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A Validated RP-HPLC Method for Estimation of Related Compounds in Hydroxy Naproxen

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A simple, linear gradient liquid chromatographic method (RP-HPLC) is proposed for the simultaneous estimation of related compounds in hydroxy naproxen samples. Chromatographic separation was achieved on Zorbax SB C8 (150 × 4.6) mm, 3.5 µm particle size RRLC short column and eluent A used as 0.1% v/v trifluoroacetic acid in water and eluent B used as acetonitrile using Agilent RRLC (UHPLC) system. The mobile phase flow rate was 1.0 mL/min and the eluted compounds were monitored at 235 nm for related substance method and assay method. The excellent resolution was obtained between hydroxy naproxen and its related compounds, which were eluted within 25 min. The performance of the method was validated with respect to ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision and robustness. The correlation coefficient(r) was > 0.995 for both the methods from linearity data and percentage of recovery is 98.0 to 102.0 for assay method and 80.0 to 120.0% for related substance method. Sensitivity of the method was found be less than 0.5 µg/mL. Peak homogeneity data for naproxen in the chromatograms from the selectivity solution obtained by use of the photodiode array detector demonstrated the specificity of the method for analysis of hydroxy naproxen in presence of the related compounds.

Keywords: Hydroxy naproxen, Ultra-high performance liquid chromatography, Rapid resolution liquid chromatography.

INTRODUCTION

Naproxen is a non-steroidal drug having analgesic, non-narcotic, antipyretic and anti-inflammatory activities. It belongs to a group of arylpropionic acids or arylalkanoic acids, which includes naproxen, flurbiprofen, benoxaprofen, pirprofen, suprofen, ibuprofen, ketoprofen, fenoprofen and carprofen. Each of the compounds of this group are related in that they are propionic acid derivatives [1-3]. From this group, one derivative was chosen for study *i.e.*, sodium salt of 2-hydroxy-2-(2-methoxynaphthalene-6-yl) propanoic acid is key intermediate of naproxen (hydroxy naproxen). Several synthetic process for generating aryl propionic acids and in particular, naproxen have been proposed.

The first synthetic route described a mixture of optical isomers or enantiomorphs and the process required resolution of mixture to obtain the more active isomer with glucamine or cinchonidine [4] and further attempts have been described in US patent pharmaceutically useful optical isomer in excess of the physiologically inactive isomer so that the resolution procedure could be simplified [5] and it revealed that the process for the preparation of α-aryl propionic acids in particular, for naproxen preparation, which involves in absence of catalytic rearrangement *i.e.* thio-ketal or ketal of 2-hydroxy-1-(6′-methoxy-2′-naphthyl)-propan-1-one during the activation of α-hydroxy moiety with an esterifying agent to form the corres-ponding substrate of aryl or alkyl ketal or thio-ketal ester [6]. Similarly hydrolysis of ester produces the corresponding aryl propionic acid, 2-(6′-methoxy-2′-naphthyl)propionic acid using this hydroxy aryl propanoic acid and also preparation of naproxen by asymmetric reduction using chiral catalysts to avoid the resolution process (Fig. 1) [7] and hydroxy aryl propanoic acid was synthesized from 2-naphthol [8-10] and during the synthesis of this compound is having a plausible contamination of process related impurities.

As this kind of intermediates in active pharmaceutical ingredient (API) synthesis repeatedly afford several impurities affecting the quality of the final drug product, their structure

