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Development and validation of an LC–MS/MS method for the determination of tolvaptan in human plasma and its application to a pharmacokinetic study

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ABSTRACT

Tolvaptan is a selective vasopressin V_2-receptor antagonist mainly used for the treatment of hyponatremia. This study described the development and validation of an LC–MS/MS method for the determination of tolvaptan in human plasma. Sample preparation involved protein precipitation with acetonitrile containing 2-demethyl tolvaptan (internal standard, IS). Chromatographic separation was performed on a Zorbax XDB C_{18} column with an isocratic mobile phase consisting of water (containing 0.1% formic acid) and methanol (25:75, v/v). Determination of the analytes was achieved by tandem-mass spectrometry with positive electrospray ionization. The multiple reaction monitoring (MRM) transitions were performed at m/z 449.2 → 252.1 for tolvaptan and m/z 435.2 → 238.1 for IS. The assay was linear over the concentration range of 0.457–1000 ng/mL, with a lower limit of quantification of 0.457 ng/mL. The intra- and inter-day precisions at three concentration levels (0.914, 111 and 800 ng/mL) were less than 15% and their accuracies were within the range of 97.7–107.8%. The mean recovery ranged from 99.2 to 104.6% and the matrix effect from 89.3 to 99.5%. Tolvaptan was stable under all tested conditions. This validated method was successfully applied to a pharmacokinetic study in healthy volunteers after oral administration of single-dose tolvaptan tablets.

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1. Introduction

Tolvaptan is an oral non-peptide selective arginine vasopressin (AVP) V_2-receptor antagonist indicated in the United States for the treatment of hypervolemic and euvolemic hyponatremia associated with heart failure, cirrhosis, and Syndrome of Inappropriate Antidiuretic Hormone (SIADH) [1].

Pharmacokinetic studies revealed that T_{max} (time to maximum plasma concentration) of tolvaptan after oral administration is about 2–4 h without any effects from food [2] and the absolute bioavailability is about 56% [3]. Tolvptan is mainly metabolized in the liver by CYP3A4 and eliminated in feces. Ketoconazole (a CYP3A4 inhibitor) and rifampicin (a CYP 3A4 inducer) both significantly influenced the pharmacokinetics of tolvaptan [4]. Tolvaptan is also a P-glycoprotein substrate which was firstly found *in vitro* transport study and confirmed by a drug–drug interaction study between tolvaptan and digoxin *in vivo* [5]. Several studies [6–8] have investigated the pharmacokinetic characteristics of tolvaptan in healthy subjects, with consistent results showing that AUC_{inf} (area under the plasma concentration–time curve from zero to infinity) of tolvaptan increased dose-proportionally.

Tolvaptan (Samsca®) was developed by Otsuka Pharmaceutical Company, and successively approved in the United States, Europe, Japan, China and so on. Recently, another manufacturer's tolvaptan tablet has been approved by the State Food and Drug Administration of China for carrying out the pharmacokinetic study. Therefore, a suitable method for determination of tolvaptan is required. To our knowledge, although a number of studies about tolvaptan pharmacokinetics have been reported [2–10], the LC–MS/MS methods for determination of tolvaptan described in these papers had several obvious shortcomings. Firstly, the LLOQs in these reports were 2 ng/mL [7], 5 ng/mL [2–6,9,10] and 10 ng/mL [8], respectively. The low sensitivity could not meet the requirement of the pharmacokinetic study of low dose tolvaptan. Secondly, the time-consuming and expensive solid-phase extraction was used for pretreatment of samples in these methods [2–10], which was not suitable for high-throughput determination in pharmacokinetic study. Finally, chromatography and validation details were not provided in these

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reports [2–10], which might have little guiding significance for reproducing or developing the determination methods of tolvaptan.

In this paper, we developed and validated a sensitive, simple and specific LC–MS/MS method for determination of tolvaptan in human plasma using protein precipitation for the first time. The method has been successfully applied to a pharmacokinetic study.

