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Bio-Analytical Liquid Chromatographic Method for the Determination of Linezolid in Plasma

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An isocratic selective and sensitive high performance liquid chromatographic method for the estimation of linezolid in human plasma by precipitation of plasma proteins with 5 % perchloric acid, after precipitation the supernatant was separated and injecting 10 µL of sample volume. The chromatography separation was achieved on a Hypersil BDS C_{18} (4.6 ID × 250 mm, 5 µ particle size) column using a mixture of 20 Mm potassium dihydrogen phosphate buffer (pH 3.5 ± 0.1) and acetonitrile in a ratio of 85:15 (v/v) as the mobile phase. The column elutes were monitored at 252 nm. The total chromatographic run time was 12.5 min and the elution of linezolid and zidovudine (IS) retention times 10.5 and 5.2 respectively. The method was found to be linear in the concentration range of 50.0 to 20059.9 ng/mL. The method was successfully applied to *in vitro* pharmacokinetic-bioequivalence studies.

Key Words: HPLC, Human plasma, Linezolid, Precipitation.

INTRODUCTION

Linezolid[1,2] (LZL), the chemical name is *N*-[[(5S)-3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] acetamide. Linezolid is a synthetic antibiotic, the first of the oxazolidinone class, used for the treatment of infections caused by multi-resistant bacteria including streptococcus and methicillin-resistant *Staphylococcus aureus* (MRSA). The drug works by inhibiting the initiation of bacterial protein synthesis. Linezolid is a synthetic antibacterial agent of the oxazolidinone class of antibiotics. Therefore, Linezolid has the potential for interaction with adrenergic and serotonergic agents. Linezolid is primarily metabolized by oxidation of the morpholine ring, which results in two inactive ring-opened carboxylic acid metabolites: The aminoethoxy acetic acid metabolite (A) and the hydroxyethyl glycine metabolite. Literature survey reveals the report of a few methods of determination for linezolid in pharmaceutical dosage forms[3,4], plasma and in other biological fluids based on HPLC[5-10] and mass spectrometric[11] are available. The authors now propose a fast eluting, accurate and precise HPLC method for the determination of Linezolid in human plasma. The entire results obtained in the present study comply with the acceptance criteria of regulatory requirements[12].

Few methods are available for determination of linezolid in different biological fluids. Boubakar *et al.*[6] reported the linezolid in growth media, but this method involves online extraction technique and economically high cost. Determination of linezolid in human breast milk reported by Sagirli *et al.*[7]. Cattaneo *et al.*[9] reported linezolid in human plasma with high LLOQ. In this report, we have introduce a new method for the determination of linezolid in human plasma with economically low cost and convenient precipitation method with low LLOQ.

EXPERIMENTAL

Linezolid (Fig. 1) and naproxen procured from Aurabindo Pharma Limited, Hyderabad and HPLC grade acetonitrile, methanol (HPLC grade) obtained from Rankem, India. Analytical grade potassium di-hydrogen phosphate, orthophosphoric acid, ethylene diamine tetra acetic acid (EDTA) disodium salt purchased from Qualigens, India. All the aqueous solutions including buffer for the mobile phase, were prepared with milli-Q (Millipore, millford, USA) grade water. The control K2EDTA human plasma was purchased from Cauvery Diagnostics and Blood bank Secunderabad, India.

A Shimadzu HPLC equipment LC-2010 HT, equipped with four pumps, a VP CTO-10AS VP column oven, SPD-10A variable-wavelength programmable UV-visible detector. A chromatography separation was achieved with an isocratic mobile phase consisting a mixture of 20 mM of potassium di-hydrogen phosphate buffer (pH 3.5 ± 0.1) and acetonitrile in a ratio of 85:15 (v/v) by using a Hypersil BDS C_{18} (4.6 ID × 250 mm, 5µ particle size) column. A mobile phase flow rate at 1.2 mL/min was getting sharp peaks in this method. The data was acquired and processed with LC solution software.

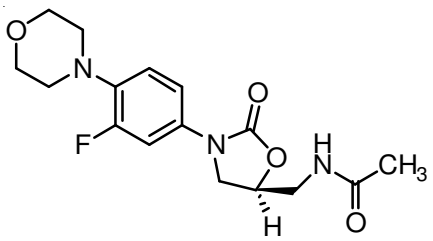

Fig. 1. Structure of linezolid

Working standard solutions and the calibration curve: The stock solutions of the linezolid and internal standard were prepared in a milli-Q water and methanol respectively. These solutions were stored below 10 ºC. The primary stock solutions at a free base concentration of 5 mg/mL for analyte and 1 mg/mL for internal standard. The stock solutions were used to prepare the calibration curve standards and quality control samples with mixture of methanol and water (20:80 v/v). The solutions for obtaining the eight point standard calibration curve were prepared by spiking the screened blank plasma with appropriate amounts of linezolid dilutions. The calibration curve was linear in the range of 50.0-20059.9 ng/mL. The quality control samples were prepared at four concentration levels of 50.1 ng/mL (LLOQ QC), 149.9 ng/mL (LQC), 8234.2 ng/mL (MQC) and 15248.6 ng/mL (HQC) and 300 µL spiked plasma volumes were aliquoted in different labeled polypropylene vials which were then capped properly and stored in a freezer at -70 ºC.

