In vitro metabolic stability and metabolite profiling of TJ0711 hydrochloride, a newly developed vasodilatory β-blocker, using a liquid chromatography–tandem mass spectrometry method

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ABSTRACT

In this paper, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous analysis of metabolic stability and metabolite profiling of 1-[4-(2-methoxyethyl) phenoxy]-3-[[2-(2-methoxyphenoxy) ethyl]amino]-2-propanol hydrochloride (TJ0711 HCl), a new vasodilatory β-blocker. Multiple reaction monitoring (MRM) was used as a survey scan to quantify the parent compound and to trigger the acquisition of enhanced product ions (EPI) for the identification of formed metabolites. In addition, comparison between MRM-only and MRM-information dependent acquisition-EPI (MRM-IDA-EPI) methods was conducted to determine analytical variables, including linearity, limit of detection (LOD), lower limit of quantification (LLOQ), as well as intra-day and inter-day accuracy and precision. Results demonstrated that MRM-IDA-EPI quantitative analysis was not affected by the addition of EPI scans to obtain qualitative information during the same chromatographic run, compared to MRM-only method. Thereafter, metabolic stability and metabolite identification of TJ0711 HCl were investigated using human liver microsomes (HLM) by the MRM-IDA-EPI method. The *in vitro* metabolic stability parameters were calculated and $t_{1/2}$, microsomal intrinsic clearance (CL_{int}), as well as hepatic CL, were 13.0 min, 106.5 μL/min/mg microsomal protein, and 1082.2 mL/min, respectively. The major formed metabolites were also simultaneously monitored and the metabolite profiling data demonstrated that this MRM-IDA-EPI method was capable of targeting a large number of metabolites, in which demethylation and hydroxylation were the principle metabolism pathways during the *in vitro* incubation with HLM.

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1. Introduction

In the early stage of drug discovery, many new chemical entities (NCE) are found to be metabolically unstable due to metabolism mediated by cytochrome P450 enzymes. Therefore, there is an increasing need to characterize their metabolic properties, such as metabolic stability, metabolite profiling, and enzyme inhibition/induction properties. Metabolic stability is a critical property of drug candidates because it plays an important role in oral bioavailability and overall pharmacokinetic performance [1]. Metabolic stability is usually quantified by monitoring the disappearance of the parent compound over time in liver preparations [2–4]. Currently, commercially available human liver microsomes (HLM) have been the most widely used *in vitro* model for metabolic stability studies [4]. Metabolite profiling studies involve qualitative and quantitative detection of metabolites formed *in vitro* and *in vivo*. Identifying the metabolic sites on the molecules helps medicinal chemists synthesize more metabolically stable compounds [5].

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become increasingly popular in the analysis of aforementioned metabolic properties. In contrast to luminescence, fluorescence, and radiometric analytical techniques, LC–MS/MS usually provides superior sensitivity and selectivity in supporting quantitative drug metabolism studies. More recently, hybrid triple quadrupole/linear ion trap (QqQ_{LIT}) mass spectrometer has been applied for metabolic property analysis with an addition of a high duty cycle in terms of the scanning capabilities using the linear ion trap [6,7]. This newly developed system has been used not only for quantitative analysis, but also for acquiring structural information data (product ion spectra) in the same analytical run. Therefore, it becomes possible for

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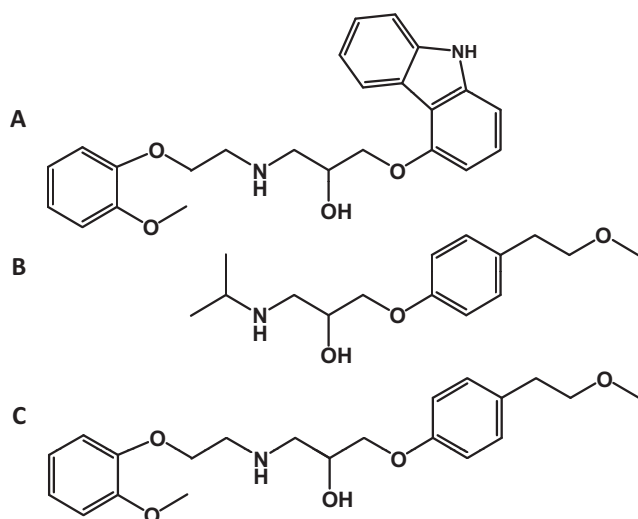
Fig. 1. Chemical structures of (A) carvedilol, (B) metoprolol, and (C) TJ0711.

the high-throughput metabolic stability assay to keep pace with screening requirements in drug discovery, especially in the lead optimization stage. Multiple reaction monitoring (MRM) has been widely used as a survey scan to trigger the acquisition of enhanced product ions (EPI) for drug metabolite identification [2,8]. To demonstrate the quantitation of the parent compound is not negatively affected by the addition of survey MRM channels and the EPI scans, a comparison between MRM-only and MRM-information-dependent acquisition (IDA)-EPI methods needs to be performed. However, to our best knowledge, only a single paper has been previously published by us [9], in which linearity of both methods was compared using praziquantel as a model compound. Therefore, there is a need to develop and validate a MRM-IDA-EPI method, using the MRM-only method as a control, to ensure both methods provide the same selectivity, sensitivity, accuracy and precision required to support the metabolic stability studies.

