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Short communication

Development of HPLC method for the determination of levosulpiride in human plasma

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Abstract

A rapid and simple high performance liquid chromatography (HPLC) method was developed and validated for determination of levosulpiride in human plasma. After extraction with ethylacetate/methylene chloride (5:1, v/v), analysis of levosulpiride in plasma samples was carried out using a reverse phase C_{18} column with fluorescence detector (maximum excitation at 300 nm and maximum emission at 365 nm) for separation and quantification. A mixture of methanol—20 mM phosphate buffer (pH 3.5, 16:84, v/v) was used as a mobile phase. The method was specific and sensitive with a limit of quantification of 5 ng/ml. This HPLC method was validated by examining the precision and accuracy for inter- and intra-day analysis in the concentration range of 5–150 ng/ml. The relative standard deviation (R.S.D.) in inter- and intra-day validation were 8.16–19.75 and 3.90–11.69%, respectively. In stability tests, levosulpiride in human plasma was stable during the storage and assay procedure. The method was applied to the bioequivalence study of two levosulpiride tablet formulations (25 mg) after a single oral administration.
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1. Introduction

Levosulpiride is a levo-enantiomer of racemic sulpiride belonging to the substituted benzamide group (Fig. 1). It is a typical neuroleptic drug with sulpiride and inhibits dopaminergic D_2 receptors at the trigger zone both in the central nervous system and in the gastrointestinal tract. Developed as an anti-emetic drug, sulpiride soon generated the interest for its antipsychotic properties and low potential to cause extrapyramidal side effect [1]. At low doses, sulpiride acts on the pre-synaptic D_2 receptors and increases dopamine turnover in dopamine terminal areas [2], this effect produces a behavioral and generalized motor and mental arousal, which is therapeutically useful in depressed patients. At high doses, sulpiride exerts its D_2 receptor blocking activity at both pre-synaptic and post-synaptic D_2 receptor sites, eliciting an antipsychotic effect. Levosulpiride acts on the central nervous system at lower doses than needed with sulpiride. Therefore, it is safe to use [3,4].

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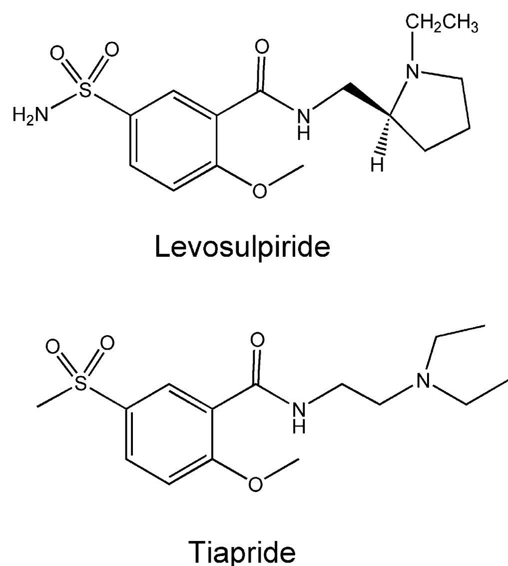

Fig. 1. Chemical structures of levosulpiride and tiapride as an internal standard.

Levosulpiride is a basic drug and has a low bioavailability [5]. Therefore, development of more effective analysis method is demanded for the routine analysis of levosulpiride in biological fluid. In order to carry out pharmacokinetic studies and monitor blood levels of patients treated with levosulpiride, a selective and sensitive analytical method was needed. Several methods have been described in the literature, including gas chromatography [6], high performance liquid chromatography (HPLC) with ultraviolet [7], fluorescence [8,9] or mass spectrometric detection [10], and chrial HPLC method [11,12]. Specially, HPLC methods are routinely used for analysis of levosulpiride in biological fluid. Most of HPLC methods require a sample pretreatment step such as liquid–liquid extraction (LLE) [13,14] or solid-phase extraction (SPE) [14–16] to obtain higher sensitive and specific results. However, there are some difficulties such as low efficiency, sensitivity, and reproducibility. Therefore, more sensitive and simpler HPLC methods are still needed to apply the clinical tests.

