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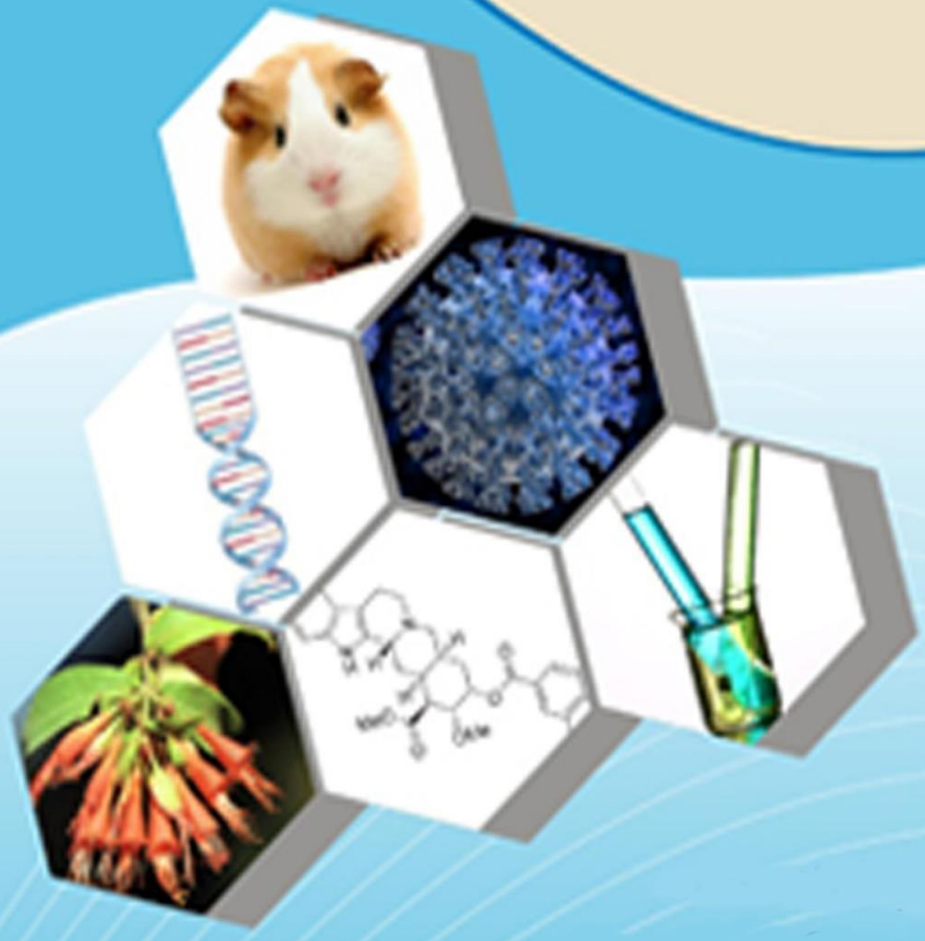

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CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR NEPAFENAC AND DEXAMETHASONE IN COMBINATION

