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Validation of a Simple, Rapid and Sensitive LC Method for Quantification of Riluzole in Rat Plasma and its Pharmacokinetic Application

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Abstract

A simple, sensitive and rapid high-performance liquid chromatography method with ultraviolet (UV) detector was developed and validated for the analysis of riluzole (RLZ) in rat plasma. The plasma sample, spiked with nebivolol as an internal standard (IS), was subjected to a single step protein precipitation prior to analysis. Chromatographic separation was achieved on a Phenomenex C18 (250 mm× 4.6 mm, 5 µm) column. A combination of methanol and phosphate buffer (25 mM KH_2PO_4, pH 3.5), in the ratio of 70:30% v/v was used as the mobile phase in isocratic mode. RLZ and IS were monitored at wavelengths of 264 nm and 280 nm respectively. No interference was observed from plasma components in the analysis of RLZ and IS. The calibration curve was linear over the range of 50–4000 ng/mL (r^2 = 0.999). The drug was found to be stable under various processing and storage conditions. The assay provided good reproducibility, accuracy and proved to be suitable for oral pharmacokinetic studies of RLZ in rats.

Keywords: Liquid chromatography; Riluzole; Rat plasma; Protein precipitation; Internal standard; Method validation; Pharmacokinetic studies

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive, fatal, neurodegenerative disease caused by the deterioration of voluntary muscle controlling motor neurons [1]. Currently, Riluzole (RLZ) is the only available, clinically approved drug for treatment of ALS [2]. Three mechanisms are proposed for action of RLZ: inhibition of excitatory amino acid release, inhibition of events following stimulation of excitatory amino acid receptors and stabilization of the inactivated state of voltage-dependent sodium channels [3]. RLZ offers neuro-protective action and prolongs the life of ALS patient.

RLZ is chemically designated as 6-(trifluoromethoxy) benzothiazol-2-amine. It is very slightly soluble in water and is weakly basic (pKa 3.5) in nature [4]. Post oral administration, it is rapidly absorbed from gastro–intestinal tract and has an absolute bioavailability of about 60% in humans [5]. It is extensively metabolized, primarily by cytochrome P450 1A2 [6].

Due to its moderate bioavailability and plethora of adverse effects, great potential exists to improve the pharmacokinetic properties of RLZ. Localizing RLZ in brain tissue by brain targeted delivery can overcome some of the adverse effects associated with RLZ. From the literature, it is evident that a few research groups have explored pharmacokinetic [7,8] and formulation [9,10] approaches for enhancing the effectiveness of RLZ in animal models. Assessment of pharmacokinetic properties for RLZ in animal models requires determination of plasma-concentration–time profile and such research endeavors need a simple, reproducible and cost effective bioanalytical method.

Previously reported methods for determination of RLZ in human plasma by high-performance liquid chromatography (HPLC) [11,12] or by liquid chromatography-coupled tandem mass spectrometry [13] indicated two major drawbacks: (i) Reported HPLC methods required higher volume of plasma sample (1 mL) for determination of RLZ and were hence, not well suited for rat studies. (ii) The LC-MS method reported by Chandu et al. though sensitive involves complicated processing steps and is expensive, limiting its use in rat studies.

Further literature survey into HPLC methods for estimation of RLZ in pre–clinical animal models revealed a method each in rat brain [14] and in mouse plasma, brain and spinal cord [15]. Since the rat brain matrix is significantly different in composition to rat plasma matrix, the same method cannot be used for estimation of RLZ in rat plasma matrix. The reported mouse plasma matrix method has long run time (over 20 min) and lesser sensitivity (LOQ = 100 ng/mL) which is again undesirable for routine sample analysis.

Hence, the aim of the present study was to develop and validate a rapid and sensitive method for the determination of RLZ in rat plasma which could be easily applied for bioavailability enhancement or drug-drug/food interaction studies of RLZ in rats.

