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Short communication

Liquid chromatography–tandem mass spectrometry quantification of levosulpiride in human plasma and its application to bioequivalence study

Prasad B. Phapale[a,b,c,1], Hae Won Lee[a,b,1], Mi-sun Lim[a,b,c], Sook Jin Seong[a,b,c], Eun-Hee Kim[b], Jeonghyeon Park[a,b,c], Miran Lee[a,b,c], Sung-Kyu Hwang[b], Young-Ran Yoon[a,b,c,*]

[a] Department of Molecular Medicine, Kyungpook National University School of Medicine, Daegu, South Korea
[b] Clinical Trial Center, Kyungpook National University Hospital, 200 Dongduk-Ro, Jung-gu, Daegu 700-422, South Korea
[c] BK21 Program, Kyungpook National University School of Medicine, 101 Dongin-2ga, Jung-gu, Daegu 700-422, South Korea

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ABSTRACT

An improved method for determining levels of levosulpiride in human plasma using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed and validated. The protein precipitation method was used for plasma sample preparation. Levosulpiride and an internal standard (IS) were isocratically separated on a UPLC BEH C_{18} column with a mobile phase of ammonium formate buffer (1 mM, adjusted to pH 3 with formic acid) and acetonitrile (60:40, v/v). MS/MS detection was performed by monitoring the parent → daughter pair of levosulpiride and the IS at *m/z* 342 → 112 and 329 → 256, respectively. The method was linear from 2.5 to 200 ng/mL and exhibited acceptable precision and percent recovery. The method was successfully demonstrated in pharmacokinetic and bioequivalence studies of two levosulpiride oral formulations administered to healthy volunteers. When compared to the previous LC–MS methods, the proposed method is faster, well-validated, and uses lesser plasma volume and a similar sensitivity. The use of UPLC allowed rapid and sensitive quantification of levosulpiride, making this method suitable for high-throughput clinical applications.

1. Introduction

Levosulpiride, N-[[(2S)-1-ethylpyrrolidin-2-yl] methyl]-2-methoxy-5-sulfamoylbenzamide, is a substituted benzamide antipsychotic and is the *levo*-enantiomer of racemic sulpiride (Fig. 1a). Levosulpiride is used to treat anxiety disorders, schizophrenia, depression, and peptic ulceration [1,2]. It acts by selectively blocking the dopaminergic D_2 receptor in both the central nervous system and gastrointestinal tract [3]. The pharmacokinetic parameters of levosulpiride have been studied in healthy volunteers and show a linear relationship with dose [4,5]. However, slow and incomplete absorption from the gastrointestinal tract and low bioavailability (~27%) [4] require a sensitive, specific, and rapid means of determining levosulpiride levels in the blood following administration.

Several methods, including spectrofluorometry [6], liquid chromatography with fluorescence [7–12], and ultraviolet detection [13,14], have been reported for determining levosulpiride levels in human biofluids. High-performance liquid chromatography (HPLC) is a well-established technique, but is limited by poor specificity and long analysis times. Gas chromatographic methods suffer from laborious sample derivatization procedures [15,16]. Improvements in sensitivity, analysis time, and specificity have been achieved using liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods [17–19]. A mass spectroscopic method employing hydrophilic interaction liquid chromatography (HILIC–MS/MS) improved the detection sensitivity of levosulpiride to 1 ng/mL, but resulted in a longer runtime, of 7 min [20]. A recently developed LC–MS/MS method, boasting short analysis times and analyte elution within 1 min, has yet to be validated with regard to percent recovery, specificity, and stability [21]. Thus, there is still a continuing need for a specific, simple, rapid, and well-validated method that is suitable for high-throughput bioanalytical applications.

Ultra-performance liquid chromatography (UPLC) is a relatively new chromatographic technique using smaller particle sizes (1.7 μm) to improve chromatographic efficiency. When coupled with tandem mass spectrometry, UPLC–MS/MS becomes a better tool for bioanalytical applications. This technique has been employed in pharmaceutical analyses [22], drug metabolism studies [23], and metabolite profiling [24], but has yet to be used in clinical pharmacokinetic studies of levosulpiride.

