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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DICLOFENAC Na AND PARACETAMOL IN BULK AND PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC

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Abstract:

A new simple, accurate, economic, rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Paracetamol and Diclofenac Na, in its pure form as well as in pharmaceutical dosage form. Chromatography was carried out on X bridge C18 (4.6×150mm) 5 µ column using a mixture of Methanol: Phosphate Buffer pH-3.6 (30:70v/v)as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 260nm. The retention time of the Paracetamol and Diclofenac Na was2.669, 3.855±0.02min respectively. The method produces linear responses in the concentration range of 10-50µg/ml of Paracetamol and 10-50µg/ml of Diclofenac Na. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Paracetamol and Diclofenac Na, RP-HPLC, Validation.*

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INTRODUCTION:

Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. In fact, analytical chemistry is the science of chemical identification and determination of the composition (atomic, molecular) of substances, materials and their chemical structure.

Chemical compounds and metallic ions are the basic building blocks of all biological structures and processes which are the basis of life. Some of these naturally occurring compounds and ions (endogenous species) are present only in very small amounts in specific regions of the body, while others such as peptides, proteins, carbohydrates, lipids and nucleic acids are found in all parts of the body. The main object of analytical chemistry is to develop scientifically substantiated methods that allow the qualitative and quantitative evaluation of materials with certain accuracy. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components. [1]

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also quality is important in every product or service, but it is vital in medicines as it involves life.

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, a physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance. [2]

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are:

1. The drug or drug combination may not be official in any pharmacopoeias.
2. A proper analytical procedure for the drug may not be available in the literature due to Patent regulations.
3. Analytical methods for a drug in combination with other drugs may not be available.
4. Analytical methods for the quantitation of the drug in biological fluids may not be available.
5. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable. [1, 2]

DIFFERENT METHODS OF ANALYSIS:

The following techniques are available for separation and analysis of components of interest.

Spectral methods:

The spectral techniques are used to measure electromagnetic radiation which is either absorbed or emitted by the sample.

E.g. UV-Visible spectroscopy, IR spectroscopy, NMR, ESR spectroscopy, Flame photometry, Fluorimetry.2

Electro analytical methods:

Electro analytical methods involved in the measurement of current voltage or resistanceas a

property of concentration of the component in solution mixture.

E.g. Potentiometry, Conductometry, Amperometry. [2]

Chromatographic methods:
Chromatography is a technique in which chemicals in solutions travel down columns or over surface by means of liquids or gases and are separated from each other due to their molecular characteristics.

E.g. Paper chromatography, thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC). [2]

Miscellaneous Techniques:
Mass Spectrometry, Thermal Analysis.

Hyphenated Techniques:
GC-MS (Gas Chromatography – Mass Spectrometry), LC-MS (Liquid Chromatography – Mass Spectrometry), ICP-MS (Inductivity Coupled Plasma- Mass Spectrometry), GC-IR (Gas Chromatography – Infrared Spectroscopy), MS-MS (Mass Spectrometry – Mass Spectrometry).

INTRODUCTION TO HPLC:

HPLC is also called as high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

1. Improved resolution of separated substances
2. column packing with very small (3,5 and 10 µm) particles
3. Faster separation times (minutes)
4. Sensitivity
5. Reproducibility
6. continuous flow detectors capable of handling small flow rates
7. Easy sample recovery, handling and maintenance. [6]

Types of HPLC Techniques
Based on Modes of Chromatography
These distinctions are based on relative polarities of stationary and mobile phases

Reverse phase chromatography:
In this the stationary phase is non-polar and mobile phase is polar. In this technique the polar compounds are eluted first and non polar compounds are retained in the column and eluted slowly. Therefore it is widely used technique.

