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Hydrophilic interaction liquid chromatography–tandem mass spectrometry for the determination of levosulpiride in human plasma

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Abstract

A rapid, sensitive and selective hydrophilic interaction liquid chromatography–tandem mass spectrometric (HILIC–MS/MS) method for the determination of levosulpiride in human plasma was developed. Levosulpiride and internal standard, tiapride were extracted from human plasma with ethyl acetate at pH 11 and analyzed on an Atlantis HILIC silica column with the mobile phase of acetonitrile–ammonium formate (190 mM, pH 3.0) (94:6, v/v). The analytes were detected using an electrospray ionization tandem mass spectrometry in the multiple-reaction-monitoring mode. The standard curve was linear (r = 0.999) over the concentration range of 1.00–200 ng/ml. The lower limit of quantification for levosulpiride was 1.00 ng/ml using 100 μl plasma sample. The coefficient of variation and relative error for intra- and inter-assay at three quality control (QC) levels were 3.8–9.1 and −2.9 to −0.1%, respectively. The recoveries of levosulpiride ranged from 80.5 to 87.4%, with that of tiapride (internal standard) being 84.6%. This method was successfully applied to the pharmacokinetic study of levosulpiride in humans.

Keyword: Levosulpiride

1. Introduction

Levosulpiride, the *levo*-enantiomer form of racemic sulpiride, 5-(aminosulfonyl-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide (Fig. 1), possesses antipsychotic, antidepressive and antiulcer effects. Levosulpiride has been used in the treatment of depression, schzophrenia and psychopathology of senescence. It is also used in the treatment of gastric or duodenal ulcers and irritable colon due to psychosomatic stress and various vertigo syndromes [1]. Levosulpiride is slowly and poorly absorbed from the gastrointestinal tract, with peak serum concentrations at 2–6 h and its bioavailability ∼27% [2]. Levosulpiride does not appear to be metabolized, showing that 70–90% of an intravenous dose and 15–25% of an oral dose is excreted unchanged in the urine [3].

A number of methods for the determination of levosulpiride in biological fluids were reported using spectrofluorometry [4], gas chromatography (GC) [5,6] and high-performance liquid chromatography (HPLC) methods with UV [7,8] or fluorescence detection [9–14]. Those methods use a large amount of plasma (more than 1 ml) [4–14] or include time-consuming extraction procedures such as solid-phase extraction [12] and the combination of liquid–liquid extraction and back extraction [14], and relatively long run time (12–30 min). Most of these methods do not provide good sensitivity for pharmacokinetic studies of levosulpiride at the single oral dose of 25 mg [4–10,13]. A sensitive, simple, fast

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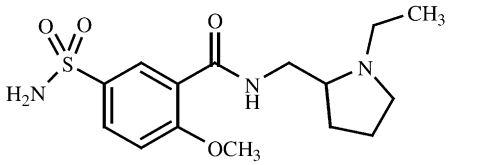
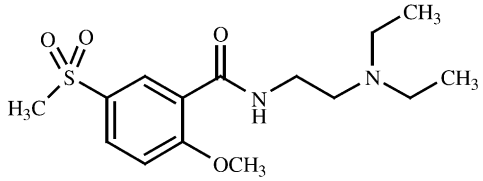


Levosulpiride



Tiapride

Fig. 1. Structures of levosulpiride and tiapride (internal standard).

and reliable bioanalytical method is required for human pharmacokinetics and bioequivalence studies of levosulpiride. Hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) method is recognized as a powerful tool for the determination of polar compounds in biological fluids [15–17]. HILIC method using fluorescence detection for the analysis of levosulpiride in plasma provided good chromatographic resolution but showed low sensitivity (20 ng/ml) using 1 ml of plasma and had a long analysis time (30 min) [13].

The purpose of this paper was to develop and validate a rapid, robust and sensitive HILIC–MS/MS method using liquid–liquid extraction at pH 11 for the quantitative analysis of levosulpiride using 100 μl human plasma. The present method has been successfully applied to the evaluation of levosulpiride pharmacokinetics in humans.