2. Experimental

2.1. Chemicals and reagents

Tolvaptan (purity 99.9%) and 2-demethyl tolvaptan standard (purity 99.8%) were supplied by Chongqing Libang Pharmaceutical Co., Ltd. (Chongqing, China). Acetonitrile and methanol of HPLC grade were obtained from Merck Co., Ltd. (Germany). HPLC grade formic acid was purchased from ROE Scientific INC (Newark, USA). Deionized water was produced with a SMART ultra-pure water system (Shanghai Canrex Analytic Instrument Co., Ltd. Shanghai, China). All other reagents were of analytical grade. Tolvaptan tablets (15 mg per tablet, Chongqing Libang Pharmaceutical Co., Ltd. China) were used in this study. Blank heparinized human plasma was collected from healthy volunteers and stored at −80 °C before use.

2.2. Instrumentation and LC–MS conditions

The LC–MS/MS system used consisted of an Shimadzu LC-20A chromatographic system including a CBM-20A system controller, two LC-20AD pumps, a SIL-20AC$_{HT}$ auto sampler and a CTO-20AC column oven (Shimadzu, Japan), and an API 4000 mass spectrometer (Applied Bio-systems, Singapore) equipped with an electrospray ionization (ESI) source system. The system control and data analysis were performed by AB Sciex Analyst software (version 1.4).

Chromatographic separation of tolvaptan and 2-demethyl tolvaptan (internal standard, IS) was achieved on a Zorbax XDB C_{18} column (150 mm × 2.1 mm, 5 μm) with a Phenomenex guard column. The mobile phase was a mixture of water (containing 0.1% formic acid) and methanol (25:75, v/v) at a flow rate of 0.3 mL/min for 3.5 min. 5 μL of sample was injected into the system by auto sampler set at 20 °C, and the column temperature was maintained at 40 °C.

The mass spectrometer was operated in an ESI positive ion mode. The multiple reaction monitoring (MRM) transitions were performed at *m/z* 449.2 → 252.1 for tolvaptan and *m/z* 435.2 → m/z238.1 for IS. Optimized values for declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP) were 80 V, 25 eV, 10 V, 15 V and 85 V, 25 eV, 10 V, 15 V for tolvaptan and IS, respectively. Other ion source conditions were as follows: Curtain Gas was 25 psi, Ion source Gas 1 was 45 psi, Ion source Gas 2 was 55 psi, IonSpary Voltage was 5500 V, source temperature was 650 °C.

2.3. Analytical procedure

2.3.1. Preparation of stock solutions, calibration standard and quality control samples

Stock solutions of tolvaptan (0.5 mg/mL) and IS (0.4 mg/mL) were prepared in methanol:water (1:1, v/v) and stored at 4 °C. A series of working standard solutions of tolvaptan ranging from 0.0457 to 10.0 μg/mL and an IS solution at 4.00 μg/mL were prepared by dilutions of their stock solutions with methanol:water (1:1, v/v) and acetonitrile, respectively. Calibration standard plasma samples were prepared as follows: 20 μL each working standard solution was mixed with 200 μL blank human plasma to obtain the concentration of 0.457, 1.37, 4.10, 12.3, 37.0, 111, 333, 1000 ng/mL. QC solutions and QC plasma samples (0.914, 111 and 800 ng/mL) were prepared in the same way.

2.3.2. Samples preparations

Plasma sample was prepared with protein precipitation. 20 μL methanol:water (1:1, v/v) or 20 μL working standard solutions and 400 μL IS solution (4.00 μg/mL) were added into 200 μL plasma samples in 2 mL Eppendorf tube. After a thorough vortex mixing for 2 min, the mixture was centrifuged at 15,700 × *g* for 10 min, and then 5 μL of the supernatant was injected into the LC–MS/MS system.

2.4. Method validation

The method validation included the determinations of specificity, matrix effects, extraction recovery, linearity, lower limit of quantitation (LLOQ), precision, accuracy, and stability.

2.4.1. Specificity and selectivity

The specificity and selectivity of the method were performed by comparing chromatograms of human blank plasma samples from six different sources, blank plasma spiked with standard, and human plasma sample after oral administration of tolvaptan tablets.

2.4.2. Matrix effect and extraction recovery

The matrix effect was determined at three QC levels (0.914, 111 and 800 ng/mL) of tolvaptan and one concentration level of IS by comparing the peak areas of analyte (A) spiked in post-protein precipitated blank plasma from six different batches with those of the pure standard solution containing an equivalent amounts of compound (B). The ratio ($A/B \times 100$) % was used to evaluate the matrix effect.

