Department of Pharmacy[1], Chinese PLA General Hospital; Department of Pharmaceutical Sciences[2], Beijing Institute of Radiation Medicine, Beijing, China

# Validation of a LC/MS/MS method for the quantitation of a new gastroprokinetic agent SHR116958 and its metabolite in rat plasma

Xin Li[1], Lina Du[2], Chenyun Wang[1]

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*Lina Du, PhD, Department of Pharmacy, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China*
*dulina@188.com*

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A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was succesfully developed for the simultaneous determination of SHR116958 (a new gastroprokinetic agent) and its metabolite in rat plasma under the condition that tamsulosin was used as the internal standard. The analytes and internal standard were extracted by liquid–liquid extraction (LLE). After electrospray ionization, positive ion fragments were detected in the multiple reaction monitoring (MRM) mode with a triple quadrupole tandem mass spectrometer. The method was linear in the concentration range of 2.71–5560 ng · $mL^{-1}$ with an average correlation > 0.99 for both SHR116958 and its metabolite. Moreover, the method was validated according to FDA guidance in terms of accuracy and precision, in the meanwhile stability of compounds was well established in a battery of studies, i.e., bench-top, autosampler and long-term storage stability as well as freeze/thaw cycles. Therefore, the method proved to be suitable for pharmacokinetics study of SHR116958 and its metabolite in rat.

## 1. Introduction

It's well known that 5-hydroxytryptamine (5-HT) has proven to be an important mediator of gastrointestinal motility. Selective 5-$HT_4$ agonist, such as mosapride, can be served as gastroprokinetic agent(Kato et al., 1995), which accelerates gastric emptying and is used for the treatment of acid reflux(Ruth et al., 1998), irritable bowel syndrome and functional dyspepsia(Mizuta et al., 2006). However, mosapride has side effect such as cardiotoxicity in clinical use. A domestic pharmaceuticals enterprise has developed a new 5-HT4 agonist SHR116958, an analogue of mosapride. It was reported that SHR116958 was well tolerated within the pharmacodynamic dose limits (Zhao et al.), and it was likely to be reported to a higher authority as a new drug with prokinetic effects on gastrointestinal tracts. From the results of the twitch inhibition mediated by 5-HT4 receptor in electrically stimulated ileum of guinea pig, it turned out that the mechanism of SHR116958, similar to which of mosapride, would be to facilitate the release of acetylcholine by stimulating 5-HT4 receptors from cholinergic interneurons and myenteric nerve plexus in gut, to increase gastrointestinal activity, and eventually to improve the conditions of the patients suffered from functional dyspepsia. The metabolite of SHR116958 is also active compound. However, there was no reports on pharmacokinetics study of SHR116958. In this paper, a liquid chromatography–tandem mass spectrometry (LC/MS/MS) method for quantitation of SHR116958 and its active metabolite in rat plasma was validated. This method has been used to successfully support pharmacokinetics study of i.v. formulations of SHR116958.

## 2. Investigations, results and discussion

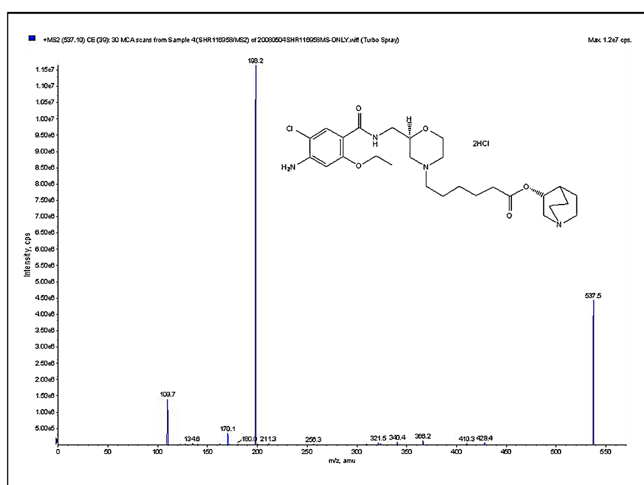
### 2.1. Bio-analytical method validation

