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Improvement and validation of a liquid chromatographic method for the determination of levosulpiride in human serum and urine

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Abstract

A rapid, selective and highly sensitive reversed-phase high-performance liquid chromatography (HPLC) method was developed for the determination of levosulpiride, 5-(aminosulfonyl)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxy benzamide, in human serum and urine. The method involved the extraction with a dichloromethane followed by back-extraction into 0.025 M sulfuric acid. HPLC analysis was carried out using reversed-phase isocratic elution with a Luna C_{18}(2) 5 μm column, a mobile phase of acetonitrile–0.01 M potassium hydrogen phosphate (30:70, v/v, adjusted to pH 8.5 with triethylamine), and a fluorescence detector with excitation at 300 nm and emission at 365 nm. The chromatograms showed good resolution and sensitivity and no interference of human serum and urine. The calibration curves were linear over the concentration range 0.25–200 ng/ml for serum and 0.2–20 μg/ml for urine with correlation coefficients greater than 0.997. Intra- and inter-day assay precision and accuracy fulfilled the international requirements. The mean absolute recovery for human serum was 89.8 ± 3.7%. The lower limits of quantitation in human serum and urine were 0.25 ng/ml and 0.2 μg/ml, respectively, which were sensitive enough for pharmacokinetic studies. Stability studies showed that levosulpiride in human serum and urine was stable during storage, or during the assay procedure. This method was successfully applied to the study of pharmacokinetics of levosulpiride in human volunteers following a single oral administration of levosulpiride (25 mg) tablet.

Keywords: Levosulpiride

1. Introduction

Levosulpiride, 5-(aminosulfonyl)-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxy benzamide (Fig. 1), is the levo-enantiomer form of racemic sulpiride, a benzamide derivative selectively inhibiting dopaminergic D_2 receptors at the trigger zone both in the central nervous system and in the gastrointestinal tract [1]. Levosulpiride has been widely prescribed because it is effective in abolishing or greatly reducing chemotherapy-induced emesis and nausea, accelerates gastric emptying and improves gastrointestinal symptoms in patients with functional dyspepsia and diabetic gastroparesis [2].

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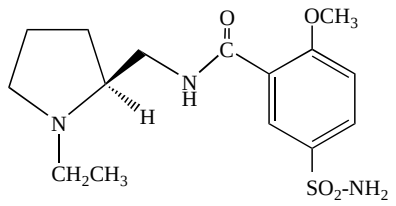
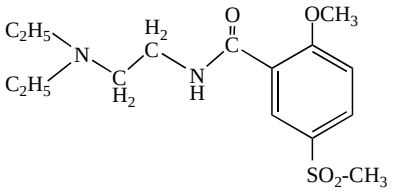


Levosulpiride



Tiapride

Fig. 1. Chemical structures of levosulpiride and tiapride (I.S.).

After oral administration of levosulpiride at the dose range of 100–200 mg, the bioavailability is weak (20–30%) with great inter- and intra-individual variability [3,4]. The time peak plasma concentration is approximately 3 h, while the plasma elimination half-life ranges from 6 to 19 h depending on the dosage and route of administration. Metabolism does not occur and the drug is excreted unchanged in the urine. The lack of hepatic metabolism makes metabolic interactions with cytochrome P450 (CYP450) substrate drugs very unlikely [5].

The earlier methods of analysis for levosulpiride were based on spectrofluorometric method [6], gas chromatography (GC) [7,8], high-performance liquid chromatography (HPLC) with UV detector [4,9,10] and fluorescence detector [11–14]. Most of these methods involve tedious [7,8], time-consuming extraction procedures using a relatively large sample volume [9,11] and do not provide good assay sensitivity for pharmacokinetic studies of levosulpiride [6–14] at the single oral dose of 25 mg. A simple, rapid, sensitive and reliable method for the determination of levosulpiride in human serum and urine was required for further investigations of its pharmacokinetics.