Normal phase chromatography:
In this the stationary phase is polar and mobile phase is non-polar. In this technique least polar compounds travel faster and are eluted first where as the polar compounds are retained in the column for longer time and eluted. [4]

Based on Principle of Separation:
Liquid/solid chromatography (Adsorption):
LSC, also called adsorption chromatography, the principle involved in this technique is adsorption of the components onto stationary phase when the sample solution is dissolved in mobile phase and passed through a column of stationary phase. The basis for separation is the selective adsorption of polar compounds; analytes that are more polar will be attracted more strongly to the active silica gel sites. The solvent strength of the mobile phase determines the rate at which adsorbed analytes are desorbed and elute. It is widely used for separation of isomers and classes of compounds differing in polarity and number of functional groups. It works best with compounds that have relatively low or intermediate polarity. [3]

Liquid/Liquid chromatography (Partition Chromatography):
LLC, also called partition chromatography, involves a solid support, usually silica gel or kieselguhr, mechanically coated with a film of an organic liquid. A typical system for NP LLC column is coated with ß, ß'-oxy dipropionitrile and a non-polar solvent like hexane as the mobile phase. Analytes are separated by partitioning between the two phases as in solvent extraction. Components more soluble in the stationary liquid move more slowly and elute later. [1,2]

Ion exchange:
In this the components are separated by exchange of ions between an ion exchange resin stationary phase and a mobile electrolyte phase. A cation exchange resin is used for the separation of cations and anion

exchange resin is used to separate a mixture of anions. [3,16,17]

Size exclusion:
In this type, the components of sample are separated according to their molecular sizes by using different gels (polyvinyl acetate gel, agarose gel). ex: separation of proteins, polysaccharides, enzymes and synthetic polymers. [3,15]

Chiral chromatography:
In this type of chromatography optical isomers are separated by using chiral stationary phase.

Affinity chromatography:
In this type, the components are separated by an equilibrium between a macromolecular and a small molecule for which it has a high biological specificity and hence affinity. [3]

Based on elution technique
Isocratic separation: In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

Gradient separation:
In this technique, a mobile phase combination of lower polarity or elution strength is followed by gradually increasing polarity or elution strength. [3]

Based on the scale of operation
Analytical HPLC: Where only analysis of samples are done. Recovery of samples for reusing is normally not done, since the sample used is very low. Ex: µg quantities.

Preparative HPLC:
Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. Ex: separation of few grams of mixtures by HPLC. [4]

Based on type of analysis:
Qualitative analysis: Which is used to identify the compound,detect the presence of impurities to find out the number of components. This is done by using retention time values.

Quantitative analysis: This is done to determine the quantity of individual or several components of mixture. This is done by comparing the peak area of the standard and sample. [3]

Instrumentation of hplc:

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

MATERIALS AND METHODS:

Paracetamol -Sura labs, Diclofenac Na-Sura labs, Water and Methanol for HPLC-LICHROSOLV (MERCK), Anhydrous di hydrogen phosphate-Finar chemicals.

HPLC METHOD DEVELOPMENT:
Mobile Phase Optimization:
Initially the mobile phase tried was Water: Methanol and ACN: Methanol with varying proportions. Finally, the mobile phase was optimized to phosphate buffer (pH 3.6), Methanol in proportion 70:30 v/v respectively.

Optimization of Column:
The method was performed with various columns like C18 column ODS column, Zodiac column, and Xterra C18 column. Xbridge C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters HPLC with auto sampler and PDAdetector 996 model.
Column	:	X bridge C18 (4.6×150mm) 5 µ
Buffer	:	Phosphate buffer (pH-3.6)-Dissolve 1.1998g of anhydrous di hydrogen phosphate in sufficient water to produce 1000ml. Adjust the pH 3.6 by using ortho phosphoric acid.
pH	:	3.6
Mobile phase	:	Methanol: Phosphate Buffer pH-3.6 (30:70v/v)
Flow rate	:	1.0 ml per min
Wavelength	:	260 nm
Injection volume	:	10 µl
Run time	:	10 min.

Optimized chromatogram, blank, System suitability parameters are shown in the figure and the results are shown in Table.

PREPARATION OF BUFFER AND MOBILE PHASE:
Preparation of Phosphate buffer (pH-3.6):

Dissolve 1.1998g of anhydrous di hydrogen phosphate dissolved in sufficient HPLC Grade water to produce 1000mL. Adjust the pH 3.6 by using ortho phosphoric acid.