2. Experimental

2.1. Materials

Levosulpiride (99.77% purity) was kindly given from Kuhnil Pharmaceutical Co. (Seoul, Korea). Tiapride hydrochloride (internal standard, >99.0% purity) was obtained from Sigma (St. Louis, MO, USA). Acetonitrile and ethyl acetate (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of levosulpiride and tiapride (1 mg/ml) were prepared in acetonitrile and water, respectively. Working standard solutions of levosulpiride were prepared by diluting each primary solution with acetonitrile. The working solution for internal standard (100 ng/ml) was prepared by diluting an aliquot of stock solution with water. All levosulpiride and tiapride solutions were stored at ca. 4 °C in polypropylene bottles in the dark when not in use.

Human plasma calibration standards of levosulpiride (1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 100 and 200 ng/ml) were prepared by spiking appropriate amount of the working standard solutions into a pool of 10 lots of drug-free human plasma. Quality control (QC) samples at 2.50, 25.0 and 150 ng/ml were prepared in bulk by adding 250 μl of the appropriate working standard solutions (0.05, 0.5 and 3 μg/ml) to drug-free human plasma (4750 μl). The QC samples were aliquoted (100 μl) into polypropylene tubes and stored −20 °C until analysis.

2.3. Sample preparation

One hundred microliters of blank plasma, calibration standards and QC samples were mixed with 10 μl of internal standard working solution and 100 μl of 100 mM NaOH to adjust pH of samples to more than 11. The samples were extracted with 900 μl of refrigerated ethyl acetate in 1.5 ml polypropylene tubes by vortex-mixing for 2 min at high speed and centrifuged at 5000 × *g* for 5 min at 4 °C. The organic layer was pipette transferred and evaporated to dryness under nitrogen at 35 °C. The residues were dissolved in 50 μl of 100% acetonitrile by vortex-mixing for 2 min, transferred to injection vials, and 10 μl were injected onto the LC–MS/MS.

2.4. LC–MS/MS analysis

For LC–MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on an Atlantis HILIC silica column (5 μm, 3 mm i.d. × 50 mm, Waters Co., Milford, MA, USA) using a mixture of acetonitrile–ammonium formate (190 mM, pH 3.0) (94:6, v/v) at a flow rate of 0.5 ml/min. The column and autosampler tray temperature were 30 and 4 °C, respectively. The analytical run time was 7.0 min. The eluent was introduced directly onto the tandem quadrupole mass spectrometer (API 2000, Applied Biosystems/MDS SCIEX, Foster City, CA, USA) through the turbo ionspray source with typical settings as follows: curtain gas, 35 psi; nebulizer gas, 50 psi; turbo gas, 65 psi; ionspray voltage, 5500 V; temperature, 380 °C. Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. The molecular ions of levosulpiride and tiapride were fragmented at collision energy of 37 and 25 V by collision-activated dissociation with nitrogen as the collision gas at a pressure setting of 7 on the instrument. Detection of the ions was performed by monitoring the transitions of *m*/*z* 342 to 112 for levosulpiride and *m*/*z* 329 to 256 for tiapride. Peak areas for all components were automati-

cally integrated using Analyst software version 1.4 (Applied Biosystems/MDS SCIEX).

2.5. Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 2.50, 25.0 and 150 ng/ml were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision.

The absolute recoveries of levosulpiride were determined by comparing the peak area of six extracted samples at the concentrations of 2.50, 25.0 and 150 ng/ml with the mean peak area of recovery standards. Three replicates of each of the recovery standards were prepared by adding levosulpiride and internal standard to blank human plasma extracts.

To evaluate the three freeze–thaw cycle stability and room temperature matrix stability, six replicates of QC samples at each of the low and high concentrations (2.50 and 150 ng/ml, respectively) were subjected to three freeze–thaw cycles or were stored at room temperature for 24 h before processing, respectively. Six replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h were assayed to assess post-preparative stability.