The aim of this study was to improve and validate an HPLC method applicable to bioassay and available to pharmacokinetics of levosulpiride in human serum and urine by means of not only simple extraction procedures but also improving sensitivity. The applicability of the method was proved in the study of the pharmacokinetics of levosulpiride in human volunteers following a single oral administration of levosulpiride (25 mg) tablet.

2. Experimental

2.1. Chemicals and reagents

Levosulpiride (99.77%) was kindly supplied from Kuhnil Pharmaceuticals (Seoul, Republic of Korea). Tiapride hydrochloride (internal standard, Fig. 1) was purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from Fischer Scientific (Fair Lawn, NJ, USA) and the other chemicals were of HPLC grade or highest quality available. HPLC grade water was obtained from a Milli-Q water purification system (Millipore Co., Milford, MA, USA) and used throughout the study. The mobile phase components such as acetonitrile, potassium dihydrogenate phosphate were filtered through a 0.45 μm pore size membrane filter prior to mixing and ultrasonically degassed after mixing.

2.2. Instruments

The HPLC system consisted of a model LC-10AD isocratic pump (Shimadzu, Kyoto, Japan), equipped with a Rheodyne 7725 injection valve (Rheodyne, Cotati, CA, USA) and a model RF-10A$_{XL}$ fluorescence detector (Shimadzu, Kyoto, Japan). The separation was performed on a Luna C$_{18}$(2) column (5 μm particle size, 250 mm × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA) with security guard cartridge (KJ0-4282, 5 μm particle size, 4 mm × 2.0 mm i.d.; Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile–0.01 M potassium dihydrogen phosphate (30:70, v/v, adjusted to pH 8.5 with triethylamine) at a flow rate of 0.8 ml/min with a column inlet pressure of about 9 MPa. Detector output was quantitated on a model Class LC-10 integrator (Shimadzu, Kyoto, Japan). The column was operated at 30 °C in column oven (ThermaSphere™ TS-130, Phenomenex, Torrance, CA, USA), and the effluent was monitored at a wavelength of 300 nm for excitation and 365 nm for emission.

2.3. Calibration standards and quality control samples

Stock solutions of levosulpiride and tiapride, the I.S., were prepared in methanol at concentration of 1 mg/ml and kept at 4 °C. Calibration standard serum samples of levosulpiride were prepared at concentrations of 0.25, 0.5, 1, 2, 5, 10, 50, 100 and 200 ng/ml in drug-free pooled serum, and 0.2, 0.5, 1, 2, 5, 10 and 20 μg/ml in drug-free pooled urine obtained from 12 different volunteers. In the same manner, quality control (QC) samples at low (2 ng/ml for serum and 0.5 μg/ml for urine), medium (10 ng/ml for serum and 2 μg/ml for urine) and high (100 ng/ml for serum and 10 μg/ml for urine), respectively, were prepared to evaluate accuracy and precision.

2.4. Extraction procedures

2.4.1. Serum

To 1 ml of blank serum, calibration standards and QC samples, 100 μl of I.S. (tiapride, 1 μg/ml in methanol), 200 μl of 0.1 M sodium hydroxide and 200 μl of 0.1 M sodium borate were added to clean test tubes. The samples were extracted with 4 ml of dichloromethane by vortex-mixing for 2 min and centrifuged at 3000 × *g* for 10 min. The aqueous layer (upper phase) was discarded by aspiration with a Pasteur pipette and organic layer (lower phase) was transferred into a clean test tubes. The organic layer was back-extracted with 200 μl of 0.025 M sulfuric acid by vortex-mixing for 1 min and centrifuged to separate the phase at 3000 × *g* for 5 min. Subsequently, a 150 μl aliquot of the supernatants was transferred into clean Eppendorf tubes and 20 μl of 0.5 M sodium hydroxide was added, and then 50 μl of the solution was injected directly onto the HPLC system.

2.4.2. Urine

To 1 ml of blank urine, calibration standards and QC samples, 50 μl of I.S. (tiapride, 1 mg/ml in methanol) was added to clean test tubes and mixed by vortexing for 30 s. The samples were diluted to 1:100 with mobile phase and 50 μl of the solution was injected onto the HPLC system.

