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RESEARCH ARTICLE
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ANALYTICAL DEVELOPMENT AND VALIDATION OF A LC-MS METHOD FOR ESTIMATION OF N-NITROSO DICLOFENAC IN DICLOFENAC SODIUM GEL 3%

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ABSTRACT
A new liquid chromatography-mass spectrometry (LC-MS) method that is selective and sensitive was developed and validated for the identification and quantification of N-Nitroso Diclofenac Impurity in Diclofenac Sodium Gel by LC-MS. The method is specific, accurate and precise. Chromatographic separation was accomplished on a symmetry C18 column 250x4.6mm, 5µ, using gradient type of separation. In which two different composition of mobile phase were used i.e. Mobile Phase A is a mixture of buffer (ammonium formate and formic acid) and acetonitrile (75:25) whereas Mobile Phase B is Acetonitrile (100%) with run time of 35 minutes. Linearity range of 0.000048 to 0.010055 µg/mL and 0.06 to 12.57 µg/mL (concentration with respect to sample). The standard solution of N-Nitroso Diclofenac is stable up to 44 hours and sample solution of Diclofenac Sodium Gel 3% is stable for 35 minutes. The method is validated according to ICH Q2 (R1).

Keywords: N-Nitroso Diclofenac Impurity, Diclofenac Sodium, LC-MS, ICH, Validation.

INTRODUCTION
Diclofenac is a non-steroidal anti-inflammatory drug (NSAID). Diclofenac is a derivative of phenyl acetic acid, is a strong inhibitor of the activity of the cyclooxygenase enzyme. It is also used in rheumatic disorders, including osteoarthritis, ankylosing spondylitis, and rheumatoid arthritis.[1]

The health and safety of people are seriously jeopardized by nitrosamine contaminants found in medication items. Regulatory organizations and manufacturers are extremely concerned when even trace amounts of nitrosamine contaminants are found in pharmaceutical products. In order to comply with regulatory standards, the detection and quantification of these nitrosamines in APIs and medicinal products can be difficult and need the use of sophisticated and delicate instruments.[2]

The method is simple and with small run time.

Nitrosamine Impurity- Nitrites and other nitrogen-containing compounds react with secondary carbamates of amides, amines and urea derivatives to generate nitrosamine. A drug may include nitrosamine for several reasons. The production and packaging of pharmaceuticals is the source of nitrosamine. Nitrosamine may be present in other active ingredients and pharmaceutical products

due to the use of processes and materials that may be sensitive to nitrosamine. We have general methods for detecting nitrosamine impurities: GC-MS, LC-MS/MS.[3,4]

Table 1: Acceptance Intake Limit[3,4]

Nitrosamine	Limit (ng/day)
NDMA	96
NDEA	26.5
NDIPA	26.5
NIPEA	26.5
NDBA	26.5

MATREIALS AND METHODS

Table 2 (a): Chromatographic Method

Equipment	Shimadzu LC-MS 8050
Polarity	Positive
Acquisition Mode	MRM
Column	Kromasil C18 250 X 4.6 mm, 5µ
Column Oven Temperature	45ºC
Mobile Phase (Pump A)	Buffer : Acetonitrile (75:25)
Mobile Phase (Pump B)	Acetonitrile
Injection Volume	80 µL
Flow rate	0.7 mL/min
Auto sampler Temperature	10ºC
Run Time	35 minutes
Mode	Binary Gradient

Table 2 (b): Mass Method

Start Time	16.0 min
End Time	20.0 min
Event Time	0.409 sec
Q1 Resolution	Unit
Q3 Resolution	Unit
Interface	ESI
Interface Heater	ON
Interface Temperature	400ºC
Desolvation Temperature	650ºC
DL Temperature	250ºC
Nebulizing gas flow	3.00 L/min
Heating gas	ON
Heating gas flow	10.00 L/min
Heat Block	400ºC
Drying gas	ON
Drying gas flow	10.00 L/min
Probe Position	4

Table 2 (c): Gradient Program

TOTAL TIME (min)	FLOW RATE (mL/min)	MOBILE PHASE A (%)	MOBILE PHASE B (%)
0.01	0.7	100.0	0.0

3.00	0.7	100.0	0.0
7.00	0.7	75.0	25.0
15.00	0.7	50.0	50.0
20.00	0.7	35.0	65.0
25.00	0.7	35.0	65.0
27.00	0.7	0.0	0.0
35.00	0.7	0.0	0.0

Table 2 (d): Volco Valve Conditions

TOTAL TIME (min)	MODULE	COMMAND	VALUE
0.01	Column Oven	Oven Valve 2	0
16.0	Column Oven	Oven Valve 2	1
20.0	Column Oven	Oven Valve 2	0
35.0	Controller	Stop	
1: To Mass & 0: To Waste			

Table 2 (e): MRM Conditions

ANALYTE NAME		PRECURSOR m/z	PRODUCT m/z	DWELL (msec)	Q1 PRE BIAS (V)	CE (V)	Q3 PRE BIAS (V)
N-NITROSO DICLOFENAC	QUANTIFIER	294.90	242.10	200	-36.0	-18.0	-26.0
	QUALIFIER	294.90	214.05	200	-16.0	-26.0	-16.0

Reagents and Chemicals

Ammonium formate, formic acid, acetonitrile, methanol and glacial acetic acid used were of LCMS grade and were used for preparation of buffer, mobile phases and diluent.