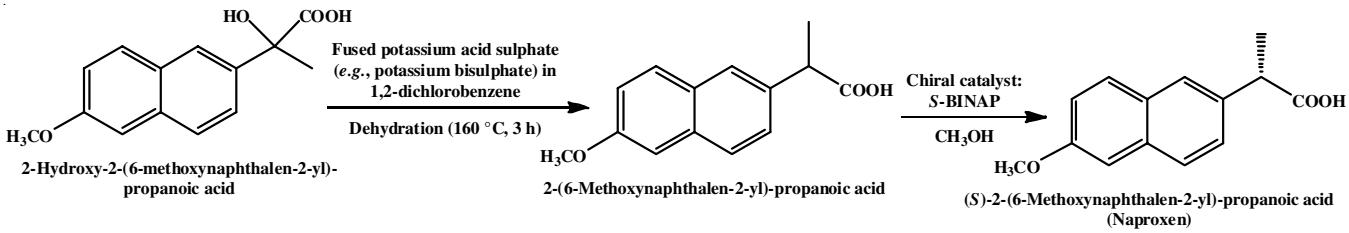


Fig. 1. Synthetic route of hydroxy naproxen

explanation is essential for impurities identification, characterization and quantification, not only for an API, but also of its key intermediates has recently become a requirement of both in the U.S. FDA, European Medicine Agency and ICH guidelines (EMA) [11-16] and these regulatory bodies are keenly reviewing and serious about the chemistry and manufacturing controls on respective intermediates and raw materials should be demonstrated to show the process capability and also method capability. Therefore, it was necessary to have a suitable method for the impurity identification and quantification and also potency of the compound of such key intermediates used in the process. During literature review, an extensive number of analytical methods were reported on naproxen drug substance and drug product by HPLC, LC-MS, LC-MS/MS [17-24] and unfortunately few intermediate HPLC methods were reported for the public interest but the research scientists spending more time on HPLC method development of key intermediates rather than the drugs. Electro-carboxylation of 2-acetyl-6-methoxynaphthalene process monitoring analytical method was reported [25]. However, this reported method is not suitable for described impurities *i.e.*, ethyl ester and dimeric impurities elution and also have a notable limitations analysis time, cost and compatible method for LC-MS. Therefore, it is essential to have a suitable method for the intermediates quality which are used for respective drugs. Hence, the research work was also attempted on key intermediates method development and validation of HPLC method for estimation of assay and its related compounds as per ICH requirement [26].

EXPERIMENTAL

Working in-house reference standard of hydroxy naproxen and related impurities were synthesized and further confirmation was performed by using spectral techniques (NMR, MASS, IR & UV) . The samples were obtained from ecoLogic Technologies limited (Hyderabad, India). Acetonitrile and Trifluoro acetic acid (HPLC-grade) were purchased from Merck Fine Chemicals (Mumbai, India). Milli Q water was obtained from Millipore direct 8L/h system (Massachusetts, USA).

The ultra-high performance liquid chromatographic method (UHPLC) system was used model of Agilent 1260 Infinity series with a diode array detector (quaternary pump: G1311B, column thermostat: G1316B, auto sampler with cooler: G1329B & G1330B and detector: G4212B) for method deve-lopment and validation, the chromatographic data was recorded by using open lab EZ chrom CDS (Agilent Technologies, Clara, US). A column Zorbax SB C8, (150 × 4.6) mm 3.5 µm RRLC column manufactured by Agilent (Agilent Technologies, Clara, US) was procured from LCGC India.

Optimized chromatographic conditions: The method was optimized on Zorbax SB C8, (150 × 4.6) mm, 3.5 µm (Agilent, US) HPLC short column and 0.1% trifluoroacetic acid was used as eluent A and eluent B used as acetonitrile. In these conditions, all related impurities were well resolved from hydroxy naproxen peak.

Chromatographic conditions: The details of the optimized method conditions are follows: Column Zorbax SB C8 (150 × 4.6) mm, 3.5 µm particle size. Eluent A: 0.1% v/v trifluoroacetic acid inwater; Eluent B: acetonitrile. Flow rate: 1.0 mL/ min; Column temperature: 40 ± 2 ºC; Wavelength of detection: 235 nm; Injection volume: 10.0 µL; Run time: 30 min; Retention time of hydroxy naproxen: about 5.0 min: Sample concentration: 500 µg/mL; Diluent: Eluent A + Eluent B (50:50).

Gradient programme		
Time (min)	Eluent-A (%)	Eluent-B (%)
0	70	30
5	70	30
20	10	90
24	10	90
25	70	30
30	70	30

Preparation of solutions

Standard solution: Hydroxy naproxen in-house standard solution was prepared 1003.7 µg/mL for assay determination and the same solution was diluted 10 µg/mL concentration for related impurities determination.

Impurities stock solutions: Related compounds stock solutions were prepared 100 µg/mL concentration in acetonitrile diluent medium, which were used for related substances determination.

Sample solution: Hydroxy naproxen sample solutions were prepared 1000 µg/mL for assay and related impurities determination.

Method validation

System suitability and selectivity: The system suitability test is an integral part of system to measure the system and method performance while beginning of analysis in routine quality control testing laboratories. Therefore, system suitability test was verified spiking of all known impurities in hydroxy naproxen test sample solution injected into the as described in above optimized chromatographic system.

