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RESEARCH ARTICLE

Determination of Riluzole in Human Plasma by Ultra Performance Liquid Chromatography – Tandem Mass Spectrometry (UPLC – MS/MS) and its Application to a Pharmacokinetic Study

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ABSTRACT:
A rapid, sensitive and selective ultra-performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method was developed and validated for the estimation of Riluzole in human plasma using Olanzapine as an internal standard. Riluzole and Internal standard were extracted from 0.5 mL plasma by solid phase extraction method. The analytical separation was carried out in a reverse liquid chromatography by using C18 (50 x 4.6mm 1.8,) 10mMAmmonium Acetate: Methanol (10:90) v/v at 0.5 mL/min at isocratic mode . The detection was performed on a triple quadruple tandem mass spectrometer by multiple reactions monitoring (MRM) mode via electro spray ionization (ESI) source. Analytes were monitored in multiple reactions monitoring mode using the respective $[M+H]^+$ions, *m/z* 234.84/137.63 for Riluzole and *m/z* 313.15/256.14 for the internal standard, respectively. The proposed method was validated with linear range of 5 -1000ng/ml for Riluzole with a runtime 2.5 minutes. The %R.S.D of intra-day and inter-day assay was lower than 15%. For its sensitivity and reliability, the proposed method is particularly suitable for pharmacokinetic studies.

KEYWORDS: Riluzole, UPLC-MS/MS, human plasma, SPE; Bioanalysis.

INTRODUCTION:

Riluzole is a member of the benzothiazole class used to treat amyotrophic lateral sclerosis (Fig.1). It delays the onset of ventilator-dependence it preferentially blocks TTX-sensitive sodium channels, which are associated with damaged neurons.. Chemically, Riluzole is 2-amino-6-(trifluoromethoxy) benzothiazole. Its molecular formula is $C_8H_5F_3N_2OS$ with a molecular weight is 234.2.[1] Extensive literature[2-6] survey reveals there is no stability indicating UPLC method for quantitative determination.

Hence, an attempt has been made to develop and accurate, rapid, specific and reproducible method for the determination of Riluzole using UPLC Tandem mass spectrometry (UPLC – MS/MS) along with method validation as per ICH norms.The stability tests were also performed on both drug substances and drug product as per ICH norms. This paper describes an Ultra performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method, which enables quantitative determination of Riluzole with high speed and good accuracy at concentrations in human plasma as low as 5.00 ng/mL. The total run time of 2.5 min per sample was reported which promised the high throughput analysis of biological samples.

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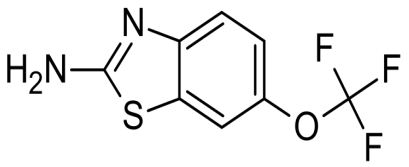

Fig.1 Structure of Riluzole

MATERIAL AND METHOD:

Chemicals and Reagents:

Riluzole (purity 99.5%), Olanzapine (purity 99.5%) were obtained from Orchid Chemicals and pharmaceuticals ltd. Methanol (HPLC grade, manufactured by J.T. Baker), Acetonitrile (HPLC grade, manufactured by J.T. Baker), Water (Milli Q water), Ammonium acetate (AR grade, manufactured by Merck India. ltd),Blank human plasma received from private blood bank.

Preparation of Standard and Quality Control Samples:

The stock solution of Riluzole, and Olanzapine internal standard was prepared by dissolving the accurately weighed reference compounds in Methanol to give a final concentration of 1 mg/mL, stored at 2-8°C in the refrigerator and is used for a maximum of 8 days. The solutions were then serially diluted with Methanol–water (50:50, v/v) to obtain standard working solutions separately. All the solutions were stored at 2-8°C and were brought to room temperature before use. Calibration solutions were prepared by spiking blank human plasma with standard solutions of each drug standard to give concentrations of 5.0, 10.0, 50.0, 100.0, 200.0, 400.0, 600.0, 800.0, and 1000.0 ng/ml. Quality control (QC) samples, which were used both in pre study validation and during each experimental run of the validation study, were prepared by spiking control human plasma with standard solutions of each drug standard solutions to give concentrations of 5.0,15.0,500.0 and 700.0 ng/ml.

