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Research Article

Pharmaceutical analysis for Novel drug formulation

Application Of Liquid Chromatography With Tandem Mass Spectrometric Method For Quantification Of Safinamide In *Invitro* Samples

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Abstract: Safinamide is a novel drug with both dopaminergic and non-dopaminergic effects, approved first by the European Commission and more recently by the US Food and Drug Administration (FDA) as an adjunctive treatment to carbidopa/levodopa in patients with mid- to late-stage Parkinson's disease (PD) and motor fluctuations. The validated analytical method was applied for the estimation of Safinamide in aqueous solution and human plasma with Safinamide-D4 as an internal standard by using UPLC-MS/MS. The chromatographic separation was achieved with 0.1% formic acid solution: Methanol, 30:70% v/v using CORTECS C18 with 100 x 4.6, 2.7 µ. The total analysis time was 4 min, and the flow rate set to 0.8 mL/min. The mass transitions of Safinamide and Safinamide-D4 obtained were m/z 303.3 and 215.2 and 307.3 and 215.2. The standard curve shows a correlation coefficient (r2) greater than 0.998 with a range of 113.0-338.0 pg/mL using the linear regression model. The method has shown good reproducibility, as intra- and interday precisions were within 10% and accuracies were within 8% of nominal values for both analytes. The method was successfully applied for the forced degradation studies at various stress conditions.

Keywords: Safinamide; human plasma; aqueous; bioanalysis; method validation.

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1. INTRODUCTION

Safinamide is a new neuroprotectant with antiparkinsonian and anticonvulsant activity for the treatment of Parkinson disease, and is a novel sodium and calcium channel blocker endowed with selective and reversible inhibition of monoamine oxidase type B (MAO-B). Chemically, Safinamide is (S)-(+)-2[4-(3-fluorobenzyloxybenzylamino) propanamide] methanesulfonate (1:1 salt) [1,2]. The chemical structure of Safinamide and Safinamide-D_4 shown in Figure 1.

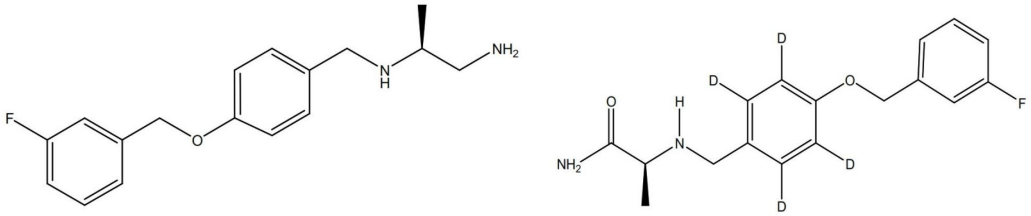

Fig 1. Chemical Structures of A) Safinamide B) Safinamide-D_4

As we know, due to the high selectivity and sensitivity of liquid chromatography tandem mass spectrometry (LC–MS/MS) method, it has been proved to be one of the most powerful tools for the determination of trace amounts of drugs and also it is essential that, an internal standard was preferred when performing analysis with mass spectrometry detection. An appropriate internal standard will give a measure of control for extraction, HPLC injection and ionization variability. It is an essential component for a high throughput analytical method[3,4].Although few analytical methods have been reported for the determination of Safinamide, but the sensitivity of published HPLC, HPTLC, LC-MS/MS methods are inadequate for stability studies, pharmacokinetic studies and therapeutic drug monitoring. HPLC and HPTLC methods[5-6] been reported with increased recovery by sample extraction, but those are time-consuming process with lack of sensitivity. Although the bioassay of Safinamide in biological fluids of humans and various animals have been reported by LC-MS [7-12] , the sensitivity was improved, but these methods possess lack of recovery. The developed method has numerous advantages over other existing methods. The pros of the developed method include the following: less aliquot volume and simple sample treatment with short elution time compared to methods developed by LC-MS/MS and better sensitivity compared to methods developed by HPLC and HPTLC. To the best of our knowledge for accurate estimation of Safinamide, till now there is no publication regarding the quantification of Safinamide using deuterated internal standard (Safinamide-D_4) has been described by UPLC–MS/MS. Therefore, considering the advantages of LC-MS/MS, the development of a UPLC-MS/MS method for the analysis of Safinamide is considered an important contribution to support the upcoming drug products for stability studies, clinical pharmacokinetic or drug-drug interaction studies in bio-samples. The aim of this study is to develop an effective, simple and rapid UPLC-MS/MS method for quantification of Safinamide and validated according to the International Conference on Harmonization (ICH) guidelines (Q2 (R1))[13-14].

