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Development and validation of LC–MS/MS methods for the determination of mirabegron and its metabolites in human plasma and their application to a clinical pharmacokinetic study

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ABSTRACT

Mirabegron is being developed for the treatment of overactive bladder. To support the development of mirabegron, including pharmacokinetic studies, liquid chromatography/tandem mass spectrometry methods for mirabegron and eight metabolites (M5, M8, M11–M16) were developed and validated for heparinized human plasma containing sodium fluoride. Four separate bioanalytical methods were developed for the analysis of: (1) mirabegron; (2) M5 and M16; (3) M8; and (4) M11–M15. Either solid-phase extraction or liquid–liquid extraction was used to extract the analytes of interest from matrix constituents. For mirabegron, an Inertsil C_8-3 analytical column was used and detection was performed using a triple-quad mass spectrometer equipped with an atmospheric pressure chemical ionization interface. For the metabolite assays, chromatographic separation was performed through a Phenomenex Synergi Fusion-RP C_{18} analytical column and detection was performed using a triple-quad mass spectrometer equipped with a Heated Electrospray Ionization interface. The validation results demonstrated that the developed liquid chromatography/tandem mass spectrometry methods were precise, accurate, and selective for the determination of mirabegron and its metabolites in human plasma. All methods were successfully applied in evaluating the pharmacokinetic parameters of mirabegron and metabolites in human plasma.

1. Introduction

Mirabegron [2-(2-amino-1,3-thiazol-4-yl)-*N*-[4-(2-{[(2*R*)-2-hydroxy-2-phenylethyl]amino}ethyl)phenyl]acetamide] is a potent and selective human β_3-adrenoceptor agonist and is the first of a new class of compounds under development for the treatment of overactive bladder [1]. Overactive bladder has been defined by the International Continence Society as "urgency, with or without urge incontinence, and usually with increased daytime frequency and nocturia, in the absence of local or metabolic factors" [2]. Mirabegron has a novel mechanism of action compared with the available therapeutic products for overactive bladder. β_3-Adrenoceptors have been shown to play a role in the relaxation of the urinary bladder detrusor smooth muscle [3]. Mirabegron activates β_3-adrenoceptors on the detrusor muscle of the bladder to facilitate filling of the bladder and urine storage [4]. Mirabegron does not directly inhibit voiding bladder contractions, and may therefore represent a promising choice for the treatment of overactive bladder with or without lower urinary tract symptoms such as those seen with benign prostatic hypertrophy [1].

The pharmacokinetic properties of mirabegron have been characterized in several clinical studies (unpublished data). Human plasma metabolites of mirabegron (M5, M8, M11–M16) were identified during a mass balance study using radiolabeled mirabegron (Fig. 1). The major metabolic routes of mirabegron in humans were

Abbreviations: APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; HESI, Heated Electrospray Ionization; HQC, high quality control; LC, liquid chromatography; LLE, liquid–liquid extraction; LLOQ, lower limit of quantitation; LQC, low quality control; MCX, mixed-mode cation exchange; MQC, medium quality control; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NaF, sodium fluoride; QC, quality control; RSD, relative standard deviation; SPE, solid-phase extraction; TBME, *t*-butylmethyl ether; ULOQ, upper limit of quantitation.

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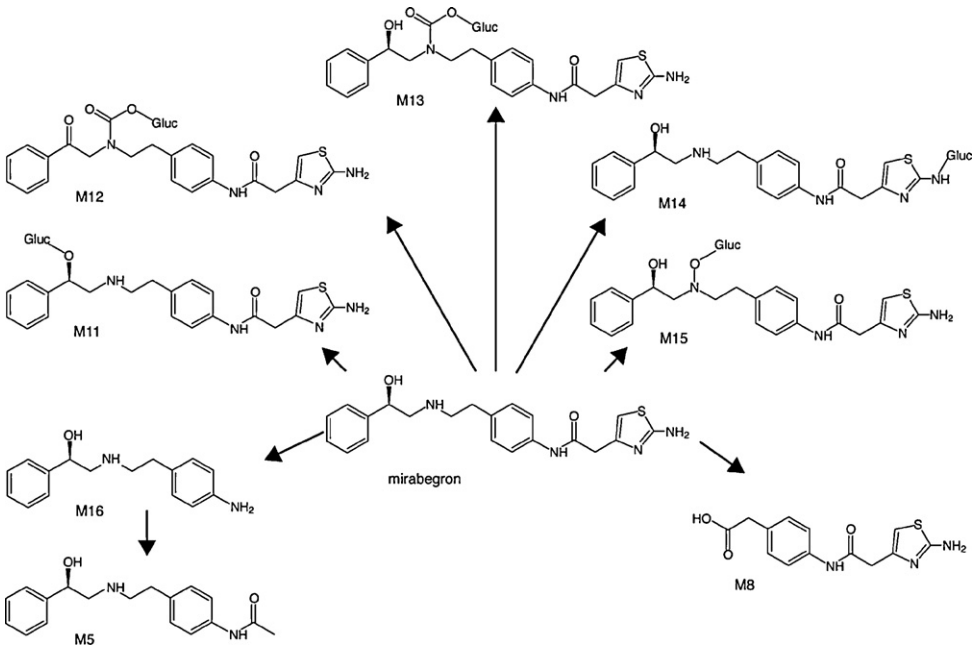

Fig. 1. Chemical structures of mirabegron and its metabolites. Gluc, glucuronide.

predicted to be amide hydrolysis by endogenous esterases (M5 and M16), glucuronidation (M11–M14), and *N*-dealkylation or oxidation of the secondary amine (M8 and M15) [5].

New guidance for drug safety metabolite testing was recently issued by the US Food and Drug Administration and the International Conference on Harmonisation [6,7]. Generally, metabolites identified only in humans or present at disproportionately higher levels in humans than in animal test species should be considered for safety assessment. Human metabolites that might raise safety concerns are those formed at greater than 10% of the total systemic exposure of drug-related materials at steady state [7]. This guidance and the fact the above-mentioned mass balance study did not provide steady-state metabolite data required the development of quantitative assays for mirabegron metabolites. For a quantitative determination of mirabegron and its metabolites in human plasma, bioanalytical methods were developed to support the analysis of clinical study samples. In the present study, four separate bioanalytical assays were developed for the analysis of: (1) mirabegron; (2) M5 and M16; (3) M8; and (4) M11–M15. All methods were fully validated according to criteria reported in internationally accepted recommendations for bioanalysis by evaluating linearity, precision, accuracy, selectivity, recovery, and stability [8,9]. The methods were successfully used to assess the pharmacokinetics of mirabegron and its metabolites in healthy volunteers and patients with overactive bladder.

