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Article

Quality by Design Approach for the Separation of Naproxcinod and its Related Substances by Fused Core Particle Technology Column

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This paper describes the development of a rapid, novel, stability-indicating gradient reversed-phase high-performance liquid chromatographic method and associated system suitability parameters for the analysis of naproxcinod in the presence of its related substances and degradents using a quality-by-design approach. All of the factors that affect the separation of naproxcinod and its impurities and their mutual interactions were investigated and robustness of the method was ensured. The method was developed using an Ascentis Express C8 150 × 4.6 mm, 2.7 μm column with a mobile phase containing a gradient mixture of two solvents. The eluted compounds were monitored at 230 nm, the run time was 20 min within which naproxcinod and its eight impurities were satisfactorily separated. Naproxcinod was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Naproxcinod was found to degrade significantly in acidic and basic conditions and to be stable in thermal, photolytic, oxidative and aqueous degradation conditions. The degradation products were satisfactorily resolved from the primary peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

Introduction

Naproxcinod (NAP), chemically known as 4-(nitrooxy)butyl-(2s)-2-(6-methoxy-2-naphthyl) propanoate (Figure 1A), is a nitric oxide-donating cyclooxygenase inhibitor in development for osteoarthritis. Its molecular formula is $C_{18}H_{21}NO_6$ and its molecular weight is 347.4 g/mol (1–2).

Quality by design (QbD) is a key principle that has gained much discussion since its initiation as part of the U.S. Food and Drug Administration's vision for 21st century current Good Food Manufacturing Practices (cGMPs) and the guidance by the International Conference on Harmonization (ICH) on pharmaceutical development (3, 4). The fundamental principle of the initiative is to demonstrate both understanding and control of pharmaceutical processes to deliver high-quality pharmaceutical products while affording opportunities for continuous improvement. Although it is clear that the initiative is primarily intended for pharmaceutical product development, its use in the development of an integrated control strategy that involves analytical technology and methods should not be underestimated (5).

QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management." The scientific understanding gained during the method development process can be used to devise method control elements and to manage the risks identified (6).

Very few methods have appeared in the literature for the determination of the enantiomeric purity of NAP on high-performance liquid chromatography in normal phase mode (NP-HPLC) (7–8). A stability-indicating NP method was reported in the literature for NAP that only captured impurities B, G and H (9).

To the best of the authors' knowledge, there is no reported reversed-phase (RP)-HPLC method for the separation and quantification of related substances (Imp-A to Imp-H) and degradation products of NAP. Hence, an effort has been made to develop a rapid RP-HPLC method using the QbD concept.

A rapid, reproducible, stability-indicating RP-HPLC method was developed for the quantitative determination of NAP and its eight impurities; namely, Imp-A, B, C, D, E, F, G and H (Figures 1B-1I). The synthetic process for the preparation of NAP involved the reaction of naproxen (Imp-B) with 1-bromo-4-chlorobutane to form an intermediate, (*S*)-4-chlorobutyl 2-(6-methoxynaphthalen-2-yl) propanoate (Imp-F) along with Imp-G as a by-product. This intermediate (Imp-F), upon reaction with silver nitrate in a suitable solvent medium, gave NAP. Imp-C was a key starting material for the synthesis of Imp-B, and the reduction of the same gave Imp-A. Because methanol and isopropyl alcohol were used during the synthesis of Imp-B, the corresponding esters, i.e., Imp-D and Imp-E, were captured. Imp-H was a dimer, formed due to the addition of Imp-B to Imp-F. Impurities A, C and D were listed in the European Pharmacopoeia as part of a monograph on naproxen (Imp-B) (10).

This method was successfully validated according to ICH guidelines (12).

Experimental

Materials and reagents

Active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories (Hyderabad, India). The HPLC-grade acetonitrile, potassium dihydrogen phosphate and ortho-phosphoric acid (approximately 88%) purchased from Merck (India). Water was prepared by using a Millipore Milli-Q Integral 5 water purification system.

