegron were excreted via urine and feces, respectively, with a total recovery of radioactivity of 89.2% of the administered dose. In the preclinical ADME studies, urinary and fecal recoveries of orally administered [^{14}C]mirabegron were 18.8% and 75.3% in rats and 46.8% and 54.2% in monkeys, respectively (unpublished observations), suggesting that orally administered mirabegron was almost completely excreted via urine and feces, despite differences in the main excretion route among the species. In feces of humans, the radioactivity was recovered almost entirely as the unchanged form. In addition to unabsorbed mirabegron, part of the 34.2% excreted unchanged in feces of humans is

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likely to represent direct biliary excretion of mirabegron. Studies in bile duct-cannulated rats suggest that unchanged mirabegron is directly excreted in rat bile (unpublished observations). Some mirabegron recovered in the feces may also have been generated from deconjugation of glucuronide metabolites of mirabegron in the intestine. Of the administered dose, 25% was excreted as unchanged mirabegron in urine, indicating that urinary excretion of unchanged form is one of the major elimination pathways of mirabegron in humans. No excretion of radioactivity was shown in expired air. Together, the results from the present study suggest that the elimination of mirabegron is through renal and possibly biliary excretion of unchanged drug and metabolism.

After oral administration to humans, mirabegron underwent different metabolic transformations, including amide hydrolysis (M5, M16 and M17), *O*-glucuronic acid conjugation (M11), *N*-glucuronic acid conjugation (M14), carbamoyl glucuronic acid conjugation (M12 and M13), oxidation or *N*-dealkylation of the secondary amine (M8, M9 and M15) and oxidation of the hydroxyl group to carbonyl group (M12) (Fig. 6), indicating the involvement of at least three kinds of drug metabolizing enzymes: esterases, UDP-glucuronosyltransferases and some oxidation enzymes, presumably cytochrome P450, in the first metabolic reaction of mirabegron. A significant percentage (approximately 75%) of the radioactivity recovered in urine was characterized by mirabegron and these ten metabolites in the radiochromatogram. The

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remaining radioactivity (about 25%) excreted in urine probably corresponds to multiple other metabolites (as indicated by multiple small peaks in the urine radiochromatograms), each of which accounts for a trace levels of drug-related substances in urine. On the basis of the metabolites found in the urine, major primary metabolic reactions of mirabegron in humans were estimated to be amide hydrolysis (M5, M16 and M17), accounting for 48% of the identified metabolites, followed by glucuronidation (M11, M12, M13 and M14) and *N*-dealkylation or oxidation of the secondary amine (M8, M9 and M15), accounting for 34% and 18% of the identified metabolites, respectively (Fig. 4; Table 3). In plasma, eight of the metabolites characterized in urine were also observed. The ratio of a mixture of M12/M13 (and M14) and M11 (and M14) to the total profiled radioactivity (% of profiled radioactivity) accounted for approximately 10% or more at all time points and the other metabolites (M5, M8, M15 and M16) seemed to be less. Therefore, direct glucuronic acid conjugates (M11, M12, M13 and/or M14) seemed to be abundant among metabolites in plasma. As in the urine, considerable amounts of multiple other metabolites, each of which accounts for a trace level of drug-related substances, seemed to exist in plasma, as shown as others in Table 3.

Poor extraction recoveries of radioactivity from plasma, especially at the later sampling times, were observed in this study. Low extraction recoveries from 8 and 12-h plasma samples were considered partly due to low radioactivity levels in these

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samples as compared to 2 and 4-h plasma samples. A small portion of the samples after extraction was used for the evaluation of extraction recovery.

In conclusion, the present study clarified the absorption and elimination kinetics of mirabegron and the characteristics of metabolites in the excreta and plasma in four healthy male subjects after a single oral administration of 160 mg of [¹⁴C]mirabegron as a solution. The results indicate that mirabegron is rapidly absorbed after oral administration, and circulates in the plasma as the unchanged form, its glucuronic acid conjugates and other metabolites. Of the administered dose, 55% is excreted in urine, mainly as the unchanged form, and 34% is recovered in feces, almost entirely as the unchanged form. Among metabolites, hydrolyzed metabolites were most abundant in urine. Mirabegron is cleared by multiple mechanisms (renal and possibly biliary excretion and metabolism) and drug-metabolizing enzymes, with no single predominating clearance pathway. The present study indicates that mirabegron is metabolized to at least 10 metabolites by multiple enzymes. Therefore, co-administered drugs that have the potential to inhibit/induce a specific enzyme or a transporter are expected to have a low propensity to affect the pharmacokinetics of mirabegron.