2. Materials and methods

2.1. Materials

2.1.1. Reference standards and chemicals

Mirabegron, its metabolites (YM-538852 (M5) hydrochloride, YM-538853 (M8) trifluoroacetate, YM-382984 (M11), YM-538858 (M12), YM-538859 (M13), YM-554028 (M14) formate, YM-9636324 (M15), and YM-208876 (M16) hydrochloride), and the internal standards (mirabegron-d5 (YM-9661388), M5-d5 (YM-9727590), IS-M8 (YM-9718763), IS-M11-M15 (YM-9674146), and M16-d5 (YM-9661658)) were supplied by Astellas Pharma Inc. (Tokyo, Japan). Acetonitrile, methanol, formic acid, ammonium acetate, ammonia solution, diethyl-ether, *t*-butylmethyl ether (TBME), isopropyl alcohol, (ortho-) phosphoric acid and dimethylsulfoxide were HPLC grade and purchased from Merck (Darmstadt, Germany). Ultra-pure water from a Milli-Q system (Millipore, Bedford, MA, USA) and ULC/MS water (Biosolve BV, Valkenswaard, The Netherlands) were used. The chemical structures of mirabegron and its metabolites are shown in Fig. 1. The internal standards used for the analyses of mirabegron and its metabolites are shown in Fig. 2.

2.2. Instrumentation

The mirabegron assay was developed and validated using an Acquity ultra-performance liquid chromatography system (Waters, Milford, MA, USA). The samples were detected using an API 4000 Q-Trap mass spectrometer (AB Sciex, Toronto, Canada) equipped with an atmospheric pressure chemical ionization (APCI) interface using positive ion mode. The chromatographic data system used was Analyst version 1.4.2 (AB Sciex). All of the metabolite methods were developed and validated on a Surveyor Plus HPLC system (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer used was a TSQ (Triple Stage Quadrupole) Quantum Ultra equipped with a Heated Electrospray Ionization (HESI) interface (Thermo Fisher Scientific). The chromatographic data systems used were Xcalibur™ version 2.0.7 and LCquan version 2.5.6 (Thermo Fisher Scientific).

2.3. Method conditions

2.3.1. Stock solutions

Stock solutions containing 1.0 mg/mL of the reference compound were prepared in 50% acetonitrile (M5, M8 and M16), 50% methanol (M11), 100% methanol (mirabegron, M14 and M15) or 10% dimethylsulfoxide in methanol (M12 and M13). All stock solutions were kept at −20 °C. Working solutions, ranging from 5.0 to 2500 ng/mL (mirabegron, M11–M13, and M15), 5.0 to 1000 ng/mL (M5 and M16), 10.0 to 5000 ng/mL (M14), or 10.0 to 2000 ng/mL

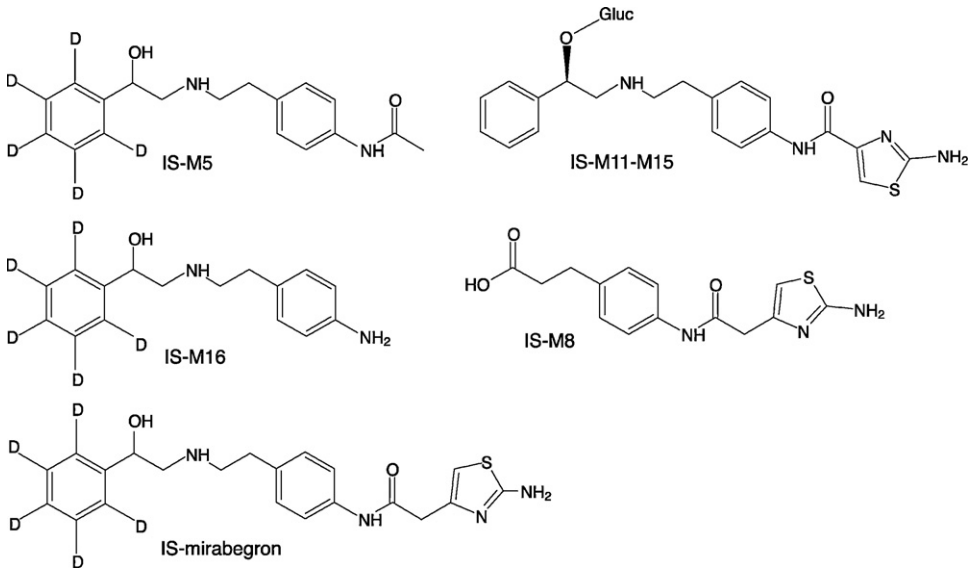

Fig. 2. Chemical structures of the internal standards used for the analyses of mirabegron and its metabolites.

(M8), were prepared by serial dilution with 50% acetonitrile (M5, M8, and M16), 15% methanol (mirabegron), or 50% methanol (M11–M15). Internal standard stock solutions were prepared in 50% acetonitrile (IS-M5 and IS-M16) or methanol (IS-mirabegron, IS-M8, and IS-M11–M15) at a concentration of 1.0 mg/mL. Internal standard working solutions were prepared by dilution with 50% acetonitrile (IS-M5, IS-M8, and IS-M16), 15% methanol (IS-mirabegron) or 50% methanol (IS-M11–M15) at concentrations of 1000 ng/mL (IS-mirabegron), 200 ng/mL (IS-M5, IS-M8, and IS-M16), or 100 ng/mL (IS-M11–M15).

2.3.2. Liquid chromatographic and mass spectrometry conditions

2.3.2.1. Mirabegron in human plasma. The analytical column used was an Inertsil™ C_8-3 (50 mm × 2.1 mm, 3 μm) purchased from GL Sciences Inc. (Tokyo, Japan). A C_8 security guard cartridge (4 mm × 2.0 mm) obtained from Phenomenex (Torrance, CA, USA) was used as a guard column. A binary isocratic separation was performed at a flow rate of 0.3 mL/min and a column temperature of 40 °C. The mobile phase consisted of 20 mmol/L ammonium acetate and acetonitrile at a 70/30 (v/v) ratio. The total run time was 2.5 min. The ultra-performance liquid chromatography autosampler was sequentially rinsed using strong and weak washes consisting of 1% formic acid in Milli-Q water/methanol/acetonitrile/isopropyl alcohol (25/25/25/25, v/v/v/v) and 20 mmol/L ammonium acetate/acetonitrile (70/30, v/v), respectively. The autosampler temperature was set at 10 °C. The effluent from the HPLC column was directed into the APCI probe for between 0.5 and 1.5 min. Mass spectrometer conditions were optimized to obtain maximal sensitivity. Ionization conditions were optimized as follows: ion source gas 1 (nitrogen), 50 psi; curtain gas, 20 psi; needle current, 3 μA; ion source temperature, 450 °C. The collision gas pressure was set at 4 units and the declustering potential at 60 V. The collision energy for mirabegron and its internal standard (IS-mirabegron) was optimal at −30 V.