Sample extraction procedure: 200 µL each of the spiked plasma calibration curve standards and the quality control samples were transferred to pre-labeled micro-centrifuge tubes containing 50 µL of the internal standard, Zidovudine (5000 ng/mL dilution). The tubes were vortexed for ten seconds. Each of the tube was added with 100 µL of 5 % perchloroic acid solution. Once again all the micro-centrifuge tubes were vortexed for thirty seconds on a Spinex unit and the tubes were further vortexed for 10 min at 2500 rpm on a vibramax unit. All the samples were loaded into the refrigerated micro-centrifuge and then centrifuged at 12000 rpm for 10 min at 10 ºC. From the each micro centrifuged tube approximately 150 µL of supernatant was transferred to pre-labeled auto-sampler vials and then analyzed with HPLC unit by injecting 10 µL of sample volume.

Validation procedures: A full validation according to the FDA guidelines was performed for the assay in human plasma.

Specificity and selectivity: The specificity of the method was evaluated by analyzing human plasma samples from at least six different sources to investigate the potential interferences at the analyte and IS.

Calibration curve: The calibration curve was acquired by plotting the ratio of sum of peak area of Linezolid to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 50, 100, 500.1, 2005.2, 5013, 10230.6, 15045 and 20059.9 ng/mL. The results were fitted to linear regression analysis by using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient of 0.999 or better. The acceptance criteria for each back-calculated standard concentration were ± 15 % deviation from the nominal value except at LLOQ, which was set at ± 20 %.

Precision and accuracy: The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different quality control levels, *i.e*. 50.1, 149.9, 8234.2 and 15248.6 ng/mL. The inter-assay precision was determined by analyzing the four levels quality control samples. The criteria for acceptability of the data included accuracy within ± 15 % standard deviation from the nominal values and a precision of within ± 15 % relative standard deviation, except for LLOQ, where it should not exceed ± 20 % of accuracy as well as precision[12].

Stability experiments: The stability of linezolid and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 24 h (auto-sampler) after the initial injection. The peak areas of the linezolid and IS obtained at 0 h on day 1 were used as the reference to determine the relative stability of the analyte at subsequent points. In all stability studies two quality control concentrations were used, *viz*. LQC and HQC. Stability of linezolid in the biomatrix during 8 h exposure at room temperature in human plasma (bench-top) was determined at ambient temperature (25 ± 1 ºC) in six replicates at each concentration. Freezer stability of linezolid in human plasma was assessed by analyzing the quality control samples stored at -70 ± 10 ºC for at least 15 days. The stability of linezolid in human plasma following repeated freeze-thaw cycles was evaluated by using quality control samples spiked with linezolid. The samples were stored at -70 ± 10 ºC between freeze-thaw cycles. The stability of linezolid was assessed after the third freeze thaw cycle. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (*i.e.* ±15 % SD) and precision (*i.e.* ±15 % RSD).

RESULTS AND DISCUSSION

In order to remove the interfering peaks from the bio matrices and to increase the selectivity and sensitivity of the analytical method different methods of sample pre-treatment were investigated. Protein precipitation with various organic solvents and their mixtures resulted in reproducible recoveries and no interferences from the sample matrix with the chromatography of the analyte and IS (data not shown). In pursuit of symmetric peak shape and shorter run time, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, sodium dihydrogen phosphate, potassium dihydrogen phosphate and formic acid with variable pH range of 3-6, along with altered flow-rates (in the range of 0.5-1.5 mL/min) were tested for complete chromatographic resolution of linezolid and IS (data not shown). The resolution of peaks was achieved with 20 mM potassium di hydrogen phosphate (pH 3.5 ± 0.1):acetonitrile (15:85, v/v) with a flow rate of 1.2 mL/min, on a Hypersil BDS C_{18}, (4.6 ID × 250 mm, 5 µ particle) column was found to be suitable for the determination of Linezolid and IS. The UV absorption spectrum of the drug was taken in methanol and the λ_{max} was found to be at 252 nm. A model chromatogram showing the separation of Linezolid is presented in Fig. 5 under the above optimized conditions retention times of 10.5

and 5.2 min were obtained for linezolid and zidovudine respectively.

