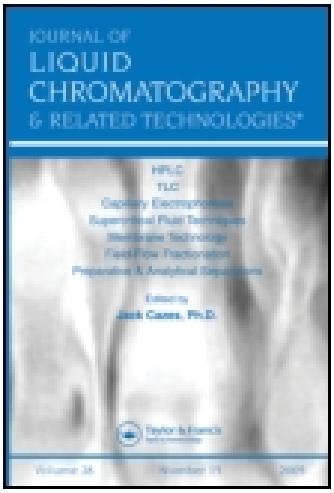

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DETERMINATION OF URAPIDIL HYDROCHLORIDE IN RABBIT PLASMA BY LC-MS-MS AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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□ *A sensitive and selective liquid chromatography tandem mass spectrometry (LC-MS-MS) method for determination of urapidil hydrochloride in rabbit plasma was developed and validated. After addition of doxapram hydrochloride as the internal standard (IS), protein precipitation by 10% trichloroacetic acid was used as the sample preparation. Chromatographic separation was achieved on a Zorbax SB-C18 (2.1 mm × 50 mm, 3.5 μm) column with acetonitrile-water as the mobile phase with gradient elution. Electrospray ionization (ESI) source was applied and operated in positive ion mode; multiple reaction monitoring (MRM) mode was used to quantification using target fragment ions m/z 387.9→ 204.6 for urapidil hydrochloride and m/z 378.9→ 291.8 for the IS. Calibration plots were linear over the range of 5–1000 ng/mL for urapidil hydrochloride in plasma. Lower limit of quantitation (LLOQ) for urapidil hydrochloride was 5 ng/mL. Mean recovery of urapidil hydrochloride from plasma was in the range 93.5%–96.4%. RSD of intra-day and inter-day precision were both less than 12%. This developed method is successfully used in pharmacokinetic study of urapidil hydrochloride in rabbit.*

Keywords LC-MS-MS, pharmacokinetics, plasma, urapidil hydrochloride

INTRODUCTION

Urapidil, 6-({3-[4-(2-methoxyphenyl)piperazin-1-yl]propyl}amino)-1,3-dimethylpyrimidine-2,4(1H,3H)-dione, is a phenylpiperazine-substituted uracil derivative.[1] It is a selective alpha 1-adrenoceptor antagonist that also has central antihypertensive action.[2–4] Urapidil given orally is effective and well tolerated when used as second-line therapy in patients with

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blood pressure inadequately controlled with other agents. Urapidil is also well suited for the treatment of hypertension in the elderly.[5,6] Intravenous urapidil is effective in the treatment of hypertensive crises.[7,8] Furthermore, lipid levels and glucose metabolism are not adversely affected and may improve with urapidil in patients with lipid or glucose abnormalities.[2]

There have been several methods published for the determination of urapidil in different biological matrices such as high performance liquid chromatography (HPLC)[9–11] and chemiluminescence (CL).[12] Veltkamp et al. developed an on-line radiometric method for determination of [14C]-urapidil in rat plasma using post-column ion-pair extraction. However, the method was not fully validated.[9] Zech et al. developed a rapid and simple method, i.e., direct injection of 100 μL of serum into the automated pre-column system, followed by HPLC with electrochemical detection, gave a detection limit for urapidil in serum of 5 ng/mL.[10] Nieder et al. reported a rapid and sensitive HPLC-method for quantitation of urapidil in human serum by liquid-liquid extraction with LLOQ of 5 ng/mL using 1.0 mL sample.[11] Yue et al. described a sensitive flow-injection (FI) chemiluminescence (CL) for the determination of urapidil in human serum and urine with a detection limit of 0.03 ng/mL.[12]

In recent years, the high sensitivity and selectivity of tandem mass spectrometry (MS-MS) had led to a growing trend of developing fast analytical methods. The mass spectrometry (MS) detection method has a much higher selectivity than ultraviolet, chemiluminescence, and electrochemical detectors, and can separate analytes from co-eluents based on their mass-to-charge ratios. In this paper, a selective and sensitive LC-MS-MS method for the determination of urapidil hydrochloride in rabbit plasma using one-step protein precipitation was developed and validated. The LC-MS-MS method was successfully applied to a pharmacokinetic study of urapidil hydrochloride after intravenous administration to rabbits.