Preparation of Plasma Samples for estimation:

To 500 µL of spiked plasma sample and 500 µL of water in a clean vial, 10µl of Internal solution was admixed and vortexed for 60 sec. The analytes were separated in OASIS HLB solid phase extraction cartridges using 1ml of Methanol and 2 mL of Water as eluent. Separated mixture was transferred and 20 µL of the supernatant was directly injected onto the UPLC/MS/MS system.

Instrumentation:

UPLC-and mass spectrometric conditions Acquity binary solvent manager and an Acquity sample Manager were used for solvent and sample delivery. Chromatographic separation was achieved by using Hypersil Gold C18 (50 x 4.6 mm, 5µm, and 20 µL at column temperature 40°C. The mobile phase consisted of 10 mM Ammonium Acetate buffer: Methanol (10:90) v/v pumped at a flow rate of 0.5mL/min. Total run time was 2.5 min for each injection. A Waters Micro mass Quattro premier mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode (ESI+) and set up in the Multiple reaction monitoring (MRM) mode. Nitrogen was used as desolvation gas (700L/Hr) and cone gas (50 L/Hr). The capillary temperature was 3.80kV. Cone voltage was 42 V. Argon was used as the collision gas and the collision energy used for Riluzole was 35 V and25V for Internal standard. Based on the full-scan mass spectra of the analytes, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as *m/z* 234.84/137.63 for Riluzole and *m/z* 313.15/256.14 for the internal standard. The scan time for each analyte was set to 0.1 s. Full-scan mass spectra of $[M^{+}H]^{+}$ of selected analytes and its respective product ion spectra are shown in Fig. 2-5. Data acquisition, Peak integration and calibration were performed with MassLynx 4.0 software.

Method Validation

The method was validated for Specificity, Accuracy, Precision, Matrix effect, sensitivity, bench Top stability, Auto sampler stability, Freeze thaw stability, Recovery, and Linearity according to the FDA guideline for Validation of bio analytical methods[8-10]. The Specificity was investigated by preparing and analyzing six individual human blank plasma samples at LLOQ level. The LLOQ was defined as the lowest concentration of the analyte measured with acceptable precision and accuracy [relative standard deviation (RSD) and relative error ≤20%, and the analytes response at this concentration level was NMT 5 times the baseline noise. Linearity was assessed by analyzing Analyte standards (5–1000 ng /ml) in human plasma. Calibration curves were analyzed by weighted linear regression (1/*x*) of assayed–nominal drug peak area ratios. Accuracy and precision were assessed by determining QC samples at three concentration levels (six samples each concentration) on three different validation days. The precision as determined as %RSD and the accuracy was expressed as a percentage of the nominal concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD should not exceed 15% and the accuracy should be within 85 - 115%. Furthermore, the recovery (extraction efficiency) of analyte from human plasma was determined by comparing the areas of spiked plasma samples before and after sample processing. The stability of analyte was assessed by determining QC samples at three concentrations (six samples each). The stability studies included: (a) stability at room temperature (22–25°C) for

6 h; (b) stability after two freeze–thaw cycles; (c) stability of the extracted samples at room temperature (22–25°C) for 12 h; and (d) the long-term stock solution stability at -20°C for 5 days. During routine analysis, each analytical run included blank plasma, blank plasma with internal standard, a set of calibration samples, a set of QC samples and unknowns.

