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Short communication

Determination of TJ0711 hydrochloride in rat plasma by high performance liquid chromatography with fluorescence detection and its application to pharmacokinetics

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ABSTRACT

In the present study, a simple and sensitive high performance liquid chromatography with fluorescence detection (HPLC-FD) method was developed to determine TJ0711 hydrochloride, a novel α- and β-receptor blocker. TJ0711 hydrochloride and verapamil hydrochloride (the internal standard) were separated on Knauer Eurospher C_{18} (250 mm × 4.0 mm i.d., 5 μm) column at 50 °C. The mobile phase was methanol:perchloric acid (12 nM, aq) (56:44, v:v), with a flow rate of 1.0 mL/min. The wavelengths of FD were set at 246 nm for excitation and 300 nm for emission. For plasma samples of rats, the analytes were extracted with acetic ether from alkalinized plasma, and then back-extracted into 10 mM dilute sulfuric acid. The linearity was over a concentration range of 20–10,000 ng/mL. The intra- and inter-day precisions referred by relative standard deviation were less than 2.0% and 4.3%, respectively. The mean analytical recoveries of TJ0711 hydrochloride at different concentrations (50, 1000 and 8000 ng/mL) ranged from 88.3% to 92.9%. The lower limit of quantification (LLOQ) was 20 ng/mL. Finally, this method was successfully applied to the estimation of pharmacokinetic parameters of TJ0711 hydrochloride after intravenous doses of 4, 8 and 16 mg/kg in rats.

1. Introduction

β-Blockers are widely used in clinical practice for a variety of cardiac and noncardiac indications. However, some adverse reactions of traditional β-blockers, such as respiratory and/or gastrointestinal disturbances, erectile and/or ejaculation dysfunction, hypoglycemia during diabetic therapy and peripheral edema, have limited their clinical use [1]. Furthermore, their application to patients with uncomplicated hypertension has widely led to a controversy over the past few years [2–5]. Therefore, many efforts have been devoted to developing new β-blockers to eliminate/reduce the aforementioned side effects but without compromising their curative effects. Recently, some new β-blockers with vasodilator properties appeared to be advantageous in the treatment of cardiovascular diseases and glaucoma as compared with conventional β-blockers. They may prove more effective in reducing cardiovascular events, but can still reduce blood pressure to a similar degree as other types of antihypertensive drugs [6]. Their vasodilator properties were found to be mediated by NO, β-adrenoceptor stimulation, reactive oxygen species scavenging, Ca^{2+} entry blockade, α_1-blockade and improving endothelial function [7,8]. Much attention has been received from β-blockers mediated by α_1-blockade with additional α-adrenoceptor blocking activity. The US Food and Drug Administration had approved α/β-blockers (carvedilol and labetalol) for use, which encouraged us to synthesize a combinatorial compound with possessing both α- and β-adrenoceptor blocking activities.

1-[4-(2-Methoxyethyl) phenoxy]-3-[[2-(2-ethoxyphenoxy) ethyl] amino]-2-propanol (named as TJ0711, shown in Fig. 1), has an aminopropanol group, a characteristic structure of most β-adrenergic blocking agents [9,10]. Our previous studies showed that TJ0711 hydrochloride was a novel α_1/β_1 receptor-blocking agent [11]. Its blockade on α_1-adrenoceptor, similar to that on β_1-adrenoceptor, is responsible for the relatively strong vasodilator properties of TJ0711 hydrochloride, which is distinguished from other new vasodilating β-blockers such as carvedilol and nebivolol [12].