Precision: For related compound method six solutions containing hydroxy naproxen were spiked with related compounds solutions 1.5 µg/mL (0.15% level of impurities with respect to the test concentration of hydroxy naproxen). Precision

solution were injected and calculated %RSD content of each impurity in hydroxy naproxen test sample. Also intermediate precision of the method was evaluated by another analyst, different system on a different day in the same laboratory.

For assay method, six individual sample solutions were prepared hydroxy naproxen at three different levels 50%, 100% and 150% of the test conc. (*i.e.* 1000 µg/mL). Each solution was injected and calculated %RSD for the assay of compound against standard solution at each level. Similarly, intermediate precision of the method was also evaluated by another analyst, on a different day in the same laboratory.

Limit of detection and limit of quantitation: The limit of detection (LOD) and limit of quantitation (LOQ) for hydroxy naproxen and related compounds were determined. Precision study was also carried out at the LOQ level by injecting six (n = 6) individual preparations and calculating the %RSD considering peak area for hydroxy naproxen and each related compound.

Linearity: For the related compound determination method linearity was checked for related compound and hydroxy naproxen at lower concentration levels LOQ to 0.5% (*i.e.* LOQ to 5 µg/mL). The responses were measured as peak areas and plotted against concentration.

The similar experiment was performed for assay method linearity by preparing the standard concentrations 50 to 150% at assay concentration level (*i.e.* 500 to 1500 µg/mL). The calibration curve was drawn by plotting the each impurity peak area *versus* its corresponding concentration. The correlation coefficient, slope and Y-intercept for each impurity was determined.

Accuracy: The accuracy of the assay method was evaluated in triplicate (n = 3) at the concentration levels of hydroxy naproxen 500, 1000 and 1500 µg/mL (50%, 100% and 150%) and the % recovery was calculated at each level. Similarly accuracy of the related substances method evaluated in triplicate (n = 3) at the concentration levels of each related compound about 0.3, 0.7 and 1 µg/mL level and the % recovery was calculated for each related compound.

Stability of the solution: A sample solution of assay method and related substance method was checked at different time intervals up to 48 h by keeping solution at 5 ºC and checked cumulative %RSD for the peak area of hydroxy naproxen and its related compounds.

RESULTS AND DISCUSSION

Method development: The main aim of the method was to develop a rapid and single chromatographic method for estimation of assay and its related compounds in hydroxy naproxen from bulk commercial samples. Initial trials were performed under acidic conditions using C18 and C8 columns to separate the related impurities from hydroxy naproxen peak. During method development trials dimer impurity eluting late retention time, considering that the method should be rapid and reduce the run time of analysis. The method optimized in such way to monitor the impurities and potency of the compound and this will help to development and optimization of the manufacturing process. Efficient chromatographic separation was achieved on above described in optimized chromatographic conditions method section. Hydroxy naproxen UV spectrum is shown in Fig. 2.

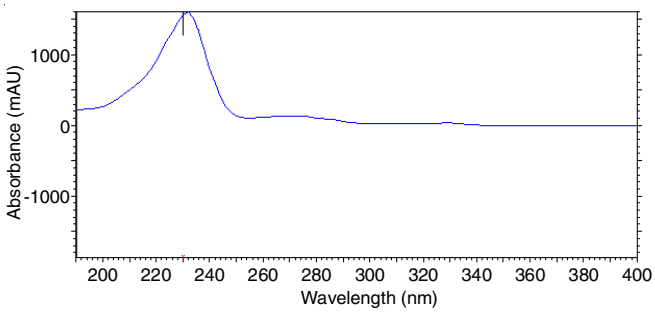


Fig. 2. UV spectra of hydroxy naproxen

Specificity/system suitability: There was no interference from the blank also from the related compounds at the retention time of hydroxy naproxen and related compound peaks. The peak purity data shows the peaks were pure and no co-eluting peak at the retention time of hydroxy naproxen peak and related compound peaks observed. The system suitability test results are given in Tables 1 and 2. A typical blank, system suitability and specificity chromatograms are given in Figs. 3-5.