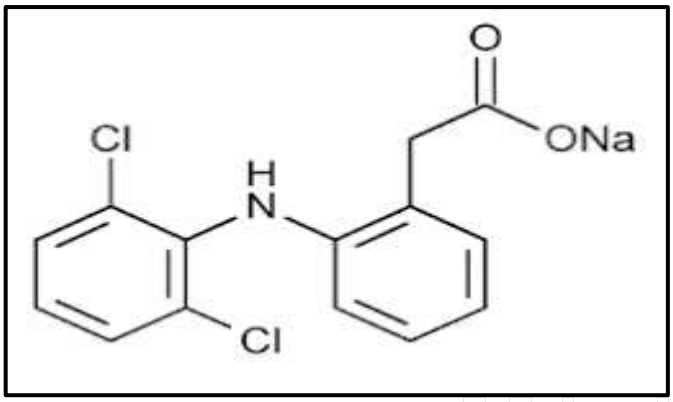

Figure 1 (a): Diclofenac Sodium $C_{14}H_{10}Cl_2NNaO_2$

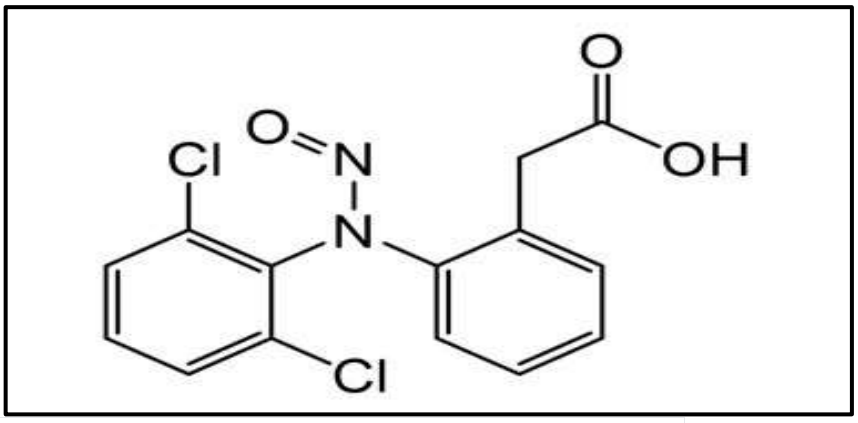

Figure 1(b): N-Nitroso Diclofenac Impurity Structure $C_{14}H_{10}Cl_2N_2O_3$

Preparation of Mobile Phase
Preparation of Buffer: Weigh and transfer about 0.126 g of Ammonium Formate and 0.5 mL of Formic Acid in 1000 mL of LCMS grade water.

Mobile Phase A: Mix Buffer and Acetonitrile in the ratio 75:25 (v/v).
Mobile Phase B: LCMS grade Acetonitrile.
Diluent: Mixture of LCMS grade Methanol and Water in the ratio 65:35 (v/v) and add 0.1% Acetic Acid.

Preparation of Standard Stock Solution
Weigh and transfer about 10 mg of N-Nitroso Diclofenac standard into 10 mL volumetric flask, add 5 mL methanol, sonicate till complete solubility and make up the final volume with methanol (Concentration 1000 ppm).

Preparation of Standard Stock Solution-I:
Transfer 1.0 mL of N-Nitroso Diclofenac standard stock solution (1000 µg/mL) into a 100 mL volumetric flask, add 5 mL diluent and make up the final volume with diluent, vortex well and label (Concentration 10 µg/mL).

Preparation of Standard Stock Solution-II
Transfer 1.0 mL of standard stock solution-I (10 µg/mL) into 10 mL volumetric flask, add 5 mL diluent and make up the final volume with diluent, vortex well and label (Concentration 1.0 µg/mL).

Preparation of Standard Stock Solution-III
Transfer 1.0 mL of standard stock solution-II (1.0 µg/mL) into 50 mL volumetric flask, add 5 mL diluent and make up the final volume with diluent, vortex well and label (Concentration 0.020 µg/mL)

Preparation of Standard Stock Solution-IV
Transfer 5.0 mL of standard stock solution-IV (0.020 µg/mL) into 50 mL volumetric flask, add 5 mL diluent and make up the final volume with diluent, vortex well and label (Concentration 0.0020 µg/mL).

Preparation of Sample Solution
Approximately weigh sample equivalent to 8 mg into a 15 mL centrifuge tube. Add 10 mL diluent, vortex for 2 minutes followed by 2 minutes sonication. Mix well and filter the solution through 0.22 µ hydrophilic PVDF syringe filter and collect in HPLC vial without discarding any volume.

