Development of Method and Validation of Related Substances In NAPROXEN Injection by Applying Stability Indicating HPLC Methodology

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Abstract

A validated HPLC method was developed for the determination of Naproxen in pharma formulation. Isocratic elution at a flow rate of 1.0 ml/min was employed on NOVA-PAK C18 4µm (3.9 x 150 mm) or equivalent, or similar is used for this chromatography analysis. Acetonitrile as 500v/v, water as 490 v/v furthermore Glacial Acetic acid as 10v/v is used a mobile phase. The UV detection wavelength was 254nm and 10.0µl sample was injected. The run time is About 120minutes for Sample, Unmarked, Placebo, System suitability, Sensitivity solution and 60minutes for diluted Regular. The Approximate retention time was founded for Naproxen is ± 3 minutes. The% R.S.D Naproxen was identified. The mean Percentage recovery for Naproxen is found within the specification limit. The method was validated as per the ICH guidelines. Thus, the proposed HPLC method can be successfully applied for routine quality control analysis of formulations. This method developed is simple and is better than the methods reported in the literature.

Key words: RP-HPLC Refractive index detector, Naproxen, flow rate, column, ICH Guidelines, USP reference.

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1. Introduction

Molecular formula for Naproxen (NP) is $C_{14}H_{14}O_3$. Molar mass is 230.263 $g \cdot mol^{-1}$. This drug Naproxen is sold as “Aleve” with remaining. This is utilized as for treating pain, cramps in menstrual, diseases like rheumatoid arthritis, gout as well as fever are known as inflammatory.[1] NP is acting as nonselective inhibitor for COX .[1] Just like to that of NSAID, this drug exert as anti-inflammatory action by decreasing mediators as inflammatory referred as prostaglandins [2] By liver to inactive metabolites it undergoes the metabolization.[1] In the year 1967 this Naproxen is authorized for usage as medical in country USA in the year 1976.[3][4] 2019 it occupied as 61st medical drug furthermore

consists of about 11 million prescriptions.[5][6] The sodium Naproxen sodium utilized as a "bridge therapy".[7] Utilization of Naproxen those are complimentary for its mechanism of action as anti-inflammatory substance.[3] This naproxen consists of properties neither reducing fever nor antipyretic in addition to anti-inflammatory activity.[3] S. Ashutosh Kumar et.al.,[8] is drifted this work by Kromosil-C_{18} ODS column (150mmX4.6mm;5µ). The authors are used buffer composition as ammonium acetate buffer 40v/v which is altered to pH as 4.0 help of 1 % Triethyl amine along with methanol as 60v/v latter subjected to filtration concluded by filters of cellulose nitrate capacity 0.45 µ. Rate of flow is 1.00 mL/min. 210 nm is wavelength. 7.00min is run time. 3.063min is retention time. Manidipa Debnath et.al.,[9] are used Kromosil -ODS C18(250X4.6mm;5µ). Mobile phase is composition of both Water as 45v/v in which pH is maintained at 2.5 help of OPA along with Methanol as 55 v/v. FR is 1.00mL/min. 277 nm is the wavelength. Rt for both Sumatriptan as well as NP are 2.790 min, 3.481min. % recovery are 99.02- 100.75, 99.85 - 100.22. 20µg/mL -80µg/mL is linearity range. LOD is 0.56µg/mL, 0.57µg/mL. LOQ is 1.69µg/mL, 1.74µg/mL. Pakhuri Mehta et.al., [10] conducted over a YMC-ODS A Pack (5µ as particles size) (250mm × 4.6mm) column with help of mobile phase contains Acetonitrile as well as 10mm Ammonium acetate buffer which contains pH as 3.8 in ratio 550 v/v:450 v/v. Rate of flow is 0.80 ml/min. 254 nm is wave length. Rt as 5.9 min ± 0.01 min. linear regression curve is drawn in between strength, response and identified as 0.25µg/ml -3.00µg/ml. 1.000 is coefficient of regression. LOD as 0.13µg/ml, LOQas0.25µg/ml.