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Authorship Contributions

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Footnotes

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Legends for figures

Fig. 1

Chemical structures of [¹⁴C]mirabegron (A) and the IS for determination of unchanged mirabegron in plasma and urine (B). *Position uniformly labeled with ¹⁴C.

Fig. 2

(A) Individual concentration–time profiles of the unchanged mirabegron in plasma (linear scale) and (B) mean concentration–time profiles of radioactivity in plasma and blood, and those of the unchanged mirabegron in plasma after a single oral administration of 160 mg of [¹⁴C]mirabegron to healthy volunteers (semilogarithmic scale). For (B), each point represents the mean ± SD of four subjects.

Fig. 3

Urinary and fecal recovery of total radioactivity after a single oral administration of 160 mg of [¹⁴C]mirabegron to healthy volunteers. Each point represents the mean ± SD of four subjects.

Fig. 4

Representative radiochromatograms of urine collected for 0–6 h (A), 6–12 h (B), 12–24 h (C) and 24–48 h (D) after a single oral 160-mg administration of

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[¹⁴C]mirabegron to healthy volunteers.

Fig. 5

Mass chromatograms at m/z 397 of total ion scans of authentic reference compound of mirabegron (A), representative urine sample (B), and pooled plasma sample (C), and product ion spectra at m/z 397 at the 78-min peak in authentic reference compound of mirabegron (D), representative urine sample (E), and pooled plasma sample (F): electrospray ionization, positive ion mode, collision energy set at 30%, and single-stage mass separation under basic LC conditions.

Fig. 6

Postulated metabolic pathways of mirabegron in humans.

Fig. 7

Representative radiochromatograms of fecal extracts collected for 0–24 h (A), 24–48 h (B), 48–72 h (C) and 72–96 h (D) after a single oral 160-mg administration of [¹⁴C]mirabegron to healthy volunteers.

Fig. 8

Radiochromatograms of plasma extracts collected at 2 h (A), 4 h (B), 8 h (C) and 12 h (D) after a single oral 160-mg administration of [¹⁴C]mirabegron to healthy volunteers.

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Fig. 9

Mass spectrometric characterization and key ROE correlations of mirabegron
metabolite M17 purified from human urine.

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Table 1 Pharmacokinetic parameters of mirabegron in plasma and urine and for radioactivity in plasma, blood and urine after a single oral administration of 160 mg of [¹⁴C]mirabegron to healthy volunteers.

Parameter	Mirabegron		Radioactivity		
	Plasma	Urine	Plasma	Blood	Urine
t _{max} (h)	1.00 ± 0.71	-	2.25 ± 1.44	2.13 ± 1.44	-
C _{max} (ng/mL)	371 ± 96	-	879 ± 279 ^a	777 ± 211 ^a	-
AUC _{inf} (ng·h/mL)	2285 ± 250	-	10443 ± 2328 ^a	13896 ± 2979 ^a	-
t _{1/2} (h)	47.9 ± 8.1	72.9 ± 13.0	28.2 ± 5.4	30.5 ± 4.0	84.5 ± 11.6
CL/F (L/h)	70.7 ± 7.63	-	-	-	-
V _Z /F (L)	4824 ± 501	-	-	-	-
CL _R (L/h)	17.7 ± 2.14	-	-	-	-

Values of PK parameters are mean ± SD, n = 4.

^a Radioactivity data were transformed into mirabegron equivalent concentrations (ng eq./mL) by multiplying with the specific activity of [¹⁴C]mirabegron.

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Table 2 Cumulative excretion of mirabegron in urine and that of radioactivity in urine, feces and expired air after a single oral administration of 160 mg of [¹⁴C]mirabegron to four healthy male subjects.

	Mirabegron	Radioactivity			
	Urine	Urine	Feces	Expired air	Total
Time period (h)	0 to 408	0 to 408	0 to 408	0 to 96	0 to 408
Amount excreted (% of dose)	25.0 ± 0.8	55.0 ± 2.7	34.2 ± 2.3	ND	89.2 ± 2.7

ND, not detected (below the detection limit).

Values are mean ± SD, n = 4.

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Table 3 Compositions of mirabegron and its metabolites in urine and plasma after a single oral administration of 160 mg of [¹⁴C]mirabegron to four healthy male subjects. Components are listed in the order of elution.