#### 2.1.1. LC-MS-MS optimization

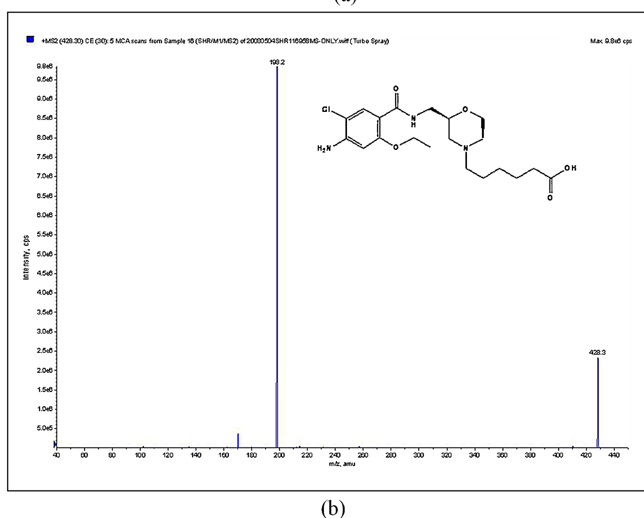
SHR116958 and its metabolite are both alkaline compounds, therefore, the mass spectrometer was operated in the positive ion multiple reaction monitoring mode during LC-MS-MS analysis. ESI spectra revealed high signals for *m/z* 537.5 (SHR116958) and *m/z* 428.5 (metabolite). The full scan spectra were dominated by protonated molecules $[M+H]^+$. The product ion mass spectrum of two protonated molecular ions is shown in Fig. 1, in which the most intense product ions were observed at *m/z* 198.2 (SHR116958) and *m/z* 198.2 (metabolite). By monitoring the product ions, a highly sensitive assay for SHR116958 and its metabolite was developed. The additional tuning of ESI source parameters onto the transition *m/z* 537.5→198.2 (SHR116958) and *m/z* 428.5→198.2 could further improve the sensitivity.

#### 2.1.2. IS optimization

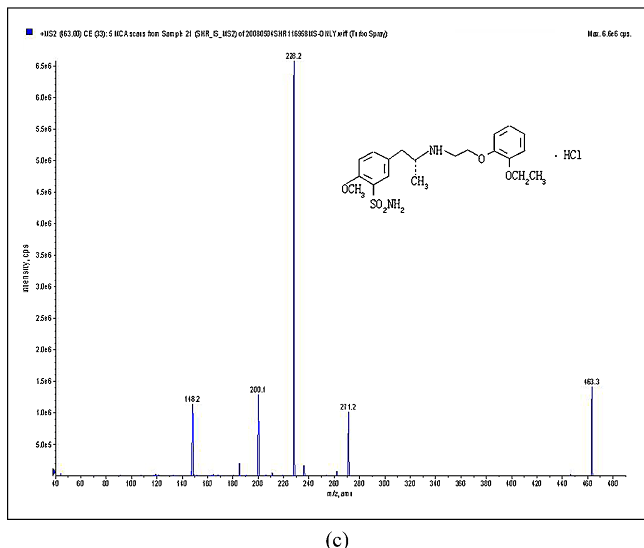
Stable isotope labeled compounds of the analytes would be the ideal internal standards, which should be commercially available with a high chemical similar chromatographic behaviors, mass spectrometric behaviors and extraction characteristics. Therefore, tamsulosin hydrochloride was chosen as I.S. for SHR116958 and its metabolite. The transition 462.8$[M+H]^+$→ 228.2 was selected (Fig. 1), considering that it showed the highest selectivity and signal-to-noise ratio. In the Fig. 2, no I.S.




