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Short communication

In vitro enantioselective metabolism of TJ0711 hydrochloride by human liver microsomes using a novel chiral liquid chromatography–tandem mass spectrometry method

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ABSTRACT

A novel liquid chromatography–tandem mass spectrometry (LC–MS/MS) method employing chiral analytical techniques was developed and validated for *in vitro* enantioselective metabolic stability study of racemic 1-[4-(2-methoxyethyl) phenoxy]-3-[[2-(2-methoxyphenoxy) ethyl]amino]-2-propanol hydrochloride (TJ0711 HCl), a newly developed vasodilatory β-blocker. Robust enantiomeric separations were achieved on a chiral SUMICHIRAL OA-2500 column using ethanol and hexane (40:60, v/v) as a mobile phase. Metabolic stability results demonstrated that both TJ0711 enantiomers underwent a rapid phase I metabolism, but preferential metabolism of R-TJ0711 was observed. Our previously reported ultra-performance liquid chromatography-multiple reaction monitoring-information dependent acquisition-enhanced product ion (UPLC-MRM-IDA-EPI) method was finally chosen for metabolite profiling study of TJ0711 enantiomers, because the newly developed HPLC-based method resulted in compromised chromatographic separation, particularly for TJ0711 metabolites. A number of metabolic products were detected and the structures of formed metabolites were predicted. Similar to racemic TJ0711 HCl, demethylation and hydroxylation were proposed to be the principle metabolism pathways during *in vitro* incubations of each enantiomer with human liver microsomes.

1. Introduction

Two classes of β-blockers have been defined according to their vasodilating properties: nonvasodilatory and vasodilatory β-blockers. 1-[4-(2-Methoxyethyl) phenoxy]-3-[[2-(2-methoxyphenoxy) ethyl]amino]-2-propanol hydrochloride (TJ0711 HCl) was synthesized by us as a new vasodilatory β-blocker for hypertension and other related syndromes [1,2]. As stated previously, TJ0711 possesses a single chiral center and the two enantiomers exhibited differing pharmacokinetic properties in preclinical pharmacokinetic studies [3]. We have reported that elimination rate of the enantiomer with R-configuration is faster than that with S-configuration after oral administration in rats [3]. However, it is unknown whether or not enantioselective pharmacokinetics results from significantly different liver metabolism. Thus, the aim of this investigation was to test the hypothesis that *in vitro* metabolism of TJ0711 HCl is enantioselective.

In 2011, we developed and validated an achiral ultra-performance liquid chromatography-multiple reaction monitoring-information dependent acquisition-enhanced product ion (UPLC-MRM-IDA-EPI) method for the simultaneous determination of racemic TJ0711 HCl as well as its metabolites [2]. The racemic mixture underwent a rapid *in vitro* phase I metabolism mediated by human liver microsomes (HLM) and a number of metabolites including demethylated and/or hydroxylated products were identified during *in vitro* incubations. However, this UPLC-based method is not applicable for enantiomeric determination of TJ0711 HCl, because it only quantified the concentration of total TJ0711 HCl, rather than both TJ0711 enantiomers.

So far, a chiral HPLC-UV assay using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a derivatization reagent has been developed by our group [3]. Nevertheless, the low sensitivity and selectivity of UV detector do not allow full characterization of enantioselective metabolic stability and metabolite profiling studies of TJ0711 HCl. Hence, there is a need to develop and validate a chiral LC–MS/MS method to chromatographically separate the two enantiomers of TJ0711 HCl. In the present work, the newly developed chiral LC–MS/MS method as well as the

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previously reported UPLC-MRM-IDA-EPI approach was employed for *in vitro* enantioselective metabolic stability and metabolite profiling studies of TJ0711 HCl, respectively.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol, ethanol, hexane, formic acid, ammonium hydroxide, and potassium phosphate monobasic were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Magnesium chloride, D-glucose-6-phosphate (G6P), D-glucose-6-phosphate dehydrogenase (G6P-DH), β-nicotinamide adenine dinucleotide phosphate (NADP+) disodium salt and metoprolol tartrate (Internal Standard, I.S.) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Racemic TJ0711 HCl and its R- and S-enantiomers were synthesized by our team. Pooled HLM (n = 200 livers) were purchased from XenoTech, LLC (Lenexa, KS, USA).