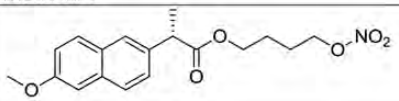
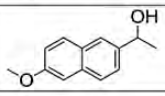
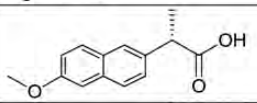
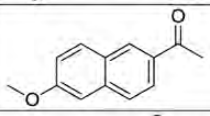
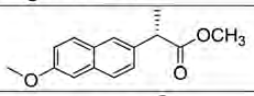
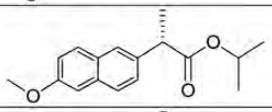
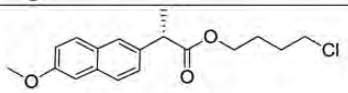
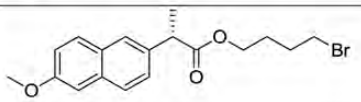
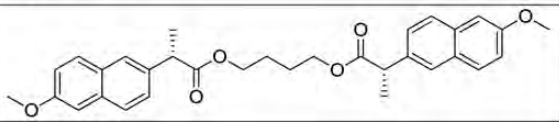
Figure	Name	Structure	Chemical name
A	Naproxcinod		(*S*)-4-(nitrooxy)butyl 2-(6-methoxynaphthalen-2-yl)propanoate
B	Impurity-A		1-(6-methoxynaphthalen-2-yl)ethanol
C	Impurity-B		(*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid
D	Impurity-C		1-(6-methoxynaphthalen-2-yl)ethanone
E	Impurity-D		(*S*)-methyl 2-(6-methoxynaphthalen-2-yl)propanoate
F	Impurity-E		(*S*)-isopropyl 2-(6-methoxynaphthalen-2-yl)propanoate
G	Impurity-F		(*S*)-4-chlorobutyl 2-(6-methoxynaphthalen-2-yl)propanoate
H	Impurity-G		(*S*)-4-bromobutyl 2-(6-methoxynaphthalen-2-yl)propanoate
I	Impurity-H		(2*S*,2'*S*)-butane-1,4-diyl bis(2-(6-methoxynaphthalen-2-yl)propanoate)

Figure 1. Structures of NAP and its eight impurities.

Chromatographic conditions and equipment

LC was conducted on a Waters Alliance 2695 HPLC with a 2998 photodiode array detector. The output signal was monitored and processed using Empower Software. All experimental design work was conducted using Design Expert 8.0.6 software by Stat-Ease (Minneapolis, MN). The chromatographic column used was an Ascentis express C8 column (150 × 4.6 mm, 2.7 μm particle size). The separation was achieved using a gradient method. KH_2PO_4 (0.02M) and 0.2% ortho phosphoric acid were used as buffer solution. Solvent A contained a mixture of buffer and acetonitrile in the ratio of 80:20 (v/v) and the solvent B contained a mixture of buffer and acetonitrile in the ratio 20:80 (v/v).

The flow rate of the mobile phase was 1.0 mL/min. The HPLC gradient program (time/percentage of solvent B) was set as: 0.01/32, 17.0/90, 17.01/32 and 20.0/32. The column temperature was maintained at 35°C and the detection was monitored at a wavelength of 230 nm, because all impurities and NAP exhibited λ_{max} at 230 nm. The injection volume was 10 μL. The diluent was a solution of acetonitrile and water (3:1)

Preparation of standard solutions

A stock solution of NAP (150 μg/mL) was prepared by dissolving an appropriate amount of the sample in diluent. Working solutions containing 0.15 μg/mL were prepared from this stock solution for the determination of related substances. A mixed stock solution (15 μg/mL) of the impurities (Imp-A to Imp-H) was also prepared in diluent.

Stress studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (11). The specificity of the developed LC method for NAP was conducted in the presence of its impurities. Stress studies were performed at an initial concentration of 150 μg/mL of NAP to provide an indication of the stability-indicating properties and the specificity of the proposed method. Intentional degradation was attempted under stress conditions of ultraviolet (UV) light (254 nm), heat (90°C), acid (0.1N HCl at room temperature), base (0.1N NaOH at room temperature), aqueous (at room temperature) and oxidation (3.0% H_2O_2 at room temperature) to evaluate the ability of the proposed method to separate NAP from its degradation products. For heat and light studies, the study period was 10 days, whereas for base, it was 1 h; for hydrolytic, acid and oxidation, it was 24 h.