(a)



(b)



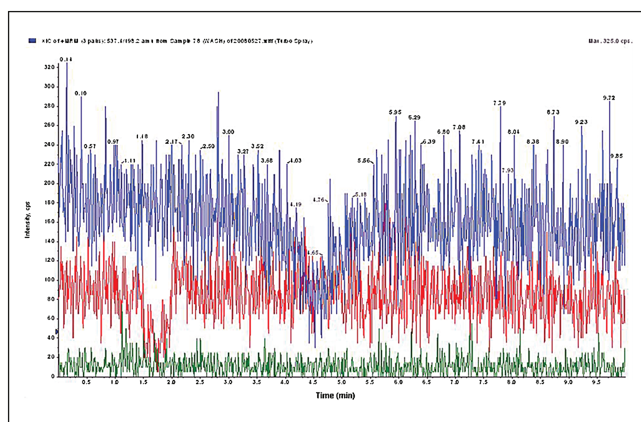

(c)

Fig. 1: Positive ESI mass spectrum of SHR116958 (a), its metabolite (b) and tamsulosin hydrochloride-IS (c)

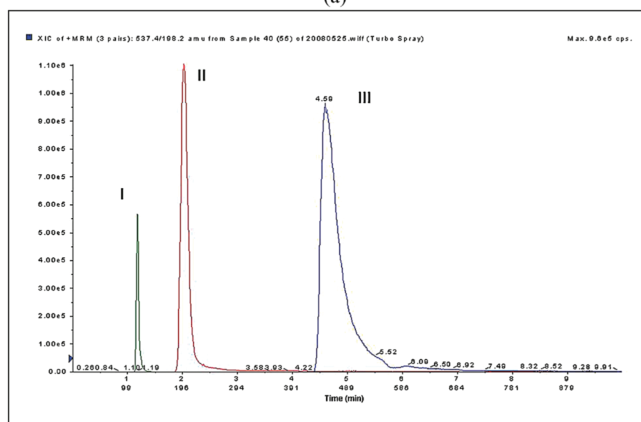
interference and no additional ion suppression were observed. Thus, it is a suitable I.S. for quantitative analysis in this assay.

### 2.1.3. *Specificity*

No significant interfering peaks were observed at the retention times of SHR116958, its metabolite and I.S. in drug-free human plasma samples for analysis. Representative chromatograms of drug-free blank plasma were depicted in Fig. 2.




(a)




(b)

Fig. 2: Representative chromatogram (a) blank plasma: SHR116958 (blue), its metabolite (green), tamsulosin hydrochloride-IS (red), (b) subject sample, (I): metabolite; (II): tamsulosin hydrochloride (IS); (III): SHR116958

### 2.1.4. *Sensitivity and linearity*

For this method, the lower limit of quantitation (LLQQ) was 2.71 ng · $mL^{-1}$ for both SHR116958 with a S/N above 6 and its metabolite with a S/N above 8. Linearity was evaluated by performing linear regression with $1/x^2$ weighing using all acceptable calibration standard curve points. The mean coefficient of determination ($r^2$) during the validation (n = 6) was 0.994 (0.001 S.D.) for SHR116958 ($y = (6 \pm 4)10^{-3}\ x + 0.13 \pm 0.08$) and 0.998 (0.001 S.D.) for metabolite ($y = (5.44 \pm 0.5)10^{-4}\ x + (4.67 \pm 0.9)10^{-3}$), respectively. The calibration range was from 2.71–5560 ng · $mL^{-1}$ for both SHR116958 and its metabolite. Representative chromatograms of samples are presented in Fig. 2. The peak III in this figure exhibited as slowly resolute peaks with big tailing factor. It should be noted that such results may be caused by contamination of column head, overload, unsuitable mobile phase, and so on.

### 2.1.5. *Recovery from plasma matrix*

The absolute extraction recoveries of the analytes from plasma were estimated by calculating the ratios of the peak areas of the samples spiked after extraction to the raw peak areas of spiked samples before extraction. The mean extraction recoveries of SHR116958 and its metabolite were more than 35.26% and 36.42%, respectively. The results are shown in Table 1.

**Table 1: Extraction recoveries of SHR116958, its metabolite and I.S. from rat plasma[a]**

| | QC1 | | QC2 | | QC3 | |
|---|---|---|---|---|---|---|
| | SHR116958l | Metabolite | SHR116958l | Metabolite | SHR116958l | Metabolite |
| Concentration | 5 ng/ml | 5ng/ml | 300 ng/ml | 300ng/ml | 3000ng/ml | 3000ng/ml |
| Peak area[b] (A) | 122000 | 9980 | 1040000 | 1320000 | 7520000 | 3710000 |
| Peak area[c] (B) | 43017 | 3720 | 366912 | 172947 | 2764352 | 1351182 |
| Recovery[d] (%) | 35.26 | 37.28 | 35.28 | 39.04 | 36.76 | 36.42 |
| CV (%) | 7.83 | 10.84 | 9.51 | 5.39 | 9.83 | 13.43 |