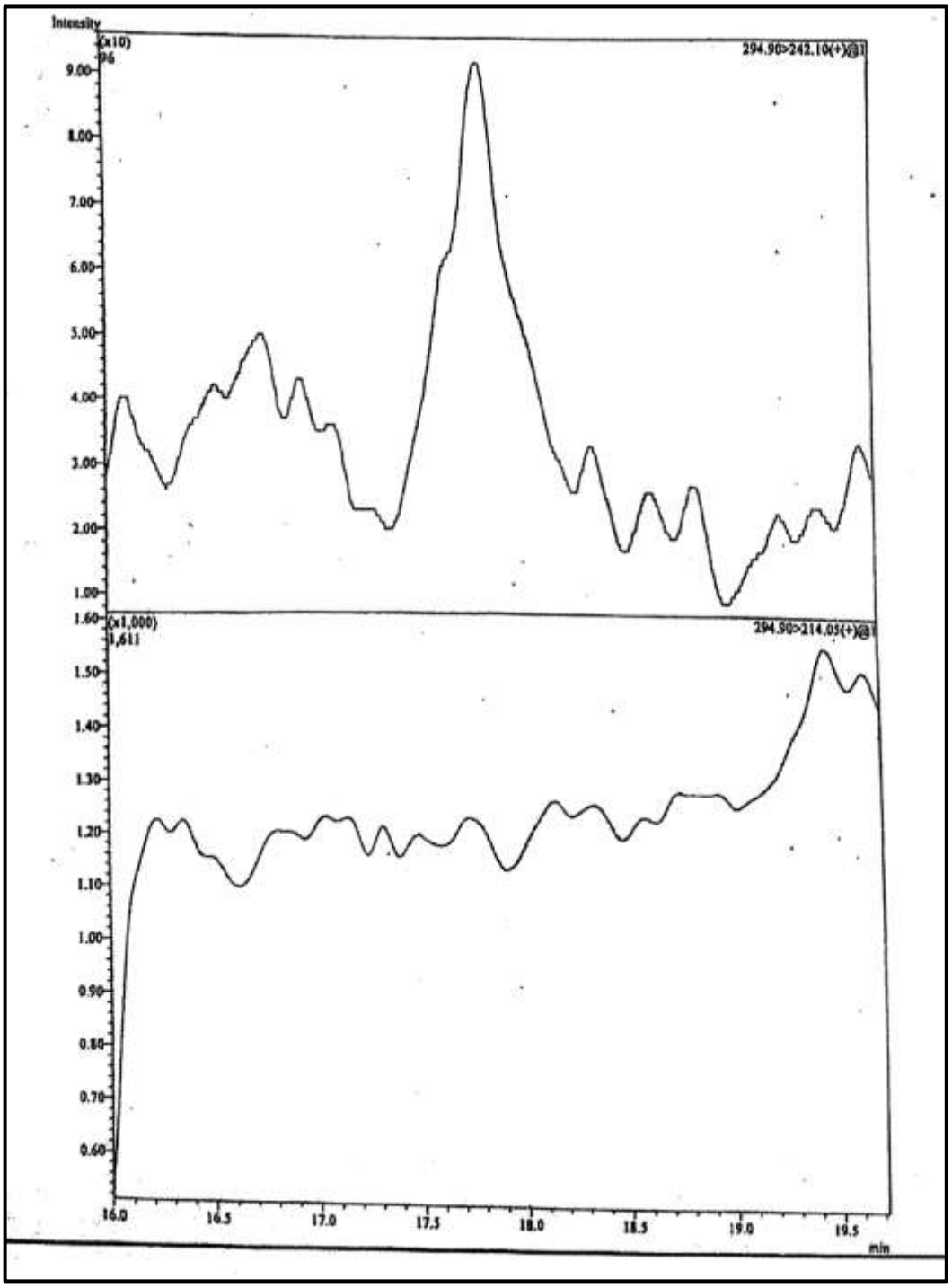

Figure 2 (a): Chromatogram of Blank

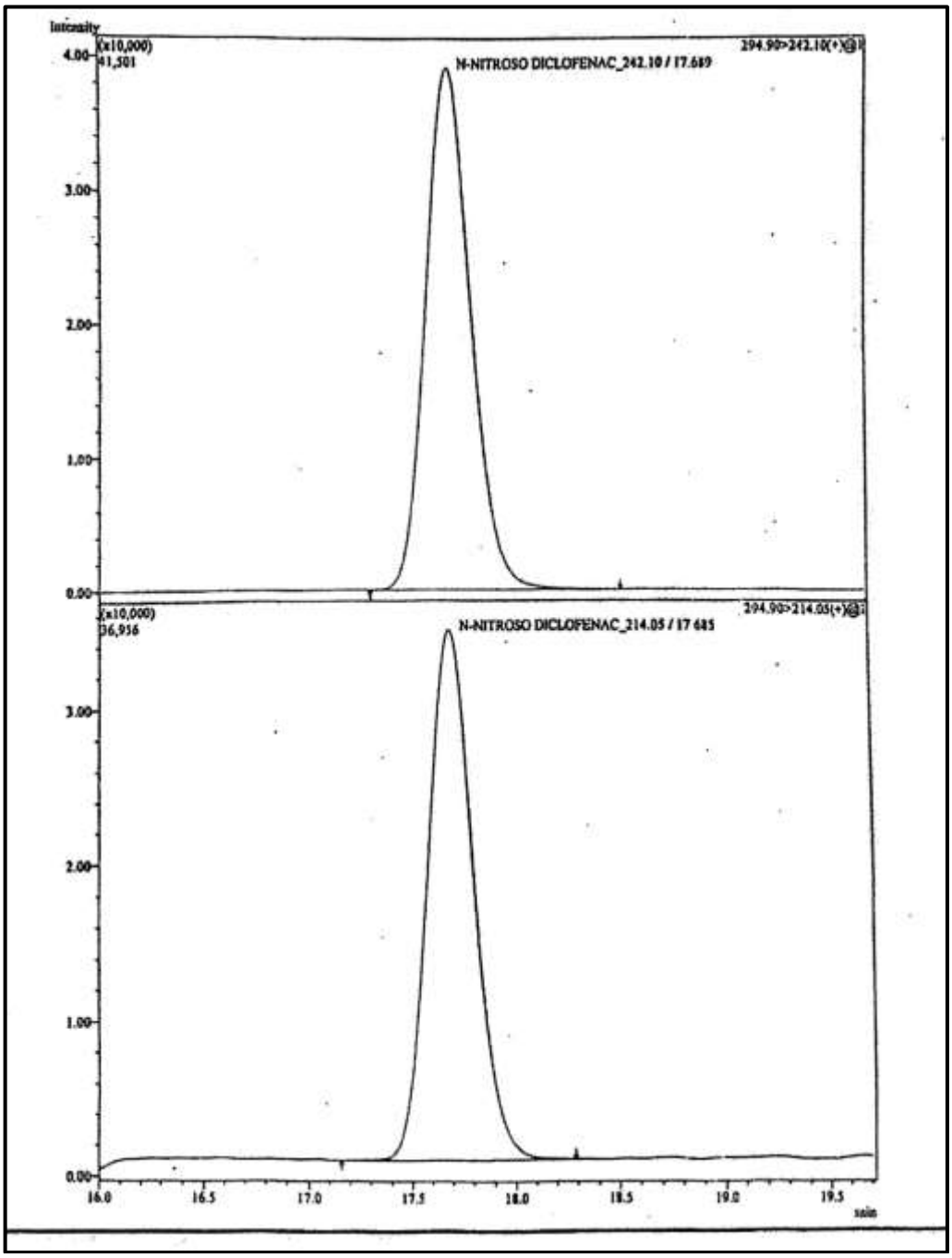

Figure 2 (b): Chromatogram of Standard Solution (N-Nitroso Diclofenac)