2.2. Instruments and analytical conditions

A Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) consisting of a binary solvent delivery system, an autosampler and a column manager was used throughout. The UPLC system is controlled by Empower Pro 6.0 software. Enantioseparation was achieved on a SUMICHIRAL OA-2500 column (5 μm, 250 mm × 4 mm) (Sumika Chemical Analysis Service Ltd., Osaka, Japan) equipped with a guard column packed with SUMICHIRAL OA-2500 material (5 μm, 10 mm × 4.6 mm). The mobile phase consisted of 40% ethanol and 60% hexane, and the flow rate was 0.8 mL/min, with a post column flow splitter (1:1) to divert 0.4 mL/min to waste. The injection volume was 10 μL and the column temperature was maintained at 20 °C. Mass spectrometric detection was carried out, using the same source and mass spectrometer conditions as previously reported [2], on an Applied Biosystem 4000 QTRAP® hybrid triple quadrupole/linear ion trap (QqQ_{LIT}) mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source. The 4000 QTrap was calibrated semiannually using polypropylene glycol (PPG) for mass accuracy. Analyst 1.4.2 software was used for data acquisition and processing.

2.3. Preparation of standard solutions

The standard stock solutions of R-TJ0711, S-TJ0711 HCl, and I.S. were prepared in methanol at a concentration of 10 mg/mL. Calibration standard spiking solutions containing 10, 20, 40, 100, 200, 400, 1000 ng/mL of each TJ0711 enantiomer and 400 ng/mL I.S. were prepared by diluting the stock solution with methanol. Quality control (QC) standard solutions were prepared similarly at four concentration levels. All standard stock and working solutions for spiking were stored at −80 °C until use.

2.4. Method validation

2.4.1. Limit of detection and lower limit of quantification

The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level. The lower limit of quantification (LLOQ) for both TJ0711 enantiomers was experimentally chosen as the minimal concentration in metabolic incubation mixtures which could be determined with less than 20% intra- and inter-day accuracy and precision.

2.4.2. Linearity, accuracy and precision

Seven calibration points in the range of 5–500 ng/mL for each TJ0711 enantiomer were used to determine linearity in three independent validation runs using $1/x$ weighting scheme. Intra-day accuracy and precision of the method were assessed by assaying five replicates of each QC point with four QC levels distributed throughout the calibration curve range. Inter-day accuracy and precision were calculated similarly using the 15 replicates of each QC point from the three validation runs. Accuracy and precision were considered to be acceptable when it was found to be less than 15%, except for LLOQ where 20% deviation was permitted.

2.4.3. Recovery and matrix effect

Extraction recoveries were determined for each QC point from the ratio of the analyte peak area in samples spiked before extraction compared to the corresponding peak area in untreated samples prepared in neat solution. The degree of matrix effect was examined for each QC point from the ratio of the analyte peak area in samples spiked post extraction compared to the corresponding peak area in untreated samples.

2.4.4. Stability and carry-over

Stability of TJ0711 enantiomers during the analytical process was assessed on the laboratory bench at room temperature, in the autosampler at 4 °C or in the freezer at −80 °C. The carry-over was assessed by the analysis of blank samples after the analysis of the highest calibration point. Peak area of the resulting carry-over peaks should not exceed 20% of the peak area at the LLOQ level.

2.5. Microsomal incubations

Incubations were carried out as described previously [2] except that 2 μM of racemic TJ0711 HCl was incubated with 1 mg/mL HLM. According to the well-stirred model approach, the *in vitro* metabolic stability parameters, such as $t_{1/2}$, microsomal CL_{int}, hepatic CL_{int}, extraction ratio (ER), and hepatic CL, were calculated using appropriate scaling factors as previously described [2,4–7]. The free fraction (f_u) of both enantiomers in blood was determined by a previously reported equilibrium dialysis method [8].

2.6. Metabolite profiling of TJ0711 enantiomers

A slightly modified MRM-IDA-EPI method was used [2]. Briefly, the 4 most intense product ions of the parent compound ($[M+H]^+$ = 376), m/z 252, 100, 91 and 224, were used to generate MRM transitions for 'guessed' metabolites using an Analyst script. The same DP, CE, and CXP values optimized for the parent compound were used for the MRM transitions of its 'guessed' metabolites. Molecular ion for the I.S. (m/z 268.2) was placed in the exclusion list.