[a] n = 5
[b] Standard spiked before extraction
[c] Standard spiked after extraction
[d] Extraction recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma after extraction (B) to the mean peak area of the analytes spiked into plasma before extraction (A)

**Table 2: Intra- day and inter-day accuracy and precision of SHR116958 and its metabolite in rat plasma**

| | QC1 | QC2 | QC3 | | | |
|---|---|---|---|---|---|---|
| | SHR116958l 5 ng/ml | Metabolite 5 ng/ml | SHR116958l 300 ng/ml | Metabolite 300 ng/ml | SHR116958l 3000 ng/ml | Metabolite 3000 ng/ml |
| Intra-day (n = 6) | | | | | | |
| Mean conc. founded | 5.13 | 5.138 | 329.6 | 293.8 | 3146 | 3066 |
| CV (%) | 7.84 | 9.64 | 8.34 | 4.9 | 6.12 | 7.79 |
| Accuracy (%) | 102.68 | 102.76 | 109.87 | 97.93 | 104.87 | 102.2 |
| Inter-day (n = 3) | | | | | | |
| Mean conc. founded | 5.15 | 5.64 | 308.93 | 296.93 | 3036.33 | 3062 |
| CV (%) | 7.85 | 10.0 | 8.45 | 5.0 | 6.09 | 7.8 |
| Accuracy (%) | 103.01 | 102.11 | 102.98 | 98.98 | 101.21 | 102.07 |

### 2.1.6. *Accuracy and precision*

Data for intra- and inter-day precision and accuracy of the assay are summarized in Table 2. The intra-day accuracy ranged between 102.68 and 109.87% with a precision of 0.16–5.95%, while the inter-day between 101.21 and 103.01% with a precision of 6.09–8.45% for SHR116958. Moreover, the intra-day accuracy ranged between 97.93 and 102.76% with a precision of 4.90–9.64%, while the inter-day between 98.98 and 102.11% with a precision of 5.0–10.0% for the metabolite (Table 2).

### 2.1.7. *Stability*

The results in Table 3 demonstrated that the SHR116958 and its metabolite were stable under these conditions compared to control samples that had not been subjected to the exposure.

## 2.2. *Application of the developed LC-MS/MS method*

The method described in this paper was applied to the pharmacokinetics study with over 500 plasma samples, in case that the blood samples were drawn over a period of 24 h after *i.v.* administration of SHR116958 to rats. Samples were stored at −70 °C until analyzed. Comparison of peak area ratios from the unknown samples with those from the calibration curve allowed the quantitation of the assayed samples. The examples of time-dependent concentration for SHR116958 and its metabolite were plotted in Fig. 3.

# 3. Experimental

### 3.1. *Chemicals and reagents*

Working standard of SHR116958, its metabolite and internal standard (IS) - tamsulosin hydrochloride were provided by Hengrui pharmaceutical plant (Shanghai, China). The chemical structures of analytes and IS are shown in Fig. 1. Methanol and acetonitrile (HPLC grade) were purchased from Fisher chemicals (Loughborough, UK). Ethyl acetate and ammonium acetate (analytical grade) were purchased from Hongxing chemical factory (Beijing, China). Distilled water prepared from demineralization was used throughout the study.

### 3.2. *Chromatographic conditions*

An Agilent 1100 system (Wilmington, DE, USA), consisting of a vacuum degasser, a binary pump, a column oven and an autosampler, was used for solvent and sample delivery. Chromatography was carried out using a 150 mm × 2.1 mm, particle size 5 µm Agilent Zorbax Eclipse XDB-C18 column with a flow rate of 0.3 mL · min$^{-1}$ at room temperature. The mobile phase consisted of methanol:acetonitrile:water (47:27:26, v/v) and 5 mmol · L$^{-1}$ ammonium acetate.