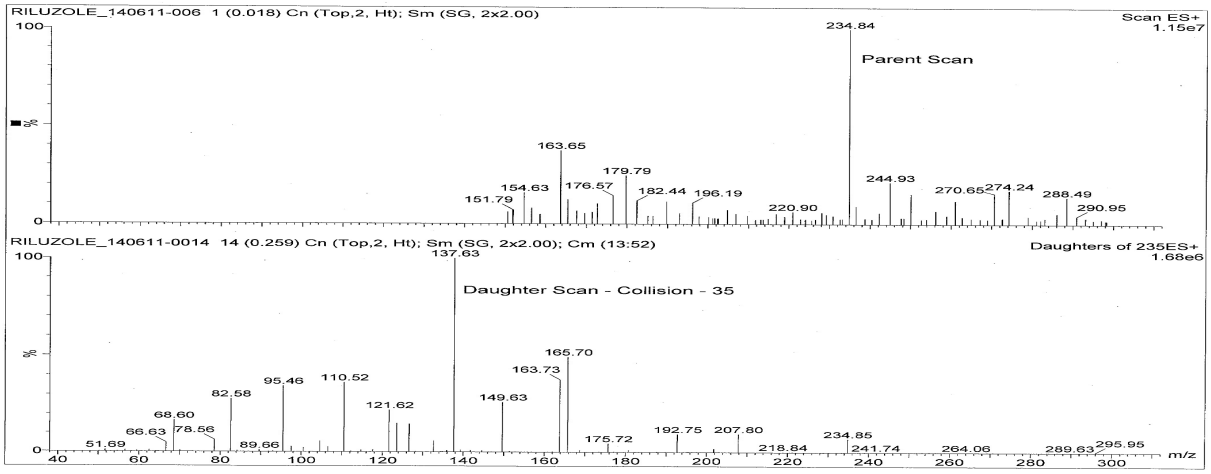

Fig.2

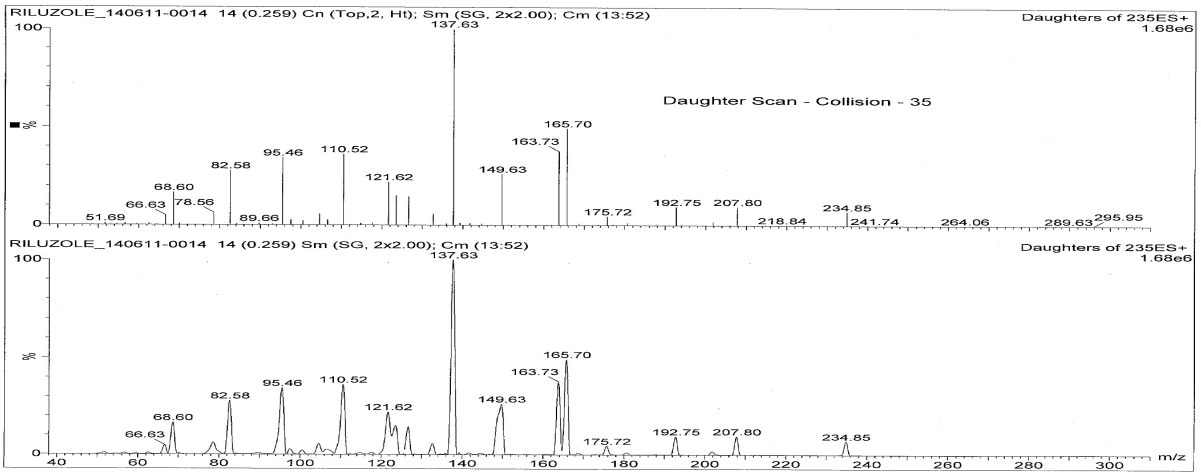

Fig.3

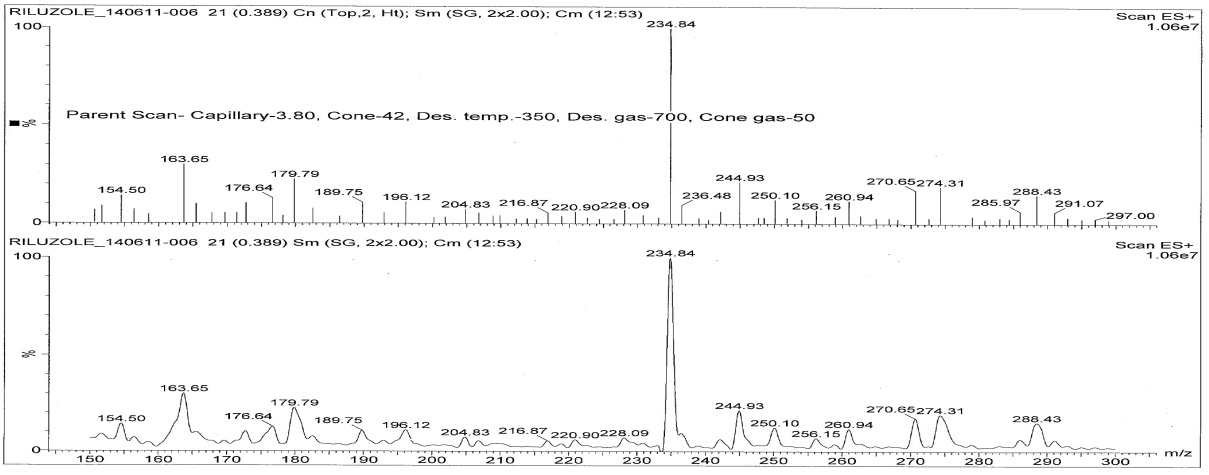

Fig.4

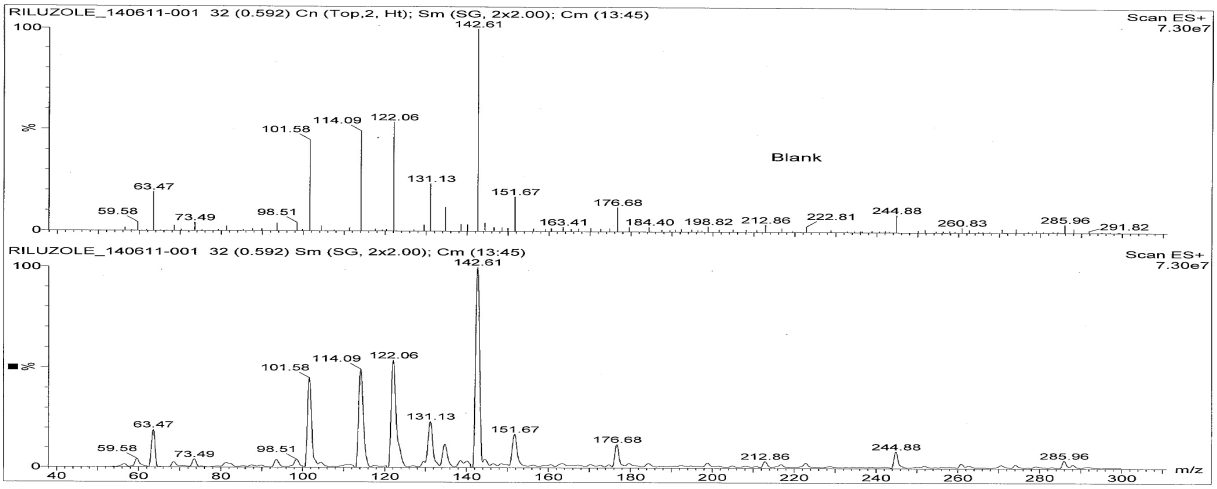

Fig.5

Fig 2-5. Full-scan mass spectra of $[M^+H]^+$ of selected analytes and its respective product ion spectra

RESULTS AND DISCUSSION:

Method Development:

In this study, ESI was chosen as the ionization source. It was found that the signal intensity of analytes in human plasma was high using ESI source and the ESI source provided satisfactory data on method validation and subsequent quantitation for plasma samples from healthy volunteers. By ESI, the analytes formed predominantly protonated molecules $[M^+H]^+$ ions in full-scan spectra. To determine glutamatergic antagonist and neuroprotective analytes using MRM mode, full-scan and product ion spectra of the analyte were investigated. The most abundant ion in the product ion mass spectrum was at 234.84 for Riluzole and 313.15 for the internal standard which is presented in Fig. 2-5. It was found that the capillary temperature and the spray voltage did not significantly influence the MS behaviour of the analyte and remained unchanged at the recommended value of 350°C and 3.8 kV. Therefore, the SRM transition of *m/z* [234.84/137.63 ,313.15/256.14] was selected to obtain maximum sensitivity.(Fig 6 and 7 represents typical chromatogram). In the present study, a simple Solid Phase Extraction technique was used. All selected analyte were not detectable with protein precipitation and inconsistent with liquid-liquid extraction during our method development. On the other hand, it was found that the extraction efficiency was increased when Solid phase Extraction (SPE) using Methanol solution as extraction solvent. A mobile phase consisting of 10 mM Ammonium Acetate buffer : Methanol (10:90) v/v) was finally used. Each chromatographic run was completed within 2.5 min.

