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Short communication

Simultaneous determination of citalopram and its metabolite in human plasma by LC–MS/MS applied to pharmacokinetic study

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ABSTRACT

A simple sensitive and robust method for simultaneous determination of citalopram and desmethylcitalopram was developed using liquid chromatography tandem mass spectrometry (LC–MS/MS). A 200 μL aliquot of plasma sample was employed and deproteinized with methanol and desipramine was used as the internal standard. After vortex mixing and centrifugation, the supernatant was diluted with water (1:1, v/v) and then directly injected to analysis. Analytes were separated by a Zorbax XDB C_{18} column with the mobile phase composed of acetonitrile and water (30:70, v/v) with 0.25% formic acid and monitored in MRM mode using a positive electrospray source with tandem mass spectrometry detection. The total run time was 3.5 min. The dynamic range was 0.2–100 ng/mL for citalopram and 0.25–50 ng/mL for desmethylcitalopram, respectively. Compared to the best existing literatures for plasma samples, the same LOQ for CIT (0.5 ng/mL) and lower LOQ for DCIT (0.25 vs 5 ng/mL) were reached, and less sample preparation steps and runtime (3.5 vs 10 min) were taken for our method. Accuracy and precision was lower than 8% and lower than 11.5% for either target. Validation results and its application to the analysis of plasma samples after oral administration of citalopram in healthy Chinese volunteers demonstrated the method was applicable to pharmacokinetic studies.

1. Introduction

The global incidence of depression has increased to the point where it will be second in the International Burden of Disease ranking by 2020. Citalopram (CIT) is a widely used non-tricyclic antidepressant for its better acceptability and fewer discontinuations. It is already known that polymorphism of CYP2C19 plays an important role in citalopram N-demethylation to its main metabolite desmethylcitalopram (DCIT) [1,2]. Due to the polymorphism of CYP2C19, great interspecies and intraspecies variations occur in individual pharmacokinetic properties. The relative proportion of CIT and DCIT could reflect the activity of CYP2C19 and its polymorphism. Thus the measurement of CIT and DCIT is useful in monitoring compliance or overdose [3], optimizing the therapeutic effect, and researching gene-dose effect.

Many assays have been reported, such as coupled with UV [4–9], photodiode array [10–12], fluorescence [13–18], and mass spectrometry [19–22] detection. All of these methods involved multi-step extractions, large volumes of organic solvent and intensive labor to prepare sample, which became the rate limiting steps. As reported [4–22], the lower concentrations of CIT and DCIT in plasma after dosing are magnitude of ng/mL, the high sensitivity and high selectivity of triple quadrupole can easily detect the lower drug concentration after dosing even diluted by several times. In this study, protein precipitation without concentration was employed to shorten processing time and reduce labor. Hence a simple sensitive and robust method suitable for the pharmacokinetic research and routine TDM of CIT and DCIT was developed.

2. Experimental

2.1. Chemicals and reagents

Citalopram hydrobromide and demethylcitalopram hydrobromide of pharmaceutical purity, and citalopram tablet formulation (Cipramil® H. Lundbeck A/S, Copenhagen, Denmark) were supplied by Salutas Pharma GmbH (Barleben, Germany). The internal standard (IS), desipramine hydrochloride of pharmaceutical purity (>98%), was purchased from Sigma–Aldrich (St. Luis, USA). Acetonitrile and methanol of HPLC grade were supplied by Burdick

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& Jackson (New Jersey, USA). Formic acid was HPLC grade and purchased from Tedia (Ohio, USA). Water was deionized and further purified using an arium 611VF from Sartorius AG Gottingen (Goettingen, Germany), and was filtered through a 0.22 μm filter prior to liquid chromatographic procedure.

2.2. Standard stock solutions and working solution preparation

Standard stock solutions of CIT, DCIT and IS, desipramine were made up at 1.0 mg/mL in methanol as free forms. All stock standard solutions were stored under freeze (−20 °C). A series of working solutions were obtained by further dilution with appropriate amount of 100% methanol at 7 concentration levels, freshly prepared every week and stored at 4 °C away from light.