Experimental

Chemicals

RLZ and Nebivolol (internal standard, IS) were obtained as gift samples from Apotex Research Pvt. Ltd., Bangalore, India and Vesta Pharmachem Pvt. Ltd., Surat, India respectively. HPLC grade acetonitrile, methanol, potassium di-hydrogen orthophosphate (KH_2PO_4) and sodium citrate were purchased from Merck laboratories, Mumbai, India. Methyl cellulose and tween 80 were purchased from S.D. Fine Chem Ltd., Mumbai, India. Milli–Q water purification system (Millipore®, MA, USA) was used for obtaining high quality HPLC grade water. Male Wistar rats were purchased from Sainath agencies, Hyderabad, India.

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Instruments and chromatographic conditions

The liquid chromatography system employed was Shimadzu HPLC (Shimadzu, Japan) with solvent delivery system of two pumps (Model LC–20AD, Prominence Liquid Chromatograph, Shimadzu, Japan), auto injector (Model SIL–20A HT, Prominence Auto Sampler, Shimadzu, Japan) and photo diode array (PDA) UV detector (Model SPD–M20A, Prominence Diode Array Detector, Shimadzu, Japan). Data collection and integration was accomplished using LC Solutions, 1.25 version software.

An endcapped C18 reverse phase column (Luna®, 250 mm long and 4.6 mm internal diameter, particle size 5 µm, Phenomenex, CA, USA) equipped with a guard column of same packing material was used for the study. A combination of methanol and phosphate buffer (25 mM KH_2PO_4, pH 3.5) in the ratio of 70:30% v/v was used as the mobile phase. Buffer was filtered through 0.22 µm Millipore filtration membrane. The HPLC system was stabilized for 1 h at 1 mL/min flow rate, through baseline monitoring prior to actual analysis. The RLZ and IS were monitored at fixed wavelengths of 264 nm and 280 nm respectively with a mobile phase flow rate of 1 mL/min in isocratic mode. An injection volume of 50 µL was optimized for final method.

Preparation of stocks and working standard solutions

Primary stock solutions (1000 µg/mL) of RLZ and IS were prepared in a volumetric flask by dissolving accurately weighed amount of RLZ and IS in methanol separately. Working standard solutions for both RLZ and IS were prepared by appropriately diluting the respective stock solutions with a pre–mixed solvent containing methanol and water in the ratio of 1:1. Working standard solution of 20 µg/mL was prepared for IS. All stock and working standard solutions were stored at 4°C until used for analysis.

Preparation of calibration standard solutions

To 90 µL of drug-free plasma, 10 µL of appropriate working standard solution of RLZ was added to achieve standard solutions containing 50, 100, 250, 500, 750, 1000, 2000, 3000 and 4000 ng/mL of RLZ in plasma. These concentrations were used to construct standard calibration curves. Of which, the concentrations of 250, 750 and 3000 ng/mL were chosen for lower quality control (LQC), medium quality control (MQC) and higher quality control (HQC) samples respectively.

Extraction technique

A simple, single–step protein precipitation method was followed for extraction of RLZ from Wistar rat plasma. To 100 µL of each plasma sample, 10 µL of IS (20 µg/mL) was added in a 1.5 mL microfuge tube. The mixtures were vortexed for 1 min and then subjected to protein precipitation by adding 350 µL of acetonitrile and thorough mixing for 1 min. Samples were then centrifuged at 7826 × *g* at 4°C for 20 min. A clean and clear supernatant (150 µL) was transferred to a sample loading vial and injected into the HPLC system.

Method validation

The developed method was validated statistically as per the guidelines given by the International Conference on Harmonization [16] and United States Pharmacopoeia [17]. Various validation parameters of the developed method were determined using the following procedures:

Linearity, accuracy, precision and specificity

Linearity of analytical method for RLZ was determined by developing a nine–point calibration curve in the range of 50 ng/mL to 4000 ng/mL, analyzed in six independent runs. Calibration curves were constructed by least-square linear regression of the peak area ratio of analyte (RLZ) to the IS versus the nominal concentrations of RLZ spiked to drug–free plasma samples.