The purity of the peaks obtained from stressed samples was checked by use of a photodiode array (PDA) detector. The purity angle was within the purity threshold limit obtained in

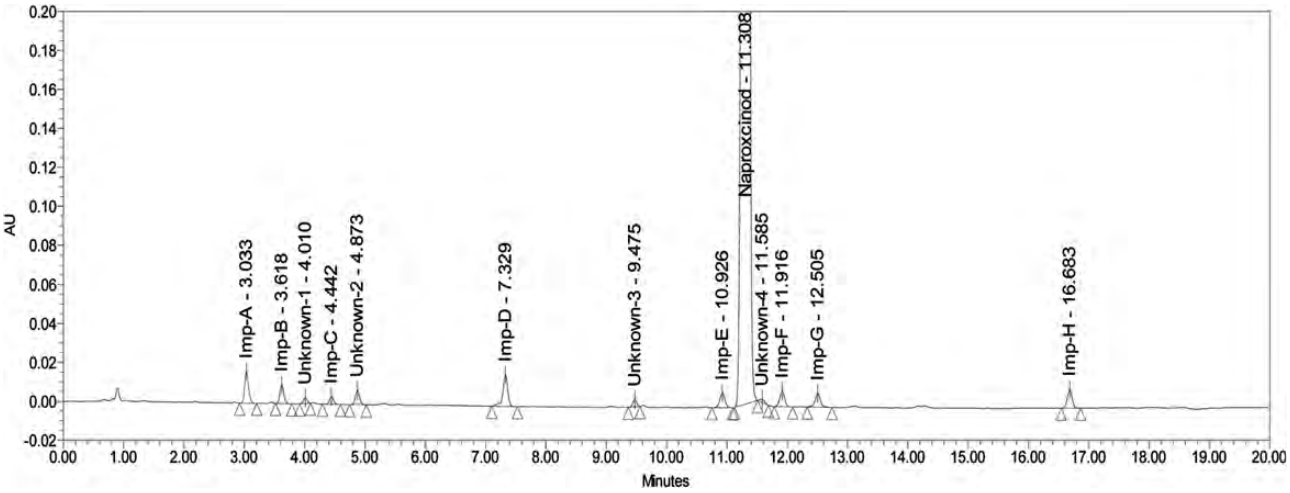


Figure 2. Naproxcinod spiked with 0.15% of all impurities.

all stressed samples and demonstrates the analyte peak homogeneity.

Method Validation

The described method was extensively validated for related substances by HPLC determination (12).

Precision

The precision of the method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual preparations of NAP spiked with 0.15% of its eight impurities (Figure 2) with respect to the test concentration (150 μg/ mL).

The percent relative standard deviation (RSD) of the area was calculated for each impurity.

The intermediate precision of the method was checked by injecting six individual preparations of NAP spiked with 0.15% of its eight impurities with respect to the test concentration (150 μg/mL) by a different analyst using a different column on a different day.

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) for NAP and its impurities were determined at signal-to-noise ratios of 3:1 and 10:1, respectively, by injecting a series of dilute solutions of known concentrations. A precision study was also conducted at the LOQ level by injecting six ($n = 6$) individual preparations and calculating the RSD (%) of the area for each impurity.

Accuracy

The accuracy of an analytical procedure expresses the agreement between the true value and the found value. For impurities, recovery was determined in triplicate at 0.075, 0.15, and 0.225% of the analyte concentration (150 μg/mL) on the drug substance and the recovery of the impurities were calculated.

Linearity of response

Linearity experiments were conducted by preparing and injecting solutions containing Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, Imp-H and NAP covering the range from 25 to 150% (0.0375, 0.075, 0.1125, 0.15, 0.1875 and 0.225%) with respect to the specification limit (0.15%). The correlation coefficients, slopes and *Y*-intercepts of the calibration curve were determined.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage.