2.3. Calibration standards and quality control sample preparation

The standard solutions of CIT (10 μL) and DCIT (10 μL) were added to tubes and immediately evaporated to dryness under nitrogen gas (Hengao, Tianjin, China). Then 200 μL of blank human plasma was added into the tubes followed by vortex mixing for 2 min. The resulting plasma concentrations were 0.2, 1, 2, 5, 20, 50, and 100 ng/mL for CIT and 0.25, 0.5, 1, 2.5, 10, 25, and 50 ng/mL for DCIT. The upper concentration levels of the two analytes were set according to the available clinical data, while the lower levels were set at the lower limit of quantification (LLOQ) of the proposed method. Quality control (QC) samples were made up in blank human plasma at three levels (low 0.5 ng/mL, 0.5 ng/mL, medium 5 ng/mL, 2.5 ng/mL, and high 100 ng/mL, 50 ng/mL, for CIT and DCIT, respectively) by a certain staff. All QC samples were stored at −70 °C.

2.4. Sample pretreatment

QC samples, calibration standard and clinical plasma samples were prepared employing a protein precipitation technique. To each tube containing 200 μL plasma, 10 μL of IS working solution (500 ng/mL) was added. After vortexing for 2 min, a 600 μL methanol was added, vortex-mixed for 5 min and centrifuged at 15,493 × *g* for 10 min. A 100 μL aliquot of the supernatant and a 100 μL aliquot of water were transferred to a clean autosampler insert following by being vortex-mixed for 30 s. 10 μL of the sample was injected into the LC–MS/MS system.

2.5. Liquid chromatography/mass spectrometry

LC was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc., USA) and separation was carried out at 35 °C using a Zorbax XDB C_{18} column (50 mm × 2.1 mm, 3.5 μm; Agilent Technologies, Inc., USA). The analytical column was protected by a XDB C_{18} narrow–bore guard column (12.5 mm × 2.1 mm, 5 μm; Agilent Technologies, Inc., USA). The mobile phase consisting of acetonitrile–water (30:70, v/v) with 0.25% formic acid was set at a flow rate of 300 μL/min. Separations were conducted under isocratic conditions. HPLC system was coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Inc., USA) using multiple reaction monitoring (MRM). An electrospray interface in positive ionization mode was used. ESI source parameters were as follows: High purity drying-gas (N_2) flow rate 8 L/min, temperature 350 °C, capillary voltage 3500 V, nebulizer pressure 172.4 kPa. MRM was used to quantify CIT (*m*/*z* 325 $[M+H]^+ \rightarrow$ 109 fragmentor 110 eV collision energy 30 eV), DCIT (*m*/*z* 311 $[M+H]^+ \rightarrow$ 109 fragmentor 80 eV collision energy 25 eV), and despramine (*m*/*z* 267 $[M+H]^+ \rightarrow$ 208 fragmentor 140 eV collision energy 20 eV). The three pairs of precursor-product ions were monitored simultaneously within the analytical procedure. MS data were processed using the MassHunter software package (Agilent Technologies, Inc., USA) containing a qualitative and a quantitative software.

2.6. Method validation procedure

The method was validated with respect to selectivity, linearity, accuracy, precision, recovery, matrix effect and stability.

Calibration curves were constructed at the range of 0.2–100 ng/mL for CIT and 0.25–50 ng/mL for DCIT by determining the best-fit of peak area ratios of analyte to IS (*y*) vs nominal concentration (*x*), and fitted to the equation $y = bx + a$ by using $1/x$ weighted least-squares regression. Concentrations of QCs and samples were calculated using the equation of the calibration curve. Intra-day and inter-day precision and accuracy were evaluated by assaying six replicates of each of spiked QCs at the low, middle and high concentrations on three separate days. The precision was expressed as relative standard deviation (RSD). Accuracy was calculated as the percent error in the calculated mean concentration relative to the nominal concentrations (RE). For the assay to be considered acceptable, the precision and accuracy at each QC level was required to be within 15%. Absolute recoveries at middle and high plasma concentrations of QC were determined in triplicate by comparing the peak area of either analyte in spiked post-protein precipitated plasma with the corresponding spiked sample. Matrix effect was investigated by comparing the deproteinized samples of blank plasma from six different drug-free volunteers spiked with low and high concentrations of QC with the direct injection of mobile phase spiked with the two analytes. Stability under the experimental condition was investigated at three levels of QC. The short-term stored stabilities of analytes after being processed were evaluated by testing their stabilities after being protein precipitated and stored for 24 h at room temperature. The long-term stability was examined for 40 days at −70 °C. Freeze/thaw stability testing was determined after freezing (at −70 °C) and thawing (at room temperature) three times.