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INTRODUCTION

Nepafenac is a nonsteroidal anti-inflammatory drug (NSAID), usually sold as a prescription eye drop 0.1% solution or 0.3% solution. It is used to treat pain and inflammation associated with cataract surgery. Dexamethasone is a steroid that prevents the release of substances in the body that cause inflammation. Dexamethasone is used to treat many different conditions such as allergic disorders, skin conditions, ulcerative colitis, arthritis, lupus, psoriasis, breathing disorders, inflammatory eye conditions, blood cell disorders, leukemia, or endocrine disorders. Topical Nepafenac and Dexamethasone together decreased complaints, inflammation, and macular swelling after cataract surgery, suggesting that this anti-inflammatory therapy is effective. The typical drug classes prescribed include topical antibiotics, NSAIDs, and corticosteroids. The combination of topical Nepafenac and Dexamethasone reduced subclinical macular swelling and inflammation as well as subjective complaints, indicating it is an efficient anti-inflammatory regimen after cataract surgery. 3) Patent of United States Cagle et al.Patent No.: US 6,716,830 B2 prove that the combination of Nepafenac and Dexamethasone was effectively used. Clinical trial approved that the combination of Nepafenac with Dexamethasone is appropriate for the prophylaxis of Irvine-Gass syndrome, also for the control and the reduction of the postoperative inflammation. This combination of Nepafenac and Dexamethasone was approved for the safety and efficacy in treatment of muscular swelling after cataract surgery. Literature survey reveals that no Spectrophotometric and Chromatographic method are reported yet for the determination of Nepafenac and Dexamethasone in combination. Therefore research work, is to develop a novel, simple, specific, accurate, precise and economic methods for estimation of Nepafenac and Dexamethasone in combination.

ABSTRACT

As per the overlain spectrum the wavelength selected for estimation of Nepafenac was 378 nm, where Dexamethasone has no significant absorbance and for Dexamethasone it was 237 nm, where absorbance of Dexamethasone is corrected. **correlation coefficient** of **0.999** and **0.999** for **Nepafenac and Dexamethasone** respectively. The suggested approach was assessed as

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wellby the Assay of Synthetic mixture containing **Nepafenac and Dexamethasone**. The % assay was observed **99.94 % for Nepafenac** and **100.06% for Dexamethasone** in mixture respectively. The % recovery for Nepafenac was got to be 99.39 to 99.71% and for Dexamethasone was observed 99.65 to 99.97% **LOD** and **LOQ** was got 0.014µg/ml and 0.043 µg /ml for Nepafenac, respectively and 0.012µg/ml and 0.036µg/ml for Dexamethasone, respectively.

Implications and Applications

Analytical chemistry is a Branch of Chemistry that determines the nature and identityof a substance and its composition.The need of the sophisticated analytical instruments and determination using them isalmost a routine process for the modern analytical laboratories. Further all themanual techniques in the line of analytical studies had steadily been transferred to theinstrumental techniques.

Nepafenac is a nonsteroidal anti-inflammatory drug(NSAID), usually sold as a prescriptioneye drop 0.1% solution or 0.3%solution. The developed method impact the treatment and management of post-cataract surgery patients by measuring the concentration of substances and to establish the identity, purity, physical characteristics, and potency of drugs in combione, before it reaches to the market.

The developed method provides potential advantages over existing methods for identifying Nepafenac and Dexamethason. Because, yet no Spectrophotometric and Chromatographic method are reported yet for the determination of Nepafenac and Dexamethasone in combination.

APPROACH FOR DEVELOPMENT OF RP-HPLC METHOD

➢ Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better perform is in terms of efficiency, stability and reproducibility.

EXPERIMENTAL WORK

1. APPARATUS AND INSTRUMENT

Spectrophotometric Method Development

List of Instrument and Apparatus

Component	Model/Software	Manufacturer
HPLC	Waters	Waters
Analytical Balance		
Volumetric Flask	-	Borosil
Pipettes	-	Borosil
Beaker	-	Borosil

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- HPLC water (RANCAM).
- pH tutor was used for pH measurement.
- **Sonication** of solutions was done using Ultrasonic cleaner.
- All **volumetric glassware** used was calibrated.
- Column: C_{18} (250mm×4.6mm) 5µm.
- Pipettes of 1, 2, 5 and 10 ml capacity.
- Measuring cylinders of 10, 100 ml and 500 ml capacity.
- Class 'B' volumetric glassware.
- All apparatus and instrument were calibrated before use.