Levosulpiride has a low bioavailability (20–30%) following oral administration. After oral administration of levosulpiride (100–200 mg), the bioavailability is reduced significantly with high individual variation [5,9]. Although the pharmacokinetics of levosulpiride has been reported [4–11], very few of them focused on the bioequivalence issue. Bioequivalence study was therefore needed for monitoring and comparing with plasma concentration of two levosulpiride formulations in human volunteers.

The aim of this study was to develop more sensitive and reproducible HPLC method with 5 ng/ml of limit of quantification (LOQ) using simpler pretreatment of one-step LLE in order to analyze levosulpiride in plasma. Moreover, this developed method was applied for the bioequivalence study of two tablet formulations of levosulpiride in healthy volunteers.

2. Experimental

2.1. Drugs and reagents

Standard levosulpiride (Fig. 1) was obtained from Dai Han Pharm. Co. Ltd. (Seoul, South Korea). Tiapride as an internal standard, sodium hydroxide, and monobasic potassium phosphate were purchased from Sigma (St. Louis, MO, USA). Methanol, acetonitrile, ethyl acetate, and methylene chloride were HPLC grade obtained from Burdick & Jackson (Muskegon, MI, USA). All other reagents were reagent grade or better. Levopride® tablets (25 mg) were obtained from SK Pharma. Levride® tablets (25 mg) made by Dai Han Pharm. Co. Ltd. were used as a test product.

2.2. Preparation of standard solutions

Stock solutions (1 mg/ml) of levosulpiride and tiapride as an internal standard were prepared by dissolving them in methanol and kept at 4 °C. Standard solutions of levosulpiride in human plasma were prepared from stock solutions by spiking the suitable volume (>10 μl/ml) of various diluted stock solutions for daily calibration.

2.3. Preparation of plasma samples

Each 8 μl of internal standard solution was spiked to 1 ml of plasma in glass tube (tiapride, 75 μg/ml in methanol) and 100 μl of 1N NaOH solution. After vortex mixing thoroughly for 5 s, the mixture was extracted with 6 ml of ethylacetate/methylene chloride

(5:1, v/v), then vortex-mixed for 10 s, and centrifuged at 3000 × *g* for 10 min. The supernatant was transferred to another clean glass tube and evaporated under a steam of nitrogen gas at 30 °C to remove traces of organic phase. Then, 200 μl of the mobile phase was added to dissolve the residue, and 60 μl of aliquot was automatically injected into the HPLC system for analysis.

2.4. Instruments

An HPLC system (Hitachi, Tokyo, Japan) equipped with a pump (L-6000 Intelligent, Hitachi) and an autosampler (L-7200, Hitachi) was used. A C_{18} column (250 mm × 4.6 mm, 5 μm) was employed (Phenomenex, CA, USA). The plasma samples were separated by isocratic elution of the mobile phase (methanol—20 mM phosphate buffer, pH 3.5, 16:84, v/v) at a flow-rate of 600 μl/min at room temperature. The eluents were monitored with fluorescence detector (F-1050, Hitachi) at 300 nm for excitation and 365 nm for emission. The HPLC system was controlled with dsChrom software (Donam Int., Seoul, South Korea).

2.5. Validation of assay method

2.5.1. Specificity

The interference of endogenous compounds was assessed by analyzing standard levosulpiride, drug-free serum, plasma spiked with levosulpiride, and plasma obtained from subjects given levosulpiride.

2.5.2. Sensitivity

The limit of quantification was defined as the low concentration yielding a precision less than 20% (relative standard deviation, R.S.D.) and accuracy between 80 and 120% of the theoretical value.