* Corresponding author at: Clinical Trial Center, Kyungpook National University Hospital, 200 Dongduk-Ro, Jung-gu, Daegu 700-422, South Korea.
Tel.: +82 534204950; fax: +82 534224950.
E-mail addresses: yry@knu.ac.kr, pphapale@yahoo.co.in (Y.-R. Yoon).
[1] Both authors contributed equally to this work.

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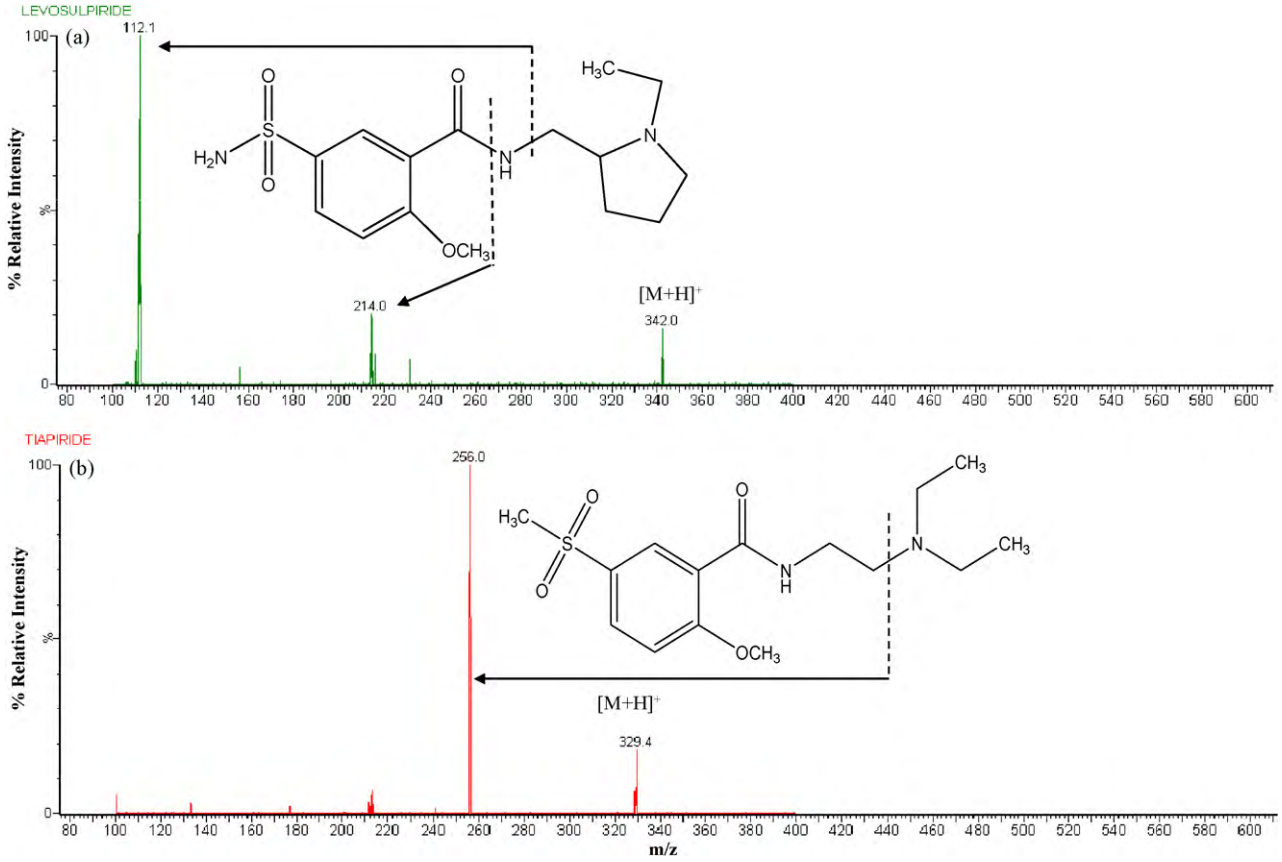


Fig. 1. Mass–mass spectra and chemical structures of (a) levosulpiride and (b) tiapiride.