Metabolites	% of dose		% profiled radioactivity			
	Urine ^a	Plasma ^b				
	0–48 h	2 h	4 h	8 h	12 h	
Unidentified	1.1 ± 0.2	2.4	1.7	1.8	3.1	
M9	0.6 ± 0.1	ND	ND	ND	ND	
M8	1.3 ± 0.3	2.2	1.4	ND	ND	
M17	2.0 ± 0.6	ND	ND	ND	ND	
M12, M13 (and M14 ^c)	1.4 ± 0.3	14.0	15.0	13.8	13.4	
M11 (and M14 ^c)	3.2 ± 0.6	13.3	14.2	13.8	11.2	
M15	0.6 ± 0.1	6.9	5.9	3.5	ND	
M16	1.7 ± 0.5	3.3	4.3	6.2	7.6	
M5	2.9 ± 0.8	3.7	4.9	9.4	13.0	
Mirabegron	18.4 ± 1.6	46.4	30.9	28.6	31.0	
Others	9.7 ± 0.9	7.8	21.7	22.9	20.7	
Total	43.0 ± 3.3	100	100	100	100	

ND, not detected (below the detection limit).

^a Values are mean ± SD, n = 4.

^b Values were obtained using pooled samples of 4 subjects.

^c Metabolite M14 was found as the structural isomer of metabolite M11 between the mixture peak of M12/M13 and the M11 peak.

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Table 4 Mass spectral data on mirabegron and its metabolites in urine and plasma. Data are from LC-MS runs of prepared mirabegron and metabolites. Components are listed in the order of elution. Proposed chemical structures are shown in Fig. 6: electrospray ionization, positive ion mode, collision energy set at 30%, and single-stage mass separation under basic LC conditions. Some of the expected product ions may be missing because of low intensities and/or high background of coeluting endogenous components.

Component	Matrix	Parent ion	Product ions			Authentic reference compounds
			[M+H] ⁺ - H ₂ O ⁺	[M+H] ⁺ - 2H ₂ O ⁺	Other characteristic fragment ions	
		<i>m/z</i>	<i>m/z</i>	<i>m/z</i>	<i>m/z</i>	
M9	Urine	194	ND	ND	148	YM-340790
M8	Urine, Plasma	292	274	ND	178, 159 , 141, 113, 106	YM-538853
M17	Urine	449	431	413	312, 273^b , 255, 238, 136	NA
M13	Urine, Plasma	617	ND	ND	441 , 397 ^b	YM-538859
M12	Urine, Plasma	615	ND	ND	439, 395^b	YM-538858
M14	Urine, Plasma	573	555	537	421, 379, 277	YM-554028
M11	Urine, Plasma	573	555	537	493, 475, 397 ^b , 379 , 260	YM-382984
M15	Urine, Plasma	589	571	ND	395 , 379, 260	YM-9636324
M16	Urine, Plasma	257	239	ND	120, 103	YM-208766
M5	Urine, Plasma	299	281	ND	ND	YM-538852
Mirabegron	Urine, Plasma	397	379	ND	260	Mirabegron

ND, not detected.

NA, not available.

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Product ion in boldface; base peak in product ion spectrum.

^a Measured exact mass in agreement with proposed structure.

^b Aglycone of glucuronide.

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Table 5 ¹H NMR chemical shifts of mirabegron metabolite M17 purified from human urine.

Nuclei Number*	$\delta^1\text{H}$ (integral, multiplicity, coupling constant)
1	7.30 (1H, dd, $J=7.2, 7.2$ Hz)
2	7.37 (2H, dd, $J=7.5, 7.5$ Hz)
3	7.40 (2H, dd, $J=7.2, 7.2$ Hz)
5	4.94 (1H, dd, $J=3.3, 10.5$ Hz)
6	3.07 (1H, dd, $J=10.8, 12.6$ Hz)
	3.15 (1H, dd, $J=3.6, 12.6$ Hz)
7	2.89 (2H, m)
8	2.89 (2H, m)
10	7.14 (1H, br.d, $J=1.4$ Hz)
13	6.74 (1H, d, $J=7.9$ Hz)
14	6.77 (1H, dd, $J=1.5, 7.9$ Hz)
1'	4.71 (1H, d, $J=7.2$ Hz)
2'	3.52 (1H, m)
3'	3.48 (1H, m)
4'	3.55 (1H, m)
5'	3.64 (1H, d, $J=9.6$ Hz)

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*: tentatively assigned (see Fig. 9), d: doublet, dd: double doublet, m: multiplet, br.: broad.