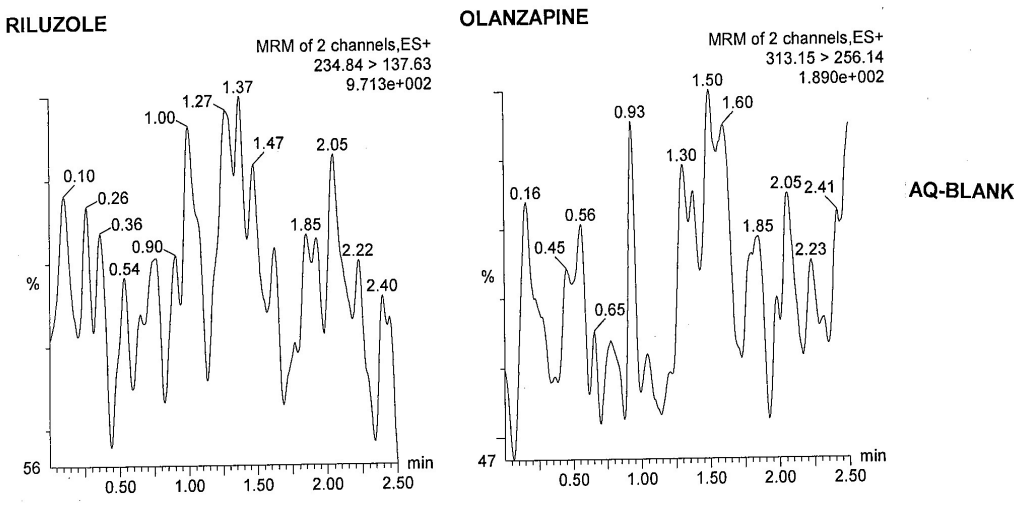

Fig 6. Blank sample of Riluzole and Olanzapine

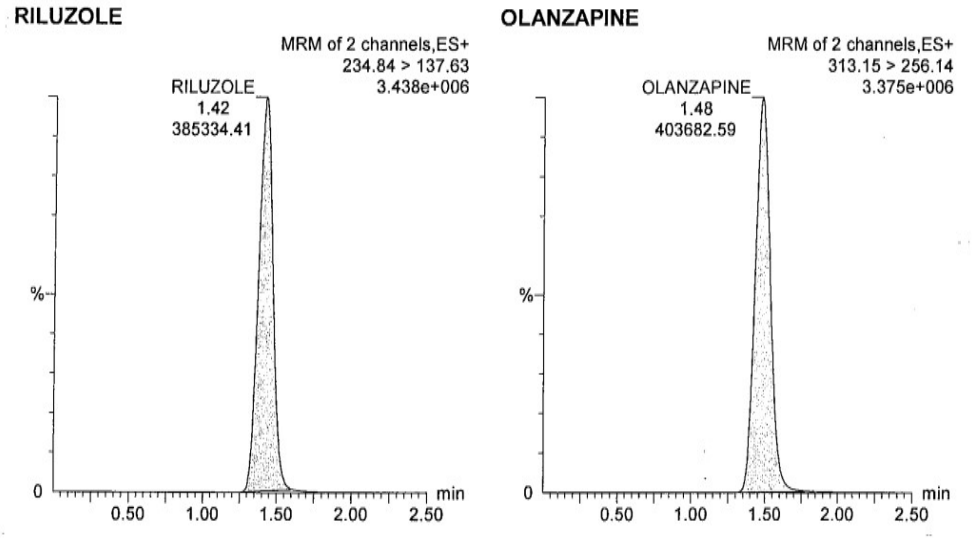

Fig 7. Typical Chromatogram of Riluzole and Olanzapine

METHOD VALIDATION:

Specificity:

The UPLC/MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity towards endogenous plasma matrix was tested in six different batches of human plasma samples by analyzing blanks and samples at LLOQ levels (Table 1 and 2). Observing the chromatographs indicated no significant visible interference at the expected retention times of the analyte since Riluzole was modified to elute in a region where visible interference is not observed. The method had the shortest total running time (2.5 min) for determination of Glutamatergic Antagonist, Neuroprotective drugs in human plasma compared with those reported in the literature[7] Matrix effects

Table -1 Specificity study for drug Riluzole

S.No	Name	Area in Blank plasma	Area of LLOQ	% of Drug Interference
1	Plasma sample – 1	0	0.000	NIL
2	Plasma sample – 2	0		NIL
3	Plasma sample – 3	0		NIL
4	Plasma sample – 4	0		NIL
5	Plasma sample – 5	0		NIL
6	Plasma sample – 6	0		NIL

Table -2 Specificity study for the internal standard Olanzapine

S.No	Plasma Lot ID	Area in Blank plasma	Area of LLOQ	% of IS Interference
1	Plasma sample – 1	0	0.000	NIL
2	Plasma sample – 2	0		NIL
3	Plasma sample – 3	0		NIL
4	Plasma sample – 4	0		NIL
5	Plasma sample – 5	0		NIL
6	Plasma sample – 6	0		NIL

To evaluate the absolute matrix effect, i.e. the potential ion suppression or enhancement due to the matrix components, six different batches of blank plasma were eluted by elute solution and then spiked with the analyte at QC concentrations. The corresponding peak areas of the analyte in spiked plasma post-extraction (*B*) were then compared with those of the aqueous standards in mobile phase (*A*) at equivalent concentrations. The ratio is defined as the ME(Matrix Effect). A ME value of 100% indicates that the response in the mobile phase and in the plasma extracts was the same and no absolute matrix effect was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The result of ME at QC concentrations of selected analytes in five different lots of human plasma shows that there was ME, as indicated by values of >100% in the area of the analyte in spiked plasma samples post-extraction. This indicated ionization enhancement for selected analytes under the present chromatographic and extraction conditions when ESI interface was employed. Fortunately, the ionization enhancement observed was similar and kept consistent over the QC concentration ranges of the analyte (5 – 700 ng/ml) without showing any analyte concentration-dependence as well as for different lots of human plasma. Moreover, such ionization did not affect the slopes and linearity of the established calibration curves over the whole analytical period. The assessment of the relative ME was made by a direct comparison of the analyte peak area values between different lots (sources) of plasma. The variability in the values, expressed as RSDs (%), is a measure of the relative ME for the target analyte. The variability was acceptable with an CV value of 2.0 % at different concentrations of Riluzole in five different lots of human plasma, indicating that the relative ME for the analyte was minimal in this study. In

the present study, an ionization enhancement effect due to the undetected matrix components in human plasma was observed. However, such ionization enhancement remained consistent over the QC concentration ranges of the analyte without showing any analyte concentration-dependence and did not significantly affect the behaviours of calibrations curves, precision and accuracy data. Thus, despite the presence of the ME, the present analytical method was reliable.

Linearity and Lower Limit of Quantification:

The slope, the intercept and the correlation coefficient (*r*) for each standard curve from each analytical run were determined automatically by Mass Lynx software programme. The representative standard curve for Riluzole was Y = 0.00661 , 18882.9*X. The mean squared correlation coefficients (r^2) for the daily calibration curves were all ≥0.998 (n=15) for riluzole and the within- and between-run CVs of the response factors for each concentration assayed were lessthan or equal to10%. For each point on the calibration curves for the analyte, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ±15%. Overall, Riluzole drug gave linear response as a function of the concentration ranges studied and showed excellent linearity over 5 - 1000 ng/ml (Table -3). The lowest concentration on the calibration curve was 5.00 ng/ml. The analyte response at these concentration levels was >20 times the baseline noise. The precision and accuracy at these concentration levels were acceptable and within the acceptance criteria. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. However, the LLOQ could be lowered by injecting a more concentrated solution into the UPLC/MS/MS system. However, the current LLOQ (an LLOQ of 5.0 ng/ml was achieved for 0.5 mL samples) was already sufficient for the estimation of Riluzole in human plasma.