2.7. Pharmacokinetic study

The proposed analytical method was tested in a Chinese healthy volunteers pharmacokinetic study approved by the Stated Food and Drug Administration (SFDA, China) and Shanghai Ruijin Hospital Ethics Committee of Clinical Investigation. Blood samples (3 mL) from 12 healthy Chinese volunteers were collected before (0 h) and 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120 and 144 h after administration of a single dose of 20 mg one CIT tablet formulation. Plasma was separated by centrifugation at 765 × *g* for 10 min at 4 °C, and stored at −70 °C until analyzed.

3. Results and discussion

3.1. Standard curve and method validation

The method was validated according to the FDA guidance for industrial bioanalytical method validation with respect to selectivity, linearity, accuracy, precision, recovery, matrix effect and stability.

The product mass spectra of CIT, DCIT and IS are shown in Fig. 1. Ion chromatograms from a blank sample (non-spiked blank plasma), a zero sample (only spiked with IS), a blank sample spiked at LOQ of two analytes and a sample after administration showed no significant interference peak at the retention time of CIT, DCIT and IS (Fig. 2.) The standard curves demonstrated an excellent linearity: for CIT, the mean slope was 5.4686 ± 0.1132 ($n = 6$), the

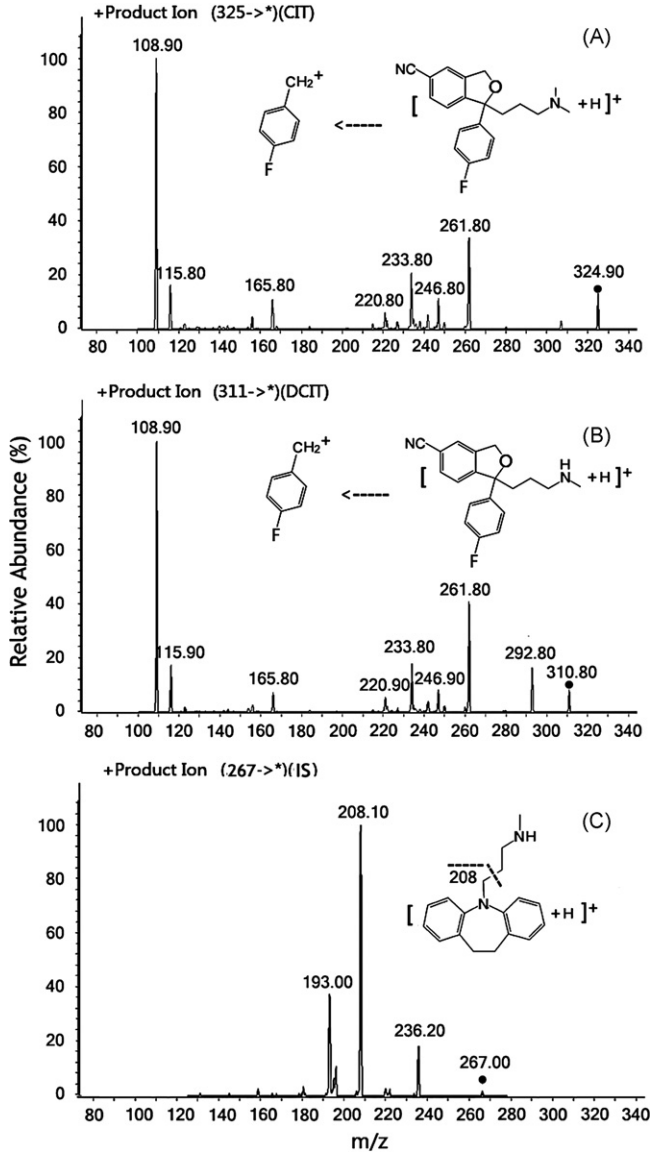

Fig. 1. Collision-induced dissociation mass spectra of CIT (A), DCIT (B) and IS (C).