2.5.3. Linearity

The linearity of calibration curve for levosulpiride was assessed in the range of 5–150 ng/ml in plasma samples. Standard samples were prepared by adding levosulpiride to blank plasma at concentrations of 5, 10, 20, 50, 100, and 150 ng/ml with 600 ng tiapride and these were extracted and analyzed as described above. Peak area ratios of each levosulpiride to tiapride were measured and the calibration curve was obtained from the least-squares linear regression (no weighing factor) presented with their correlation coefficients. The regression line was used to calculate the respective concentrations of levosulpiride in the plasma samples from volunteers.

2.5.4. Precision and accuracy

The inter- and intra-assay relative standard deviation and standard errors of mean were used to validate the precision and accuracy of the assay by determining standard samples of levosulpiride in plasma. For inter-day validation, five sets of control samples at six different concentrations (5–150 ng/ml) were evaluated on five different days. The range of relative standard deviation was reported. For intra-day validation, five sets of controls at four different drug concentrations were assayed with one standard curve on the same run. The range of the relative standard deviation was also reported. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

2.5.5. Recovery

To assess the absolute recoveries of levosulpiride extracted from plasma, the peak area ratios of analytes to internal standard from the extracted quality control (QC) samples were compared with those obtained from a mobile phase having the same concentration. The mean recoveries were determined at low, medium, and high concentrations in three replicates.

2.5.6. Stability

To test the short- and long-term stability of the basic drug such as levosulpiride, three QC samples, containing low (5 ng/ml), medium (50 ng/ml) and high (150 ng/ml) concentrations, were stored under different conditions: at 4 °C, room temperature, −20 °C at 6, 12, and 24 h after preparation; −70 °C for 2 months. Moreover, the stabilities of stock solutions were tested at room temperature for 6 h in the daylight. The compounds were considered stable if the variation of assay was less than 10% of initial time response.

2.6. Bioequivalence study

Assay of levosulpiride in plasma samples was performed by this validated HPLC method. The assay was completed within 2 months or a period with

acceptable sample stability following the last day of medication. Quantitative analysis of levosulpiride in plasma and suitable quality controls was performed.

2.6.1. Subjects

The protocol of bioequivalence study was approved by the Korean Food and Drug Administration. A total of 24 healthy male subjects participated in this study after signing a consent form. The subjects had an age of 24 ± 2.5 years (21–33 years), body weight of 68 ± 9.6 kg (55–94 kg) and height of 174 ± 5.0 cm (164–184 cm). Subjects with a history of drug allergies or idiosyncrasies, renal or hepatic impairment or drug or alcohol abuse were excluded. Subjects who used medications of any kind within 2 weeks of the start or during the study were also excluded.

2.6.2. Drug administration

Subjects were advised not to take any medication for 2 weeks before the study and were requested to fast for at least 10 h overnight the day before each treatment. A single dose (75 mg) consisting of three Levopride® tablets or Levride® tablets according to the randomized plan was given to each subject in a fasting state for each treatment period. Fasting continued for further 4 h after drug administration. The drug was administered with 240 ml of water. Subjects were provided a standard meal at 4 h (lunch) and 8 h (supper) after drug administration in each treatment. The washout period between the two treatment periods was 1 week, which is 10 times longer than the elimination half-life of this drug.

2.6.3. Blood sampling

Heparinized blood samples (5–10 ml) were withdrawn from the forearm vein according to the time schedule, which included a blank before drug administration and then at 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, and 36 h post-dosing. Blood samples were transferred to Vacutainer® tube (BD, NJ, USA) and immediately separated by centrifugation at 2000–3000 × g for 10 min. Following centrifugation, plasma samples were separated and stored at −70 °C prior to analysis.

2.6.4. Pharmacokinetic data

The following parameters were assessed for the period of 0–36 h: the area under the plasma concentration-time curves from time zero to the last measurable levosulpiride sample time ($AUC_{0-36\,h}$); maximum plasma concentration (C_{max}); time to reach the C_{max} (T_{max}) were obtained directly from the concentration-time curve data by visual inspection from each volunteer's plasma concentration-time plots for levosulpiride. The elimination rate constant (K_e) was obtained as the slope of the linear regression of the log-transformed concentration-time curve data in the terminal phase. The half-life ($T_{1/2}$) was calculated from ln 2 divided by K_e.