To determine the robustness of the method, the experimental conditions were deliberately changed. The resolution of NAP and its eight impurities were evaluated. The mobile phase flow rate was 1.0 mL/min; to study the effect of flow rate on resolution, it was changed to 0.80 and 1.2 mL/min. The effect of column temperature was studied at 30 and 40°C. The effect of acetonitrile content in solvent B was studied at levels of 90 and 110%.

Solution stability and mobile phase stability

NAP solutions (spiked) prepared in diluent were injected at 0, 12 and 48 h time intervals, the impurity content (Imp-A to Imp-H) was calculated and the consistency was checked in the percent area of the principal peak at each interval. The prepared mobile phase was kept constant during the study period.

The mobile phase stability was demonstrated by injecting the freshly prepared solution of NAP and its impurities at different time intervals (0, 12 and 48 h).

Results and Discussion

Method development and optimization

Defining the method objectives

An important consideration in developing impurity profiling methods is to appropriately define the requirements of the method. In a QbD approach, this involves establishing what impurities need to be separated and eluted from the chromatographic column, followed by detection. Examination of the route of synthesis for the compound of interest and structurally similar compounds is often a good starting point to define the impurities that may be considered in method development. The objectives for development can be defined as (i) to retain Imp-B, a polar degradation product, and to elute the potential non-polar reaction by-product, Imp-H, in a reasonable analysis time; (ii) to resolve known and potential process-related impurities from the active pharmaceutical ingredient (API); and (3) to determine a user-friendly methodology for quality control laboratories.

Column screening and method optimization

According to the chemical structures of the impurities and NAP, it is evident that they are hydrophobic in nature. Because the objective was to develop a rapid HPLC method, a C8 stationary phase was selected to reduce the hydrophobic interactions between the stationary phase and the impurities. The development started with 0.1% orthophosphoric acid (OPA) as mobile phase A, and methanol and water in the ratio of 75:25 (v/v) was used as mobile phase B. The selected wavelength was 230 nm and the column oven was maintained at 35°C. The gradient program was (time/percentage of mobile phase B) 0.01/50, 15/85, 30/100, 54/100, 55/50 and 60/50. NAP was retained up to 23 min and Imp-H was retained up to 40 min. Trials were made with different C8 columns and with different mobile phases (Table I). None of the trials met the objective of rapid resolution of impurities and NAP.

Trials were made with an Ascentis Express C8 150 × 4.6 mm, 2.7 μm column employing a mixture of 0.02M KH_2PO_4 and 0.2% OPA as buffer (pH of the solution: ~2.5). Mobile phase A was buffer and acetonitrile in the ratio of 80:20 (v/v). Mobile phase B was buffer and acetonitrile in the ratio 20:80 (v/v). The initial gradient program was (time/percentage of mobile phase B) 0.01/20, 25/100, 27/20 and 30/20. The column oven was maintained at 35°C. The effect of the pH of buffer on the elution of impurities was studied (Figure 3) and based on the results, a pH of 2.5 was selected. All of the peaks were separated with resolution (R_s) >1.5. Different gradient programs were attempted to optimize the retention of NAP and resolution between the impurities. The final optimized gradient program was (time/percentage of mobile phase B) 0.01/32.0, 17.0/90.0, 17.01/32.0 and 20.0/32.0.

Table I
Trials on Different C8 Columns

Sample	Column name	Resolution: Imp-C/Unknown-2	Resolution: Imp-E/NAP
1	Symmetry Shield RP8: 150 × 4.6 mm, 3.5 μm	Less than 1.0	2.28
2	Zorbax Eclipse C8: 150 × 4.6 mm, 5 μm	1.6	Less than 1.0
3	Inertsil C8-3: 150 × 4.0 mm, 5.0 μm	1.1	Less than 1.0
4	Betasil C8: 150 × 4.6 mm, 5 μm	1.2	Less than 1.0
5	YMC Pak Pro C8: 150 × 4.6 mm, 5 μm	1.7	Less than 1.0
6	Xterra RP8: 150 × 4.6 mm, 5 μm	Less than 1.0	2.0
7	Ascentis Express C8: 150 × 4.6 mm, 2.7 μm	3.5	2.5