mean intercept was 0.0091 ± 0.0044 (n = 8) and regression coefficients (Rs) were greater than 0.999; for DCIT the mean slope was 4.1254 ± 0.1812 (n = 6), the mean intercept was 0.0043 ± 0.0018 (n = 6) and Rs were greater than 0.999. Accuracy and precision of intra- and inter-assay were summarized in Table 1. The absolute recoveries were 98.1% (mean) for CIT and 98.2% for DCIT at the middle QC concentration and in the range of 98.9% for CIT and 100.2% for DCIT at high QC concentration. The relative matrix effect was 95.6 ± 6.8% (0.5 ng/mL) and 98.7 ± 8.5% (100 ng/mL) for CIT, 98.5 ± 5.1% (0.5 ng/mL) and 96.4% ± 8.0% (50 ng/mL) for DCIT. CIT and DCIT were proved to be stable in processed samples at room temperature for at least 24 h, for 40 days in plasma at −70 °C and in plasma after three freeze and thaw cycles. These results indicate that the method was reliable and robust within the analytical range (Table 2).

3.2. Application to a pharmacokinetic study

The present method was successfully applied to the pharmacokinetics studies after an oral administration of 20 mg citalopram hydrobromide tablets to 12 healthy Chinese healthy volunteers. Mean concentration-time profiles of CIT and DCIT were displayed in Fig. 3. The parameters were calculated by a non-compartmental model. The values of C_{max} and T_{max} were obtained directly from experiment observations. The mean of C_{max} was 33.0 ng/mL for CIT and 3.0 ng/mL for DCIT, respectively; the median of T_{max} in 12 volunteers was found to be 3.0 h for CIT and 24.0 h for DCIT; the AUC_{0-t} and $AUC_{0-\infty}$ calculated by the linear trapezoidal method were found to be (1417.0 ± 323.9) (mean ± SD, n = 12, the same below) and (1615.8 ± 408.5) ng h/mL for CIT, and (279.4 ± 50.4) and (397.8 ± 85.2) ng h/mL for DCIT; the elimination half-life ($t_{1/2}$) was estimated as (49.6 ± 5.7) h for CIT and (76.2 ± 20.6) h for DCIT. In the experiment, the last sampling concentrations (t = 144 h) varied from 1.1 to 4.9 ng/mL for CIT and 0.4 to 1.3 ng/mL for DCIT in 12 volunteers. The present LLOQ of 0.2 ng/mL for CIT and 0.25 ng/mL for DCIT was proved to be sufficient for clinical pharmacokinetic studies after an oral dose of 20 mg.

3.3. Discussion

Liquid liquid extraction (LLE), solid phase extraction (SPE) and protein precipitation (PP) are the most common preparation techniques for plasma samples analysis. The former two methods were employed frequently because of cleaner removal endogenous interference and their capabilities to concentrate some analytes. However, lower recovery due to the transfer of a fraction of the organic extract after the extraction, time-consuming and labor intensive may be disadvantages of the two techniques. In this methodologic development, we adopted PP for its time and labor saving. Furthermore, the high sensitivity and high selectivity of triple quadrupole can easily detect the lower drug concentration after dosing even diluted 6 times, and the matrix effect was tolerable via investigation. Methanol was chosen as PP agent compared with acetonitrile, acetone and different proportional mixture of methanol and acetonitrile (25:75; 50:50; 75:25, v/v), for it produced better peak shape and bet-

Table 1
The accuracy and precision of intra- (n = 5) and inter-assay (n = 3), respectively.