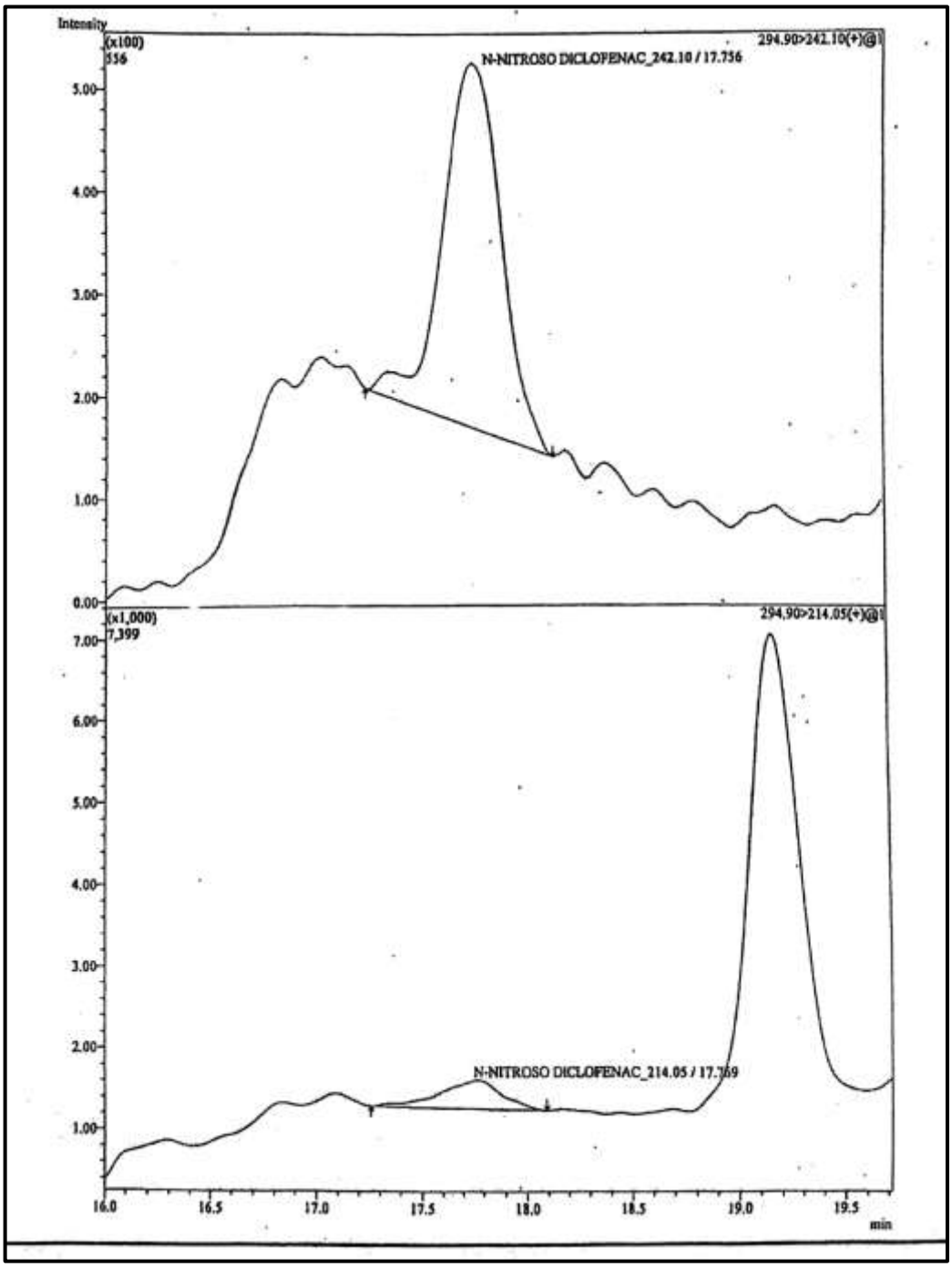

Figure 2 (c): Chromatogram of Sample (Diclofenac Sodium Gel 3%) As Such Solution

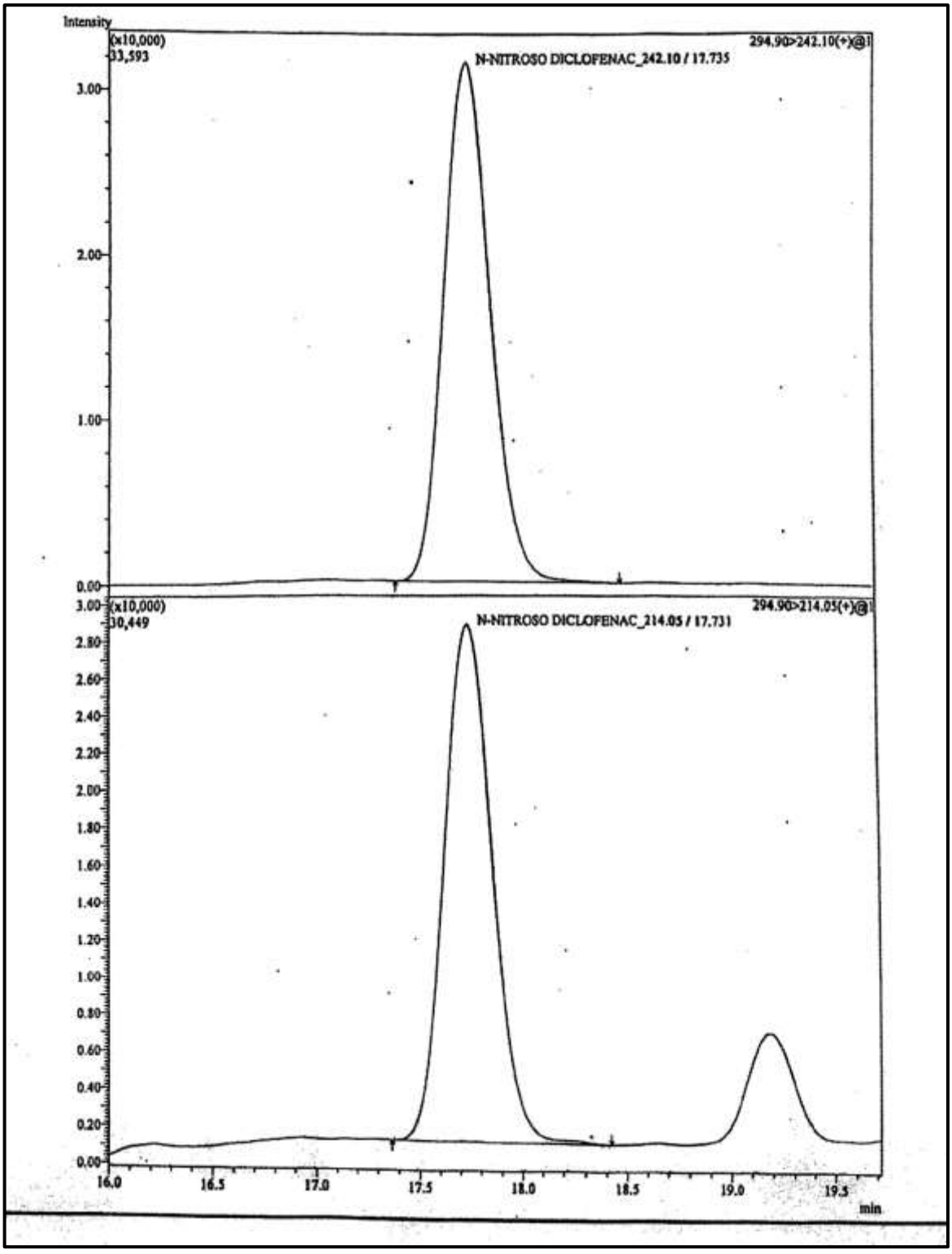

Figure 2 (d): Chromatogram of Sample (Diclofenac Sodium Gel 3%) Spike Solution

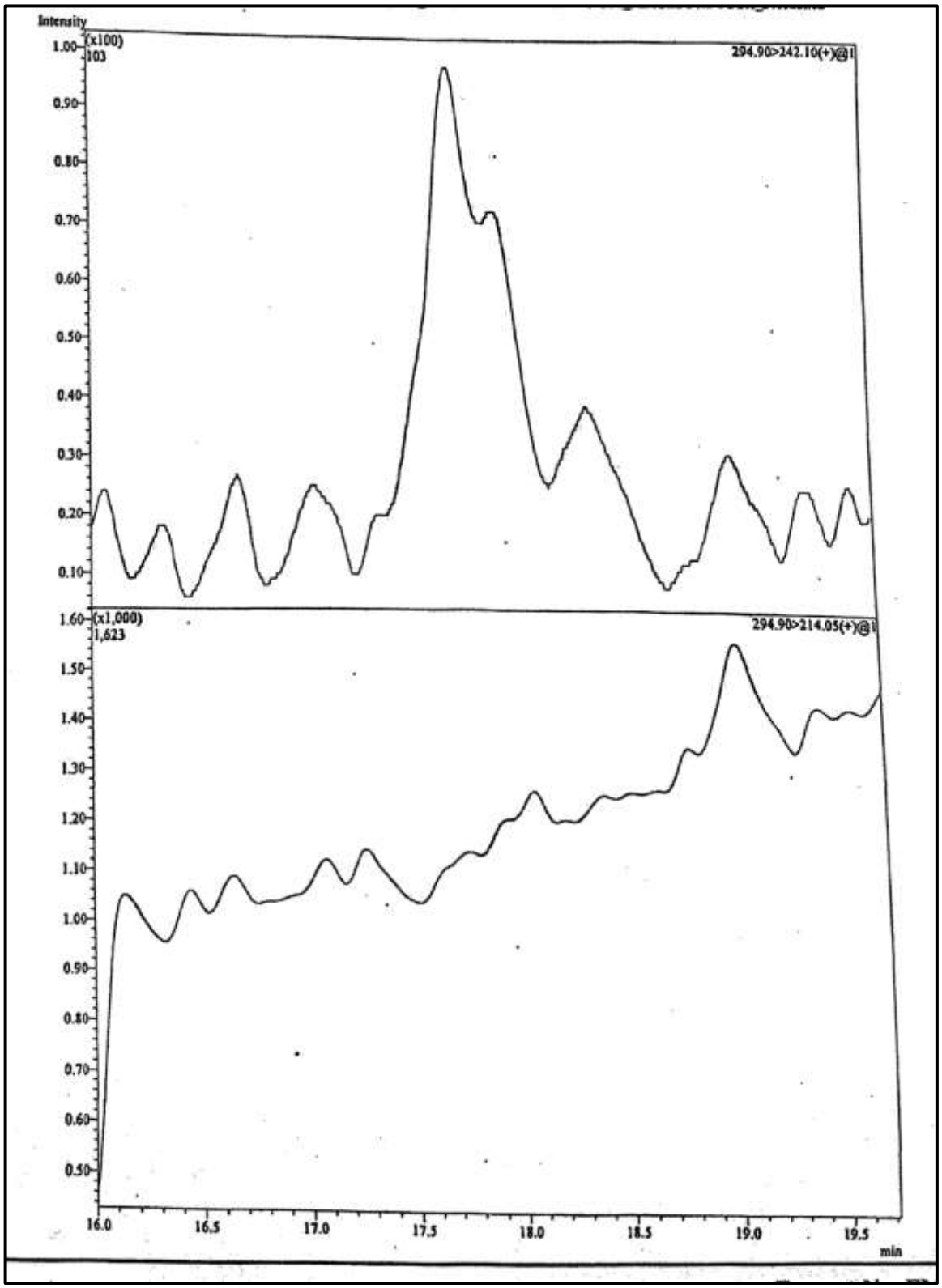

Figure 2 (e): Chromatogram of Placebo As Such Solution

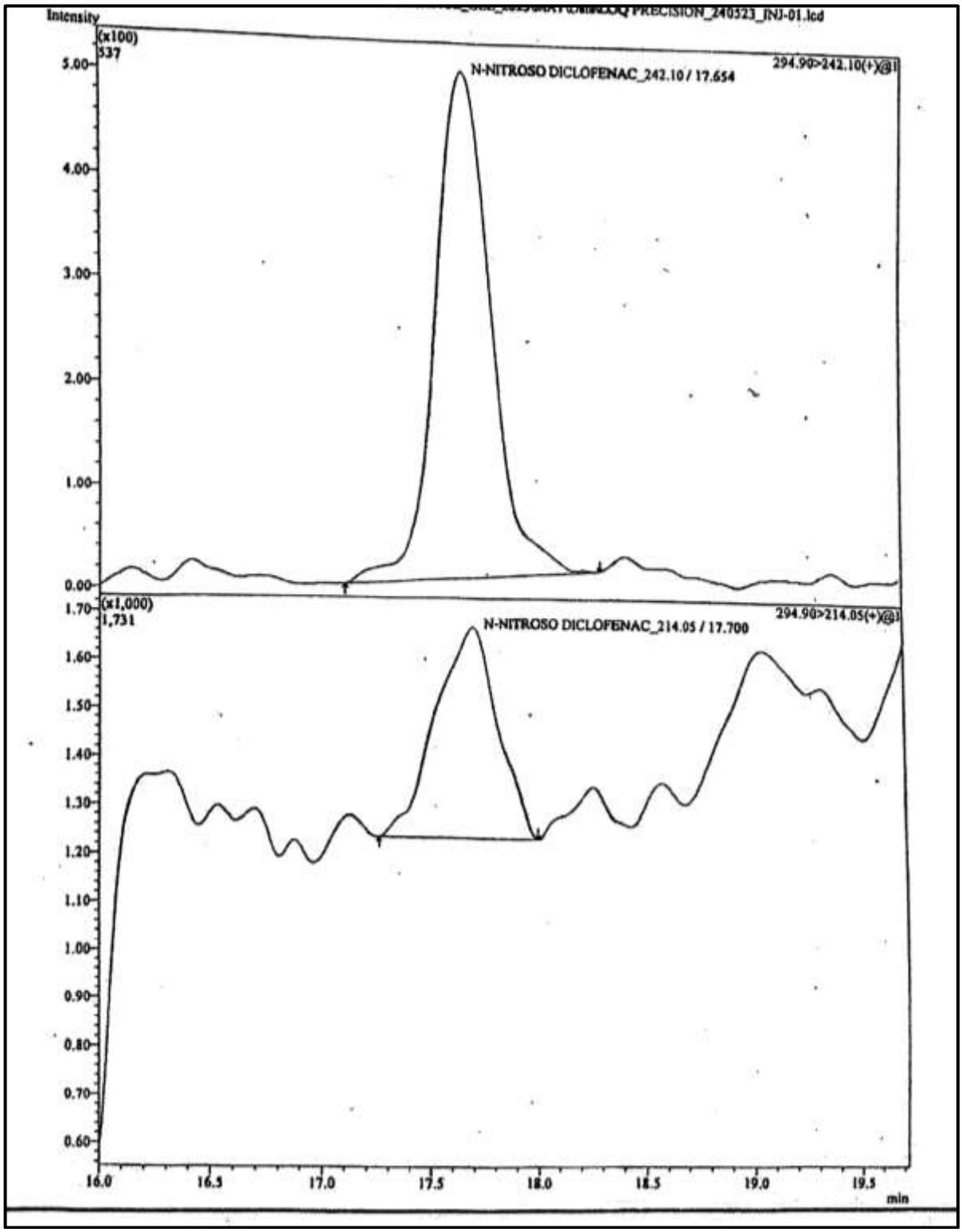

Figure 2 (f): Chromatogram of Placebo Spike Solution

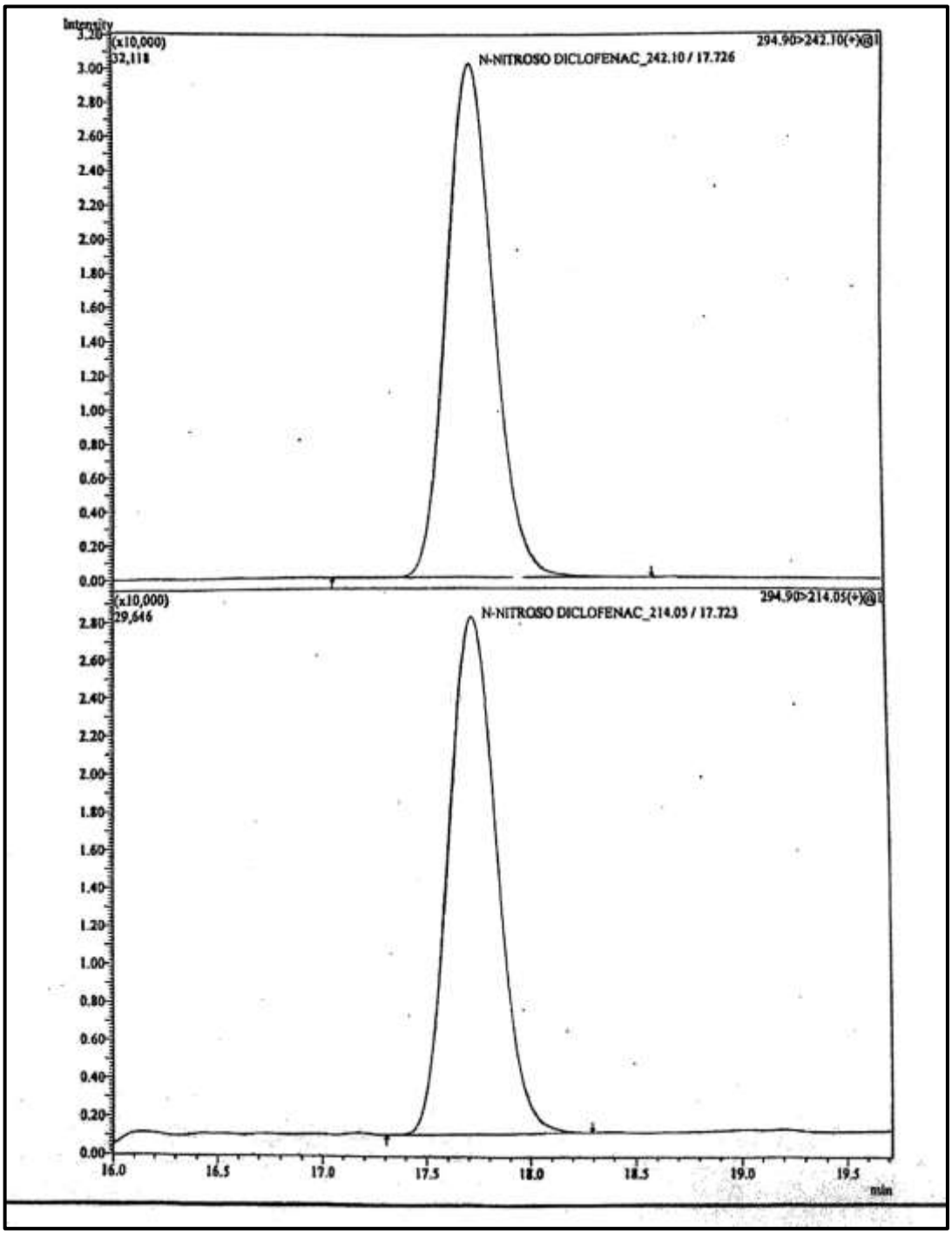

Figure 2 (g): Chromatogram of LOQ Solution

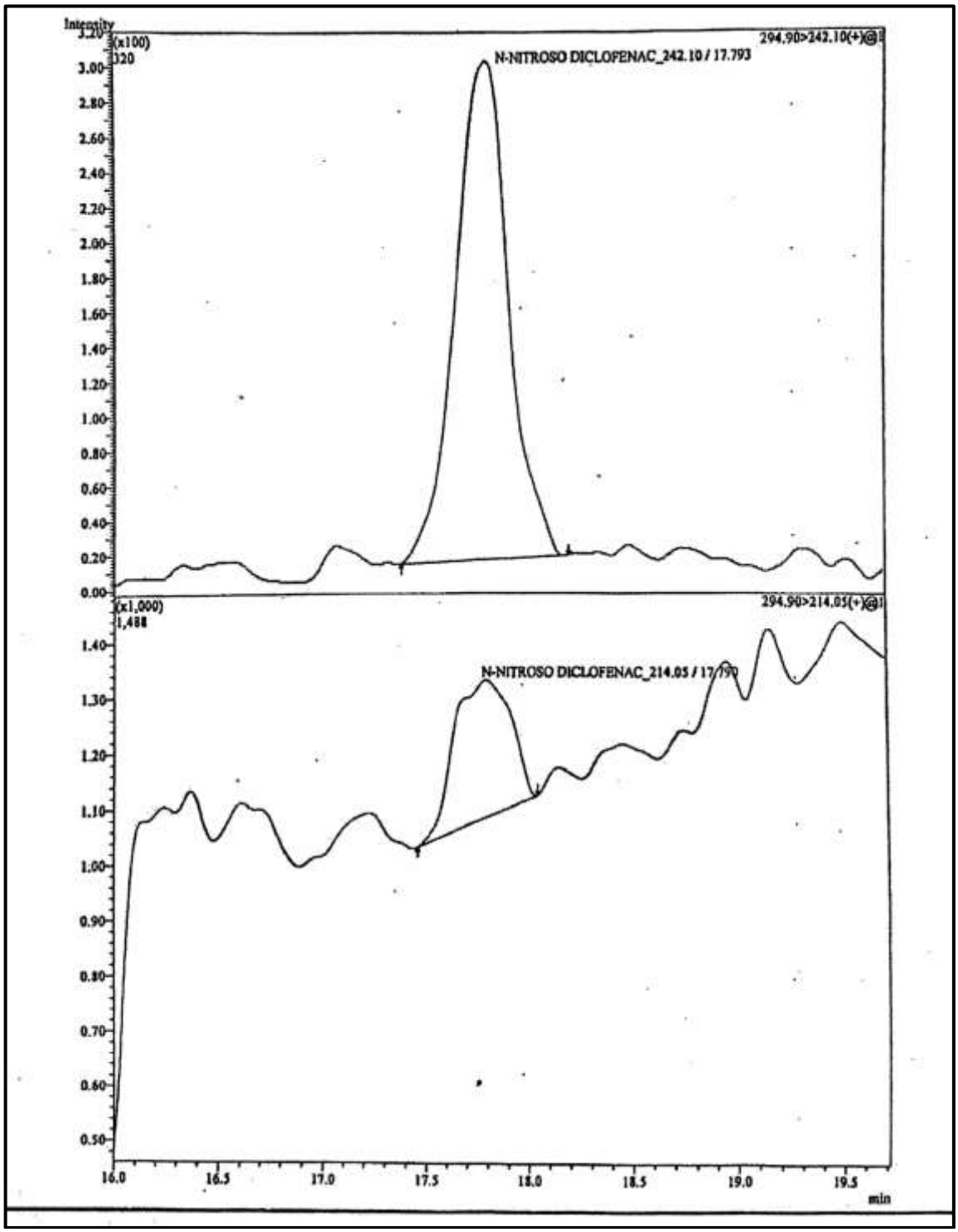

