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Development and Validation of Reverse-phase High-performance Liquid Chromatography Method for Novel Synthetic Pyridine Derivative

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ABSTRACT

The study was focused toward synthesis, characterization and quantification of Pyridine derivative by Reverse Phase High Performance Liquid Chromatography method. The synthesis of novel pyridine derivative was carried out by condensation reaction in presence of inert catalyst K_2CO_3 and THF solvent. Characterization was done by I.R, 1H-NMR,and HPLC. Based on the spectral data, the structure of novel pyridine derivative was characterized as 5 - (4 - Substituted amino) - 3- Nitrobenzene - 1 - Sulfonyl) - 4, 5, 6, 7 – Tetrahydrothieno[3, 2 - C] Pyridine The method validation including precision, accuracy, LOD, robustness, Linearity, and range was developed as per ICH guideline through an efficient isocratic RP-HPLC.It was determined that the above procedure is specified, accurate, precise, robust, and sturdy.

Keywords : RP-HPLC, Pyridine Derivative, Method Development, Validation

I. INTRODUCTION

The contaminant is defined in the biomedical industry as any additional organic molecule, other than the medicinal compounds or factors, which originates from synthesis or undesirable compounds which persist with Active Pharmaceutical Ingredients (APIs).The contaminant might arise mostly during the formulation phase or after the APIs and compositions have aged. Medication analysis is critical in drug discovery, manufacturing, and clinical usage. Contaminants in small quantities are unavoidable in drug material or dosage form. As a result, their level must be maintained and controlled. They either enhance or reduce the therapeutic properties of the Active Pharmaceutical Ingredient.[1]The HPLC technique is widely used to predict pharmaceutical's unadulterated and dose forms because it is easy, accurate, and repeatable. The International Conference on Harmonization (ICH) defines an impurity characteristic of a drug material as "a representation of the recognized and undiscovered impurities presents in a novel medicinal substance" Contaminants are described in pharmaceutical drugs as "substances inside the product which are not the API itself as well as the

active ingredient utilized to make it. "Contaminants are identified using a number of chromatographic and spectroscopic approaches, potentially alone or in conjunction with other techniques. [2-4]

Many of the most common heterocyclic compound obtained from natural goods, medicines, including conducting polymers hasthe **Pyridine backbone**.[5]Most effective methods of synthesizing such heterocycles depend upon amine-carbonyl compound condensation or cycloaddition processes.[6]The present study is centralized on synthetic approach of pyridine derivative and its identification as well as validation using RP-HPLC technique. There are multiple methodologies for identifying and analyzing contaminants, such as TLC, HPTLC, and HPLC.Among others, HPLC has indeed been extensively used throughout the field of contamination monitoring because of the large choice of detectors and stationary phases, as well as its sensitivity and cost-effective separation, have contributed to its many uses.

II. MATERIALS AND METHODS

Materials reagents and chemicals:

4,5,6,7-Tetra hydrothieno [3,2-c] pyridine hydrochloride, THF, 4-chloro-3-nitro benzene sulfonyl chloride, K_2CO_3Acetonitrile (HPLC Grade),Trifluoroacetic acid, and, DMSO were from Merck Chemicals Pvt. Ltd.Melting points were determined by open capillary method. The NMR spectra were recorded on Bruker model NMR.IR spectra were recordedon Shimadzu FTIR-8400S spectrophotometer using KBr discmethod. Method validation was performed on Agilent HPLC model.

Synthetic approach:

In a round bottom flaskTransfer 1.0 mL (5-4-chloro-3-nitrobenzene-1-sulfonyl) -4,5,6,7-tetrahydrothieno [3,2-C] pyridine was mixed with DMF, K2CO3, and 2.0mole morpholin and allowed to sit at room temperature for 45 minutes. The reaction mixture was agitated at 75C for 3-4 hours at reflux temperature before the solvent was evaporated under reduced pressure. Because the pH of the combination was higher than 7.0, diluted HCl was added to lower the pH to 6.5 and the reaction mixture was stirred for another 2 hours at room temperature. Then, to the resulting residue, add Water and stir for 1 hour at room temperature. The solid was filtered away, then the mixture was triturated with ethanol and strained to obtain the product.The solid was dried under reduced pressure. Yield: 68 %, M.P 185 ^{0}C.in Figure 01.

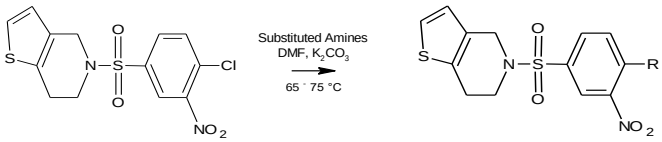


Figure 01 : Synthesis scheme of 5 –[4- (morpholin-4-yl)- 3- nitrobenzene - 1 – Sulfonyl] - 4, 5, 6, 7 – Tetrahydrothieno [3, 2 - C] Pyridine.

Characterization of synthesized compound:

Advanced Quantitative Methodologies such as I.R spectra, 1H-NMR, 13C-NMR, and Mass spectrometry were used to describe the synthesized compound. The functional group characterization was carried out on SHIMADZU FTIR 8400 Spectrophotometer withFrequency range of 4000-400 cm^{-1} through KBr disc method. NMR data were recorded for 1H NMR with deuterated solvent and 13C NMR.

HPLC Method development:

To create a rapid HPLC method for quantifying drug substances with the maximum selectivity, precision, and accuracy different ratio of mobile phases were used. Agilent's chromatographic equipment was utilized to design and validate this method, and it included a Sartorius digital balance and a Mettler Toledo branded balancer, as well as an oven for weighing and heating, and a Spinco ultrasonic bath for degassing and sonication.