### 3.3. *Mass spectrometric conditions*

The analytes and I.S. were detected using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Ontario, Canada) equipped with a Turbo spray interface. Zero-air was used as

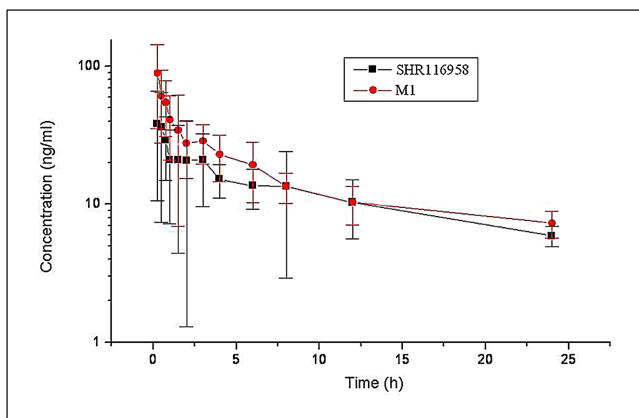



Fig. 3: Concentration-time profile for rats after *i.v.* 36 mg · kg$^{-1}$ dosing of SHR116958

**Table 3: Summary of stability of SHR116958 and its metabolite in rat plasma**

| | QC1 | | QC2 | | QC3 | |
|---|---|---|---|---|---|---|
| | SHR116958l ng/ml | Metabolite ng/ml | SHR116958l 00 ng/ml | Metabolite 00 ng/ml | SHR116958l 000 ng/ml | Metabolite 000 ng/ml |
| Room temp. (12h) | | | | | | |
| Mean conc.founded (n = 5) | 4.31 | 5.37 | 289 | 312 | 2890 | 3107 |
| CV (%) | 3.14 | 0.006 | 2.27 | 8.13 | 4.49 | 3.52 |
| Bias (%) | −13.80 | 7.40 | −3.67 | 4.00 | −3.67 | 3.57 |
| Autosampler (48h) | | | | | | |
| Mean conc.founded (n = 5) | 4.89 | 5.08 | 290 | 281 | 3103 | 2879 |
| CV (%) | 4.48 | 0.815 | 3.10 | 6.69 | 4.18 | 1.67 |
| Bias (%) | −2.20 | 1.60 | −3.33 | −6.33 | 3.43 | −4.03 |
| 4 °C (48h) | | | | | | |
| Mean conc.founded (n = 5) | 5.32 | 5.18 | 304 | 312 | 3111 | 3241 |
| CV (%) | 7.31 | 4.39 | 5.21 | 7.57 | 7.7 | 2.88 |
| Bias (%) | 6.40 | 3.60 | 1.33 | 4.00 | 3.70 | 8.03 |
| Freeze-thaw | | | | | | |
| Mean conc.founded (n = 5) | 5.13 | 5.21 | 295.8 | 307.62 | 3066 | 2895 |
| CV (%) | 5.79 | 2.63 | 3.82 | 8.43 | 1.73 | 3.03 |
| Bias (%) | 2.60 | 4.20 | −1.40 | 2.54 | 2.20 | −3.50 |
| Long term (30 day) | | | | | | |
| Mean conc.founded (n = 5) | 5.24 | 5.32 | 303 | 321 | 3002 | 3021 |
| CV (%) | 8.52 | 5.11 | 5.81 | 4.23 | 3.90 | 7.68 |
| Bias (%) | 4.80 | 6.40 | 1.00 | 7.00 | 0.07 | 0.70 |

**Table 4: Optimized parameters for MRM analysis of R116958, its metabolite and IS**

| Compound | Transition (*m/z*) | Dwell time (ms) | DP (V) | CE (eV) | EP (V) | CXP (V) |
|---|---|---|---|---|---|---|
| SHR116958 | 537.5/198.2 | 100 | 68 | 42 | 10 | 10 |
| Metabolite | 428.5/198.2 | 100 | 60 | 26 | 10 | 10 |
| Tamsulosin hydrochloride (IS) | 462.8/228.2 | 100 | 55 | 32 | 10 | 10 |

source gas while ultra high pure (UHP) nitrogen was used as both curtain and collision gases. The mass spectrometer was operated in a positive ion multiple reaction monitoring (MRM) mode. The MRM transitions for analytes and I.S. are shown in Table 1. The ion source parameters were set as follows: Collision gas = 4 p.s.i., curtain gas = 16 p.s.i., gas1 = 46 p.s.i., gas2 = 60 p.s.i., ionspray voltage = 5500 V, temperature = 600 °C. The declustering potential (DP), collision energy (CE) and other compound parameters for the analytes as well as I.S. were optimized individually as shown in Table 4.