2.3.2.2. M5 and M16 in human plasma. The analytical column used was a Synergi Fusion-RP C_{18} (150 mm × 2.0 mm, 4 μm) purchased from Phenomenex. A C_{18} guard column (4 mm × 2.0 mm) obtained from Phenomenex was used as a guard column. A quaternary gradient separation was performed at a flow rate of 0.4 mL/min and a column temperature of 40 °C. Mobile phase A consisted of methanol, mobile phase B was ULC/MS water, mobile phase C was 100 mM ammonium acetate, and mobile phase D was 0.1% formic acid. Mobile phases C and D were both kept at 10% during the complete run. The linear gradient profile was as follows: (a) 0 min, 30% A; (b) 3 min, 80% A; (c) 3–4.5 min, 80% A; and (d) 4.6 min, 30% A. The total run time was 7.5 min. The autosampler temperature was set at 15 °C. The effluent from the HPLC column was directed into the HESI probe for between 1.5 and 3.5 min. Mass spectrometer conditions were optimized to obtain maximal sensitivity. Ionization conditions were optimized as follows: capillary temperature at 300 °C, vaporizer temperature at 300 °C, HESI spray voltage at 3.0 kV, sheath gas pressure at 60 psi, auxiliary gas pressure at 40 units, and ion sweep gas pressure at 10 units. The collision gas pressure was set at 1.5 mTorr and the source collision-induced dissociation (CID) at −10 V. The collision energies for M5, M16, and their internal standards (IS-M5 and IS-M16, respectively) were optimal between −20 and −30 V.

2.3.2.3. M8 in human plasma. A quaternary gradient separation was performed at a flow rate of 0.4 mL/min and a column temperature of 40 °C. Mobile phase A consisted of methanol, mobile phase B was ULC/MS water, mobile phase C was 100 mM ammonium acetate, and mobile phase D was 0.2% formic acid. Mobile phases C and D were both kept at 10% during the complete run. The linear gradient profile was as follows: (a) 0 min, 30% A; (b) 2.5 min, 80% A; (c) 2.5–3.4 min, 80% A; and (d) 3.5 min, 30% A. The total run time was 6.5 min. The autosampler temperature was set at 15 °C. The effluent from the HPLC column (same as used for the M5 and M16 assay) was directed into the HESI probe for between 1.0 and 3.5 min. Mass spectrometer conditions were optimized to obtain maximal sensitivity. Ionization conditions were optimized as follows: capillary temperature at 300 °C, vaporizer temperature at 300 °C, HESI spray voltage at 3.0 kV, sheath gas pressure at 60 psi, auxiliary gas pressure at 40 units, and ion sweep gas pressure at 10 units. The collision gas pressure was set at 1.5 mTorr and the source CID at −10 V. The collision energies for M8 and its internal standard (IS-M8) were optimal between −25 and −30 V.

2.3.2.4. M11–M15 in human plasma. A quaternary gradient separation was performed at a flow rate of 0.4 mL/min and a column temperature of 40 °C. Mobile phase A consisted of acetonitrile, mobile phase B was Milli-Q water, mobile phase C was 50 mM ammonium acetate, and mobile phase D was 0.1% formic acid.

Mobile phases C and D were both kept at 10% during the complete run. The linear gradient profile was as follows: (a) 0 min, 20% A; (b) 3.5 min, 40% A; and (c) 3.6 min, 20% A. The total run time was 7.5 min. The autosampler temperature was set at 15 °C, and the HPLC column used was the same as for the M5 and M16 assay. Mass spectrometer conditions were optimized to obtain maximal sensitivity. Ionization conditions were optimized as follows: capillary temperature at 300 °C, vaporizer temperature at 300 °C, HESI spray voltage at 3.0 kV, sheath gas pressure at 60 psi, auxiliary gas pressure at 40 units, and ion sweep gas pressure at 0 units. The collision gas pressure was set at 1.5 mTorr and the source CID at −10 V. The collision energies for M11–M15 and their internal standards (IS-M11–M15) were optimal between −15 and −25 V.

2.3.3. Sample preparation

2.3.3.1. Mirabegron human plasma. The samples for calibration curves were prepared in glass tubes by spiking 500 μL of blank plasma (Richmond Pharmacology Ltd., London, UK) containing 4 mg/mL sodium fluoride (NaF) with 20 μL of the appropriate working solutions to yield the following concentration range: 0.2 (lower limit of quantitation [LLOQ])–100.0 ng/mL. For the quality control (QC) samples, concentrations of 0.6 ng/mL (low QC [LQC]), 8.0 ng/mL (medium QC [MQC]), and 80.0 ng/mL (high QC [HQC]) were prepared. For quantitation, 20 μL of internal standard working solution (IS-mirabegron) was added. After preparing the samples, 400 μL of 20 mmol/L ammonium acetate solution was added and the samples were thoroughly mixed. Then, a liquid–liquid extraction (LLE) step was applied to extract mirabegron and its internal standard from human plasma. For this, 3 mL of diethyl-ether was added, followed by shaking for 10 min. The solutions were centrifuged for 5 min at 2361 × *g*, and the upper organic layer was transferred to a new tube and evaporated to dryness under a stream of nitrogen at 40 °C. The residues were reconstituted in 200 μL of mobile phase (starting conditions) and 10 μL was injected into the liquid chromatography/tandem mass spectrometry (LC–MS/MS) system.

2.3.3.2. M5 and M16 in human plasma. Samples for calibration curves were prepared in glass tubes by spiking 500 μL of blank plasma (Richmond Pharmacology Ltd.) containing 4 mg/mL NaF with 50 μL of the appropriate working solutions to yield the following concentration range: 0.5 (LLOQ)–100.0 ng/mL. For the QC samples, concentrations of 1.5, 15.0, and 80.0 ng/mL were obtained. For quantitation, 50 μL of internal standard working solution (IS-M5 and IS-M16) was added. Processing of the samples was performed on melting ice until the addition of TBME to avoid *in situ* formation of M16. After sample preparations, 500 μL of 2% ammonia solution was added and the samples were thoroughly mixed. An LLE step was applied to extract the two analytes and their internal standards from human plasma. For this, 3 mL of TBME was added, followed by shaking for 10 min. The solutions were centrifuged for 5 min at 2656 × *g*, and the upper organic layer was transferred to a new tube and evaporated to dryness under a stream of nitrogen at 40 °C. The residues were reconstituted in 200 μL of mobile phase (starting conditions) and 5 μL was injected into the LC–MS/MS system.

2.3.3.3. M8 in human plasma. The samples for calibration curves were prepared in glass tubes by spiking 500 μL of blank plasma (Richmond Pharmacology Ltd.) containing 4 mg/mL NaF with 50 μL of the appropriate working solutions to yield the following concentration range: 1.0 (LLOQ)–200.0 ng/mL. For the QC samples, concentrations of 3.0, 20.0, and 160.0 ng/mL were obtained. For quantitation, 50 μL of internal standard working solution (IS-M8) was added. After sample preparations, 500 μL of 0.5 M (ortho-) phosphoric acid was added and the samples were thoroughly mixed. A solid-phase extraction (SPE) procedure was applied to extract the analyte and its internal standard from human plasma. For this, 1 mL of the sample was transferred into a properly conditioned (1 mL of acetonitrile followed by 1 mL of Milli-Q water) Oasis® MCX (Mixed-mode Cation Exchange) SPE (30 mg, 1 mL) cartridge. Before eluting the cartridge with 1 mL of 70% acetonitrile in 1% ammonia solution, the cartridge was washed with 1 mL of 2% formic acid followed by 1 mL of acetonitrile. The eluent was evaporated to dryness under a stream of nitrogen at 40 °C. The residues were reconstituted in 200 μL of mobile phase (starting conditions) and 5 μL was injected into the LC–MS/MS system.