Preparation of standard solution:

In 25ml volumetric flask 50mg standard is transferred after accurately weighed and made up the volume up to the mark with diluent (water:Acetonitrile - 30:70) and mixed well. 1.3 ml solution from the stock solution were taken and made up to 50ml with mobile phase.

Preparation of Sample Solution:

The sample solution was prepared by dissolving accurately weighted 50mg standard active pharma ingredient (API) of synthesis drug in 25ml volumetric flask up to the mark using diluent (water:Acetonitrile - 30:70).

Chromatographic conditions:

The separation was done through J'Sphere ODS-H80 (150 mm x 4.6 mm id, 5µ particle size) column with gradient mode using two different mobile phases. The gradient system was set using 0.1% Trifluoroacetic acid as mobile phase -A and Acetonitrile as mobile phase- B in different proportion. The column flow rate and temperature were set correspondingly at 1.0 mL/minute and 25 °C.The chromatogram was obtained by measuring the UV absorbance of the eluted analytes with a diode array detector at 220 nm. The injection volume of the auto-sampler was set 20µL and the run time was 60 minutes. The gradient program of system was shown in table 01.

Table 01: The gradient program of RP-HPLC system

Time	Mobile Phase-A	Mobile Phase-B
0	55	45
5	55	45
15	45	55
25	40	60
30	35	65
35	15	85
40	55	45
45	55	45

Method Validation:

Existing analytical methods must be tested in accordance with the ICH criteria to demonstrate that the proposed procedures are suitable for quality management.The proposed methodologies were validated for linearity, quantification limit, detection limit, accuracy, and precision. [7-14]

(i) System Suitability:

The system suitability and specificity of the RP-HPLC technique was investigated by keeping the standard concentration constant against blank and stress (forced degradation) application.Peak area, tailing factor, theoretical plate, retention duration, and resolution were all determined in triplicate.

(ii) Precision:

The precision of analytical separation approach was stabilized by using injecting replicates of standard. Six independent injection replications were performed to determine the inter-day precision, and the area response of the drug material was measured. The precision of the approach has indeed been termed as relative standard deviation (RSD).

(iii) Accuracy:

The accuracy was tested using a standard solution of synthesized chemical in formulations.The recovery of the recommended approach was examined using the pseudo stimulation technique with three unique measurement range of 50%, 100%, and 150% for this previously identified sample solution having a specific quantity of synthesized chemical.Triplicate assessments of these three levels were reported to estimate the percent recovery and percent relative error.

(iv) Limit of Detection and Limit of Quantification:

Limit of Detection (LOD) is the smallest amount of drug content that the proposed technology can detect, whereas Limit of Quantification(LOQ) is the smallest quantity that the method can quantify.The guidelines

recommend a minimal signal-to-noise ratio (S/N) of greater than 3.3 for LOD and greater than 10 for LOQ.The given formula can hypothetically determine it on the basis of linearity data.

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

Where σ = Residual Standard Deviation of regression line, S = Slope of regression line.

(v) Linearity and Range:

The linearity of the analytical method was demonstrated from LOQ to 150 percent of the specification level concentration for the specified degradation and from LOQ to 150 percent of the standard concentration for the respective analyte.Spiking specified degradation products in the range of LOQ to 150 percent of specification limit and analyte in the range of LOQ to 150 percent of standard concentration in diluents yielded a linearity solution level. Six concentrations range from 0.832 to 29.64 µg/ml (0.832, 10.40, 15.08, 19.76, 25.48 and 29.64mL / 50mL) were used to detect linearity.

III.RESULTS AND DISCUSSION

The synthesized drug was identified through spectrometric methods and validated by RP-HPLC system. The physicochemical properties of drug were properly shown in Table 02,

Physicochemical properties

Table 0 2 : Physicochemical Properties

Comp.	Substitution R	Molecular Formula/Weight	M.P. ^{0}C	Yeild %	% Composition Calculated/Found		
					C	H	N
1B	Morpholine	$C_{17}H_{19}N_3S_2O_5$ 409	185	68	49.86 49.85	4.68 4.65	10.26 10.23

The synthesized drug was identified as 5 –[4-(morpholin-4-yl)- 3- nitrobenzene - 1 – Sulfonyl] - 4, 5, 6, 7 –Tetrahydrothieno [3, 2 - C] Pyridine. from different spectroscopic data. The physicochemical properties are shown in table 02.The molecular formula of compound was settled as $C_{17}H_{19}N_3S_2O_5$ which was further verified by mass spectrometric analysis.The ESI-MS data (Figure 06) of compound showed a molecular ion peak at m/z409 $[M^+H]^+$. The IR (KBr) spectrum of synthesized compound showed the absorption bands (Table 03) at ν_{max}1458,2916,2854,1350,3092,1535,1458,833 cm^{-1} indicating the presence of thiophene group, aliphatic C-H bond, and aromatic ring in its molecule.A calculation of double bond equivalence revealed the presence of eight degree of unsaturation which was established by the presence of one aliphatic and two aromatic ring and a nitro group in the molecule.The 1H NMR spectrum (Table 04, Figure03) showed the signals for the presence of one aromatic proton at δH 8.293-8.288 (1H, s), the singlet nature of the signals has suggested them to be aromatic in nature and the aromatic ring mustbe trisubstituted. In order to confirm the nature of carbon atoms present in compound its 13C NMR spectrum (Table 05, Figure 04) was measured. The multiplicity of carbons determined and found the presence of eight methylene, one methine carbons.

I.R Spectra

IR spectra were recorded on Shimadzu FTIR-8400S spectrophotometer using KBr disc method shown in figure 02.

