ORIGINAL ARTICLE

Determination of Mirabegron in rat plasma by UPLC–MS/MS after oral and intravenous administration

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SUMMARY

Mirabegron is a kind of β3 adrenergic receptor agonist which is an effective drug for the treatment of overactive bladder. In this research, a UPLC–MS/MS method is developed and validated for the study of mirabegron pharmacokinetic in rats. A protein precipitation method is applied for sample preparation with acetonitrile. m/z 397.3→379.6, m/z 326.4→121.0 for mirabegron, tolterodine (IS), respectively in the positive ion mode was performed for quantitation. The method is reliable and reproducible in our study (intra-day precision≤11.06%, inter-day precision≤11.43%) with concentration curves linear from 5 to 2500 ng/mL(R2>0.999). Stability studies demonstrated that mirabegron was stable under a variety of storage conditions. This method was successfully applied for determining mirabegron in rats after oral and intravenous administration.

KEYWORDS: *Urinary bladder, overactive. Adrenergic beta-3 receptor agonists. Plasma. Administration, intravenous. Administration, oral. Rats.*

INTRODUCTION

Overactive bladder (OAB) is a kind of syndrome characterized by symptoms of urinary urgency, frequency, nocturia and urge incontinence[1-3]. It is estimated that in 2018 there were 546 million people suffering from OAB worldwide. Irwin et al.[4] revealed that the prevalence of OAB worldwide is estimated as being greater in women than in men in 2008 (11.6% vs. 9.7%, respectively), 2013 (11.7% vs. 9.8%) and 2018 (11.9% vs. 10.0%) (Irwin and others 2011).

Mirabegron, namely (2-(2-amino-1,3-thiazol-4-yl)-N-[4-(2-{[(2R)-2-hydroxy-2-phenylethyl]amino} ethyl)phenyl]acetamide), trade name Myrbetriq meer-bet-trick in the US and Betmiga in Europe, is a novel, effective and highly selective β3 receptor agonists for the treatment of OAB[5,6]. It was approved in the United States in July 2012[7]. Astellas Pharma Inc. found it is an agonist of the human β3-adrenoceptor[1]. The activation of the β3 adrenergic receptor in the detrusor can relax the muscle in the bladder and increase bladder capacity. Takusagawa et al.[8] suggested that CYP3A4 and CYP2D6 are involved in the oxidative metabolism of mirabegron in vitro, although Sawamoto et al.[9] indicated that these isozymes play a limited role in vivo. Before further pharmacological and pharmacokinetic research is fully possible, it is necessary to first develop an an-

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alytical method for the effective determination of Mirabegron in biological fluids.

A literature survey reveals that several pieces of literature reported the analytical methods for the determination of this β3 adrenoceptor agonist mirabegron in biological fluids have been established. Raymond van Teijlingen et al.[10] developed and validated a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determining mirabegron and its metabolites. Zhou et al.[11] was the only group using Liquid Chromatographic (LC) to Separate Mirabegron Enantiomers on a Chiralpak AY-H Column. However, no piece of literature focused on the ultra performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) method for determining mirabegron in biological fluids. Compared with LC and LC-MS/MS, UPLC–MS/MS shows a dramatic enhancement in speed, sensitivity, selectivity, robustness, resolution as well as sample throughput.

In the present study, the development of a sensitive and rapid method for estimating mirabegron in plasma was achieved and successfully applied to the pharmacokinetic study of mirabegron after oral and intravenous administration. Acetonitrile precipitation was applied in our work. Separation and quantitation of mirabegron in plasma samples were performed with UPLC-MS/MS. The column was packed with C18 particles of 1.7 µm, which contribute to higher column performance, efficient separation and short analysis time. The total run time for each sample was shorter than 3min (2.5min). Our method behaved faster and more sensitively compared with that of previous studies[10,11].