2.3.3.4. M11–M15 in human plasma. The samples for calibration curves were prepared in glass tubes by spiking 500 μL of blank plasma (Richmond Pharmacology Ltd.) containing 4 mg/mL NaF with 50 μL of the appropriate working solutions to yield the following concentration ranges: 0.5 (LLOQ)–250.0 ng/mL for M11–M13, and M15; and 1.0 (LLOQ)–500.0 ng/mL for M14. For the QC samples, concentrations of 1.5 (3.0 for M14), 15.0 (30.0), and 200.0 (400.0) ng/mL were obtained. For quantitation, 50 μL of internal standard working solution (IS-M11–M15) was added. After sample preparations, 500 μL of 0.5 M (ortho-) phosphoric acid was added and the samples were thoroughly mixed. An SPE procedure was applied to extract the analytes and internal standards from human plasma. For this, 1 mL of the sample was transferred into a properly conditioned (1 mL of methanol followed by 1 mL of Milli-Q water) Oasis® MCX SPE (30 mg, 1 mL) cartridge. Before eluting the cartridge with 1 mL of 2% ammonia/methanol (20/80, v/v), the cartridge was washed with 1 mL of 2% formic acid followed by 1 mL of methanol and 1 mL of 2% ammonia. The eluent was evaporated to dryness under a stream of nitrogen at 40 °C. The residues were reconstituted in 200 μL of mobile phase (starting conditions) and 20 μL was injected into the LC–MS/MS system.

2.4. Method validation

2.4.1. Specificity

The specificity of the methods was determined by analyzing eight different lots of blank control matrix for the presence of potential interferences in the retention window of the peaks of interest. The influence of the presence of interferences in blank control matrix with and without internal standard was determined by comparing the peak areas with blank control matrix containing the analyte at LLOQ level. The acceptance criterion for the ratio between the peak area of interference and the analyte at LLOQ level was 0.2. For interference in the retention window of the internal standard, a criterion of 0.05 was considered acceptable.

2.4.2. Linearity and lower limit of quantitation

Standard curves (consisting of 9 to 11 concentration levels) from the four methods were extracted and assayed with weighted ($1/x^2$) linear regression. Blank samples were analyzed to confirm the absence of interference and the LLOQ was set as the lowest amount of analyte in a sample that could be quantitatively determined with acceptable precision and accuracy (*i.e.*, 20% relative standard deviation [RSD] and ±20% in these assays, respectively).

2.4.3. Precision and accuracy

The precision of the methods was determined from the QC samples by replicate analyses at four concentration levels (LLOQ, LQC, MQC, and HQC). Batch precision and accuracy were determined by repeated analyses of the QC samples in three different batches ($n = 6$ per batch). The concentration of each sample was determined using the calibration curve prepared and analyzed in the same batch. The acceptance criteria for precision and accuracy for LQC, MQC, and

HQC were 15% RSD and ±15%, respectively. For LLOQ, the acceptance criteria for precision and accuracy were 20% RSD and ±20%, respectively.

2.4.4. Extraction recovery

The extraction recovery was determined by dividing the peak areas of the analytes added into blank biological matrix and extracted using the appropriate extraction procedure with those obtained from the analyte spiked into an equivalent volume of redissolving solution which was added to extracted plasma. This procedure was repeated for six replicates at three QC levels (LQC, MQC, and HQC). There were no acceptance criteria for the extraction recovery. The acceptance criterion for the precision of the recovery samples at each level was 15% RSD.

2.4.5. Matrix effect

The matrix effect was evaluated by preparing samples at two QC levels (LQC and HQC) and two concentration levels above the upper limit of quantitation (ULOQ) (8 × ULOQ and 18 × ULOQ) using eight different lots of blank control matrix. The samples above ULOQ were diluted using pooled biological matrix to obtain a concentration within the validated range of the calibration curve. For the validation of the mirabegron assay in human plasma, six different lots of human plasma were used and dilution integrity of 8 × ULOQ was evaluated. The concentration of each sample was determined using the calibration curve prepared and analyzed in the same batch. The acceptance criteria for the precision and accuracy for LQC, HQC, and the samples above ULOQ were 15% RSD and ±15%, respectively.

2.4.6. Stability

2.4.6.1. Freeze and thaw stability. QC samples at two concentration levels (LQC and HQC) were stored at −20 °C and −70 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for at least 12 h under the same conditions. The freeze–thaw cycles were repeated at least two times.

2.4.6.2. Short-term room temperature stability. QC samples at two concentration levels (LQC and HQC) were kept at room temperature for a period that exceeded the preparation time of the samples (around 24 h).

2.4.6.3. Long-term stability. QC samples at two concentration levels (LQC and HQC) were kept at −20 °C and −70 °C for a period that exceeded the time from blood (sample) collection to the time of processing the clinical samples (at least 1 year).

2.4.6.4. Whole blood stability. Whole blood samples (obtained from healthy donors) were pre-incubated at 37 °C for 10 min after spiking the samples with the appropriate amount of analyte working solution. Plasma was immediately prepared from whole blood after pre-incubation as a control ($t = 0$), and after storage for 0.5, 1, and 2 h at room temperature. After storage at room temperature, the plasma samples were processed according to the methods described in Section 2.3.3. Stability was evaluated by comparing the measured concentrations of samples after pre-incubation ($t = 0$) and after storage.

The concentrations of the stability QC samples were determined using the calibration curve prepared and analyzed in the same batch. The acceptance criteria for the precision and accuracy for the QC samples were 15% RSD and ±15%, respectively.

2.5. Application to a clinical study

The bioanalytical methods were applied to measure the plasma concentration levels and evaluate the pharmacokinetics of mirabegron and its metabolites in 12 healthy young male subjects who received daily oral dosing of 100 mg mirabegron. The subjects were part of a clinical study evaluating the pharmacokinetic characteristics of mirabegron and its metabolites in healthy young and elderly male and female subjects. Written informed consent, as per institutional guidelines, was obtained from all subjects.

At scheduled time points, a 5 mL blood sample was taken *via* venipuncture or cannulation from a forearm vein. Blood samples for analysis of mirabegron and its metabolites were collected into tubes containing sodium-heparin as an anticoagulant and 20 mg NaF as a stabilizer (Becton Dickinson catalog number 367764). Following blood sampling, sample tubes were gently inverted (six- to eight-fold), followed by centrifugation at 2000 × *g* and 4 °C. The separated plasma was immediately (within 60 min of collection) stored at −70 °C to await analysis. Plasma concentration data of mirabegron and its metabolites were analyzed using WinNonlin software version 5.1 or higher (Pharsight Corp., Mountain View, CA, USA).