2. REAGENTS AND MATERIAL

Working Standard API

Standard	Purpose	Source
Nepafenac	Analysis	Pure Chem Pvt. Ltd. Ankleshwar
Dexamethasone	Analysis	Farbe Firma Ankleshwar

- All the Reagents and Solvents used wereof AR orHPLCgrades.
- Acetonitrile HPLC Grade, Merck Ltd.
- Formic Acid
- Ultra-pure water
- Membrane filter: 0.22 µm nylon membrane filter (RANKEM)

3. STANDARD SOLUTION PREPARATION

- **Preparation Standard solution of NEPAFENAC**

A standard stock solution of NEPA (1000 µg/ml) was prepared separately by dissolving 100 mg of drug in 100 ml mobile phase - Acetonitrile: Water (60:40, v/v).

- **Preparation Standard solution of DEXAMETHASONE**

A standard stock solution of DEXA (1000 µg/ml) was prepared separately by dissolving 100 mg of drug in 100 ml mobile phase - Acetonitrile: Water (60:40, v/v).

- **Preparation Standard solution of NEPAFENAC and DEXAMETHASONE in Combination**

1.0 ml from working standard stock solutions of NEPA (1000 µg/ml) and 1.0 ml from working standard stock solutions of DEXA (1000 µg/ml) were taken in a common volumetric flask diluted up to 10ml with mobile phase - Acetonitrile: Water (60:40, v/v) to make final concentration NEPA (100µg/ml) and DEXA (100µg/ml).

- **Preparation of Linearity Solution:**

Stock solution was prepared. Concentration of NEPA was 1000 µg/ml and DEXA was 1000 µg/ml. From this solution, take 0.1, 0.5, 1.0, 1.5, 2.0 ml diluted upto 10 ml Methanol to make

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the concentration 10, 50, 100, 150, 200 µg/ml &10, 50, 100, 150, 200 µg/ml of NEPA and DEXA respectively.

4. SELECTION OF ELUTION MODE

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to polar compounds. It is not only simple, convenient but also better performs in terms of efficiency, stability and reproducibility. C_{18} column was selected because it is least polar compared to C_4 and C_8 columns. C_{18} column allows eluting polar compounds more quickly in comparison to non-polar compounds.

In addition to this, UV detector is used, which allows easy detection of the compounds in UV transparent organic solvents. A 250 x 4.6 mm column of 5µm particles packing was preferred as a starting point for method development. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability. This configuration provides a large number of theoretical plate's values for most separation.

5. MOBILE PHASE OPTIMIZATION

Various solvent ratios of acetonitrile and Water were tried as mobile phase for separation of NEPAFENAC and DEXAMETHASONE.

6. PREPARATION OF MOBILE PHASE

The mobile phase consisted of mixture of Acetonitrile and Water in ratio of (40:60, v/v). The mode for was isocratic. The mobile phase was filtered through a 0.22 µm nylon membrane filter and degassed prior to use.

CHROMATOGRAPIC SEPERATION

Standard and sample solutions were injected in column using ultra fast auto sampler. The chromatogram was run for appropriate time duration with degassed mobile phase, mixture of Acetonitrile and Water in ratio of (60:40, v/v), using UV detector (SPD-20AV) at wavelength 237 nm. The chromatogram was stopped after separation was achieved completely. Data related to peak like area, height, retention time, resolution etc. was recorded using EMPOWER 3 software.

CHROMATOGRAPHIC CONDITION

Table: Chromatographic Condition

Parameter	Condition
Method	Isocratic reverse phase technique
Stationary Phase	Inertsil C18 column (250 mm x 4.6 mm) 5 µm
Mobile Phase	Acetonitrile : water (60 : 40 V/V)
Flow Rate	1.0 min/ml
Column Temperature	25°C
Wavelength	237 nm
Total Run Time	10 min

VALIDATION OF PROPOSED METHOD

Parameters to be considered for the validation of method are:

1. SYSTEM SUITABILITY STUDIES

The system suitability was evaluated by five replicate analyses of NEPA and DEXA mixture at concentration of 100 µg/ml of NEPA and 100 µg/ml of DEXA. The column efficiency, resolution, and peak asymmetry were calculated for the standard solutions.