1-[4-(2-Methoxyethyl) phenoxy]-3-[[2-(2-methoxyphenoxy) ethyl]amino]-2-propanol (TJ0711), a 'hybridized' compound derived from carvedilol and metoprolol (shown in Fig. 1), was synthesized as a new vasodilatory β-blocker for hypertension and other related syndromes [10]. Our previous preclinical pharmacokinetic results showed that a rapid in vivo clearance (half life <30 min) was observed in rats following a single intravenous dose of TJ0711 hydrochloride (TJ0711 HCl) [11]. One plausible explanation for the fast elimination of TJ0711 HCl is that it might undergo an extensive metabolism in rat liver. In addition, both carvedilol and metoprolol are subject to a rapid first-pass drug metabolism, resulting in demethylated and/or hydroxylated metabolites [12–14]. Moreover, most data show that those metabolites are less effective or inactive compared to the parent molecules [15,16]. Thus, an attempt was made to investigate the in vitro metabolic stability and metabolite profiles of TJ0711 HCl using HLM.

In this paper, a LC–MS/MS method employing an IDA approach using MRM as survey scans and EPI as dependent scans was developed for simultaneous assay of the parent compound and formed metabolites. Furthermore, comparison between MRM-IDA-EPI and MRM-only methods was conducted to provide proof of principle of the use of MRM-IDA-EPI for simultaneous analysis of metabolic stability and metabolite profiling. Finally, MRM-IDA-EPI method was successfully applied to an in vitro metabolic stability and metabolite profiling study of TJ0711 HCl.

2. Experimental

2.1. Chemicals and reagents

TJ0711 HCl was synthesized by our team and the purity was determined to be higher than 99.8% by HPLC. The internal standard (IS), celiprolol hydrochloride, was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Magnesium chloride, D-glucose-6-phosphate (G6P), D-glucose-6-phosphate dehydrogenase (G6P-DH) and β-nicotinamide adenine dinucleotide phosphate (NADP+) disodium salt were obtained from Sigma–Aldrich (St. Louis, MO, USA). Potassium phosphate monobasic and HPLC-grade methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pooled HLM (n = 200 livers) were purchased from XenoTech, LLC (Lenexa, KS, USA).

2.2. Preparation of standard solutions

The stock solutions of TJ0711 HCl and IS were prepared in methanol at a concentration of 10 mg/mL. Working standard solutions containing 2, 4, 10, 20, 40, 100, 200, 400, 1000 ng/mL TJ0711 HCl and 400 ng/mL IS were prepared by diluting the stock solution with methanol.

2.3. Microsomal incubations

Incubations were carried out in triplicate in a final volume of 100 μL. TJ0711 HCl was pre-incubated with 0.5 mg/mL HLM in 50 mM potassium phosphate monobasic buffer (pH 7.4) containing 3 mM MgCl$_2$ for 5 min at 37 °C. TJ0711 HCl was also prepared in the same potassium phosphate monobasic buffer and the final concentration of TJ0711 HCl was 1 μM. Reactions were initiated by adding warm NADPH regenerating system (1 mM NADP+, 5 mM G6P, 1 U/mL G6P-DH) and terminated by adding 100 μL ice-cold methanol containing 400 ng/mL IS at various pre-defined intervals (0, 1, 2, 5, 10, 15, 20, 30, 45, and 60 min). The samples were centrifuged at 16,000 × g for 10 min to remove precipitated protein and 10 μL of the supernatant was directly injected for LC–MS/MS analysis.

2.4. Instruments and conditions

Ultra-performance liquid chromatography (UPLC) separation of TJ0711 HCl and its metabolites was performed on an ACQUITY UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters, Milford, MA, USA) at room temperature using a Waters ACQUITY UPLC system (Waters). The mobile phase consisted of A (7.5 mM ammonium acetate, pH 7.0) and B (methanol). The gradient profile was held at 15% B for 3 min, increased to 95% B linearly in 13 min, and then held at 95% B for another 2 min and switched back to the initial condition in 0.25 min, followed by a 1.75-min equilibration. The flow rate was set at 0.2 mL/min.