3. Results and discussion

3.1. Method development

3.1.1. Optimization of mass spectrometry conditions

The MS conditions of all analytes were determined by an infusion of 10 μL/min into the source of the mass spectrometer using a variety of different solvent conditions. After having obtained the optimal solvent conditions, the parameters on MS were varied to obtain stable and intense daughter fragments from the analytes of interest. All MS analyses were performed in positive mode. The mirabegron and M11–M15 assay were evaluated on the API4000 QTrap and the Quantum Ultra. Both systems gave comparable sensitivity, accuracy and precision. Throughout the development of the methods, the specificity, reproducibility, and robustness of the MS methods were monitored and adjusted to establish methods that could be validated and applied to clinical study samples. The following selected reaction monitoring transitions were found to be stable and intense for the analysis of unchanged mirabegron and its metabolites (all *m/z* masses are $[M+H]^+$): mirabegron (*m/z* 397.2 to *m/z* 260.2); M5 (*m/z* 299.1 to *m/z* 120.1); M8 (*m/z* 292.1 to *m/z* 113.0); M11 (*m/z* 573.1 to *m/z* 379.1); M12 (*m/z* 615.1 to *m/z* 395.1); M13 (*m/z* 617.1 to *m/z* 379.1); M14 (*m/z* 573.1 to *m/z* 379.1); M15 (*m/z* 589.1 to *m/z* 395.1); M16 (*m/z* 257.1 to *m/z* 120.1); IS-mirabegron (*m/z* 402.2 to *m/z* 260.3); IS-M5 (*m/z* 304.1 to *m/z* 120.1); IS-M8 (*m/z* 306.1 to *m/z* 113.0); IS-M11–M15 (*m/z* 559.1 to *m/z* 365.1); and IS-M16 (*m/z* 262.2 to *m/z* 120.1).

3.1.2. Optimization of liquid chromatography conditions

The Synergi Fusion-RP C_{18} column was selected for the analysis of all metabolites since this column provided a symmetrical peak shape and, in combination with the mobile phase used, the highest intensity and most favorable selectivity. For the M11–M15 assay it was important to have sufficient resolution between M11 and M14 and to have M12 separated from M13. Even though M12 and M13 have different MS/MS transitions, M12 interfered in the M13 trace and could cause an overestimation of M13 when overlaid. The following retention times were obtained for the metabolites: M5 (2.9 min); M8 (2.2 min); M11 (2.1 min); M12 (3.6 min); M13 (3.3 min); M14 (1.7 min); M15 (3.5 min); M16 (2.4 min); IS-mirabegron (0.9 min); IS-M5 (2.9 min); IS-M8 (3.0 min); IS-M11–M15 (2.8 min); and IS-M16 (2.4 min).

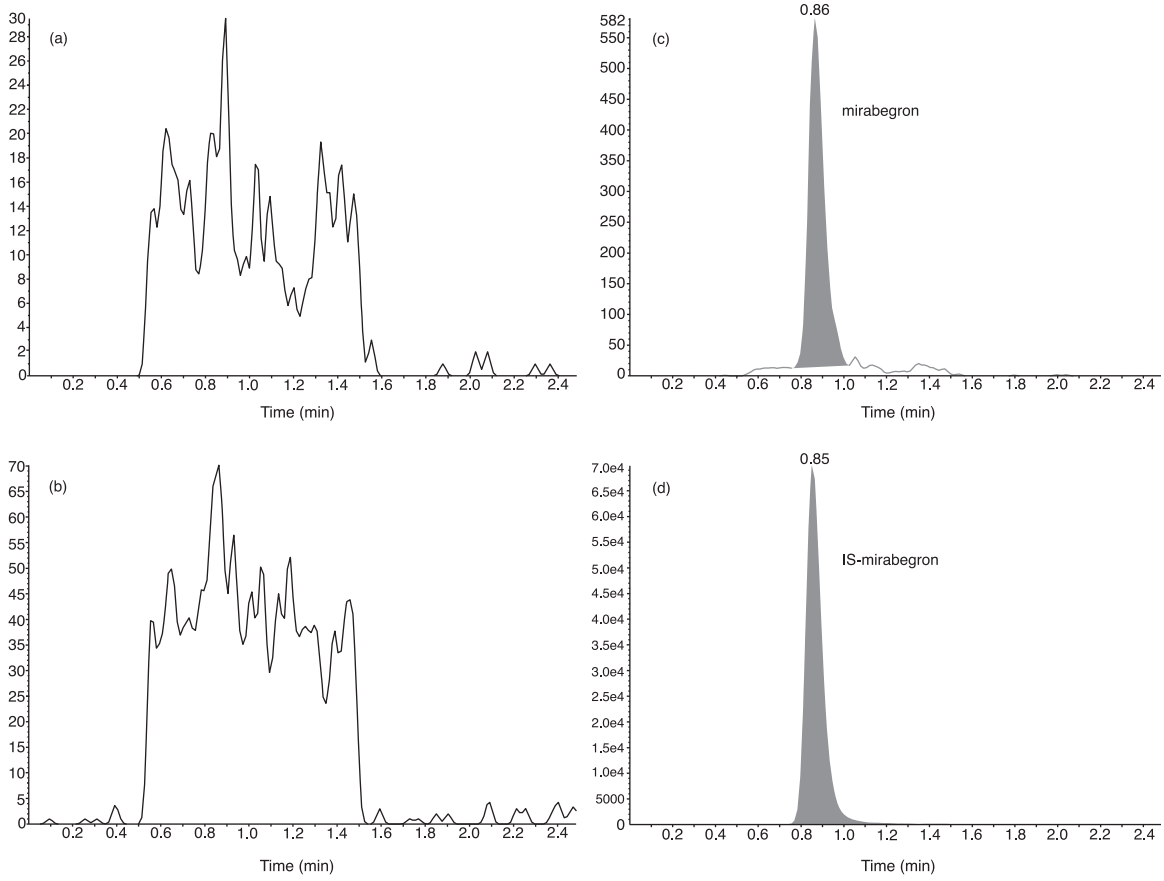

Fig. 3. Multiple-reaction monitoring (MRM) chromatograms of blank human plasma (a and b) and blank human plasma containing unchanged mirabegron at the lower limit of quantitation and its internal standard (c and d).

3.1.3. Sample preparation

For the quantitative determination of mirabegron and its metabolites M5, M8, M11–M16 in human plasma, it was necessary to develop and validate four separate bioanalytical methods for: (1) mirabegron; (2) M5 and M16; (3) M8; and (4) M11–M15.

A method had been developed for the determination of unchanged mirabegron in human plasma using LLE and APCI interfaces. Due to poor extraction recoveries of the metabolites M8 and M11–M15 using LLE and differences in ionization efficiency (HESI *vs* APCI), and due to the difference in chemical properties between