2.5. Method validation

2.5.1. Specificity

The interference of endogenous compounds was assessed analyzing standard levosulpiride, drug-free serum and urine samples, serum and urine spiked with levosulpiride, and serum and urine samples obtained from subjects given levosulpiride. And also, all the peaks presenting a retention time of levosulpiride were investigated using a diode array detector (2017 Daiode Array, Shiseido, Tokyo, Japan).

2.5.2. Sensitivity

The lower limit of quantitation (LLOQ) was defined as the lowest concentration yielding a precision less than 20% (coefficients of variation, CV) and accuracy between 80 and 120% of the theoretical value. It was determined at 0.25 ng/ml for serum and 0.2 μg/ml for urine in five replicate samples.

2.5.3. Linearity

The linearity of calibration curve for levosulpiride was assessed in the range of 0.25–200 ng/ml in serum and 0.2–20 μg/ml in urine samples. The straight-line regression equations were treated statistically (weighting factor: 1/concentration) and are presented with their correlation coefficients.

2.5.4. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, QC samples were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficients of variation for the analysis of QC samples in five replicates, and inter-day precision was determined through the analysis of QC samples on five consecutive days. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

2.5.5. Recovery

The absolute recoveries from human serum were assessed by comparison of the peak area from extracted QC samples to the area of standard corresponding to respective concentrations followed by application of a correction factor. The relative recoveries from human serum were assessed by comparison of the peak area from extracted QC samples to those from extracted

samples containing water instead of serum. The mean recoveries were determined at low, medium and high concentrations in five replicates.

2.5.6. Stability

To test the short- and long-term stability of the basic drug such as levosulpiride, two QC samples, containing low (2 ng/ml for serum and 0.5 μg/ml for urine) and high (100 ng/ml for serum and 10 μg/ml for urine) concentrations, were stored under different conditions: at room temperature at 0, 1, 2, 4, 12 and 24 h in the acidic condition during the back-extraction; at −70 °C for 1 month. And also, 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. Pharmacokinetic studies of levosulpiride

Twelve normal healthy male Korean volunteers (22–29 years, 56.0–72.8 kg) participated in the pharmacokinetic study of oral levosulpiride after giving written informed consent. All subjects fasted at least 10 h before drug administration and continued to fast up to 4 h thereafter. They abstained from consumption of alcohol or xanthine-containing foods and beverages during the study. Each volunteer received a single oral dose of a levosulpiride tablet (25 mg) with 240 ml of spring water.

Blood samples were withdrawn from the forearm vein before oral administration and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 36 h after the oral administration, transferred to Vacutainer® (5 ml, Becton Dickinson and Company, USA) tubes, and centrifuged. Following centrifugation (3000 × *g*, 20 min, 4 °C), serum samples were transferred to polyethylene tubes and immediately stored at −70 °C until analysis.

Urine samples were collected before oral administration and for the following intervals after the oral administration: 0–4, 4–12, 12–24 and 24–36 h. The excreted urine was collected, the total volume measured, and an aliquot was placed at −70 °C in polyethylene tubes until analysis. The serum and urine samples were analyzed for levosulpiride content by the proposed HPLC method.

Pharmacokinetic parameters were calculated by non-compartmental analysis of serum concentration–time curve data using WinNonlin software (Pharsight Corporation, CA, USA) [15]. The peak concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined by the inspection from individual serum concentration–time profiles for levosulpiride. The area under the serum concentration–time curve (AUC_{0-t}) was calculated by the linear trapezoidal rule from 0 to 36 h. The area under the plasma concentration–time curve from zero to time infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/\lambda_Z$, where C_t is the last measurable concentration. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_Z$, where λ_Z is terminal rate constant. The urinary excretion profiles were used to determine the cumulative excreted amounts in 36 h.