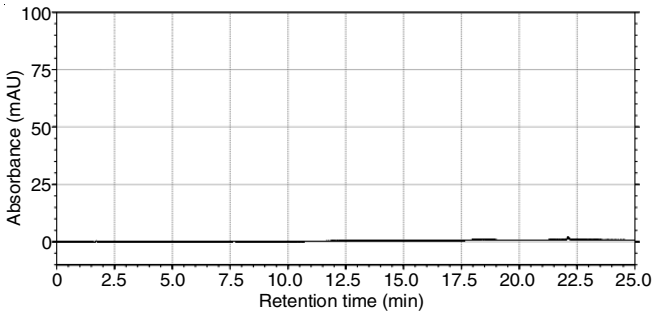


Fig. 3. Typical blank chromatogram

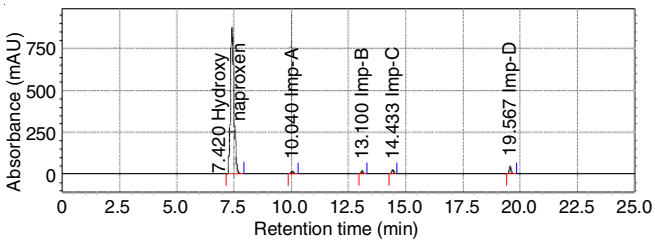


Fig. 4. System suitability chromatogram

Precision: The precision of the method for assay determination, the %RSD was found to be below 0.51 and well within the acceptance value. For related compound, the %RSD showed a value less than 2.6%. The %RSD of hydroxy naproxen assay results obtained in the intermediate precision was with in 1.0 and related substance method was found be less than 5%. The method precision results are given in Tables 3 and 4.

Limit of detection and limit of quantitation: The LOD and LOQ for hydroxy naproxen and related compounds were found be 0.01 and 0.05 µg/mL. The results are given in Table-5. Method sensitivity chromatograms are shown in Figs. 6 and 7.

Linearity: Calibration curve obtained by least square regression analysis between peak areas *versus* concentration showed linear relationship with correlation coefficient of 0.999

TABLE-1
SYSTEM SUITABILITY RESULTS

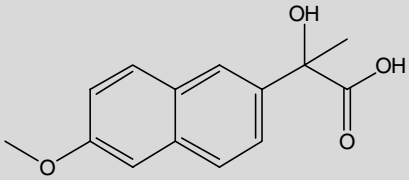
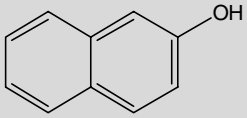
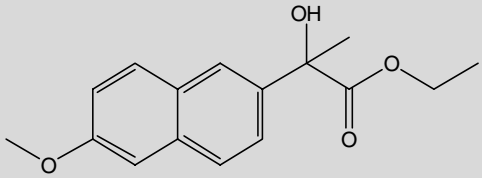
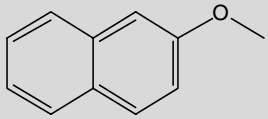
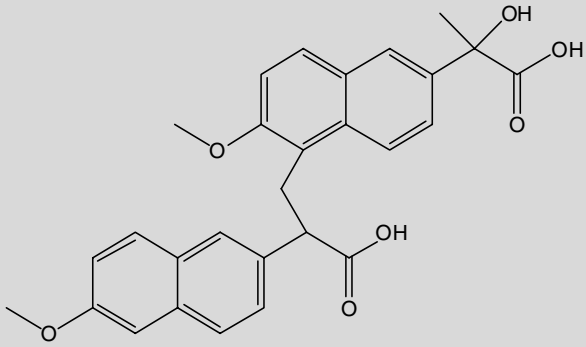
Compound	Structure	RT (min)	~ RRT	USP theoretical plates	USP tailing factor (T)	USP resolution
m.f.: $C_{14}H_{14}O_4$; m.w.: 246.26	 2-Hydroxy-2-(6-methoxynaphthalen-2-yl)propanoic acid (Hydroxy naproxen)	7.420	1.00	10772	1.16	–
Impurity-A m.f.: $C_{10}H_8O$; m.w.: 144.17	 2-Naphthol	10.040	1.35	47731	1.19	11.15
Impurity-B m.w.: $C_{16}H_{18}O_4$; m.w.: 274.32	 Ethyl 2-hydroxy-2-(6-methoxynaphthalen-2-yl)propanoate	13.100	1.77	134346	1.18	18.73
Impurity-C m.f.: $C_{11}H_{10}O$; m.w.: 158.20	 2-Methoxynaphthalene	14.433	1.95	135655	1.14	8.90
Impurity-D m.f.: $C_{28}H_{26}O_7$; m.w.: 474.51	 3-(6-(1-Carboxy-1-hydroxyethyl)-2-methoxynaphthalen-1-yl)-2-(6-methoxynaphthalen-2-yl)propanoic acid	19.567	2.64	374769	1.22	36.07