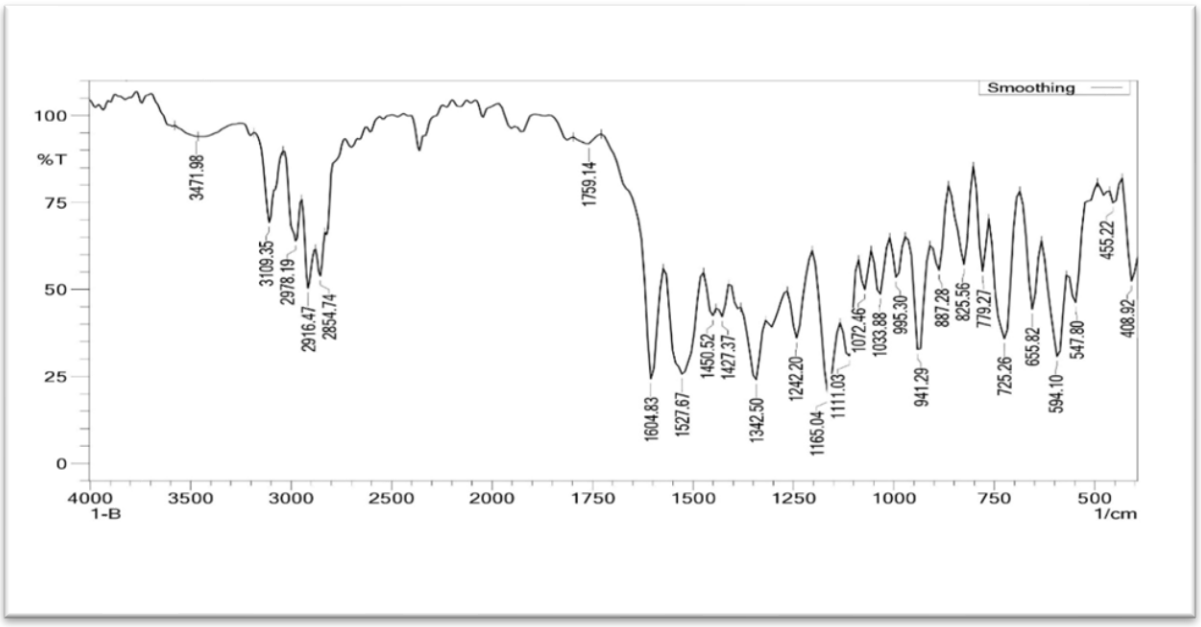


Figure 02 : IR spectrum for synthesized drug

Various peaks starting from 779 cm^{-1} to 3092 cm^{-1} were observed. C-H stretching was observed at 3109, 2978,2916, 2854 and, 1604 cm^{-1} which are indicating the aromatic and aliphatic compounds respectively. The identification of functional group is shown in table 03.

Table 03: Functional Group detection through IR Frequency

Vibrating frequency (cm^{-1})	**Functional group**
3109	Aromatic C –H Stretching
2978 2916 2854	Aliphatic C –H Stretching
1604	C = C Stretching
1527	NO_2 Stretching
1342 1242	S=O Stretching

NMR- Spectra

The 1H NMR spectrum was recorded for synthesized drug (Figure 03) and interpretation is shown in table 04.

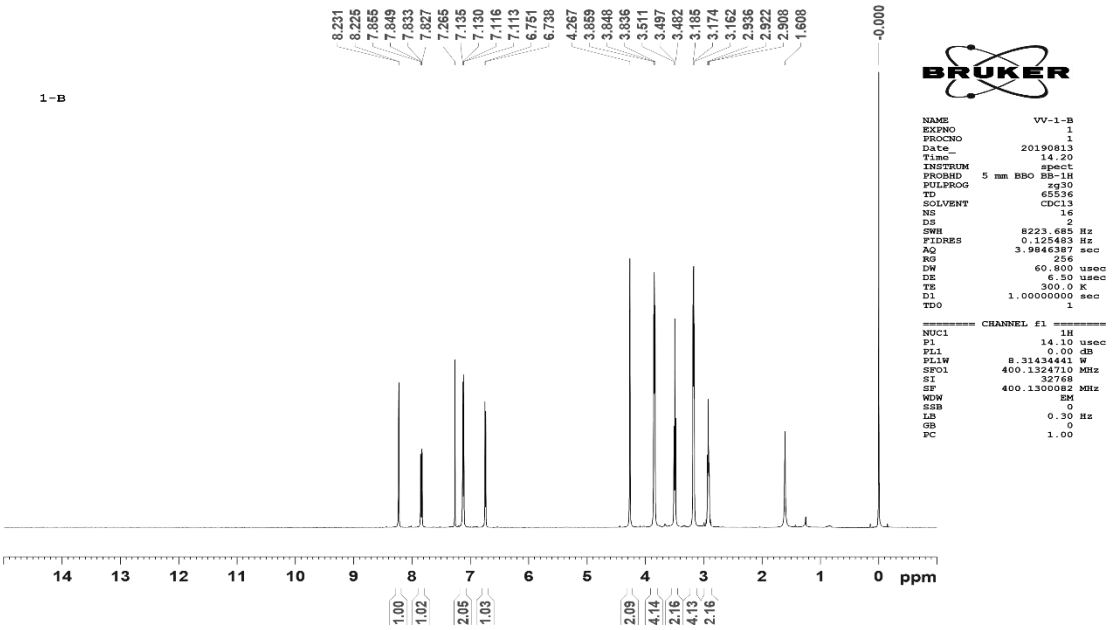


Figure 03 : 1H NMR spectrum

In 1H NMR, eight types of protons were identified among which most protons were found to be doublet. Four triplet protons were identified between 2.904-2.932 ppm and maximum chemical shift were reported 7.943-7.917 ppm. The 1H interpretations are shown in table 04.