2. MATERIALS AND METHODS

2.1 Materials

Safinamide (ALSACHIM), safinamide-D4 (ALSACHIM), HPLC grade methanol, acetonitrile (J.T.Baker, Phillipsburg, NJ, USA), formic acid (Merck Pvt. Ltd, Worli, Mumbai), ultrapure water (Milli - Q system, Millipore, Bedford, MA, USA), human plasma (Doctors Pathological Labs). The chemicals and solvents are used in this study analytical and HPLC grade.

2.2 Preparation of standard solution and internal standard solutions

The standard stock solution of Safinamide (1.0 mg/mL) and Safinamide-D_4 (1.0 mg/mL) were prepared in Methanol. Standard solutions of different concentrations of Safinamide were prepared from Safinamide stock solution (1000 µg/mL) in the mobile phase of 0.1 % Formic Acid solution: Methanol, 30:70% v/v. To each aqueous standard solution, 100 µL of 75.00 pg/mL of Safinamide-D_4 was added and vortexed for 5 min. Then the sample was injected into the UPLC-MS/MS for analysis. Therefore, Safinamide-D_4 was selected as an internal standard. For the selection of internal standard; Carbidopa, Levodopa, Pramipexole,Ropinirole were tried with optimized mobile phase and column conditions. The Safinamide-D_4 (internal standard) spiking solution (75.0 pg/mL) was prepared from standard stock solution of Safinamide-D_4 (1000.0 µg/mL) in mobile phase (0.1 % Formic Acid solution: Methanol, 30:70% v/v).

2.3 Instrumentation

Chromatographic separation was performed on a QSight Triple Quad UPLC-ESI-MS/MS system (Perkin Elmer) Combined with QSight LX50 UHPLC, data acquisition and processing were accomplished using Simplicity™ 3Q software. The chromatographic separation was achieved with 0.1 % Formic acid solution: Methanol 30:70% v/v using the CORTECS C_{18} with 100 x 4.6, 2.7 µ since the best peak shape and low baseline noise was observed. This could be due to lower carbon loading of the column and enabling the selectivity by base material of the column. Whereas, lower carbon load reduced retention time of analyte and increased high through put. In addition, mobile phase with high organic solvent ratio increased the ionization efficiency. The absence of co-eluting peaks as well as interference from matrix ions was minimal with optimize column and mobile phase. . The peak elution times for Safinamide and Safinamide-D_4 were found as 2.22±0.01 min.

2.4 Method application to Bioanalysis of Safinamide in plasma spiked samples (Plasma Sample Extraction and Cleanup Procedure)

Various organic solvents were optimized to extract Safinamide and Safinamide-D_4 from the spiked plasma

samples. After a series of trials, acetonitrile was selected as appropriate due to high recovery efficiency and matrix-free interference. To each labelled polypropylene tube, 100 µL of internal standard (Safinamide-D_4; 75.00 pg/mL) was mixed with the 100 µL screened plasma spiked sample and 3.0 mL of acetonitrile was added, vortexed for 10 min and centrifuged at 55000 rpm for 15 min at 25 °C. Finally, supernatent liquid was transferred to a clean polypropylene tube and evaported with the nitrogen gas at 50 °C and residue was reconstituted with 100 µL mobile phase, injected into the UPLC-MS/MS. Inconsistency in peak area response of Safinamide and Safinamide-$D_4$was observed during analysis of extracted samples. This could be due to the low solubility of Safinamide and Safinamide-D_4in the buffer solution that was finalized during chromatographic optimization. Low solubility of Safinamide and Safinamide-D_4could be due to the high hydrophobic nature of these compounds, which lead to suppressed Safinamide and Safinamide-D_4peak area response in the extracted samples. Hence, mobile phase was ideal for Safinamide andSafinamide-D_4.

3. STATISTICAL ANALYSIS

In vitro results were expressed as mean ± SD of at least six replicates. The HPLC-MS results of analytes were calculated using linear regression without weighting, according to the equation: Y = mx+c, where Y is the area under the peak (AUP) ratio of the drug and X is the concentration of Internal standard. The % RSD was calculated for all values. Student's *t*-test was used to inspect the concentration difference at each day and one-way analysis of variance (ANOVA) was used to assess the reproducibility of the assay using Graph Pad Prism-8 software. The level of confidence was 95%.