TABLE-2
SYSTEM SUITABILITY/SYSTEM PRECISION RESULTS

Preparation	Hydroxy of naproxen	Impurity-A	Impurity-B	Impurity-C	Impurity-D
1	46605	29210	19940	21165	22845
2	45875	28656	18587	23780	22402
3	45820	25505	19988	22809	23289
4	47390	24870	20869	21225	22668
5	46855	24800	20528	21476	22579
6	46085	28800	20038	20892	22801
Average	46438.3	26973.6	19991.8	21891.1	22764.1
St dev	621.5	2120.1	779.2	1145.0	302.7
%RSD	1.3	7.9	3.9	5.2	1.3

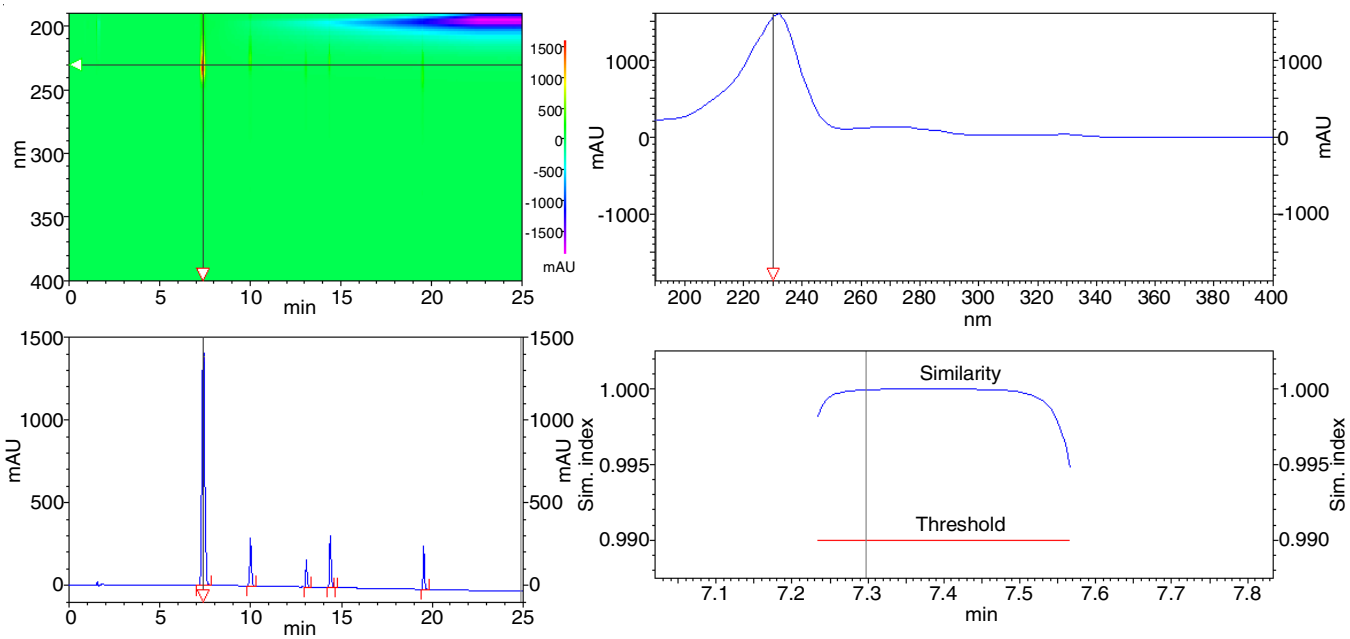


Fig. 5. Specificity chromatogram

TABLE-3
METHOD PRECISION RESULTS FOR RELATED SUBSTANCES METHOD

Preparation	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Total Imp's
Preparation-1	0.145	0.151	0.161	0.143	0.851
Preparation-2	0.138	0.153	0.159	0.141	0.869
Preparation-3	0.142	0.159	0.160	0.141	0.848
Preparation-4	0.149	0.153	0.160	0.141	0.859
Preparation-5	0.142	0.152	0.161	0.143	0.849
Preparation-6	0.145	0.153	0.158	0.140	0.841
Average	0.144	0.154	0.160	0.142	0.853
St dev.	0.004	0.003	0.001	0.001	0.010
%RSD	2.60	1.83	0.73	0.87	1.64

