Sahoo et al., 1:11
http://dx.doi.org/10.4172/scientificreports.523

Open Access Scientific Reports

Research Article Open Access

Determination of Levosulpiride in Human Plasma Using HPLC Method and its Application to Bioequivalence Study

Bijay Kumar Sahoo*, Ayan Das, Jayanti Mukherjee, Soumendra Darbar and Tapan Kumar Pal

Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India

Abstract

An accurate, precise and sensitive high performance liquid chromatographic method was developed for the determination of levosulpiride in human plasma samples and the developed method was successfully applied for analyzing levosulpiride in plasma samples for a bioequivalence study with twelve healthy volunteers. Peak area ratio of the analyte to internal standard was used for the quantification of serum samples. The study was conducted using an open, randomized crossover design to determine relative bioavailability of levosulpiride tablets (test and reference preparations) in twelve healthy male volunteers following single oral administration. The pharmacokinetic parameters like area under the plasma-concentration-time curve from zero to the last measurable levosulpiride sample time and to infinity (AUC_{0-t} and $AUC_{0-\infty}$), maximum concentration (C_{max}), time to maximum concentration (T_{max}), elimination rate constant (K_e) and elimination half-life ($T_{1/2}$) were determined by non compartmental method. The bioequivalence between the two formulations was assessed by calculating individual peak plasma concentration (C_{max}) and area under the curve (AUC_{0-t}) ratio (Test/Reference). The assay showed excellent relationships between peak height ratios and plasma concentrations ($r^2 \geq 0.9925$). The geometric mean of Levosulpiride 100 mg tablet (test/reference) individual percentage ratio was 100% for AUC_{0-t} and 99% for C_{max}. The 90% confidence intervals were 99.2-100.1% and 98.4-99.9%, respectively. The relative bioavailability between test and reference was 99.54%. Since the 90% CI for both AUC_{0-a}, and C_{max} lies within the 80-125% proposed by the FDA, it was concluded that both preparations of levosulpiride 100 mg tablets were bioequivalent in terms of both the rate and extent of absorption.

Keywords: Bioequivalence; Levosulpiride; Internal standard; High performance liquid chromatography; Amisulpride

Abbreviations: CAN: Acetonitrile; AUC: Area Under the Plasma Concentration-Time Curve; CPU: Clinical Pharmacological Unit; CI: Confidence Interval; C_{max}: Maximal Observed Plasma Concentration; CV: Coefficient of Variation; FDA: Food and Drug Administration; HPLC: High Performance Liquid Chromatography; K_e: Elimination Rate Constant; IS: Internal Standard; LOD: Limit of Detection; QC: Quality Control; RE: Relative Error; SD: Standard Deviation; $T_{1/2}$: Half-life; T_{max}: Time Taken to Reach C_{max}

Introduction

Chemically, Levosulpiride is [(S)-(-)-5-(aminosulfonyl)-N-[(1-ethyl-2-pyrrolinyl) methyl]-2 methoxyIbenzamide] (CAS No.23672-07-3.), a new antipsychotic agent belonging to the substituted benzamide group (Figure 1). Levosulpiride is only a weak D_2 dopamine receptor antagonist. Furthermore, in the D_2 receptor family (which includes D_2, D_3 and D_4 receptors), the affinity of levosulpiride for the D_2 receptor is only 2-3 times greater than that for the D_3 receptor (this contrasts with typical antipsychotics, which are 10-20 times more potent at D_2 than at D_3) [1]. At low doses (50-200 mg/day), levosulpiride preferentially blocks dopamine autoreceptors which are located on presynaptic neurons. At these doses, levosulpiride is therapeutic for negative and cognitive symptoms of schizophrenia and for depressive and somatoform disorders. At high doses (400-800 mg/day), levosulpiride blocks both dopamine presynaptic and postsynaptic D_2 receptors and may therefore be effective for the positive symptoms of schizophrenia. Its low incidence of extrapyramidal side effects (EPS) is characteristic of a typical antipsychotic [2,3].