A 4000 QTrap® hybrid QqQ$_{LIT}$ mass spectrometer equipped with an electrospray ionization (ESI) source (Applied Biosystems, Foster City, CA, USA) was coupled to the UPLC system described above. Analyst 1.4.2 software was used for data acquisition and processing. All MS experiments were conducted under positive MRM mode for both MRM-only and MRM-IDA-EPI methods. Mass spectrometric conditions, such as ion source voltage, temperature, gas pressure, declustering potential (DP), collision energy (CE), and cell exit potential (CXP), were optimized by infusing 1 μg/mL solutions of the analytes dissolved in 50% methanol at 5 μL/min using a Harvard 22 standard infusion syringe pump (Harvard Apparatus, South Natick, MA, USA). Source parameters were set as follows: curtain gas: 30 psi; ion source:

Table 1
MRM transitions (based on the 4 most abundant fragment ions) and MS parameters for TJ0711.

	MRM transition	Declustering potential (V)	Collision energy (eV)	Cell exit potential (V)
TJ0711	$376.2 \rightarrow 252.1$	76	31	12
	$376.2 \rightarrow 100.1$	76	39	16
	$376.2 \rightarrow 91.1$	76	71	16
	$376.2 \rightarrow 224.1$	76	57	12
IS	$380.2 \rightarrow 251.0$	71	35	12
	$380.2 \rightarrow 74.0$	71	51	12

5500 V; temperature: 500 °C; nebulizer gas (Gas 1): 30 psi; auxiliary gas (Gas 2): 20 psi; CAD gas: high; interface heater: on. Mass spectrometer parameters for each transition are shown in Table 1.

For the MRM-only method, a total of six MRM transitions for the parent compound and IS were used for metabolic stability quantitation (Table 1). The dwell time for each transition was set at 20 ms. The MRM-IDA-EPI method was described in detail previously [8,9]. Briefly, MRM channels for the 'guessed' metabolites generated by an analyst script based on the most common 12 biotransformation pathways, along with the original MRM transitions used for MRM-only method, were used as survey scans to obtain quantitative information of the parent compound and qualitative information of metabolites by triggering EPI scans. In this case, the 4 most intense product ions of TJ0711 ([M+H]$^+$ = 376) were m/z 252, 100, 91 and 224. Thus, 8 MRM survey channels for demethylated (M+H-14) metabolites at $362 \rightarrow 252$, $362 \rightarrow 238$, $362 \rightarrow 100$, $362 \rightarrow 86$, $362 \rightarrow 91$, $362 \rightarrow 77$, $362 \rightarrow 224$, $362 \rightarrow 210$, were generated. This guaranteed the detection of the demethylated metabolites derived either from dealkylation in product or neutral loss parts of the molecule. A total of 100 MRM survey channels were created for TJ0711 HCl, as well as two dedicated MRM channels to monitor IS, with a dwell time of 5 ms/channel and a pause time of 5 ms.

The same DP, CE, and CXP values optimized for the parent compound were used for the MRM transitions of its 'guessed' metabolites. The IDA threshold was set at 500 counts per second (cps), above which EPI spectra were triggered and collected for the parent mass of that particular channel. The EPI scan rate was 4000 amu/s and the scan range was 75–500 amu. The CEs were set at 42 eV with a CE spread of 10 eV. Dynamic exclusion was enabled and the repeat count was set at 3 with an exclusion time of 8 s. Dynamic fill time (DFT^{TM}) function was used to prevent overfilling of the LIT. The total cycle time for the MRM-IDA-EPI was 1.7 s/cycle.

2.5. Determination of analytical variables

To compare analytical variables between both methods, calibration curves in the range of 1–500 ng/mL were prepared for TJ0711 HCl by spiking 100 μL aliquots of incubation mixture with 100 μL of appropriate working solutions, followed by centrifugation at $16,000 \times g$ for 10 min. Each supernatant was divided into two equivalent aliquots for MRM-only and MRM-IDA-EPI analyses. Measurements were performed in three independent validation runs in order to obtain analytical variables, such as linearity, limit of detection (LOD), lower limit of quantification (LLOQ), intra-day and inter-day accuracy and precision. Quality control (QC) samples of TJ0711 HCl with the concentrations of 1, 2, 100, and 450 ng/mL were prepared and analyzed by both methods. Accuracy and precision of the method were determined by assaying five replicates of each QC point using freshly prepared calibration curves in three separate runs. Intra-day accuracy and precision were calculated from the % bias [% (Measured − Theoretical)/Measured concentration] and relative standard deviation [% RSD = % Standard Deviation/Mean], respectively. Inter-day precision was 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 allowed. Linear regression analyses were conducted using $1/x$ weighting scheme. The LOD was defined as the concentration that produced a signal three times above the noise level, and the LLOQ was defined as the lowest calibration standard concentration at which intra- and inter-day accuracy and precision were less than or equal to 20%. In addition, the recoveries of TJ0711 over the whole concentration range were determined by comparing peak areas obtained from QC samples and those found by direct injection of neat solution prepared at the same concentration using 50% methanol in H$_2$O.