4. RESULTS AND DISCUSSION

The developed method was validated according to the recommendations of the International Conference on Harmonization guideline Q2R1[14]. Specificity, linearity, accuracy, precision, detection & quantitation limits and robustness of the developed method were investigated. Besides, the stability of analyte was shown. In Selectivity parameter, no significant response was observed at retention times of Safinamide and Safinamide-D4 in mobile phase and blank plasma samples. The linearity of the calibration curve for Safinamide was assessed at 50 % to 150 % of the target concentration and the results demonstrated good linearity. Precision of the method was tested using a standard solution with concentrations of 169, 225 and 293 pg/mL. These data exhibited that the present analytical method was precise and accurate. Detection and quantification limits calculated were found this method is sensible for the estimation of Safinamide in pharmaceutical formulations. The robustness of the analytical method was tested by evaluating the influence of minor modifications in HPLC conditions and the results were within the acceptable limits (RSD less than 2.0 %). Stability of the method was assessed by considering stock solution stability for Safinamide and Safinamide-D4. The analytes were stable under all studied conditions (Ambient and Refrigerated conditions), since the % difference values were lesser than 2%. Percentage recovery for the three different concentrations of Safinamide was 98.58 %, 98.28 % and 99.36 % respectively. The overall mean % recovery and % RSD of Safinamide across three concentration levels were 99.07 % and 1.15 % accordingly. For the Safinamide-D4 (internal standard) the mean % recovery and % RSD was 99.55 % and 0.87 % respectively. These results illustrated that the developed method had high recovery and no matrix effect under the tested conditions. Under -30 °C storage conditions upto 20 days, the stability of Safinamide in plasma samples was investigated. From the results, it was found that the analyte was stable.

4.1 Method development

The mass parameters of analyte & internal standard optimized showed the abundant intensity of ions. The MS optimization was performed by direct injection of Safinamide and Safinamide-D_4 into the mass spectrometer. The mass parameters were optimized to obtain better ionization of Safinamide and Safinamide-D4 molecules. The full scan mass spectrum dominated by protonated molecules [M+H] m/z 303.3 and 307.3 for Safinamide and Safinamide-D_4 and the major fragment ions observed in each product spectrum were at m/z 215.2 and 215.2, respectively. The mass spectra of parent and productions are shown in Figure 2.