For determining the intra–day accuracy and precision, five replicates of each quality control (QC) samples of RLZ were analyzed twice on the same day. The inter–day accuracy and precision were assessed by analysis of five precision and accuracy batches on three different days across all the QC levels. In order to study the specificity of the present method, six different lots of drug–free plasma samples were subject to the same analytical procedure to check the potential chromatographic interference from plasma matrix.

Sensitivity

Lowest limit of quantification (LLOQ) was determined as the minimum concentration of analyte that produced relative standard deviation (RSD) within 20% of the nominal value for both precision and accuracy [16]. Quantitation limit and detection limit were also determined using standard deviation of response and slope of the calibration curve obtained from the linear regression analysis.

Recovery

Recovery of RLZ was determined by comparing the peak area of analyte (RLZ) obtained from plasma (extracted) samples with analytical standard (unextracted) samples at the same nominal concentration. Recovery study was performed across the QC levels and precision of RLZ recovery at each level (n = 5) was determined.

Stability

Bench top stability of RLZ in rat plasma was assessed by preparing and storing five sets of each QC samples at room temperature and analyzing then every 3 h up to a period of 6 h on the day of preparation. Freeze thaw stability of RLZ in rat plasma was determined across the QC levels for three freeze–thaw cycles. A total of four sets at each QC level (n = 5) were prepared and one set of the prepared concentrations was analyzed on the day of preparation (no freeze thaw cycle) and the remaining three sets were frozen at -20°C for 24 h. All the frozen samples were thawed and one set of QC samples was analyzed. The remaining two sets were kept at -20°C for freezing and were analyzed after two and three freeze thaw cycles. Long–term stability of RLZ in rat plasma was determined across the QC samples. A total of four sets at each QC level (n = 5) were prepared and one set of the prepared concentrations was analyzed on the day of preparation. The remaining three sets were frozen at -20°C. Each set of stored samples was analyzed after 7, 15 and 30 days of sample preparation. The percentage deviation from the mean concentrations observed at zero time was calculated in all the stability studies.

Pharmacokinetic study

Male Wistar rats, weighing 180 to 220 g were used in the study. The experimental protocol was approved by the Institutional Animal Ethics Committee (Approval No.: IAEC–14/09–11). All animals were fasted overnight (12 h) before dosing and continued till 4 h after administration of test items, thereafter rat chew diet was provided ad libitum.

A freshly prepared aqueous suspension of RLZ (containing 0.5% methyl cellulose and 0.1% tween 80) was administered at a dose of 10 mg/kg through the oral route in rats (n = 5) in the pharmacokinetic

study. Blood samples (0.5 mL) were collected from retro–orbital plexus into the microfuge tubes containing sodium citrate as anticoagulant (3.8% w/v) at pre dose, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 16 h post dose and kept on ice till further processing. These samples were further harvested for plasma by centrifuging at 4°C for 10 min at 905 × ***g*** and then stored at -20°C till further analysis. All samples were processed according to the procedure described earlier and analyzed using the validated HPLC method.

Results and Discussion

Method development

The effects of various buffers and buffer compositions with varying pH conditions on the retention time and peak symmetry of RLZ were investigated. Mobile phase was selected based on the short run time, symmetric factor, sensitivity, ease of preparation and the economy of the method for in vivo studies in rats.

A significant effect of mobile phase composition was observed on retention time of RLZ. Retention time of RLZ was increased from 4.1 min (drug peak merged with plasma junk) to 11.5 min (broader peak with decreased height was observed) with a decrease in organic phase (methanol) composition from 80% to 50% *v/v* in the mobile phase. Mobile phase consisting 70% of methanol was found to provide retention time of 6.8 ± 0.11 min for RLZ. No additional advantage was observed in using acetonitrile in comparison with methanol on RLZ peak properties including sensitivity and peak symmetry. Hence, methanol was selected as an organic as it is cost effective for routine sample analysis.

