WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 10, Issue 5, 1234-1253. Research Article ISSN 2277– 7105

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE DETERMINATION OF POTENTIAL IMPURITIES OF NEFOPAM

Bharathi Tejas G. J.*[1] and Bhadre Gowda D. G.[2]

[1]Analytica Chemie Inc, VITC, Peenya II Stage, Bangalore – 570008, Karnataka, India.
[2]Chemistry Department, Yuvaraja's College, University of Mysore, Mysore-570005, Karnataka, India.

Article Received on
28 Feb. 2021,
Revised on 18 March 2021,
Accepted on 08 April 2021
DOI: 10.20959/wjpr20215-20215

***Corresponding Author**
Bharathi Tejas G. J.
Analytica Chemie Inc,
VITC, Peenya II Stage,
Bangalore - 570008,
Karnataka, India.

ABSTRACT

The objective of the study was to develop and evaluate the reverse phase liquid chromatography (RP-HPLC) method for the quantitative determination of potential impurities of Nefopam active pharmaceutical ingredient. The method uses a Puratis RP-18 column (250 × 4.6mm, 5µm) with mobile phase A consisted, 0.1% trifluoro acetic acid in water and mobile phase B consisted of acetonitrile with a gradient programme. The column temperature was maintained at 25 °C and the detection was carried out at 220 nm. Efficient and reproducible chromatographic separation was achieved on Puratis RP-18 stationary phase in gradient elution profile. The newly developed HPLC method was validated according to ICH guidelines considering four impurities to demonstrate precision, linearity, accuracy and robustness of the method. The developed HPLC method was found to be accurate and sensitive. The correlation coefficient values are greater than 0.99 for Nefopam and its four impurities. Detection limit and quantitation limit was 0.40ppm and 1.23ppm respectively, indicating the high sensitivity of the newly developed method. Accuracy of the method was established based on the recovery obtained between 99.78% and 102.07% for all impurities. The result of robustness study also indicates that the method is robust and is unaffected by small variation in chromatographic conditions. The proposed HPLC method provides reliable, reproducible, accurate and sensitive for the quantification of Nefopam related substances.

KEYWORDS: Nefopam; Impurities; RP-HPLC; Validation.

INTRODUCTION

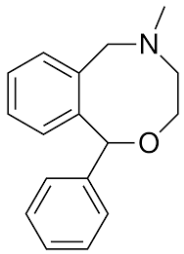
Nefopam is a medication used to treat moderate pain, for example after an operation or a serious injury, dental pain, joint pain or muscle pain, or pain from cancer. It's also used for other types of long-standing pain when weaker painkillers no longer work. Nefopam is a centrally acting, non-opioid painkilling medication, that is primarily used to treat moderate to severe pain. Nefopam acts in the brain and spinal cord to relieve pain via novel mechanisms: antinociceptive effects from triple monoamine reuptake inhibition, and antihyperalgesic activity through modulation of glutamatergic transmission.

Nefopam is effective for prevention of shivering during surgery or recovery from surgery. Nefopam was significantly more effective than aspirin as an analgesic in one of the clinical trial, although with a greater incidence of side effects such as sweating, dizziness and nausea, especially at higher doses. Nefopam tends to produce fewer side effects, does not produce respiratory depression, and has much less abuse potential, and so is useful either as an alternative to opioid analgesics, or as an adjunctive treatment for use alongside opioid (or other) analgesics. Nefopam is also used to treat severe hiccups.

Nefopam is cyclized analogue of orphenadrine, diphenhydramine, and tofenacin, with each of these compounds different from one another only by the presence of one or two carbons. The ring system of nefopam is a benzoxazocine system.

The mechanism of action of nefopam and its analgesic effects are not well understood, although inhibition of the reuptake of serotonin, norepinephrine, and to a lesser extent dopamine (that is, acting as an SNDRI) is thought to be involved. It also reduces glutamate signaling via modulating sodium and calcium channels.

Chemical structure of Nefopam is given in figure 1.



Molecular weight = 253.345 $g \cdot mol^{-1}$

Molecular formula = $C_{17}H_{19}NO$

Fig. 1: Structure of Nefopam.

Several analytical methods have been reported to determine Nefopam in bulk drug, formulation and in biological matrices. These methods include spectrophotometry[5,7], thin layer chromatography (TLC)[1,8], high performance liquid chromatography (HPLC)[2,3] and liquid chromatography tandem mass spectrometry (LC/MS).[6] Nefopam is an official drug in USP, EP, BP, and IP. Extensive literature survey reveals that no HPLC methods have been reported for the analysis of Nefopam drug substance. Hence it was felt necessary to develop an accurate, rapid and sensitive HPLC method for the determination of Nefopam impurities.

Objective of the current study was to develop a HPLC–UV method for the quantitative determination of impurities in Nefopam and check the suitability of the method as per ICH guidelines. No Pharmacopeial methods are available for Nefopam. The newly developed HPLC method separates all impurities with short run time.

MATERIALSAND METHODS

Reagents and Chemicals

Samples of Nefopam and standards of Imp-1, Imp-2, Imp- 3 and Imp-4 (Table 1) were received from Analytica Chemie Inc Bangalore, India. HPLC grade methanol and acetonitrile was purchased from Rankem, Mumbai, India. Deionized water was prepared using a Milli- Q plus water purification system from Millipore (Bedford, MA, USA). Analytical reagent grade of trifluoroacetic acid, sodium hydroxide, hydrogen peroxide and hydrochloric acid were purchased from Merck India Limited (Mumbai, India).

Instruments

The LC method development and validation were done using Waters Allaiance 2695/2996, Empower 2/2006 equipped with PDA detector. The data were collected and the peak purity of the Nefopam peak was checked using empower software.

Chromatographic conditions

The chromatographic separations were achieved on Puratis RP-C18 column (250 mm length × 4.6 mm ID with 5µm particle size, Chromachemie). Mobile phase A consisted, 0.1% trifluoroacetic acid in water and mobile phase B consisted acetonitrile with a gradient programme (T_{min}A:B) $T_0$95:05, T_{15}5:95, T_{17}05:95, T_{18}95:05, T_{20}95:05. The column temperature was maintained at 25 °C and the detection was carried out at 220 nm. The flow rate was set to 1.0 mL/min. The test concentration was about 100 and the injection volume was 10µL. A degassed mixture of acetonitrile and water (8:2) was used as diluent for standard and sample

preparations.

Preparation of stock solutions for method validation

A test preparation of 100 ppm of Nefopam API sample was prepared by dissolving in diluent. A stock solution of impurities was prepared by dissolving 25 mg each of Imp-1, Imp-2, Imp-3, Imp-4 and 25 mg of Nefopam in diluent and made up to 50 mL with diluent. Transferred 10 mL of each individual stock solution into a 50 mL volumetric flask and made up to volume with diluent.

Sample preparation for forced degradation studies

Stress study is a complementary part of stability testing wherein influence of environmental factors like pH, temperature, humidity, oxygen and light are evaluated on a drug substance and products. Stress testing of the drug substance was performed as per ICH guidelines Q1 (R2) and it can help to identify the likely degradation products, which can in turn help to establish the degradation pathways, the intrinsic stability of the molecule and specificity of the proposed method. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Acid hydrolysis was performed in 1N HCl at 30 °C for 24hrs. The study in basic solution was carried out in 1N NaOH at 30 °C for 24hrs. For study in neutral solution, the drug dissolved in water and was kept at 40 °C for 24hrs. Oxidation studies were carried out at ambient temperature in 10% hydrogen peroxide for 24hrs. Samples were withdrawn at appropriate times and subjected to LC analysis after suitable dilution (100 µg/mL) to evaluate the suitability of the proposed method to separate Nefopam from its degradation products. The excess of acid or base in volumetric flasks were neutralized and made up to the volume with diluent. Corresponding blank solutions were prepared following the sample procedure without Nefopam sample. Thermal degradation was done at 80°C for 24 hrs on the solid sample. Photodegradation studies were carried out according to option 2 of Q1B in ICH guidelines. Photolytic degradation was performed by keeping 150 mg of each sample in two separate losses on drying (LOD) bottles in a photo stability chamber model TP 0000090G (Thermo Lab equipments Pvt. Ltd., Mumbai, India). One bottle was covered with lid and then with aluminium foil (dark control) whereas another bottle (photolytic exposed sample) was covered with lid to get a minimum exposure of 24 hours for light and 200 Wh/m2 for ultraviolet region. A 0.1 mg/mL samples were prepared for thermal degradation and photolytic degradation samples.