Specificity and selectivity: The specificity and selectivity of the present method was evaluated by checking the blank EDTA (dipotassium ethylene di-amine tetra acetic acid) plasma (without spiking with linezolid) obtained from different blood donors. Six different lots of blank plasma were screened and all of them were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard. According to chromatograms in Fig. 2 represented no interference at anlayte and ISTD from the extracted blank plasma sample, Fig. 3 shows there is no interference in the presence of extracted blank +ISTD plasma sample, Fig. 4 represented extracted STD-1 (LLOQ) plasma sample and Fig. 5 showed the extracted STD-8 (ULOQ) plasma sample of linezolid.

Recovery: The percent recoveries were determined by comparing the areas of the extracted quality control samples with equivalent post spiked samples. Recovery for linezolid ranges from 73.2 % to 75.6 % (mean recovery: 74.35 %) and recovery for IS 68.8 %.

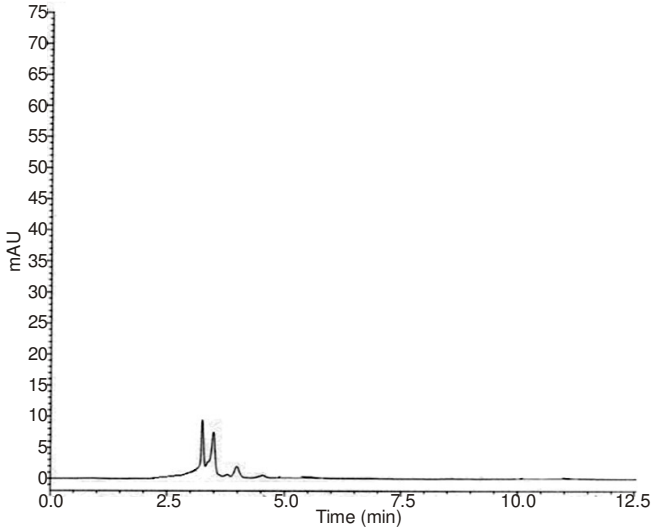

Fig. 2. A chromatogram of the extracted blank plasma sample of linezolid

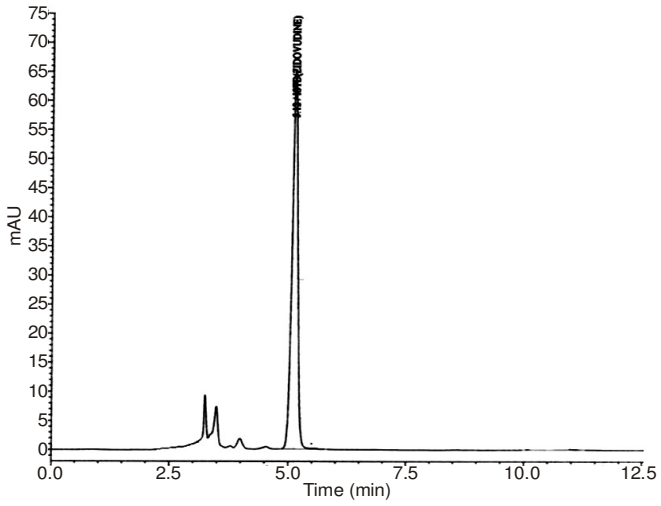

Fig. 3. A chromatogram of the extracted blank +ISTD plasma sample of linezolid

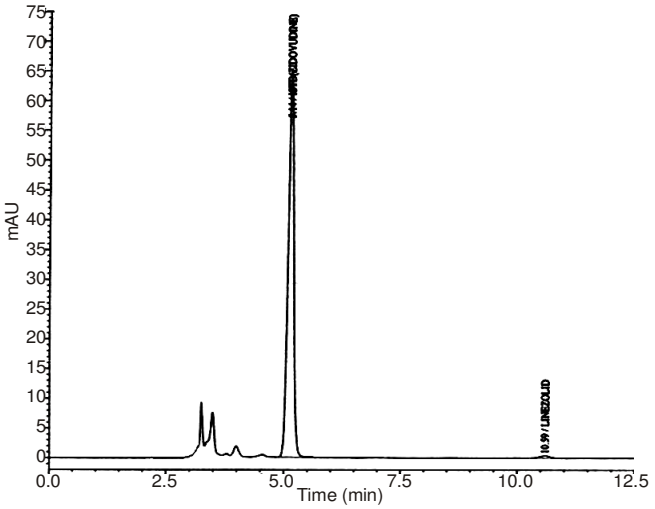

Fig. 4. A chromatogram of the extracted STD-1(LLOQ) plasma sample of linezolid

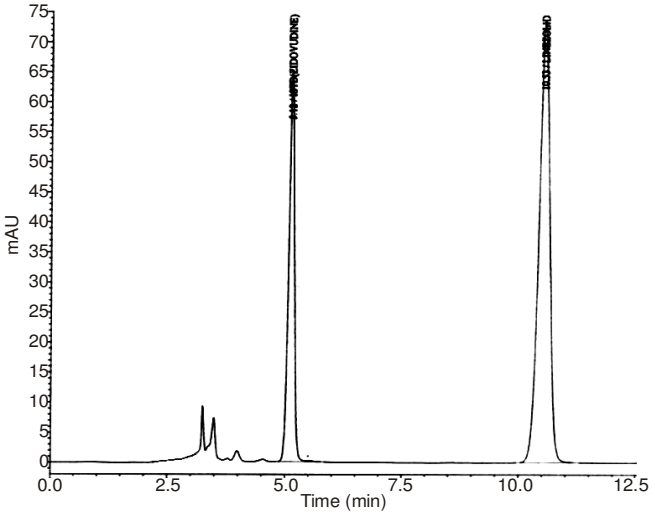

Fig. 5. A chromatogram of the extracted STD-8(ULOQ) plasma sample of linezolid

Calibration curve: The plasma calibration curve was constructed using 8 calibration standards (*viz.*, 50.0-20059.9 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) *vs.* concentration and fitted to the y = mx + c using weighing factor ($1/X^2$). The correlation coefficient (r) ranged from 0.9930 to 0.9999.

Precision and accuracy: The accuracy and precision data for intra-day and inter-day plasma samples are presented in Table-1. The assay values on both the occasions (intra-day and inter-day) were found to be within the accepted variable limits.

