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"DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF NAPROXEN SODIUM AND CODEINE PHOSPHATE IN BULK AND PHARMACEUTICAL FORMULATIONS"

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ABSTRACT
RP-HPLC method were developed and validated for the simultaneous estimation of Naproxen Sodium (NAP) and Codeine Phosphate (COD) in bulk and in its tablet dosage forms as per ICH guidelines. In the developed RP-HPLC method, NAP and COD were detected at 254 nm. 10mM Ammonium acetate and Acetoritrile in the ratio of 55:45 is used as a mobile phase. In RP-HPLC method LOD and LOQ value of NAP is 0.265 µg/ ml and 0.805 µg/ ml and for COD is 0.078 µg/ ml and 0.239 µg/ ml. The developed methods were validated according to ICH guidelines. The developed methods were found to be simple, accurate and precise for the routine analysis of the NAP and COD in bulk and tablet dosage forms.
Index terms: Naproxen Sodium, Codeine Phosphate, Validation and RP-HPLC.

I.INTRODUCTION:

Naproxen is indicated for the management of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, polyarticular juvenile idiopathic arthritis, tendinitis, bursitis, acute gout, primary dysmenorrhea, and for the relief of mild to moderate pain Further, it is first-line therapy for osteoarthritis, acute gouty arthritis, dysmenorrhea, and musculoskeletal inflammation and pain. It is chemically known as (+)-(S)-2-(6-Methoxynaphthalen-2-yl) propanoic acid with molecular formula $C_{14}H_{13}NaO_3$. As with other non-selective NSAIDs, naproxen exerts it's clinical effects by blocking COX-1 and COX-2 enzymes leading to decreased prostaglandin synthesis. Although both enzymes contribute to prostaglandin production, they have unique functional differences. The COX-1 enzymes is constitutively active and can be found in normal tissues such as the stomach lining, while the COX-2 enzyme is inducible and produces prostaglandins that mediate pain, fever and inflammation. The COX-2 enzyme mediates the desired antipyretic, analgesic and anti-inflammatory properties offered by Naproxen, while undesired adverse effects such as gastrointestinal upset and renal toxicities are linked to the COX-1 enzyme[1-3].

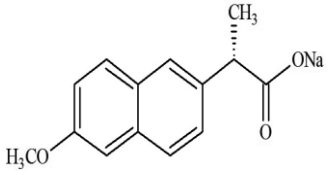

Fig. 1: Structure of Naproxen sodium.

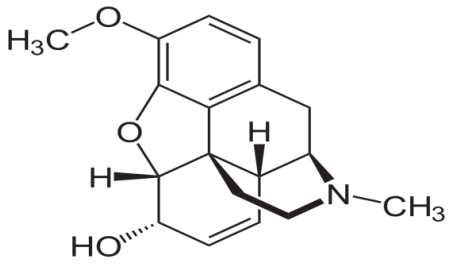

Fig. 2: Structure of Codeine phoshphate.

Codeine is used to treat mild to moderate pain and also to reduce coughing. It is usually combined with other medicines, such as acetaminophen, in prescription pain medicines. It is frequently combined with other drugs in prescription and over-the-counter (OTC) cough and cold medicines. It is desiganated chemically 5α,6α)-7,8-didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol. With molecular formula $C_{18}H_{21}NO_3.H_3PO_4.$ ½ H_2O.

Although the exact mechanism of action of codeine is still unknown, it is generally thought to be mediated through the agonism of opioid receptors, particularly the mu-opioid receptors.Morphine was previously postulated to contribute to the analgesic effect of codeine due to the O-demethylation of codeine to morphine by CYP2D6. Particularly, CYP2D6 poor metabolizer did not experience the analgesic effect of codeine. However, this is unlikely to be the main mechanism of action of codeine as only 5% of codeine is metabolized to morphine. Other hypotheses also postulate that codeine-6-glucuronide, the main metabolite of codeine, mediates the analgesic effect of codeine as it not only has an affinity to the mu receptors as codeine but also can be metabolized to morphine-6-glucuronide, which was observed to be more potent than morphine.