2.6.5. Statistical analysis

For the purpose of bioequivalence analysis a two-way ANOVA performed with the K-BE Test 2002 program [17] at a significant level of 0.05. The test and reference treatments of each study were compared with respect to relevant pharmacokinetic variables using an analysis of variance with subject, treatment, and period effects with the raw data. Bioequivalence of the test treatment to the reference treatment was assessed on the basis of the confidence intervals for the "test/reference" mean ratios of these raw variables in relation to the bioequivalence range of 80–120% for the raw data.

3. Results and discussion

3.1. Specificity

Reproducible chromatographic separation between levosulpiride and tiapride were established after examining various chromatographic conditions by injecting the extracted plasma sample. Under our experimental conditions reproducible chromatographic separations were obtained at methanol-20 mM phosphate buffer, pH 3.5 (16:84, v/v). Representative chromatograms of levosulpiride and tiapride analyzed in human plasma were presented in Fig. 2 and specificity of levosulpiride was determined. The extraction and HPLC assay resulted in symmetrical peak shape and good baseline resolution of levosulpiride and tiapride. Plasma matrix components did not interfere with the analysis. Using this system, the retention times for levosulpiride and tiapride were 8.1 and 11.6 min, respectively. The retention time was faster than those reported by Xu and Stewart [12] and Huang et al. [13]. The total analysis time for each run was 20 min.

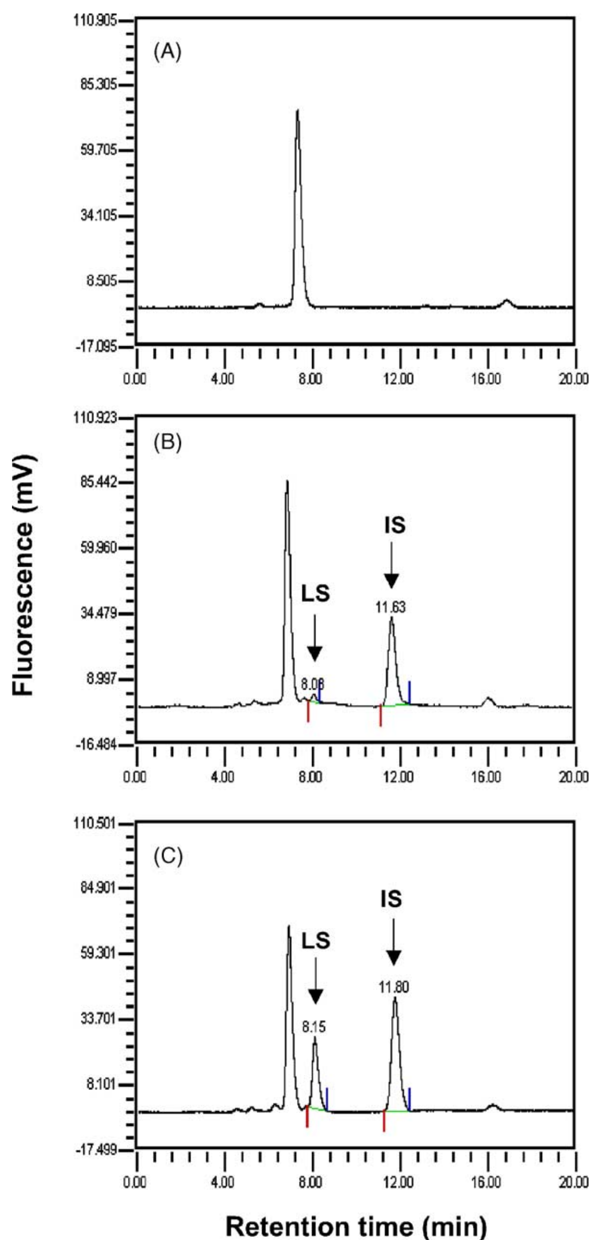

Fig. 2. Chromatograms of (A) blank plasma, (B) blank plasma spiked with 5 ng/ml (LOQ) of levosulpiride (LS), and (C) 50 ng/ml of levosulpiride (600 ng/ml of tiapride, IS).