Figure 2 (h): Chromatogram of LOD solution

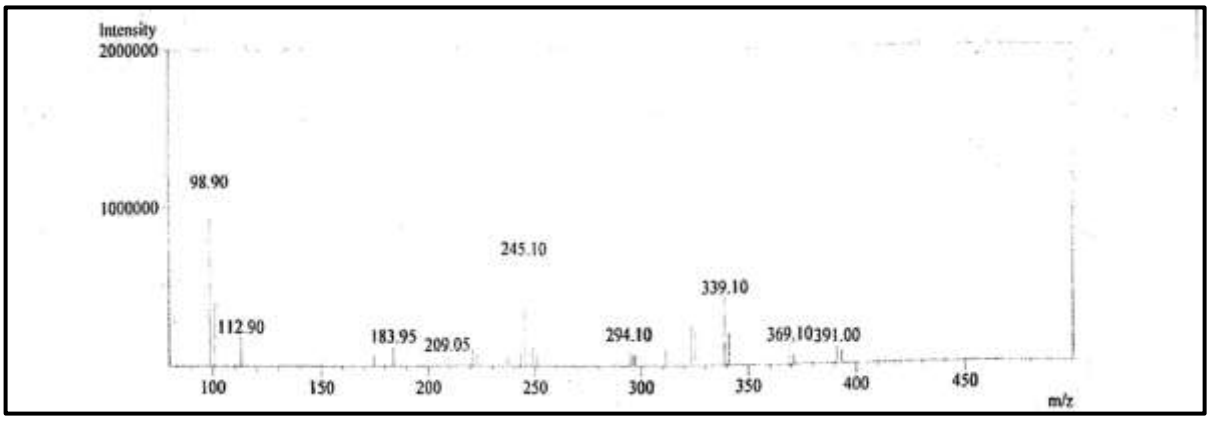

Figure 2 (i): Mass Spectrum of N-Nitroso Diclofenac

Table 3: Results of Specificity for N-Nitroso Diclofenac

Sample Name	Area	Content (ppm)
Standard	610584	NA
LOQ Solution	9144	NA
Blank	Not detected	NA
Placebo	Not detected	Not detected
Placebo spiked at 100% Specification Level	487414	4.85
Sample Solution	7165	0.07
Sample spiked at 100% Specification Level	509361	4.78

Table 4: Results of System Precision

Injection No.	Area N-Nitroso Diclofenac
1	610584
2	621735
3	626402
4	632740
5	628982
6	634083