Table 04: 1H NMR data interpretation

Sr. No.	Signal Position (δ ppm)	Relative No. Of Protons	Multiplicity
1	8.231-8.225	1H	Doublet
2	7.855-7.827	1H	Doublet
3	7.135-7.113	2H	Doublet
4	6.751-6.738	1H	Doublet
5	4.267	2H	Singlet
6	3.859-3.836	4H	Triplet
7	3.511-3.482	2H	Triplet
8	3.185-3.162	4H	Triplet
9	2.936-2.90	2H	Triplet

13C NMR were recorded for synthesized drug in Bruker base NMR model which is shown in figure 04 and interpretation is recorded in table 05.

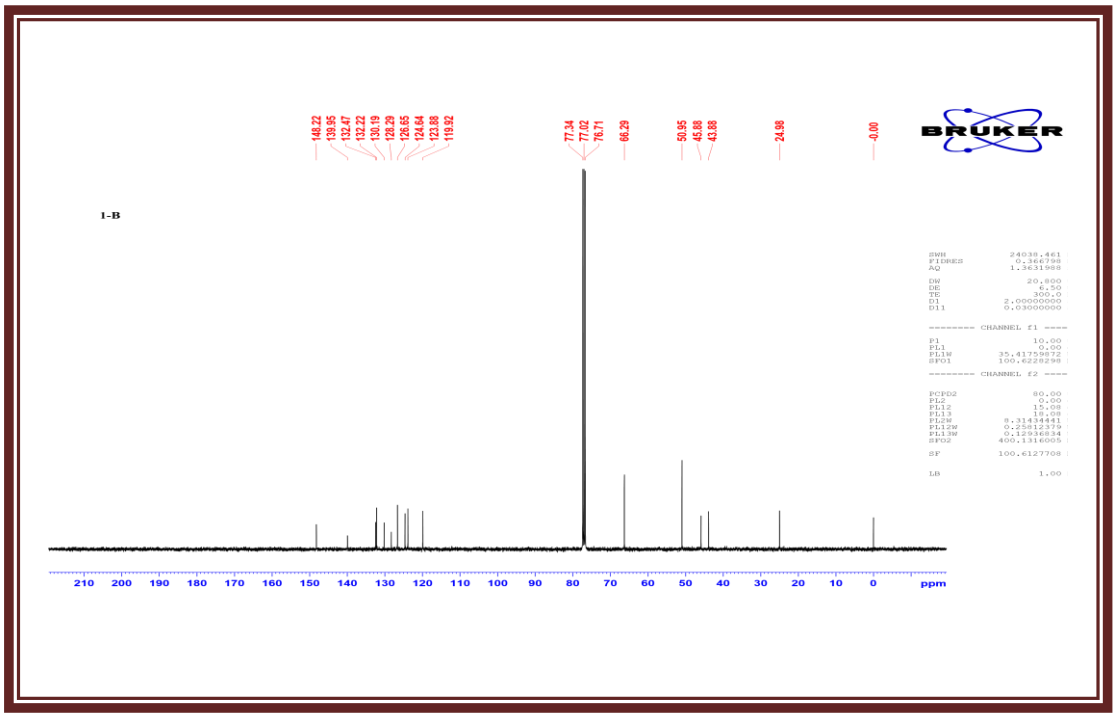


Figure 04 : 13C NMR spectrum

Table 05: 13C NMR data interpretation

δ value	Carbon Assignment
24.98	14
43.88	15
45.88	11
50.95	1, 4
66.29	2, 3
119.92	6
123.88	9
124.64	7
126.65	16
128.29	12
130.19	13
132.22	17
132.47	8
139.95	5
148.22	10

Mass spectroscopy:

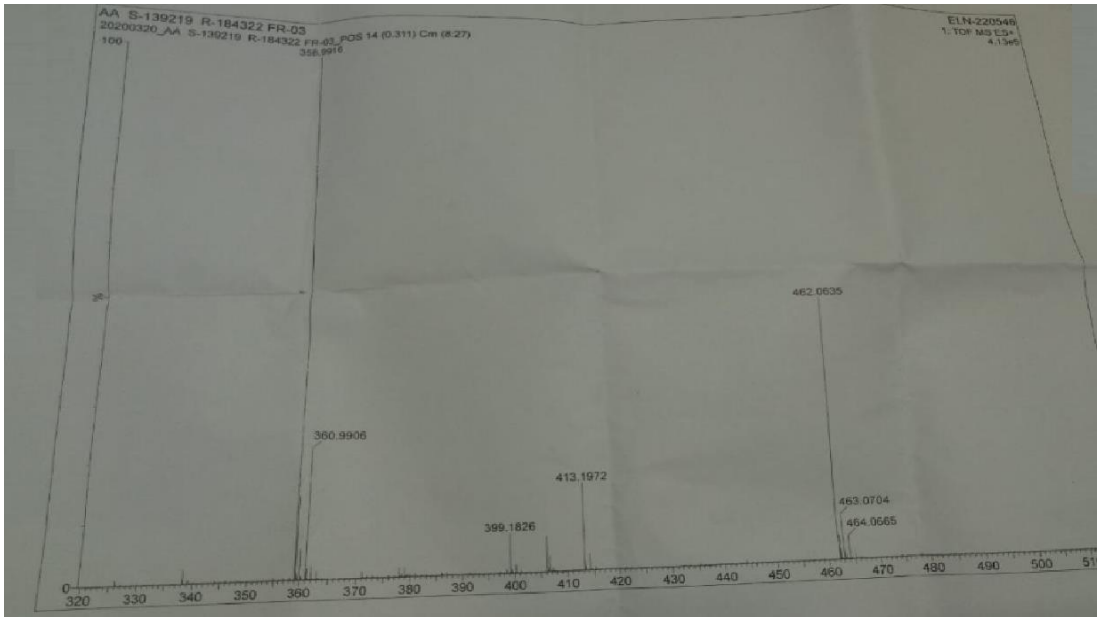
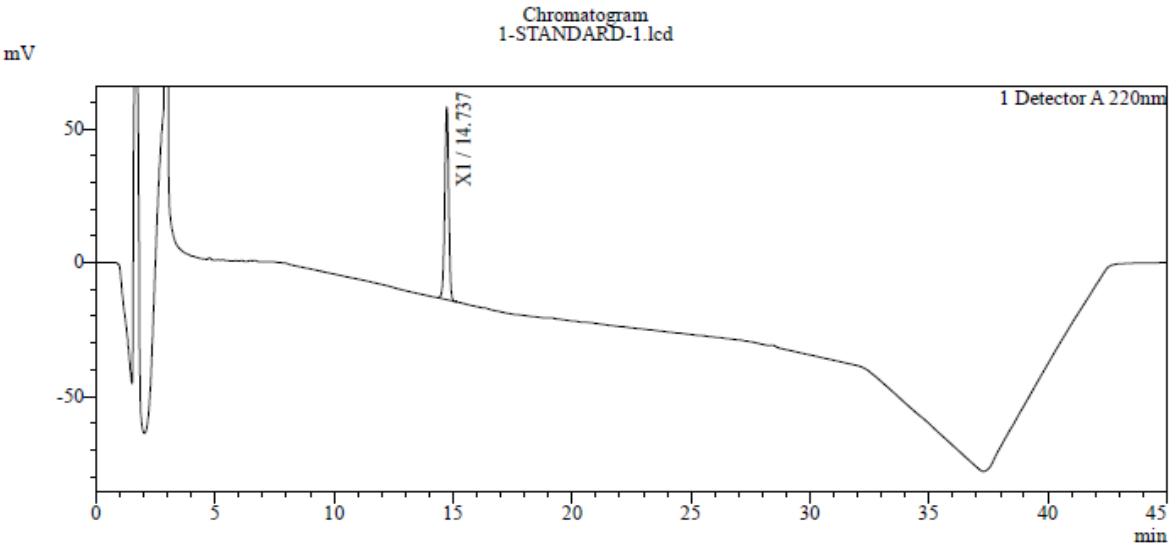


Figure 06 : Mass spectral study of pyridine derivative

HPLC method validation

A number of test runs were conducted utilizing reversed columns of C18, varied proportions of mobile phases and varying rates of circulation for the synthesized pyridine derivative separation with appropriate chromatographical characteristics such as resolution, theoretical plate, and the tail factor.The chromatogram ofthe optimized method is presented in Figure 07.



Peak Table 1-STANDARD-1.lcd

Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%
1	X1	14.737	833491	100.000
Total			833491	100.000

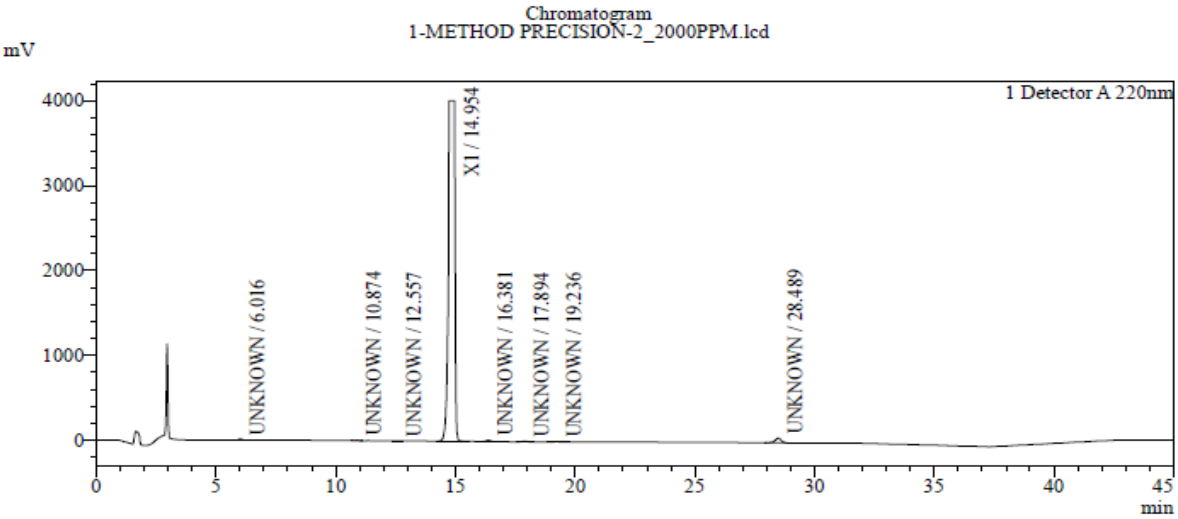
Figure 07 : HPLC Chromatogram

Precision Study

Repeatability (intra-day precision) was determined by injecting six replicate (n = 6) solutions of the standard concentration which is shown in various sections. The system precision is shown in table 06, method precision is shown by figure 08 and table 07.