EXPERIMENTAL

Chemicals and Reagents

Urapidil hydrochloride water solution (5 mg/mL) was purchased from Nycomed Deutschland GmbH (Singen, Germany) and doxapram hydrochloride water solution (20 mg/mL) was purchased from Jiangsu Nhwa Pharmaceutical Corporation Limited (Xvzhou, China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA). Trichloroacetic acid (analytical grade) was purchased from Shanghai Haoshen Chemistry Reagent Corporation Limited (Shanghai, China).

Instrumentation and Conditions

All analyses were performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany), and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software (Version B.01.03 [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm × 50 mm, 3.5 µm) column at 40°C, with acetonitrile–water as mobile phase. The flow rate was 0.3 mL/min. A gradient elution program was conducted for chromatographic separation with mobile phase A (water), and mobile phase B (acetonitrile) as follows: 0–1.0 min (10–90% A), 1.0–6.0 min (90–90% A), 6.0–7.0 min (90–10% A), and 7.0–10.0 min (10–10% A).

Drying gas flow and nebulizer pressure was set at 6 L/min and 20 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350°C and 3500 V, respectively. LC-MS-MS was performed with MRM mode using target ions at m/z 387.9 → 204.6 for urapidil (Figure 1a) with fragmentation energy of 0.35 v and m/z 378.9 → 291.8 for doxapram (IS, Figure 1b) with fragmentation energy of 0.25 v, in positive ion electrospray ionization interface, respectively.

Calibration Standards and Quality Control Samples

Individual stock solutions of urapidil hydrochloride (1.0 mg/mL) and doxapram hydrochloride (internal standard, IS) (400 µg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol. 0.4 µg/mL working standard solution of IS was prepared by dilution of the IS stock solution with methanol. All of the solutions were stored at 4°C and were brought to room temperature before use.

Urapidil hydrochloride calibration standards were prepared by spiking blank rabbit plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range 5–1000 ng/mL for urapidil hydrochloride in rabbit plasma (concentrations 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards, three different plasma concentrations (10, 100, and 800 ng/mL). The analytical standards and QC samples were stored at −20°C.

Sample Preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard

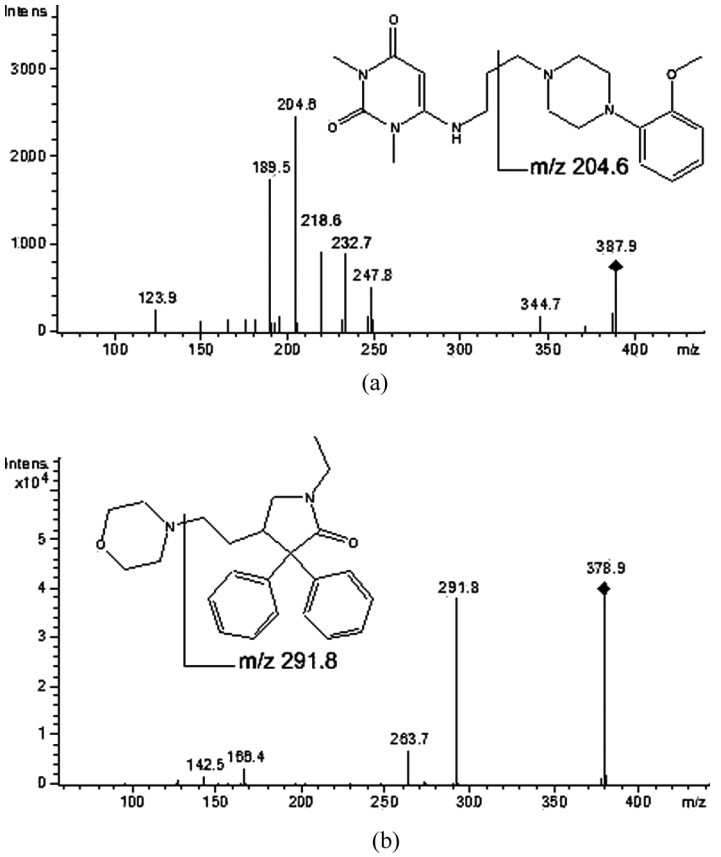

FIGURE 1 MS-MS product-ion spectrum of urapidil (a) and doxapram (IS, b) with $[M+H]^+$ at m/z 387.9 and 378.9 as the precursor ion, respectively.

working solution (0.4 μg/mL) was added to 100 μL of collected plasma sample followed by the addition of 100 μL 10% trichloroacetic acid. The tubes were vortex mixed for 0.5 min. After centrifugation at 15000 rmp for 10 min, the supernatant (10 μL) was injected into the LC-MS-MS system for analysis.