Peak tailing has been a major concern for ionizable basic compounds like RLZ (with free amino group). Secondary interactions, via an ion exchange mechanism, between protonated base (BH^+) of analyte and acidic, ionized silanol ($Si–O^-$) groups on the surface of silica support particles are primarily responsible for the tailing phenomenon. These interactions and thereby tailing can be reduced by using acidic buffers (up to pH 3) at higher buffer concentration and choosing buffer cations that are strongly held by the silanols (18–19). Initially, buffers like ammonium acetate and ammonium formate at higher concentrations of up to 30 mM were tried, however, phosphate buffer (25 mM KH_2PO_4) was found to be suitable in reducing the tailing factor and in improving peak shape of RLZ.

Final optimization was carried out by changing the pH of the selected buffer. A decrease in buffer pH from 5.8 to 3.5 caused further improvement on peak tailing of RLZ to the acceptable range (< 1.2). Decreasing the pH of buffer reduced the ionization of acidic silanols groups and thereby reducing the interaction between protonated RLZ and silanols groups.

In HPLC analysis, selection of proper internal standard significantly impacts precision, accuracy and recovery of the method. Different compounds including acyclovir, diclofenac, nebivolol and raloxifene were tested for selection as internal standards in optimized chromatographic conditions. Peaks of acyclovir and diclofenac showed short retention (retention factor < 0.5) and were found to merge with plasma components. Raloxifene showed high retention time (15.5 min) and hence contributed to increase in total run time for each sample that belied our objective of developing a rapid method. Only nebivolol could be well separated from plasma junk and RLZ within short run time of 10 min. Therefore nebivolol was selected as IS. Under the optimal chromatographic conditions, the retention times for IS and RLZ obtained were 5.3 ± 0.09 and 6.8 ± 0.11 min respectively with good separation (resolution > 2) between the analyte peaks.

Method Validation

Specificity: Potential interference of plasma components in the analysis of RLZ and IS was checked at two wavelengths, 264 nm and 280 nm. No significant interference, from endogenous substances in plasma was observed either at the retention time of RLZ or at IS, indicating specificity of the method. Results for specificity are shown in Figure 1A and Figure 1B.

Linearity: The mean regression equation for calibration curve of RLZ in rat plasma matrix was Y = 0.00085 (± 0.00007) X–0.03835 (± 0.0012) (r^2 = 0.999), where 'Y' represents the ratio of peak area of RLZ to IS, and 'X' represents concentration of RLZ. The calibration curve was linear in the concentration range of 50–4000 ng/mL.

Accuracy and precision: All three quality control samples (LQC = 250 ng/mL, MQC = 750 ng/mL and HQC = 3000 ng/mL) showed an accuracy ranging from –1.22% to 3.25% with maximum %RSD of 4.11 across the entire QC range, establishing the accuracy of method for RLZ estimation in rat plasma (Table 1).

From the results obtained in intermediate precision studies, the %RSD values for intra–day variation and inter–day variation were found to be not more than 4.10 and 4.02 respectively (Table 1). Lower %RSD values indicated repeatability and intermediate precision of the proposed method.