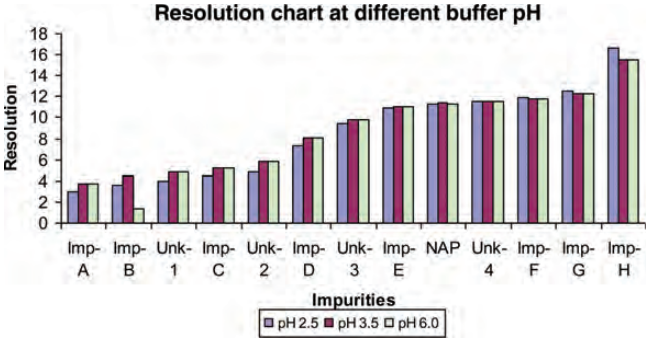


Figure 3. Effect of buffer pH on the resolution.

Table II
Factors and their Levels

Factors	Levels		
	−1	0	+1
Factor A (flow rate)	0.8 mL/min	1.0 mL/min	1.2 mL/min
Factor B (temperature)	30°C	35°C	40°C
Factor C (percentage of acetonitrile in solvent B)	72%	80%	88%

Design of experiments and identification of critical analytical method factors

On the basis of the preliminary experiments, the largest influences on the separation of compounds were flow rate (Factor A), column temperature (Factor B) and percentage of acetonitrile in solvent B (Factor C).

Factors and their low (−), high (+) and center point (0) levels are presented in Table II. To screen the relative influence of these factors and their possible interactions in the experimental domain, a central composite design (CCD) was chosen, which studies the effects of the selected three factors in 20 runs, including six center points. As the parameters to define chromatographic behavior of investigated substance and its impurities, resolution between two critical pairs; i.e., Unknown-1, Impurity-C (R_s-1) and Impurity-E, NAP (R_s-2) was chosen. The design and the responses obtained by the experiments are presented in Supplementary Table I. The information gathered from CCD experiments also provided an evaluation of the method's robustness.

According to the analysis of variance (ANOVA) for the response of R_s-1, the model's *F*-value was 74.36, which implies that the model is significant. Values of Prob > *F* for the terms A, B, C, AC and BC were less than 0.0500, which indicated that these terms of the model are significant. The fraction of explained variation by means of coefficient of determination, R^2, was also evaluated. The Pred *R*-Squared of 0.8650 is in reasonable agreement with the Adj *R*-Squared of 0.9508.

According to the ANOVA for the response R_s-2, the model's *F*-value was 204.85, which implies that the model is significant. Values of Prob > *F* for the terms A, B, C, AC and C2 were less