The extraction efficiency was measured by comparing the peak area of analyte (C) added into blank plasma and pretreated by protein precipitation with that of analyte (D) spiked in post-protein precipitated blank plasma at the same nominal concentrations. The ratio ($C/D \times 100$) % was defined as the extraction efficiency.

The extraction recovery of tolvaptan was carried out at the three QC levels (0.914, 111 and 800 ng/mL) in five replicates, and that of IS was determined with the same method.

2.4.3. Linearity, carry-over effect and LLOQ

Each calibration curve included a blank sample, a zero sample and eight calibration concentration levels, but blank and zero samples were only analyzed to confirm the absence of interferences and not to construct the calibration function. The blank sample was injected immediately following the calibration standard at the highest concentration in each run to monitor the carry-over effect of tolvaptan or IS. Calibration curves were fitted by the peak area ratio *vs* analyte concentrations using a 1/X weighted linear least-squares regression model. LLOQ was defined as the lowest drug concentration on the calibration curve. The back calculated standard concentrations should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. The LLOQ was evaluated by analyzing five replicates of spiked samples at the concentration of 0.457 ng/mL, and the precision and accuracy ($n = 5$) should be within ±20%.

2.4.4. Accuracy and precision

Intra- and inter-day precisions were assessed by assay of five replicates of QC plasma samples at low, medium, and high concentrations (0.914, 111 and 800 ng/mL) on the same day and on three different days. Precision was expressed as relative standard deviation (RSD, %) and accuracy was evaluated by comparing the measured concentration with the nominal concentration. For the

precision and accuracy, the acceptance criteria should be within 15% RSD and 85–115% of nominal concentration, respectively.

2.4.5. Stability

The stability of stock solutions of tolvaptan and the IS was tested at room temperature for 6 h and in 4 °C for 30 days. Stabilities of tolvaptan in human plasma were estimated by assay of three replicates of QC samples at three concentrations under the following conditions: short-term stability after storage at room temperature (25 °C) for 6 h; long-term storage stability after storage at −80 °C for 120 days; freeze–thaw stability through three freeze–thaw cycles (−80 °C to 25 °C). The post-preparative stability was examined after 24 h in the autosampler maintained at 20 °C. The measured concentrations of stabilities are compared to the nominal concentrations. The deviation should be within ±15%.

2.5. Application to pharmacokinetic study

The single-dose pharmacokinetic study was approved by the Ethical Committee of Third Xiangya Hospital of Central South University. All subjects signed the informed consent before any screening item being performed. In this clinical study, there were 6 healthy Chinese male volunteers with age of 22.5 ± 2.1 years and weight of 57.3 ± 3.6 kg, and 6 healthy Chinese female volunteers with age of 24.0 ± 0.6 years and weight of 49.8 ± 2.7 kg. They were randomly assigned to three groups and took a single oral dose of 15 mg, 30 mg and 60 mg tolvaptan tablets, respectively, with 200 ml of water after an overnight fast (10 h). Blood samples (5 mL) were collected pre-dose (0 h) and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 24, 36 and 48 h after administration. The blood samples were immediately centrifuged at 4000 rpm for 5 min and the supernatants were stored frozen at −80 °C until analysis.

The main pharmacokinetic parameters of tolvaptan were calculated by non-compartmental model using Drug and Statistics Software (DAS, version 2.1, Mathematical Pharmacology Professional Committee of China). In order to assess the dose-proportionality of C_{max} and AUC_{last}, linear regression analysis was performed on log-transformed C_{max} and AUC_{last} *versus* log-transformed dose and 95% confidence intervals (CI) of the slope were calculated. Dose proportionality was concluded if the 95% CI of the slope included the value 1. Statistical analyses were performed with SPSS software (Version 17.0 for Windows; SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Mass spectrometry

Mass spectrometric detection was performed by an API 4000 mass spectrometer equipped with an ESI. Tolvaptan and IS gave better response in the positive mode than in the negative mode. Under full-scan mode, the analytes yielded predominantly $[M+H]^+$ ions at *m/z* 449.2 for tolvaptan and *m/z* 435.2 for IS. Different DP (1–200 V) and CE (5–130 eV) values were tested, the most sensitive mass transitions were monitored from *m/z* 449.2 → 252.1 for tolvaptan with DP of 80 V and CE of 25 eV, and from *m/z* 435.2 → 238.1 for IS with DP of 85 V and CE of 25 eV (Fig. 1).