2.6. Calculation of in vitro metabolic stability parameters

The rate constant for the disappearance of parent compound (k) was calculated from the slope of the terminal phase of the percent turnover vs. time plot. The percent turnover was calculated as the percentage of analyte concentrations in incubated samples relative to those of 0-min samples. The *in vitro* metabolic stability parameters including $t_{1/2}$, microsomal CL$_{int}$, hepatic CL$_{int}$, extraction ratio (ER), and hepatic CL, were calculated using appropriate scaling factors as previously described [4,9,17,18]. The free fraction in blood (f_u) for TJ0711 HCl was determined to be 91.7% using equilibrium dialysis method [19].

3. Results and discussion

3.1. Method development

During *in vitro* metabolic stability studies, analytical methods with very high sensitivity and selectivity are required for the detection of the trace concentration of parent compounds and their metabolites. Compared to UV and fluorescence detectors, the assay sensitivity for the quantitation of TJ0711 HCl has been enhanced by at least 100-fold using the LC–MS/MS method [11,20]. In this study, the [M+H]$^+ \rightarrow$ 252 transition was used for the parent compound quantification. Although the hybrid QTrap QqQ$_{LIT}$ mass spectrometer is capable of performing EMS (enhanced MS), EPI (enhanced product ion), PI (precursor ion), NL (neutral loss), or MRM (multiple reaction monitoring) scans, MRM transitions were selected as survey scans to trigger the acquisition of EPI spectra in this study, since our previous study demonstrated that MRM-based IDA methods were able to detect a larger number of metabolites than other survey scan-base IDA analyses due to the superior sensitivity and selectivity of MRM scans [9]. In addition, a full-scan based EMS-IDA-EPI method was conducted to ensure no unexpected metabolites were missed. However, only two major metabolites were identified by this full-scan based IDA method and no additional metabolites other than those detected by MRM-IDA-EPI were found (data not shown). To the best of our knowledge, the effect of the addition of survey MRM channels for 'guessed metabolites' and the IDA-EPI scans on quantitation of the parent compound has not yet been fully investigated. Thus, a MRM-based IDA method was developed and validated in the current study to ensure the sensitivity, accuracy, and precision required for TJ0711 HCl quantification, using a simple MRM-only method as a standard control. In addition, the importance of adequate and satisfied chromatographic separation should also be addressed especially when multiple metabolites elute with the similar retention time. Different from most reports using HPLC for metabolic stability screening and metabolite characterization, we employed UPLC methods for an improved baseline resolution [2,8,21,22]. Moreover, the gradient change from 15% to 95% was carried out more smoothly in 13 min, allowing the parent

Table 2

Calibration curves of TJ0711 HCl in a concentration range of 1.0–500 ng/mL using MRM-only and MRM-IDA-EPI methods.

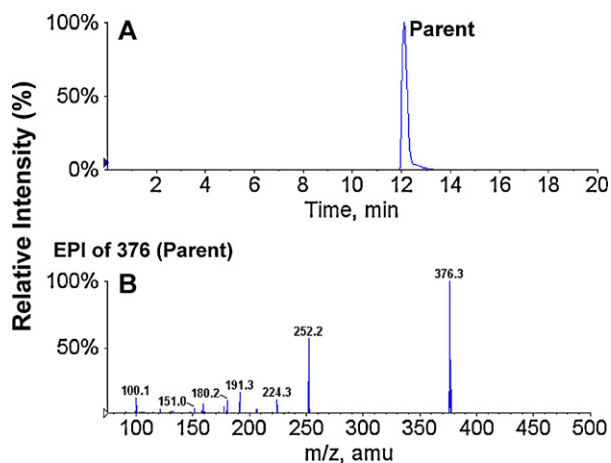|	MRM-only method	MRM-IDA-EPI method
Slope	0.0088 ± 0.0005	0.0090 ± 0.0007
Intercept	0.0006 ± 0.0003	0.0007 ± 0.0003
r	0.9995 ± 0.0004	0.9990 ± 0.0005
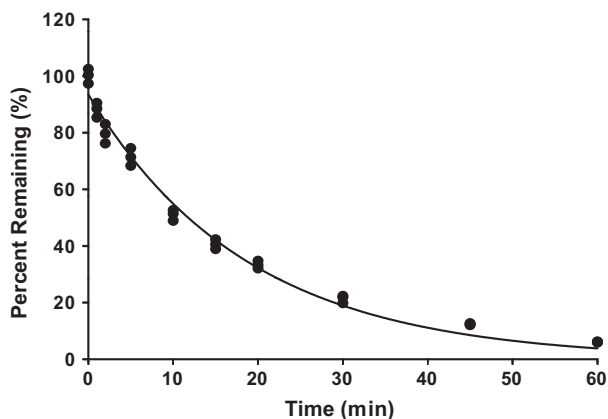
Fig. 2. Metabolic elimination profile (% turnover or amount remaining vs. incubation time) for TJ0711 hydrochloride obtained using MRM-IDA-EPI method.

compound and all metabolites to be eluted within the analytical run.