STABILITY OF ANALYTICAL SOLUTION

In order to demonstrate the stability of both the standard and sample solutions during analysis, both solutions were analyzed over a period of 24 h at room temperature. The results indicated that for both the solutions, the retention time and peak area of NEPA and DEXA did not show much variation (% RSD less than 2.0). There was no significant degradation within the indicated period. Hence, it was concluded that both the solutions were stable for 24 h at room temperature.

LINEARITY AND RANGE

The linearity response was determined by analyzing 5 independent levels of concentrations in the range of 10-200µg/ml and 10-200µg/ml for NEPA and DEXA, respectively.

Preparation of Calibration Curve for NEPA and DEXA

Calibration curve for NEPA

- The calibration curve was constructed with five concentrations ranging from 10-200µg/ml for NEPA (10µl injected). The data of peak area versus concentration were treated by linear least square regression analysis.

Calibration curve for DEXA

- The calibration curve was constructed with five concentrations ranging from 10-200µg/ml for DEXA (10µl injected). The data of peak area versus concentration were treated by linear least square regression analysis.

2. PRECISION

I.Intraday precision

- The precision of the developed method was assessed by analyzing samples of the same batch in nine determinations with three Standard solutions containing concentrations 50, 100, 150µg/ml for NEPA and 50, 100, 150µg/ml for DEXA and three replicate (n=3) each on same day.
- The % RSD value of the results corresponding to the peak area was expressed for intra-day precision.

II.Interday precision

- The precision of the developed method was assessed by analyzing samples of the same batch in nine determinations with three Standard solutions containing concentrations 50, 100, 150µg/ml for NEPA and 50, 100, 150µg/ml for DEXA and three replicate (n=3) each on different day.
- The % RSD value of the results corresponding to the peak area was expressed for inter-day precision.

3. ACCURACY

- From the Synthesis Mixture weigh accurately equivalent about 100mg of NEPA and 100mg of DEXA.
- Take Four 100 ml Volumetric Flask and in each flask add synthetic mixture equivalent to 10mg of NEPA and 10mg DEXA respectively. Flask 1 form as a Placebo and remaining flask

2, 3, 4 spike with 80, 100 and 120 % of Solid API.

- Take content in 100 ml volumetric flask dissolved in 25 ml Methanol and Sonicate for 15min. make up the volume with mobile phase- Acetonitrile: Water (60:40%v/v) up to 100 ml. The solution was filtered through Whatman filter paper No. 42.
- Finally the solution had concentration 100µg/ml for NEPA and 100µg/ml for DEXA.
- Data from nine determinations over three concentration levels covering the specified range was determined and % recovery was calculated.

Table: Spiking of Synthetic Mixture in the Accuracy

Concentration of Synthetic mixture (µg/ml)		Concentration of API in spiking solution (µg/ml)		Total concentration of (µg/ml)	
				NEPA	DEXA
NEPA	DEXA	NEPA	DEXA		
100	100	-	-	100	100
100	100	80	80	180	180
100	100	100	100	200	200
100	100	120	120	220	220

4. LOD (LIMIT OF DETECTION) and LOQ (LIMIT OF QUANTIFICATION)

The LOD and LOQ are estimated from the set of 5 calibration curves used to determine method linearity.

The LOD and LOQ may be calculated as

$$LOD = 3.3 \times \frac{SD}{Slope}$$

$$LOQ = 10 \times \frac{SD}{Slope}$$

Where, SD = SD of Five intercept of calibration curve

Slope = the mean slope of the five calibration curves

5. ROBUSTNESS

Robustness and Ruggedness of the method was determined by subjecting the method to slight change in the method condition, individually, the:

- Flow rate,
- Change in Mobile phase ratio,

Three replicates were made for the same concentration (100µg/ml of NEPA and 100µg/ml of DEXA).

% RSD was calculated.