Table 06: System precision

Injection No.	Area response of Drug Substance
1	833491
2	834007
3	834123
4	834248
5	834183
6	834284
Mean	834056
%RSD	0.035



Peak Table 1-METHOD PRECISION-2_2000PPM.lcd

Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%
1	UNKNOWN	6.016	154392	0.201
2	UNKNOWN	10.874	38360	0.050
3	UNKNOWN	12.557	38760	0.051
4	X1	14.954	75259584	98.061
5	UNKNOWN	16.381	189369	0.247
6	UNKNOWN	17.894	84522	0.110
7	UNKNOWN	19.236	55092	0.072
8	UNKNOWN	28.489	927623	1.209
Total			76747702	100.000

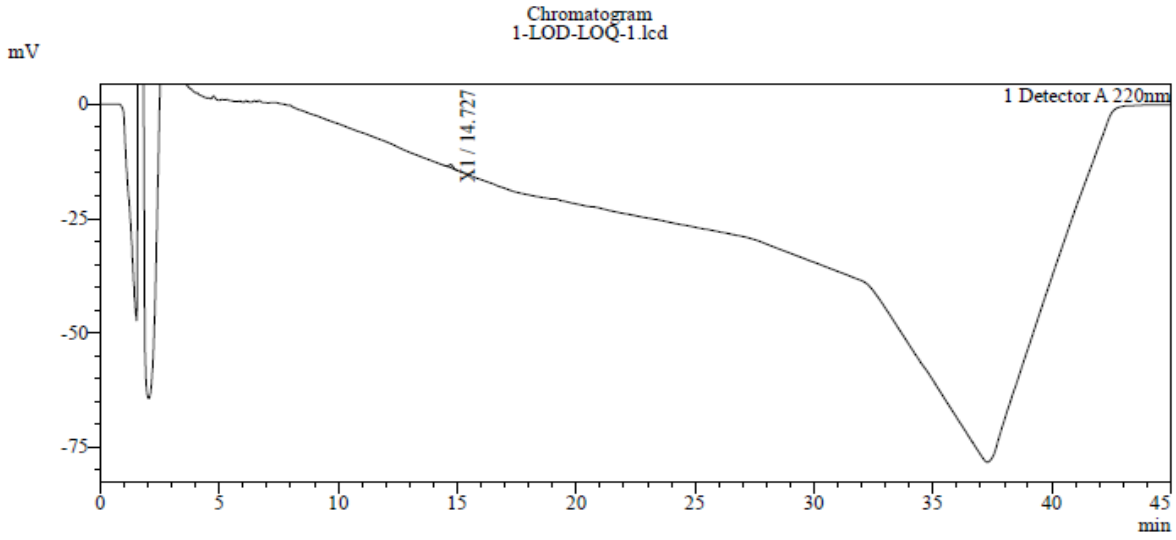
Figure 08 : Chromatogram of the optimized method

Table 07: method precision

Name	Un Known (RRT-0.40)	Un Known (RRT-0.73)	Un Known (RRT-0.84)	Un Known (RRT-1.09)	Un Known (RRT-1.20)	Un Known (RRT-1.29)	Un Known (RRT-1.91)
Set-1	153734	38655	38902	189657	84320	54986	927955
Set-2	154392	38360	38760	189369	84522	55092	927623
Set-3	154366	38407	38652	189451	84093	55704	928243
Set-4	154426	38576	38535	189662	84092	55956	928721
Set-5	154321	38283	38808	189717	84236	55699	928590
Set-6	154383	38379	38817	189613	84143	56488	929112
Mean	154270	38443	38746	189578	84234	55654	928374
% RSD	0.17	0.37	0.34	0.07	0.20	1.00	0.06

LOD and LOQ

The LOD-LOD chromatogram for test samples are shown in figure 09,10,11,12 as well as in 13 and, the data are indicating in table 08, 09 and, 10.

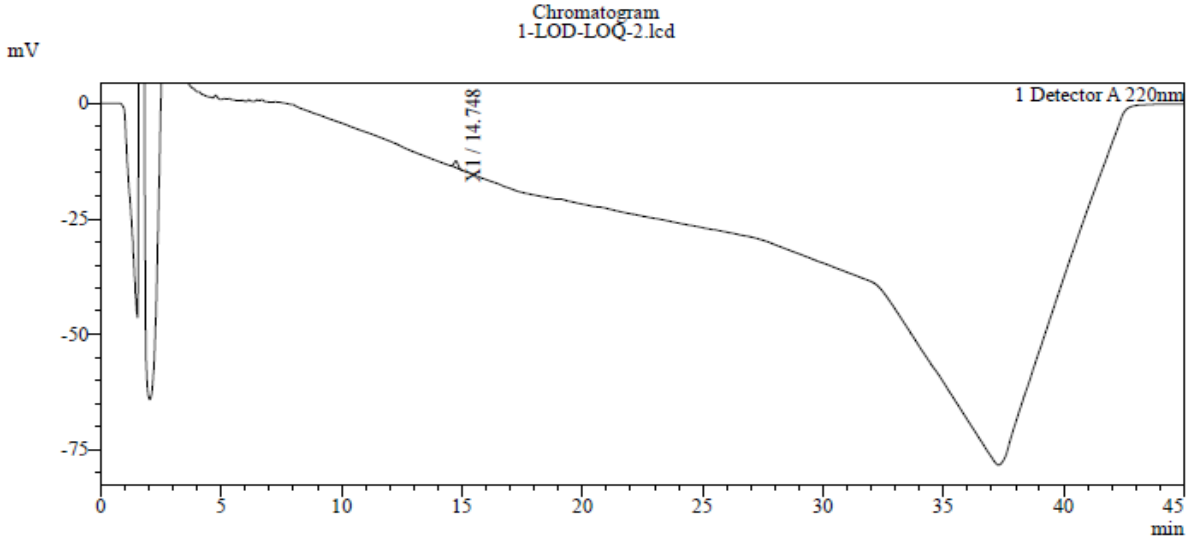


Peak Table 1-LOD-LOQ-1.lcd

Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%
1	X1	14.727	7562	100.000
Total			7562	100.000