3. Results and discussion

3.1. HPLC method improvement

Luna $C_{18}(2)$ column with the mobile phase of acetonitrile and 0.01 M potassium dihydrogen phosphate (pH 8.5) resulted in the short chromatographic run time (8 min) with satisfactory separation of levosulpiride and I.S. The retention time for levosulpiride and I.S. were 5.5 and 7.0 min, respectively.

Various other octadecylsilica columns such as Spherisorb ODS and Xterra™ RP_{18} were tried for the quantitation of levosulpiride in serum and urine. These columns provided inadequate resolution.

3.2. Specificity

Fig. 2 shows representative chromatograms of the human serum with back-extraction using 0.025 M sulfuric acid after the extraction with dichloromethane. There were no peaks of interfering with levosulpiride and I.S. at their retention times in the blank human serum (Fig. 2A). We could confirm the each identical peak spectrum with the diode array detector. This back-extraction step was essential to minimize interfering peaks, especially when analysing in the lower nanogram range. Therefore, the extraction procedures using a dichloromethane and back-extraction with 0.025 M sulfuric acid was selected.

Fig. 3 shows representative chromatograms of the human urine samples. In non-diluted urine samples severe interferences from endogenous compounds were observed at the retention time of levosulpiride, there-

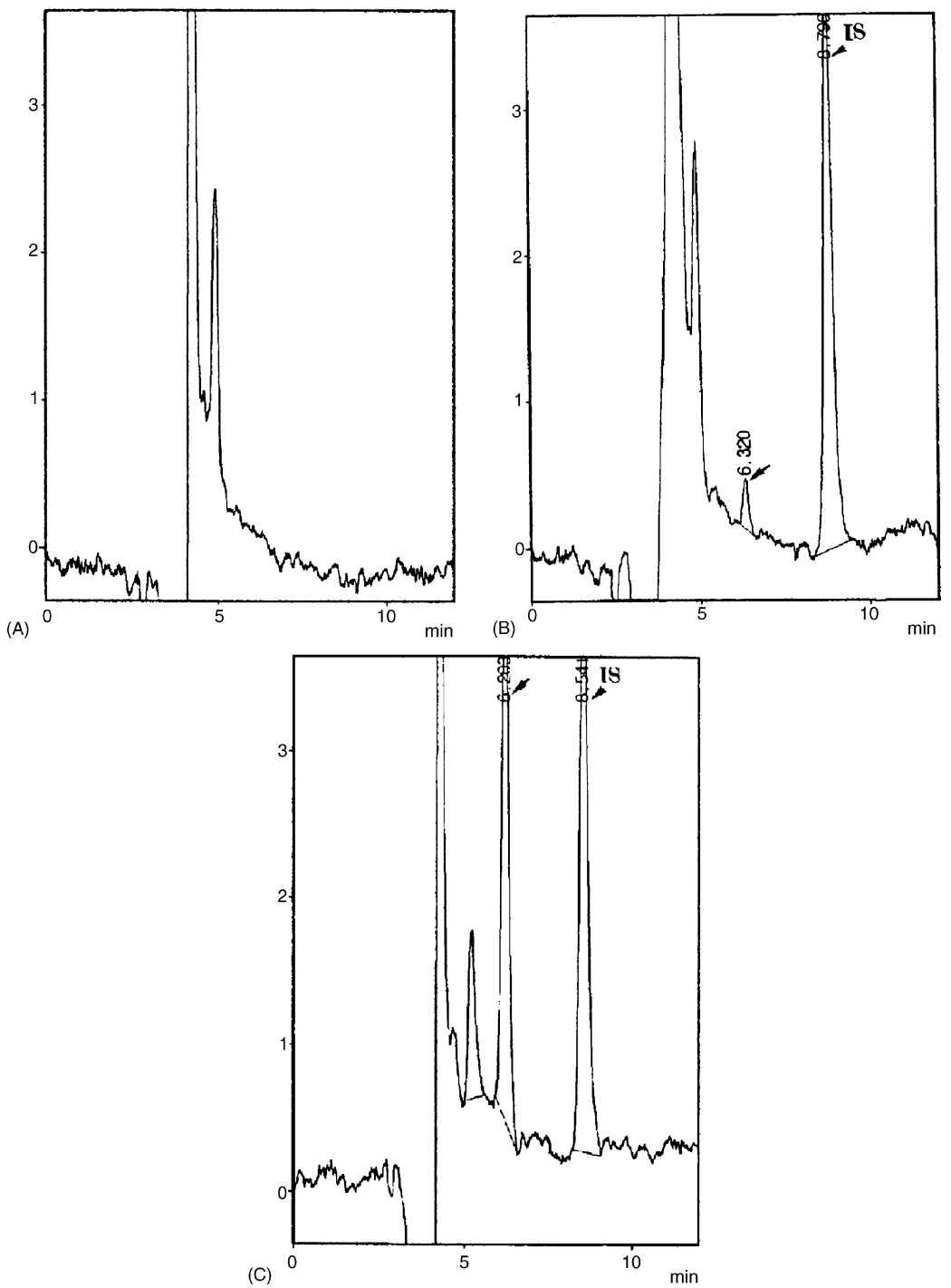


Fig. 2. Chromatograms of (A) blank human serum, (B) blank human serum spiked with levosulpiride (0.5 ng/ml, LOQ) containing internal standard (I.S., tiapride 100 ng/ml), and (C) human serum sample (15.86 ng/ml) at 1 h after a single oral dose of levosulpiride (25 mg) tablet to a volunteer. (Arrow head) Levosulpiride peak.