High performance liquid chromatography (HPLC) and gas chromatography (GC) have been used to quantify β-blockers in plasma and evaluate pharmacokinetics in preclinical and clinical studies [13–15]. It is necessary to determine TJ0711 hydrochloride in biological samples and study its pharmacokinetics. In our previous study [16], HPLC-UV method failed to detect TJ0711 hydrochloride directly because of its poor ultraviolet absorption. As a solution, a derivatization approach, using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as derivatization reagent,

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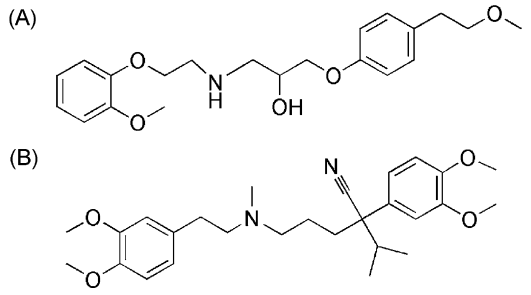

Fig. 1. Chemical structures of (A) TJ0711 and (B) verapamil (I.S.).

was developed. However, the sample preparation procedure was very time consuming.

For our continuous effort, fluorescence detector (FD) was used to determine TJ0711 hydrochloride considering its suitable fluorescence in the present study. It is the first time HPLC-FD was used to measure TJ0711 hydrochloride in biological samples and was applied to its pharmacokinetics study. Compared to HPLC-UV method, it was a direct detection methodology without derivatization, and greatly improved detection sensitivity.

2. Experimental

2.1. Materials and reagents

TJ0711 hydrochloride (≥99.7% purity) was synthesized by our team. The internal standard (I.S.), verapamil hydrochloride, was purchased from Sigma Laboratories (Saint Louis, USA). All other reagents were of analytical grade or HPLC grade.

2.2. Preparation of stocks, calibration standards and quality control samples

Stock solutions were prepared by dissolving TJ0711 hydrochloride and I.S. in distilled water to obtain 100 and 3 μg/mL, respectively. Calibration standards and quality control samples were prepared by adding different concentrations of stock solution into drug-free plasma.

2.3. Instrumentation and chromatographic conditions

A Hitachi HPLC System (Kyoto, Japan) was equipped with a quaternary gradient low-pressure mixing pump (L-2130), an L-2485 fluorescence detector and a column compartment.

A Knauer Eurospher-100 C_{18} (250 mm × 4.0 mm i.d., 5 μm) was used for separation, with a C_{18} (4 mm × 10 mm i.d., 10 μm) precolumn from Knauer (Berlin, Germany). The mobile phase was methanol:perchloric acid (12 nM, aq) (56:44, v:v). Separations were performed at 50 °C with a flow rate of 1.0 mL/min and 50 μL was injected for quantitative analysis. The wavelengths of fluorescence detection were set at 246 nm for excitation and 300 nm for emission.

2.4. Pretreatment for plasma samples

For plasma samples, a two-step extraction method was used to extract target analyte. It was carried out as follows: (1) 10 μL of I.S. stock solution (3 μg/mL) and 40 μL of 0.1 M sodium hydroxide were added into 0.15 mL rat plasma sample successively, then vortexed for 30 s. (2) 0.5 mL acetic ether was added into the above alkalinized sample solution and vortexed for 5 min followed by centrifugation at 13,800 × *g* for 10 min. (3)The upper organic phase was transferred into a new tube containing 0.15 mL of 10 mM dilute sulfuric acid and vortexed for 10 min then centrifuged at 13,800 × *g* for 10 min. (4) The organic phase was discarded; 50 μL aqueous phase was collected and injected into the HPLC system.

2.5. Method validation

The selectivity of the assay was checked by comparing the chromatograms of six batches of drug-free rat plasma samples with the corresponding spiked plasma.

In our pilot study, the maximum concentration expected was about 6000 ng/mL. Therefore, a slight wide concentration range from 20 to 10,000 ng/mL was chosen for the linearity of TJ0711 hydrochloride, which was calculated by plotting peak area ratio of TJ0711 and I.S. (*y*) versus TJ0711 concentrations (*x*, ng/mL). Three different concentrations of QC samples (50, 1000 and 8000 ng/mL) in six replicates were analyzed in 3 consecutive days to determine the intra- and inter-day accuracy and precision.

The recoveries of TJ0711 hydrochloride were evaluated in six replicates at three QC levels (50, 1000 and 8000 ng/mL). Limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated at S/N > 3 and S/N > 10, respectively. Besides the criteria of calculation, six replicates were needed to evaluate the intra-day precision and accuracy in determination of LLOQ.