### *3.4. Calibration standards and quality control (QC)*

Standard sample, QC sample stock solutions and I.S. were prepared from solid powders that dissolved into mobile phase. Stock solutions were stored at approximately 4 °C. Standard curve and QC samples were prepared by diluting stock solutions with blank rat plasma. The concentrations of standard curve and QC samples were summarized in Table 5.

### *3.5. Sample preparation*

Prior to assay, frozen rat plasma samples were thawed at ambient temperature and centrifuged at 3000 rpm for 5 min at 4 °C to precipitate solids. In the following order, 0.025 mL of the I.S. solution (0.2 μg · $mL^{-1}$) was added into each 2 mL Eppendorf tube except for blank plasma. 0.1 mL of standard samples, QC samples, study samples and blank plasma were transferred into the eppendorf tubes. Ethyl acetate 1.2 mL was then added to each tube. The mixture was vortexed for approximately 8 min, then centrifuged at 8000 rpm for 5 min to separate the organic phase from the aqueous layer. The supernatant was evaporated to dryness under a slightly heated stream of nitrogen at approximately 40 °C. The residue was reconstituted with 0.05 mL mobile phase, and transferred to an autosampler vial, in which 25 μL of sample was injected into the LC-MS-MS system.

### *3.6. Data acquisition and analysis*

Data acquisition was performed using Analyst 1.4 software (Applied Biosystems-SCIEX). Calibration curves were constructed using the peak area ratios of analytes to I.S. by weighted ($1/x^2$) least-square linear regression. Test samples and quality control samples were then interpolated from the calibration curve to obtain the concentrations of the respective analytes.

### *3.7. Method validation*

Analytical method validation was performed in accordance with the recommendations published by the FDA. In brief, specificity was ascertained by analyzing five blank human plasma samples. Five sets of calibration curves ranging from 2.71 to 5560 ng · $mL^{-1}$ for both SHR116958 and its metabolite were constructed by plotting the peak-area ratios of analyte/I.S. *versus* analyte concentrations in blank human plasma. The intra-day pre-

**Table 5: Standard solution concentrations**

| Standard and QCs | SHR116958 (ng/ml) | Metabolite (ng/ml) |
|---|---|---|
| Standard 1 | 2.71 | 2.71 |
| Standard 2 | 5.42 | 5.42 |
| Standard 3 | 10.85 | 10.85 |
| Standard 4 | 21.7 | 21.7 |
| Standard 5 | 43.4 | 43.4 |
| Standard 6 | 86.8 | 86.8 |
| Standard 7 | 174 | 174 |
| Standard 8 | 347 | 347 |
| Standard 9 | 694 | 694 |
| Standard 10 | 1390 | 1390 |
| Standard 11 | 2780 | 2780 |
| Standard 12 | 5560 | 5560 |
| QC1 | 5 | 5 |
| QC2 | 300 | 300 |
| QC3 | 3000 | 3000 |

cision and accuracy were estimated by analyzing five replicates at three different QC levels. The inter-assay precision was determined by analyzing QC samples of three levels on five different runs. The accuracy was expressed by (mean observed concentration)/(spiked concentration) $\times$ 100% and the precision by relative standard deviation (RSD). The extraction recoveries of SHR116958 and its metabolite of three QC levels were determined by comparing peak area of the analytes obtained from plasma samples with the analytes spiked before extraction to those spiked after the extraction. QC samples prepared to test stability were subjected to short-term (12 h) room temperature, 48 h autosampler at 18 °C, 48 h refrigeration at 4 °C, two freeze-thaw cycles at $-70 \pm 5$ °C, and long term at $-70 \pm 5$ °C for 30 days stability tests.

### 3.8. Application of the assay

Plasma samples from eighteen rats were collected predose (blank plasma) and after i.v. administration of SHR116958.

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