RESULTSAND DISCUSSION

Method development

The determination of the suitability of a HPLC method is based upon the level of development. However, at a minimum HPLC method should provide baseline separation of starting materials, desired products, known impurities, and expected by products. The chromatographic conditions should also be chemically compatible with the analytes. The main objective of the HPLC method development for Nefopam was to achieve efficient separation of impurities.

Selection of wavelength

The optimum wavelength of detection is the wavelength that gives the highest sensitivity for the significant related substances and minimizes the difference in response factors between those of the active pharmaceutical ingredient and the related substances. Nefopam and its impurities give good detector response at 220 nm, therefore the final absorption wavelength for detection was chosen at 220 nm.

Mobile phase selection

In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. Choosing a right buffer and pH is very critical for method development. Buffers are generally recommended to control the pH stability of the mobile phase. Buffers like ammonium acetate, dipotassium hydrogen orthophosphate, potassium hydrogen phosphate, diammonium hydrogen orthophosphate, ammonium hydrogen phosphate, and its combination were studied for HPLC method development. At sufficiently low pH, basic analytes are in ionised form and will elute more quickly but with improved peak shape. Conversely, at higher pH basic compounds will be more retained. Peak splitting may be observed if the pH of the mobile phase is similar to the pKa of the compound and the analyte elutes as both a charged and uncharged species. Acetonitrile was chosen as organic modifiers. The principle difference in the behavior of acetonitrile and methanol is that where acetonitrile forms a thick multi-molecular adsorbed layer on the surface of reverse phase adsorbent (C_1-C_{18} and phenyl phases), while methanol is adsorbed only in monomolecular fashion. This brings a principal difference in the analyte retention mechanism in these two hydro-organic systems.

Column selection

The heart of a HPLC system is the column. Changing a column will have the greatest effect

on the resolution of analytes during method development. Silica-based packing materials dominate in applications for RP separations in the pharmaceutical industry. The vast majority of RP LC separations take place on column that contain C_{18} bonded stationary phases due to their stability, retentivity and reproducibility. In addition, these hydrophobic ligands provide the desired separation most of the time. However, screening several different types of stationary phases during method development for a particular separation is often useful because different columns usually have different selectivity for components in a sample. Several experiments were conducted to get a baseline resolution between Nefopam and impurities. water miscible organic solvent.

Use of RP_{18} column with a 250 mm length × 4.6mm ID column and 5µm particle size, use of 0.1% trifluoro acetic acid in water as mobile phase A and acetonitrile as mobile phase-B was significant in achieving the desired resolution of Nefopam and its impurities. After several trials for gradient profile, chromatographic conditions were finalized as described under section chromatographic conditions.

Results of forced degradation

Nefopam was found to be stable under stress conditions such as thermal, photolytic and hydrolysis conditions. The developed method was able to separate other impurities generated during the oxidation, acid & base hydrolysis from Nefopam and other known impurities. Significant degradation of the drug substance was observed under acidic, oxidation, hydrolysis, thermal & photolytic stress conditions leads to a degradation product at RRT 1.20 & basic stress conditions leads to a degradation products at RRT 0.94,1.07,1.15,1.20 &1.32. LC/MS analysis was carried out to identify this degradation product of Nefopam using Agilent 6140 single quadrupole mass spectrometer. Acid hydrolysis stressed sample shown in the chromatogram, indicating that the degradation products formed may between and Nefopam and hydrochloric acid, base hydrolysis stressed sample shown in the chromatogram indicating that the degradation products formed may between and Nefopam and sodium hydroxide. Chromatograms of forced degradation study have been depicted in Figure 3 and degradation studies are depicted in Table 3.

Degradation studies and peak purity test results derived from PDA detector and LC/MS confirmed that the Nefopam peak was homogenous. The method was found to be specific in the presence of Imp1, Imp-2, Imp-3, Imp-4 and their degradation products confirmed the stability indicating power of the newly developed method.

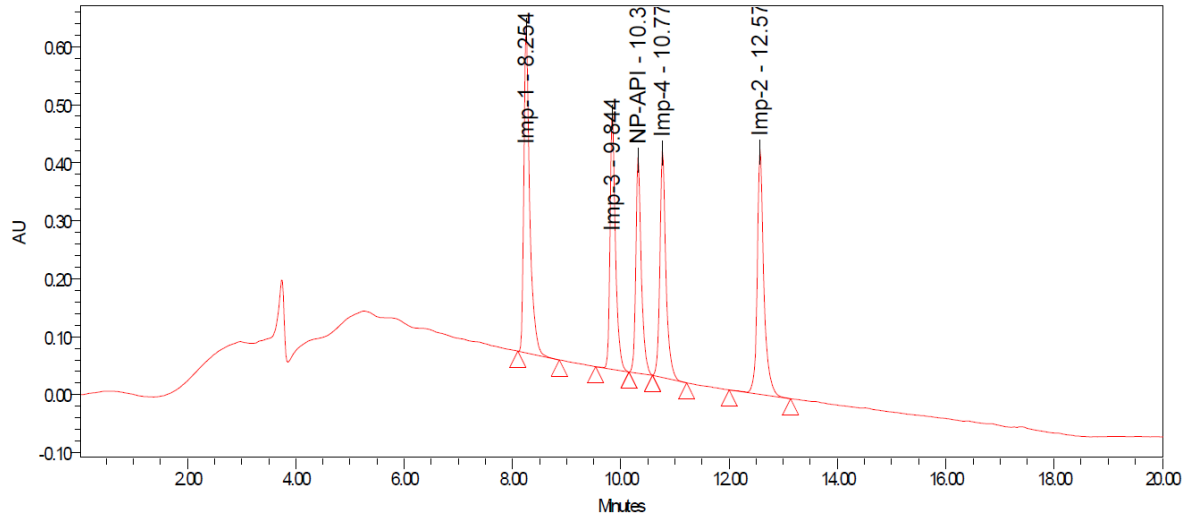


Fig. 2: Chromatogram of Nefopam spiked with impurities.

METHODVALIDATION

The newly developed method was validated for sensitivity, linearity, precision and accuracy, robustness and system suitability according to ICH guidelines[9]. Validation study was carried out for Imp-1, Imp- 2, Imp-3 and Imp-4. The system suitability and selectivity were checked by injecting 100ppm of Nefopam solution containing 100ppm of all impurities monitored throughout the validation. Method validation results are summarized in Table 2.

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection and limit of quantitation were determined for Nefopam and for each of the related substances as per ICH $Q2R_1$ guideline. The LOD and LOQ for Imp-1, Imp-2, Imp-3, Imp-4 and Nefopam were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration. The limit of detection and the limit of quantitation for Imp-1, Imp-2, Imp-3, Imp-4 and Nefopam were about 0.40ppm and 1.23 of analyte concentration i.e. 100ppm respectively. Precision study was also carried at the LOQ level by injecting six individual preparations of all impurities and the relative standard deviation for LOQ concentration for all impurities were below 2%.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. A linearity test solution for related substance method was prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 150% of the permitted maximum level of the impurity (i.e. LOQ, 0.41 ppm

,0.30 ppm, 1.09 ppm, 0.84 ppm and 1.23 ppm) was subjected to linear regression analysis. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The correlation coefficient obtained was greater than 0.99 for all impurities. The result showed an excellent correlation between the peak and concentration of all impurities. The range of the method was from LOQ to 0.60 ppm of the analyte concentration (100 ppm).

Precision

Precision of the method was studied for method precision and intermediate precision. Method precision was checked by injecting six individual preparations of (100 ppm) Nefopam spiked with 100ppm of each impurity. In the intermediate precision study, the similar procedure of method precision was carried out by a different day. % RSD of areas of each impurity was within 2.0, confirming good precision at low level of the developed analytical method.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method was evaluated in triplicate at LOQ,50% level (0.06 mg/mL), 100% level (0.13 mg/mL) and 150% level (0.18mg/ mL). The percentage recovery of all impurities in drug substance has been calculated. Chromatogram of Nefopam spiked with four impurities was depicted in Figure 2.

Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and the resolution between closely eluting peaks, impurities were evaluated. Close observation of analysis results of deliberately changed chromatographic conditions viz; flow rate (0.2±0.05 mL/min), mobile phase composition (± 2% acetonitrile) and column temperature (25±5°C) shown that no significant change in relative retention time for all impurities in spiked sample illustrating the robustness of the method.

Solution stability and mobile phase stability

The solution stability of Nefopam and its related impurities was carried out by leaving both spiked and unspiked sample solutions in tightly capped HPLC vials for 72 h in an auto sampler. Content of each impurity was determined against freshly prepared standard solution. No significant changes were observed in the content of any of the impurities. The solution

stability and mobile phase stability experiment data confirms that the sample solutions and mobile phase used during related substance determination were stable for at least 72 hour.