EXPERIMENTAL PROCEDURE

Chemicals

Mirabegron (Cat: BD 256310, Purity>99%) was obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). Tolterodine (internal standard, IS) (Cat: CLS-BD17869, Purity>95%) was purchased from J&K Scientific Ltd. (Beijing, China). Acetonitrile was obtained in HPLC grade from Merck KGaA (Darmstadt, Germany), as well as methanol. Formic acid, obtained from Tedia Company (Cincinnati, OH, USA) was HPLC-grade. Millipore Milli-Q purification system (Bedford, MA, USA) was used to prepare ultra-pure water. All other reagents used in the whole experiment were of analytical or HPLC grade.

Ultra performance liquid chromatography and tandem mass spectrometry conditions

UPLC-MS/MS with ACQUITY UPLC H-Class and XEVO TQD triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) interface were used to analyze mirabegron and tolterodine. Data were processed using Masslynx 4.1 (Waters Corp., Milford, MA, USA) software. Water (containing 0.1% formic acid) was chosen as the aqueous portion of the mobile phase, and acetonitrile was selected as the organic phase in our study. Separation was achieved in 2.5 min by gradient elution using a UPLC® BEH C18 column (2.1mm × 50 mm, 1.7 µm). The column was kept at 40°C with a flow rate of 0.35 mL/min. The injection volume was of 2 µL. Elution was in a linear gradient. In the progress of elution, acetonitrile content was changed from 45 to 95% between 0.3 and 1.3 min. Organic phase acetonitrile was maintained at 95% for 1 min. Then in the next 0.2 min, it was decreased to 45%. The positive ionization mode was used for compound ionization with nitrogen (flow rate of 800 L/h, cone gas of 50 L/h) as the desolvation gas. Argon was used as the collision gas. Table 1 showed the main working parameters of the mass spectrometer. The MRM transitions were m/z 397.3→379.6 and m/z 326.4→121.0 for Mirabegron and IS, respectively (Fig.1).

Standard solutions, quality control (QC) sample, and calibration standards

A total of 10 mg Mirabegron and tolterodine were dissolved respectively in methanol to obtain a concentration of 2.00 mg/mL and 1.0mg/mL standard stock solution. The stock solutions of the standards were further diluted in methanol to produce combined standard working solutions at a series of concentrations. The concentrations of Mirabegron QC samples in rat plasma were 25, 500, 2000 ng/mL. The IS working solution was diluted from the 1.00 mg/mL tolterodine stock solution to make the final concentration at 250 ng /mL. The lower limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curves. The LOD was defined as a signal/noise ratio > 3. The concentration levels of plasma calibration standards were 5, 10, 50, 100, 250, 500, 1000, and 2500 ng/mL. They were prepared by spiking 10 µL working standard solutions into 90µL blank plasma (vortexed for 30 s). All stock solutions were kept at −40 °C until to use.

TABLE 1. MASS-TO-CHARGE (M/Z) VALUES FOR PROTONATED MIRABEGRON AND TOLTERODINE OBTAINED BY ESI+ AND MASS TRANSITIONS USED FOR QUANTIFICATION IN THE MRM MODE.

Drug	[M+H]+	Mass transition	Capillary	Cone voltage	Collision
	(m/z)	(m/z to m/z)	(KV)	(V)	energy
Mirabegron	397.34	397.34→379.67	0.7	35	15
Tolterodine	326.47	326.47→121.07	1	50	30