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6. ANALYSIS OF NEPA AND DEXA IN SYNTHETIC MIXTURE

The preparation of synthetic mixture was as per patent:

- Nepafenac-100
- Dexamethasone-100
- Benzalkonium Chloride- 0.005%
- Purified Water ……q.s

➢ Prepare different concentration of synthetic mixture as follow:

I.Nepafenac and Dexamethasone (10:100µg/ml)

Take Powder equivalent to 1 mg Nepafenac and 100mg of Dexamethasone of Synthetic Mixture in 100 ml Volumetric Flask. Dissolve in 25 ml of Methanol. Sonicate for 15 min. Dilute up to the 100 ml with Solvent. Shake Vigoursly. Filter the solution & further dilute.

II.Nepafenac and Dexamethasone (100:100µg/ml)

Take Powder equivalent to 10 mg Nepafenac and 100mg of Dexamethasone of Synthetic Mixture in 100 ml Volumetric Flask. Dissolve in 25 ml of Methanol. Sonicate for 15 min. Dilute up to the 100 ml with Solvent. Shake Vigoursly. Filter the solution & further dilute.

III.Nepafenac and Dexamethasone (1000:100µg/ml)

Take Powder equivalent to 100 mg Nepafenac and 100mg of Dexamethasone of Synthetic Mixture in 100 ml Volumetric Flask. Dissolve in 25 ml of Methanol. Sonicate for 15 min. Dilute up to the 100 ml with Solvent. Shake Vigoursly. Filter the solution & further dilute.

CHROMATOGRAPHIC METHOD

1. SELECATION OF WAVELENGTH

Selection of Wavelength was carried out using UV Spectrophotometer. Both drug detected at 276nm.

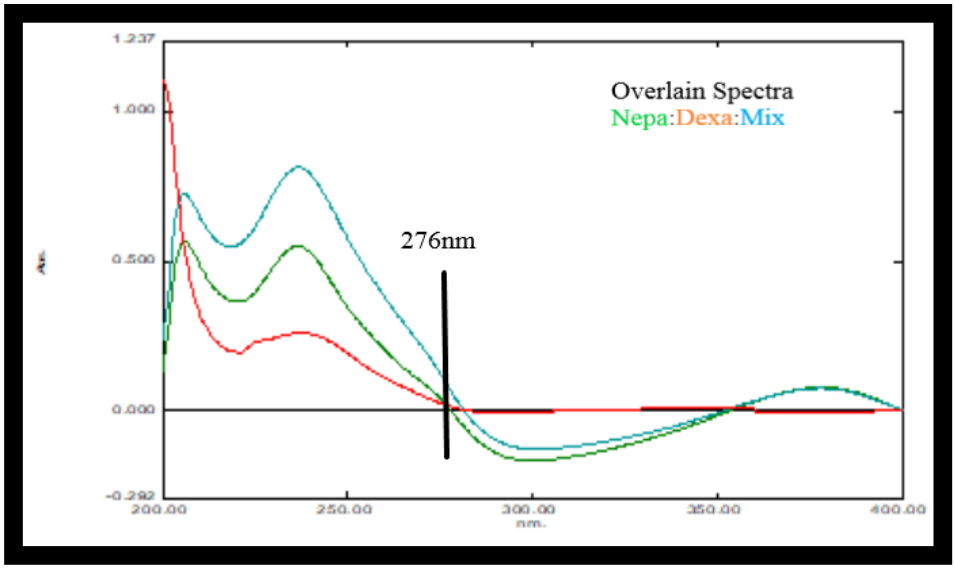

Figure 9.11:Overlain Spectra taken in UV Spectrophotometer

2. MOBILE PHASE SELECTION

Various mobile phases with different ratio of different solvent and pH were used shown in

table. The mixture of Acetonitrile and Water in ratio of (60:40, v/v) provided optimum polarity for proper migration, separation and resolution of NEPA and DEXA peaks. Under these conditions, the eluted peaks were well defined, resolved and free from tailing.