3.2. Chromatography

Various combinations of methanol, acetonitrile and buffers (formic acid and ammonium formate) on several C_{18} columns (Zorbax Eclipse XDB, Zorbax TC, Luna and AGT Venusil) were carried out to obtain suitable retention times, symmetrical peak shapes, high responses and lower matrix effects. Methanol presented higher responses than acetonitrile. Formic acid can promote the ionization of analytes and improve peak shapes. Eventually, the most suitable combination of retention times, responses and peak shapes were achieved on Zorbax XDB C_{18} column (150 mm × 2.1 mm, 5 μm) with an isocratic mobile phase consisting of water (containing 0.1% formic acid) and methanol (25:75, v/v).

Matrix effects may be reduced with a small-volume injection of supernatant of protein precipitation [11]. So, only 5 μL of the supernatant was injected into LC–MS/MS system with an adequate sensitivity. The retention times of tolvaptan and IS were 2.1 min and 2.4 min, respectively, under above chromatographic conditions. The run time was only 3.5 min, which was appropriate for determination of a large number of samples.

3.3. Sample preparation

Comparing with SPE for sample preparation presented in previous reports [2–10], one-step protein precipitation was more economical, convenient and non-time consuming, which is suitable for pharmacokinetic study with a large number of plasma samples. Acetonitrile was chosen as the protein precipitant in this study due to excellent precipitation, more symmetrical peak shape and fewer matrix effect compared with methanol and trifluoroacetic acid.

3.4. IS selection

OPC-41100 (an analog containing an additional methyl group) supplied by Otsuka Pharmaceutical Company was used as an internal standard in previous studies [2–10]. The manufacturer (Chongqing Libang Pharmaceutical Co. Ltd.) synthetized another analog of tolvaptan, 2-demethyl tolvaptan (Fig. 1). Because of their similar structure, tolvaptan and 2-demethyl tolvaptan shown the approximate chromatographic behavior, extraction recovery and matrix effect. Therefore, 2-demethyl tolvaptan was selected as the IS and high accuracy and precision of LC–MS/MS assay was presented in this study.

3.5. Method validation

3.5.1. Specificity and selectivity

The representative chromatograms of a human blank plasma, a human blank plasma spiked with tolvaptan (LLOQ, 0.457 ng/mL) and IS, and a plasma sample collected at 3 h after single oral administration of 30 mg tolvaptan tablets to a volunteer were shown in Fig. 2. Although a peak at 2 min, maybe a metabolite peak, was observed in Fig. 2C, it did not influence the quantification of tolvaptan because of its good separation from the peak of tolvaptan. Therefore, no interference from endogenous substances was observed at the retention times of tolvaptan and IS, indicating good specificity and selectivity of the method.

3.5.2. Matrix effect and recovery

The matrix effects of tolvaptan were 98.5 ± 5.4%, 93.5 ± 3.5% and 92.2 ± 2.9% at three concentrations of 0.914, 111 and 800 ng/mL, respectively. The matrix effect of the IS was 94.3 ± 4.8%. The results indicated that the matrix effects on the ionization of tolvaptan and IS were not obvious under these conditions.

The recoveries of tolvaptan at the concentrations of 0.914, 111 and 800 ng/mL were 102.5 ± 5.5%, 104.6 ± 4.1% and 99.2 ± 2.5%, respectively. The recovery of IS was 99.4 ± 4.6%. These data manifested that the recovery of tolvaptan and IS in human plasma was high and reproducible.