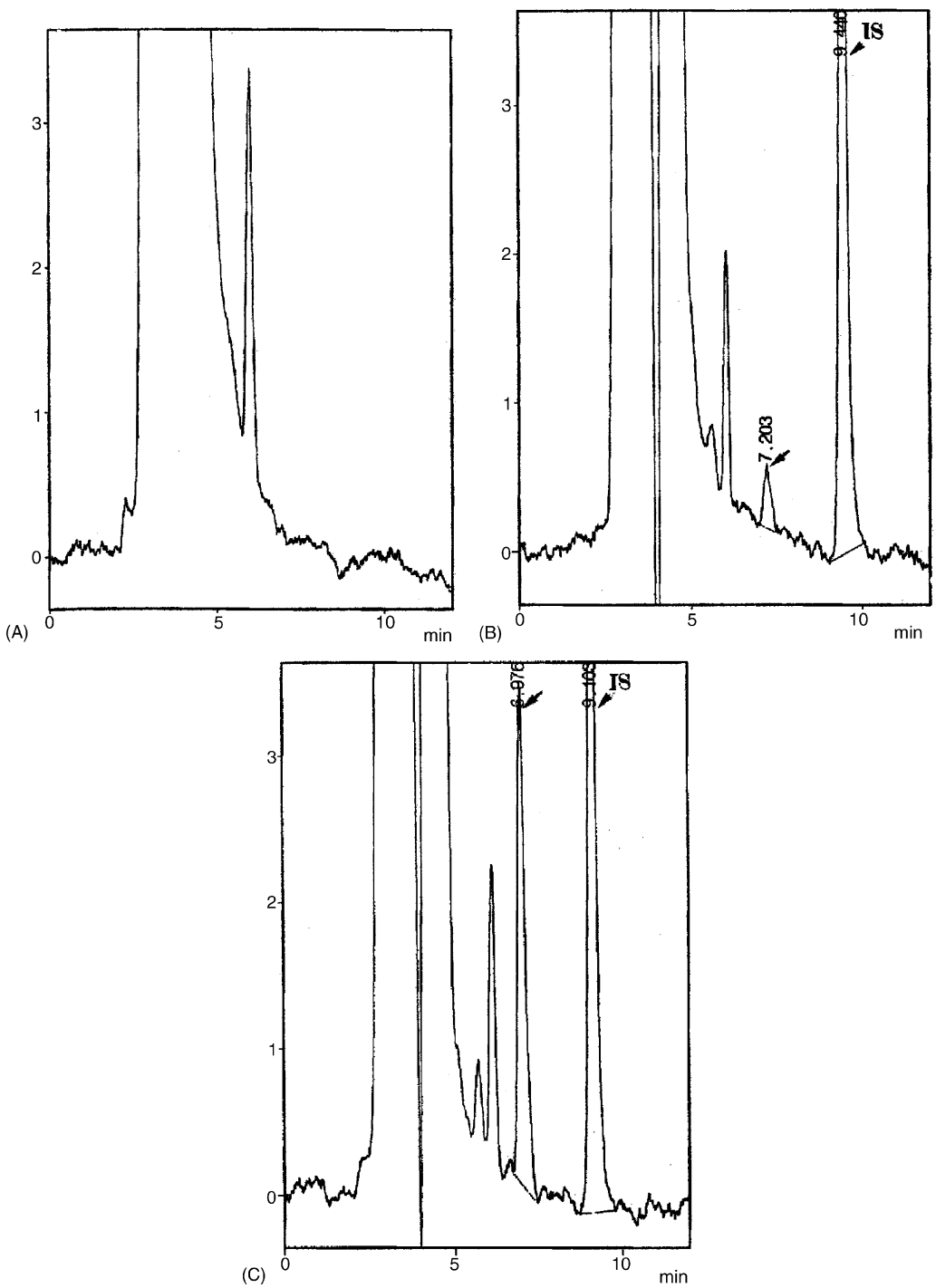


Fig. 3. Chromatograms of (A) blank human urine, (B) blank human urine spiked with levosulpiride (0.5 μg/ml, LOQ) containing I.S (tiapride 50 μg/ml), and (C) human urine sample (6.39 μg/ml) at 8–12 h after a single oral dose of levosulpiride (25 mg) tablet to a volunteer. (Arrow head) Levosulpiride peak.

fore, a dilution pretreatment of the samples was used as a clean-up step. This simple dilution treatment with mobile phase resulted in a sufficient cleaning step, free from interfering peaks at the retention time of levosulpiride and the I.S.

3.3. Sensitivity

The lower limit of quantitation was defined as those quantities producing a background noise of approximately 10 times that could be estimated with an intra- and inter-day precision less than 20% (CV), and accuracy between ±20% (bias). The LLOQ was found to be 0.25 ng/ml for serum and 0.2 μg/ml for urine samples injected on-column with a 50 μl loop, respectively. The mean percent accuracy values for serum and urine samples were 88.00 and 105.00% with CV of 15.91 and 14.29% at the LLOQ, respectively. Particularly, the LLOQ value for serum was 40-fold or more lower than those reported by Bressolle and Bres [9], Alfredsson et al. [11], Nicolas et al. [12] and Huang et al. [14], and 4-fold lower than that reported by Tokunaga et al. [13], respectively.

3.4. Linearity

The calibration curves for levosulpiride were linear over the concentration range of 0.25–200 ng/ml in human serum and 0.2–20 μg/ml in human urine, respectively. The least squares regression equations were treated statistically (weighting factor: 1/concentration) and presented with their correlation coefficients (r). The mean (±S.D.) regression equations from nine replicate calibration curves on different days for human serum and urine: $y = (0.03946 \pm 0.00330)x + (0.02461 \pm 0.00283)$ and $y = (0.08650 \pm 0.00531)x + (0.01260 \pm 0.00108)$ (where, y is the peak area ratio and x the concentration), showed significant linearities ($r = 0.9979$ and 0.9983, $P < 0.001$, respectively).

3.5. Precision and accuracy

Table 1 shows a summary of intra- and inter-day precision and accuracy. The intra-day accuracies for serum and urine samples were 88.50–100.00% and 98.00–101.50% at QC samples with the precision (CV) less than 11.86 and 12.24%, respectively. The inter-day accuracies for serum and urine samples ranged from 82.00 to 102.55% and 96.00 to 100.50% at QC samples with the precision (CV) less than 9.76 and 12.50%, respectively. These results indicated that the present method has a satisfactory accuracy, precision and reproducibility.