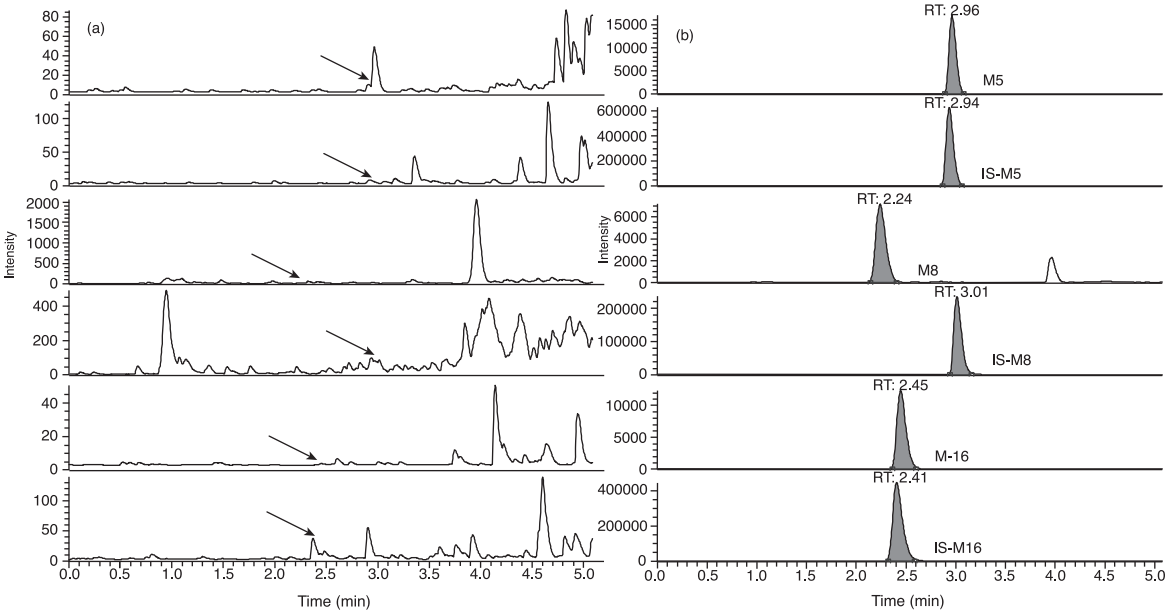

Fig. 4. Selected reaction monitoring chromatograms of blank human plasma (a) and blank human plasma containing mirabegron metabolites M5, M8, and M16 at the lower limit of quantitation and their internal standards (b). Arrows indicate the retention times of the individual metabolites and internal standards.

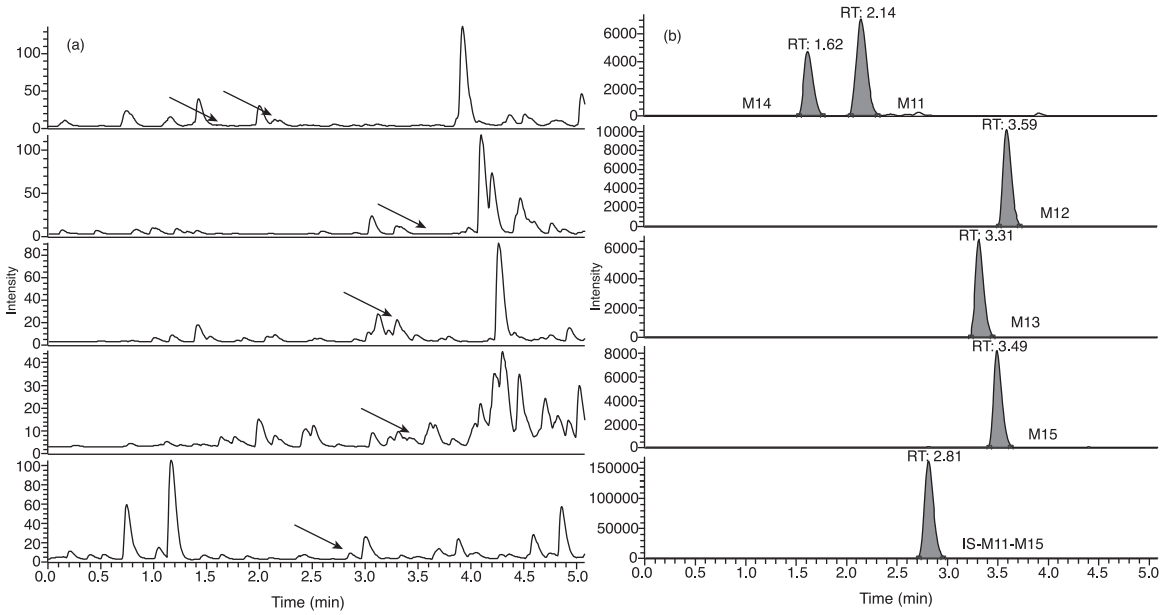

Fig. 5. Selected reaction monitoring chromatograms of blank human plasma (a) and blank human plasma containing mirabegron metabolites M11–M15 at the lower limit of quantitation and their internal standards (b). Arrows indicate the retention times of the individual metabolites and internal standards.

M5, M16 and unchanged mirabegron, it was difficult to combine the determination of unchanged mirabegron with the analysis of its metabolites. The recovery of unchanged mirabegron using LLE is <50%. Incurred sample reproducibility was investigated in this study for unchanged mirabegron. 97% of the samples passed the ISR acceptance criteria, showing good reproducibility for the entire range.

Initially, the aim was to develop one SPE method for all metabolites. However, due to significant differences in extraction recoveries, two separate SPE methods were developed: one for the analysis of M11–M15 (all glucuronides) and one for the analysis of M5, M8, and M16. Both methods used Waters Oasis MCX SPE (1 cc, 30 mg) cartridges, but different washing procedures were required. For M5, M8, and M16, washing using 2% ammonia was not possible because M16 was lost during this step; for M11–M15, it was necessary to wash with 2% ammonia to be able to wash out interfering matrix substituents and obtain the required LLOQ for those metabolites. Waters Oasis HLB (Hydrophilic Lipophilic Balanced) cartridges were also tested during the development of the assays, but it was not possible to apply selective washing to the samples to obtain the desired LLOQ for the metabolites.

After applying the methods to the analysis of clinical study samples, it was observed that during the processing of the human plasma samples M16 was overestimated because of *in situ* formation of M16. It is hypothesized that M16 is formed due to back-conversion of direct conjugates (glucuronides, *etc.*) of M16 during the Oasis MCX extraction method under acidic conditions. To prevent this, the sample extraction method for M16 was changed from SPE to LLE under weak alkaline conditions and the samples were kept on melting ice until the addition of TBME required the LLE step. Because the change from SPE to LLE was not suitable for the determination of M8 in human plasma, the analysis of M5, M8, and M16 was split into two separate methods.

Initially, methanol was used in the extraction procedure for the analysis of M8, but this caused conversion of M8 into its methylester. This methylester of M8 had the same parent mass and daughter fragment mass as the analog internal standard of M8 (IS-M8), which caused the formation of an M8-related peak in the internal standard trace. Although the conversion did not have any influence on the quantitation of M8, the extraction procedure was adjusted using acetonitrile instead of methanol.

3.1.4. Internal standard selection

An ideal internal standard for the quantitative analysis of analytes should be a structurally similar analog or stable isotope-labeled compound, according to the US Food and Drug Administration guideline [9]. For the analysis of unchanged mirabegron, M5, and M16, deuterated internal standards were available. An analog of M8 was chosen as the internal standard for quantitation of M8 based on similarities in structure, extraction recovery, and mass spectrometric behavior. For the analysis of M11–M15, one glucuronide analog was selected for the quantitation of all five metabolites. This internal standard showed similar behavior by means of extraction recovery and mass spectrometric behavior. Chromatographically, the internal standard of IS-M11–M15 elutes in between the five metabolites to be quantified.