Table 1: Potential impurities of Nefopam (NP).

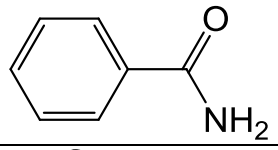
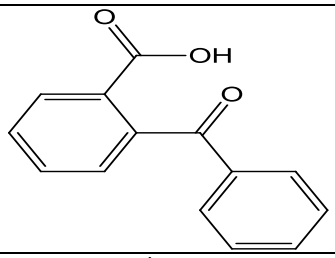
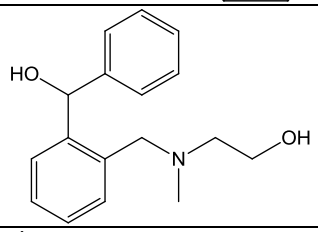
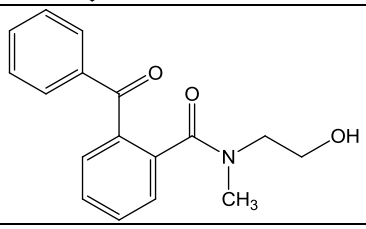
Sl no	Structure	Mol wt	IUPAC name	Code
01		121.14 g/mol	Benzamide (Benzoic acid amide)	Imp-1
02		226.23 g/mol	Benzophenone-2-carboxylic Acid	Imp-2
03		271.35 g/mol	2-((2-(Hydroxy(phenyl)methyl)benzyl)(methyl)amino)ethanol	Imp-3
04		283.32 g/mol	2-Benzoyl-N-(2-hydroxyethyl)-N-methylbenzamide	Imp-4

Table 2: Method validation summary report.

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	NP
System suitability					
RT	8.25	12.57	9.84	10.77	10.31
RRT	0.80	1.21	0.95	1.04	
Rs	-	8.67	8.16	2.28	2.56
N	30942	60774	49157	50420	54333
T	1.7	1.5	1.4	1.4	1.3
Linearity					
r^2	0.9996	0.9989	0.9975	0.9994	0.9991
Slope	111496.7	65365.	49089.5	55924.6	48224.6
Detection limit (ppm)	0.13	0.10	0.36	0.27	0.40
Quantitation limit (ppm)	0.41	0.30	1.09	0.84	1.23
Precision					
% RSD (n 6)	0.98	0.39	0.10	0.22	0.34
Repeatability (intraday)					
% RSD (n 6)	0.20	0.21	0.55	0.40	0.35
Intermediate precision (interday)					
% RSD (n 6)	0.82	0.98	0.98	0.57	0.30

Accuracy at 50% level (n 3)					
Amount added (mg)	0.0530	0.0546	0.0622	0.0576	0.062
Amount recovered mg	0.0533	0.0547	0.0626	0.0579	0.0694
% Recovery	100.52	100.23	100.71	100.50	102.07
Accuracy at 100% level (n 3)					
Amount added (mg)	0.1060	0.1092	0.1244	0.1152	0.1380
Amount recovered (mg)	0.1074	0.1101	0.1265	0.1171	0.1386
% Recovery	101.35	100.83	101.69	101.66	100.44
Accuracy at 150% level (n 3)					
Amount added (mg)	0.1590	0.1638	0.1866	0.1728	0.1880
Amount recovered (mg)	0.1587	0.1651	0.1864	0.1738	0.1872
% Recovery	99.78	100.77	99.87	100.59	99.60

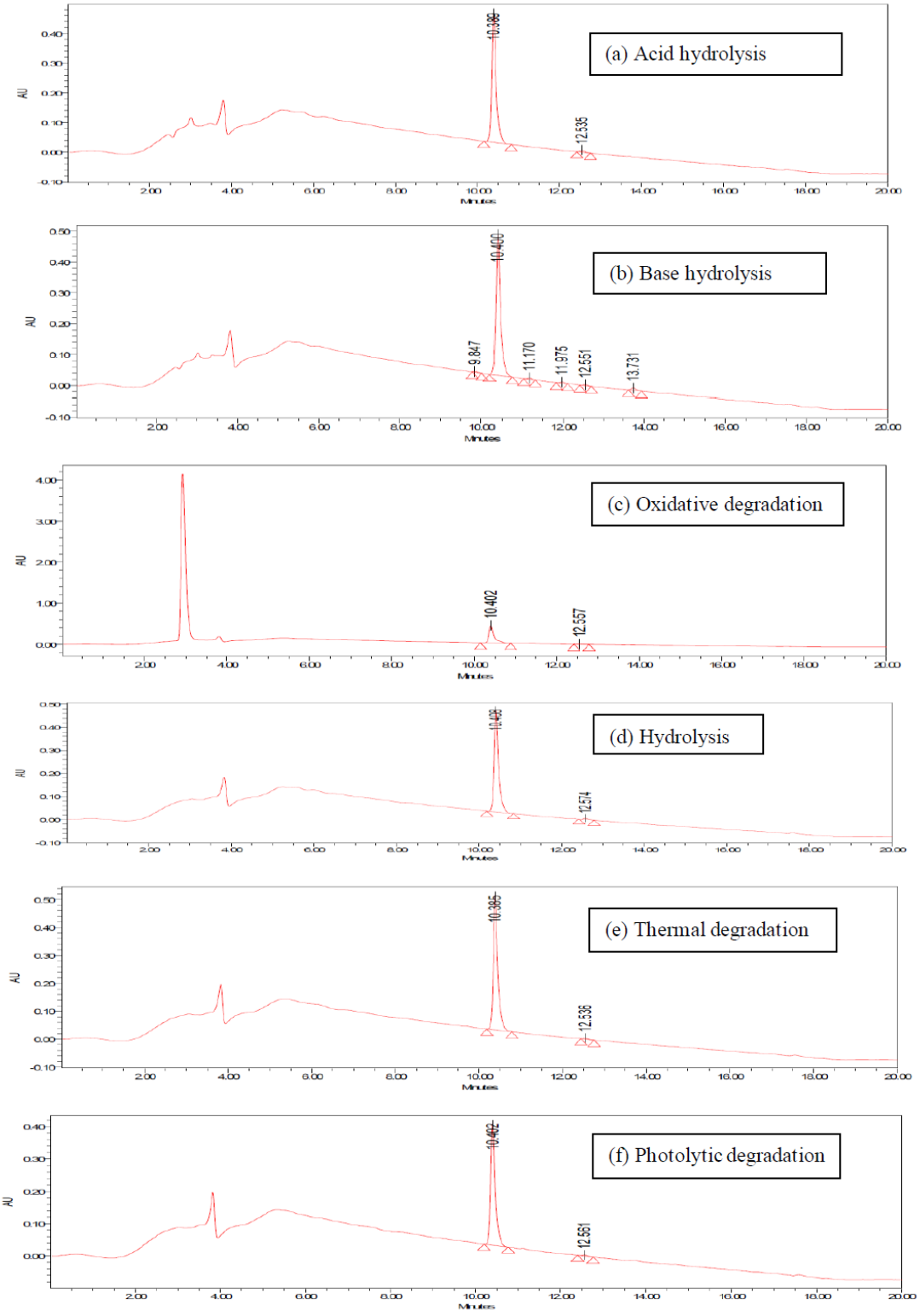
n- number of determinations; RT- retention time; RRT- relative retention time; Rs- USP resolution;

N- number of theoretical plates; T- USP tailing factor; r- correlation coefficient.

Table 3: Degradation studies report.

Stress condition	Time(hrs)	Temp(°C)	% Assay of active substance	% Area
Acid Hydrolysis (1N HCl)	24	30	98.23	98.58
Basic Hydrolysis (1N NaOH)	24	30	96.64	95.70
Oxidation (10% H_2O_2)	24	30	98.66	98.89
Hydrolysis (30 °C)	24	40	99.58	98.41
Thermal	24	80	99.67	99.39
Photolytic	24	30	99.50	98.74

Figure-3: Typical chromatogram of Nefopam under stress conditions: (a)acid hydrolysis, (b)base hydrolysis, (c) oxidative degradation, (d) hydrolysis, (e)thermal degradation and (f)photolytic degradation



Appendix:

Figure-I: Nefopam (NP) Mass spectra – 254.22$(M+H)^+$.