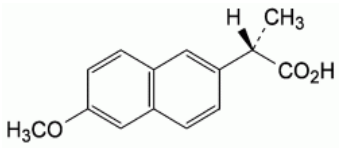


Figure: 2.1 Structure of Naproxen

2. EXPERIMENTAL

Chemicals and Equipment:

NOVA-PAK C18 4µm (3.9 x 150 mm) or equivalent is utilized to this proposed chromatography analysis. Regular balance is utilized for measuring the drug. Ammonium acetate, triethyl amine, Acetonitrile, glacial acetic acid is procured as grade AR furthermore water is from HPLC grade. Author procured from Merck Specialties Private Limited, Mumbai, India. 3.90gm of ammonium acetate is taken in 1lit. beaker 875.00ml of HPLC grade water is added to the dissolving the buffer. Later, volume is adjusted to 1000ml, pH is

altered as 4.5 by triethyl amine. Buffer 500v/v, water 490v/v along with Glacial Acetic Acid as 10v/v are used a buffer. For this experimental work authors are used different solutions includes Blank solution, Standard; Sample, Diluted Standard - 2 PPM; System Suitability Solution; Sensitivity solution - 0.4ppm; Sample– 20.00mg; stock; NP Standard Stock;

3. METHOD DEVELOPMENT

To this case disparate parameters were studied as well as considered to this NP in tablet dosage form of strength as 15.00mg. For 10ppm solution of NP by using UV spectrophotometer the spectrum in Acetonitrile was recorded separately. Peak for maximum absorbance wavelength deliberated in the range of 254. By using NOVA-PAK C18 4µm (3.9 x 150 mm) or equivalent. In this method Acetonitrile as 500v/v water as 490v/v, Glacial Acetic acid as 10v/v were utilized as mobile phase this proved that this is very good combination by comparing by variable peaks of chromatography those were obtained by different mixtures. Finally, noted from experiment is 1.00ml/min rate of flow is most suitable for eluting analyte.

4. Validation of Proposed Method and requirements

System Suitability

From results obtained, decided as proposed system acting as more suitable to this validation of analytical method. Total six replicate injections of API working standard solution were injected with help of method analysis. Percentage relative standard deviations (% RSD) for the peak responses were determined. % RSD of the peak responses due to NP for total six injections should be less than or equal to 5.0 %. By changing conditions of flow rate as 0.80mL/min., 1.00mL/min along with 1.20mL/min standard areas as well as tailing factors were calculated and those values are represented in the table: 2.2 and 2.3 by changing flow rate of NP.

Sample	Naproxen Area
1	9628
2	9348
3	9864
4	9765
5	9776
6	9360
Mean	9624
% RSD	2.3

Table 2.2: System suitability results

	0.80mL/min as Flow		1.00mL/min as Flow		1.20mL/min as Flow	
	Std. Area	**Tailing factor**	**Std. Area**	**Tailing factor**	**Std. Area**	**Tailing factor**
	6089.30	1.115	4990.29	1.119	4069.13	1.132
	5884.59	1.009	4890.64	1.109	4170.59	1.119
	5929.40	1.110	4899.20	1.220	4140.29	1.122
	6069.44	1.120	4932.73	1.009	4139.28	1.122
	6048.59	1.120	4718.25	1.109	4089.19	1.119
Avg	6045.092	1.100	4889.79	1.009	4131.09	1.1338
SD	73.866	0.0033	63.003	0.00086	32.689	0.0007
% RSD	1.137	0.3908	1.217	0.0839	0.7919	0.0065

Table 2.3: System Suitability Results for NP by changing Flow Rate

Specificity

Each and every of products obtained by degradation furthermore impurities should be exactly resolved by active compound peak as at least baseline resolution which is greater than to value as 1.5 furthermore should elute within specified assay run time. Threshold angle is less to that of angle of purity. No further components were observed for co-elute by peak of NP. Purity values indicate as this drug NP peak is contemplated as spectrally pure. Finally, this process is method engaged as specific to API – NP in product formulation. Chromatogram for solvent placebo, API, product along with detergent chromatogram peaks are represented in the figures 2.2 to 2.6. The peak purity for NP is represented in figures 2.7 to 2.8.