3.2. Method validation

3.2.1. Specificity

The specificity of the methods was evaluated and the chromatograms of the LLE and SPE methods produced extracts with no or minimal interferences at the retention times of mirabegron, its metabolites, and their internal standards. Fig. 3 shows representative chromatograms of blank human plasma and mirabegron at the LLOQ with its internal standard. Fig. 4 shows chromatograms of blank human plasma and M5, M8, and M16 at the LLOQ with their internal standards. Fig. 5 shows chromatograms of blank human plasma and M11–M15 at the LLOQ with their internal standard.

3.2.2. Linearity and lower limit of quantitation

All calibration curves were linear within the concentration range described in Section 2.3.3 using weighted linear least-square regression: $1/x^2$. Table 1 shows the average determination coefficients with standard deviations for mirabegron and its metabolites. The calibration curve ranges for mirabegron and its metabolites were as follows: mirabegron, 0.2–100 ng/mL; M5

Table 1
Average determination coefficients with standard deviations (SDs) for mirabegron and its metabolites in human plasma.

Analyte	Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
$r^2 \pm$ SD	0.9988 ± 0.0011	0.9993 ± 0.0003	0.9996 ± 0.0003	0.9992 ± 0.0003	0.9987 ± 0.0006	0.9982 ± 0.0005	0.9988 ± 0.0008	0.9981 ± 0.0006	0.9994 ± 0.0007
n	8	6	6	11	11	11	11	11	6

and M16, 0.5–100 ng/mL; M8, 1–200 ng/mL; M11–M13, and M15, 0.5–250 ng/mL; and M14, 1–500 ng/mL.

The LLOQ samples for all methods were analyzed, with acceptable precision and accuracy. Table 2 shows the accuracy and precision (% RSD) for unchanged mirabegron and its metabolites at the LLOQ, as described in Section 2.3.3. LLOQ concentration levels for mirabegron and its metabolites were as follows: mirabegron, 0.2 ng/mL; M5, M11–M13, M15, and M16, 0.5 ng/mL; M8 and M14, 1 ng/mL.

3.2.3. Accuracy and precision

Data for the batch accuracy and precision (% RSD) for unchanged mirabegron and its metabolites in human plasma at three different concentration levels are presented in Table 3.

LQC concentration levels for mirabegron and its metabolites were as follows: mirabegron, 0.6 ng/mL; M5, M11–M13, M15, and M16, 1.5 ng/mL; and M8 and M14, 3 ng/mL. MQC concentration levels were as follows: mirabegron, 8 ng/mL; M5, M11–M13, M15, and M16, 15 ng/mL; M8, 20 ng/mL; and M14, 30 ng/mL. HQC concentration levels were as follows: mirabegron, M5 and M16, 80 ng/mL; M8, 160 ng/mL; M11–M13, and M15, 200 ng/mL; M14, 400 ng/mL.

At each concentration level, the precision (% RSD) was lower than 5% and the accuracy did not exceed ±13% for any of the analytes.

3.2.4. Extraction recovery

The mean extraction recoveries of unchanged mirabegron and its metabolites from human plasma at three different concentration levels are presented in Table 4. The mean recoveries for mirabegron and its metabolites ranged from around 40% to 90%. The internal standards had similar recoveries when compared with their reference compounds. The precision (% RSD) of the extraction procedures for all analytes was lower than 13%.

3.2.5. Matrix effect

The matrix effect on the determination of mirabegron and its metabolites from eight different human plasma lots was within the acceptance criteria at each level evaluated, as shown in Table 5. The ULOQs for mirabegron and its metabolites were as follows: mirabegron, M5, and M16, 100 ng/mL; M8, 200 ng/mL; M11–M13, and M15, 250 ng/mL; and M14, 500 ng/mL.

The accuracy deviation values were within ±12% of the actual values. The precision (% RSD) values of the analyses were lower than 8%.

3.2.6. Stability

Table 6 summarizes the freeze–thaw stability at −20 °C and −70 °C, short-term stability at room temperature, whole blood stability at room temperature and long-term stability at −20 °C and −70 °C. Good stability was demonstrated under all the conditions tested.

3.3. Application to a clinical study

The validated LC–MS/MS methods were successfully used to measure the plasma concentration levels in study samples from a clinical pharmacokinetic study. Plasma concentrations of mirabegron, M5, M8, M11–M16 were used to assess the plasma pharmacokinetics of mirabegron and its metabolites after multiple oral dose administration of 100 mg mirabegron to healthy young male subjects. Dosing was 100 mg mirabegron twice daily on day 1 and 100 mg once daily from day 2 to day 7, a full PK profile was evaluated from day 7 to day 14 at pre-dose and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 16, 24, 36, 48, 72, 96, 132, 168 h post-dose. A summary of the exposure parameters C_{max} and AUC_{tau} is presented in Table 7. The pharmacokinetic evaluation demonstrated that two

Table 2
Accuracy and precision (% relative standard deviation [RSD]) for mirabegron and its metabolites at the lower limit of quantitation in human plasma.

Analyte	Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
Accuracy (% RSD)	96.1 (4.7)	101.6 (4.5)	96.6 (5.6)	100.1 (9.5)	110.7 (5.8)	96.4 (6.6)	101.3 (7.9)	89.8 (7.6)	101.4 (6.4)
n	18	6[a]	6	18	18	18	18	18	6

[a] *n* = 6 because the experiment was performed as part of a partial validation.

Table 3
Accuracy and precision (% relative standard deviation [RSD]) for the analysis of mirabegron and its metabolites in human plasma. HQC, high quality control; LQC, low quality control; MQC, medium quality control.

Analyte	Accuracy (% RSD)								
	Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
LQC	100.6 (2.6)	99.7 (3.8)	88.3 (2.5)	98.8 (4.1)	110.1 (3.0)	100.7 (3.8)	100.9 (3.8)	93.7 (3.1)	97.1 (2.3)
MQC	95.0 (1.6)	99.6 (2.5)	96.3 (1.1)	100.7 (2.0)	110.2 (2.6)	103.2 (1.9)	102.6 (1.9)	97.2 (1.8)	99.8 (1.7)
HQC	98.1 (0.8)	98.2 (2.2)	95.5 (1.7)	96.6 (2.2)	102.2 (2.3)	95.9 (2.3)	95.8 (1.9)	87.7 (2.8)	100.9 (1.9)
n	18	6[a]	6	18	18	18	18	18	6

[a] *n* = 6 because the experiment was performed as part of a partial validation.

Table 4
Mean extraction recovery (%) and extraction precision (% relative standard deviation [RSD]) of mirabegron and its metabolites in human plasma. HQC, high quality control; LQC, low quality control; MQC, medium quality control.

Analyte	% mean extraction recovery (% RSD)								
	Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
LQC	40.0 (5.6)	63.9 (7.2)	85.5 (4.8)	81.7 (6.0)	81.2 (2.2)	83.1 (2.0)	74.0 (6.0)	85.8 (4.0)	75.1 (8.3)
MQC	38.1 (5.3)	54.0 (10.8)	87.2 (4.8)	82.6 (1.0)	89.2 (2.3)	86.8 (1.9)	83.2 (1.3)	88.1 (1.8)	58.7 (12.7)
HQC	40.4 (7.8)	56.0 (6.4)	88.7 (2.4)	84.1 (1.0)	87.9 (1.1)	87.6 (1.0)	83.2 (1.4)	91.3 (1.4)	57.5 (7.2)
n	6	6	6	6	6	6	6	6	6

Table 5
Matrix effect on the determination of mirabegron and its metabolites in human plasma, expressed in accuracy and precision (% relative standard deviation [RSD]). HQC, high quality control; LQC, low quality control; NA, not applicable; ULOQ, upper limit of quantitation.