Various analytical methods have been described earlier to quantify levosulpride in biological fluids of both animals and humans. Though they were effective, they were quite complex. Gas chromatography [4], both high performance liquid chromatographic with fluorescence or mass spectrometric detection approaches has been reported in the literature [5,6]. To understand the pharmacokinetic behavior of levosulpride in humans, a reliable quantitative method is needed. An HPLC – UV method has described for measurement of Levosulpiride in human plasma with UV detection [7]. But the method suffered from lack of clinical application. This paper describes a HPLC method with a rapid and simple sample preparation using liquid-liquid extraction technique requiring smaller sample volumes and enabling low limit of quantification. Levosulpiride is a basic drug and has a low bioavailability (20-30%) following oral administration [8]. The bioequivalence study

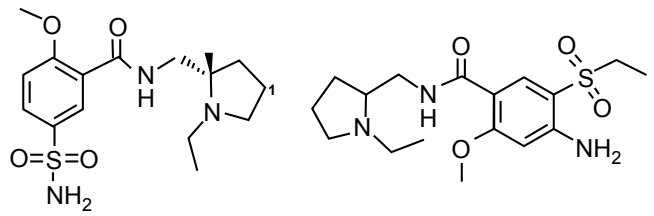

Figure 1: Structures of Levosulpiride and Amisulpride

***Corresponding author:** Bijay Kumar Sahoo, C/O Prof. (Dr.) T.K. Pal, Department of Pharmaceutical Technology, Jadavpur University, Kolkata–700 032, India, Fax: +91 33 2414 6967; E-mail: bijaysahoo14@gmail.com

Received November 04, 2009; **Published** November 05, 2012

Citation: Sahoo BK, Das A, Mukherjee J, Darbar S, Pal TK (2012) Determination of Levosulpiride in Human Plasma Using HPLC Method and its Application to Bio-equivalence Study. 1:523. doi:10.4172/scientificreports.523

of two formulation (Test & Reference) is carried out by comparing equivalence with respect to the rate and extent of absorption, while the area under concentration time curve (AUC) generally serves as the characteristic of the extent of absorption [9,10]. No single parameter reliably measures the rate of absorption; for instance, the maximal drug concentration (C_{max}) has been widely used, but it depends more on the fraction absorbed than the rate of absorbed; the time of maximal concentration (t_{max}) depends on both absorption and elimination rates.

The main purpose of this study was to develop more sensitive and reproducible HPLC method to analyze levosulpride in plasma. Moreover, this developed method was applied for the bioequivalence study of two tablet formulations of levosulpride in healthy Indian volunteers.

Material and Methods

Drugs and chemicals

HPLC- grade Acetonitrile (Purity not less than 99.80%), potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck India Ltd. (Mumbai, India). Water was purified by a Milli-Q gradient system of Millipore (Elix, Milli-Q A10 Academic) until a resistivity of 18 MΩ was achieved.

Levosulpiride was obtained from M/s Psycho-Remedies Ltd. (Latton danna, Ludhiana, India). Amisulpride used as internal standard (IS) was obtained from M/s Optimus pharma Pvt Ltd., (Hyderabad, India).

The blank human plasma with EDTA-K3 anticoagulant was collected from Clinical Pharmacological Unit (CPU) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India.

Standard solutions

The stock solutions of analyte and IS were prepared by dissolving the accurately weighted standard compound in water to give final concentration of 1 mg/ml. Successive dilutions of 100 and 10 µg/ml were made from the stock solutions, which were used to prepare the calibration curve and quality control samples. A seven-point standard curve was prepared by spiking the appropriate amounts of working solution into the blank plasma to obtain final concentrations of 10, 25, 50, 75,100, 150 and 200 ng/ml for the analyte. The concentration of IS in plasma sample was 300 ng/ml. All stock solutions and working standard solutions were stored in polypropylene vials at -20°C freezer. The linear regression of the peak area ratio of analyte/IS *vs.* concentration was used to obtain calibration curve. The regression equation of the calibration curve was then used to calculate the plasma concentration. The back calculated values of the concentrations were statistically evaluated.