Fig. 1

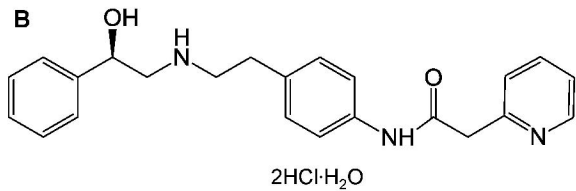
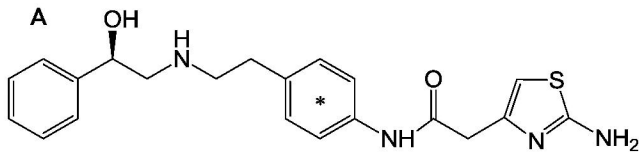


Fig. 2

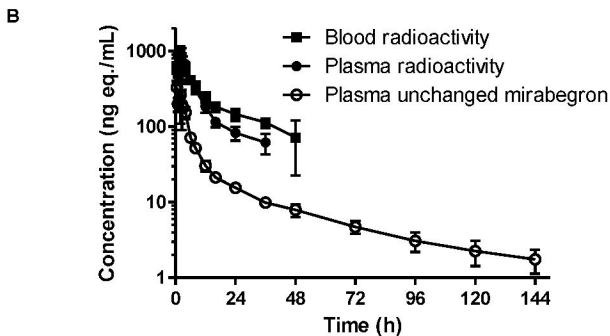
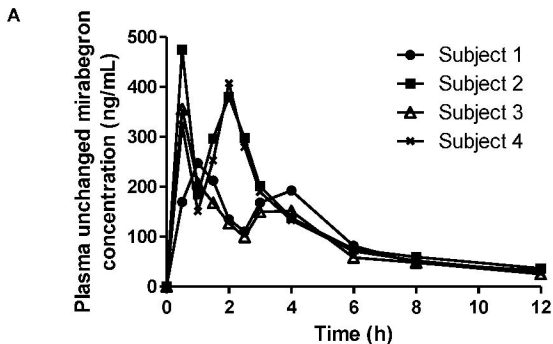