Average	625754
SD	8663.6896
%RSD	1.38

Table 5 (a): Signal to Noise ratio of LOD and LOQ level

Injection No.	S/N ratio of N-Nitroso Diclofenac standard	
	LOD	LOQ
1	28.46	33.24
2	26.41	38.71
3	28.79	38.78
4	28.16	35.70
5	24.43	22.13
6	27.88	34.55
Average	27.35	33.85

Table 5 (b): Results of LOD and LOQ Confirmation

Injection No.	%RSD of N-Nitroso Diclofenac standard	
	LOD	LOQ
1	5258	9144
2	5307	9353
3	5564	7897
4	5986	8054
5	6063	7776
6	6740	8564
Average	5819.70	8464.67
SD	561.70	667.18
%RSD	9.65	7.88

Table 6: Results of Linearity for N-Nitroso Diclofenac

S. No.	Linearity Level	Obtained Concentration (ppm)	Concentration w.r.t Sample (ppm)	Average Area
1	LOQ Level Solution	0.000048	0.06	9298
2	1.9% Specification Level Solution	0.000097	0.12	15362
3	25% Specification Level Solution	0.001257	1.57	159765
4	50% Specification Level Solution	0.002514	3.14	319499
5	100% Specification Level Solution	0.005027	6.28	634821
6	150% Specification Level Solution	0.007541	9.43	948819
7	200% Specification Level Solution	0.010055	12.57	1292448
Slope				127389246
y-Intercept				-110.17
Correlation Coefficient (r)				0.999
Squared Coefficient Correlation (r^2)				0.999