QC samples were made using the stock solution. Four levels of QC samples in plasma were 10.0 (lower limit of quantitation, i.e. LLOQ), 30 (low-), 100 (medium-), and 180 (high-) ng/ml for the analyte. QC samples were prepared in a 50 ml pool, then aliquoted into pre- labeled 2 ml polypropylene vials and stored at -20°C until used.

Extraction procedure

For calibration standards, an aliquot of 0.1 ml for each spiking solution was spiked into 0.9 ml of control blank plasma in polypropylene tube. Then 0.1 ml of IS (Amisulpride 300 ng/ml) were vortex-mixed for 2 mins. Then 6 ml Dichloromethane: Chloroform (50:50, v/v) was added and mixed for 15 min. All the samples were centrifuged for 15 min at 4000 rpm. The organic layer was separated and dried under nitrogen atmosphere at 40-45°C, The residue was reconstituted by 200 µl mobile phase and injected into the HPLC system.

HPLC conditions

The HPLC system was Knauer, Berlin, Germany, and it consisted of a solvent delivery pump (1000), a Rheodyne injector and an UV-visible detector (2500). Integration was done using Chemitochrom 2000 software. HPLC was carried out isocratically at room temperature using an analytical column, Hypersil C18 (250×4.6, 5 µ particle size) from phenomenex, USA. The mobile phase was 10 mM phosphate buffer and acetonitrile (HPLC grade) in the ratio of 85:15 (v/v) and eluted at a flow rate of 1 ml/min. The sample was injected through the Rheodyne injector system fitted with 20 µl fixed loop. The effluent was monitored using UV detection at 237 nm. The method was validated for linearity range, accuracy, and precision and system suitability parameter as per the standard guidelines [11].

Analytical method validation

Validation was accomplished through determination of linearity, quantification limit, detection limit, accuracy, precision, specificity and stability. The human plasma pool employed for the validation of the analytical methods was initially used to determine the absence of interfering peaks with the retention time equal or close to that of levosulpiride.

Calibration curve and linearity

The calibration curves were constructed using 0.9 ml of blank plasma spiked with 0.1 ml of internal standard (300 ng/ml) and 0.1 ml of levosulpiride of various concentrations. The linear regression equations were obtained from the peak area ratio of analyte and internal standard plotted against their respective plasma concentrations (10 to 200 ng/ml).

Recovery

The recovery of levosulpiride was evaluated by comparing the peak areas obtained after plasma extraction with the peak areas obtained after direct injection of the standard solutions.

Limit of quantification and limit of detection

The limit of quantitation (LOQ) was determined as the lowest levosulpiride concentration that could be analysed with a precision of less than 20% (C.V) and with accuracy between ± 20%, as determined in the inter-day analytical runs. The LOQ was found to be 20 ng/ml. The limit of detection (LOD) was defined as the sample concentration of levosulpiride resulting in a peak height of three times the signal – to – noise ratio (S/N).

Specificity

The specificity of the method was evaluated by running the blank plasma through assay procedure and comparing the retention times of endogenous compounds in plasma with those of levosulpiride and internal standard.

Accuracy and precision

Precision was expressed as coefficient variation (CV), while accuracy as the percentage relative error (RE). Three validation batches were processed on three separate days. Each batch included one set of calibration standards and six replicates of LLOQ, low-, medium-, and high-concentrations of QC samples. Inter-batch and intra-batch

precision and accuracy evaluations were based on back-calculated concentrations.

Stability

Stability of analyte in plasma was assessed from spiked samples at low, medium and high quality control samples (LQC, MQC, HQC) at room temperature for 8 h, at 2-8°C for 48 h and at -20°C for one month. Each determination was performed in duplicate. The compounds were considered stable if the variation of assay was less than 10% of initial time response.

Application to bioequivalence study

The above mentioned validated method was successfully used to analyze plasma sample for a bioequivalence study of levosulpiride. Test preparation was 100 mg tablet manufactured by Psycho-Remedies, Lattondanna, Ludhiana, India. Tablet nexipride containing 100 mg of levosulpiride, manufactured by Sun Pharmaceutical Industries Ltd., Mumbai, India was used as Reference preparation.