The current study represents the first application of UPLC–MS/MS for the quantification of levosulpiride in human plasma in a clinical environment. The aim of this work was to develop a validated, rapid, sensitive, and specific method for the quantification of levosulpiride in human plasma. Sample preparation procedures were kept simple to minimize the overall analysis time, as this is desirable in high-throughput clinical applications [18,19]. This method was successfully demonstrated in a pharmacokinetic study of two 75-mg levosulpiride tablet formulations administered to 23 healthy male volunteers.

2. Experimental

2.1. Reagents and chemicals

Levosulpiride standard (Lot No. 25AG0100) (Fig. 1a) and the internal standard (IS), tiapiride (Lot No. 080H0354) (Fig. 1b) were purchased from SK Chemicals Co. Ltd. (Seoul, South Korea) and Sigma (St. Louis, MO, USA), respectively. HPLC-grade acetonitrile and other solvents were purchased from Merck (Darmstadt, Germany). ACS reagent-grade ammonium formate, formic acid, and other chemicals were purchased from Sigma. Blank human plasma samples were obtained from healthy Korean male volunteers. Water for chromatography was purified through a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Liquid chromatography

An ACQUITY^{TM} UPLC system, equipped with a micro-vacuum degasser, thermostated autosampler, binary gradient pumps, and thermostated column compartment were obtained from Waters Corp., Milford, MA, USA. The analytes were separated on an ACQUITY^{TM} UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters Corp.) maintained at 30 °C. A 60:40 (v/v) mixture consisting of ammonium formate buffer (1 mM, adjusted to pH 3 with formic acid) and acetonitrile was used in isocratic mode as the mobile phase. All solvents and the mobile phase were filtered through a 0.22-μm membrane filter (Millipore, Dublin, Ireland). The mobile phase was delivered at a flow rate of 0.2 mL/min. The autosampler temperature was kept at 5 °C for longer analysis times (see post-preparation stability data in Table 2). The sample injection volume was 5 μL (partial-loop mode). The mass spectrometric data acquisition runtime was 3 min. Data were collected using MassLynx software and processed with QuanLynx software (Waters Corp., Milford, MA, USA).

2.3. Mass spectrometry

A Quattro Premier XE^{TM} micromass triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) was interfaced with an ESI probe in positive ionization mode. MS conditions were: ESI source temperature 80 °C, desolvation temperature 250 °C, capillary voltage 3.3 kV, cone voltage 35 V, cone gas flow 50 L/h of nitrogen, desolvation gas flow 900 L/h of nitrogen. The collision gas (Ar) for MS/MS was maintained at 2.8×10^{-3} mbar. The optimized collision energy for both levosulpiride and IS was 30 eV. The MS was operated in multiple-reaction monitoring (MRM) mode and the MRM transition sets for levosulpiride and IS were *m/z* 342 → 112 and 329 → 256, respectively, with a dwell time of 0.10 s per transition and a *m/z* tolerance of ±0.1 Da.

2.4. Preparation of standards and quality controls

Stock 1-mg/mL solutions of levosulpiride and IS were prepared separately in methanol. Working standard solutions containing 25, 50, 100, 200, 500, 1500 and 2000 ng/mL of levosulpiride and a 50-ng/mL IS solution were prepared by serial dilutions of the stock. The diluent consisted of a mixture of ammonium formate buffer (1 mM, pH 3.0) and acetonitrile (50/50, v/v). Drug-free blank plasma was spiked with working standard solutions to prepare plasma calibration standards with final concentrations of 2.5, 5, 10, 20, 50, 150, and 200 ng/mL of levosulpiride. Similarly, quality control (QC) samples were prepared at four concentrations: 2.5 ng/mL (lower limit of quantitation, LLOQ), 5 ng/mL (Low, LQC), 20 ng/mL (middle, MQC), and 150 ng/mL (high, HQC) of levosulpiride. All standard stock solutions were kept at −20 °C until analysis. Plasma calibration standards and quality controls were extracted daily before analysis using the procedure described below.