Figure 09 : Chromatogram of LOD-LOQ-01

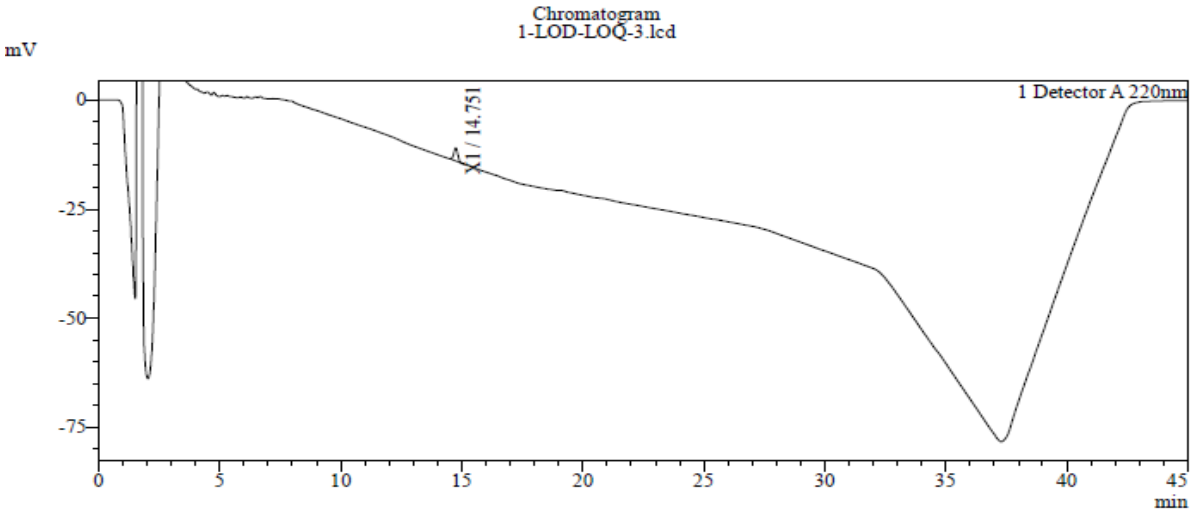


Peak Table 1-LOD-LOQ-2.lcd

Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%
1	X1	14.748	16346	100.000
Total			16346	100.000

Figure 10 : Chromatogram of LOD-LOQ-02

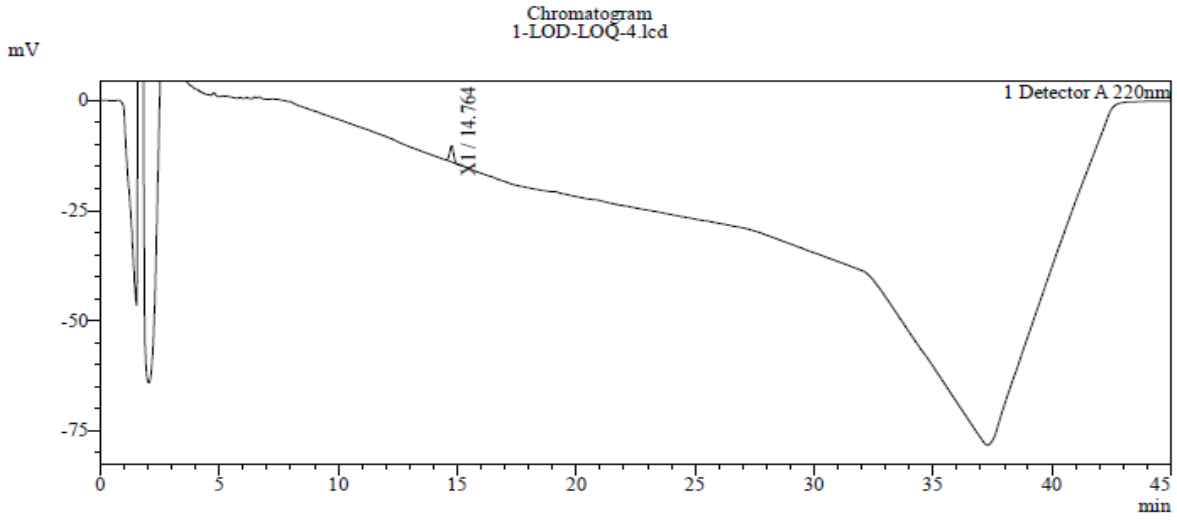


Peak Table 1-LOD-LOQ-3.lcd

Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%
1	X1	14.751	33568	100.000
Total			33568	100.000