Sensitivity: Analysis of six independent samples of 50 ng/mL

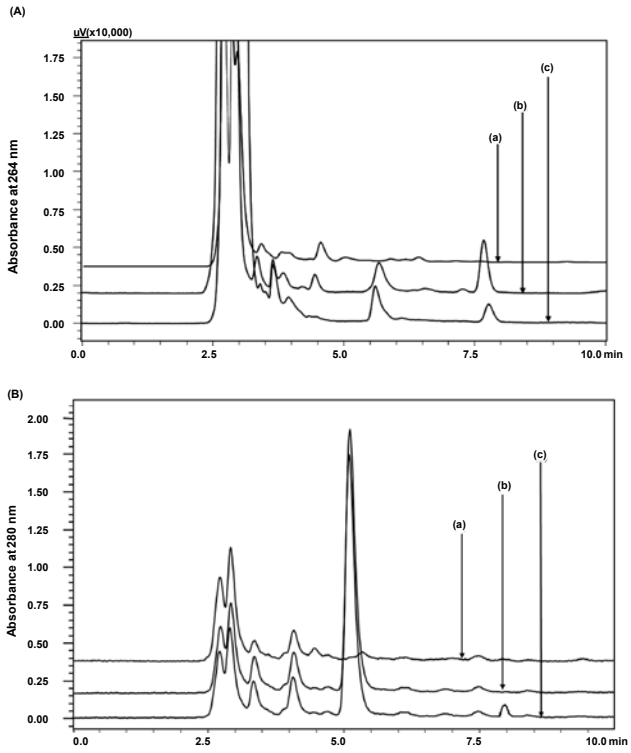

Figure 1: (A) Overlaid chromatograms (extracted at 264 nm) of (a) blank plasma (b) plasma spiked with IS (2000 ng/mL) along with RLZ (250 ng/mL) and (c) in vivo test sample taken 16 h after oral administration of RLZ (10 mg/kg). (B) Overlaid chromatograms (extracted at 280 nm) of (a) blank plasma and (b) plasma spiked with IS (2000 ng/mL) alone and (c) plasma spiked with IS (2000 ng/mL) along with RLZ (250 ng/mL).

had shown accuracy in the range of to –3.81% to 5.52% with intra–day and inter–day precision values of 5.83% and 6.02% respectively. Quantification limit and detection limit determined using standard deviation of the response and the slope of the calibration curve were found to be 49.09 ng/mL and 16.21 ng/mL respectively. Hence, LLOQ of 50 ng/mL was considered to be reliable, reproducible and accurate for the proposed method. The LLOQ value obtained for RLZ in the present study is significantly improved compared to previously reported method (100 ng/mL) by Colovic *et al.* in mouse plasma.

Extraction recovery: Recovery of RLZ from the spiked rat plasma samples, when compared with analytical standards of same concentration, for all the QC levels tested was in the range of 81.3% to 85.5% with %RSD less than 4.11% at each of QC levels.

Stability: No significant degradation of RLZ in plasma was observed under various stress conditions: bench top storage at room temperature for 6 h, three freeze and thaw cycles and long–term storage at -20°C for 30 days. The deviation from the zero time concentration was found to be in the range of –1.81% to 0.84% and -2.61% to 2.05% in bench top stability and freeze thaw stability studies respectively. In case of long–term storage stability study, deviation from the zero day concentration was found to be in the range of –3.90% to 2.70%. Results obtained from stability studies are summarized in Figure 2.

Pharmacokinetic application: The proposed analytical method was successfully applied to study the pharmacokinetics of RLZ after single dose oral administration of an aqueous suspension of the drug in rats. The mean plasma concentration versus time profile of RLZ obtained following oral administration is given in Figure 3.

The pharmacokinetic parameters obtained from the study using non–compartmental analysis (WinNonlin(R), 5.1 version, Pharsight Inc., CA, USA) were area under the curve (AUC) = 8460.13 ± 560.25 h ng/mL, area under the first–moment curve (AUMC) = 55075.38 ± 3609.33 h^2 ng/mL, mean retention time (MRT) = 6.51 ± 0.12 h, time to reach the maximum plasma concentration (t_{max}) = 1.0 h and maximum plasma concentration (C_{max}) = 1680.46 ± 30.42 ng/mL. Samples collected till 16 h post oral administration of the drug were analyzed in the study, indicating sensitivity and the applicability of the proposed method to in vivo pharmacokinetic studies of drug in rats.

Conclusions

A rapid, precise, specific, sensitive and cost effective HPLC method was developed and validated for estimation of RLZ in rat plasma. The drug was found to be stable under various processing and storage conditions. The developed method allows high sample throughput due to the simple procedure for sample preparation and relatively short run time. The method was successfully employed in determining the pharmacokinetic parameters of the drug following oral administration in rats.