2.5. Plasma sample preparation

Plasma samples were stored at −80 °C and allowed to thaw gradually at room temperature before processing. A 20-μL aliquot of IS solution (50 ng/mL) was vortexed with 100 μL of plasma sample/standard and 300 μL of acetonitrile in a polypropylene tube for 5 min. This mixture was then centrifuged (16,100 rcf, 5 min, 5 °C). The supernatant was filtered through a PVDF filter (Millipore, 0.2 μm, 4 mm) into the injection vial.

Samples having concentrations above the calibration curve range were diluted with blank plasma and appropriate dilution factor was incorporated while calculating their concentrations.

2.6. Method validation

The method was validated with regard to linearity, specificity, accuracy, precision, percent recovery, and stability according to the guidelines of United States Food and Drug administration [25]. Calibration curves with seven calibration standards spanning the concentration range of 2.5–200 ng/mL were generated daily prior to analysis. Calibration curves were plotted using the peak area ratio of levosulpiride to that of IS as a function of the nominal concentration. Curves were fitted by a weighted ($1/x$) least squares regression.

The lower limit of quantification (LLOQ) was used to determine sensitivity and was defined as the concentration of levosulpiride at which the signal to noise ratio (S/N) was greater than 10, with a precision less than 20% and accuracy between 80 and 120% of the theoretical value. The LLOQ was set as the lowest concentration in the calibration curve.

Intra-day accuracy (%DEV) and precision (%RSD) were assessed by replicate analyses ($n = 5$) of four QC samples (LLOQ, LQC, MQC, HQC) on the same day. Inter-day accuracy and precision were determined by analyses of these same QC samples on five different days. Accuracy was calculated as the percent deviation (%DEV) between the mean calculated concentration and the nominal concentration.

$$\mathrm{DEV}(\%) = \left[\frac{\text{mean calculated concentration}}{\text{nominal concentration}}\right] \times 100$$

Precision was determined by the relative standard deviation (%RSD), defined as

$$\mathrm{RSD}(\%) = \left[\frac{(\mathrm{SD})}{M}\right] \times 100$$

where SD is the standard deviation and M is the mean observed concentration within replicates.

Endogenous interference from blank plasma was assessed by analyzing drug-free blank plasma from six individuals and blank plasma spiked with levosulpiride (2.5 ng/mL) and IS (50 ng/mL). MRM chromatograms were compared to identify any background interference at the retention times of levosulpiride and IS.

The percent extraction recovery of levosulpiride at three concentrations (LLOQ, LQC, MQC, HQC) was determined in triplicate and calculated as the peak area ratio of levosulpiride spiked in blank plasma sample before and after extraction. The plasma sample preparation procedure was same as mentioned previously.

Stability was assessed using the low (5 ng/mL) and high (150 ng/mL) QC concentrations in triplicate. Four stability conditions were tested were: post-preparative stability at 5 °C for 24 h, freeze–thaw stability over three cycles, short-term temperature stability at room temperature for 6 h, and long-term temperature stability at −20 °C for 69 days. Additionally, the stability of stock solutions of levosulpiride and IS, kept at −20 °C for 14 days was evaluated after diluting it to 100-ng/mL solutions with diluent.