The stability of TJ0711 hydrochloride in rat plasma was estimated using quality control samples (50, 1000 and 8000 ng/mL) with six replicates for each concentration. Stabilities of TJ0711 hydrochloride of three freeze–thaws, long-term (−20 °C, 30 days), short-term (25 °C, 24 h) and post-preparation (25 °C, 24 h) in plasma were tested respectively. The stabilities of stock solutions of drug and the internal standard were also evaluated respectively at room temperature for 24 h comparing the instrument response with that of freshly prepared solutions.

2.6. Application to pharmacokinetic study

Approval was obtained from Animal Care and Ethics Committee of Tongji Medical College (Wuhan, China). Adult male Sprague–Dawley rats (190–220 g), purchased from the Laboratory Animal Center of Tongji Medical College, were fasted for 12 h with free access to water. The minimum curative dose of 4 mg/kg for intravenous injection was obtained from our previous pharmacodynamic study in rats and 16 mg/kg was tolerance dose. Based on the previous data and guidelines of State Food and Drug Administration, different doses (4, 8 and 16 mg/kg) were chosen to study their pharmacokinetics on rats.

TJ0711 hydrochloride solution was injected intravenously into a lateral tail vein of rats at different doses (4, 8 and 16 mg/kg, respectively). Serial blood samples were collected in heparinized Eppendorf tubes from the retro-orbital plexus before administration and after intravenous injection at time-points of 3, 5, 10, 20, 30, 45, 60, 90, 120 and 180 min, respectively. After each sampling, the removed volume of blood (0.4 mL) was supplemented with an equal volume of normal saline.

3. Results and discussion

3.1. Method development

pH of a mobile phase plays an important role in influencing the reproducible separation of ionic analytes by reversed-phase HPLC. It was reported that the pH of mobile phase below 3 can inhibit ionization of silanols and will minimize interactions between silanols and a protonated basic compound [17]. Good peak shape is possible when pH of the mobile phase is 2 units above or below the pK_a of an analyte, in which the analyte reaches its single form by 99%. Based on this condition, good peak shape of TJ0711 was also