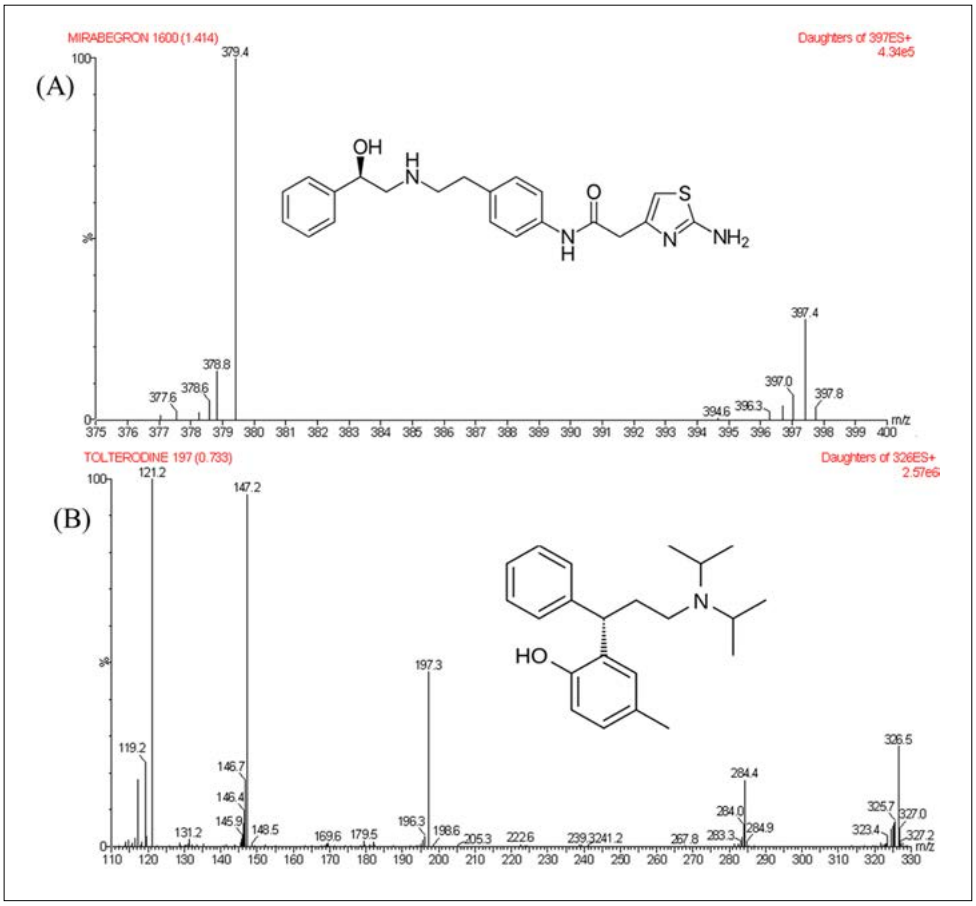

FIGURE 1. The mass spectrum and chemical structures of Mirabegron and IS in the present study: (A) Mirabegron; (B) Tolterodine (IS).

Sample preparation

Deproteinization using acetonitrile was applied as a reliable and straightforward technique for sample preparation. All frozen plasma samples were thawed and vortex-mixed before analysis. Plasma 100 µL followed by 30µL IS (50 ng /mL) and 200 µL acetonitrile were added into fresh 1.5 mL clean EP tubes. The tubes were vortexed thoroughly for 2.0 min to mix well and then spun in a centrifuge at 13000 rpm for 10 min. The upper organic phase 100 µL was carefully transferred into 0.5mL clean glass tubes and was diluted with an equal volume of water. Then 2 µL supernatant was put for analysis by UPLC–MS/MS system after vortex mixed.

Method validation

According to the Guidance for Industry, Bioanalytical Method Validation of the Food and Drug Administration (USFDA)[12] and European Medical Agency (EMA) guidelines[13] of bioanalytical method validation, selectivity, linearity, precision, accuracy, recovery, stability and matrix effect were the items of validation.

Mixed blank plasma from ten rats was used for the evaluation of the selectivity of the method towards endogenous plasma matrix. The chromatograms of a blank sample, a blank plasma sample spiked with Mirabegron and IS, and a rat plasma sample 15 min after oral administration of a single dosage of 20.0 mg/kg Mirabegron are shown in Fig.2.

To evaluate the linearity, concentrations with seven points of Mirabegron were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least squares linear regression on three consecutive days.