	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	RSD (%)	RE (%)
Intra-day	0.50	0.54	9.6	8.0
CIT	5.00	5.12	4.7	2.4
	100.00	105.30	4.8	5.3
DCIT	0.50	0.46	6.4	−8.0
	2.50	2.65	7.2	6.0
	50.00	50.52	9.0	1.0
Inter-day	0.50	0.51	11.4	2.0
CIT	5.00	4.97	4.1	−0.6
	100.00	101.49	3.0	1.5
DCIT	0.50	0.51	7.0	2.0
	2.50	2.70	6.8	8.0
	50.00	51.70	8.3	3.4

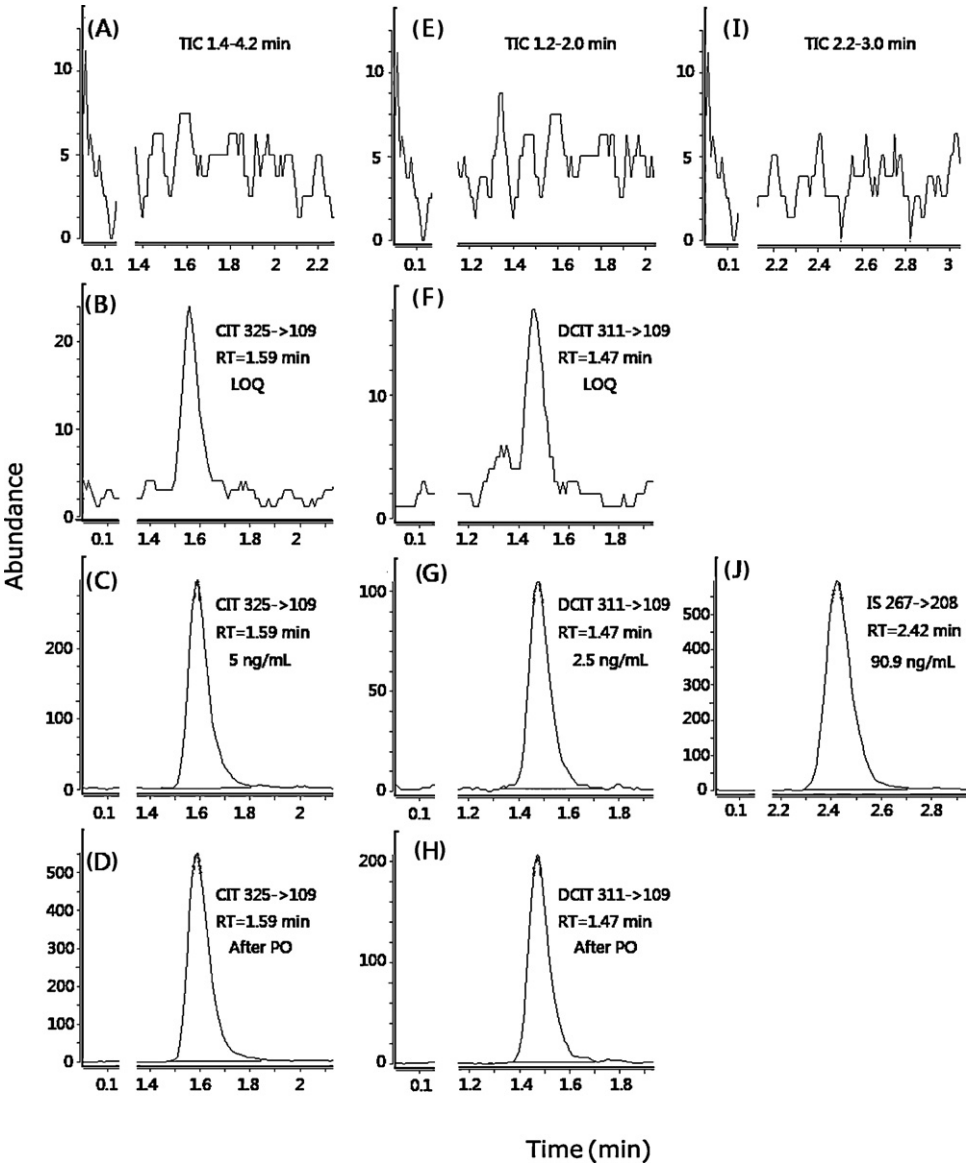

Fig. 2. Representative MRM chromatograms blank human plasma (non-spiked): (A) RT = 1.4–4.2 min (E) RT = 1.2–2.0 min and (I) RT = 2.2–3.0 min; CIT (B) spiked in blank human plasma (LOQ); (C) spiked in blank human plasma (5 ng/mL); (D) in sample after oral adminstration of 20 mg, DCIT (F) spiked in blank human plasma (LOQ); (G) spiked in blank human plasma (2.5 ng/mL); (H) in sample after oral adminstration of 20 mg, (J) IS spiked in plasma sample.

ter reproducibility. And the method would be further simplified by employing 600 μl methanol with appropriately spiked IS in advance as a precipitation solvent. After extraction and centrifugation, the supernatant was diluted 2 times by deionized water to get consistent with the strength of mobile phase for symmetrical peak shape.