Binding to the mu receptors by codeine activates the G-proteins $G\alpha_i$, causing a decrease in intracellular cAMP and Ca^{2+} level. This causes hyperpolarization of nociceptive neurons, thus imparing the transmission of pain signals.

The combination of NAP and COD is more effective in increasing threshold and tolerance to electrically induced pain[4-6].
Literature survey reveals that, till date no UV-Spectrophotometric method has been reported for the determination Nap and COD. In this study attempts has been made to develop and validate simple, accurate and economical UV methods for simultaneous estimation of NAP and COD[7].

II.EXPERIMENTAL:

Chemicals and Reagents:

Acetonitile used is of HPLC grade, Ammonium acetate of AR grade is taken and its 10 mM strength is prepared by using HPLC grade water. All chemicals and reagents used were of HPLC grade.

Instrumentation:

Shimadzu make HPLC unit accomplished with SPD-20AD UV-Visible detector, Enable C18 (250*4.6*5) Column (Shimadzu), LC-20 AD Pump was used for the analysis. Quantitative HPLC was performed on a isocratic mode with 20 µl injection volume of sample loop (manual). The output signal was monitored and integrated using software LAB SOLUTIONS (Shimadzu).

III.Method: RP-HPLC method:

Preparation of Mobile phase:

Preparation of buffer solution: 0.7708 g of ammonium acetate was dissolved in to the 1000 mL of HPLC water and its pH was adjusted to 3.8 by using O-Phosphoric acid
The mobile phase (1000 mL) was prepared by mixing of ammonium acetate and acetonitrile in the ratio of 55:45 v/v .
The mobile phase was sonicated for 20 min, and then it was filtered through 0.45 µm nylon filter paper.

Preparation of standard stock solution:

100 mg each of COD and NAP were weighed separately and transferred in two different 100 mL volumetric flasks. Both the drugs were dissolved in 100 mL of mobile phase by sonication and then volume was made up to the mark with mobile phase to get a concentration of 1000 µg/mL of each component (stock A and A' solution).
From the above stock A solution 10 mL of aliquot was pipetted out into a 100 mL volumetric flask and the volume was made up to the mark with mobile phase to obtain a concentration of 100 µg/mL of COD each component (stock B solution).
The above stock A^1 solution is itself is used as the standard stock solution for NAP.

Preparation of calibration curves:

Appropriate dilutions were prepared separately and 20 µl of each was injected into the HPLC system and their chromatograms were recorded under the same chromatographic conditions as described below. Peak areas were recorded for all the peaks and a standard calibration curve of AUC against concentration was plotted.

Chromatographic condition:

The mobile phase containing both buffer and acetonitrile in the ratio of 55:45 was selected as the optimum composition of mobile phase, because it was found that this solvent system resolved both the components ideally. The flow rate was set to 1.2 ml/min and UV detection was carried out at 254 nm. The mobile phase and samples were degassed by sonication for 15 min and filtered through 0.45 µm membrane filter. All determinations were performed at room temperature (25°C).

Selection of analytical concentration range:

Appropriate aliquots were pipetted out from the standard stock solution (B for COD) and standard stock solution (A for NAP) into a series of 10 ml volumetric flasks. The volume was made upto the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 50-250 µg/ml and 5-25 µg/ml of NAP and COD respectively. Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µl volume of the drug was injected into the HPLC system and their chromatograms were recorded under the same chromatographic conditions as described above. Peak areas were recorded for all the peaks and a standard calibration curve of peak area against concentration was plotted.

Analysis of Tablet formulation:

Twenty tablets of NAP and COD (APRANAX PLUS) in combination were weighed and their average weight was determined. The tablets were crushed to fine powder and from the triturate, tablet powder equivalent to 100 mg of NAP is weighed and transferred to 100 ml volumetric flask (Which also contain 54.5 mg of COD) and dissolved in the solvent and the content was kept in sonicator for 15 min. The solution was filtered through Whatmann filter paper No.41 and this solution was used as stock A solution.