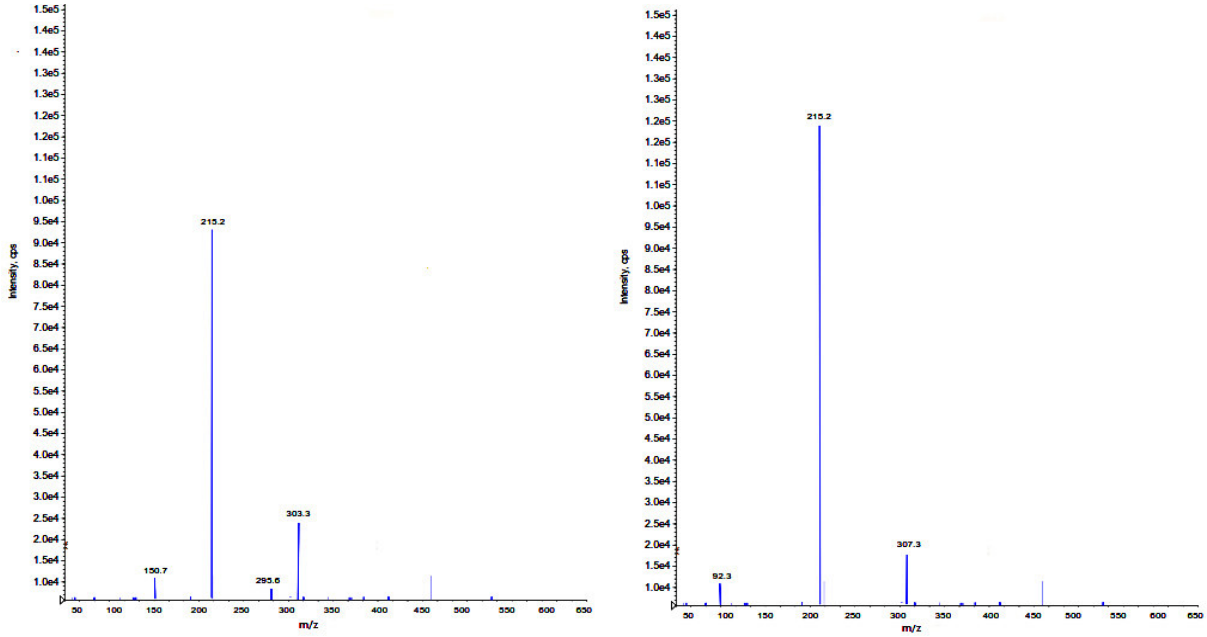

Fig 2. Parent ion mass spectra (Q1) and (Q3) of Safinamide & Safinamide -D_4

4.2 Method validation

4.2.1 System suitability

System suitability was done with respect to injection repeatability (relative standard deviation of retention time and peak area response) using six replicate injections of aqueous standard solutions of 100 % level (225.0 pg/mL) along with internal standard (75.0 pg/mL) were injected into UPLC-MS/MS and % RSD was calculated

4.2.2 Selectivity

Selectivity of the method was assessed by checking that no interference peaks were found at the retention times of Safinamide and Safinamide-D_4 with mobile phase and blank plasma sample solutions. For this, chromatogram of standard solution (225.0 pg/mL) and blank samples (mobile phase and blank plasma) are compared. In Figure 3, no peak was observed at the retention times of Safinamide and Safinamide-$D_{4\ in}$ the chromatogram of blank samples. Thus, the method was proved selective.

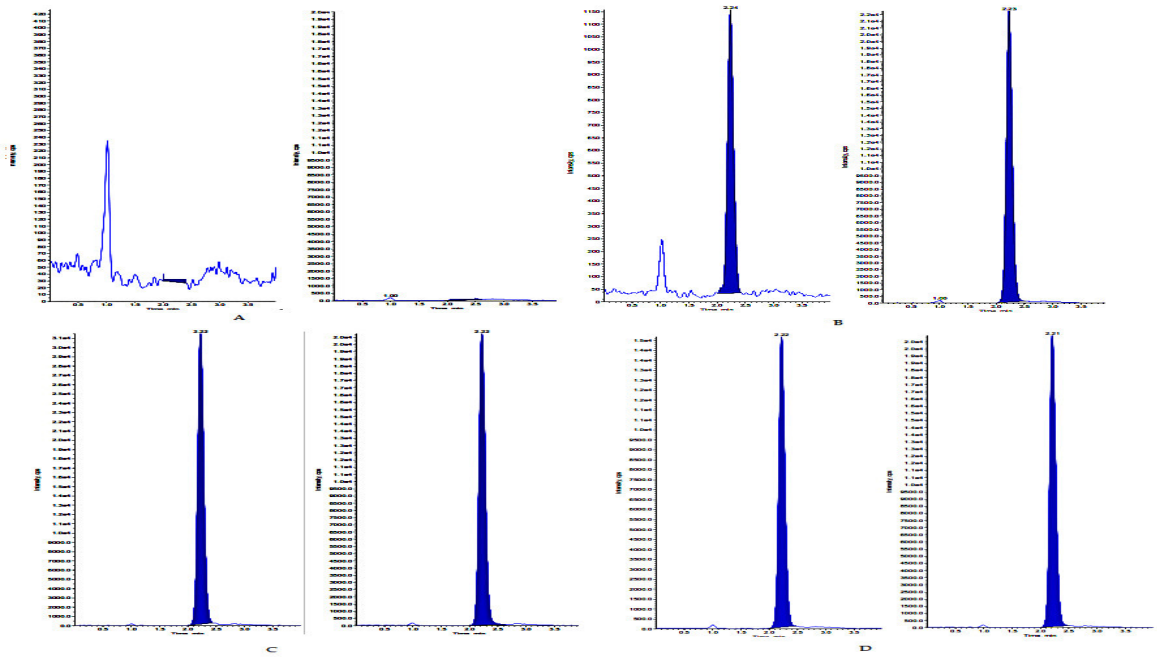

Fig 3.Chromatograms of Safinamide& Safinamide-D_4 (A): Mobile Phase and Blank plasma (B): Standard solution (containing 113.0 pg/mL) (C): Standard solution (containing 338.0 pg/mL) (d): Plasma spiked Standard sample.