In IDA methods, measures should be taken to ensure the acquisition of the EPI spectra of co-eluting metabolites. We set our exclusion criteria at 8 s after 3 consecutive appearances to avoid continuous triggering of the dependent scan acquisition of metabolites with higher concentrations after 3 consecutive dependent scans. Therefore, the MRM-IDA-EPI method leaves the opportunity (8 s) for other co-eluting metabolites with lower abundance to trigger the acquisition of their own EPI spectra. In addition, the IS (m/z 380.2) was not placed in the exclusion list of IDA criteria across the full LC run to ensure that any metabolites with the same mass as IS were not missed.

It's worth noting that this MRM-IDA-EPI method is unable to detect unexpected metabolites, which are not 'guessed metabolites' [22]. The effectiveness of our method relies on metabolite prediction. For this reason, 4 most intensive MRM transitions were chosen to reduce the possibility of unpredicted fragmentation.
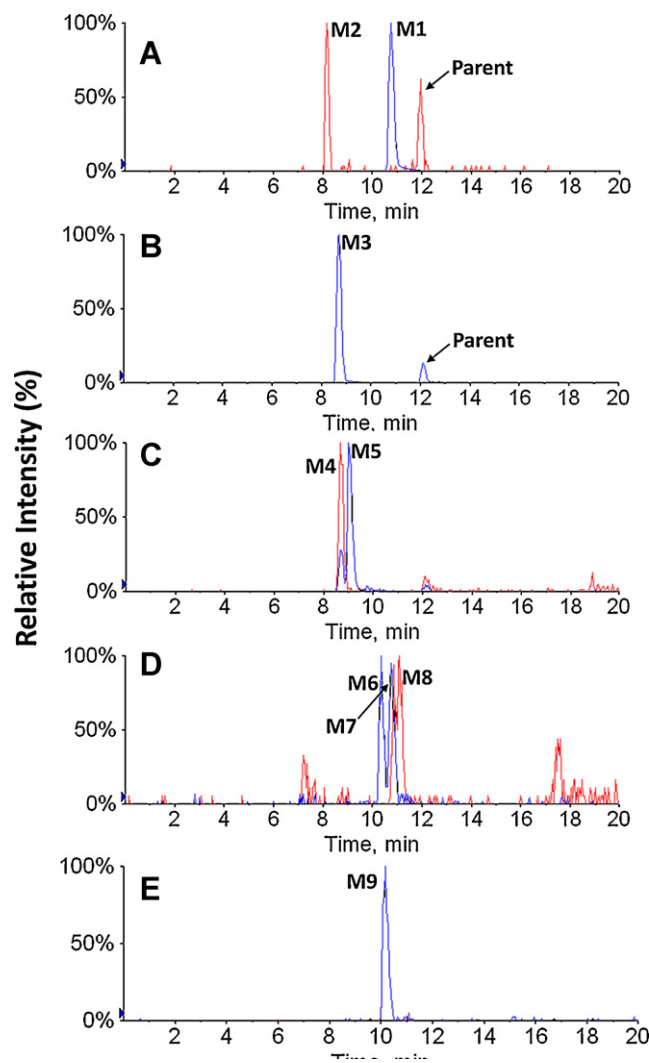

Fig. 3. Representative extracted ion chromatogram (EIC) (A) and enhanced product ion (EPI) spectra (B) obtained from a control sample of TJ0711 hydrochloride without incubation with HLM using the MRM-IDA-EPI method.

Table 3

Summary of the intra-day and inter-day accuracy and precision of TJ0711 using MRM-only and MRM-IDA-EPI methods.

	MRM-only method		MRM-IDA-EPI method	
	%Bias	%RSD	%Bias	%RSD
Intra-day ($n = 5$)				
LLOQ-QC (1 ng/mL)	−6.5	12.5	5.8	8.8
Low-QC (2 ng/mL)	4.1	9.7	−7.2	10.5
Mid-QC (100 ng/mL)	6.9	4.2	5.0	7.5
High-QC (450 ng/mL)	−5.4	7.6	2.8	6.4
Inter-day ($n = 15$)				
LLOQ-QC (1 ng/mL)	3.8	9.8	−3.6	13.4
Low-QC (2 ng/mL)	−7.4	11.7	−5.0	8.3
Mid-QC (100 ng/mL)	8.5	7.4	7.3	9.1
High-QC (450 ng/mL)	−4.1	4.9	−6.4	7.5

Although non-targeted survey scans, such as EMS and NL, do not require pre-selecting metabolism pathway, in fact, they are more likely to miss some minor metabolites with low signal intensity, due to the high background signal from the matrix, as reported in literature [2,9]. On the other hand, our approach was efficient in