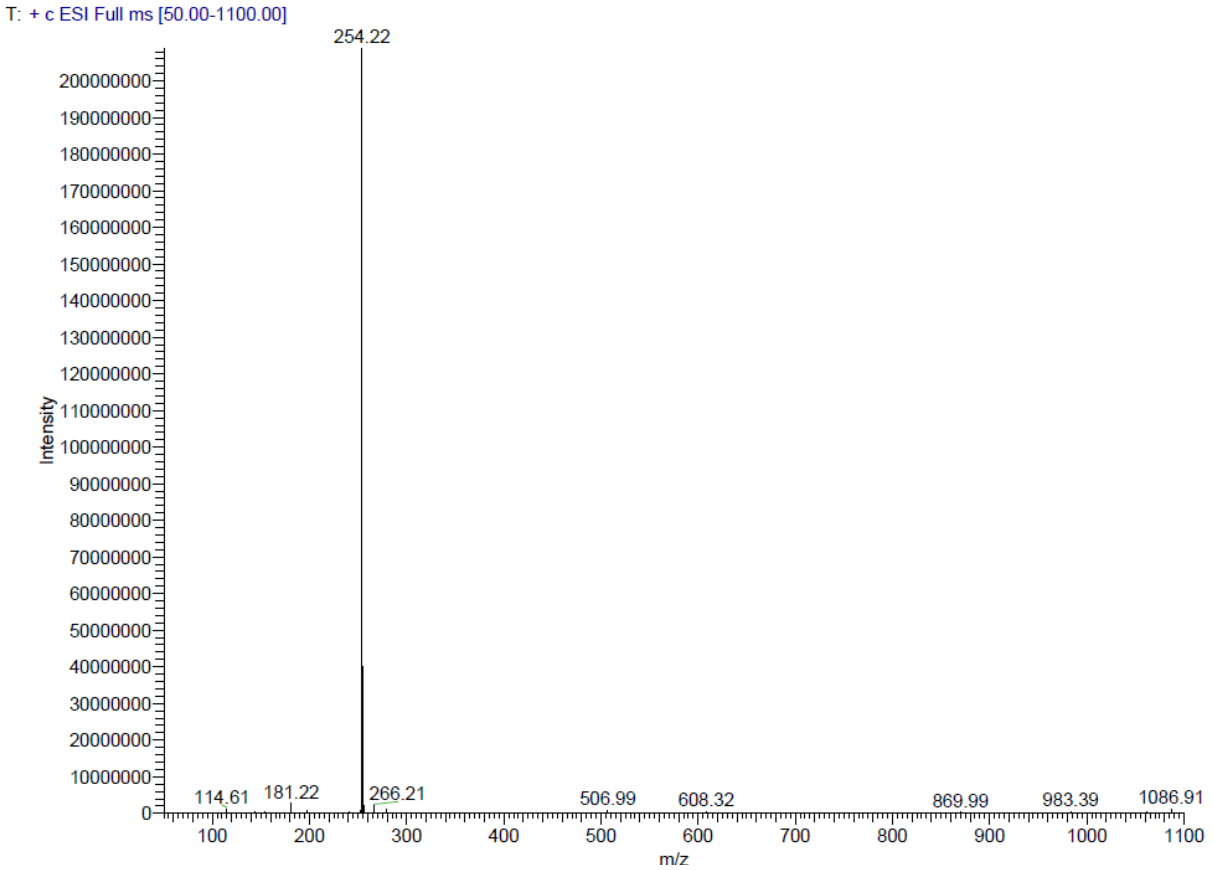


Figure II: Nefopam (NP)-IR spectra.

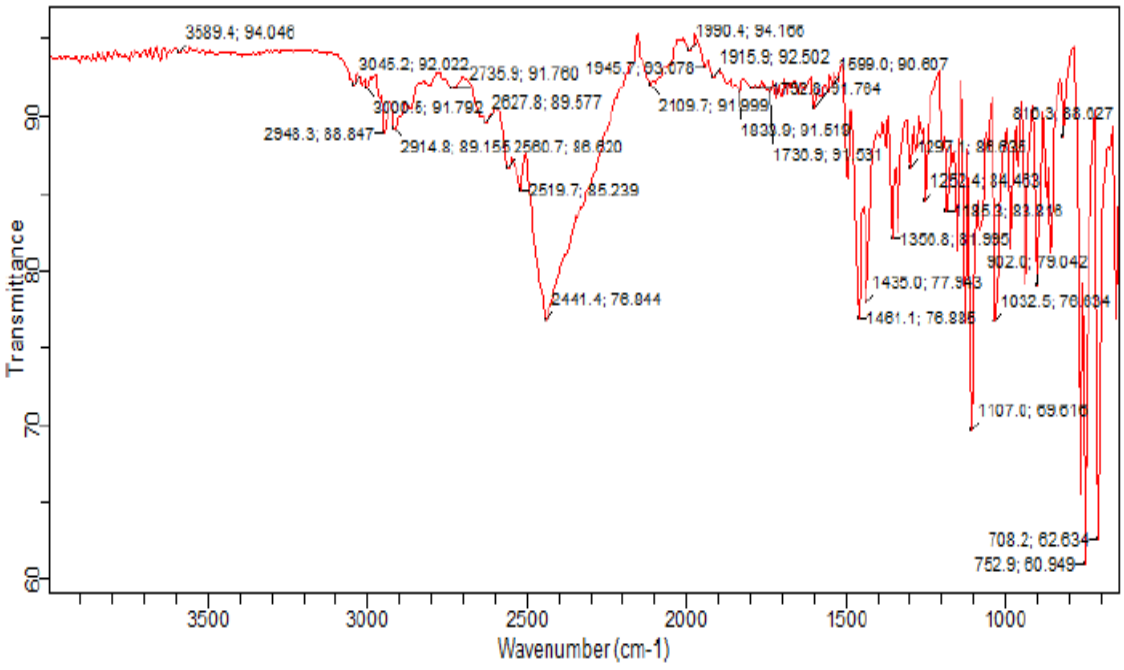


Figure-III: Nefopam (NP)-1H NMR spectra.