4.2.3 *Linearity and Range*

In order to investigate the linearity of the method, the calibration curves were constructed using different concentrations ranging from 113.0 to 338.0 pg/mL. Linearity graph plotted as a peak area ratio (Safinamide peak area / Safinamide-D_4 peak area) on the Y-axis against Safinamide concentration (pg/ml) on the X-axis. The method was linear in the range of 113.0 to 338.0 pg/mL (Table 1 and Figure 4). The regression equation obtained by least squares regression was, y = 0.00296x + 0.00621 for safinamide, where 'y' indicates the peak area ratio of the analyte to its internal standard and 'x' indicates the concentration of the analyte. Linearity was found to be quite satisfactory and reproducible.

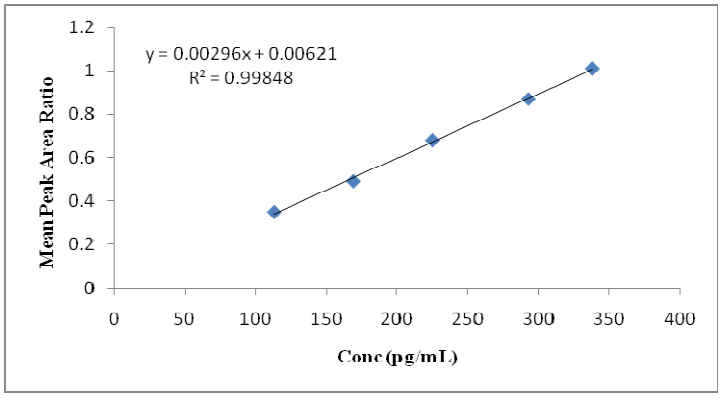

Fig 4. Calibration curve for Safinamide

Table 1. Calibration curve details of Safinamide

Linearity Level (%)	Nominal Conc. (pg/mL)	Safinamide Mean Peak Area (n=3)	Safinamide-D_4 Mean Peak Area (n=3)	Mean Peak Area Ratio (n=3)
50	113.00	17412	51264	0.35
75	169.00	26117	51134	0.49
100	225.00	34823	52124	0.68
130	293.00	45270	51454	0.87
150	338.00	52235	51203	1.01
			Correlation coefficient	**0.9993**
			Y-Intercept	**0.002243**
			Slope	**0.00298**
			Standard Error	**0.01067**

4.2.4 *Precision*

The precision of the method was determined by calculating the coefficient of variation (% CV) for each concentration level (169, 225 and 293 pg/mL). Intra-day precision was tested using a standard solution with concentration 169, 225 and 293 pg/mL. Data summarized in Table 2 revealed satisfactory values for intra-day precision. The % RSD and accuracy were found to be 0.33 to 0.52 and 99.93 to 100.17 % for intra-day precision. For intermediate precision, % RSD and accuracy was found to be 0.26 to 0.58 and 99.61 to 99.94 %. Mean and the percent relative standard deviation values are summarized in Table 2.

Table 2. Precision and accuracy of Safinamide at three different concentrations

(pg/mL)	*Mean Concentration measured (n=10;pg/mL; Mean±S.D)	% RSD	% Accuracy	**Mean Concentration measured (n=30;pg/mL; Mean±S.D)	% RSD	% Accuracy
169.00	169.30±0.83	0.49	100.17	168.76±0.45	0.26	99.86
225.00	224.85±1.16	0.52	99.93	224.85±1.30	0.58	99.94
293.00	293.08±0.97	0.33	100.03	291.86±1.22	0.42	99.61

**** Within-run (Intra-day); ** Between-run (Inter-Day)***

4.2.5 *Robustness*

Robustness was carried out by varying the method parameters like flow rate (±5 %) and column temperature (± 5%) was made to evaluate the impact on the system suitability parameters of the developed method. Replicate injections of aqueous standard solutions of 225.0 pg/mL along with internal standard was injected into UPLC-MS/MS and % RSD was calculated. Results are presented in Table 3.