3. Results and discussion

3.1. Method development

A typical extracted ion chromatogram using the final chromatography and detection conditions is shown in Fig. 1. The retention times of R-TJ0711, S-TJ0711 and I.S. were 12.7, 10.6, and 5.5 min, respectively. To gain enantiomeric separation of TJ0711 HCl, initial experiments were performed on variant reverse phase or normal phase HPLC and UPLC columns. However, enantiomeric separations were not achieved regardless of the composition of the mobile phases. Subsequently, commercial chiral columns were investigated with various solvent systems. Among them, the SUMICHIRAL OA-2500 column can completely separate R- and S-TJ0711, when a mobile phase consisting of ethanol and hexane (40:60, v/v) is used. It is worthy to note that column temperature plays a critical role in enantiomeric separation of TJ0711 HCl.

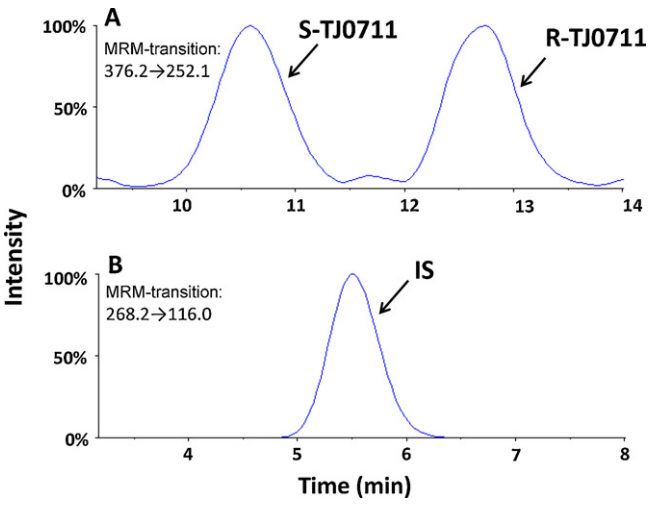

Fig. 1. Representative LC–MS/MS chromatograms of (A) TJ0711 enantiomers and (B) I.S. under the final chromatography and detection conditions.

The enantioselectivity increased with decreasing column temperature (data not shown). That is, poor baseline resolutions of the two enantiomers were observed at a column temperature of 30 °C or higher, and sufficient separations were attained when column temperature was lower than 20 °C. Finally, a column temperature of 20 °C was chosen for enantioselective determination of TJ0711 HCl. These findings were in good agreement with previous reports using SUMICHIRAL chiral columns for enantiomeric separation [9,10]. In addition, individual injections of each enantiomer solution verified the chromatographic elution order of the TJ0711 enantiomers.

A liquid–liquid extraction (LLE) method using ethyl acetate has been reported for plasma sample clean-up [11], however, this method included multiple time-consuming procedures, and sample throughput was therefore greatly reduced. Recently, we developed a simple deproteinized procedure for *in vitro* metabolic incubation samples [2]. Accordingly, the same deproteinized approach was applied for enantioselective determination of TJ0711 HCl. Additionally, no racemization of the enantiomers occurred during sample preparation, because only a single peak was shown on the chromatogram when TJ0711 enantiomers were individually extracted (data not shown).

3.2. Method validation

The current assay offered a LOD of 0.5 ng/mL and a LLOQ of 5 ng/mL for each TJ0711 enantiomer. These LLOQ values are lower than those obtained from ultraviolet or fluorescence detection [3,11], but they are higher than those reported by a UPLC–MS/MS method [2]. Linearity was determined with the use of a weighting factor. Regression coefficients (R) are 0.9997 for R-TJ0711 and 0.9994 for S-TJ0711.

Intra-day and inter-day accuracy and precision results for both analytes are summarized in Table 1. All accuracy and precision data were less than 15% at all concentration levels. Table 2 summarizes recoveries of TJ0711 enantiomers in the matrix of interest using methanol-based protein precipitation. The recovery was consistently higher than 80% throughout the dynamic range for both analytes. In addition, no appreciable matrix effect was found for analyzed compounds and I.S. (data not shown).