Due to the non-polar nature of the stationary phase more polar component DEXA will be eluted first because of its more affinity towards the polar mobile phase and less polar component NEPA will be eluted later due to its more affinity towards non-polar stationary phase.

3. SYSTEM SUITABILITY DATA

Table : Observed values for system suitability test *(n=6)

PARAMETERS	Observed Values		IP'2010 Specification
	NEPA	DEXA	
Retention Time (min)	6.561	2.838	-
Peak Area			-
Theoretical plates			Not less than 2000
Asymmetry (10%)			Not greater than 2
Resolution			>2

VALIDATION PARAMETERS

1. Linearity and Range

Linearity in the concentration range was **10-200µg/ml** and **10-200µg/ml** for NEPA and DEXA, respectively.

Correlation coefficient (r^2) for calibration curve of NEPA and DEXA was found to be0.999and 0.999, respectively.

The regression line equation for NEPA and DEXA are as following,

y = 266684x + 544049---------- (1)

y = 102251x - 178069---------- (2)

Table : Calibration data for NEPA and DEXA*(n=6)

Sr. No	Concentration(µg/ml)		Peak Area* ± SD NEPA	Peak Area* ± SD DEXA
	NEPA	DEXA		
1.	10	10	2821942 ±1028	989430±438
2.	50	50	14108580±1333	4948821±641
3.	100	100	27216974±890	9898167±1311
4.	150	150	41324743±402	14863450±13982
5.	200	200	53256977±657	20557909±18593

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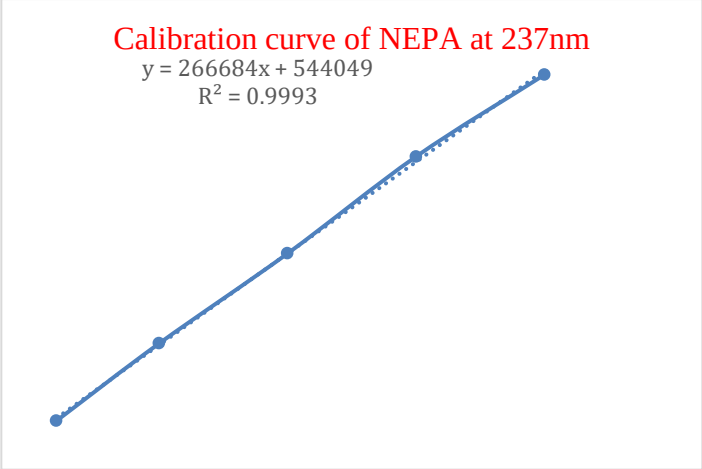

Figure: Calibration Curve for NEPA

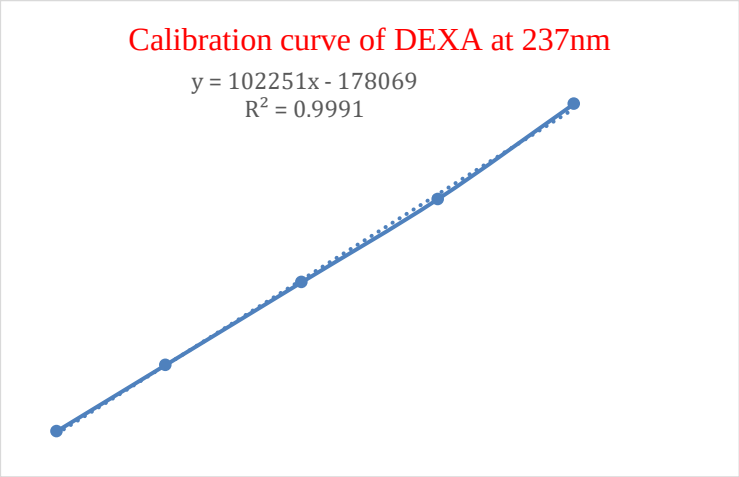

Figure: Calibration curve for DEXA

2. Precision

I.Intraday Precision

The data for Intraday precision for combined standard solution of NEPAand DEXA is presented. The % R.S.D was found to be 0.011-0.019 % for NEPA and 0.011-0.0.06 % for DEXA. This % RSD value was found to be less than 1.0 indicated that the method is precise.