Table 1
Precision and accuracy of HPLC analysis of levosulpiride in human serum and urine samples

Target concentration (ng/ml)	Concentration found (mean ± S.D.)	CV (%)	Accuracy (%)
In human serum			
Intra-day ($n = 5$)			
2	1.77 ± 0.21	11.86	88.50
10	10.0 ± 1.1	10.70	100.00
100	97.3 ± 5.7	5.84	97.26
Inter-day ($n = 5$)			
2	1.64 ± 0.16	9.76	82.00
10	9.40 ± 0.71	7.55	94.00
100	102.6 ± 6.5	6.32	102.55
In human urine			
Intra-day ($n = 5$)			
0.5	0.490 ± 0.060	12.24	98.00
2	2.03 ± 0.21	10.34	101.50
10	9.91 ± 0.93	9.38	99.10
Inter-day ($n = 5$)			
0.5	0.480 ± 0.060	12.50	96.00
2	2.01 ± 0.14	6.97	100.50
10	9.84 ± 0.82	8.33	98.40

3.6. Recovery

The extraction recoveries of levosulpiride were determined at low (2 ng/ml), medium (10 ng/ml), and high (100 ng/ml) concentrations in five replicates (Table 2). The mean absolute recoveries of levosulpiride for human serum were found to be 89.8 ± 3.7%. The mean relative recoveries of levosulpiride for human serum were found to be

Table 2
Absolute and relative recoveries of levosulpiride for human serum

Concentration (ng/ml)	Absolute recovery (%, mean ± S.D., $n = 5$)	Relative recovery (%, mean ± S.D., $n = 5$)
2	88.1 ± 4.6	89.5 ± 7.3
10	90.2 ± 3.3	93.4 ± 7.9
100	91.2 ± 3.2	92.1 ± 4.7