The precision and accuracy of the method were assessed by determining QC samples in rat plasma at different concentrations (25, 500, 2000 ng/mL for Mirabegron in rat plasma) on three separate days. Precision was expressed by the intra- and inter-day relative standard deviation (RSD), required to be less than 15%. Accuracy was expressed as the relative error (RE = measured value/true value-1) with an acceptance criterion of±15% for all QC samples.

Peak area ratios of Mirabegron from plasma samples spiked with a known concentration of unextracted samples and the extraction of the blank plasma at the same concentration at three QC levels (25ng/mL, 500ng/mL, 2000ng/mL) were used as the percent extraction recoveries.

Stability was examined in all matrices at 3 QC concentrations (n=5) under different conditions: at room temperature for 12 h, in the autosampler at room temperature for 12 h, on storage at −40 °C for 30 days, and through three complete freeze-thaw cycles. All of the stability testing QC samples were determined by using the calibration curve of freshly prepared standard samples.

The matrix effect (ME) was evaluated by comparing the ratio (A/B *100%) of peak response of Mirabegron containing an equivalent amount both extracting from blank plasma (A) and dissolved in pure standard solution (B).

Application to a pharmacokinetic study

The pharmacokinetic study was carried out in ten healthy male Sprague-Dawley rats (250 ± 20 g) obtained from the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China). All ani-

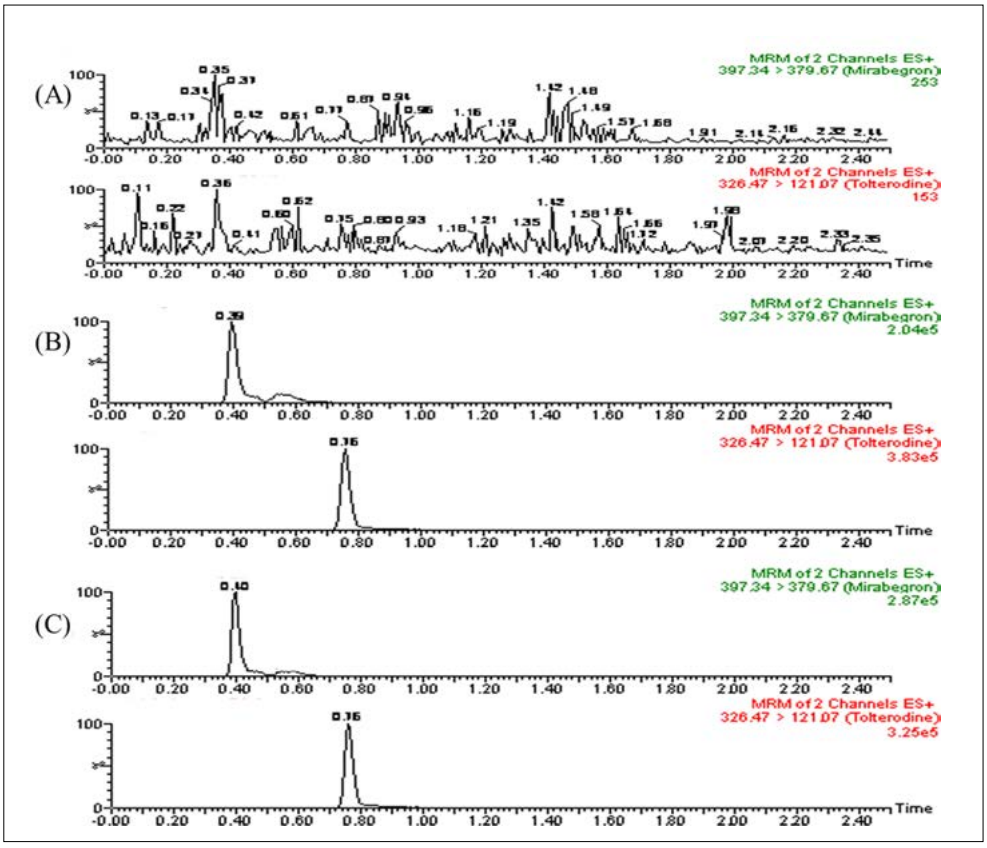

FIGURE 2. Representative chromatograms of Mirabegron and IS in rat plasma samples. (A) a blank plasma sample; (B) a blank plasma sample spiked with 50 ng/ml Mirabegron and IS; (C) a rat plasma sample 15 min after oral administration of a single dosage of 20.0 mg/kg Mirabegron.