The results of stability studies showed that TJ0711 enantiomers were stable for at least 4 h on the laboratory bench at room temperature and for 24 h in the 4 °C autosampler (data not shown). Furthermore, standard stock and working solutions for spiking were stable for at least 30 days in −80 °C freezer (data not shown).

Table 1
Summary of the intra-day and inter-day accuracy and precision of TJ0711 enantiomers using a chiral LC–MS/MS method.

	R-TJ0711		S-TJ0711	
	%Bias	%R.S.D.	%Bias	%R.S.D.
Intra-day (n = 5)				
LLOQ-QC (5 ng/mL)	5.9	10.1	−8.6	8.9
Low-QC (15 ng/mL)	−6.8	12.3	6.0	9.1
Med-QC (150 ng/mL)	4.5	6.1	7.8	4.6
High-QC (450 ng/mL)	7.3	4.9	−4.9	5.6
Inter-day (n = 15)				
LLOQ-QC (5 ng/mL)	4.4	7.9	−5.3	14.1
Low-QC (15 ng/mL)	−7.7	9.4	−3.1	13.7
Med-QC (150 ng/mL)	−2.4	8.2	4.2	8.2
High-QC (450 ng/mL)	6.1	5.5	−5.8	3.6

Table 2
Recoveries of TJ0711 enantiomers in metabolic incubation mixtures at 4 QC levels (n = 5).

	Recovery (%)	
	(R)-TJ0711	(S)-TJ0711
LLOQ-QC (5 ng/mL)	83.7 ± 6.9	87.4 ± 7.6
Low-QC (15 ng/mL)	89.4 ± 8.7	86.3 ± 5.0
Med-QC(150 ng/mL)	86.5 ± 6.6	85.7 ± 6.8
High-QC (450 ng/mL)	84.4 ± 5.3	87.1 ± 8.4

The carry-over effect was found to be less than 1% of the LLOQ for both TJ0711 enantiomers after an injection of a 500 ng/mL sample.

Collectively, this novel chiral LC–MS/MS method was fully validated in the current study to ensure the sensitivity, accuracy, and precision required for enantiomeric quantification of TJ0711 HCl.

3.3. Metabolic stability of TJ0711 enantiomers

The validated analytical method described above was successfully applied to an enantioselective metabolic stability study of TJ0711 HCl. The disappearance of parent compounds was monitored by calculating the percent remaining of each compound relative to the concentration at 0 min and then plotted against the incubation time. Fig. 2 presents the concentration–time profile of TJ0711 enantiomers after incubation with HLM. The *in vitro* metabolic stability parameters including $t_{1/2}$, microsomal CL_{int}, hepatic CL_{int}, ER, and hepatic CL are shown in Table 3. The results indicated that both TJ0711 enantiomers underwent a rapid phase I liver metabolism and were classified as high-extraction-ratio compounds since ER values obtained were larger than 0.7. On the other hand, TJ0711 enantiomers were subject to significantly different metabolism rates *in vitro* ($p < 0.01$). Preferential metabolism of R-TJ0711 was observed during the incubation of racemic TJ0711 HCl with HLM. Therefore, S-TJ0711 was more metabolically stable compared with its R-isomer, which in turn support our aforementioned hypothesis that enantioselective pharmacokinetics of racemic TJ0711 HCl might be attributed to distinct *in vivo* metabolism of two isomers [3].

3.4. Metabolite profiling of TJ0711 enantiomers

In contrast to our previous UPLC-based approach [2], the current chiral LC–MS/MS method presented above resulted in a markedly improved baseline resolution for TJ0711 enantiomers. However, the separations of TJ0711 metabolites were greatly compromised especially for multiple metabolites derived from the same metabolism pathway. As such, this HPLC-based method employing chiral techniques significantly reduced the total number of metabolites identified in metabolic study. Thus, the previously reported UPLC-based chromatographic method was applied for

Table 3
Metabolic stability parameters of TJ0711 enantiomers as determined from incubation with HLM.