Experimental design

This randomized, single-dose, two-treatment, and two-way cross over study, with a washout period of 7 days between the two dosing sessions, was conducted in accordance with Good Clinical Practice (GCP) Guidelines and Good Laboratory Practice (GLP) Guidelines. In each dosing session, volunteers received either of the Test or the Reference preparation of levosulpride 100 mg only on the study day at a fixed time. Subjects were non-alcoholic and non-smokers, healthy Indian male volunteers mean age ± SD (25.25 ± 4.693) years and mean weight (60.50 ± 5.036) Kg, were enrolled in this study after obtaining written informed consent. Volunteers were screened for inclusion in the study within 21 days before the commencement of the study. The study protocol was reviewed and approved by Drugs Control General of India (DCGI) and the Institutional Ethical Committee of Jadavpur University prior to the start of the study. The study was in compliance with revised Declaration of Helsinki.

All the volunteers assembled in CPU ward at 6.00 a.m. on the study day of each session, after overnight fasting of 10 hrs. Their TPR, BP was recorded and an indwelling intravenous catheter was introduced with strict aseptic precautions in the suitable vein for blood collection. They received either of the study preparations according to their code nos. A total of 15 blood samples were collected at 0 hr. (before drug administration) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 12.0, 24.0, 36.0 hrs. (after drug administration) in the test tubes with EDTA at each time point. Breakfast, lunch and dinner were provided after 3 hrs, 6 hrs, and 13 hrs respectively after drug ingestion. On the study days volunteers were permitted normal activities, excluding strenuous exercise.

Collected blood samples were centrifuged immediately; plasma was separated and stored frozen at -200°C with appropriate labelling of volunteer code no., study date and collection time, till the date of analysis.

Pharmacokinetic analysis

The following Pharmacokinetic parameters were directly determined by using the non- compartmental method. Both the maximum plasma concentration (C_{max}) and time to peak plasma concentration (t_{max}) were obtained directly from the analytical data. The elimination half-life ($t_{1/2}$) was calculated from the slope of the terminal log linear phase, using the formula $0.693/K_e$; where K_e is the apparent elimination rate constant. AUC_{0-t} (where t is the time at which the last quantifiable concentration is observed) was calculated using the trapezoidal rule, $AUC_{0-\infty}$ was calculated as the sum of AUC_{0-t} and the extrapolated area determined by dividing the observed concentration at the time of the last quantifiable concentration by the slope of the terminal log linear phase [12]. $AUC_{0-\infty}$ was calculated according to the following formula:

$$AUC_{0-\infty}=AUC_{0-t}+C_{last}/K_e$$

Where C_{last} is the last quantifiable plasma level

Statistical analysis

An analysis of variance (ANOVA) was performed on the pharmacokinetic parameters AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} by using general linear model (GLM) procedures, in which sources of variation were subject, formulation and period. The 90% confidence interval (CI) of the test/reference ratio for AUC_{0-t}, C_{max} and $AUC_{0-\infty}$ (log transformed) was determined. According to the guidance from the FDA, bioequivalence between the two formulations can be concluded when 90% confidence interval (CI) for the pharmacokinetic parameters of the two products are found within the acceptable range of 80–125% (FDA) [13].