2.6. Application

The developed LC–MS/MS method was used in a pharmacokinetic study after an oral administration of levosulpiride to humans. Four healthy male volunteers, fasted for 10 h, received a single oral dose of levosulpiride (25 mg tablet) with 200 ml of water. Blood samples (1 ml) were withdrawn from the forearm vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 36 h post dosing, transferred to Vacutainer^{TM} plasma glass tubes (sodium heparin, BD, NJ, USA) and centrifuged. Following centrifugation (3000 × *g*, 20 min, 4 °C), plasma samples were transferred to polypropylene tubes and stored at −20 °C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. The peak concentration (C_{max}) and the time to peak concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time plot for levosulpiride. Area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 36 h.

3. Results and discussion

3.1. HILIC–MS/MS

The electrospray ionization of levosulpiride and tiapride produced the abundant protonated molecular ions (MH^+) at

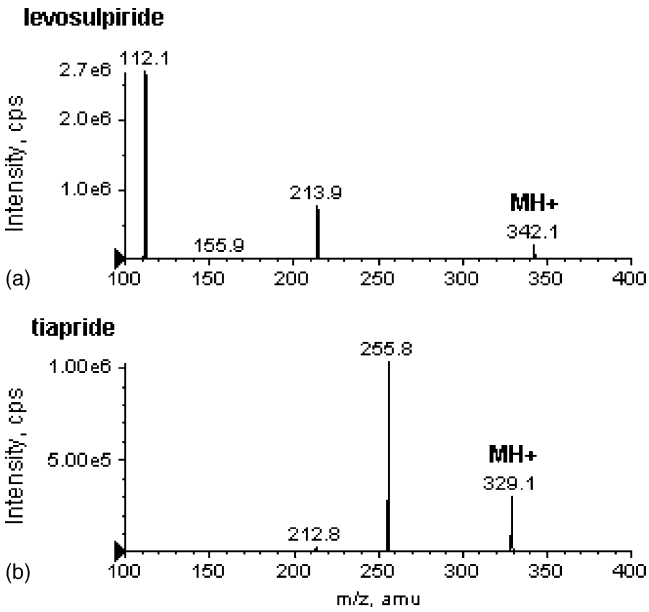


Fig. 2. Product ion mass spectra of: (a) levosulpiride; (b) tiapride (internal standard).

m/*z* 342 and 329, respectively, under positive ionization conditions, without any evidence of fragmentation. MH^+ ions from levosulpiride and tiapride were selected as the precursor ion and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 2). The fragment ions at *m*/*z* 112 (1-ethyl-2-pyrrlidinylmethylene) and 256 (the loss of diethylamino group from MH^+ ion) were produced as the prominent product ions for levosulpiride and tiapride, respectively. The quantification of the analytes was performed using the MRM mode due to the high selectivity and sensitivity of MRM data acquisitions, where the precursor and product ions are monitored. Two pairs of MRM transitions were selected: *m*/*z* 342 → 112 for levosulpiride and *m*/*z* 329 → 256 for tiapride (internal standard).

HILIC methods operated with the silica column and low aqueous–high organic mobile phase have been proved to be ideal for the analysis of polar compounds in biological fluids [15–17]. Levosulpiride was well retained on a silica column and higher acetonitrile content (94%) in the mobile phase enhanced the signal intensity of levosulpiride.

Fig. 3 shows the representative MRM chromatograms obtained from the analysis of blank human plasma, human plasma spiked with levosulpiride at 1.00 ng/ml and a plasma sample obtained from the volunteer after oral administration of 25 mg levosulpiride. The retention times of levosulpiride and tiapride were 4.9 and 4.5 min, respectively, and no interfering peaks were observed in drug-free human plasma.