Analyte	Accuracy (% RSD)								
	Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
LQC	98.6 (2.5)	101.5 (2.5)	99.9 (2.0)	105.7 (3.0)	110.7 (4.2)	106.5 (4.7)	105.5 (3.9)	100.2 (3.7)	99.3 (2.1)
HQC	99.1 (0.8)	101.3 (2.0)	101.1 (1.2)	101.9 (2.7)	107.6 (2.3)	100.1 (2.3)	101.6 (2.3)	91.4 (2.8)	99.2 (1.0)
8 × ULOQ	96.0 (7.2)	106.7 (4.6)	103.3 (2.9)	100.1 (2.1)	97.7 (2.8)	99.6 (1.7)	101.3 (1.8)	89.5 (2.2)	109.6 (4.9)
18 × ULOQ	NA	106.6 (4.5)	110.5 (6.4)	98.5 (2.6)	93.6 (1.2)	97.0 (3.1)	98.8 (2.5)	88.1 (2.6)	111.3 (3.5)
n	6	8	8	8	8	8	8	8	8

Table 6
Stability data for mirabegron and its metabolites (*n* = 6 per quality-control level) expressed in accuracy and precision (% relative standard deviation [RSD]). F/T, freeze–thaw; HQC, high quality control; LQC, low quality control; NA, not applicable.

	Analyte	Accuracy (% RSD)								
		Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
F/T–20 °C (3 cycles[a])	LQC	90.6 (3.4)	98.8 (3.3)	99.4 (2.6)	95.4 (5.5)	101.8 (5.7)	99.6 (5.9)	88.6 (5.5)	94.7 (4.3)	96.3 (1.9)
	HQC	89.1 (3.0)	95.5 (1.7)	92.9 (0.7)	91.3 (3.1)	94.2 (2.3)	92.9 (2.3)	89.5 (2.6)	85.3 (3.0)	96.5 (1.1)
F/T–70 °C (3 cycles)	LQC	96.9 (2.8)	97.2 (2.9)	96.7 (2.9)	94.4 (4.4)	100.6 (2.9)	97.5 (2.1)	91.6 (2.2)	92.1 (4.6)	97.2 (2.4)
	HQC	96.8 (1.6)	96.0 (2.2)	92.2 (1.1)	91.4 (1.8)	94.2 (2.6)	93.4 (1.6)	88.4 (1.8)	86.2 (1.9)	96.6 (1.0)
Short-term room temperature (24 h)	LQC	94.4 (4.7)	97.6 (3.0)	91.3 (2.5)	99.0 (4.6)	106.8 (4.3)	91.9 (2.3)	99.2 (3.4)	91.0 (5.7)	98.0 (2.7)
	HQC	92.0 (1.4)	97.4 (1.8)	94.6 (0.8)	98.4 (0.8)	100.0 (1.6)	89.4 (1.7)	96.7 (1.4)	87.2 (1.1)	95.0 (1.7)
Long-term–20 °C	Days	390	455	479	604	604	604	604	604	346
	LQC	96.2 (4.2)	101.6 (0.9)	95.8 (2.6)	103.7 (5.4)	105.1 (3.2)	107.6 (4.2)	104.3 (4.0)	100.1 (6.8)	89.9 (1.4)
	HQC	NA	96.2 (1.1)	92.0 (0.9)	102.6 (3.3)	100.6 (2.8)	101.8 (2.8)	102.5 (2.8)	97.9 (2.5)	93.8 (1.1)
Long-term–70 °C	Days	390	455	479	604	604	604	604	604	455
	LQC	94.3 (4.2)	102.0 (2.4)	94.0 (1.5)	104.1 (4.2)	104.1 (2.9)	106.2 (2.3)	107.8 (1.6)	105.5 (6.6)	100.3 (1.0)
	HQC	NA	97.4 (1.0)	93.6 (0.9)	102.2 (2.3)	99.9 (2.3)	102.1 (3.0)	102.0 (2.8)	97.1 (2.4)	98.5 (1.0)
Whole blood room temperature (2 h)	LQC	102.7 (2.1)	103.8 (1.7)	96.7 (2.0)	100.0 (2.8)	100.1 (2.4)	91.2 (4.1)	98.9 (2.8)	98.0 (3.4)	100.9 (2.9)
	HQC	101.0 (1.8)	104.4 (1.5)	95.2 (1.8)	96.8 (1.1)	98.7 (1.5)	100.4 (1.6)	96.9 (1.9)	102.4 (1.3)	99.3 (1.6)

[a] For M8 and M5/M16 assays in human plasma, five F/T cycles were evaluated for −20 °C and −70 °C instead of three.

Table 7
Mean (standard deviation) pharmacokinetic parameters after daily dosing of 100 mg mirabegron in healthy young males (*n* = 12). AUC_{tau}, area under the plasma concentration–time curve for a dosing interval; C_{max}, maximum plasma concentration.

Parameter	Mirabegron	M5	M8	M11	M12	M13	M14	M15	M16
C_{max}, ng/mL	134 (58.0)	8.96 (3.64)	4.69 (2.18)	34.0 (12.2)	29.7 (11.0)	5.84 (2.1)	11.8 (2.8)	9.60 (3.57)	6.62 (2.84)
AUC_{tau}, ng h/mL	947 (228.0)	145 (57.0)	38.2 (20.6)	296 (90.0)	255 (113.0)	35.3 (17.6)	127 (19.0)	68.0 (25.7)	97.4 (41.4)

glucuronide metabolites (M11 and M12) represent more than 10% of the total drug-related material exposure.

4. Conclusions

This paper describes precise and accurate LC–MS/MS methods for the determination of mirabegron and its metabolites in human plasma. Good linearity and specificity were observed for all analytes discussed, which makes the methods suitable for the determination of these analytes in clinical study samples. Stability of mirabegron and its metabolites in human plasma was demonstrated under conditions relevant for the analysis of these analytes in clinical study samples. To prevent hydrolysis of mirabegron by plasma esterases present in human plasma, blood samples were collected in commercially available tubes containing sodium fluoride and heparin. For the analysis of M16, samples were processed on melting ice to prevent *in situ* M16 formation. The methods were successfully applied to a clinical study for the assessment of pharmacokinetic parameters of mirabegron and its metabolites in human plasma. Based on ICH M3 criteria [7], two major metabolites (M11 and M12) were identified.

Role of the funding source

Astellas was actively involved in the study design; the collection, analysis, and interpretation of data; writing of reports; and the decision to submit the paper for publication.

Disclosure statement

Raymond van Teijlingen, John Meijer, Shin Takusagawa, Marcel van Gelderen, Cas van den Beld and Takashi Usui are full-time employees of Astellas. None of the authors has at any time received payment or services from a third party for any aspect of the submitted work. None of the authors has any relevant financial activities outside of the submitted work or any other relationships, conditions, or circumstances that present a potential conflict of interest.

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