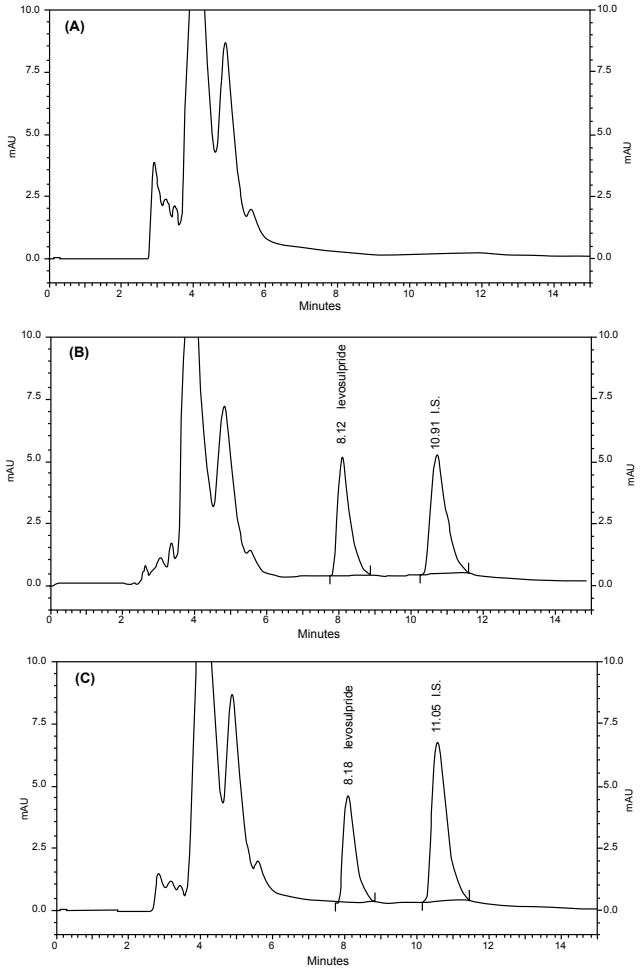

Figure 2: [A] Blank plasma; [B] Blank plasma spiked with raw drugs; [C] Human plasma containing levosulpiride and IS.

Results

The described analytical method used for the measurement of levosulpiride was shown to be accurate and sensitive. An internal standard was used in the study and the run time was 15 mins. The peaks of levosulpiride and internal standard were well resolved (Figure 2). The retention time (RT) of levosulpiride and internal standard (IS) was about at 8.12 and 10.91 mins respectively. No interferences were observed in human plasma sample.

The LOD and LOQ for levosulpride in plasma were 5 and 20 ng/ml, respectively. The relationship between concentration and peak area ratio was found to be linear within the range of 10 ng/ml to 200 ng/ml with a r^2 value=0.9925 and intercept not significantly different from zero (Figure 3).

Quality control points at low, medium, and high levels (30, 120 and 180 ng/ml) for levosulpiride were used to determine stability, absolute recovery, within-day and between-day, precision and accuracy. No significant degradation of levosulpiride was observed during the period under the storage conditions. The mean absolute recovery of levosulpiride in plasma was 82.6%, 84.9% and 86.6% at 30 ng/ml, 100 ng/ml and 180 ng/ml, respectively. The within-day and between-day precision and accuracy data are summarized in table 1.

The pharmacokinetic parameters of reference and test product obtained after administration to healthy volunteers are summarized in table 2.

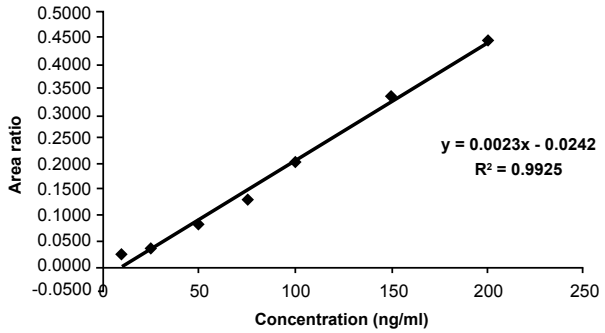

Figure 3: Calibration curve of levosulpiride.

Concentration (ng/ml)	Within day (n=6)		Between day (n=6)	
	Accuracy (%)	Precision (CV %)	Accuracy (%)	Precision (CV %)
30	80.19	9.95	81.39	12.75
100	82.8	2.65	83.77	4.75
180	84.6	7.6	85.21	4.44

Table 1: Within-day and between–day precision and accuracy of HPLC method for levosulpiride 100 mg.

Parameter	Test	Reference	90% CI (Log-transformed data)
AUC_{0-t} (ng.h/ml)	1767.61 ± 80.95	1775.84 ± 79.47	0.99404-1.00470
$AUC_{0-\infty}$ (ng.h/ml)	2175.59 ± 69.7	2199.1 ± 81.91	0.99538-1.00187
C_{max} (ng/ml)	139.18 ± 5.61	143.33 ± 8.24	0.98780-1.00068
t_{max} (h)	3.08 ± 0.73	3.16 ± 0.65	-
K_e (h^{-1})	0.052 ± 0.003	0.051 ± 0.003	-
$t_{1/2}$ (h)	13.39 ± 0.66	13.56 ± 0.74	-

Table 2: Pharmacokinetic parameters of Levosulpiride 100 mg (mean ± SD) in 12 volunteers with Test and Reference preparation.