Stability: The predicted concentrations for PPX at 149.9 and 15248.6 ng/mL samples deviated within ± 15 % of the nominal concentrations in a battery of stability tests, *viz.* ininjector (49 h), bench-top (11 h), repeated four freeze-thaw cycles and long term stability at -70 ± 10 ºC for 36 days (Table-2). The results were found to be within the assay variability limits during the entire process.

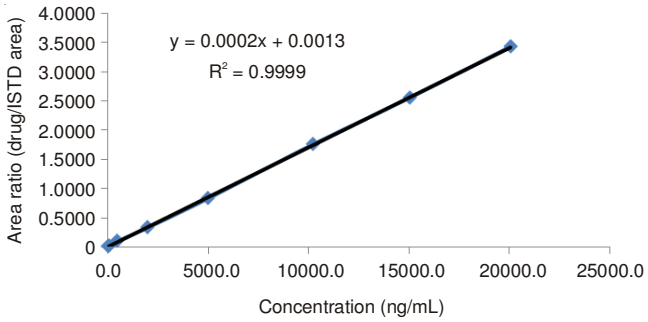

Fig. 6. Calibration curve for linezolid

TABLE-1
BACK CALCULATED CONCENTRATION OF QC SAMPLES FOR LINEZOLID (INTRA- AND INTERDAY)

Intra day QC ID	LZL LLOQ-QC1-6	LZL LQC1-6	LZL MQC1-6	LZL HQC1-6
Mean	47.00	147.05	8476.05	14911.70
SD	2.883	6.313	182.506	348.720
CV (%)	6.1	4.3	2.2	2.3
Nominal (%)	93.8	98.1	102.9	97.8
Inter day QC ID	LZL LLOQ-QC19-24	LZL LQC19-24	LZL MQC19-24	LZL HQC19-24
Mean	47.65	148.87	7716.43	15566.30
SD	2.804	8.403	439.145	405.886
CV (%)	5.9	5.6	5.7	2.6
Nominal (%)	95.1	99.3	93.7	102.1

TABLE-2
STABILITY RESULTS OF LINEZOLID

Freeze–Thaw stability (4 cycles)	Nominal (%): 98.2-104.5 % CV: 2.3-6.1 Percent stability: At LQC Level: 104.3 At HQC Level: 99.6
Bench top stability (11.00 h)	Nominal (%): 97.2-105.7 % CV: 2.9-3.9 Percent stability: At LQC Level: 104.7 At HQC Level: 106.0
In injector stability (49.00 h)	Nominal (%): 102.0-103.6 % CV: 1.4-5.1 Percent stability: At LQC Level: 101.4 At HQC Level: 100.4
Dry extract stability (39.24 h)	Nominal (%): 95.1-102.2 % CV: 1.8-6.1 Percent stability: At LQC Level: 103.2 At HQC Level: 100.5
Long term stability in plasma (36.00 days at-70 °C)	Nominal (%): 96.2-97.9 % CV: 1.1- 3.8 Percent stability: At LQC Level: 99.0 At HQC Level: 99.1

Conclusion

In summary, we have developed a new method for the determination of linezolid in human plasma, which offers the highest sensitivity (50 ng/mL) compared with other methods described in the literature using a simple precipitation procedure and commercially feasible. From those results of all the validation parameters and applicability of the assay, we can conclude that the present method is useful for clinical pharmacokinetic studies of linezolid with desired precision and accuracy.

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