3.2. Method validation

This method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation

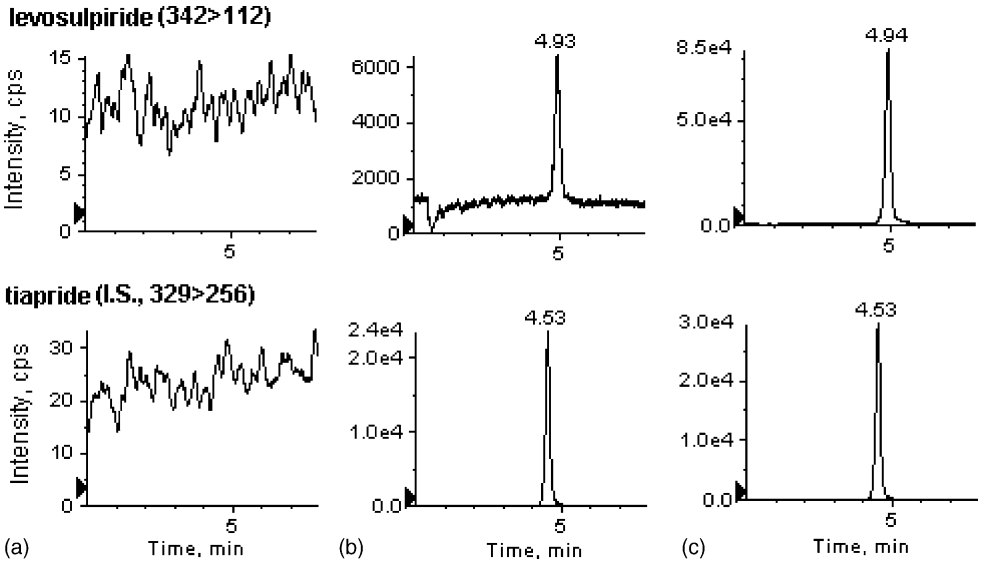


Fig. 3. MRM LC–MS/MS chromatograms of: (a) blank human plasma; (b) human plasma sample spiked with 1.00 ng/ml of levosulpiride; (c) human plasma sample obtained 3 h after oral administration of levosulpiride (25 mg).

Table 1
Calculated concentrations of levosulpiride in calibration standards prepared in human plasma (n = 9)

	Theoretical concentration (ng/ml)								Slope	Intercept	r
	1.00	2.00	5.00	10.0	20.0	50.0	100	200			
Mean	1.03	1.91	4.94	10.3	20.9	50.0	100	200	0.1289	0.0295	0.999
CV (%)	8.8	2.0	5.9	7.5	3.5	4.0	3.5	3.6	8.2		
RE (%)	3.0	−4.7	−1.2	3.4	4.4	1.2	0.2	0.0			

[18]. The analysis of blank human plasma samples from ten different sources showed that there was no interference from endogenous plasma substances, confirming the specificity of the present method. For six samples of blank plasma from six independent sources with levosulpiride at 2.50 ng/ml, CV and RE were 3.2 and 1.7%, respectively. These tight CV and RE values indicate no significant lot-to-lot variation in matrix effects.

Calibration curves were obtained over the concentration range of 1.00–200 ng/ml of levosulpiride in plasma. Linear regression analysis with a weighting of 1/concentration gave the optimum accuracy of the corresponding calculated concentrations at each level (Table 1). The low coefficients of variation value for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing levosulpiride. Both intra-and inter-assay CV values ranged from 3.8 to 9.1% at three QC levels. The intra- and inter-assay RE values for levosulpiride were −2.9 to −0.1% at three QC levels. These results indicated that the present method has an acceptable accuracy and precision.

The lower limit of quantitation (LLOQ) was set at 1.00 ng/ml for levosulpiride using 100 μl of human plasma. Representative chromatogram of an LLOQ is shown in Fig. 3b and the signal-to-noise ratio for levosulpiride is about 20 at 1.00 ng/ml. CV and RE at the LLOQ level were 3.7 and −5.0%, respectively (Table 2).