No interfering endogenous peaks were detected in chromatogram. Fluctuations in retention times occurred due to changes in temperature and column performance within 1 min. Peak shape was the same for injection of pure standards in mobile phase as for extracted plasma standards and plasma samples.

Table 1
Precision and accuracy of levosulpiride in human plasma ($n = 5$)

Concentration (ng/ml)		Precision (R.S.D., %)	Accuracy (%)
Inter-day	5	19.75	104.93 ± 12.29
	10	14.79	96.51 ± 10.66
	20	11.60	102.56 ± 5.25
	50	7.39	101.62 ± 6.04
	100	9.96	97.31 ± 4.95
	150	8.16	100.98 ± 1.75
Intra-day	5	11.69	109.48 ± 7.67
	10	3.90	88.83 ± 3.88
	50	6.04	96.05 ± 7.92
	100	8.08	106.22 ± 6.39

3.2. Sensitivity

The limit of quantification was determined as a concentration of drug giving a signal-to-noise ratio greater than 10 with an accuracy between 80 and 120% and with a precision R.S.D. (%) less than 20%. The LOQ was estimated to be 5 ng/ml as shown in Table 1. The LOQ had a four-fold or much lower value than that obtained by Bressolle and Bres [7], Nicolas et al. [8], Xu and Stewart [12] and Huang et al. [13]. In the case of comparing the method by Huang et al. [13], even though it applied a similar treatment, the sensitivity increased four-folds in our method. It suggested that the sensitivity was largely affected by a usage of different type of column and this HPLC system using reverse-phased column was suitable for the analysis of levosulpiride in plasma. Fig. 2B shows chromatogram of LOQ.

3.3. Linearity

The linearity of detector response was assessed for extracted plasma standards over the range of 5–150 ng/ml. The calibration curve of levosulpiride exhibits an excellent linearity and a correlation coefficient. The mean (±S.D.) regression equation from five replicated calibration curves was $y = 0.0071$ $(\pm 0.0006)x + 0.0009$ (± 0.0123) ($y =$ levosulpiride concentration, $x =$ ratio of peak area) with the correlation coefficient of 0.9994 (± 0.0029).

3.4. Precision and accuracy

The inter-day precision and the accuracy were determined by analyzing plasma samples spiked at 5, 10, 20, 50, 100, and 150 ng/ml. Inter-day precision was determined by analyzing five calibration curves on five different days. The intra-day precision and the accuracy were determined by analyzing plasma samples spiked at 5, 10, 100, and 150 ng/ml. The intra-day precision was determined by analyzing five replicates on the same day. The precision of levosulpiride calculated as a relative standard deviation (R.S.D.) was always below 15% except for LOQ. The accuracy of levosulpiride expressed as a percentage of the measured concentration to the theoretical concentration ranged from 96.5 to 104.9%. The results of inter- and intra-day precision and accuracy for levosulpiride in human plasma are presented in Table 1.

3.5. Recovery

LLE is a simpler method for extraction of many plasma samples and needs no more apparatus than SPE. Moreover, other extraction methods of levosulpiride using like SPE or back extraction were less efficient (exceeded 50%) [16] and needed more delicate conditions in operation [14–16]. In order to obtain the optimal extraction condition, we conducted several trials of LLE in this study. A rapid and efficient LLE of levosulpiride from plasma were achieved using ethylacetate/methylene chloride mixture (5:1, v/v) as extraction solvents according to Huang et al. [13]. Therefore, LLE by Huang et al. [13] was used among the several reported methods for the LLE of levosulpiride in biological fluid.