Method Validation

The selectivity of the method was evaluated by analyzing blank rabbit plasma, blank plasma spiked urapidil hydrochloride, and IS and a rabbit plasma sample.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of urapidil hydrochloride to IS were plotted against analyte concentrations, and standard curves were

well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) in the concentration range of 5–1000 ng/mL. The LLOQ was defined as the lowest concentration on the calibration curves.

To evaluate the matrix effect, blank rabbit plasma were protein precipitated and then spiked with the analyte at 10, 100, and 800 ng/mL (six different sources). The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect (ME). The matrix effect of IS was evaluated at the working concentration (40 ng/mL) in the same manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (10, 100, and 800 ng/mL) in three validation days. The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE).

The recoveries of urapidil hydrochloride at three QC levels ($n = 6$) were determined by comparing peak-area of the analytes in QC samples to which the analytes were added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

The stabilities of urapidil hydrochloride in rabbit plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 10 and 800 ng/mL that were exposed to different conditions.[13] These results were compared with those obtained for freshly prepared plasma samples. The short-term stability was determined after the exposure of the spiked samples at room temperature for 2 hr, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 hr. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25°C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at −20°C for 30 days. The stability of the IS (40 ng/mL) was evaluated in a similar way.

Pharmacokinetic Study

Japanese male rabbits (2.1–2.3 kg) were raised and obtained from Wenzhou Medical College Laboratory Animal Center (Wenzhou, China). All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. Rabbits were intravenously given the urapidil hydrochloride via marginal ear vein at a dose of 3 mg/kg within 0.5 min. Blood samples (0.3 mL) were collected from the marginal ear vein into heparinized 1.5 mL polythene tubes at 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hr after dosing. The samples were immediately centrifuged at

5000 rpm for 5 min. The plasma obtained (100 μL) was stored at −20°C until analysis. Plasma urapidil hydrochloride concentration versus time data for each rabbit was analyzed by DAS software (Version 2.0, Medical College of Wenzhou, China).

RESULTS AND DISCUSSION

Method Development

The preliminary investigation was focused on the selection of the ionization source. The atmospheric pressure chemical ionization (APCI) and electrospray ionization modes (ESI) were all tested. Urapidil and IS could be ionized under positive APCI or ESI conditions. It was found that ESI would offer higher sensitivity for the analyte than APCI.

The mobile phase played a critical role in achieving good chromatographic behavior (including peak symmetry and short analysis time) and appropriate ionization. Various combinations of acetonitrile, methanol, water, and 0.1% formic acid in water with changed content of each component were investigated and compared to identify the optimal mobile phase. Acetonitrile was chosen as the organic solvent because of its suitable sharper peak shape, lower pressure, and increased stability compared to methanol. Formic acid added into the mobile phase could not improve the sensitivity; therefore, acetonitrile-water was chosen as the mobile phase. Gradient elution provided better peak symmetry, proper retention time, and avoided the matrix effects for the analyte and IS, compared to isocratic elution. A flow rate of 0.3 mL/min produced good peak shapes and permitted a run time of 10 min.

Plasma protein precipitation was used for pre-treatment of rabbit plasma samples as it is rapid, widely used, and has a high recovery. The supernatant was directly injected into the LC-MS-MS system for analysis. Methanol, acetonitrile, 10% trichloroacetic acid in water (*W/V*), 10% trichloroacetic acid in methanol (*W/V*), and 10% trichloroacetic acid in acetonitrile (*W/V*) were investigated as precipitation reagents, and the recoveries were 60.3%, 66.7%, 94.3%, 85.4%, and 40.5% for urapidil hydrochloride (100 ng/mL), respectively. Trichloroacetic acid (10%) in water (*W/V*) proved to be the best reagent in terms of the peak shape obtained by LC-MS-MS. It is more simple and rapid than the liquid-liquid extraction method described in literature.[11]

Selectivity and Matrix Effect

Figure 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with urapidil hydrochloride and IS, and a