Table : Intraday precision data for estimation of NEPA and DEXA*(n=3)

Conc. (µg/ml)		Peak area* ± SD NEPA	% RSD	Peak area* ± SD DEXA	% RSD
NEPA	DEXA				
50	50	14118312±2301	0.016	4948511±551	0.011
100	100	27220211±5307	0.019	9902205±5950	0.060
150	150	41331420±4643	0.011	14848599±2560	0.017

II.

III.Interday Precision

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Conc. (µg/ml)		Peak area* ± SD NEPA	% RSD	Peak area* ± SD DEXA	% RSD
NEPA	DEXA				
50	50	14126691±3608	0.026	4956543±1820	0.037
100	100	27240212±9373	0.034	9902107±6711	0.068
150	150	41350609±7983	0.019	14857279±2588	0.017

The data for Interday precision for combined standard solution of NEPAand DEXA is presented. The % R.S.D was found to be 0.019-0.034 % for NEPA and 0.017-0.068 % for DEXA. This % RSD value was found to be less than 1.0 indicated that the method is precise.

3. Accuracy

Accuracy of the method was determined by recovery study from synthetic mixture at three levels (80%, 100%, and 120%) of standard addition.

Percentage recovery for NEPAand DEXAby this method was found in the range of 99.39 to 99.71% and 99.65 to 99.97%,respectively.

The value of %RSD within the limit indicated that the method is accurate and percentage recovery shows that there is no interference from the excipients.

Table : Recovery data of NEPA *(n=3)

Conc. of NEPA (µg/ml)	Amount of Std. NEPA added (µg/ml)	Total amount of NEPA(µg/ml)	Total amount of NEPAfound (µg/ml) Mean*± SD	% RSD	% Recovery (n=3)	% RSD
100	-	100	27254314±35643	0.13	100.16	
100	80	180	48506674±62912	0.13	99.61±0.29	0.30
100	100	200	53761160±83817	0.16	99.39±0.31	0.32
100	120	220	59162025±18971	0.03	99.71±0.06	0.06

Table: Recovery data of DEXA *(n=3)

Conc. of DEXA (µg/ml)	Amount of Std. DEXA added (µg/ml)	Total amount of DEXA(µg/ml)	Total amount of DEXAfound (µg/ml) Mean*± SD	% RSD	% Recovery (n=3)	% RSD
100	-	100	10058674±1028	0.010	-	-

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100	80	180	18210310±1023	0.036	99.65±0.08	0.08
100	100	200	20262709±29212	0.144	99.79±0.29	0.29
100	120	220	22325521±2709	0.040	99.97±0.07	0.07

4. Limit of detection and quantitation

The LOD & LOQ for NEPA was found to be 0.014 µg/ml and 0.043µg/ml, respectively.
The LOD & LOQ for DEXA was found to be 0.012 µg/ml and 0.036 µg/ml, respectively.

Table : LOD and LOQ data of NEPA and DEXA

Parameter	**NEPA**(1µg/ml)	**DEXA**(1µg/ml)
LOD (µg/ml)	0.014	0.012
LOQ (µg/ml)	0.043	0.036

5. Ruggedness and Robustness

The obtained Ruggedness and Robustness results are presented in Table. The % RSD was found to be 0.021-0.198 % for NEPA and 0.018-0.403 % for DEXA. These % RSD value was found to be less than 1.0 indicated that the method is precise. No significant changes in the retention time were observed, proving that the developed method is rugged and robust.