From the above stock A solution 2 ml volume of the solution was pipetted and the volume was made up to 10 ml with the solvent to obtain a solution with a desired concentration range of NAP and COD i,e 200 µg of NAP and 10.9 µg of COD and analysed at 400 - 200 nm[8-11].

IV.METHOD OF VALIDATION:

Linearity and Range.

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

Both the drugs obeyed Beer's Law in the concentration range of 50-250 µg/mL and 5-25 µg/mL for NAP and COD respectively.

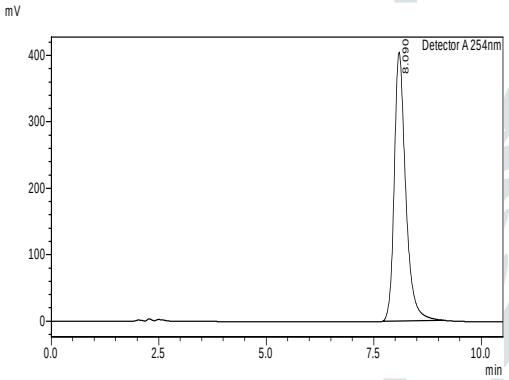

Fig 3: Chromatogram of NAP at 254 nm.

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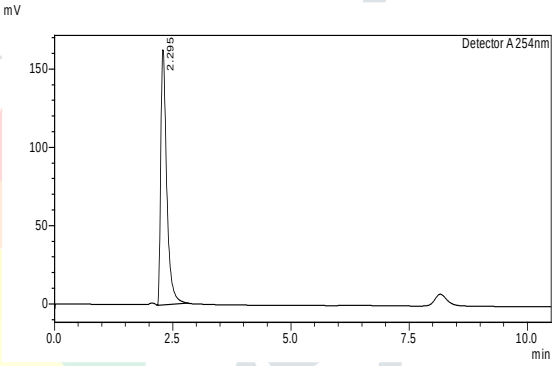

Fig 4: Chromatogram of COD at 254 nm.

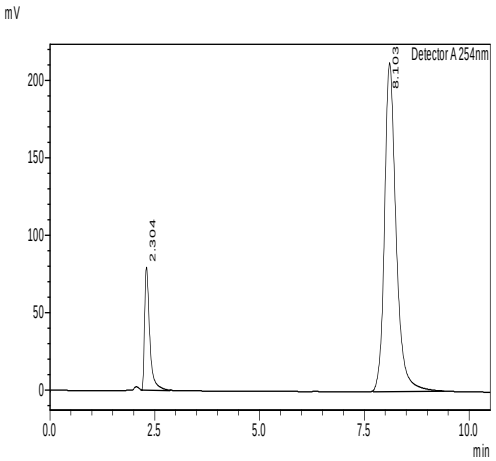

Fig 5: Chromatogram of mixture of NAP and COD at 254 nm.

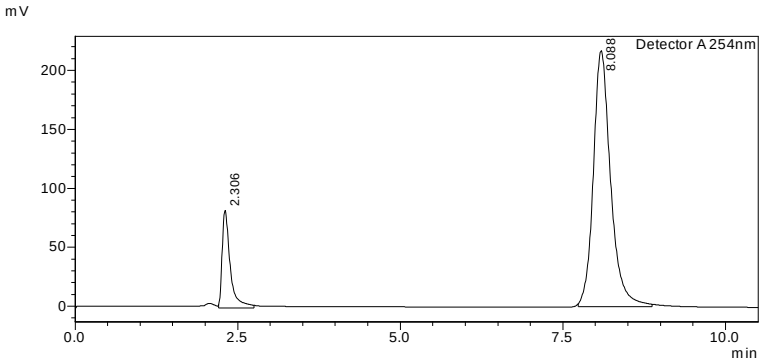

Fig 6: Chromatogram of NAP and COD from formulation at 254 nm.