TABLE-4
METHOD PRECISION RESULTS FOR ASSAY METHOD

Preparation	Weight	Area	Assay (% w/w)
1	49.779	164999152	98.47
2	49.750	163707988	97.76
3	50.182	164731032	97.52
4	50.645	167939756	98.51
5	49.953	166152087	98.82
6	50.298	166746977	98.49
		Average	98.26
		Standard deviation	0.503
		%RSD	0.510

for hydroxy naproxen and ≥ 0.997 for related compounds respectively over the calibration ranges tested. The results are demonstrated that an excellent correlation between the peak area and concentration. The linearity results are shown in Tables 6 and 7.

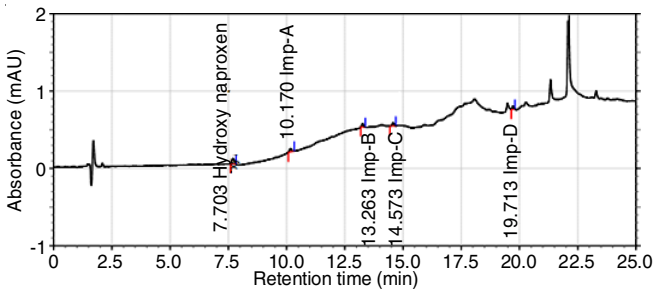


Fig. 6. Method sensitivity (LOQ) chromatogram

Accuracy: The accuracy of the assay method was determined in percentage recovery of hydroxy naproxen from bulk samples ranged from 98.6 to 101.4%. The percentage recovery of the five impurities from bulk drug samples ranged from 91.6 to 98.5% and the results are shown in Tables 8 and 9.

TABLE-5
METHOD SENSITIVITY RESULTS

Parameter	Hydroxy naproxen	Impurity-A	Impurity-B	Impurity-C	Impurity-D
LOD (%)	0.01	0.005	0.006	0.004	0.002
LOD S/N	3.1	3.1	3.4	3.4	2.3
LOQ (%)	0.05	0.02	0.02	0.02	0.01
LOQ S/N	10.4	9.7	10.1	9.6	10.1
LOQ Precision %RSD (n = 6)	1.3	9.3	3.4	6.0	8.9

TABLE-6
LINEARITY RESULTS OF RELATED SUBSTANCES METHOD

Parameter	Impurity-A	Impurity-B	Impurity-C	Impurity-D	Hydroxy naproxen
Slope (m)	341.8	354.2	253.7	150.8	265.7
Intercept (c)	1.27	1.25	-0.98	-0.56	-0.63
Correlation coefficient	0.9984	0.9986	0.9978	0.9985	0.9975
Y-intercept at 100% level	3.60%	3.50%	3.80%	3.70%	2.30%

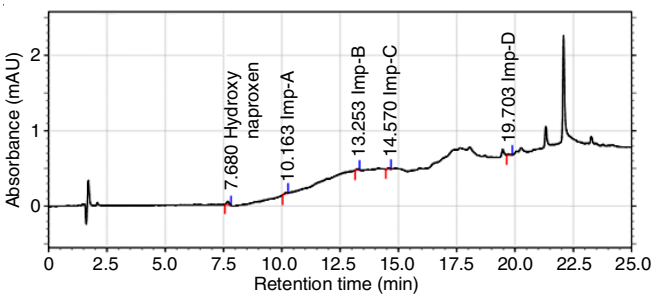


Fig. 7. Method sensitivity (LOD) chromatogram

TABLE-7
LINEARITY RESULTS OF ASSAY METHOD

Linearity level	Concentration of test sample (µg/mL)	Hydroxy naproxen
50%	510.56	84561875
75%	752.54	122036908
100%	995.58	165017106
125%	1245.16	204275924
150%	1502.76	242242240
	Correlation (r)	0.9996
	Regression (r2)	0.9991
	Slope	160467
	Y- Intercept	2948472.0
	% Y-intercept at 100% level	1.79

Solution stability: The %RSD of assay of hydroxy naproxen during solution stability and mobile phase stability experiments is within 1.0. No significant changes are observed in the content of impurity-A, impurity-B, impurity-C and impurity-D during solution stability and mobile phase stability experiments. The solution stability and mobile phase stability experiments data confirmed that the sample solutions and mobile phase used for assay and related substances determination were stable up to the study period of 48 h, auto sampler temperature at 25 ºC.