The effect of pH of human plasma on the recovery of levosulpiride using ethyl acetate was evaluated: the extraction recoveries of levosulpiride at pH 7, 9 and 11 of plasma samples were 35, 56 and 85%, respectively. Therefore, pH of plasma samples was adjusted to pH 11 using NaOH. The extraction recoveries of levosulpiride from spiked human plasma were determined at the concentrations of 2.50, 25.0 and 150 ng/ml in six replicates. The recoveries of levosulpiride ranged from

Table 2
Precision and accuracy of levosulpiride in quality control samples

	Intra-batch (n = 6)				Inter-batch (n = 18)		
QC (ng/ml)	1.00	2.50	25.0	150	2.50	25.0	150
Mean (ng/ml)	0.95	2.43	24.5	146	2.46	25.0	150
CV (%)	3.7	6.0	3.8	7.3	7.3	9.1	8.5
RE (%)	−5.0	−2.9	−1.9	−2.7	−1.5	−0.2	−0.1

Table 3
Absolute recoveries of levosulpiride and tiapride (internal standard) from spiked human plasma

Concentration (ng/ml)	Recovery (%, mean ± S.D., n = 6)	
	Levosulpiride	Tiapride
2.50	80.5 ± 3.9	–
25.0	87.4 ± 2.9	–
150	82.5 ± 3.0	–
5.00	–	84.6 ± 6.4

"–" Not assayed.

Table 4
Stability of samples (n = 6)

Statistical variable	Theoretical concentration (ng/ml)	
	2.50	150
Freeze and thaw stability		
Mean	2.63	152
CV (%)	2.1	3.6
RE (%)	5.2	1.3
Short-term temperature stability (24 h at room temperature)		
Mean	2.60	148
CV (%)	5.9	5.4
RE (%)	4.0	−1.3
Post-preparative stability (24 h at room temperature)		
Mean	2.56	147
CV (%)	8.0	9.7
RE (%)	2.4	−2.0

80.5 to 87.4%, with that of tiapride (internal standard) being 84.6 ± 6.4% (Table 3). The one-step liquid–liquid extraction with ethyl acetate at pH 11 has been successfully applied to the extraction of levosulpiride from human plasma.

Stability of levosulpiride during sample handling (freeze–thaw and short-term temperature stability) and the stability of processed samples were evaluated (Table 4).

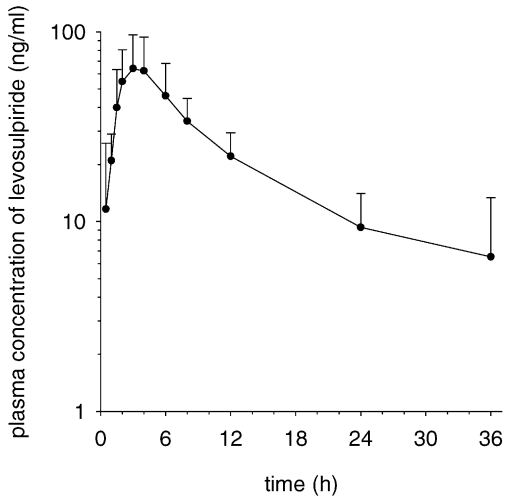


Fig. 4. Mean plasma concentration–time plot of levosulpiride after a single oral dose of levosulpiride (25 mg tablet) to four male volunteers. Each point represents the mean ± S.D.

Three freeze–thaw cycles and room temperature storage of the QC samples for 24 h before analysis had little effect on the quantification. Extracted QCs and calibration standards were allowed to stand at ambient temperature for 24 h prior to injection without affecting the quantification.

3.3. Application study

This method has been successfully used to the pharmacokinetic study of levosulpiride after a single oral dosing of levosulpiride (25 mg) to four healthy male volunteers. Fig. 4 shows mean plasma concentration profiles of levosulpiride in four healthy male volunteers. C_{max}, T_{max}, AUC and $t_{1/2}$ of levosulpiride were 77.4 ± 29.7 ng/ml, 3.1 ± 1.2 h, 872 ± 442 ng h/ml and 8.7 ± 2.8 h, respectively.

4. Conclusions

A sensitive, rapid and reliable HILIC–MS/MS method for the analysis of levosulpiride in human plasma using has been successfully developed and validated. Liquid–liquid extraction with ethyl acetate after alkaline treatment to pH 11 was used for the extraction of levosulpiride from the plasma. The LLOQ for levosulpiride was 1.00 ng/ml using 100 μl plasma. This method was successfully applied to the clinical pharmacokinetic study of levosulpiride.

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