Table 1: Statistical data of NAP and COD at 254 nm by RP-HPLC method.

Sl. No.	Concentration (µg/ml)		Area	
	NAP	COD	NAP	COD
1	50	5	1491155	261999
2	100	10	2980192	561950
3	150	15	4412795	832550
4	200	20	5835298	1059930
5	250	25	7415858	1334985

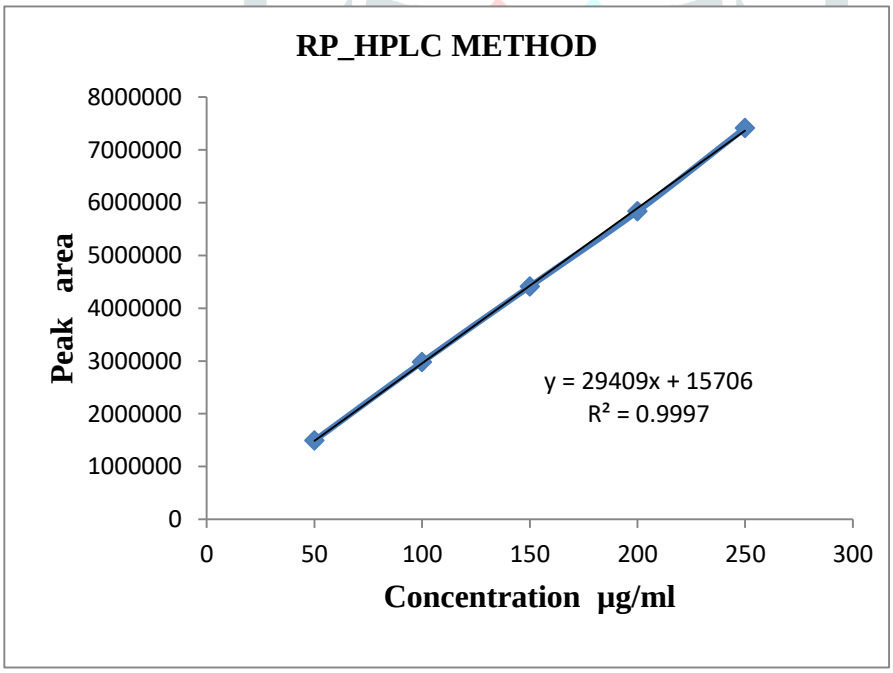

Fig 7: Calibration curve for NAP at 254 nm by HPLC Method.