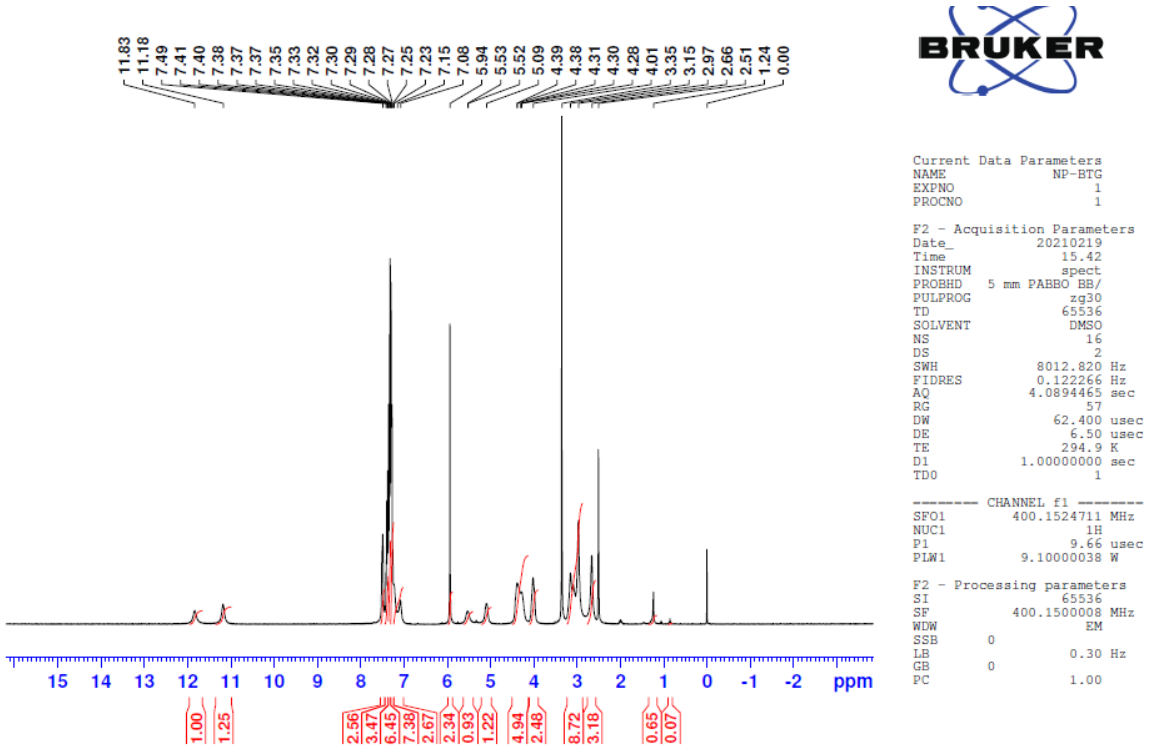


Figure-IV: Nefopam Imp-1 Mass spectra – 122.1(M+H) $^{+}$

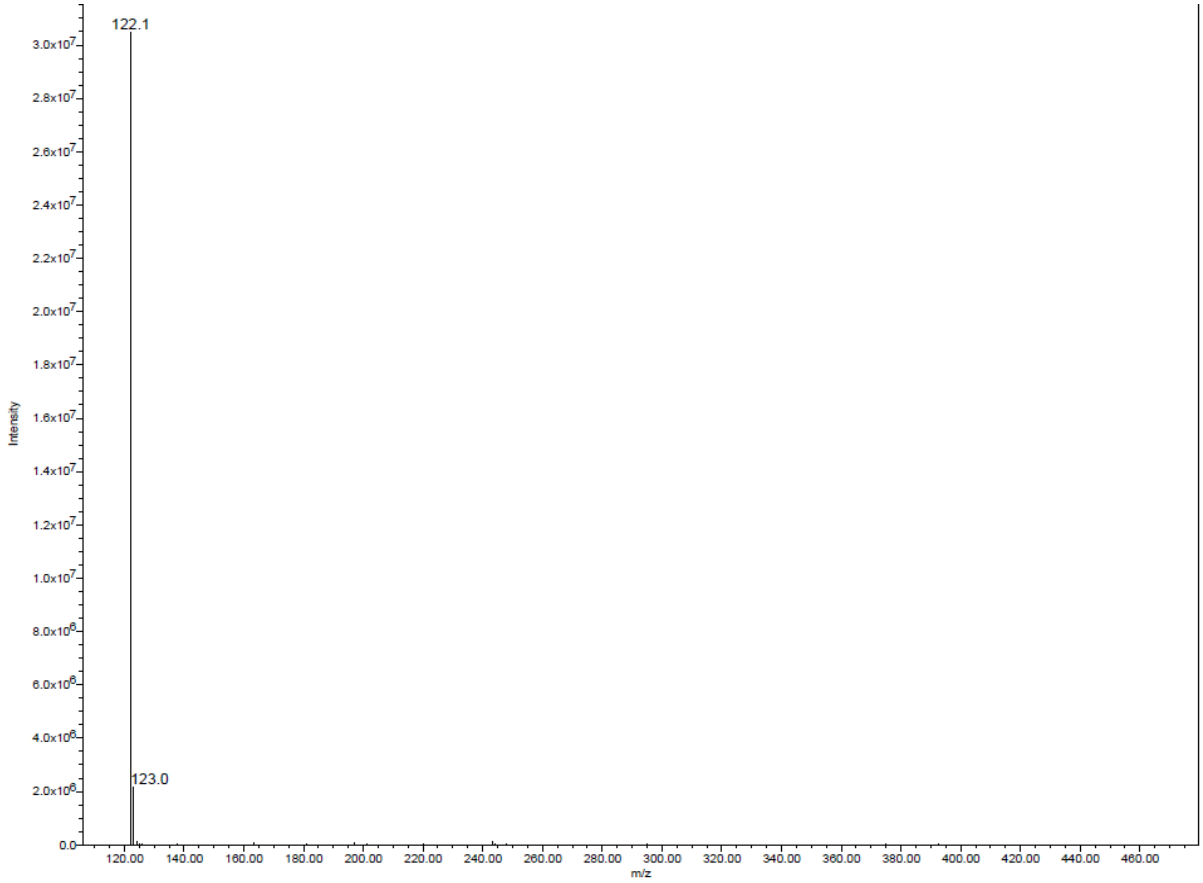


Figure-V: Nefopam Imp-1-IR spectra.

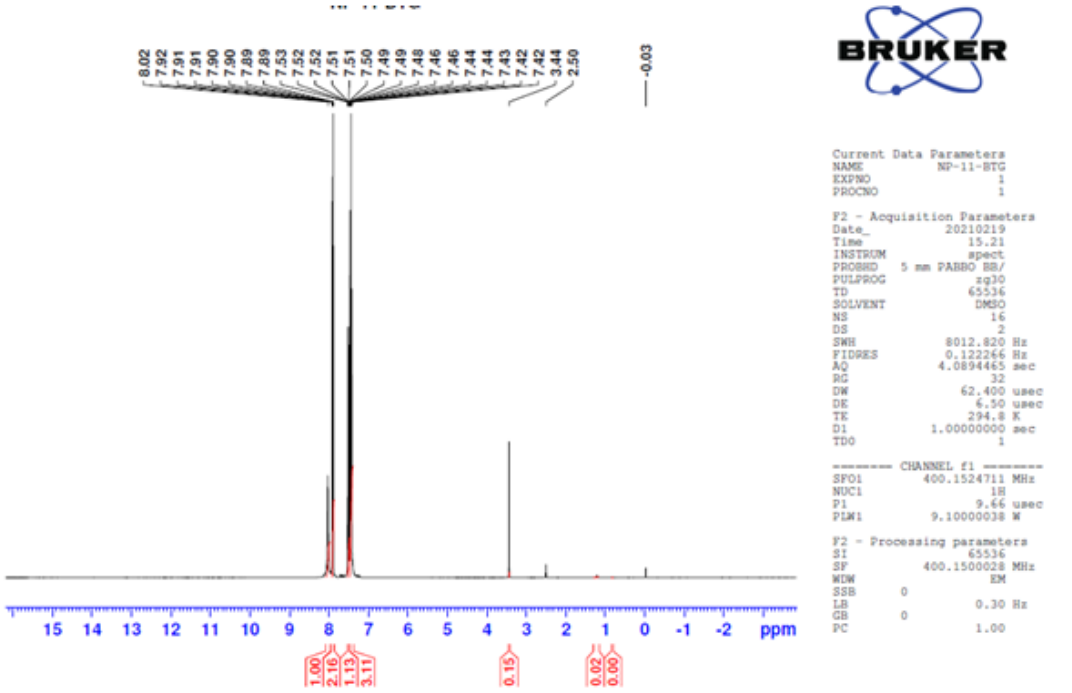


Figure-VI: Nefopam Imp-1-1H NMR spectra.