Fig. 3

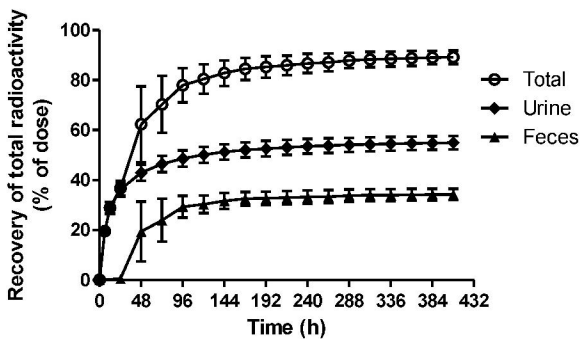


Fig. 4

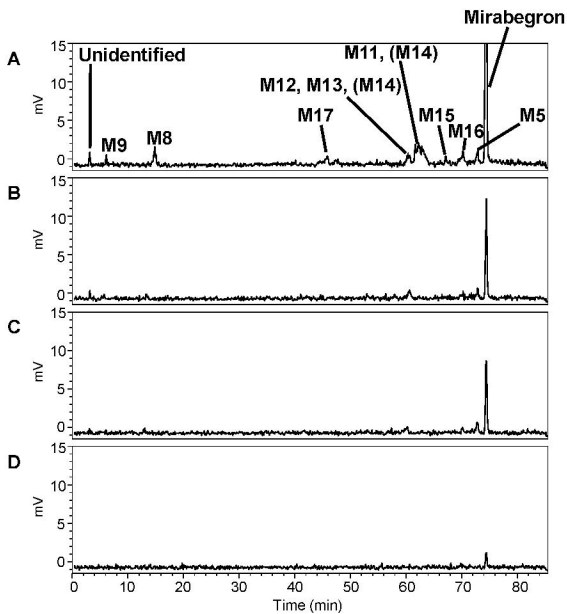


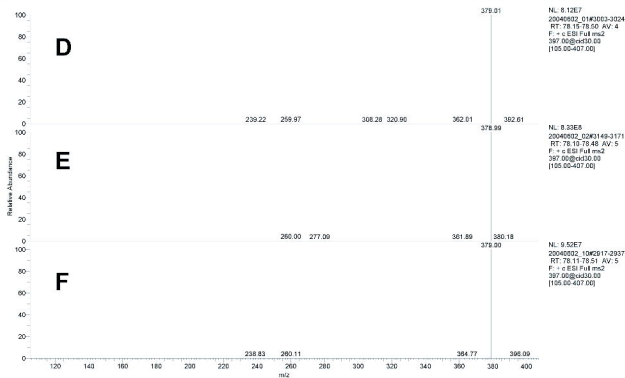
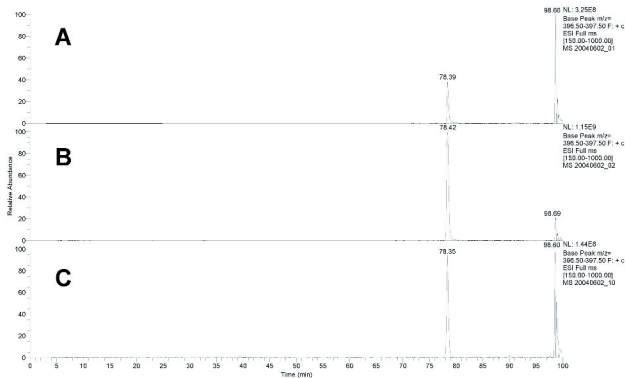
Fig. 5

Fig. 6

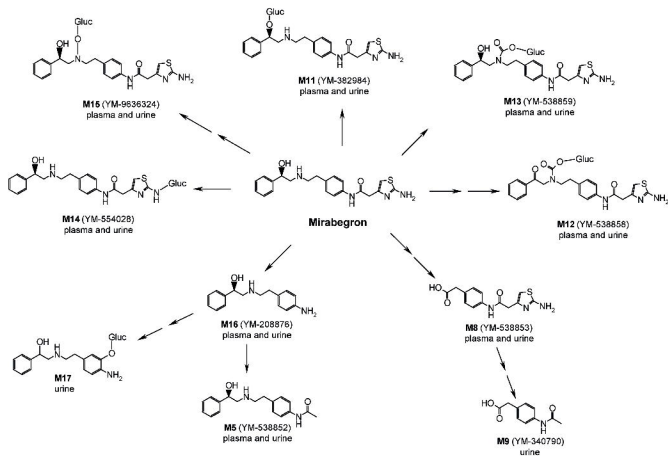


Fig. 7

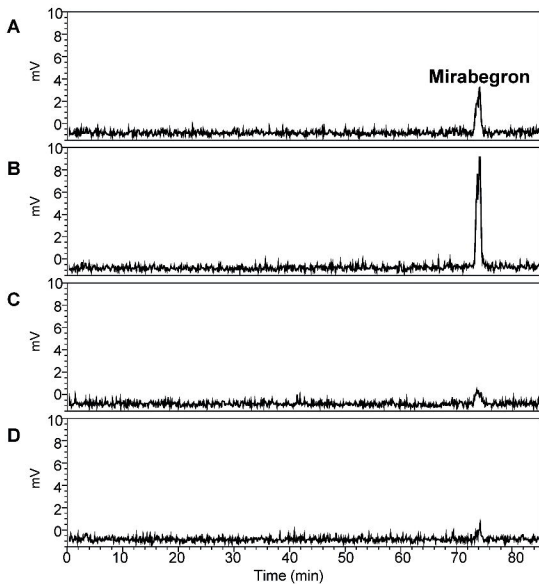


Fig. 8

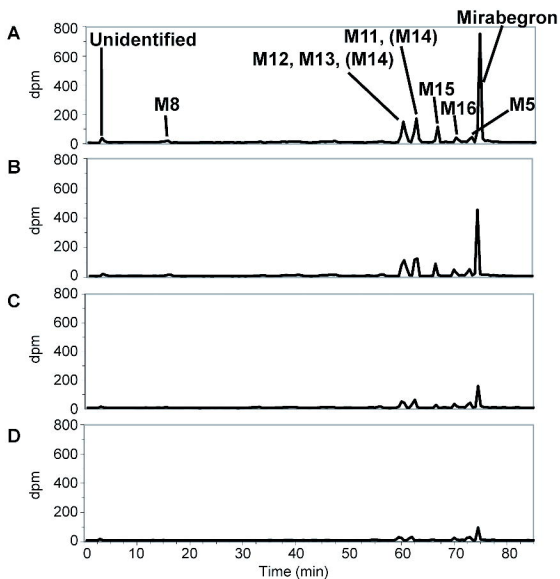


Fig. 9