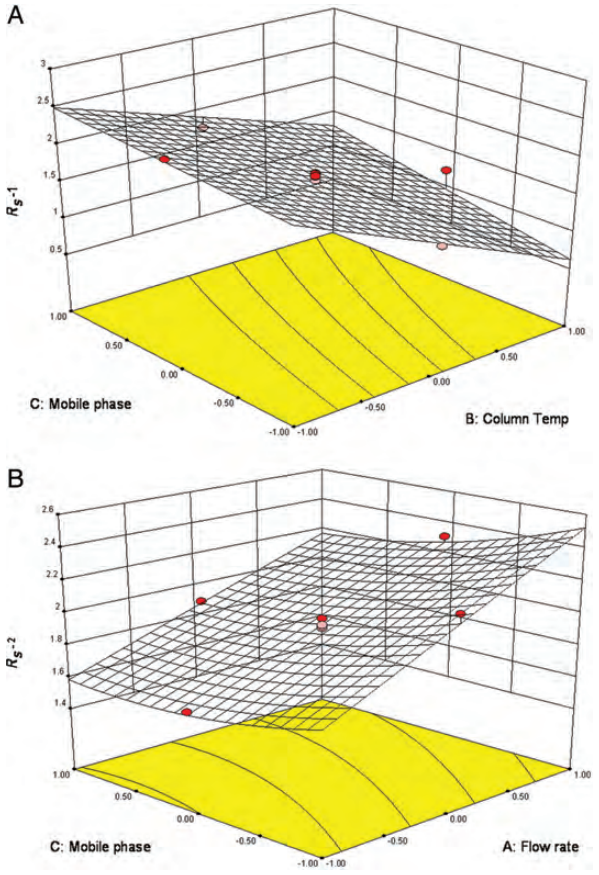


Figure 4. Three-dimensional plots of the response surface for the resolution: the variation of response (R_s-1; Unknown-1/Imp-C) as a function of acetonitrile content in solvent B and column temperature; the fixed factor is flow rate at 1.0 mL/min (A); the variation of response (R_s-2; Imp-E/NAP) as a function of acetonitrile content in solvent B and flow rate; the fixed factor is column temperature at 35°C (B).

than 0.0500, which indicates that these terms of the model are significant. The fraction of explained variation by means of R^2 was also evaluated. The Pred *R*-Squared of 0.9611 is in reasonable agreement with the Adj *R*-Squared of 0.9817. According to the values of coefficients of the polynomial model of CCD, the resolution between Unknown-1 and Imp-C (R_s-1) was significantly influenced by Factor B (column temperature) and the resolution between Imp-E and NAP (R_s-2) was affected by Factor A (flow rate), when compared to the other individual factors. The combined effect of the model term BC for the response R_s-1 and AC for the response R_s-2 was found to be more effective than the other combinations of model terms. The responses were plotted in the form of three-dimensional response surfaces to easily and more precisely define the chromatographic behavior of the investigated substances (Figure 4).

The final equation for resolution R_s-1 and R_s-2 in terms of coded factors was represented as

$$R_s\text{-}1 = 1.67 - 0.30 \text{ X A} - 0.70 \text{ X B} + 0.24 \text{ X C} + 0.10 \text{ X A X C} + 0.11 \text{ X B X C}$$

$$R_s\text{-}2 = 1.93 + 0.34 \text{ X A} - 0.077 \text{ X B} - 0.13 \times \text{C} - 0.046 \times \text{A} \times \text{C} + 0.074 \times \text{C2}$$

To achieve the best separation performances and reasonable retention of all substances in the gradient system, the data analysis led to the conclusion that the final composition of the mobile phase should contain 80% of acetonitrile and 20% of buffer. The temperature of the column should be maintained at 35°C and the flow rate should be maintained at 1.0 mL/min.

Validation of the method

Precision

The percent RSD of peak area for the eight impurities; namely, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G and Imp-H, in the study of the repeatability is shown in Table III. RSD (%)

Table III
Validation data

Parameter	NAP	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G	Imp-H
Linearity									
r	0.9998	0.9997	0.9998	0.9995	0.9998	0.9997	0.9997	0.9982	0.9996
Slope	242.4	455.3	388.7	116.7	317.7	266.0	280.7	253.3	343.1
Y-Intercept	−3.1	−519.0	−435.7	−265.9	−256.3	−358.4	−991.9	138.4	−328.2
Accuracy (%Recovery)									
LOQ (= 3)	-	99.4	99.9	100.4	112.5	84.1	94.0	105.0	101.9
50% (= 3)	-	100.3	102.5	99.6	95.7	86.1	90.7	107.8	101.7
100% (= 3)	-	99.7	100.9	99.0	97.7	89.2	93.0	102.3	100.5
150% (= 3)	-	99.9	100.7	99.0	97.0	92.6	94.9	100.8	100.5
Precision (%RSD)									
LOQ (= 6)	0.8	0.6	1.0	1.7	1.3	0.4	4.6	1.4	1.5
100% (= 6)	-	0.2	0.1	0.4	0.1	1.5	0.5	0.3	0.2
150% (= 6)	-	0.2	0.2	0.3	0.4	0.8	0.4	0.2	0.2
Rugged ness: Different day and analyst (%RSD)									
100% (= 6)	-	0.3	0.4	0.7	0.2	0.6	0.3	1.5	0.2
Robustness (Resolution)									
Different flow 0.8mL/min	1.6	-	3.5	5.0	16.4	20.5	3.2	2.9	19.7
Different flow 1.2mL/min	2.3	-	3.1	4.4	14.7	18.5	3.1	3.1	21.7
Column temperature 30°C	2.1	-	3.6	4.9	16.2	19.3	3.3	3.1	20.8
Column temperature 40°C	1.8	-	3.0	4.7	14.9	17.7	3.0	2.9	20.8
90% organic ratio	2.2	-	3.6	4.8	15.8	18.6	3.2	3.0	21.1
110% organic ratio	1.9	-	3.2	4.7	15.8	18.9	3.2	2.9	20.4
Limit of Detection (concentration in μg/mL)									
-	0.015	0.015	0.015	0.025	0.015	0.015	0.015	0.015	0.015
Limit of Quantification(concentration in μg/mL)									
-	0.045	0.045	0.045	0.068	0.045	0.045	0.045	0.045	0.045