2.7. Pharmacokinetic application

This method was successfully used to determine plasma concentration-time profiles in a pharmacokinetic study of levosulpiride. The study protocol was approved by the institutional review board of Kyungpook National University Hospital, Daegu, Korea. Twenty-three healthy Korean male volunteers with a mean age of 24.1 ± 1.7 years and a mean body weight of 69.3 ± 9.1 kg participated in this study. All volunteers were free from any infection and healthy according to their complete medical histories, physical exams, full blood counts, urinalyses, and ECG cardiograms. Volunteers were not taking any other drug or medication during the study period. After giving written informed consent, all volunteers received either a single, 75-mg dose of the test or reference formulation, followed by a 1-week washout period and the administration of the other formulation. Blood samples (6 mL) were collected into sodium heparinized Vacutainer tubes before (0 h) and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 36 h after drug administration. Plasma from all blood samples was immediately separated by centrifugation (1811 rcf, 10 min) and all plasma samples were stored at −80 °C until analysis. Pharmacokinetic (PK) parameters, calculated with WinNonlin 5.2 software (Pharsight Corporation, CA, USA), included the maximum plasma concentration of levosulpiride (C_{max}) at time T_{max}, the area under the levosulpiride plasma concentration-time profile (AUC), and the half-life ($t_{1/2}$) of levosulpiride in the terminal phase.

3. Results and discussion

3.1. Sample preparation

A protein precipitation method was employed, using acetonitrile as the extracting solvent. This procedure proved much simpler than previous LC–MS [20,21] and chromatographic methods [7–15]. The amount of plasma used (100 μL) was significantly lower than that in previous studies [5–16,21]. Earlier studies had shown a relationship between the pH of the sample and the percent recovery of levosulpiride; more basic conditions (pH 11) resulted in a higher recovery of levosulpiride from the plasma [20]. However, the aim of the present study was to develop a simple and rapid sample preparation procedure suitable for high-throughput clinical applications. The mean percent recovery of levosulpiride from plasma at concentrations of 5, 20, and 150 ng/mL was 41.3 ± 1.11, 43.1 ± 0.82, and 42.7 ± 0.69, respectively. The mean percent recovery of IS at a concentration 50 ng/mL was 62.8 ± 1.52 with an RSD of 2.42%. Although low, these recoveries were sufficient for the