3.5.3. Linearity, carry-over effect and LLOQ

The method was linear over the concentration range 0.457–1000 ng/mL for tolvaptan in human plasma. The linear regression equation ($n=6$) was $Y=(7.123 \pm 0.320) \times 10^{-3}X+$

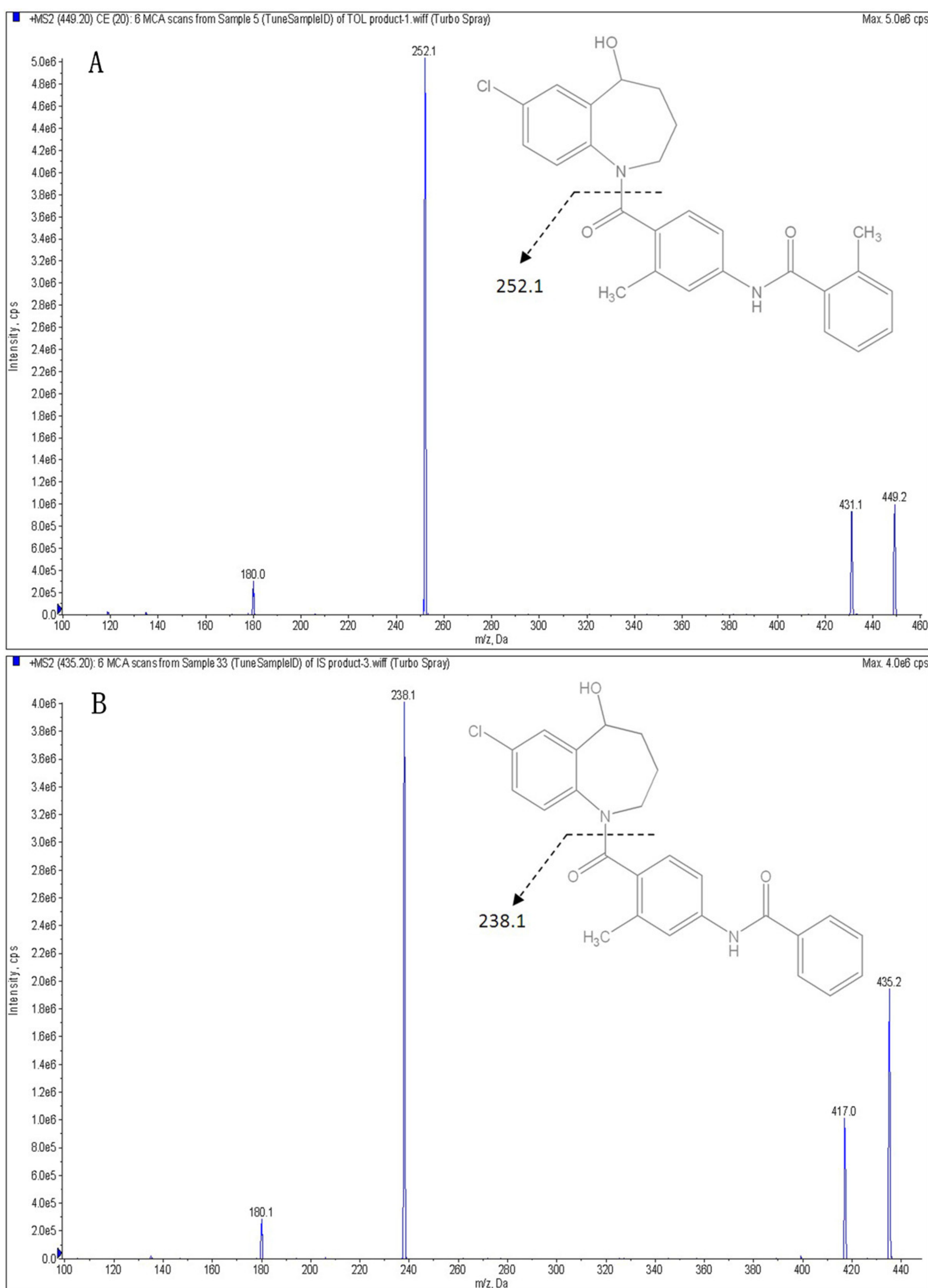

Fig. 1. The chemical structures and product ion mass spectra of the $[M+H]^+$ ions of tolvaptan (A) and IS (B).