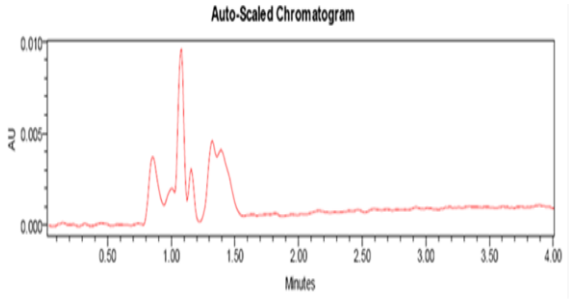


Fig 2.2: Chromatogram: Solvent – no significant peaks detected

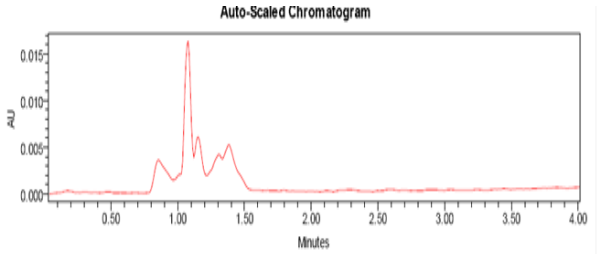


Fig 2.3: Chromatogram: Placebo – no significant peaks detected

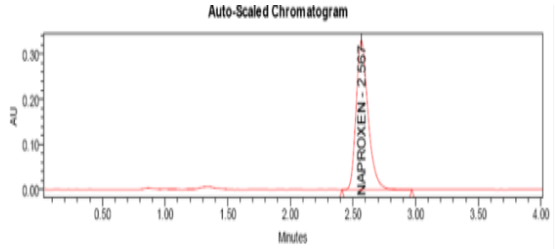


Fig 2.4: Chromatogram: API – peak due to Naproxen eluted at about 3 min

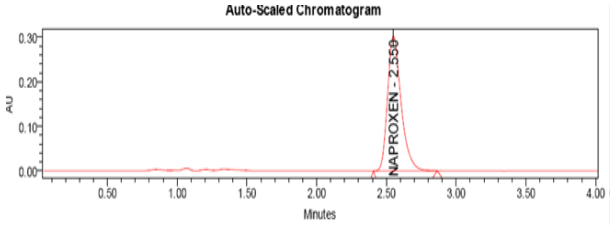


Fig 2.5: Chromatogram: Product - peak due to Naproxen eluted at about 3 min

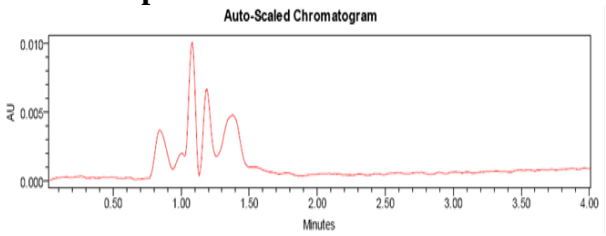


Fig 2.6: Chromatogram: Detergent - no significant peaks detected

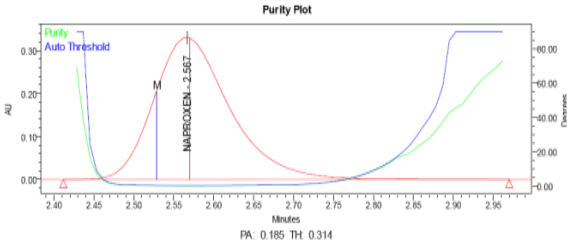


Fig 2.7: Peak purity 1

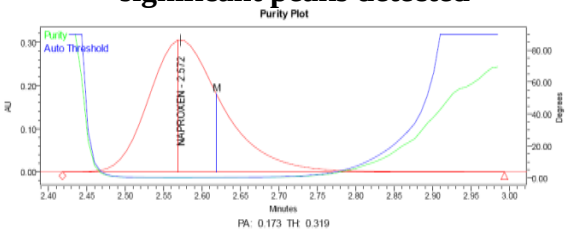


Fig2.8: Peak purity 2

FORCED DEGRADATION STUDIES:

Specificity by forced degradation:

Forced degradation study of NP injection strength has been performed to finalize that in study of stability or overall shelf life if product of degradation is observed that may not interfere in original NP peak. In addition to this these studies provides very much help for measuring which type of degradation pathway i.e., neither it is oxidative, nor it undergoes alkali

hydrolysis, acidic hydrolysis, water hydrolysis, dry heat furthermore photolytic etc., for each and every degradation. For these studies authors are designed various solutions includes sample; placebo; Acid Stressed sample 1.00N HCl; Alkali Stressed sample 1.00N NaOH; 3.0% w/v Hydrogen Peroxide Stressed sample; Neutral Stressed sample; UV light exposed sample; Sunlight light exposed sample; Thermal Stressed (Dry heat) sample. Consequently, obtained degradation substances/products were taken out from NP peak. From the obtained values and results, it is concluded that sample is degrading more in acidic furthermore alkali stressed condition i.e., 1.00N NaOH & 1.0N HCl. In Sunlight condition the NP peak is degraded slightly, no more degradation is identified either in neutral, UV, thermal along with conditions of peroxide. From this conditions author is concluded that related substances method is considered as more specific also highly stability indicating.

System Precision

Retention time **(**Rt), area for total 6 perseverance's were computed along with % RSD. To HPLC system, injected blank that is as diluent 1 injection as well as diluted standard solution as six injections. Response of peak for NP from regular preparation along with % RSD is recorded to Rt. %RSD of Rt for NP deliberated from total 6 injections of diluted standard solution is found that NMT 1.0. By using total 6 injections %RSD of area counts to NP deliberated diluted standard solution. Finally, it is found that NMT 5.0. Due to this it is finalized that Rt along with Area counts were similar which are concluded by RSD i.e., less than 1.0%, less than 5.0% respectively. Due to this reason, it could be finalized that precision of system reaches exactness for validation of method. Analytical system abides by the requirements which are specified by method precision. Values those are obtained were tabulated in the table 2.4

Sample	% Recovery
1	34
2	11
3	495
4	93
5	108
6	97
Mean	100

Table 2.4: System precision Results

Method precision (MP):

Calculated assay % of impurities to NP in preparation of sample. %RSD to various impurities 0.05% furthermore above of total number of 6 measurements is found that NMT 15. %RSD to overall impurities to total 6 measurements are found that NMT 10. By values obtained it is finalized as this method is more precise. The values are tabulated in the table: 2.5 and table: 2.6

Injection	Peak Areas	% Assay
1	4436.56	100.65
2	4438.58	100.88
3	4434.56	100.87
4	4449.15	100.60
5	4442.13	101.20
Mean	4439.796	100.60
SD	62.63	52.30
% RSD	1.25	0.06

Table 2.5: Method Precision Results

Intermediate Precision(IP):

Calculated % of impurities latter compared values those are obtained in MP latter to Intermediate Precision. Finally, calculated % RSD to total 12 measurements includes method precision as well as Intermediate Precision. %RSD to impurities 0.05 also above to total 6 measurements which is found that NMT 15.0. %RSD to total impurities for total 6 measurement is found that NMT 10.0. %RSD of total impurities for total 6 measurements is NMT 10. % RSD of impurities 0.05% as well as above to total 12 measurements includes both MP as well as IP is found that NMT 15.0. %RSD for total impurities 12 determinations MP as well as IP is found that NMT 10.0. From results finally concluded that this process is rugged. Results are tabulated in table: 2.7 and presence of total impurity is shown in table: 2.8.

Injection	Peak Areas	% Assay
1	4436.5151	100.68
2	4437.6279	100.19
3	4436.5151	100.77
4	4448.1612	100.88
5	4441.1712	100.26
6	4442.1312	101.19
Mean	4445.67	100.00
SD	58.92	44.4
% RSD	1.56	0.08

Table 2.7: Peak areas and % Assay Results

Preparation of sample	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E	Max. Unknown	Total Impurity
1	0.2039	0.0619	0.2442	0.0251	0.0179	0.0674	0.5739
2	0.0641	0.2449	0.1829	0.0249	0.0179	0. 0646	0.5586
3	0.0624	0.2469	0.1952	0.0271	0.0181	0.0684	0.5688
4	0.0619	0.2581	0.1769	0.0261	0.0182	0.0559	0.5559
5	0.0631	0.2421	0.1780	0.0271	0.0188	0.0697	0.5433
6	0.0631	0.2451	0.1940	0.0241	0.0182	0.0613	0.5717
Mean	**0.0619**	**0.2459**	**0.1879**	**0.0247**	**0.0189**	**0.0655**	**0.5621**
%RSD	**0.3**	**2.5**	**5.8**	**3.3**	**1.4**	**6.4**	**2.2**