Preparation of mobile phase:
Accurately measured 300 ml (30%) of Methanol and 700 ml of Phosphate buffer (70%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:

(Optimized):
Mobile phase : Methanol: Phosphate Buffer pH3.6 (30:70v/v)
Column : X Bridge (4.6 × 150 mm, 5 µ)
Flow rate : 1.0 ml/min
Wavelength : 260 nm
Column temp : Ambient
Injection Volume : 10 µl
Run time : 8 min

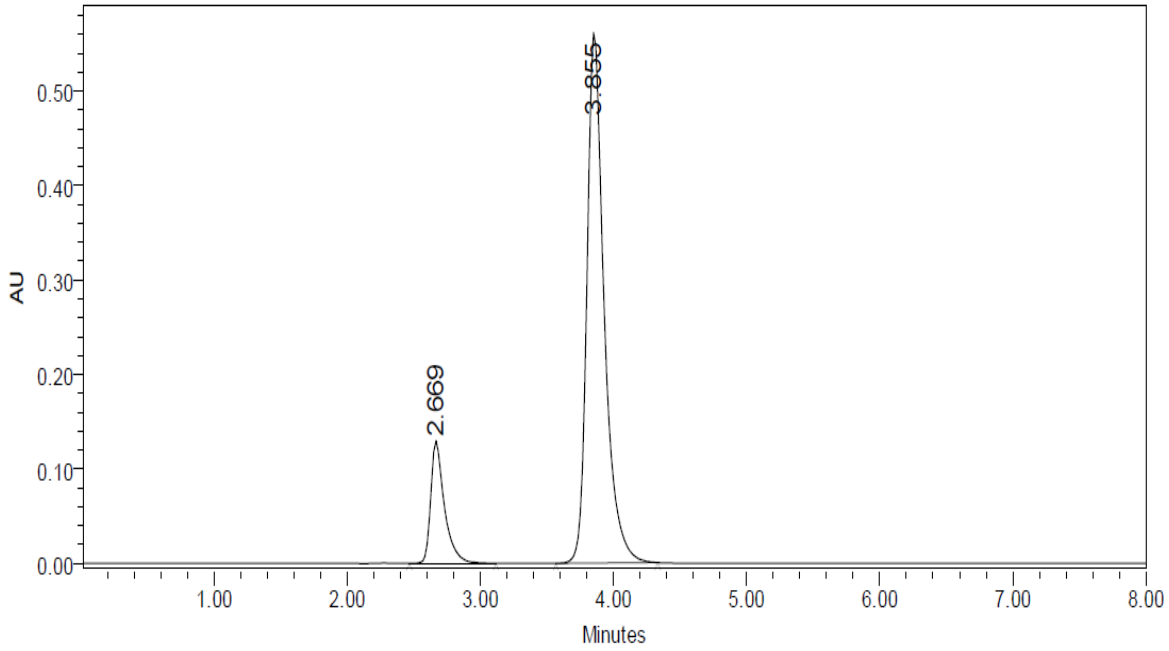

Figure: Chromatogram for trail 7

Table: - peak results for trail 7

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Paracetamol	2.669	986574	128672		1.5	3551.0
2	Diclofenac Na	3.855	5365216	562209	1.7	1.4	4675.7

Observation:
This trial shows improper separation sample peaks, baseline and show very less plate count in the chromatogram. So it's required more trials to obtain good peaks.

From the above chromatogram it was observed that the Paracetamoland Diclofenac Napeaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.
Retention time of Paracetamol – 2.669min
Retention time of Diclofenac Na –3.855min

SYSTEM SUITABILITY:

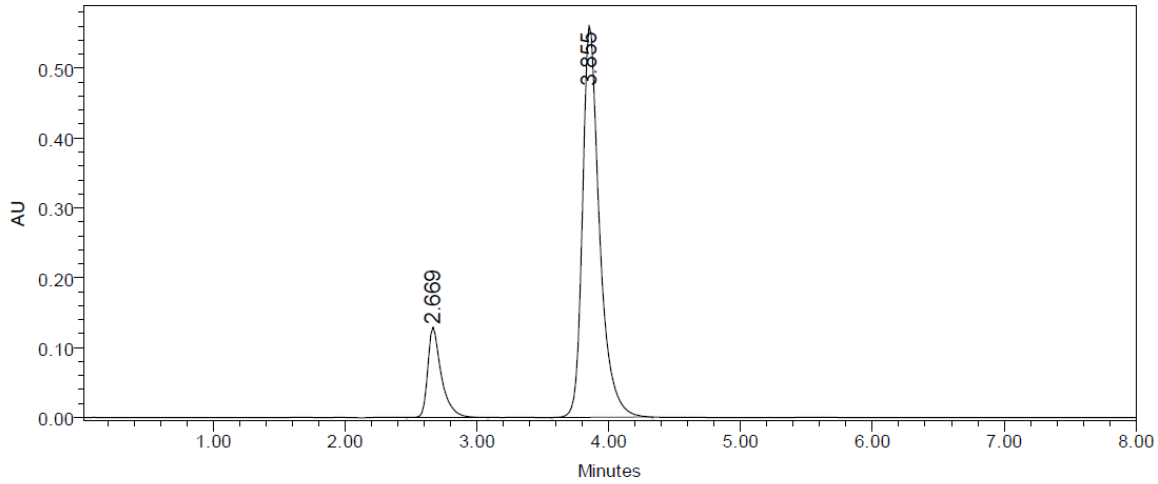

Figure: Chromatogram for system suitability

Table: Results of system suitability parameters for Paracetamol and Diclofenac Na

S.No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Paracetamol	2.669	979867	129658		1.6	3854
2	Diclofenac Na	3.855	5356471	587452	1.8	1.9	4796

Acceptance criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

Assay (Standard):

Table: Showing assay standard results

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Paracetamol	2.669	986587	127854		1.5	3552	1
2	Diclofenac Na	3.855	5387451	561414	1.7	1.4	4654	1
3	Paracetamol	2.669	987824	126985		1.5	3571	2
4	Diclofenac Na	3.855	5378475	568951	1.7	1.4	4635	2
5	Paracetamol	2.654	986541	127894		1.5	3841	3
6	Diclofenac Na	3.849	5369875	568475	1.7	1.4	4684	3

Assay (Sample):

Table: Showing assay sample results

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Paracetamol	2.669	988626	127854		1.6	3561	1
2	Diclofenac Na	3.855	5387547	568541	1.7	1.4	4874	1
3	Paracetamol	2.651	989685	127841		1.5	3658	2
4	Diclofenac Na	3.849	5392435	563524	1.7	1.4	4641	2
5	Paracetamol	2.621	989874	127856		1.5	3854	3
6	Diclofenac Na	3.840	5389854	565412	1.7	1.4	4365	3

Table-: Showing assay results

S.No	Name of compound	%purity
1	Paracetamol	99 %
2	Diclofenac Na	100%

The retention time of Paracetamoland Diclofenac Nawas found to be 2.669min and 3.855mins respectively. The % purity of Paracetamoland Diclofenac Nain pharmaceutical dosage form was found to be 99% and 100% respectively.

PRECISION:

Table: Results of method precision for Paracetamol :

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Paracetamol	2.669	986857	128231	3653	1.5
2	Paracetamol	2.659	987854	129852	3541	1.5
3	Paracetamol	2.671	985474	128145	3635	1.5
4	Paracetamol	2.669	986589	129611	3595	1.5
5	Paracetamol	2.669	985213	128321	3698	1.5
Mean			986397.4			
Std. Dev			1075.302			
% RSD			0.109013			

Table: Results of method precession for Diclofenac Na :

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Diclofenac Na	3.855	5378559	565621	4675	1.4	1.7
2	Diclofenac Na	3.842	5386231	564587	4696	1.4	1.7
3	Diclofenac Na	3.850	5385411	563651	4684	1.4	1.7
4	Diclofenac Na	3.845	5369874	563544	4763	1.4	1.7
5	Diclofenac Na	3.855	5389745	578547	4954	1.4	1.7
Mean			5381964				
Std. Dev			7880.279				
% RSD			0.14642				

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

INTERMEDIATE PRECISION/RUGGEDNESS:

Table-: Results of Intermediate precision for Paracetamol

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Paracetamol	2.669	978985	128874	3686	1.5
2	Paracetamol	2.529	975686	128365	3654	1.5
3	Paracetamol	2.669	969876	128471	3536	1.5
4	Paracetamol	2.569	975487	128698	3682	1.5
5	Paracetamol	2.569	978546	128365	3598	1.5
6	Paracetamol	2.669	976898	128241	3536	1.5
Mean			975913			
Std. Dev			3286.897			
% RSD			0.336802			

Table: Results of Intermediate precision for Diclofenac Na:

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Diclofenac Na	3.845	5352141	563658	4685	1.4	1.7
2	Diclofenac Na	3.795	5365847	564587	4665	1.4	1.7
3	Diclofenac Na	3.855	5378412	563652	4654	1.4	1.7
4	Diclofenac Na	3.840	5378543	563547	4641	1.4	1.7
5	Diclofenac Na	3.855	5363598	565811	4669	1.4	1.7
6	Diclofenac Na	3.855	5386879	562541	4658	1.4	1.7
Mean			5370903				
Std. Dev			12656.43				
% RSD			0.235648				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY:

Table-: accuracy (recovery) data for Paracetamol

%Concentration (at specification Level)	**Area**	**Amount Added (ppm)**	**Amount Found (ppm)**	**% Recovery**	**Mean Recovery**
50%	509438	15	15.041	100.273%	100.549%
100%	1010974.3	30	30.160	100.533%	
150%	1515817	45	45.379	100.842%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table-: Accuracy (recovery) data for Diclofenac Na

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	347527	15	14.933	99.553%	99.935%
100%	609753	30	29.810	99.366%	
150%	884568	45	45.400	100.888%	

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

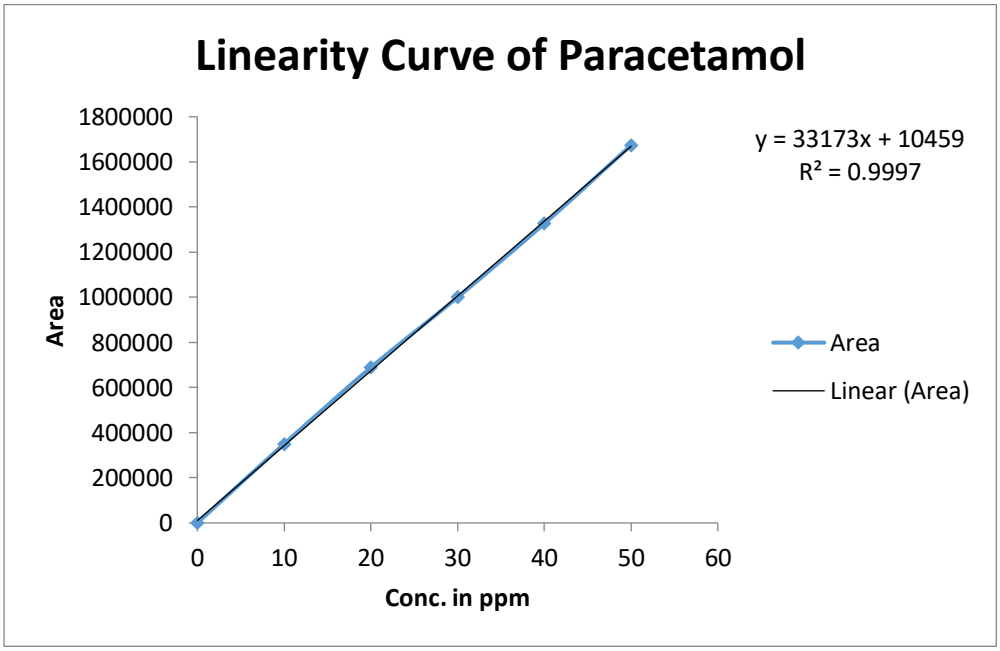

Figure 6.3.4 Calibration graph for Paracetamol

Linearity Results: (for Paracetamol)

S.No	Linearity Level	Concentration(ppm)	Area
1	I	10	349877
2	II	20	688574
3	III	30	999895
4	IV	40	1326522
5	V	50	1673877
Correlation Coefficient			0.999