Fig. 4. Representative extracted ion chromatograms (EICs) obtained from a sample incubated with HLM for 60 min for: (A) demethylated metabolites (M1, M2: 362 m/z); (B) a demethylated + ketonized metabolite (M3: 376 m/z); (C) demethylated + monohydroxylated metabolites (M4, M5: 378 m/z); (D) monohydroxylated metabolites (M6–M8: 392 m/z); (E) a di-demethylated metabolite (M9: 348 m/z).
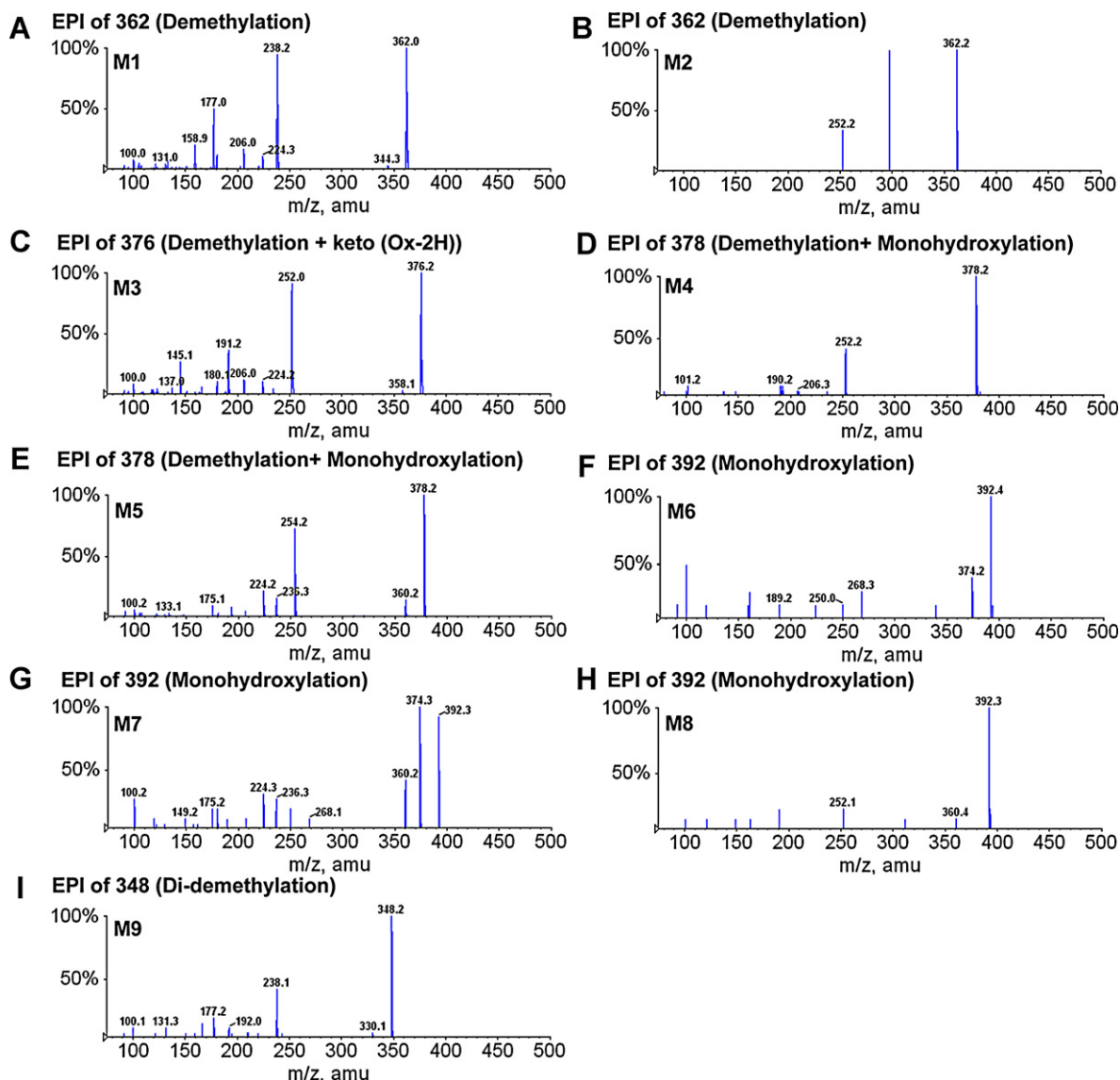
Fig. 5. Representative enhanced product ion (EPI) spectra of nine TJ0711 metabolites: (A) M1; (B) M2; (C) M3; (D) M4; (E) M5; (F) M6; (G) M7; (H) M8; (I) M9.

supporting both the metabolic stability and metabolite profiling studies by simultaneously providing quantitative turnover information and metabolite structure information despite that the list of metabolites detected is not complete.