Table : Ruggedness and Robustness data of NEPA and NEPA *(n=3)

Ruggedness data of NEPA (100 µg/ml) (n=3)*						
No	**Factor**	**Level**	**Peak area* ± SD**	**%RSD**	**R_t± SD**	**%RSD**
0.	**Mobile phase:** ACN: Water (60:40 v/v)			**Standard retention time:**6.571		
1.	Change in the Flow Rate ± 0.1 ml/min	0.9 ml/min	27251633±5806	0.021		
		1.1 ml/min	27252447±6958	0.026		
2.	Wavelength ± 2.0 nm	239nm	27873609±55144	0.198	6.571±0.001	0.011
		235nm	27868697±48197	0.173	6.574±0.004	0.054
Ruggedness data of DEXA (100µg/ml) (n=3)*						
No	**Factor**	**Level**	**Peak area* ± SD**	**%RSD**	**R_t± SD**	**% RSD**
0.	**Mobile phase:** ACN: Water (60:40 v/v)			**Standard retention time:**2.817		
1.		0.9 ml/min	9885891±15367	0.023		

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	Change in the Flow Rate ± 0.1 ml/min	1.1 ml/min	9885491±1785	0.018		
4.	Wavelength ±2.0 nm	239nm	9829678±39596	0.403	2.817±0.007	0.025
		235nm	9846810±15367	0.156	2.816±0.001	0.050

6. Assay

Chromatogram of the Test Stock solution containing 1000µg/ml of NEPA and 1000µg/ml of DEXA was prepared. From this stock solution prepared different Concentration of solution was recorded and peak areas were noted for estimation of NEPA and DEXA, respectively.

The concentration of NEPA and DEXA in synthetic mixture was determined against the standard NEPA and DEXA. The results from the analysis of synthetic mixture containing different Concentration of NEPA and DEXA in combination are presented in table.

The % assay shows that there is no interference from excipients and the proposed method can successfully Applied.

Table : % Assay of NEPA and DEXA*(n=3)

Sr. No.	Conc. (NEPA:DEXA)	%Assay*	
		NEPA	DEXA
1	10:100	99.65%	100.03%
2	100:100	100.06%	100.05%
3	1000:100	100.11%	100.09%

Summary of validation parameters

Table : Summary of validation parameters

PARAMETERS	RP-HPLC Method	
	NEPA	DEXA
Concentration range (µg/ml)	10-200	10-200
Regression equation	y = 266684x + 544049	y = 102251x - 178069
Correlation Coefficient(r^2)	0.999	0.999
Accuracy (%Recovery) (n=3)	99.39 to 99.71%	99.65 to 99.97%
Intra-day Precision (%RSD) (n=3)	0.011-0.019	0.011-0.060
Inter-day precision (%RSD) (n=3)	0.019-0.034	0.017-0.068
LOD (µg/ml)	0.014	0.012
LOQ (µg/ml)	0.043	0.036

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Robustness and Ruggedness	0.021-0.198	0.018-0.403
%Assay	99.94	100.06

CONCLUSION

All the parameters for two substances met the criteria of the ICH guide lines for the method validation and found to be suitable for routine quantitative analysis in pharmaceutical dosage forms. The result of linearity, accuracy, precision proved to be within limits with lower limits of detection and quantification. Ruggedness and Robustness of method was confirmed as no significant were observed on analysis by subjecting the method to slight change in the method condition. Assay results obtained by proposed method are in fair agreement.

Further Research

Additionally other analytical techniques can be found out for NEPA and DEXA in combination based on the findings of this study. Pharmaceutical stability testing is the process by which drug manufacturers collect data on their product over predetermined lengths of time in specific environmental conditions to determine if there is any change in the quality of the Active Pharmaceutical Ingredient (API) or Final Product (FP). Future studies might be build upon the results of this method for the determination of Nepafenac and Dexamethasoneon stability testing studies for these drugs in combine.

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