TABLE 2. PRECISION, ACCURACY, AND RECOVERY FOR MIRABEGRON OF THE QUALITY CONTROL SAMPLE IN RAT PLASMA (N = 6).

Analyte	Concentration(ng/mL)	Found(ng/mL)	CV(%)		Accuracy RE(%)	Recovery(%)
			Intra-Day	Inter-Day		
Mirabegron	25	23.8573±2.75	11.06	11.43	-4.61	84.95
	500	495.50±14.71	2.98	3.83	-0.90	92.81
	2000	2001.82±12.54	0.97	0.81	0.09	93.26

mals were housed in an environmentally controlled room which was maintained at a temperature of 20 ± 5°C and a relative humidity of 50-60%, with natural light-dark cycles. The rats were allowed to adapt to these conditions for at least one week. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical University and followed the Guide for the Care and Use of Laboratory Animals. All rats were randomly divided into two groups (n=5 per group): Group A (oral administration of mirabegron at 20 mg/kg), Group B (intravenous administration of mirabegron at 10 mg/kg). Rats fasted for at least 12 h before mirabegron administration, but water was freely available. The blood samples(0.5 ml) were collected into heparinized tubes from the tail vein at 0, 0.083, 0.167,0.333, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24h after oral or intravenous administration of mirabegron (dissolved in normal saline, homogenized at 36°C for 30 min). The samples collected were then centrifuged at 13000 rpm for 10 min immediately. Plasma samples separated from whole blood were transferred into 0.5 mL clean centrifuge tubes as soon as possible and stored at -40 °C until analysis. The pharmacokinetic parameters were evaluated by non-compartmental modeling using DAS version 3.0 (Bontz Inc., Beijing, China).

RESULTS AND DISCUSSION

Method development and optimization

Different columns, such as UPLC® BEH C18 (2.1 mm × 50 mm, 1.7μm), UPLC BEH C18 Column(2.1 mm × 100 mm, 1.7 μm), UPLC® BEH HILIC (2.1 mm × 50 mm, 1.7 μm) were compared for chromatographic separation. The UPLC® BEH C18 (2.1mm × 50 mm, 1.7 μm) column demonstrated satisfactory chromatographic results with minimal matrix effects and proper retention time for mirabegron and tolterodine over other columns. The mobile phase played a critical role in achieving good chromatographic behavior and appropriate ionization[14,15]. To produce the best response, sensitivity, separation efficiency, and appropriate ionization, several mobile phase systems were tested to identify the optimal mobile phase. The mobile phase systems of acetonitrile-water and methanol-water at various ratios were tested, and different buffers including formic acid, acetic acid, and ammonium acetate were evaluated. Finally, a mobile phase of acetonitrile and formic acid (0.1%) in water was selected as the best solvent mixture. Various compounds (oxybutynin, tolterodine, solifenacin, midazolam) were tested to decide on a suitable IS which gave satisfactory validation results of UPLC quantification. Finally, we select tolterodine as IS for its proper retention time and favorable peak shape at the optional condition. Multiple reaction monitoring (MRM) mode was used as MS method for quantification of mirabegron and IS, and the electrospray ionization (ESI) source was operated in both positive and negative modes for ion detection. The ion source parameters were optimized to get a proper response. Solid-phase extraction and Liquid-liquid extraction are effective means to improve the sensitivity and robustness of assays[16,17]. However, both of them are time-consuming and expensive and may result in environmental pollution. In our work, we offered sample preparation with a simple protein precipitation method of plasma protein by acetonitrile, which can reduce the sample preparation time when compared with that of solid-phase extraction or liquid-liquid extraction. The whole separation of the analyte and IS was completed within only 2.5 min per sample, which was much quicker than that of previously proposed methods. Mirabegron and IS were eluted at about 0.39 and 0.76 min, respectively.