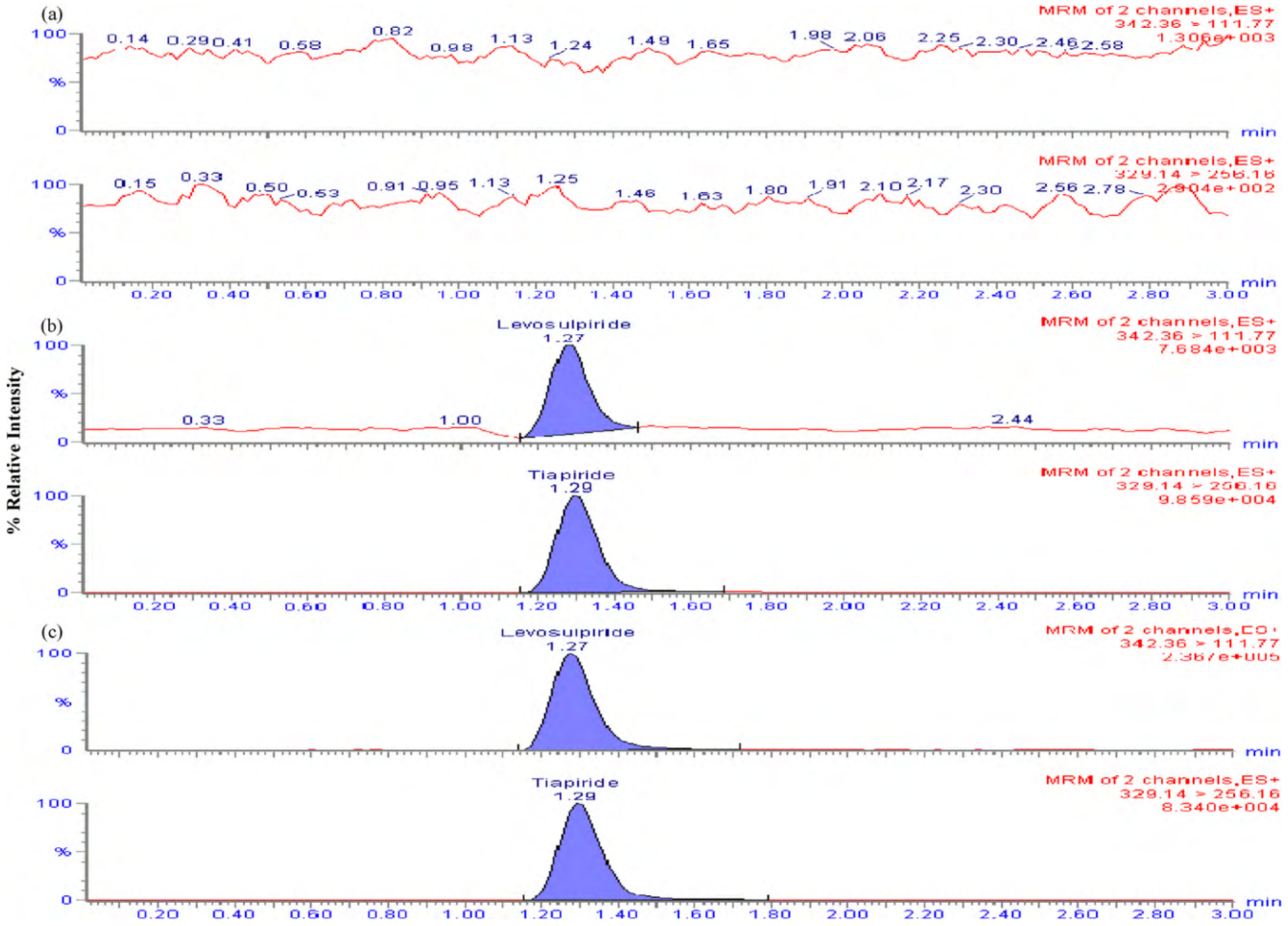


Fig. 2. Typical MRM chromatograms of levosulpiride (upper panel) and tiapiride (lower panel) from (a) blank human plasma, (b) blank human plasma spiked with levosulpiride at the LLOQ (2.5 ng/mL) and tiapiride (50 ng/mL), and (c) plasma from a volunteer 1.0 h after oral administration of a 75-mg dose of levosulpiride spiked with tiapiride (experimental conditions were same as in text).

present study and exhibited acceptable precision (RSD < 5%). And it also suggests that, tiapiride, which has been used in previous studies [20,21], was a suitable internal standard in this method.

3.2. UPLC–MS

UPLC–MS/MS MRM scans were used to determine levels of levosulpiride and IS in human plasma. Solutions of analyte and IS (1 μg/mL) dissolved in methanol were infused into the mass spectrometer along with the mobile phase (0.2 mL/min) to optimize the MS parameters. The MS spectra of levosulpiride and IS contained intense $[M+H]^+$ ions at *m/z* 342 and 329, respectively (Fig. 1a and b). When these parent ions underwent fragmentation, using the MS/MS conditions described above, the resulting mass spectra showed intense product ions at *m/z* 112 and 256 for levosulpiride and IS, respectively. Thus, the corresponding MRM modes were selected in the MS method and the analysis parameters were set to give the highest sensitivity for these ion sets. The infusion of mobile phase along with the analytes was critical for optimization of desolvation and cone gas flows, both of which depend on flow rate.

Although a recent LC–MS method [21] used a high concentration of ammonium acetate buffer (50 mM), in the current study, a lower concentration (1 mM) of ammonium formate buffer maintained at pH 3 was found suitable. The acidic pH maintained by ammonium formate buffer was appropriate for both positive-mode ionization and UPLC retention. The use of UPLC allowed separation of analyte and IS from the endogenous matrix within 1.5 min and yielded excellent chromatographic peak shapes with an isocratic mobile phase. The 3-min runtime is considerably shorter than that of previous methods [5–16], including LC–MS [20]. Recently, a rapid method was described that afforded elution of analyte and IS within the void volume (0.6 min). However, this method has not been validated with regard to recovery or matrix effect and stability, which are important concerns for early-eluting peaks [21].