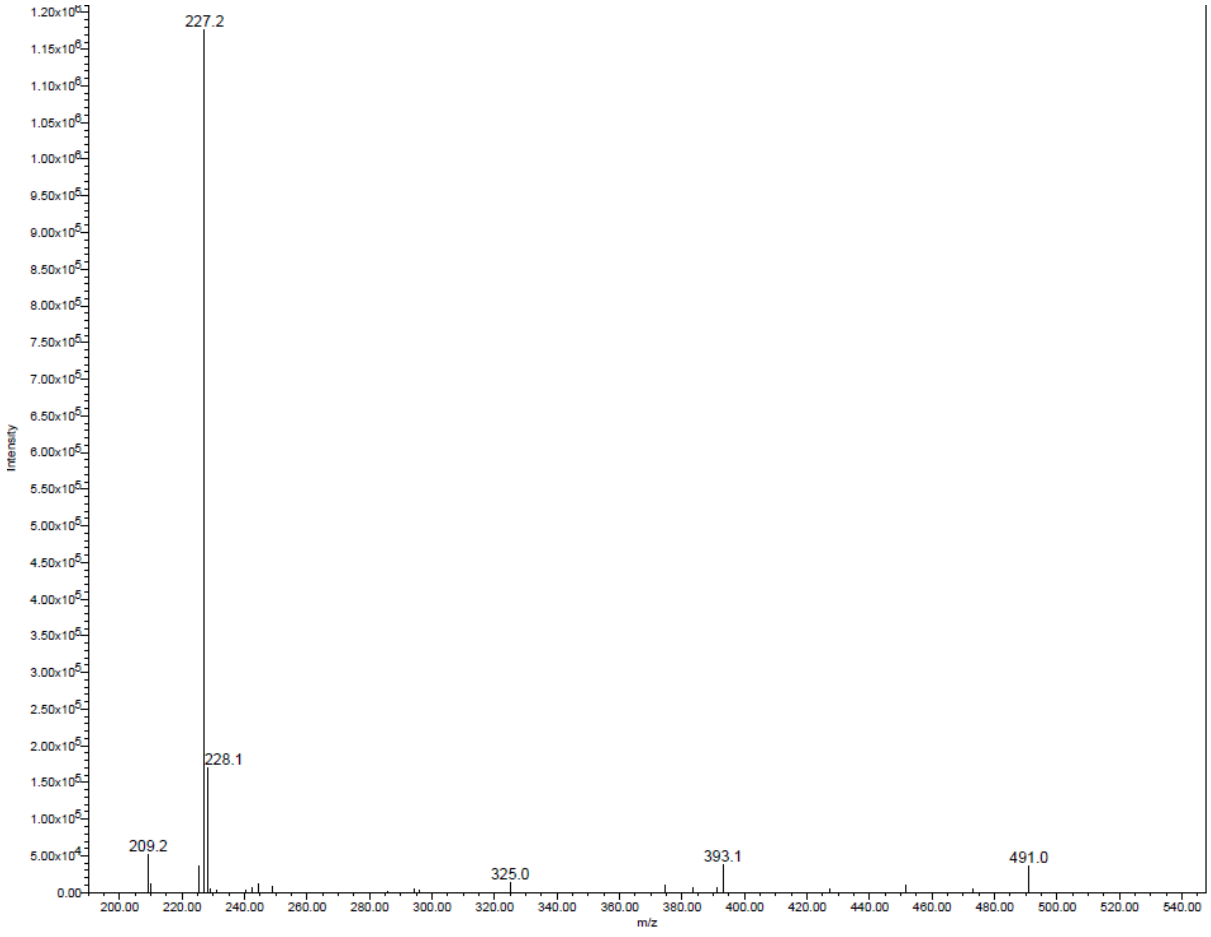


Figure VII: Nefopam Imp-2 Mass spectra –227.2(M+H)$^+$.

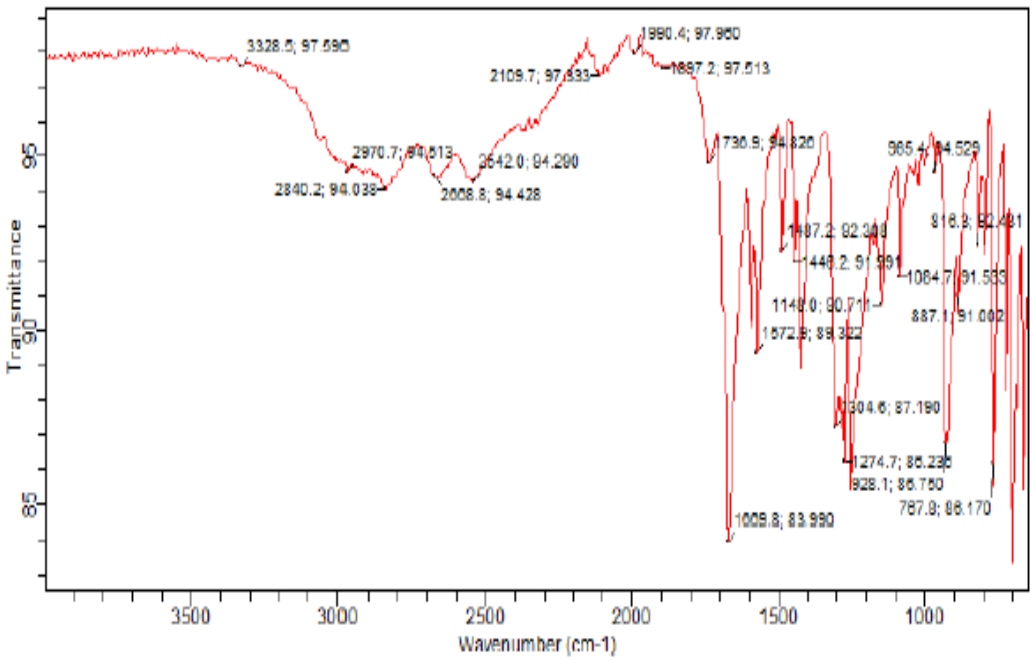


Figure VIII: Nefopam Imp-2-IR spectra.

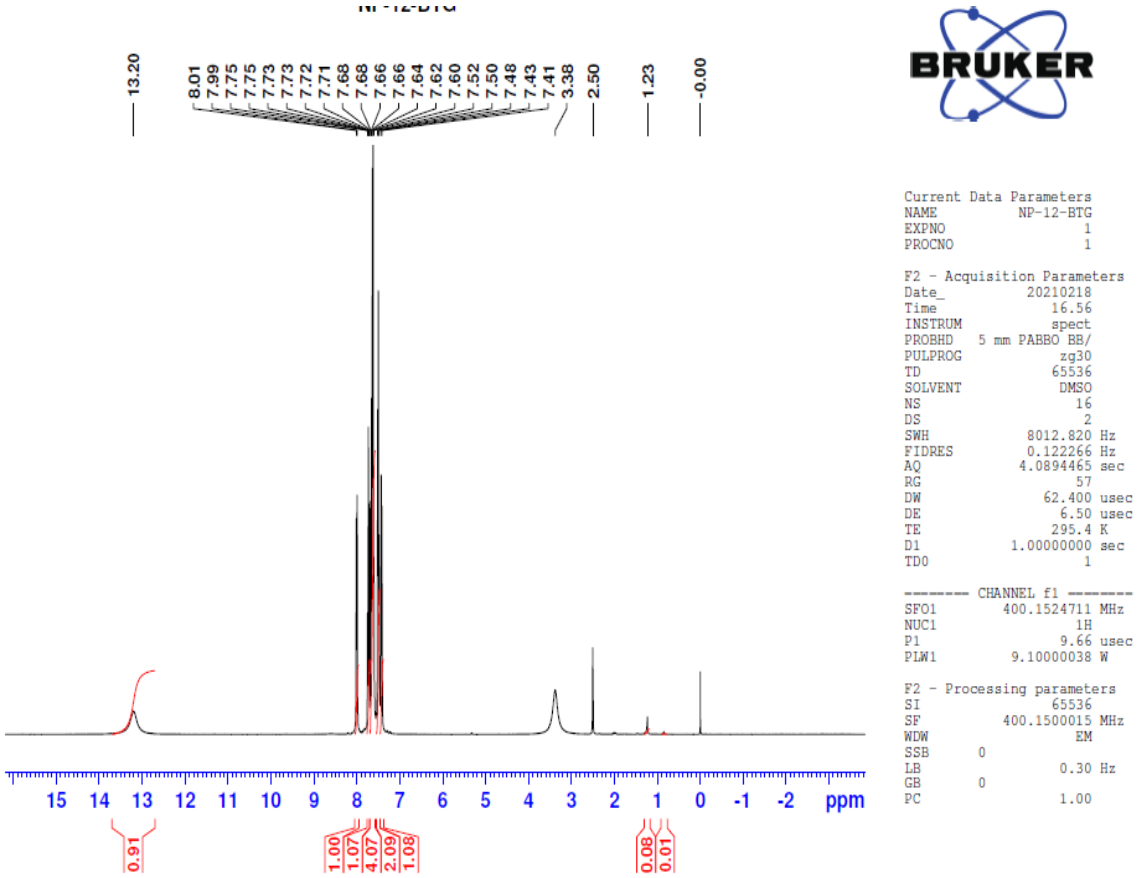


Figure IX: Nefopam Imp-2-1H NMR spectra.