QC Level	Predicted concentration[a] (ng/mL)		Mean accuracy[e] (%)	Intra-day repeatability (%RSD) (*n* = 5)			Inter-day repeatability (%RSD) (*n* = 30)
	Mean[b] ± SD[c]	%RSD[d]		Day-1	Day-2	Day-3	
LQC (250 ng/mL)	258.14 ± 10.62	4.11	3.25	4.04 3.78	4.10 3.97	4.06 4.04	4.02
MQC (750 ng/mL)	740.85 ± 23.14	3.12	-1.22	3.24 3.08	3.17 2.97	3.26 3.14	3.17
HQC (3000 ng/mL)	3037.75 ± 73.68	2.42	1.25	2.54 2.78	3.17 2.97	2.86 3.04	2.75

[a]Each value is mean of ten independent determinations (*n* = 10).
[b]Predicted concentration of RLZ was calculated from linear regression equation.
[c]Standard deviation.
[d]Percentage relative standard deviation.
[e]Accuracy is given in relative error % = [100 × (predicted concentration – nominal concentration)/nominal concentration.)].

Table 1: Results of accuracy and intermediate precision studies for the proposed method in rat plasma.

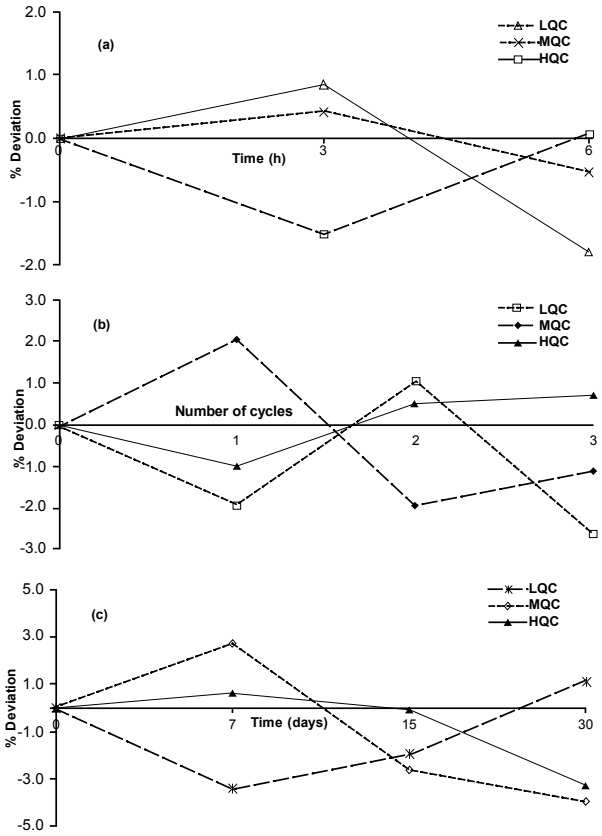

Figure 2: Stability study of RLZ in rat plasma (a) Bench top stability, (b) Freeze thaw stability and (c) Long term stability. LQC - 250 ng/mL, MQC - 750 ng/mL and HQC - 3000 ng/mL. Each point represents mean of five independent determinations.

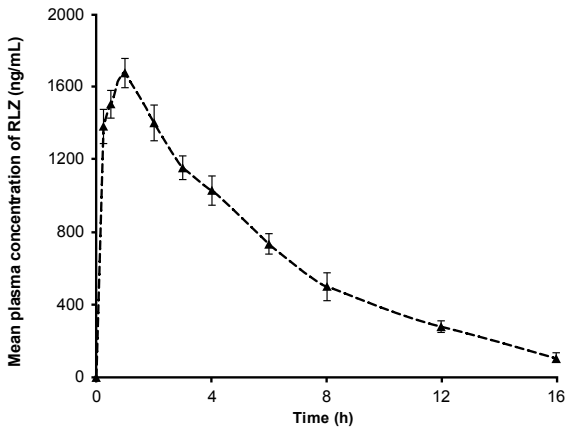

Figure 3: Mean plasma concentration–time profile of RLZ after single dose oral administration (10 mg/kg) of RLZ to Wistar rats. Each point represents the mean of five independent determinations.

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