3.3. Method validation

Analyses of blank plasma from six individuals and plasma spiked with standard and IS solutions showed no endogenous or background inference at the retention times of levosulpiride and IS (1.3 min). This demonstrates the specificity of the current method for determining levosulpiride levels from a plasma matrix (Fig. 2).

The lower limit of quantification (LLOQ) was 2.5 ng/mL. The calibration curve of levosulpiride in plasma was linear between 2.5 and 200 ng/mL levosulpiride, as evidenced by a mean ($n = 5$) correlation coefficient (R^2) of 0.9997 ± 0.0004. The slope of the calibration regression was 0.0304 ± 0.0017 with a *y*-intercept of 0.0047 ± 0.0031. The sensitivity of this method was comparable with that of previous LC–MS methods [20,21] and was suitable for the present PK study. During analysis of clinical samples we found very few samples with levosulpiride concentrations above the calibration curve range (2.5–200 ng/mL). These samples were diluted with equal volume of blank plasma and reanalyzed. Although dilu-

Table 1
Intra-day ($n = 5$) and inter-day ($n = 5$) precision and assay accuracy of quality control samples for the determination of levosulpiride at four concentrations (2.5, 5, 20 and 150 ng/ml) in plasma.

Added QC concentrations (ng/ml)	Calculated concentrations (mean ± SD, ng/ml)		Precision (RSD, %)		Accuracy (DEV, %)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
2.5	2.51 ± 0.18	2.63 ± 0.24	7.17	9.13	100.4	105.2
5	4.94 ± 0.16	5.19 ± 0.12	3.24	2.31	98.8	103.8
20	19.37 ± 0.41	20.22 ± 0.57	2.12	2.82	96.9	101.1
150	146.05 ± 3.36	153.89 ± 3.33	2.30	2.16	97.4	102.6

Table 2
Results of stability of levosulpiride standard in plasma at four different conditions ($n = 3$).

Added QC concentrations (ng/ml)		Calculated concentrations (mean ± SD, ng/ml)				
		Day 0	Post-preparation[a]	Freeze–thaw cycles ($n = 3$)	Short-term[b]	Long-term[c]
5	Mean ($n = 3$) ± SD	5.28 ± 0.114	4.98 ± 0.169	4.63 ± 0.122	4.68 ± 0.384	5.39 ± 0.11
	% Relative concentration	–	94.3	87.7	88.6	102.1
150	Mean ($n = 3$) ± SD	156.93 ± 1.963	152.06 ± 3.809	142.97 ± 8.197	158.30 ± 3.857	137.60 ± 2.509
	% Relative concentration	–	96.9	91.1	100.9	87.7
100 (Levosulpiride stock)	% Relative concentration[d]		95.9			
100 (IS stock)	% Relative concentration[d]		102.5			

[a] After 24 h at 5 °C.
[b] After 6 h at Room temperature.
[c] After 69 days at −20 °C.
[d] 14 days at −20 °C.