$(5.478 \pm 4.889) \times 10^{-4}$ ($r = 0.9997 \pm 0.0002$). Peak areas at the retention time of tolvaptan were around or less than 5% of the one for the LLOQ of tolvaptan when blank samples were injected after a highest concentration sample. Also, no signal for the IS was found at the retention time and the ion channel of IS under the same conditions. This indicated that the carry-over effect was negligible [12]. The LLOQ for tolvaptan was 0.457 ng/mL and the accuracy and precision was 112.2% and 4.7%, respectively.

The LLOQs in previous reports were 2 ng/mL (7), 5 ng/mL (2–6, 9, 10) and 10 ng/mL (8), respectively. Kim et al. [7] considered that

Fig. 2. Representative HPLC chromatograms including blank human plasma (A); blank human plasma spiked with 0.457 ng/mL tolvaptan (LLOQ) and IS (B); plasma sample (135 ng/mL) collected at 3 h after single oral administration of 30 mg tolvaptan tablets to a volunteer (C).

a dose-dependent extension in the last measurable time-points for tolvaptan may account for the dose-dependent increase in $t_{1/2}$. Hence, the more accurate $t_{1/2}$ for tolvaptan in a low dose would be obtained with the lower LLOQ determination. In our study, the LLOQ was 0.457 ng/mL and sufficient for the determination up to 48 h even after administration of a single dose of 15 mg tolvaptan.

3.5.4. Precision and accuracy

The inter- and intra-day precision and accuracy of tolvaptan in human plasma at QC concentrations are presented in Table 1. The inter- and inter-day precisions (RSD, %) were <15% and the accuracies (%) were within the range of 97.7–107.8%, which demonstrated that the present method has a satisfactory precision, accuracy and reproducibility.

3.5.5. Stability studies

In terms of the stock solution stability, tolvaptan and IS were stable at room temperature for 6 h and at 4 °C for 30 days. No significant degradation was observed in human plasma storage at room temperature for 6 h, −80 °C for 120 days, or three freeze–thaw cycles at −80 °C. The post-preparative samples were also stable in the auto-sampler maintained at 20 °C for 24 h. The results of stability tests are summarized in Table 2.

3.6. Pharmacokinetic study

The newly developed LC–MS/MS method was successfully applied to determine tolvaptan in the plasma samples collected from volunteers after oral administration of tolvaptan tablets in the single-dose pharmacokinetic study. The mean plasma concentration–time profile of tolvaptan was presented in Fig. 3 and

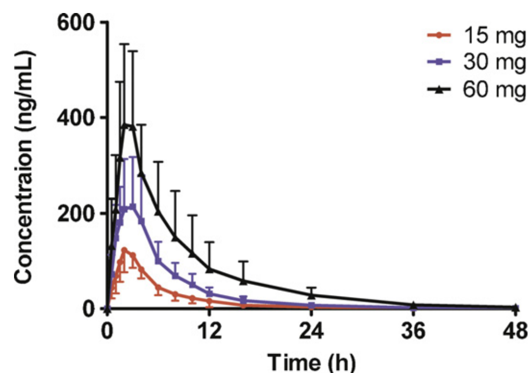

Fig. 3. Mean plasma concentration–time profiles of tolvaptan after single oral administration of 15 mg, 30 mg and 60 mg tolvaptan tablets to healthy Chinese volunteers.

Table 1
Inter- and intra-day imprecision, accuracy and recovery of tolvaptan in human plasma.

Spiked concentration (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
	Measured concentration (ng/mL)	Precision (RSD %)	Accuracy (%)	Measured concentration (ng/mL)	Precision (RSD %)	Accuracy (%)
0.914	0.893 ± 0.0362	4.1	97.7	0.940 ± 0.0467	5.0	102.8
111	119 ± 1.79	1.5	107.4	120 ± 2.53	2.1	107.8
800	841 ± 23.0	2.7	105.2	833 ± 17.6	2.1	104.1

Table 2
Stability analysis of tolvaptan under various conditions.