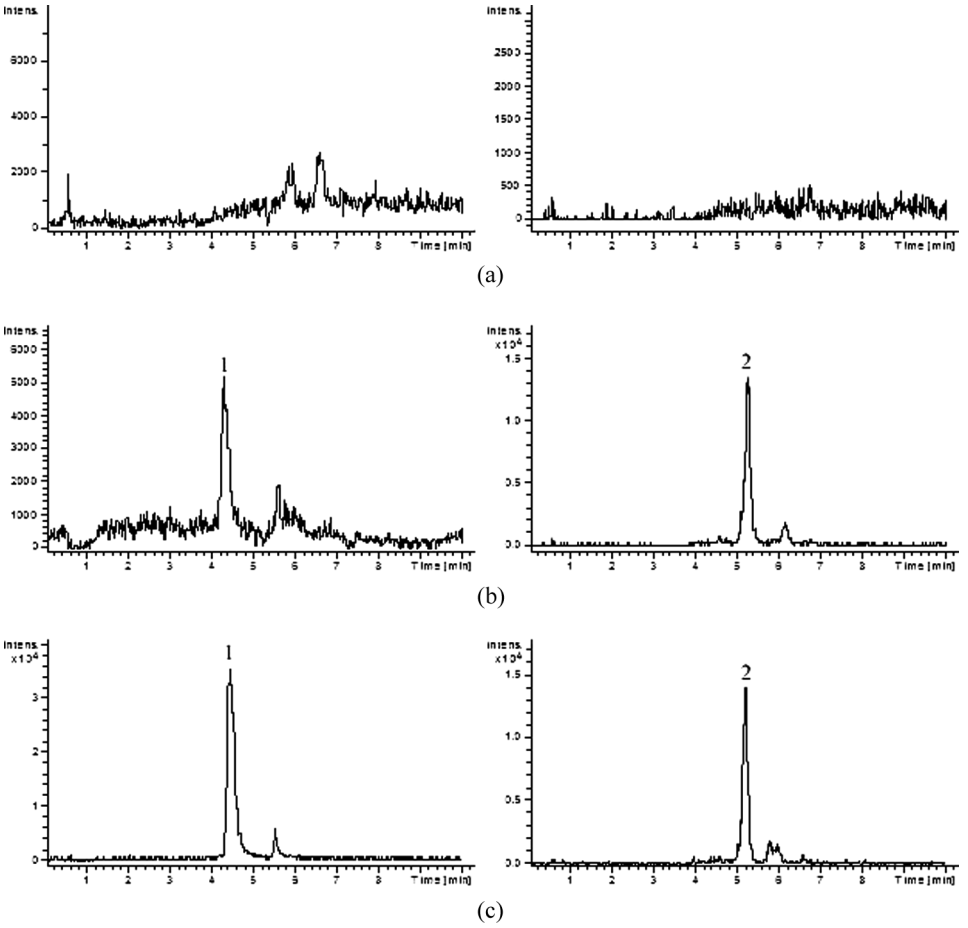

FIGURE 2 Representative LC-MS-MS chromatograms of urapidil hydrochloride (1) and doxapram hydrochloride (IS, 2), (a) blank plasma; (b) blank plasma spiked with urapidil hydrochloride (10 ng/mL) and IS (40 ng/mL); (c) a rabbit plasma sample 3 h after intravenous administration of single dosage 3 mg/kg urapidil hydrochloride.

plasma sample. No interfering endogenous substances were observed at the retention times of the analyte and IS.

The ME for urapidil hydrochloride at concentrations of 10, 100, and 800 ng/mL were measured to be 87.1, 89.5, and 85.4% ($n = 6$), respectively. The ME for IS (40 ng/mL) was 89.3% ($n = 6$).

Calibration Curve and Sensitivity

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 5–1000 ng/mL for urapidil

hydrochloride in rabbit plasma. Typical equation of the calibration curve was: $y = 0.013346x + 0.021412$, $r = 0.9963$, where y represents the ratios of urapidil hydrochloride peak area to that of IS and x represents the plasma concentration.

The LLOQ for the determination of urapidil hydrochloride in plasma was 5 ng/mL. The precision and accuracy at LLOQ were 13.8% and 92.6%, respectively. The LOD, defined as a signal–noise ratio of 3, was 2 ng/mL for urapidil hydrochloride in plasma.

Precision, Accuracy and Recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day precision and the inter-day precision both were 12% or less at each QC level. The accuracy of the method ranged from 92.6% to 109.6% at each QC level. Mean recoveries of urapidil hydrochloride were better than 93.5%. The recovery of the IS (40 ng/mL) was 83.1%. Assay performance data are presented in Table 1. The aforementioned results demonstrate that the values are within the acceptable range and the method is accurate and precise.