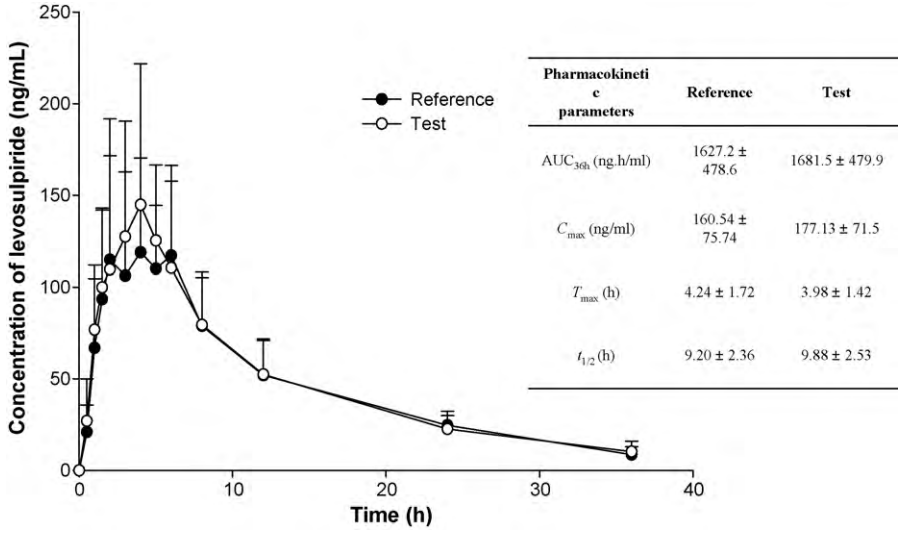
tion integrity test was not performed we found that serial dilution of QC samples (MQC to LLOQ) gives acceptable results until four-fold dilutions with blank plasma (data not shown).

Intra- and inter-day accuracy and precision are summarized in Table 1 for the four QC solutions analyzed in triplicate and ranged from 2.12 to 7.17% and from 2.31 to 9.13%, respectively. Intra- and inter-day accuracy ranged from 96.9 to 100.4% and from 101.1 to 105.2%, respectively. These results indicate acceptable levels of accuracy and reproducibility for bioanalytical applications [25].

The stability of levosulpiride in human plasma over the course of typical sample preparation procedures (free-thaw and short-term), long-term storage, and after sample extraction (post-preparation) are summarized as % relative concentration in Table 2. These four tests showed very little variation in calculated levosulpiride levels, suggesting adequate sample stability. Storage of stock solutions for up to 14 days at −20 °C yielded no significant change in chromatographic peak areas (Table 2). Although the previously reported LC–MS methods were sensitive and rapid, they were not validated with regard to long-term stability [20,21].

3.4. Pharmacokinetic application

The above validated UPLC–MS/MS method was successfully applied in a pharmacokinetic study of two levosulpiride formulations, a reference and a test formulation. Plasma concentrations of levosulpiride were determined after a single oral administration of a 75-mg dose to 23 healthy Korean male volunteers. The mean plasma concentration versus time profile for both formulations is given in Fig. 3. The error bars indicate one standard deviation. The data in Fig. 3 show that the mean estimated pharmacokinetic parameters resulting from both the plots were nearly equivalent for the two formulations. The significant overlap of the plasma concentration-time profiles and the equivalence of the pharmacokinetic parameters suggest that the two formulations were bioequivalent and that the test drug was well tolerated. The



Pharmacokinetic parameters	Reference	Test
AUC_{36h} (ng.h/ml)	1627.2 ± 478.6	1681.5 ± 479.9
C_{max} (ng/ml)	160.54 ± 75.74	177.13 ± 71.5
T_{max} (h)	4.24 ± 1.72	3.98 ± 1.42
$t_{1/2}$ (h)	9.20 ± 2.36	9.88 ± 2.53

Fig. 3. Plasma concentration as a function of time after oral administration of two tablet formulations (reference and test), each containing a 75-mg dose of levosulpiride. Data show the mean and one standard deviation from 23 healthy male volunteers. Pharmacokinetics parameters obtained were summarized inset table.

obtained parameters were in accordance with previous reports [14].

4. Conclusions

A validated UPLC–MS/MS method for rapid (3-min runtime) and sensitive (LLOQ of 2.5 ng/mL) quantification of levosulpiride levels in human plasma is described. This method has considerable advantages over other techniques, including LC–MS/MS, such as efficient chromatography and a simple sample preparation procedure, making it suitable for high-throughput clinical applications. This method was successfully applied to a clinical pharmacokinetic study of levosulpiride oral formulations.

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