Concentration (ng/mL)	6 h, Room temperature ($n = 3$)		120 days, −80 °C ($n = 3$)		Three cylces, freeze/thaw ($n = 3$)		24 h, post-preparative ($n = 5$)	
	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)
0.914	6.8	2.6	−4.5	6.4	5.4	3.3	0.4	4.7
111	10.3	2.1	−1.1	1.8	8.1	1.9	8	1.2
800	3.3	1.7	−0.6	0.7	4.2	0.2	6.3	2.5

Table 3
Pharmacokinetic parameters (mean ± SD) of tolvaptan after single oral administration of 15 mg, 30 mg and 60 mg to healthy Chinese volunteers.

Parameters	Mean ± SD		
	15 mg	30 mg	60 mg
$t_{1/2}$ (h)	7.1 ± 3.8	6.8 ± 1.6	7.0 ± 1.9
T_{max} (h)	2.3 ± 0.5	2.3 ± 0.89	2.5 ± 0.6
C_{max} (ng/mL)	129 ± 36.6	250 ± 105	418 ± 166
AUC_{0-t} (ng h/mL)	758 ± 217	1570 ± 494	3360 ± 1420
$AUC_{0-\infty}$ (ng h/mL)	768 ± 215	1590 ± 490	3400 ± 1410
V (L)	219 ± 149	208 ± 98	201 ± 84
Cl (L/h)	20.7 ± 5.0	20.6 ± 6.3	19.9 ± 6. 7

the corresponding pharmacokinetic parameters were summarized in Table 3. The C_{max} and AUC of tolvaptan in our study were similar to those reported in the literatures [6–8]. The result of log–log regression analysis of AUC_{0-t} *versus* dose showed the slope is 1.051 (95% CI, 0.845–1.257), demonstrating a dose-proportional increase in the 15–60 mg dose range. Although the result of log–log regression analysis of C_{max} *versus* dose also indicated the increase in C_{max} was dose proportional (slope, 0.819; 95% CI, 0.560–1.079), the upper limit of 95% CI was only just greater than 1, indicating a trend of non-dose proportional increase in C_{max}. These results were basically in consistent with previous reports [7,8].

4. Conclusions

A sensitive, simple and specific LC–MS/MS method has been developed and validated for quantification of tolvaptan in human plasma using protein precipitation for the first time. The main advantages of the method are simple sample preparation, short analytical run time and low LLOQ. The method has been successfully applied to a single-dose pharmacokinetic study of tolvaptan in healthy volunteers.

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References

[1] Samsca (tolvaptan tablets for oral use). http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/022275s005lbl.pdf
[2] S.E. Shoaf, S.R. Kim, P. Bricmont, S. Mallikaarjun, Eur. J. Clin. Pharmacol. 68 (2012) 1595.
[3] S.E. Shoaf, P. Bricmont, S. Mallikaarjun, Int. J. Clin. Pharmacol. Ther. 50 (2012) 150.
[4] S.E. Shoaf, P. Bricmont, S. Mallikaarjun, Br. J. Clin. Pharmacol. 73 (2012) 579.
[5] S.E. Shoaf, Y. Ohzone, S. Ninomiya, M. Furukawa, P. Bricmont, E. Kashiyama, S. Mallikaarjun, J. Clin. Pharmacol. 51 (2011) 761.
[6] S.E. Shoaf, Z. Wang, P. Bricmont, S. Mallikaarjun, J. Clin. Pharmacol. 47 (2007) 1498.
[7] S.R. Kim, T. Hasunuma, O. Sato, T. Okada, M. Kondo, J. Azuma, Cardiovasc. Drugs Ther. 25 (Suppl. 1) (2011) S5.
[8] S. Yi, H. Jeon, S.H. Yoon, J.Y. Cho, S.G. Shin, I.J. Jang, K.S. Yu, J. Cardiovasc. Pharmacol. 59 (2012) 315.
[9] S.E. Shoaf, S. Mallikaarjun, P. Bricmont, Eur, J. Clin. Pharmacol. 68 (2012) 207.
[10] S.E. Shoaf, S.L. Bramer, P. Bricmont, C.A. Zimmer, J. Cardiovasc. Pharmacol. 50 (2007) 213.
[11] D.N. Heller, Rapid Commun. Mass Spectrom. 21 (2007) 644.
[12] European Medicines Agency, Guideline on Bioanalytical Method Validation, London, United Kingdom, 2011.