Calibration curve and sensitivity

The standard calibration curves for mirabegron in rat plasma exhibited excellent linearity over the concentration range 5-2500 ng/mL (r^2> 0.999) using weighted least square linear regression analysis with a weight factor of $1/x^2$. A typical equation of the calibration curve is: y= 0.000220848 x + 0.00239281, r = 0.999942, r^2 = 0.999884, where y represents the ratios of mirabegron peak area to that of IS and x represents the plasma concentration. Fig.2 showed the representative chromatograms of mirabegron and IS in the rat plasma sample. No endogenous interference at the retention time of analytes and the IS was observed. The LLOQ and LOD for determining mirabegron in plasma was 5 ng/mL and 1 ng/mL, respectively.

Precision, accuracy, recovery, and matrix effect

Assay performance data were presented in Table 2. Intra-day precision was 11.06% or less, and the inter-day precision was 11.43% or less at three levels

of mirabegron. The accuracy of the method ranged from -4.61 to 0.09 at each QC level. The mean recoveries of mirabegron extracted from plasma were 84.95%, 92.81%, 93.26% at concentrations of 25, 500 and 2000 ng/mL (Table 2), respectively. The results demonstrated that the values were within the acceptable range and the method was accurate and precise. The matrix effects determined at concentrations of low, medium, high for mirabegron were 89.32% (RSD %, 9.16), 90.23 % (RSD %, 4.11), and 95.41 % (RSD %, 1.60), respectively. The matrix effect for IS (250 ng/ml) was 91.2% with the RSD of 4.55% (n = 6). As a result, the matrix effect from plasma was negligible in this method. These results indicated that the method was reproducible.

TABLE 3. SUMMARY OF STABILITY OF MIRABEGRON UNDER VARIOUS STORAGE CONDITIONS (N = 5).

Condition	Concentration(ng/mL)		CV(%)	Accuracy(RE%)
	Nominal	Found		
Ambient,12h	25	23.12±2.76	11.97	-7.51
	500	512.05±17.13	3.34	2.41
	2000	2017.22±22.72	1.13	0.86
Autosample, ambient, 12h	25	23.01±2.33	10.11	-7.95
	500	509.67±19.40	3.81	1.93
	2000	2017.48±15.10	0.75	0.87
Three freeze-thaw	25	22.35±2.87	12.86	-10.59
	500	505.74±18.39	3.64	1.15
	2000	2005.19±16.07	0.80	0.26
−40 °C, 50 days	25	22.94±3.14	13.71	-8.26
	500	505.14±24.29	4.81	1.03
	2000	1988.28±17.01	0.86	-0.59

TABLE 4. THE MAIN PHARMACOKINETIC PARAMETERS AFTER ORAL ADMINISTRATION OF 20.0 MG/KG MIRABEGRON AND INTRAVENOUS ADMINISTRATION OF 10.0 MG/KG MIRABEGRON IN THE PLASMA OF TEN RATSPLASMA.