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

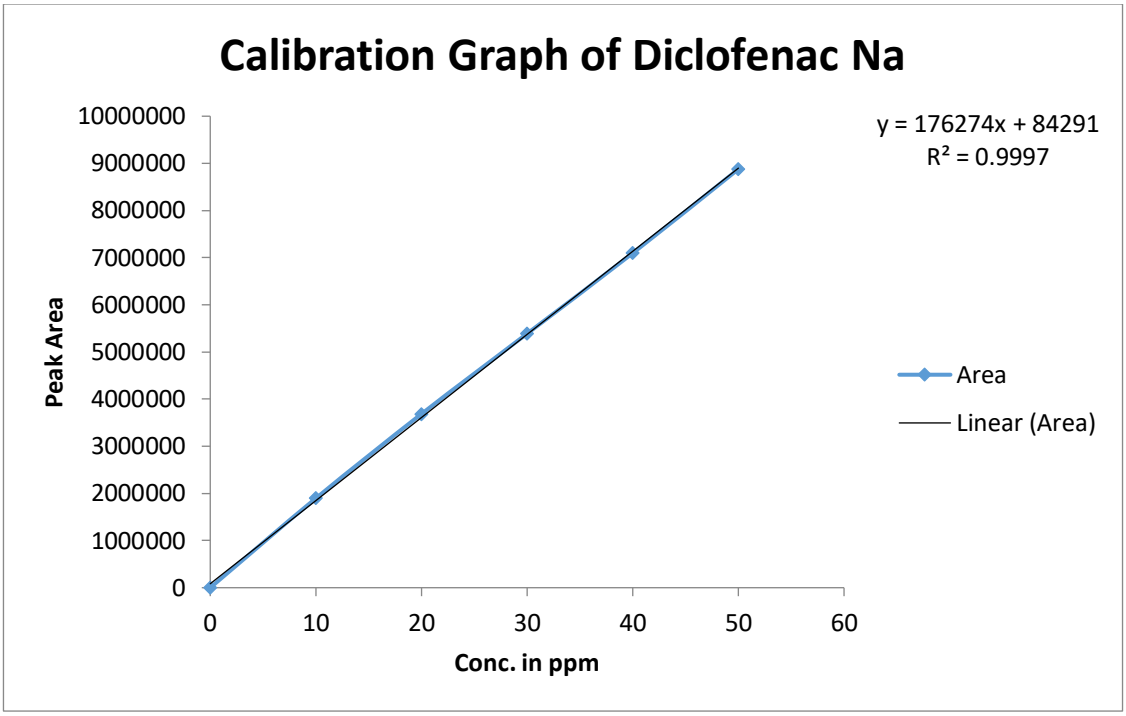

Figure 6.3.4 Calibration graph for Diclofenac Na

Linearity Results: (forDiclofenac Na)

S.No.	Linearity Level	Concentration(ppm)	Area
1	I	10	1896545
2	II	20	3685798
3	III	30	5389557
4	IV	40	7096443
5	V	50	8878478
Correlation Coefficient			0.999

Acceptance Criteria:

- Correlation coefficient should be not less than 0.99.

Table-9 Analytical performance parameters of Paracetamol and Diclofenac Na

Parameters	Paracetamol	Diclofenac Na
Slope (m)	33173	17627
Intercept (c)	10459	84291
Correlation coefficient (R^2)	0.999	0.999

Acceptance criteria:

Correlation coefficient (R^2) should not be less than 0.999.

ROBUSTNESS:

System suitability results forParacetamol:

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	3569.2	1.5
2	1.0	3551.0	1.5
3	1.1	3584.4	1.5

***** Results for actual flow (1.0 ml/min) have been considered from Assay standard.

System suitability results forDiclofenac Na:

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	4864.2	1.4
2	1.0	4675.7	1.4
3	1.1	4524.9	1.4

***** Results for actual flow (1.0ml/min) have been considered from Assay standard.

System suitability results for Paracetamol:

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	4789.4	1.5
2	*Actual	3551.0	1.5
3	10% more	4635.6	1.5

System suitability results for Diclofenac Na:

S.No.	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	5865.8	1.4
2	*Actual	4675.7	1.4
3	10% more	5342.4	1.4

***** Results for actual mobile phase have been considered from Assay standard.

SUMMARY AND CONCLUSION:

High performance liquid chromatography is at present one of the most sophisticated tools of the analysis. The estimation of Paracetamol and Diclofenac Nawas done by RP-HPLC. The Phosphate buffer was p^H 3.6 and the mobile phase was optimized with consists of Methanol: Phosphate buffer (pH-3) mixed in the ratio of 30:70 % v/ v. An Xbridge column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. The linearity range of Paracetamol and Diclofenac Na were found to be from 10-50µg/ml, 10-50µg/ml respectively. Linear regression coefficient was not more than 0.999, 0.999.

The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Paracetamol and Diclofenac Na.LOD and LOQ were found to be within limit.

The results obtained on the validation parameters met ICH and USP requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

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