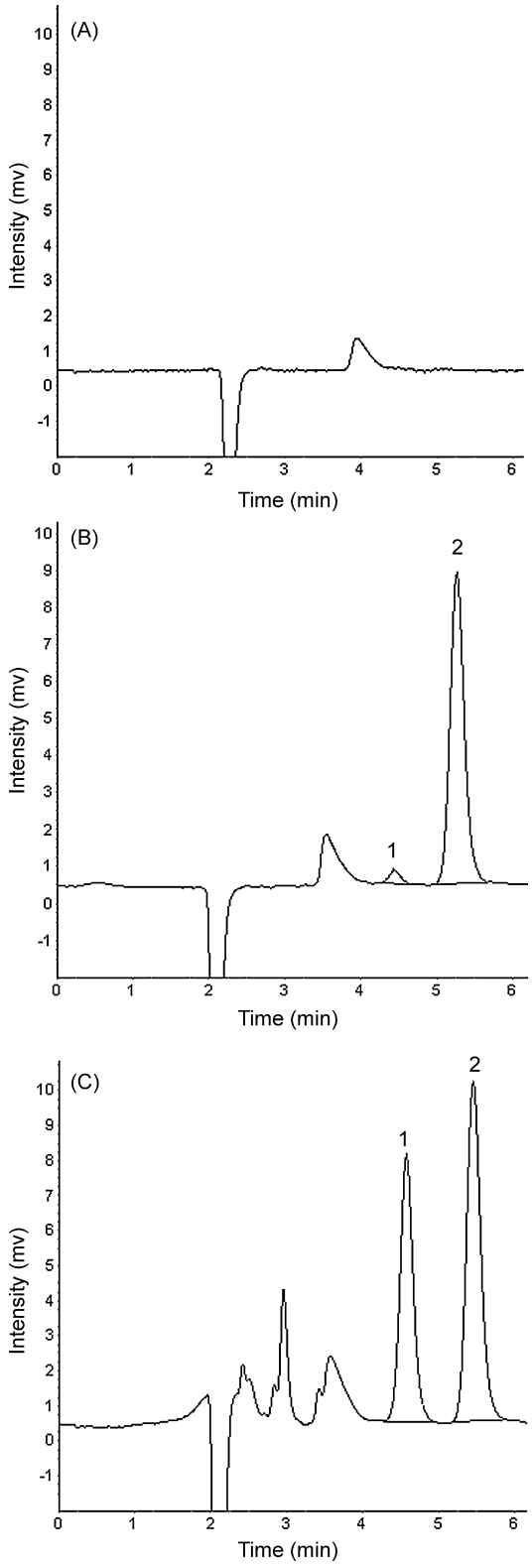

Fig. 2. Typical HPLC chromatograms of (A) drug-free plasma sample, (B) plasma spiked with TJ0711 hydrochloride (20 ng/mL) and I.S. (300 ng/mL) and (C) plasma sample at 30 min of a rat after intravenous administration TJ0711 hydrochloride (930 ng/mL) after an 8 mg/kg dose. Peak: 1, TJ0711 hydrochloride; Peak: 2, verapamil hydrochloride (I.S.).

Table 1
Intra-day ($n = 6$) and inter-day ($n = 18$) precision and accuracy of TJ0711 hydrochloride measurements in rat plasma.

QC (ng/mL)	Precision		Accuracy
	Mean ± SD	RSD (%)	Deviation (%)
Intra-day			
50	49.1 ± 0.99	1.9	−3.7
1000	978.7 ± 18.7	1.8	−3.3
8000	8040.8 ± 56.6	0.69	0.98
Inter-day			
50	47.9 ± 2.1	4.2	−3.2
1000	955.3 ± 17.2	1.9	−4.5
8000	7938.7 ± 134.6	1.6	1.1

obtained because of its pK_a is 7.89. Analysis at the low pH may cause early elution of a basic compounds due to its highly solvated and protonated property unless mobile phase additives are employed [18,19]. In this paper, the effect of several mobile phase additives (acetic acid, perchloric acid, phosphoric acid, sodium dihydrogen phosphate) on peak shape, analyte retention and column efficiency was studied. Among these additives, 12 nM perchloric acid (pH = 2.8) was selected because of its best efficiency in terms of satisfactory retention factor and tailing factor (data not shown).

Liquid–liquid extraction combining protein precipitation is one of the most common preparation methods for biological samples. However, when it was attempted herein, there was a major interfering peak around the retention time of TJ0711 hydrochloride and I.S. Hence, a two-step extraction procedure was employed to solve this problem here. Firstly, to de-protonate the analyte beneficially and facilitate transferring the analyte from aqueous to organic phase, sodium hydroxide was added to alkalinize plasma samples. Secondly, to obtain a maximum extraction recovery, acetic ether was used for the first-step extraction and an acid back extraction (organic solvent to aqueous phase) was used again, which generally provides a cleaner extract and has been employed for amine compounds before HPLC analysis [20–23]. As a result, the interfering endogenous peak was found to disappear after back extraction in this paper. In addition, to ensure effective extraction from organic solvent to aqueous phase, vortex time should not be less than 10 min.

3.2. Method evaluation

Under the chromatographic conditions mentioned above, typical chromatograms of a drug-free plasma sample, a LLOQ sample with TJ0711 hydrochloride (20 ng/mL) and sample after dosing 8 mg/kg with TJ0711 hydrochloride were shown in Fig. 2. Endogenous plasma components did not interfere with the elution of TJ0711 and I.S. The retention time of verapamil (5.3 min) was suitable for I.S. compared to that of TJ0711 (4.5 min).

The linearity of TJ0711 hydrochloride in plasma was in the range from 20 to 10,000 ng/mL. The representative regression equation was $y = 0.00091x + 0.00423$ ($r = 0.9998$). The standard deviations of slope and intercept were 0.00003 and 0.0002, respectively ($n = 6$). The LLOQ (precision referred by relative standard deviation was 1.26%, and accuracy was 95.3–107.2%) and LOD were 20 and 5 ng/mL, respectively. The present method proved to be more sensitive than the previous HPLC-UV method (LLOQ, 125 ng/mL) [16].