Table – 3 Linearity study of Riluzole

Cali. Std No	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9	Intercept	Slope	r
Nominal Conc (ng/ml)	5.0	10.0	50.0	100.0	200.0	400.0	600.0	800.0	1000.0			
PL-1	4.8	10.0	57.2	101.7	208.2	423.6	605.9	766.5	987.2	0.00177	0.00197	0.999
PL-2	4.9	10.4	53.5	104.8	211.8	440.1	606.2	749.4	984.0	0.00248	0.00213	0.998
PL-3	4.7	10.3	56.5	103.3	207.2	436.0	611.9	749.4	985.7	0.00236	0.00220	0.998
Mean	4.8	10.2	55.7	103.2	209.0	433.2	608.0	755.1	985.6	**NA**	**NA**	**NA**
±SD	0.1	0.2	2.0	1.6	2.4	8.6	3.4	9.8	1.6			
%CV	1.9	2.3	3.6	1.5	1.2	2.0	0.6	1.3	0.2			
%Nominal	96.6	102.5	111.5	103.2	104.5	108.3	101.3	94.4	98.6			

Table – 4 Precision and Accuracy study of Riluzole

Parameter	Added conc (ng/mL)	Found Conc (ng/mL)	Intra Run (% CV)	Inter Run (% CV)	Accuracy (% RE)
LLOQ	5.000	4.917 ± 0.239	5.5	5.8	1.3
Low QC	15.000	15.315 ± 0.684	3.5	3.6	2.6
Mid QC	500.000	524.646 ± 19.974	2.4	3.8	1.6
High QC	700.000	716.385 ± 14.204	2.4	3.6	1.2

Precision and Accuracy:

The intra-batch and inter batch precision and accuracy data for selected drugs are summarized in Table - 4. All values of accuracy and precision were within recommended limits (FDA, guidance) [9]. The intra-batch precision for Riluzole was % CV is 2.4 to 5.5 and accuracy was 95877 to 104.423 %.The inter-batch precisionand Accuracy for Riluzole was % CV is 3.6 to 5.8 and 98.661 to 102.642% respectively.

Recovery:

Table – 5 shows the recovery (extraction efficiency) of Riluzole drug from human plasma. The mean recovery as 82.043% to 87.993% for Riluzole at all different concentrations, which indicated that the extraction efficiency using extraction solvent used is satisfactory.

Table – 5 Summary of Recovery Study

Analyte name	Riluzole		
Analyte concentration level	LQC	MQC	HQC
%Mean recovery	82.043	82.374	87.993

Stability:

The stability of Riluzole in human plasma under different storage conditions are presented in Table – 6 . No degradation products were detected under the selected MS conditions. Hence Riluzole human plasma can therefore be stored at room temperature (25°C) for 6 h, 8 days at 2 to 8°C and after two freeze–thaw cycles.These results indicate that selected analytes are stable under routine laboratory conditions and no specific procedure (e.g. acidification or addition of organic solvents) is needed to stabilize the compounds for daily clinical drug monitoring.

Table –6 Riluzole stability data

S. No	Comparison Samples		Stability Samples	
	LQC	HQC	LQC	HQC
Nominal Conc.(ng/ml)	15.000	700.000	15.000	700.000
1	15.120	732.914	15.847	736.601
2	16.040	722.395	15.347	732.076
3	15.929	721.901	14.895	741.199
4	15.713	714.658	15.839	727.827
5	15.448	688.265	15.632	701.219
6	16.034	711.382	16.041	732.095
Mean	15.714	715.253	15.600	728.503
±SD	0.368	15.171	0.418	14.123
%CV	2.344	2.121	2.681	1.939
%Mean Stability			99.276	101.853

CONCLUSIONS:

In this study, we reported on a newly developed UPLC/ MS/MS method for the determination of Riluzole in human plasma. The sample pre-treatment was easy and extraction efficiency was more. The selected analyte was subjected to UPLC/ MS/MS analysis using ESI technique with satisfactory mass spectral response generated. Detailed validation following FDA guideline indicated that the developed method had high sensitivity, reliability, specificity and excellent efficiency with a total running time of 2.5 min per sample. The method was successfully applied to pharmacokinetic studies of Riluzole estimation human plasma.

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