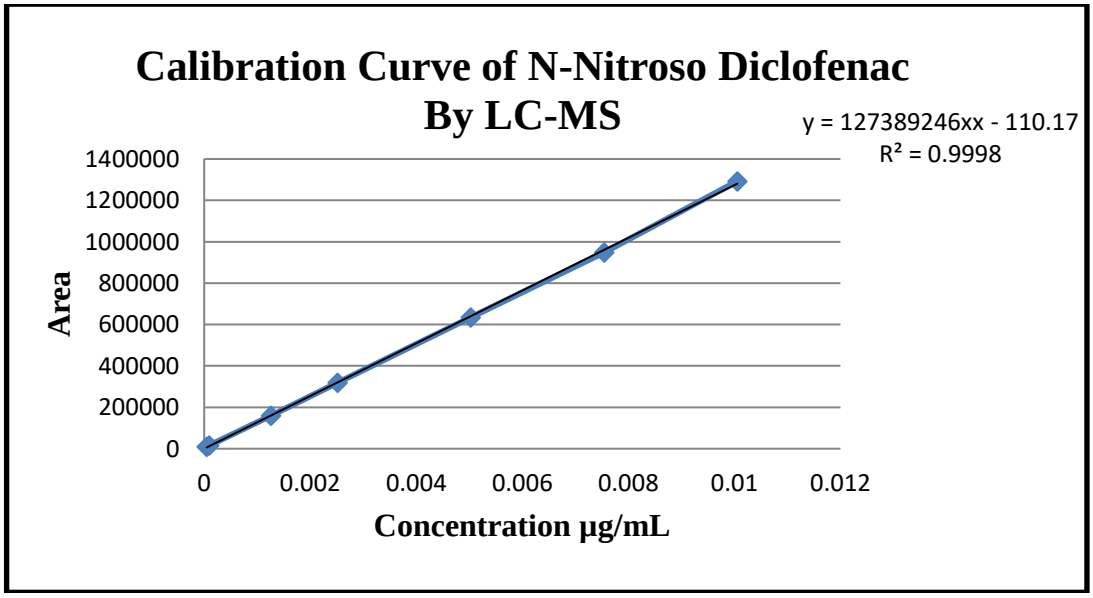

Figure 3: Results of Linearity for N-Nitroso Diclofenac

Table 7: Results of Accuracy for N-Nitroso Diclofenac

S. No.	Label	Amount of Analyte in Sample (ppm)	Amount Spiked (ppm) w.r.t Sample	% Recovery	% Mean Recovery
1	Control	0.012	NA		
2	Sample- As	0.011	NA		
3	Such Sample	0.013	NA		
1	Sample Spiked	0.048		79.38	
2	at LOQ Level	0.049	0.060	82.05	79.16
3		0.046		76.05	
1	Sample Spiked	2.503		80.15	
2	at 50% Level	2.499	3.123	80.03	80.98
3		2.585		82.76	
1	Sample Spiked	5.364		85.89	
2	at 100% Level	5.078	6.245	81.31	81.21
3		4.773		76.43	
1	Sample Spiked	7.367		81.53	
2	at 150% Level	7.527	9.368	80.35	79.73
3		7.243		77.32	

Table 8: Results of Method Precision for N-Nitroso Diclofenac

Preparation No.	N-Nitroso Diclofenac Content (ppm)		
	As Such Sample	Spike Sample at Spec Level	% Recovery
1	0.013	5.363	85.87
2	0.011	5.077	81.30
3	0.011	4.772	76.42
4	0.000	4.825	77.26
5	0.000	5.216	83.52
6	0.015	5.293	84.75
Mean	0.033	5.091	81.52
SD	NA	0.24599	3.93856
%RSD	NA	4.8	4.8

Table 9: Results of Filter Compatibility

Sample	Sample Area	Amount of Analyte w.r.t Sample (ppm)	% Recovery	% Difference
Diclofenac Gel_ As Such_ Filtered with 0.22 µ PVDF Filter	7165	0.07	NA	-20.5
Diclofenac Gel_ As Such_ Unfiltered	5694	0.05	NA	
Diclofenac Gel_ Spike at 100% Level_ Filtered with 0.22 µ PVDF Filter	509361	4.92	77.69	13.7

Diclofenac Gel_ Spike at 100% Level_ Unfiltered	579176	5.83	92.41

Table 10: Results of Solution Stability for N-Nitroso Diclofenac standard and Diclofenac Sodium Gel 3% sample

Sample Name	Area	% Difference
N-Nitroso Diclofenac standard_0 Hours	610584	
N-Nitroso Diclofenac standard_2.9 Hours	634083	3.8
N-Nitroso Diclofenac standard_12.8 Hours	626933	2.7
N-Nitroso Diclofenac standard_19.8 Hours	536139	-12.2
N-Nitroso Diclofenac standard_25 Hours	705391	15.5
N-Nitroso Diclofenac standard_32 Hours	652049	6.8
N-Nitroso Diclofenac standard_35 Hours	681631	11.6
N-Nitroso Diclofenac standard_38 Hours	666621	9.2
N-Nitroso Diclofenac standard_44 Hours	641068	5.0
Diclofenac Gel_ As Such Sample_0 Minute	6353	
Diclofenac Gel_ As Such Sample_35 Minutes	10919	71.9
Diclofenac Gel_ Spike at 100% Level_0 Minute	570075	
Diclofenac Gel_ Spike at 100% Level_35 Minutes	579765	1.7

VALIDATION STUDY

All validation parameters is done using ICH Q2 (R1) guidelines.

Specificity

The method's specificity was demonstrated by injecting blank, standard, LOQ solution, sample, sample spiked at 100% specification level, placebo and placebo spiked at 100% specification level. There was no interference detected. The mass spectrum of N-nitroso diclofenac is given in Fig 2(i) and the respective chromatograms of these is given in Figures 2(a), 2(b), 2(c), 2(d), 2(e), 2(f), and 2(g), and Table 3.

System Precision

Six replicate injections of standard solution to establish the system precision. Refer Table 4.

Limit of Detection and Limit of Quantitation (LOD & LOQ)

Precision at LOD and LOQ established by injecting six replicate injections of LOD level solutions and six replicate injections of LOQ level solutions. The S/N ratio is given in Table 5(a) and the %RSD of LOD and LOQ is given in Table 5(b) and the respective figures are shown in Figure 2(g) and 2(h)..

Linearity

Linearity was determined by injecting five different concentrations of linearity standard solutions in the range of LOQ to 200% of specification level. Calculated the correlation coefficient, slope, and y-intercept. Refer Table 6 and Figure 3.

Accuracy

Accuracy parameter was performed by spiking specified impurity of concentrations of LOQ, 50%, 100%, and 150% of the specification level. Preparations were done in triplicate. Refer Table 7.

Method Precision

Method precision was determined by carrying out analysis of six individual sample preparation and six spiked sample at specification level. As sample contains impurity level below LOQ, hence method precision performed on as spike sample. Refer Table 8.

Filter Compatibility
Filter compatibility was determined by injecting Diclofenac Sodium Gel 3% as such sample and spiked sample with and without 0.22 µ PVDF syringe filter to determine the % difference. Refer Table 9.
Solution Stability
The solution stability was determined by analyzing standard, sample and spiked sample preparations at 100% specification level kept at sampler temperature (10^{o}C). Refer Table 10.

RESULTS

Mobile phase combination of buffer and acetonitrile (mobile phase A) and acetonitrile (mobile phase B) and mixture of methanol and water with 0.1% acetic acid as diluent with gradient elution plays important role in separation. The retention time of N-Nitroso Diclofenac is 17.6 minutes and of Diclofenac is 19 minutes which is nominal and total run time of 35 minutes. The chromatographic and mass conditions are summarized in tables 2 (a), 2(b), 2(c), 2(d), and 2 (e).
0.22 µ PVDF syringe filter was found suitable for sample filtration. The stability of the standard solution was up to 44 hours and sample solution was found stable for 35 minutes at sampler temperature 10^{o}C.
All method validation parameters results were found within the acceptable criteria. The validation proved that the method is linear, precise, accurate and specific.

CONCLUSION

A new LC-MS method for the estimation of N-Nitroso Diclofenac impurity in the Diclofenac gel was developed. The method is precise accurate and fast The validation was performed as per ICH guidelines and all the results were found within the acceptance criteria. It is concluded that the method is specific, precise, linear, accurate and filter compatible. Hence, the method can be used for intended purpose.

ABREVIATIONS

NDMA- N-nitrosodimethylamine
NDEA-N-nitrosodiethylamine
NDIPA-N-nitrosodiisopropylamine
NIPEA- N-nitrosoisopropylethylamine
NDBA-N-nitrosodibutylamine
ICH- International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use

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Conflict of interest: None.

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