3.2. Comparison of analytical performance between MRM-only and MRM-IDA-EPI methods

To compare the analytical performance between MRM-only and MRM-IDA-EPI methods, nine-point calibration curves, encompassing a concentration range of 1–500 ng/mL TJ0711 HCl, were prepared and analyzed in three separate runs with each method. All calibration curves produced by both methods were linear over the concentration ranges tested, with average correlation coefficients of 0.9995 and 0.9990 for MRM-only and MRM-IDA-EPI methods, respectively (Table 2). Both methods exhibited equivalent response factors (slope and intercept values in Table 2), resulting in identical LOD and LLOQ values (0.2 ng/mL and 1 ng/mL, respectively). However, it should also be noted that sufficient data points (typically more than 10 data points) for each chromatographic peak were still achievable using the MRM-IDA-EPI method due to the fast scanning capability of the QqQ$_{LIT}$. Intra-day and inter-day accuracy and

precision results obtained from both methods are summarized in Table 3. All accuracy and precision were less than 15% at all concentration levels, indicating that both methods are accurate and precise for metabolic stability analysis. Mean recovery values ±RSD for TJ0711 and IS were 94.1 ± 4.9% and 92.3 ± 6.4%, respectively. In summary, the analytical performance obtained from both methods agreed very well. Quantification of the parent compound was not affected by the simultaneous acquisition of qualitative analysis for metabolite identification and therefore robust quantification was achieved using MRM-IDA-EPI method.

3.3. Method application

Metabolic stability of TJ0711 HCl was studied by monitoring the disappearance of the parent compound over a 60-min incubation with HLM. Concentrations of the parent compound were determined according to the peak area ratios of parent vs. IS using corresponding calibration curves prepared in HLM matrix. The disappearance of the parent compound was monitored by calculating the percent remaining of each compound relative to 0 min and was plotted against the incubation time (Fig. 2). The in vitro metabolic stability parameters were calculated and $t_{1/2}$,
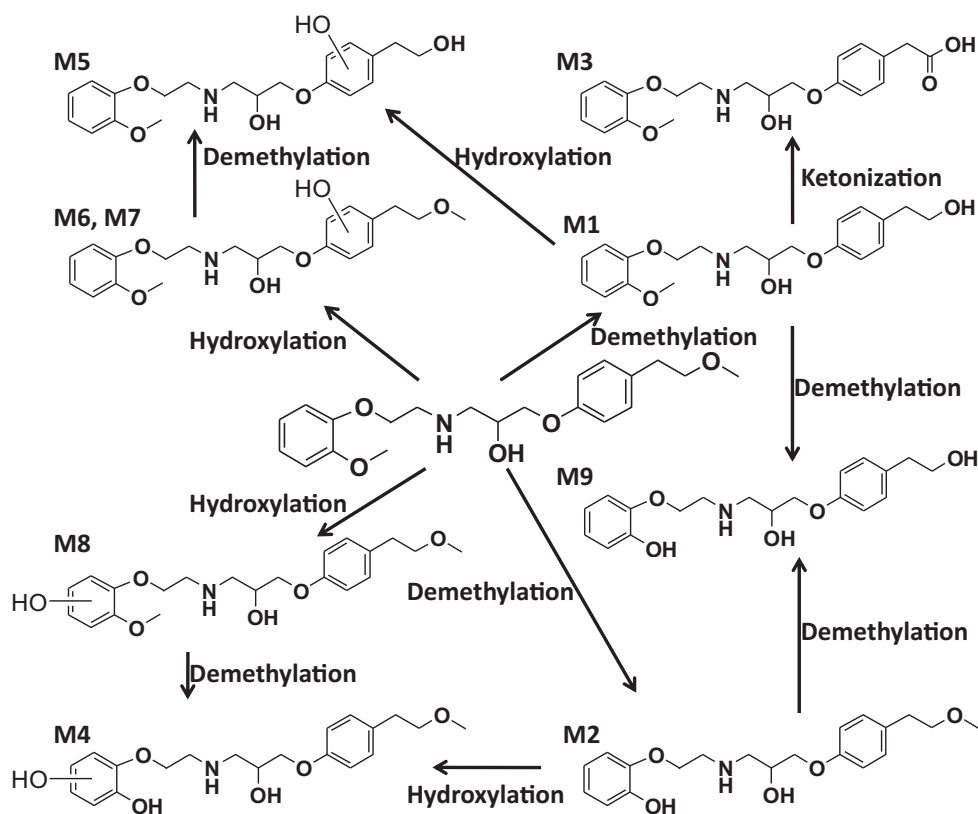
Fig. 6. Proposed metabolic pathways of TJ0711 incubated with HLM *in vitro*.

microsomal CL$_{int}$, hepatic CL$_{int}$, ER, and hepatic CL were 13.0 min, 106.5 μL/min/mg microsomal protein, 5199.8 mL/min, 0.8, and 1082.2 mL/min, respectively. As a result, TJ0711 might be classified as a high-extraction-ratio compound based on the *in vitro* human microsomal stability data [23], which verified our aforementioned hypothesis that the fast elimination of TJ0711 is ascribe to the extensive liver phase I metabolism.