Table 2.8: Total impurity

Stability is calculated with help of injection in regular intervals by day wise with help of standard room temperature as 25°C as well as 5°C. For 5°C stability % Difference for NP is – 4.1. For 3.5mg/vial % Difference for Impurity's like A, B, C, D furthermore along with unknown impurity were 11.1, -2.2, -9.7, 7.2 & -14.1 respectively. The Spiked sample solution is stable for 11days. For 3.50mg/vial. % Difference for Impurity's like A, B, C, D furthermore along with unknown impurity were 5.6,7.0,0.2,14.1,-3.3 & -2.8. For 25°C stability % Difference for NP is -3.6 to 11 days at a temperature of 25°C for 3.5mg/vial. % Difference for Impurity's named as A, B, C, D furthermore along with unknown impurity was 7.7, -13.5, -7.2, 3.1 &-13.2. Spiked sample resolution is stable for about 11 days at 3.5mg/vial. % Difference to Impurity's named as A, B, C, D along with unknown impurity were 2.3, -0.1, -4.0, -13.2 & -8.4. Total impurities, %difference in area counts among those are starting along with specified period is within below or equal to ± 10.0. The values obtained were tabulated in table: 2.9.

Diluted Standard		
Time (Hrs.)	**Area counts**	**% Difference**
Initial	47181	**-**
Day 1	47899	**-1.5**
Day 3	48296	**-2.4**
Day 7	45972	**2.6**
Day 11	49126	**-4.1**

Table2.9: Diluted standard solution and their % difference

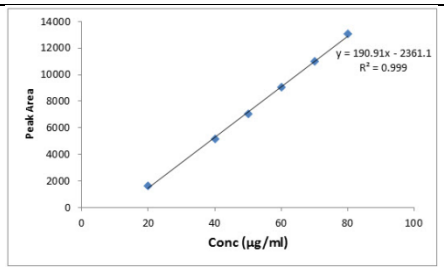
LINEARITY:

Plotted graph for NP, impurity's like A, B, C, D furthermore E along with strength which is taken in PPM over X-axis furthermore response of area over Y-axis. Correlation

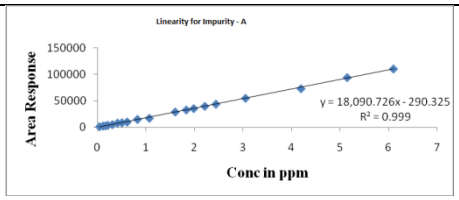
coefficient furthermore R square value is 0.995. The % intercept is below to 5.0 of response at 100 % specification area. Precision for maximum levels of %RSD is NMT 5.0. Finally, the results containing graphs are shown from fig: 2.9 to fig: 4.0 and values are represented in the table: 3.0

Concentration in µg/mL	Average Area	Statistical Analysis	
20	1621.89	Slope y-Intercept Correlation Coefficient	190.9x -2361 0.999
40	5141.73		
50	7066.67		
60	9054.19		
70	10980.2		
80	13060.1		

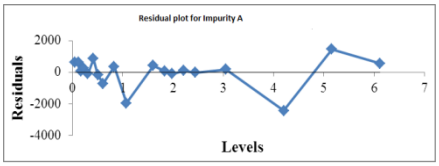
Table 3.0: Linearity results to NP



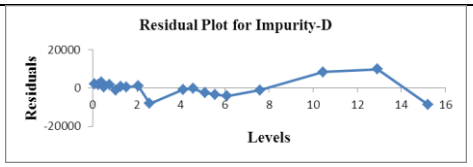
Linearity for Naproxen(NP)



Linearity plot for impurity - A



Residual plot for Impurity - A



Residual plot for Impurity - D

ACCURACY:

For this authors are prepared NP Standard Stock, Impurity-A Standard Stock, Impurity-B Standard Stock, Impurity-C Standard Stock, Impurity-D Standard Stock, Impurity-E Standard Stock, Preparation of Recovery Standard, Sample stock, Placebo stock. Individual furthermore mean recovery at each and every level is lies in between 85% to 115% to impurity of known. Individual as well as mean recovery at each and every level lies in among 80.0% to 120.0% to both foreign substance E along with unknown impurity. Finally from below values it is

finalized that recovery is good also it is within the limit as well as recovery at LOQ level is in acceptance criteria. Therefore, method is accurate**.** Results are represented in the table: 3.1

Set	(%) Level	Sample stock added in ml	Impurity-A Solution of Standard stock Added in mL	Impurity-B Solution of Standard stock Added in mL	Impurity-C Solution of Standard stock Added in mL	Impurity D Solution of Standard stock Added in mL	Impurity E Solution of Standard stock Added in mL	Make up to volume with Diluent
1	50	5	0.032	0.079	0.080	0.059	0.039	10
2	50	5	0.032	0.079	0.080	0.059	0.039	10
3	50	5	0.032	0.079	0.080	0.059	0.039	10
1	100	5	0.060	0.158	0.149	0.129	0.079	10
2	100	5	0.060	0.158	0.149	0.129	0.079	10
3	100	5	0.060	0.158	0.149	0.129	0.079	10
1	200	5	0.121	0.319	0.311	0.269	0.159	10
2	200	5	0.121	0.319	0.311	0.269	0.159	10
3	200	5	0.121	0.319	0.311	0.269	0.159	10
1	300	5	0.159	0.488	0.458	0.399	0.239	10
2	300	5	0.159	0.488	0.458	0.399	0.239	10
3	300	5	0.159	0.488	0.458	0.399	0.239	10

Table 3.1: Impurity along with unknown impurity

DETECTION LIMIT

With the help of cleaning validation matrix the maximum allowable carryover of NP is 0.008mg. Range for standard solutions above are injected two times furthermore average result is utilized for treating the values. Total seven solutions containing 0.08mg/swab, 0.04mg/swab, 0.008mg/swab, 0.004mg/swab, 0.0008mg/swab, 0.0004mg/swab, furthermore 0.0002 mg/swab of NP, those are relative to working strengths, are well prepared as well as injected by method of analysis. A linear regression curve is constructed which is shown in the fig: 4.1: Detection limit should capable of detecting API at 50% MAC. 50% MAC which is equal to value 0.0004mg/swab along with method provides linear response from 0.00001mg/swab – 0.2 mg/swab, therefore this process should detects above strength for API

0.0004mg/swab which is generally a 50% MAC required for method. Finally obtained values are represented in the table: 3.2.

Conc., in mg/swab	Area 1	Area 2	Average Area
0.08	1667844	1669515	1668680
0.04	842454	840781	841618
0.008	177074	177535	177455
0.004	94208	93966	94087
0.0008	20001	20072	20037
0.0004	9185	9067	9126
0.0002	5290	4901	5096

Table 3.2: Detection limit values

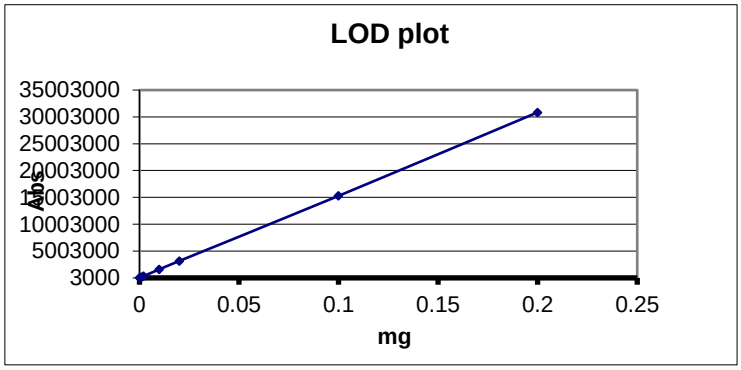
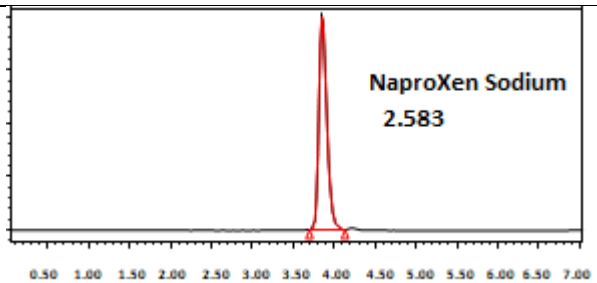