The mean analytical recoveries of TJ0711 hydrochloride ranged from 88.3% to 92.9% at three different concentrations in rat plasma.

The stock solutions of TJ0711 hydrochloride and I.S. were found to be stable at room temperature for 24 h (relative error between fresh and test solutions, TJ0711 hydrochloride: 1.2%, I.S.: 0.6%). The stability data (precision referred by RSD was less than 2.5%, and accuracy was 89.0–108.9%) of

Table 2
Pharmacokinetic parameters of TJ0711 hydrochloride following intravenous administration of 4, 8, and 16 mg/kg (mean ± SD, $n = 6$).

Pharmacokinetic parameters	Intravenous administration		
	4 mg/kg	8 mg/kg	16 mg/kg
$AUC_{(0-t)}$ (ng min/mL)	33661.0 ± 5954.4	75742.8 ± 3471.4	184266.4 ± 25721.2
$AUC_{(0-\infty)}$ (ng min/mL)	35021.8 ± 6243.9	77413.3 ± 2876.6	185430.7 ± 25663.2
$t_{1/2}$ (min)	23.1 ± 7.6	25.3 ± 3.6	25.9 ± 1.8
CL (L/min kg)	0.116 ± 0.023	0.105 ± 0.005	0.088 ± 0.012
V (L/kg)	3.5 ± 1.1	3.6 ± 0.5	3.2 ± 0.3

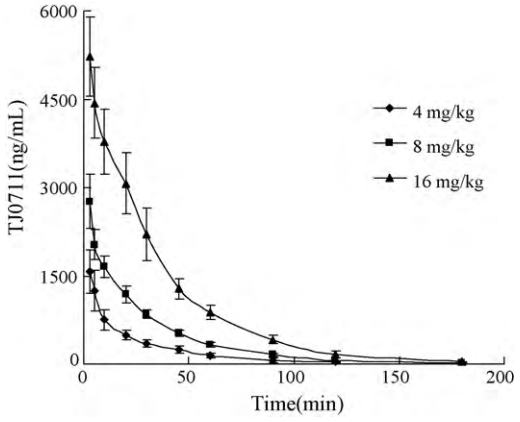

Fig. 3. Mean plasma concentration–time profiles of TJ0711 hydrochloride in rats ($n = 6$) following intravenous administration of 4, 8, 16 mg/kg of TJ0711 hydrochloride.

three freeze–thaws, long-term, short-term and post-preparation indicated that TJ0711 hydrochloride was stable under these conditions.

In addition, the precision and accuracy of this method were summarized in Table 1. The reproducibility of the method was assessed by examining both intra- and inter-day variance. The data showed that the RSD values of the intra-day and the inter-day ranged from 0.69% to 1.9% and from 1.6% to 4.2%, respectively.

3.3. Pharmacokinetic results

The above-developed HPLC-FD analytical method was successfully applied to the pharmacokinetic study in rats. The mean plasma concentration–time curves of TJ0711 hydrochloride with different doses of TJ0711 hydrochloride (4, 8 and 16 mg/kg after intravenous administration) were shown in Fig. 3. After administration, the concentrations of TJ0711 hydrochloride in different doses were found to be decreased unilaterally. The drug concentration was below LLOQ after collection of sample for 120 min in dose of 4 mg/kg.

The mean pharmacokinetic parameters (mean ± SD) were summarized in Table 2. Main pharmacokinetic parameters at different doses were analyzed by one-way ANOVA with Bonferroni post hoc test. Similar pharmacokinetic processes were observed at doses of 4 and 8 mg/kg, while a nonlinear process occurred at the dose of 16 mg/kg.

4. Conclusion

A novel HPLC-FD method was developed and validated to quantify TJ0711 hydrochloride in rat plasma. A two-step extraction procedure, including aqueous-organic solvent extraction and organic solvent-acidified aqueous back extraction, was demonstrated to afford high recovery of target analyte from rat plasma. The method was successfully applied to study the pharmacokinetics of TJ0711 hydrochloride in rats.

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