Robustness: The robustness of the method is determined as a measure of the analytical method capability to be unaffected by small variation in method parameters [27]. The different variations such as variation in flow rate by ± 0.2 mL/min, variation in column temperature by ± 5 ºC, at these changed conditions the system suitability was evaluated at each condition. In all the conditions, the resolution between each pair was greater than 2.0 and tailing factor of hydroxy naproxen peak was found be less than or equal to 1.5 (Table-10).

Conclusion

The developed gradient stability-indicating RP-HPLC method had shown an excellent selectivity between impurities along with hydroxy naproxen and objective of the development

TABLE-8
RESULTS OF ACCURACY FOR ASSAY METHOD

Accuracy	Conc. (µg/mL)	Area	Assay (%)	Recovery (%)	Mean recovery (%)
50% level-1	510.6	85803432	99.85	101.6	
50% level-2	509.7	85281042	99.42	101.2	101.4
50% level-3	510.9	85691401	99.66	101.4	
100% level-1	995.6	164999152	98.47	100.2	
100% level-2	995.0	163707988	97.76	99.5	99.7
100% level-3	1003.6	164731032	97.52	99.3	
150% level-1	1502.8	245940129	97.24	99.0	
150% level-2	1493.5	243156638	96.73	98.4	98.6
150% level-3	1498.9	244257926	96.82	98.5	

TABLE-9
RESULTS OF ACCURACY FOR RS METHOD

Compound	Level (%)	Amount added (µg/mL)	Amount recovered (µg/mL)	Recovery (%)	Mean recovery (%)
Impurity-A	50	0.365	0.341	93.4	94.1
	100	0.733	0.708	96.5	
	150	1.093	1.009	92.3	
Impurity-B	50	0.376	0.365	97.1	97.4
	100	0.753	0.742	98.5	
	150	1.123	1.085	96.6	
Impurity-C	50	0.386	0.369	95.6	97.0
	100	0.772	0.755	97.8	
	150	1.157	1.129	97.6	
Impurity-D	50	0.369	0.338	91.6	93.1
	100	0.738	0.711	96.3	
	150	1.107	1.013	91.5	

TABLE-10
RESULTS OF ROBUSTNESS EVALUATION DATA

Parameter	As such	Condition-1	Condition-2
Flow rate	1.0 mL/min	0.8 mL/min	1.2 mL/min
USP plate count	11496	24018	11154
USP tailing	1.186	1.180	1.236

Compound	RT (min)		~ RRT		Resolution	
	Condition-1	Condition-2	Condition-1	Condition-2	Condition-1	Condition-2
Hydroxy acid	9.133	6.173	1.00	1.00	–	–
β-Naphthol	11.487	8.913	1.26	1.44	11.01	12.67
Ethyl ester	14.373	12.167	1.57	1.97	17.55	20.08
2-Methoxynaphthalene	15.773	13.447	1.73	2.18	8.90	9.01
Ethyl dimer	20.773	18.687	2.27	3.02	33.24	38.15

Parameter	As such	Condition-1	Condition-2
Column oven temperature	40 °C	35 °C	45 °C
USP plate count	11496	13845	11225
USP tailing	1.186	1.170	1.212

Compound	RT (min)		~ RRT		Resolution	
	Condition-1	Condition-2	Condition-1	Condition-2	Condition-1	Condition-2
Hydroxy acid	7.747	7.147	1.00	1.00	–	–
β-Naphthol	10.360	9.740	1.34	1.36	11.76	10.82
Ethyl ester	13.253	12.973	1.71	1.82	18.98	18.83
2-Methoxynaphthalene	14.660	14.233	1.89	1.99	9.82	8.18
Ethyl dimer	19.727	19.420	2.54	2.71	36.77	34.95

is achieved on short column *i.e.*, RRLC Zorbax SB C8 150 × 4.6 mm, 3.5 µm to reduce the run time of the method to have a quick turn around time (TAT) for the routine samples analysis, which was more economic and environment friendly to minimize the HPLC effluent waste when compare to other reported HPLC methods. The developed method was 30 min of run time and, hydroxy naproxen and its related compounds were eluted within 25 min. The present method was found to be simple, linear gradient method, specific, precise, linear and accurate. Hence, it can be used successfully for the routine analysis of hydroxy naproxen API samples and for analysis of stability samples obtained during accelerated stability study.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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