The pH of the mobile phase influences both the chromatographic elution of the analytes and the intensity of the precursor ion of compound mainly related to the degree of ionization in ion source. It was found that positive ionization of analytes in ion source increases in acidic eluents. However, more than 0.025% formic acid in flow phase caused concomitant decrease in the responses of both compound and exceptional impairment in the chromatographic peak shape. Additionally, low pH will shorten the lifetime of analytical column.

Furthermore, we test different proportion of methanol and water in flow phase. If the retention time of analytes were less than 1 min, there would be severe ion suppression. Hence, an eluent consisting of ACN–water (30:70, v/v) containing 0.025% formic acid appeared to be most appropriate for CIT, DCIT and IS from both mass spectrometric and chromatographic point of view. The total running time was 3.5 min.

We have presented a simple sensitive and robust LC–MS/MS method for the quantification of CIT and DCIT in human plasma. The current method includes the shortest chromatographic run-

Table 2
The stability of short term, long term and freeze-thaw cycle ($n = 5$).

	%C_0[a]					
	Low		Mid		Hig	
	CIT	DCIT	CIT	DCIT	CIT	DCIT
Autosampler condition 24 h	93.3	110.6	97.6	104.7	98.5	92.1
40 days in plasma below 70 °C	98.6	109.9	98.9	105.5	100.1	97.6
Freeze-thaw 3 times	97.5	111.8	103.4	105.8	105.1	98.7

Results were expressed for each concentration level as the percentage of initial control concentration (C_0).
[a] The mean calculated concentration from a control set of replicates (time zero).

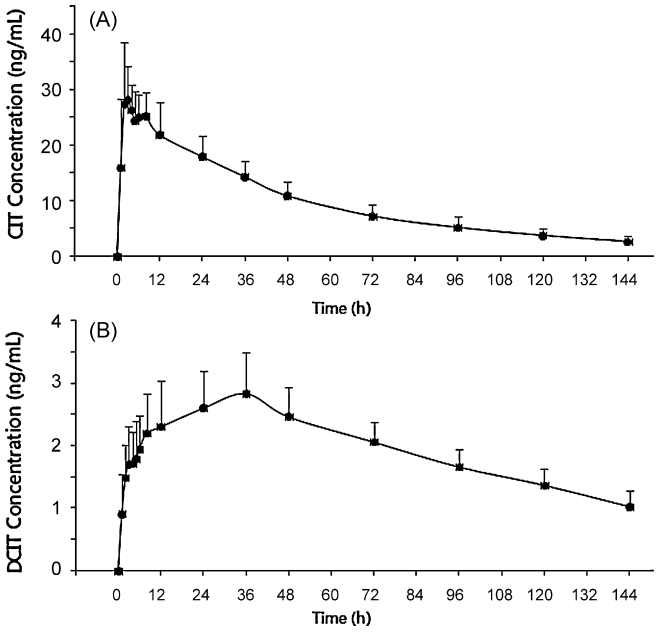

Fig. 3. Concentration (Mean ± SD)–time profiles of CIT (A) and DCIT (B) after administration up to 144 h in healthy adult male Chinese volunteers ($n = 12$).

time and the least preparation step so far proposed for CIT and DCIT quantification, as well as a relatively small volume of human plasma (200 μL) for analysis. The method was proven to be highly sensitive, accurate, precise and specific and was applied to a pharmacokinetic study (>1000 samples), which was completed in a very short period of time. This method allows for the simultaneous determination of CIT and DCIT in plasma and is adequate for pharmacokinetic study and routine TDM.

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