Stability

The stability results showed that urapidil hydrochloride spiked into rabbit plasma was stable for 2 hr at room temperature, for 30 days at −20°C, and during three freeze–thaw cycles. Urapidil hydrochloride extracts in the sample solvent on autosampler was also stable over a 24 hr period. The results of stability experiments are listed in Table 2.

Application

The developed method was applied to a pharmacokinetic study in Japanese male rabbits. The profile of the average plasma concentration

TABLE 1 Precision, Accuracy and Recovery for Urapidil Hydrochloride of Quality Control Sample in Rabbit Plasma ($n = 6$)

Concentration (ng/mL)	RSD (%)		RE (%)		Recovery (%)
	Intra-day	Inter-day	Intra-day	Inter-day	
10	11.5	11.6	9.6	−7.4	96.4
100	9.1	7.2	6.5	−2.3	94.3
800	2.7	4.3	−3.6	4.5	93.5

TABLE 2 Summary of Stability of Urapidil Hydrochloride and IS Under Various Storage Conditions ($n=3$)

Condition	Concentration (ng/mL)		RSD (%)	RE (%)
	Added	Found		
Ambient, 2 h	10	9.6	5.3	−4.0
	800	819.5	4.3	2.4
	(IS) 40	38.9	3.6	−2.8
−20°C, 30 days	10	8.7	8.7	−13.0
	800	776.4	6.5	−3.0
	(IS) 40	42.1	5.4	5.3
3 freeze thaw	10	11.1	7.9	11.0
	800	761.2	7.5	−4.8
	(IS) 40	42.5	6.8	6.3
Autosampler ambient 24 h	10	10.4	4.3	4.0
	800	823.2	2.1	2.9
	(IS) 40	38.5	2.5	−3.8

versus time after a single intravenous administration at a dose of 3 mg/kg urapidil hydrochloride was presented in Figure 3. The pharmacokinetic data for urapidil were computed from the plasma concentration-time data using each rabbit as an independent subject and using a two-compartment model. The main pharmacokinetic parameters were: the area under the plasma concentration-time curve from 0 to t h ($AUC_{0\rightarrow t}$) was 2638.82 ± 636.93 h ng/ml; the area under the plasma concentration-time curve from 0 to infinite h ($AUC_{0\rightarrow\infty}$) was 2745.6 ± 650.3 h ng/ml; the plasma clearance (CL) was 1.14 ± 0.26 L/h/kg, the distribution half-life ($t_{1/2\alpha}$) was

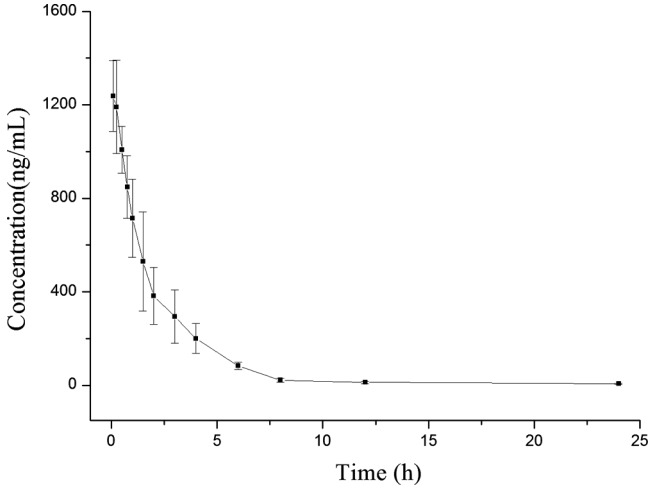

FIGURE 3 Mean plasma concentration time profile after intravenous administration of single dosage 3 mg/kg urapidil hydrochloride in 6 rabbits.

0.43 ± 0.20 h, and the apparent volume of distribution (V_d) was 2.2 ± 0.27 L/kg.

CONCLUSION

A sensitive, simple and specific LC-MS-MS method for the determination of urapidil hydrochloride in rabbit plasma was developed and validated over the concentration range of 5–1000 ng/mL. The simple and rapid protein precipitation by 10% trichloroacetic acid was used for pretreatment of plasma samples. The LC-MS-MS method successfully applied to a pharmacokinetic study of urapidil hydrochloride after intravenous administration of single dosage 3 mg/kg to rabbits.

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