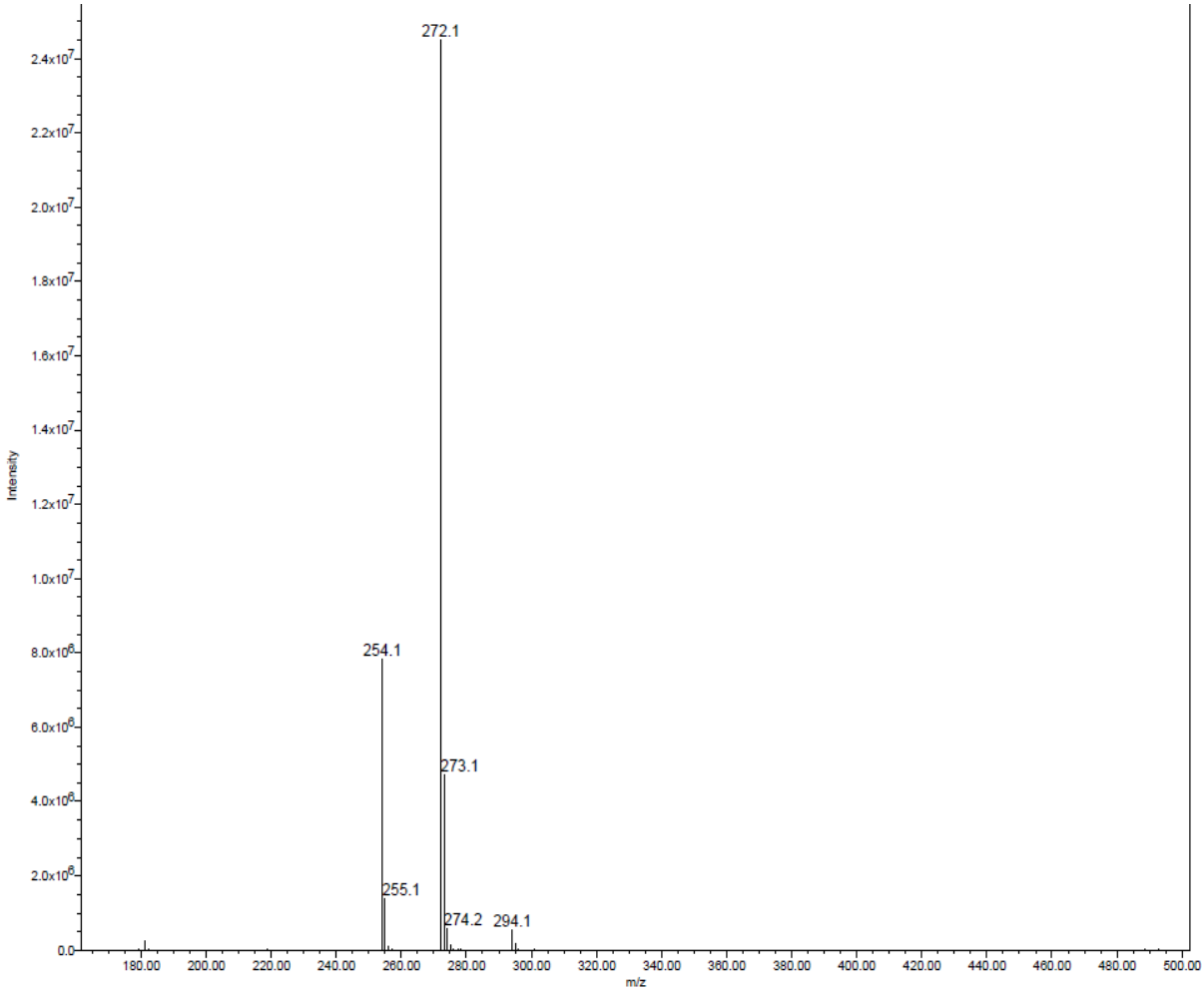


Figure X: Nefopam Imp-3- Mass spectra –272.1(M+H)$^+$.

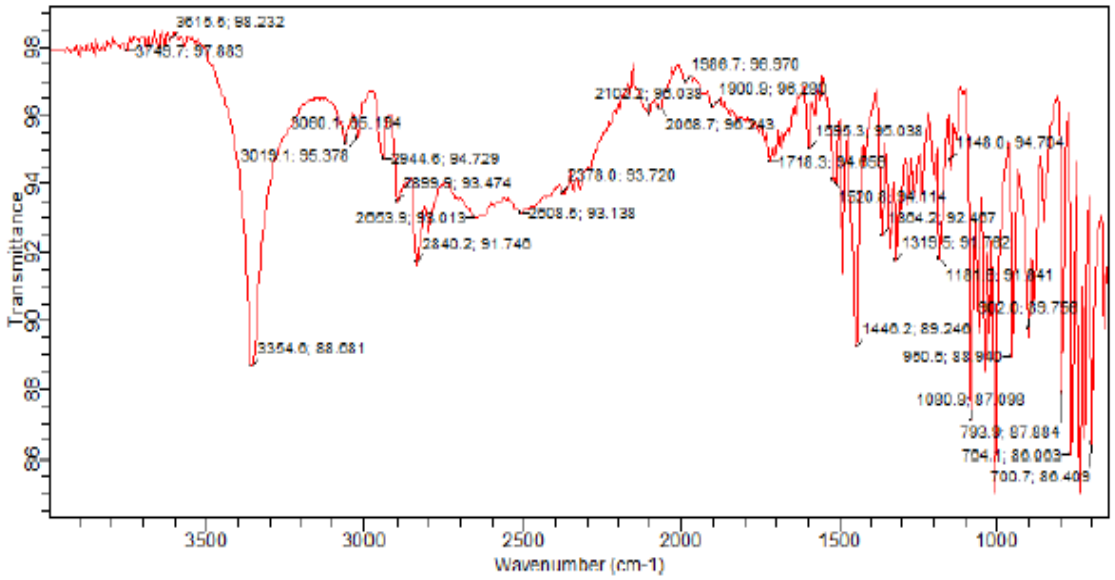


Figure XI: Nefopam Imp-3-IR spectra.

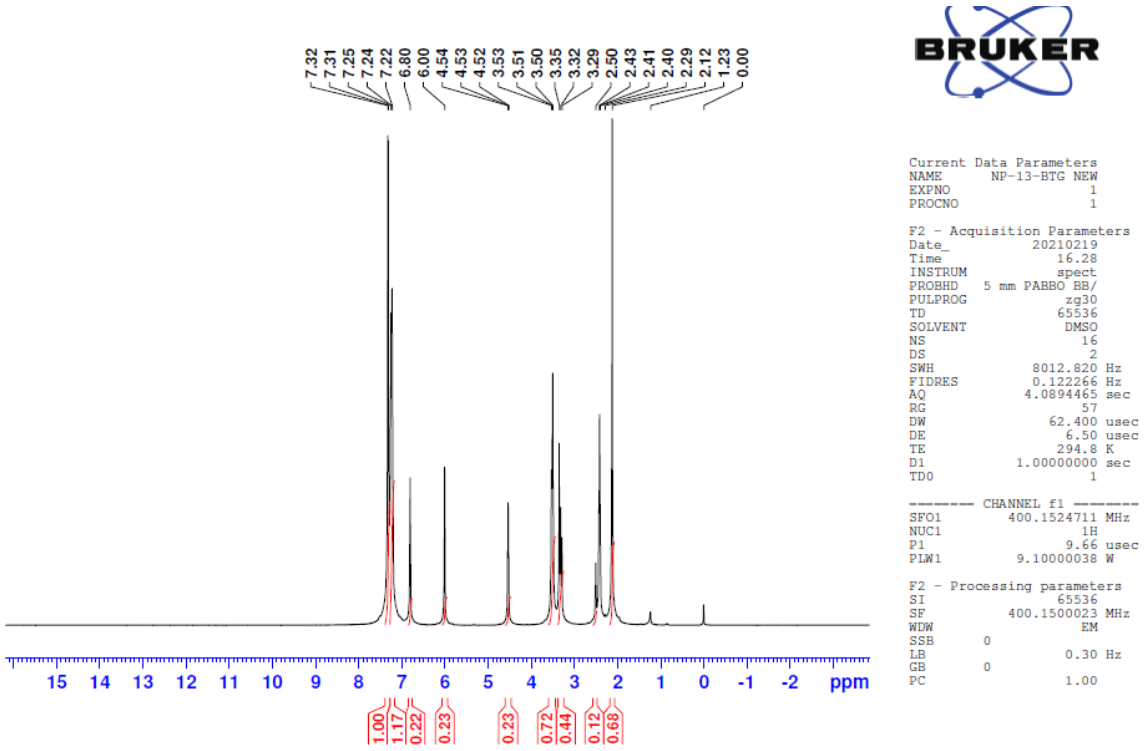


Figure XII: Nefopam Imp-3-1H NMR spectra.