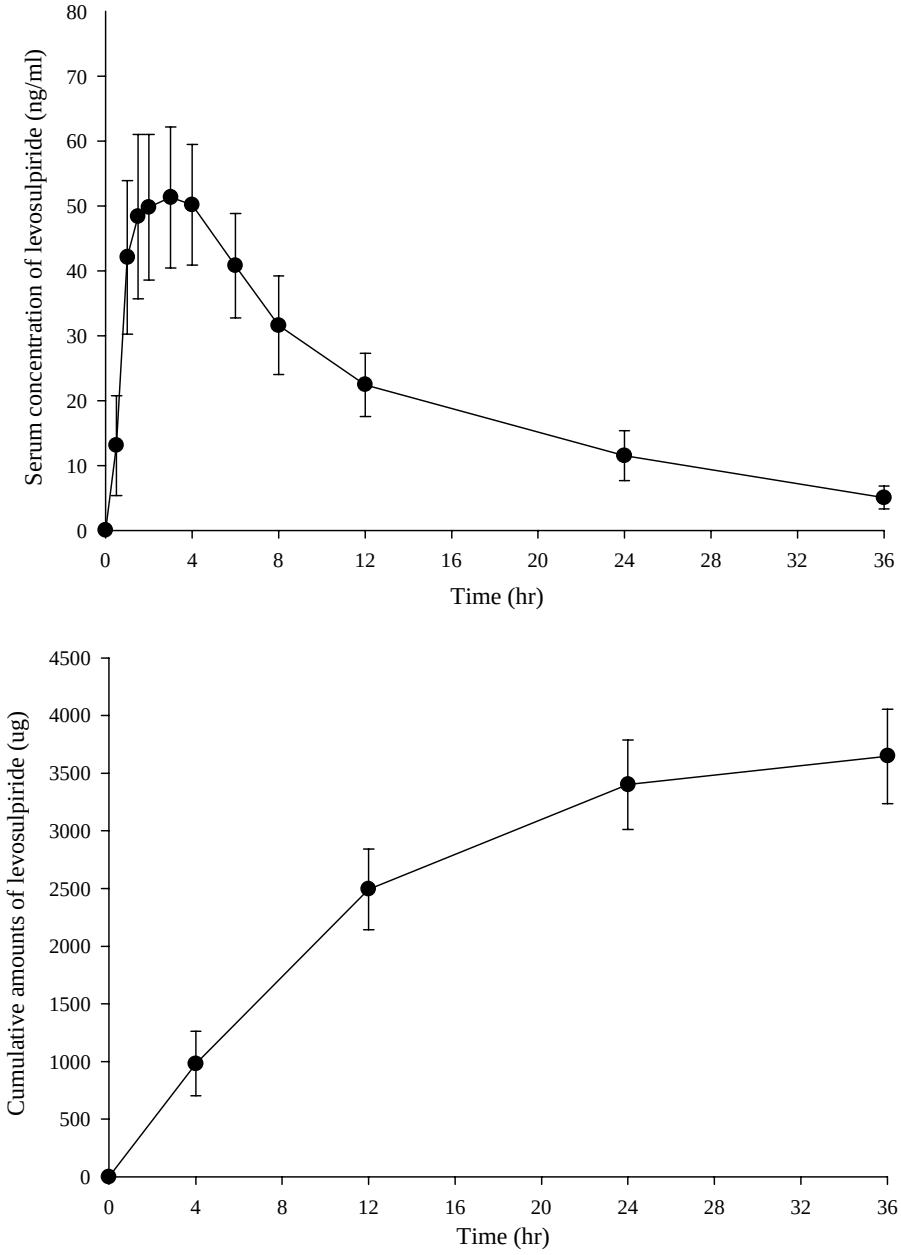


Fig. 4. Mean (±S.D., $n = 12$) serum (upper panel) and urine (lower panel) concentration–time profiles of levosulpiride following a single oral administration of levosulpiride (25 mg) tablet in 12 male volunteers. Vertical bar represents the standard deviation of the mean.

91.6 ± 6.1%. The simple liquid–liquid extraction with dichloromethane has been successfully applied to the extraction of levosulpiride from human serum.

3.7. Stability

Two QC samples of levosulpiride and I.S. were stable in 0.025 M sulfuric acid at room temperature

Table 3
Pharmacokinetic parameters of levosulpiride after single oral administration of levosulpiride (25 mg) tablet in human volunteers ($n = 12$, mean ± S.D.)

C_{max} (ng/ml)	63 ± 23
T_{max} (h)	2.8 ± 1.5
$t_{1/2}$ (h)	11.0 ± 2.2
AUC_{0-36} (ng h/ml)	742 ± 49
$AUC_{0-\infty}$ (ng h/ml)	828 ± 56
Urinary excreted amounts (mg)	3.65 ± 0.41

with quantitation variation less than 6.09% during 24 h in the daylight. It could be confirmed that these basic drugs would not be decomposed during the back-extraction. They were also stable at −70 °C during 1 month in serum and urine (CV was less than 4.60%). Finally, the storage of stock solutions at room temperature during for 6 h in the daylight produced no significant decrease of each levosulpiride and I.S. in peak area.

3.8. Pharmacokinetics of levosulpiride in human

The suitability of this method was proved in the pharmacokinetic study of levosulpiride after a single oral administration of levosulpiride (25 mg) tablet to 12 healthy male Korean volunteers. The serum and urine chromatograms of an oral administered levosulpiride are shown in Figs. 2C and 3C. Fig. 4 shows the mean (±S.D.) serum and urine concentration–time curves of levosulpiride in 12 subjects. The pharmacokinetic parameters such as C_{max}, T_{max}, AUC, $t_{1/2}$ and urinary excreted amounts of levosulpiride are shown in Table 3.

4. Conclusion

A rapid, sensitive and accurate validated HPLC method was improved and validated for the determination of levosulpiride in human serum and urine. It uses a simple solvent extraction and chromatography on a Luna column. The present method was successfully applied to the pharmacokinetic study of levosulpiride after a single oral administration of levosulpiride (25 mg) tablet to human volunteers. It has been currently used in this laboratory for a number of years for investigating the population pharmacokinetic characteristics of levosulpiride, and these results will be presented elsewhere.

Acknowledgements

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