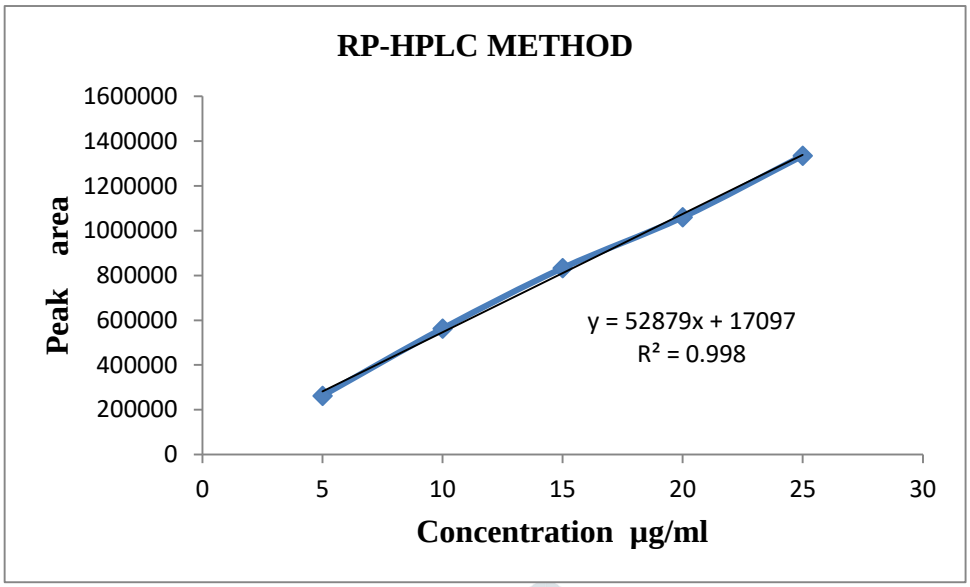

Fig 8: Calibration curve for COD at 254 nm by HPLC Method.

ACCURACY:

Procedure for determination of Accuracy.

Recovery studies were carried out by adding 80 %, 100 % and 120% of the standard drug solution of NAP and COD to the known amount of sample solution by standard addition method.

Table 2: Determination of Accuracy of NAP and COD.

Level Of % recovery	Amount taken from formulation (µg/mL)		Amount of standard drug added (µg/ml)		Total amount recovered (µg/ml)		% Recovery	
	NAP	COD	NAP	COD	NAP	COD	NAP	COD
80 %	100	5.45	80	8	179.9	13.47	99.94	100.14
	100	5.45	80	8	178.95	13.42	99.41	99.77
	100	5.45	80	8	179.96	13.44	99.97	99.92
100 %	100	5.45	100	10	199.45	15.40	99.72	99.67
	100	5.45	100	10	199.35	15.39	99.67	99.61
	100	5.45	100	10	199.32	15.41	99.66	99.74
120 %	100	5.45	120	12	219.18	17.38	99.62	99.59
	100	5.45	120	12	219.25	17.36	99.65	99.48
	100	5.45	120	12	219.22	17.37	99.64	99.54

]

Table 3 : Statistical validation data for accuracy determination.

evel of (%) Recovery	Mean*		Standard deviation*		%Coefficient of Variation*		tandard Error*	
	NAP	COD	NAP	COD	NAP	COD	NAP	COD
80 %	99.77	99.94	0.31478	0.1861	0.3154	0.186206	0.2639	0.1560
100 %	99.68	99.67	0.03403	0.0650	0.0341	0.065277	0.0285	0.0545
120 %	99.64	99.53	0.01596	0.0550	0.0160	0.055332	0.0133	0.0461

*n = 3

Procedure for addition of 80 % standard solution of NAP to the known amount of sample solution.
1 ml of sample stock solution (containing 1000 µg/ml of NAP) is transferred to the 10 ml volumetric flask and added 0.8 ml of standard stock solutions of NAP (800 µg/ml). The volume is made up to the mark with Mobile phase. Peak area was measured at 254 nm respectively.

Procedure for addition of 80 % standard solution of COD to the known amount sample solution.
1 ml of sample solution (containing 54.5 µg/ml of COD) is transferred to the 10 ml volumetric flask and added 0.8 ml of standard stock B solutions of COD (80 µg/ml). The volume is made up to the mark with Mobile phase. Peak area was measured at 254 nmrespectively.

Procedure for addition of 100 % standard solution of NAP to the known amount of sample solution.
1 ml of sample stock solution (containing 1000 µg/ml of NAP) is transferred to the 10 ml volumetric flask and added 1 ml of standard stock solutions of NAP (1000 µg/ml).The volume is made up to the mark with Mobile phase. Peak area was measured at 254 nm respectively.

Procedure for addition of 100 % standard solution of COD to the known amount of sample solution.
1 ml of sample stock solution (containing 54.5 µg/ml of COD) is transferred to the 10 ml volumetric flask and added 1ml of standard stock B solutions of COD (100 µg/ml). The volume is made up to the mark with Mobile phase. Peak area was measured at 254 nm respectively.