Extraction efficiency of levosulpiride was evaluated in blank plasma samples and mobile phase spiked with known amounts of the levosulpiride and tiapride. Plasma samples were extracted as described above and the recovery was calculated by comparing the peak area ratio of levosulpiride to tiapride obtained from the extracted working standard solutions in plasma and those resulting from the direct injection of the working standard solutions of levosulpiride prepared in the mobile phase having the same concentrations (5, 50, 150 ng/ml) of levosulpiride and tiapride. Extraction efficiency of levosulpiride measured in triplicate was $91 \pm 1.5\%$. However, recovery of LLE reported by Huang et al. [13] was almost quantitative at least 50% even though we used a similar procedure. It was demonstrated that they used a different type of column. This has shown that this

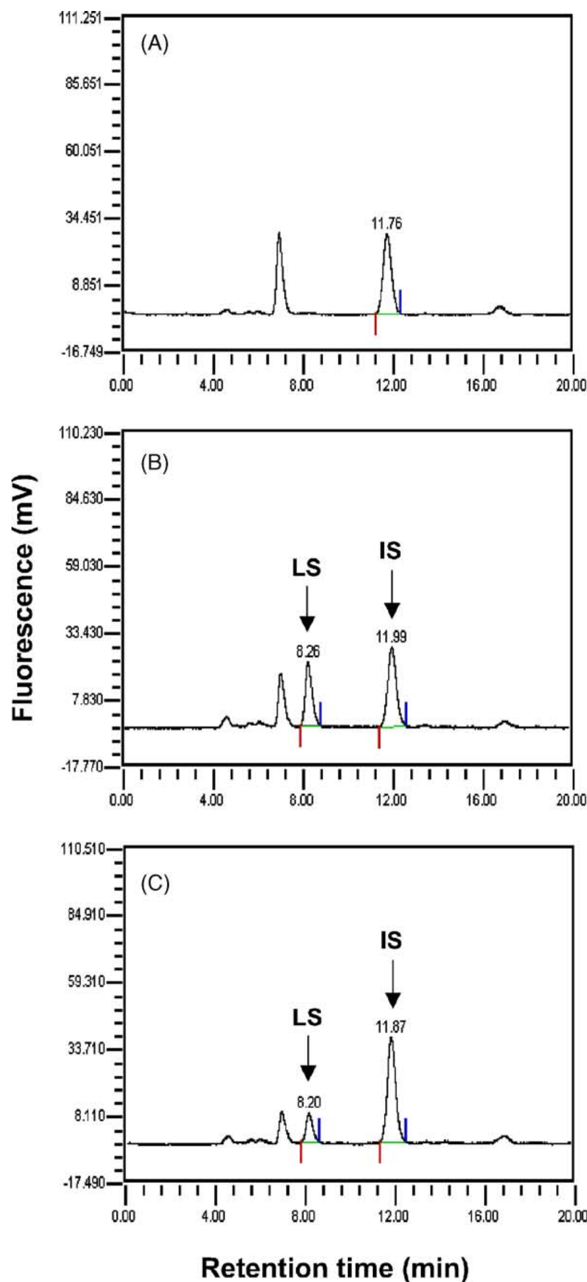

Fig. 3. Chromatograms of a volunteer plasma at (A) 0, (B) 6, and (C) 12 h after a single oral dose of 75 mg levosulpiride.