Table 3. Robustness results of Safinamide and Safinamide-D_4

Parameter	Flow Rate (+10 %) (n=5)	Flow Rate (-10 %) (n=5)	Column Temp High (at+5 °C) (n=5)	Column Temp Low (at-5 °C) (n=5)
Mean	225.16	224.28	224.44	225.39
SD	0.36	1.18	2.22	0.28
% RSD	0.16	0.53	0.99	0.12
Mean %RSD	0.34		0.56	

4.2.6 Limit of detection and limit of quantitation

The limit of detection was calculated as 11.83 pg/mL and the limit of quantitation was calculated as 35.84 pg/mL. These values indicated that the method was sensitive to quantify the drug in biological samples.

4.2.7 Stability

The storage conditions of samples need to maintain the integrity of a drug. A safinamide at a concentration of 225.0 pg/mL solution and safinamide -D4 (Internal standard) solution at 75.0 pg/ml were prepared from fresh stock solutions. A portion of the freshly prepared standard solutions (Safinamide and Safinamide -D4) were kept at ambient temperature (25°C) for 24 hours and then analyzed by the proposed method. A second portion of the freshly prepared standard solutions (Safinamide and Safinamide -D4) were stored at refrigerated temperature (between 2°C and 8°C) for 24 hours and then analyzed. The results were compared with those obtained from samples analyzed at initial moment (0.0hours). The analytes were stable under all conditions according to calculated results seen in Table 4.

Table 4. Solution stability data of Safinamide and Safinamide-D_4

Stability Sample	* % Difference at 0.0 Hours	* % Difference at 24.0 Hours	** % Difference at 0.0 Hours	** % Difference at 24.0 Hours
Safinamide (225.0 pg/mL)	0.0001	-0.1737	0.0001	-0.0280
Safinamide–D_4 (75.0 pg/mL)	0.0001	0.5897	0.0001	0.2052

**** Ambient temperature; ** Refrigerated Temperature***

4.2.8 Extraction efficiency (Recovery)

The extraction recovery of analyte (Safinamide) at three (113, 225, 338 pg/mL) different concentrations were determined by measuring the peak area responses from screened plasma samples spiked with Safinamide (Extracted samples) with those Aqueous standard samples (Un-extracted samples). The samples were extracted as per sample extraction and clean-up method. The recovery of internal standard (Safinamide-D_4) at concentration of 75.0 pg/mL was determined in the same way. The recovery of Safinamide and Safinamide-D_4 were determined using six replicates. The recovery and matrix effect data are summarized in Table 5.

Table 5. Recovery of Safinamide andSafinamide-D_4 in spiked plasma samples

Concentration (pg/mL)	Safinamide				Safinamide-D_4			
	% Recovery	% RSD	Mean % Recovery (n=18)	Mean % RSD (n=18)	% Recovery	% RSD	Mean % Recovery (n=18)	Mean % RSD (n=18)
113.00	98.58	1.74	99.07	1.15	100.33	0.80	99.55	0.87
225.00	98.28	0.96			103.31	1.00		
338.00	99.36	0.75			95.02	0.81		

4.2.9 Long-term Stability (-30°C, 20 Days)

Drug (Safinamide**)** spiked plasma samples with a concentration of 225.0 pg/mL (LT Stability samples) of six replicates were stored at -30 °C freezer up to 20 days. Stability of safinamide in plasma samples (at -30 °C upto 20 days) were assessed by comparing with freshly prepared drug (Safinamide**)** spiked plasma samples (Comparison samples) with concentration of 225.0 pg/mL (Long term Comparison samples) of six replicates. The results were reported in terms of % RSD represented in Table 6.

Table 6.Long-term Stability (-30 °C, 20 Days) of safinamide in plasma samples

Stability sample	% RSD	
	Comparison samples (0 days)	LT Stability samples (20 days)
Safinamide (225.00 pg/mL)	1.38	1.13

5. CONCLUSION

In summary, for the first time, the proposed method has been developed and validated for the measurement of Safinamide by UPLC-MS/MS using Safinamide-D_4 as an internal standard. The method showed precise recovery for both Safinamide and internal standard. The high structural selectivity of HPLC-MS seems therefore required for comprehensive and effective studies of safinamide formulations. Actually, HPLC-MS seems to be essential for bio-analytical studies, particularly for new drugs and formulations, as well as for method development.

6. ACKNOWLEDGEMENT

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7. AUTHORS CONTRIBUTION STATEMENT

Dr.B.R.Challa and Dr.P.Srinivasa Babu designed and supervised the study; Mr. T.Mohan Rao performed the experiment and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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