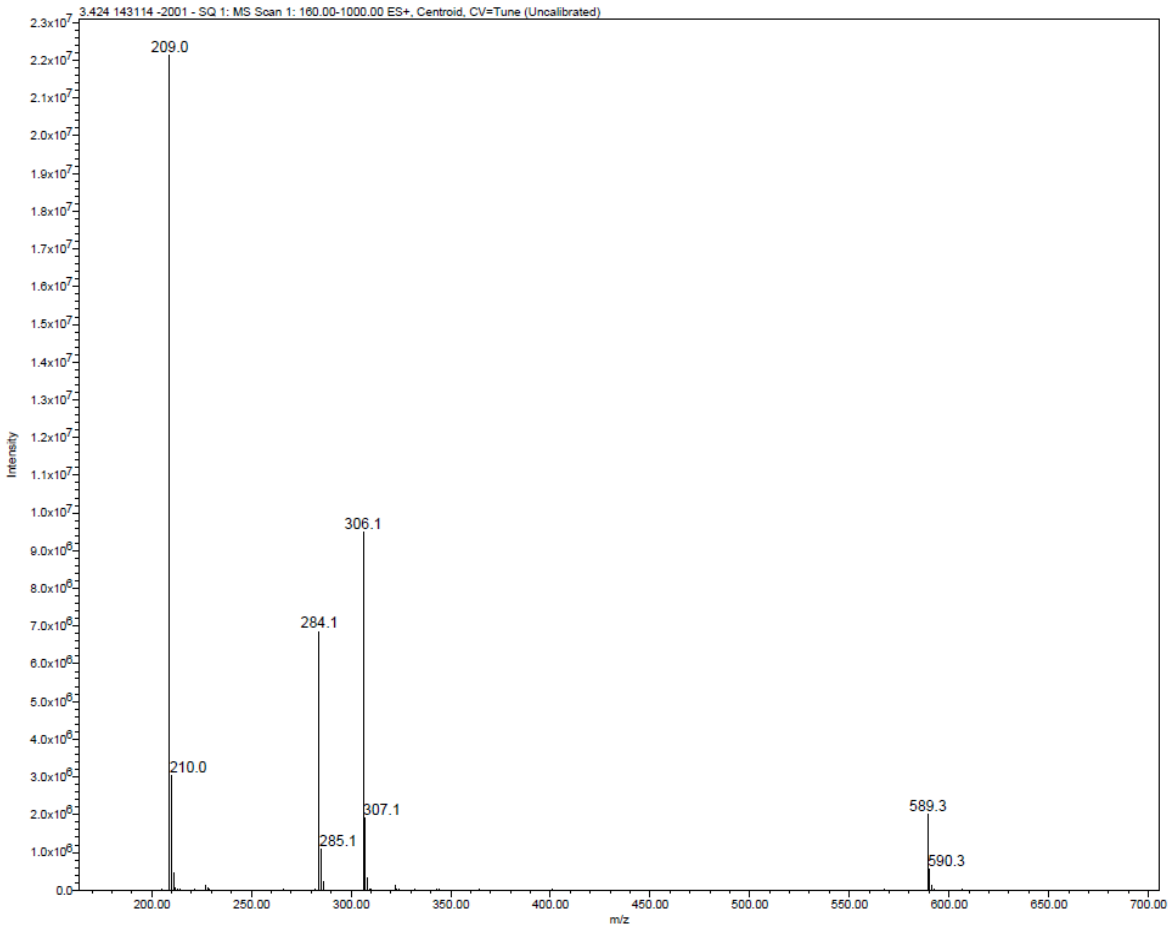


Figure XIII: Nefopam Imp-4- Mass spectra –284.1(M+H)$^+$.

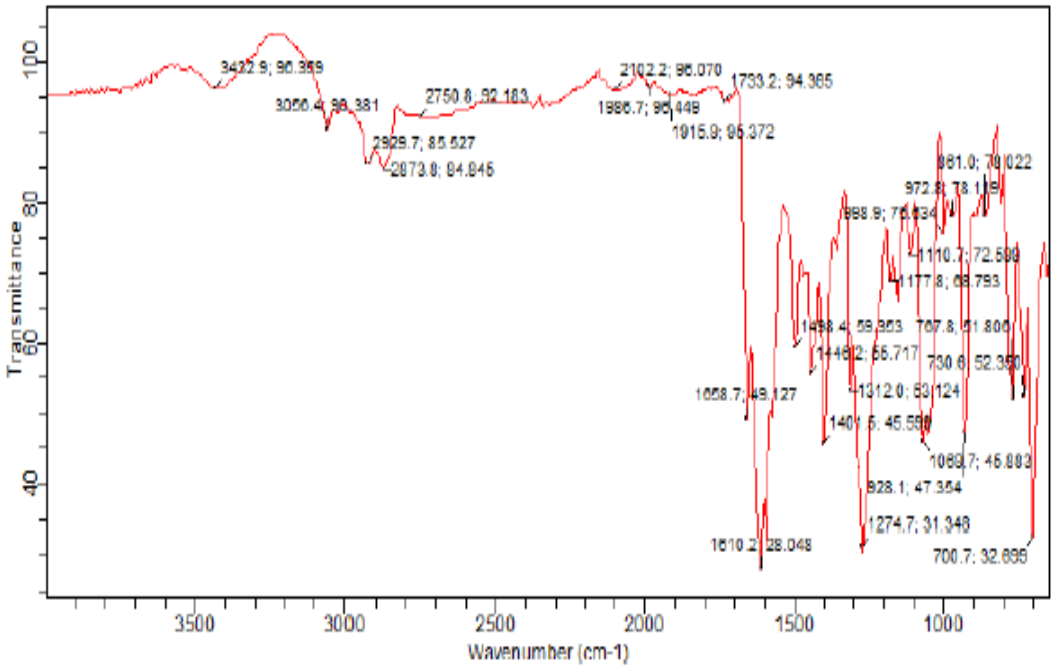


Figure-XIV: Nefopam Imp-3-IR spectra.

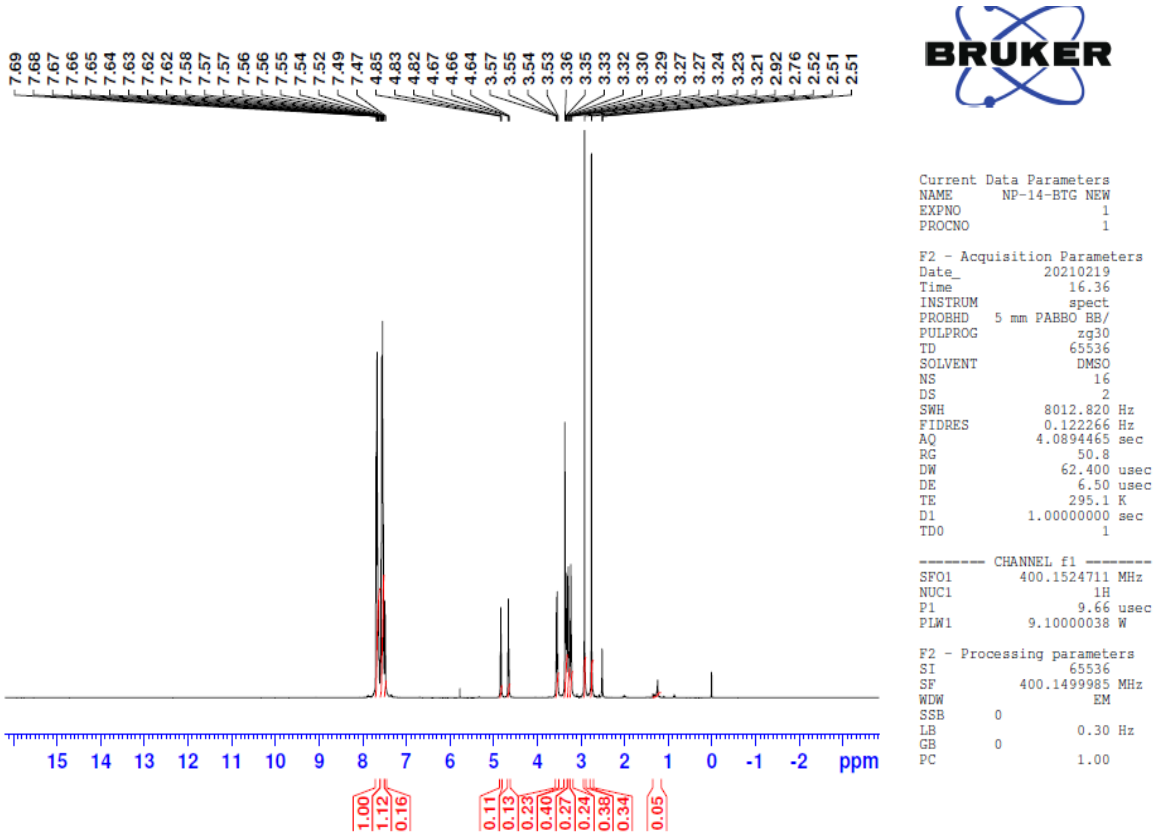


Figure XV: Nefopam Imp-4-1H NMR spectra.

CONCLUSION

The developed HPLC method provides reliable, reproducible, accurate and sensitive for the quantification of Nefopam related substances. This newly developed method has been validated as per regulatory requirements and has shown acceptable precision, accuracy and adequate sensitivity. This method can be used for the routine analysis of Nefopam active pharmaceutical ingredient related substances.

ACKNOWLEDGEMENTS

The authors are thankful to the management of Analytica Chemie Inc for providing necessary facilities for carrying out this work.

CONFLICT OF INTEREST

None.

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