Procedure for addition of 120 % standard solution of NAP to the knows amount of sample solution.
1ml of sample stock solution (containing 1000 µg/ml of NAP) is transferred to the 10 ml volumetric flask and added 1.2 ml of standard stock solutions of NAP (1200 µg/ml).). The volume is made up to the mark with Mobile phase. Peak area was measured at 254 nm respectively.

Procedure for addition of 120 % standard solution of COD to the known amount of sample solution.
1 ml of sample stock solution (containing 54.5 µg/ml of COD) is transferred to the 10 ml volumetric flask and added 1.2 ml of standard stock B solutions of COD (120 µg/ml).). The volume is made up to the mark with Mobile phase. Peak area was measured at 254 nm respectively.

The concentration of COD and NAP were calculated. At each level of recovery dies, three determinations were performed. The results obtained were compared and statistically validated.

PRECISION:

It is the procedure which express closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed condition.

Procedure for determination of intra-day precision.
In intraday precision the above sample mixture containing 50 µg/mL of NAP and 5 µg/mL of COD was analyzed six times at different time interval in the same day at their selected analytical wavelength 254 nm for NAP and COD. The variation of the results within the same day was analyzed and statistically validated.

Table 4: Intra-day precision of NAP and COD.

Sr. no	Amount used (µg/mL)		Amount obtained (µg/mL)		Amount obtained (%)	
	NAP	COD	NAP	COD	NAP	COD
1	50	5	49.49	4.96	98.98	99.20
2	50	5	49.68	4.95	99.36	99
3	50	5	50.06	5.01	100.12	100.20
4	50	5	49.99	4.98	99.98	99.60
5	50	5	49.98	4.98	99.96	99.60
6	50	5	49.91	4.99	99.82	99.80

Table 5: Statistical validation data for determination of intra-day Precision.

Components	Mean*	Standard deviation*	% Coefficient of Variation*	Standard Error*
NAP	99.70333	0.440621	0.441932	0.3694
COD	99.56667	0.427395	0.429255	0.3583

*n = 6

Procedure for determination of inter-day precision.

In inter-day precision the above sample mixture containing 50 µg/mL of NAP and 5 µg/mL of COD were prepared and analysed six times at same time on three different days of a week at their selected analytical wavelength at 254 nm for NAP and COD. The variation of the results on different days was analyzed and statistically validated.

Table 6: Inter-day precision of NAP and COD.

SI.No.	ıount used (µg/mL)		ınt obtained (µg/mL)		% Obtained	
	NAP	COD	NAP	COD	NAP	COD
DAY-1						
1	50	5	49.71	4.955	99.42	99.1
2	50	5	49.92	4.945	99.84	98.9
3	50	5	49.9	4.955	99.8	99.1
4	50	5	49.82	4.99	99.64	99.8
5	50	5	49.88	4.955	99.76	99.1

6	50	5	49.91	4.964	99.82	99.28

DAY-II

1	50	5	49.98	4.99	99.96	99.8
2	50	5	49.97	4.985	99.94	99.7
3	50	5	49.98	4.9829	99.96	99.658
4	50	5	49.87	4.982	99.74	99.64
5	50	5	49.96	4.986	99.92	99.72
6	50	5	49.92	5	99.84	100

DAY-III

1	50	5	49.93	4.995	99.86	99.9
2	50	5	49.88	4.988	99.76	99.76
3	50	5	49.86	4.987	99.72	99.74
4	50	5	49.88	4.978	99.76	99.56
5	50	5	49	4.958	98	99.16
6	50	5	49.92	4.997	99.84	99.94

Table 7: Statistical validation data for inter-day precision.

Components	**Mean***	**Standard Deviation***	**Co-efficient of Variation**	**Standard Error***
NAP	99.69889	0.442903	0.444241	0.36143
COD	99.54767	0.343722	0.345284	0.281616

ROBUSTNESS:

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters. The solutioncontaining150 µg/mL of NAP and 15 µg/mL of COD was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate and pH.

Table 8: Robustness result for variations in flow rate (mL/min) and pH.