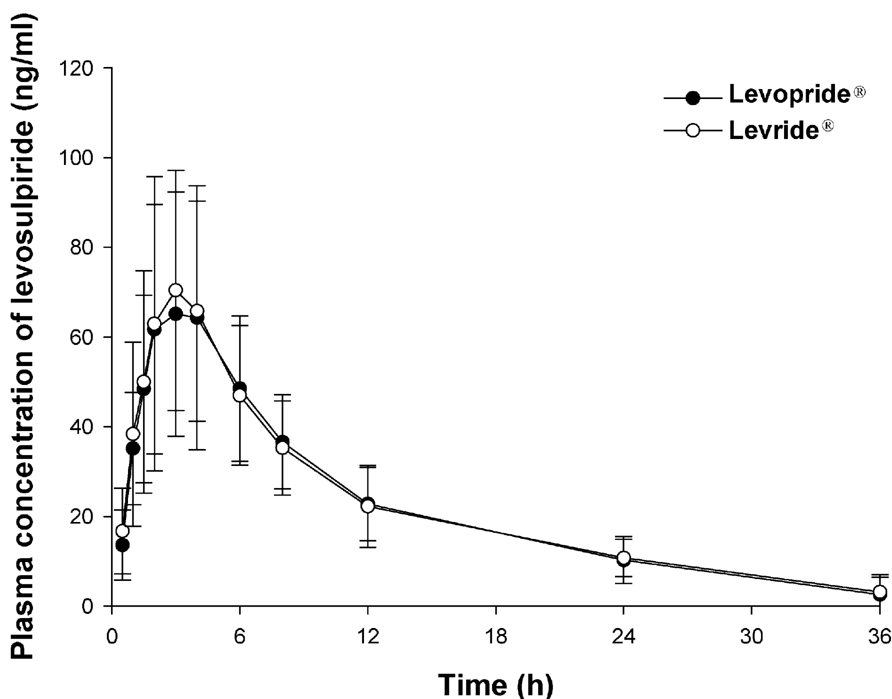

Fig. 4. Mean plasma concentration-time profiles of levosulpiride in 24 healthy subjects for the test (Levride®) and reference (Levopride®) products.

pretreatment method is suitable for the analysis of levosulpiride in plasma sample.

3.6. Stability

No tendency of degradation of levosulpiride and tiapride at room temperature under daylight for 6 h was observed. Plasma samples spiked with levosulpiride and tiapride showed no loss of analytes during 2 months at −70 °C. In short term stability study, they were also stable until 24 h at 4 °C, room temperature and −20 °C.

3.7. Bioequivalence of levosulpiride

This method was applied to a bioequivalence study of two levosulpiride tablet formulations. Twenty-four healthy volunteers were administered a single oral dose of levosulpiride tablets (25 mg, three tablets). Plasma samples were obtained during 36 h after levosulpiride administration. Fig. 3 shows the change of the plasma concentration of levosulpiride after oral administration. Fig. 4 displays similar bioavailabilities with the mean (± S.D.) of levosulpiride plasma concentration-time profiles for 24 volunteers for the test and reference products. The highest concentration of levosulpiride was observed at 3 h after administration. The area under the curve ($AUC_{0-36\,h}$) was 725 ng/ml·min. These values are comparable to the parameters reported by Mucci et al. [4]. After a single oral administration of 100 mg levosulpiride tablet to 12 healthy volunteers, $AUC_{0-\infty}$, C_{max}, and T_{max} were 2032 ng/ml·h, 184 ng/ml, and 2.6 h, respectively. The pharmacokinetic parameters of two levosulpiride formulations are shown in Table 2. From these results, analysis of variance for these parameters, after

Table 2
Pharmacokinetic parameters of levosulpiride in plasma of 24 healthy subjects after an oral administration of 75 mg levosulpiride

Parameters	Levopride®	Levride®
$AUC_{0-36\,h}$ (ng/ml·min)	718 ± 186	725 ± 183
C_{max} (ng/ml)	81 ± 184	80 ± 29
T_{max} (h)	3.21 ± 1.02	3.08 ± 1.02
K_e (h^{-1})	5.67 ± 1.74	5.21 ± 1.91
$T_{1/2}$ (h)	9.34 ± 5.29	10.9 ± 6.73

log-transformation of the data, showed no statistically significant difference between the two formulations, with a *P*-value greater than 0.05.

4. Conclusion

We established a simple and sensitive analytical method for the determination of levosulpiride in human plasma using HPLC method with fluorescence detection. A one-step LLE provided a simple, rapid, and practical procedure. This method showed excellent sensitivity, reproducibility, specificity, and velocity. The method has been successfully used to provide the bioequivalent study of levosulpiride in human plasma. Moreover, it is suggested that the present HPLC analysis method can be applied to routinely monitor the concentration of levosulpiride.

Acknowledgements

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