	$t_{1/2}$ (min)	CL_{int} (μL/min/mg microsomal protein)	Hepatic CL_{int} (mL/min)	ER	Hepatic CL (mL/min)
R-TJ0711	10.1 ± 0.2	137.4 ± 2.6	6710.6 ± 127.0	0.81 ± 0.01	5466.9 ± 122.6
S-TJ0711	17.3 ± 0.1*	80.1 ± 0.5*	3913.7 ± 22.6*	0.71 ± 0.01*	2815.4 ± 20.8*

* Statistically significant differences between R-TJ0711 and S-TJ0711 ($p < 0.01$).

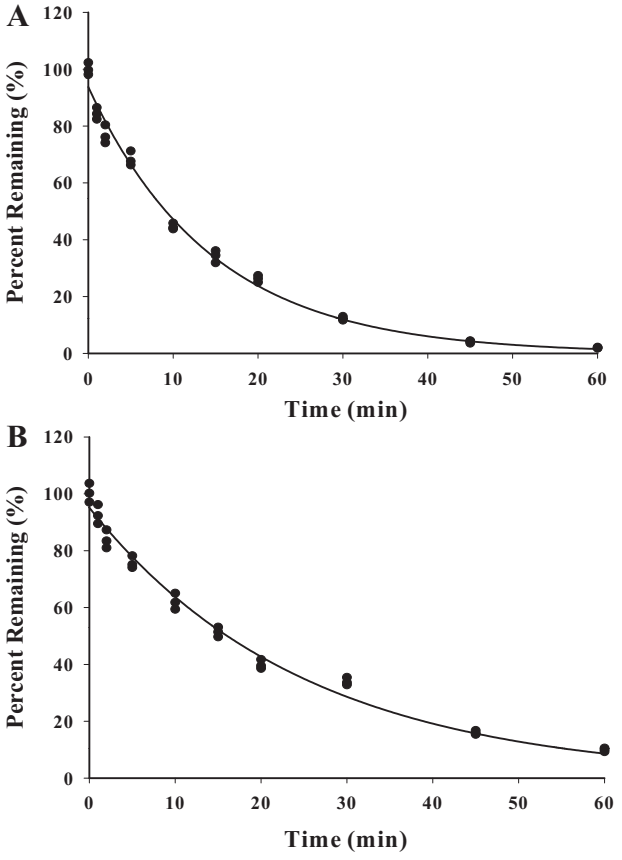

Fig. 2. Metabolic elimination profiles (% turnover or amount remaining *vs.* incubation time) for: (A) R-TJ0711 and (B) S-TJ0711.

metabolite profiling study of R- and S-TJ0711 HCl. Because enantiomeric separation of TJ0711 HCl is not able to be achieved by this UPLC-based method, TJ0711 enantiomers are required to be individually incubated with HLM in order to clarify the metabolite profiling characteristics between both enantiomers.

During *in vitro* metabolic incubation with HLM, 8 and 9 metabolites were detected for R-TJ0711 and S-TJ0711, respectively. Interestingly, an additional monohydroxylated metabolite was identified for S-TJ0711, although S-TJ0711 is more metabolically stable than R-TJ0711. Metabolism pathways of TJ0711 enantiomers, as well as the position of the metabolic modification, were proposed based on our reported criteria [2]. EPI spectra from detected metabolites confirmed that the same metabolism pathway and metabolic sites as racemic TJ0711 HCl were found for both enantiomers (data not shown). Our findings are consistent with our previous report, in which metabolite profiling of racemic TJ0711 HCl was investigated [2].

4. Conclusions

This paper presents a novel chiral LC–MS/MS method to simultaneously measure TJ0711 enantiomers in *in vitro* metabolic stability samples. This chiral method was further validated and successfully applied for an *in vitro* enantioselective metabolic stability study of TJ0711 HCl. The metabolic stability parameters obtained unveiled preferential metabolism of R-TJ0711 compared with S-TJ0711. In addition, using our previously published MRM-IDA-EPI method, 8 and 9 metabolites were detected for R- and S-TJ0711, respectively. Similar to racemic TJ0711 HCl, demethylation and hydroxylation were proposed to be the principle metabolism pathways for both R- and S-TJ0711 during *in vitro* incubations with HLM.

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