Method Parameter	**Level**	**Retention Time**		**Tailing factor**	
Flow Rate (mL/min)		**NAP**	**COD**	**NAP**	**COD**
1.1	-1	8.094	2.294	1.090	1.244
1.2	0	8.095	2.295	1.091	1.245
1.3	1	8.096	2.296	1.092	1.246
pH					

3.6	-0.2	8.093	2.293	1.090	1.242
3.8	0	8.095	2.295	1.091	1.245
4.0	0.2	8.097	2.297	1.093	1.246

RUGGEDNESS:

The evaluation of ruggedness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in analyst or instrument. The solution containing 150 µg/mL of NAP and 15 µg/mL of COD was injected to HPLC three times by different analyst.

Table 9 : Ruggedness result for variations in analyst.

Method Parameter	**Retention Time**		**Tailing factor**	
Analysts	**NAP**	**COD**	**NAP**	**COD**
Analysts 01	8.095	2.295	1.091	1.245
Analysts 02	8.096	2.296	1.093	1.247

V.RESULTS AND DISCUSSION:

The present manuscript deals with simultaneous estimation of NAP and COD in combined tablet dosage form by RP- HPLC method using suitable mobile phase. The developed method is based upon estimation of both the drugs by determining the peak area of the chromatogram at selected analytical wavelength. The linearity of the proposed method was established by least square regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 50-250 µg/ml for NAP (r2 = 0.9997) and 5-25 µg/ml COD (r2 = 0.997) respectively, along with the summary of validation and system suitability parameters as shown in the Table 11. Recovery studies were also performed to determine the accuracy and precision of the proposed method. Recovery experiments were performed at three levels, 80%, 100% and 120% of the labeled amount of both the drugs as shown in Table 2.Three replicate samples of each concentration levels were prepared and the percentage recovery at each level (n = 3), and mean % recovery (n = 3) were determined and Statistical validation data for accuracy determination summarized in Table 3. Intra-day precision as estimated by assaying samples containing 50 µg/ml of NAP and 5 µg/ml of COD, six times and the results were averaged for statistical evaluation. The assay results and statistical validation data for intra-day precision are summarized in Table 4 and 5. Inter-day precision was evaluated by analyzing a set of quality control samples containing 50µg/ml of NAP and 5 µg/ml of COD, replicates were analyzed on three consecutive days. The determination of inter-day precision and statistical validation data for inter-day precision is summarized in Table 6 and 7. Both intra-day and inter-day variation showed less % RSD value indicating high grade of precision of the method. The robustness was evaluated by analysing the samples by varying few parameters like pH and flow rate. The determination of robustness and statistical validation data is summarized in Table 8. Table 9 Ruggedness result for variations in analyst. The validation results obtained confirm the suitability of the proposed RP-HPLC method for simple, accurate and precise analysis of NAP and COD in pharmaceutical preparations. The proposed method does not need prior separation of NAP and COD before analysis.

Table 10: Summary of validation and system suitability parameters of NAP and COD.

Parameters	**NAP**	**COD**
Linear range (µg/mL)	50-250	5-25
Slope	29409	52879
Intercept	15706	17097

Regression coefficient (r^2)	0.997	0.998
Limit of Detection (µg/mL)	0.265728	0.078968
Limit of Quantification (µg/mL)	0.805237	0.239296
Retention time (min)	2.295	8.090
Tailing factor	1.09	1.2
Resolution factor	16.729	
Theoretical plate	4860	2623
HETP µm	29.969	15.521

VI.CONCLUSION:

Proposed study describes a new RP-HPLC method for the estimation of NAP and COD in combination using simple mobile phase. The method gives good resolution between the compounds with a short analysis time. The method was validated and found to be simple, sensitive, accurate and precise. So the developed method can be used conveniently for analysis of NAP and COD in combined pharmaceutical dosage forms.

VII.ACKNOWLEDGEMENT:

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