Table IV
Forced degradation data

Degradation condition	Time	RS by HPLC % degradation	Remarks/observation
HCl- 0.1N RT (Acid hydrolysis)	24hr	1.9%	Impurity-B as a degradation product
NaOH-0.1N RT (Base hydrolysis)	1 hr	3.1%	Impurity-B as a degradation product
Water hydrolysis at RT	24hrs	No degradation	No degradation observed.
Oxidation by H_2O_2- 3.0% RT	24hrs	No degradation	No degradation observed.
Thermal (90°C) for 10 days	10days	No degradation	No degradation observed.
UV at 254nm for 10 days	10days	No degradation	No degradation observed.

results of NAP and its impurities for intermediate precision (intra-day and inter-day repeatability) are within 1.5%. These results confirmed that the method was highly precise.

LOD and LOQ

The determined LOD, LOQ and precision at LOQ values for NAP and its eight impurities are reported in Table III.

Accuracy

The percentage recovery of eight impurities of NAP in bulk drug samples ranged from 86.1 to 112.5 % (Table III).

Linearity

For all eight impurities and NAP, a linear calibration curve was obtained, ranging from 0.0375 to 0.225% (25, 50, 75, 100, 125 and 150%) with respect to the specification limit (0.15%). The correlation coefficient obtained was greater than 0.99 (Table III). The results indicate excellent linearity.

Robustness

In all of the deliberate varied chromatographic conditions (flow rate, column temperature and variation of acetonitrile content in solvent B), all analyte peaks were adequately resolved and elution orders remained unchanged (Table III).

Solution stability and mobile phase stability

No significant changes in the amounts of the eight impurities were observed during solution stability and mobile phase experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable for up to 48 h during the determination of related substances.

Results from forced degradation studies

All forced degradation samples were analyzed at an initial concentration of 150 μg /mL of NAP with HPLC conditions mentioned previously, using a PDA detector to ensure the homogeneity and purity of the NAP peak. Degradation was not observed when NAP was subjected to aqueous, oxidative, light, and heat conditions. Significant degradation was observed when the drug was subjected to acidic hydrolysis (0.1N HCl for 24 h at room temperature) and in basic hydrolysis (0.1N NaOH at room temperature for 1 h). Acidic and basic degradation led to the formation of Imp-B. This was confirmed by co-injecting Imp-B standard with these degraded samples. The peak purity test results obtained from the PDA detector confirmed that the NAP peak obtained from all degradation conditions was homogenous and pure. Results from forced degradation studies are presented in Table IV.

This table shows that the ester functionality of NAP was susceptible to acidic and basic hydrolysis.

Conclusion

The rapid gradient RP-HPLC method developed for the quantitative analysis of naproxcinod and related substances in bulk drugs is precise, accurate, linear, robust and specific. Satisfactory results were obtained from the validation of the method .The retention time (approximately 10.0 min) enables rapid determination of the drug. This method exhibited an excellent performance in terms of sensitivity and speed. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples of naproxcinod.

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