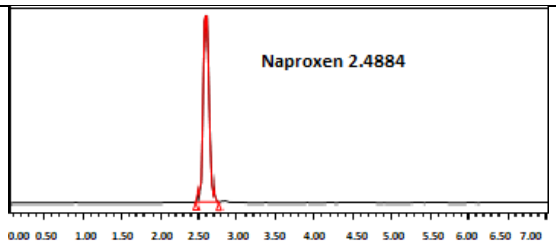
Fig 4.1: linear regression curve

ROBUSTNESS:

By varying temperature of **c**olumn as ±5°C; change in rate of flow as ±0.20ml/min, wavelength variation as ±5nm; organic variation as ±2.0%; Parameters of system suitability compulsory obey all conditions. Total known impurities which may be separated by each other as well as by NP peak in sample spiked by impurities. Above data, shows that, this Related Substances process is robust towards low variations in method parameters. The graphs obtained were represented in Fig: 4.2 to Fig: 4.4



By flow rate as 0.80mL/min flow



A typical chromatogram for robustness with flow rate (for 1.2 mL/min flow)

5. RESULTS AND DISCUSSION

Different mobile phase compositions were tried to asses RP-HPLC parameters. Finally required separation along with good peak symmetry is identified in a mixture of Acetonitrile as 500v/v, water as 490v/v, Glacial Acetic acid as 10v/v. Rate is 1.00ml/min. Optimum wavelength is 254nm. Run time as 120 minutes to Sample, blank, placebo, system suitability, sensitivity solution furthermore 60minutes to diluted Standard. From sensitivity solution, single to noise ratio for NP peak is NLT10.0. From diluted standard solution, Tailing factor to NP peak is NMT 2.0. From solution of diluted standard, theoretical Plates to NP peak is NLT 2000. %RSD to total 6 injections NP peak is NMT 5.0. There is no interference because Blank as diluent preparation, diluted standard solution, Impurity's at retention time to NP peak along with each other. From obtained values it is concluded that peaks to diluent, placebo were not interfered in Impurity's along with NP peak as well as each other. By using System precision %RSD of Rt to NP obtained from 6 injections of diluted standard solution is NMT 1.0. %RSD of Area counts for NP obtained from 6 injections of diluted standard solution is NMT 5.0 is observed that 1.5. Unknown impurity is 10.6. %RSD of total impurities for total 6 perseverance is 2.5. For Intermediate precision, %RSD of total impurities for total 12 perseverance method precision & intermediate precision founded at 7.0. For identifying stability in solution % difference to area counts for NP peaks in standard is within ± 5.0% from minimum area to after specified period. Author is concluded by values is that standard solution is stable for about 11 days at a temperature of 5°C also standard solution is stable for 11 days at a temperature of 25°C; % difference for NP is 5°C as -4.1%, 25°C as 1.0%. The percentage difference for NP is 5°C at -4.6%, 25°C at -9.6%. Spiked sample solution is stable for about 11days in 5°C also spiked sample is stable up to 11 days at 25°. % difference for NP is 5°C. For NP, Impurity's linearity results for coefficient of regression are measured and noted in the table. % intercept for NP, Impurity's is -0.9%, 0.8%,0.8%,1.0%,1.7% as well as 0.1% respectively. Method employed is specific to API Naproxen in the product. % RSD to peak responses is mainly due to Naproxen for total six injections should be neither less than nor equal to 5.0%. 50% MAC which is nearly equal to 0.0004mg/swab also this method proposed by the author provides linear response ranging as 0.00001 mg/swab to 0.2 mg/swab therefore the method can detect the above concentration of API 0.0004mg/swab which is required for this method. Finally, author is concluding that cleaning Validation method is proven to be valid and the validation test results and shows that this process complies with requirements to validation.

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