During the study of TJ0711 HCl metabolic stability, 9 metabolites were formed and detected using our approach. Fig. 3 demonstrates the extracted ion chromatogram (EIC) and the EPI spectrum of the parent compound (TJ0711 HCl). Assignment of the detected masses as metabolites was based on the following criteria: (1) the MRM signal should be detected in the sample incubated with HLM (time 60 min), but undetectable in the 0-min control sample, (2) an expected mass of the metabolite ion should be observed from EPI spectra, (3) either the same product ions or equivalent neutral losses were found from the EPI spectra of TJ0711 and its metabolites. EICs and EPI spectra of TJ0711 metabolites are shown in Figs. 4 and 5, respectively. Metabolism pathways of TJ0711 were proposed based on the mass shift of the metabolites, fragmentation pattern obtained from EPI spectra, as well as the intensity of the MRM signal (Fig. 6). For example, M1 and M2 were assigned as demethylated metabolites because the precursor ion (*m/z* 362) and/or the product ions (*m/z* 238 and/or *m/z* 177) were 14 mass units smaller than the corresponding precursor (*m/z* 376) and product ions (*m/z* 252 and *m/z* 191) of TJ0711. In addition to M1 and M2, a third peak obtained in Fig. 4A was considered as the parent compound since the same retention time was observed compared to the parent compound and the MRM signal was also detectable in the control sample (time 0 min). Similarly, M3 was assigned as a metabolite with a combination of demethylation and ketonization; M4 and M5 were assigned as metabolites with a combination of demethylation and monohydroxylation; M6–M8 were

assigned as monohydroxylated metabolites; M9 was assigned as a di-demethylated metabolite.

The position of the metabolic modification was narrowed down to a specific part or a specific 'soft spot' on the molecules, where the metabolic reaction occurs. For instance, the fragmentation pattern of M1 is quite similar to TJ0711, except that fragment ions of *m/z* 238 and *m/z* 177 rather than *m/z* 252 and *m/z* 191 were observed in the M1 EPI spectrum. These findings suggested that demethylation took place at the part of the molecule corresponding to the *m/z* 252 product ion. In addition, the highest signal among all MRM transitions for M1 was from the 362.2 → 238.1 transition. According to the structure of TJ0711, O-demethylation proposed to occur in the methoxyethyl group for M1. In contrast, M2 was identified as the product of demethylation of the 2-methoxyphenoxy group since no mass shift was observed in the 252 ion of M2 EPI spectrum and the 362.2 → 252.1 rather than 362.2 → 238.1 transition produced the highest signal. Therefore, O-demethylation occurred at both methyl groups of TJ0711 after incubation with HLM for 60 min. However, the signal intensity of M1 was 500-fold higher than M2, indicating that M1 is the predominant demethylated metabolite for TJ0711 HCl. M9 is a di-demethylated metabolite, in which O-demethylation took place at both methyl groups. Similarly, the structure of M3 was resulted from a combination of demethylation and ketonization on TJ0711 molecule (Fig. 6). For the metabolite M4, demethylation and mono-hydroxylation were proposed to occur on 2-methoxyphenoxy group since no mass shift was observed in the 252 ion of M4 EPI spectrum and the 378.2 → 252.1 rather than other transitions produced the highest signal. Similarly, the structure of M5 was resulted from a combination of demethylation and mono-hydroxylation on 2-methoxyethylphenoxy group. The structures for monohydroxylated metabolites (M6–M8) were also similarly proposed and shown in Fig. 6. Nevertheless, further studies using

deuterate exchange and nuclear magnetic resonance (NMR) techniques are required to determine the precise structure of TJ0711 metabolites.

4. Conclusion

A LC–MS/MS method using MRM-IDA-EPI approach on a hybrid QqQ$_{LIT}$ mass spectrometer could be used to support the simultaneous quantitative and qualitative analysis in metabolic stability and metabolite profiling studies. In the present study, MRM scans were used as survey scans for the higher selectivity and sensitivity than EMS, NL, MIM, and PI scans, which are more likely to miss low-abundance metabolites due to the high background signal from matrix. Quantitative determination of the parent compound disappearance was not compromised in this combined quantitative–qualitative approach. This MRM-IDA-EPI approach was successfully applied for metabolite identification while quantifying the turnover of TJ0711 HCl during the *in vitro* metabolic stability studies. EPI spectra from parent and potential metabolites were obtained within the same chromatographic run and these spectra were used to confirm the structures proposed for metabolites. Finally, demethylation and hydroxylation were found to be the principle metabolism pathways for TJ0711 HCl during the *in vitro* incubation with HLM.

Acknowledgements

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