Figure 11 : Chromatogram of LOD-LOQ-03

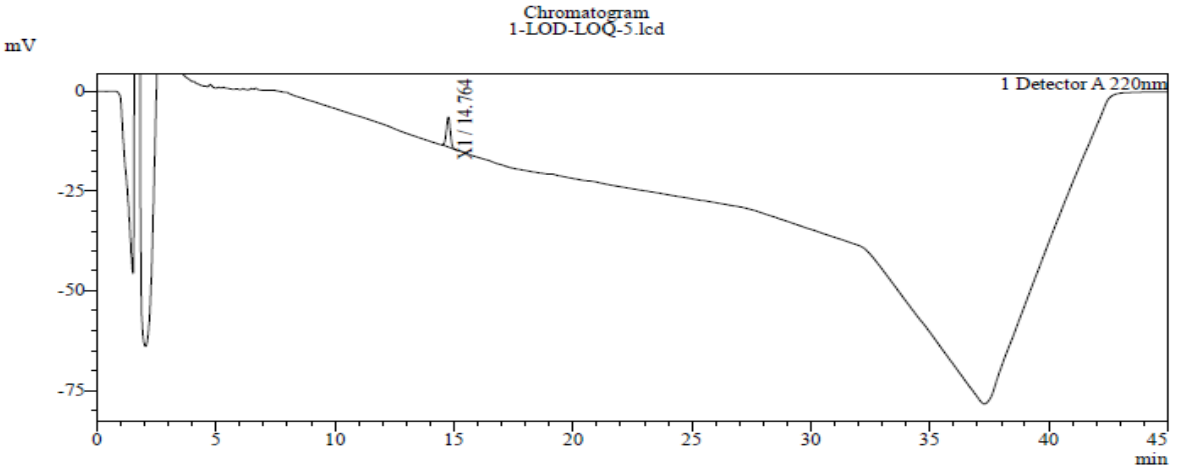


Peak Table 1-LOD-LOQ-4.lcd

Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%
1	X1	14.764	42189	100.000
Total			42189	100.000

Figure 12 : Chromatogram of LOD-LOQ-04



Peak Table 1-LOD-LOQ-5.lcd

Detector A 220nm

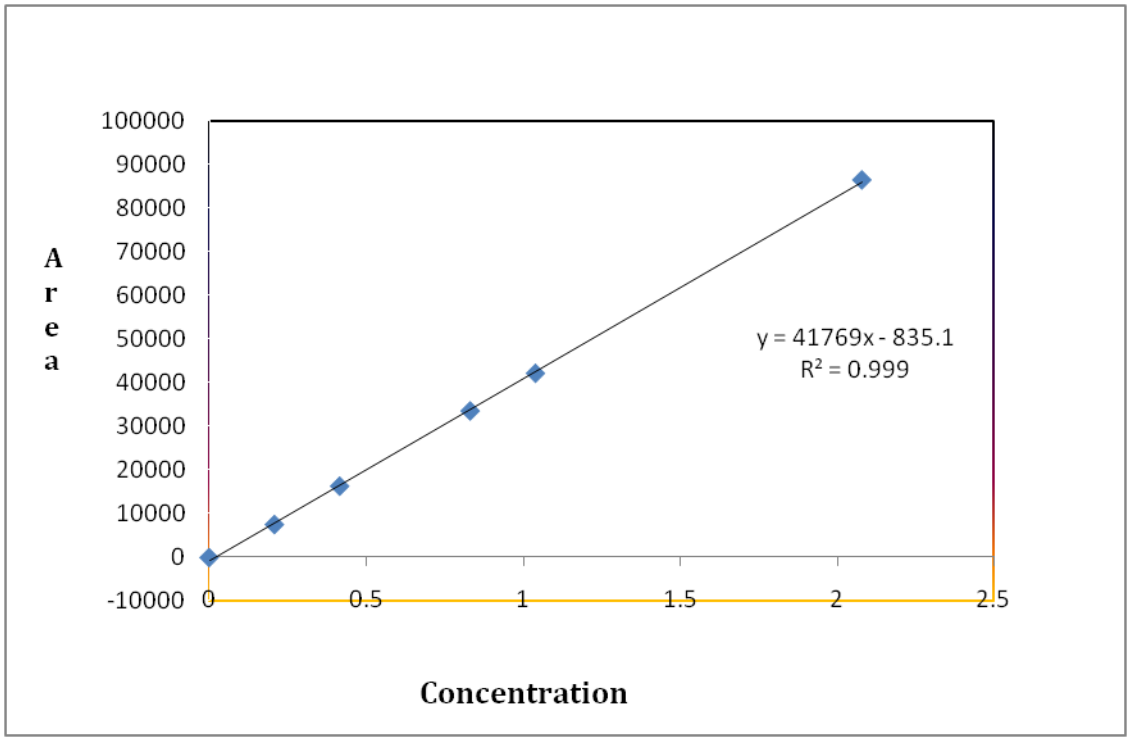
Peak#	Name	Ret. Time	Area	Area%
1	X1	14.764	86461	100.000
Total			86461	100.000

Figure 13 : Chromatogram of LOD-LOQ-05

Table 08 : LOD and LOQ Determination for standard preparation

Standard Solution		
Sr. No.	**Conc. (ppm)**	**Area Response**
1	0	0

2	0.208	7562
3	0.416	16346
4	0.832	33568
5	1.040	42189
6	2.080	86461
Slope		41769.45153
Intercept		-835.1683673
Squared CC*		0.99974



X axis =Concentration (µg/ml) Y axis = PeakArea

Figure 14 : Regression Analysis chart of linearity Study included with LOQ level

Table 09 : Limit of Detection and Limit of Quantification for standard

% Level	Reported	
	ppm	%
LOD	0.400	0.020
LOQ	0.800	0.040

Table 10 : Observation at LOD and LOQ Precision

Sr. No.	Standard	
	LOD Precision Area Response	LOQ Precision Area Response
1	16346	33432
2	16123	33626
3	16234	33795
4	16312	33576
5	16107	33548
6	16289	33638
Mean	16235	33602
%RSD	0.62	0.35

IV. CONCLUSION

A new RP-HPLC method for the accurate detection of5 - (4 –morpholin -4 – yl)- 3- Nitrobenzene - 1 - Sulfonyl) - 4, 5, 6, 7 –Tetrahydrothieno [3, 2 - C] effectively developed and validated in this work.The proposed approach is said to have been straightforward, accurate, and precise.The developed approach is devoid of excipients, contaminants, or active chemicals that could create difficulties.The

optimized formulation is also said to be accurate, genuine, and trustworthy.

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