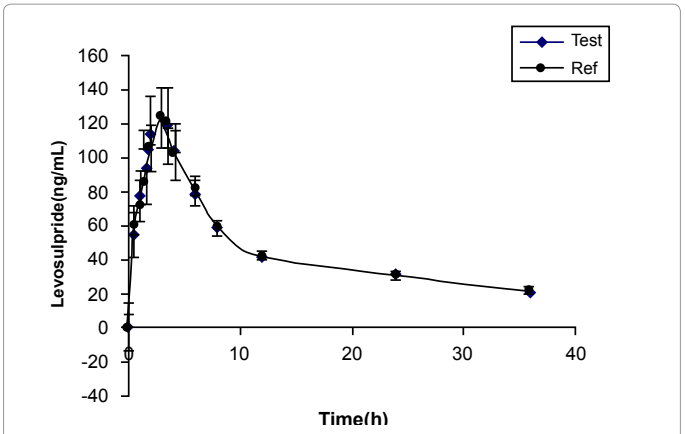

Figure 4: Curves of the mean plasma concentration of levosulpiride (Mean ± SD) of 12 volunteers *vs*. time (h).

As can be seen from table 2, 90% CI for all the compared pharmacokinetic parameters for both the drugs (ratios of C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$) were obtained within the range of 0.80-1.25. There was no statistically significant difference between the test and references formulations (P>0.05). The relative bioavailability of levosulpiride between test and reference was 99.54%. Figure 4 shows mean levosulpride plasma concentration as a function of time after the oral administration of 100 mg levosulpiride of both brands.

Discussion

Under our experimental conditions reproducible chromatographic separations were obtained at acetonitrile-10 mM phosphate buffer, pH 4.8 (15:85, v/v). The extraction and HPLC assay resulted in symmetrical peak shape and good baseline resolution of levosulpiride and amisulpride. Using this system, the retention times for levosulpride and amisulpride were 8.12 and 10.91 mins, respectively. The retention time was faster than those reported by [14] and [15]. The choice of amisulpride as an internal standard for levosulpride was based on the presence of similar functional groups in both structures in addition to their similarity in terms of elemental compositions and chemical behaviour. Several wavelengths (225-330 nm) were evaluated and 237 nm produced the best results in terms of selectivity and sensitivity.

The method presented here was sufficient to perform bioequivalence study. Twelve healthy volunteers were administered a single oral dose of levosulpride tablet. Plasma samples were obtained during 36 h. after levosulpride administration. The time to reach maximum plasma concentration (t_{max}) was 3.08 h after drug administration. The AUC_{0-36h} was 2175.59 ng/ml.h. These values are comparable to the parameters reported by Jin et al. [14]. After a single oral administration of levosulpride (25 mg, three tablets) to 24 healthy volunteers, AUC_{0-36h}, C_{max}, and T_{max} were 725 ng/ml.min, 80 ng/ml, and 3.08 h, respectively. The elimination half life ($t_{1/2}$) of levosulpiride was 13.39 h. Thus the one week washout period was sufficient due to the fact that no sample prior to administration in phase two showed any levosulpride levels.

Conclusions

The validated HPLC method employed here proved to be simple, fast, reliable, sensitive and selective enough to be used in the determination of levosulpiride in human plasma. The 90% CI of C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were in the acceptable range of 0.8-1.25. Both formulations were equal in terms of rate and extent of absorption. On the basis of pharmacokinetic parameters studied, it can be concluded that the test

product is bioequivalent with the reference product. Considering the fact that the present method involves a shorter running time and a simple sample preparation process, it may be used in similar studies as a time and cost effective alternative to other available methods.

Acknowledgements

The authors are thankful to Department of Science and Technology (DST), New Delhi, India for providing us the necessary instrumental facilities for carrying out the study and to Psycho-Remedies, Ludhiana, India for sponsoring the bioequivalence study.

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