Parameters	Mirabegron (oral)	Mirabegron (intravenous)
$t_{1/2}$ (h)	3.207±0.415	4.471±0.571
T_{max}	3.000±0.000	0.083±0.000
C_{max} (ng/mL)	829.06±38.245	9643.37±3812.36
$AUC_{0\to t}$ (ng/mL h)	4214.66±1068.33	13776.37±4409.77
$AUC_{0\to\infty}$ (ng/mL h)	4243.65±1081.86	13990.66±4465.37
$MRT_{0\to t}$ (h)	5.234±0.341	3.961±0.789
$MRT_{0\to\infty}$ (h)	5.389±0.376	4.378±0.882
CLz/F (L/h/kg)	4.99±1.367	0.774±0.237

STABILITY

All stability studies of mirabegron in rat plasma were conducted at three concentration levels (25, 500 and 2000 ng/mL). The results of stability tests of mirabegron in rat plasma are summarized in Table 3 and are well within the acceptable limit. Mirabegron was demonstrated to be stable after being placed at room temperature for 12 h, in the autosampler at room temperature for 12 h, stored at −40 °C for 30 days, and after three complete freeze-thaw cycles. Moreover, the established method was suitable for the pharmacokinetic study.

Application of the method in a pharmacokinetic study

The pharmacokinetic study dosing of mirabegron (dissolved in normal saline) via oral and intravenous administration was performed successfully to validate UPLC-MS/MS method. The plasma concentration-time curves of mirabegron are shown in Fig.3. A non-compartmental model was applied to calculate the following pharmacokinetic parameters: t1/2, Tmax, Cmax, AUC, CL. All parameters were summarized in Table 4. It was found that oral administration of mirabegron had a much lower Cmax(829.06 ± 38.245ng/mL) , AUC0-t (4214.66 ± 1068.33 ng/mL h) , AUC0→∞ (4243.65 ± 1081.86 ng/mL h) than that of intravenous administration (9643.37 ± 3812.36 ng/mL , 13776.37 ± 4409.77 ng/mL h and 13990.66 ± 4465.37) in rat plasma. According to the formula to calculate F (%) = AUC oral/AUC intravenous × 100%. The F Value of Oral administration absolute bioavailability is 15.17%. The Tmax, the clearance CL/F of oral mirabegron was 36-folds and 6.45-folds than that of intravenous administration, respectively.

CONCLUSION

A rapid, sensitive and accurate UPLC–MS/MS method for quantifying mirabegron in rats was established for the first time and validated for linearity, accuracy, precision, recovery, and stability.

The validation has proved this method is reproducible, sensitive, and robust. To the best of

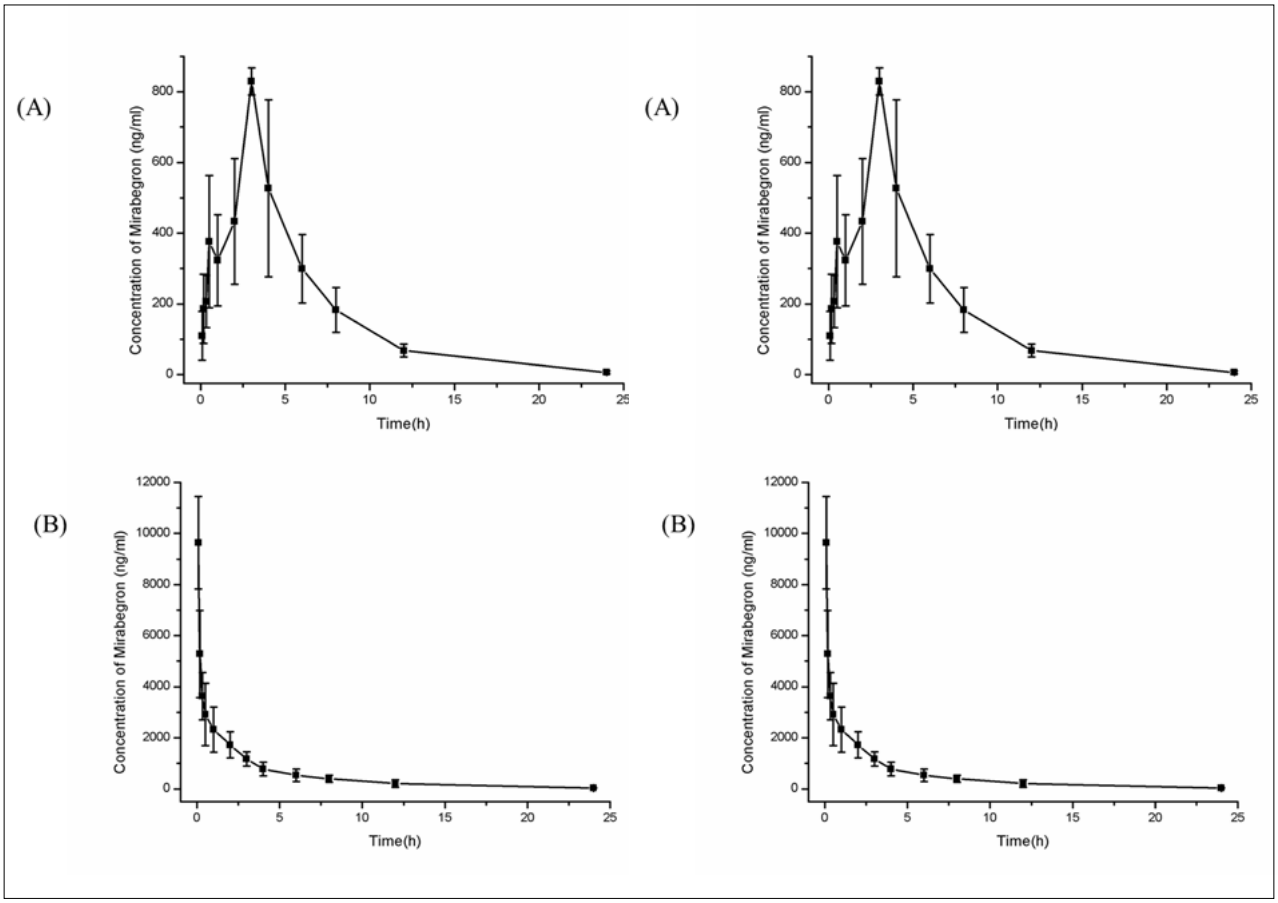

FIGURE 3. Mean plasma concentration time profile after oral administration of 20.0 mg/kg Mirabegron and intravenous administration of 10.0 mg/kg Mirabegron in ten rats.

our knowledge, this is the first report of determining the mirabegron level in rat plasma using a UPLC-MS/MS method. The LLOQ of 5 ng/mL for mirabegron in plasma was achieved, and a simple protein precipitation procedure was developed with average extraction recoveries over 84.95% for each analyte. Compared to other studies, one of the main advantages offered by the method developed is the shorter running time which meets the requirement of high throughput in bioanalysis. To sum up, the validated method has been successfully applied to the pharmacokinetic study of mirabegron in rats, and we suggest it could be applied to human pharmacokinetic studies in the near future.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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RESUMO

Mirabegron é um tipo de agonista do receptor adrenérgico beta 3 que demonstra eficácia no tratamento de bexiga hiperativa. Nesta pesquisa, o método UPLC-MS/MS é desenvolvido e validado para o estudo da farmacocinética mirabegron em ratos. Um método de precipitação de proteínas é aplicado para a preparação de amostras com acetonitrilo. 397.3 → 379.6 M / Z, M / Z 326.4 → 121.0 para mirabegron, tolterodina (IS), respectivamente, para o íon positivo foi realizado para quantificação. O método é fiável e reprodutível em nosso estudo (precisão intradia ≤ 11,06%; precisão entredia ≤ 11.43%), com curvas de concentração linear de 5 a 2 ng/ml (R2 > 0,999). Estudos de estabilidade demonstraram que mirabegron permanece estável sob uma variedade de condições de armazenamento. Este método foi aplicado com sucesso para a determinação de mirabegron em ratos após administração oral e intravenosa.

PALAVRAS-CHAVE: *Bexiga urinária hiperativa. Agonistas de receptores adrenérgicos beta 3. Plasma. Administração intravenosa. Administração oral. Ratos.*

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