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SYNTHESIS, EVALUATION OF MUTUAL PRODRUG AND ANALYTICAL METHOD DEVELOPMENT FOR SIMULTANEOUS ESTIMATION OF PARACETAMOL AND ACECLOFENAC

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Abstract

Aceclofenac and paracetamol have shown effective simultaneous separation using the RP-HPLC method. In order to develop the method, multiple preliminary trial runs were conducted on the pre-validated RP-HPLC system using varying mobile concentrations and solvents containing different ratios of methanol to phosphate buffer (85:15). Inertsil C18 (4.6 x 250mm, 5µm) was used for chromatographic separation. Prior to usage, the produced mobile phase was degassed using ultrasonication and filtered through a 0.45 µm membrane filter using a vacuum pump. The injection volume was 20 µl, the column temperature was ambient, the flow rate was 1.0 mL/min, and a UV detector was used for detection at 276 nm. Aceclofenac had a retention time of 5.768 minutes and paracetamol 3.719 minutes, respectively, were found. In present study, this simultaneous method was validated according to ICH guidelines Q2 (R1). The above developed method was validated by using validation parameters viz, linearity, accuracy, precision and robustness. Straight line calibration graph were obtained in the concentration range 200-1000 µg/ml and correlation coefficient (r 2) for proposed method was found to be 0.999 for Paracetamol and 50-250 µg/ml with r2 value of 0.9992 for Aceclofenac. Both, system as well as method precision showed the developed method is precise with respect to RSD, tailing factor, and number of theoretical plats were calculated for both solutions, all the results are within limits.

Keywords: Paracetamol, Aceclofenac, RP-HPLC Method, Linearity, Accuracy.

Introduction

The term Prodrug was introduced by Albert who used “prodrug” or “proagent” to refer to a pharmacologically inactive compound that is transformed by the mammalian system into an active substance by either chemical or

metabolic means. Another term drug latentiation, which implies a time lag element or component, was coined by Harper. Later, the concept of prodrug and latentiated drug for solving various problems was attempted and the definition of drug latentiation was extended to include non–enzymatic regeneration of parent compounds5. The prodrug approach has emerged as a tool in overcoming various obstacles to drug formulation and targeting such as chemical instability, poor aqueous solubility, inadequate brain penetration, insufficient oral absorption, local irritation and toxicity. It is justified by the fact that once the barrier to the use of parent compound has been overcome, these temporary forms can be converted to the free parent compound that can exert its pharmacological activity. A prodrug is thus defined as a biologically inactive derivative of a parent drug molecule that usually requires a chemical or enzymatic transformation within the body to release the active drug, and possess improved delivery properties over the parent molecule. Most of the limitations can be overcome by prodrug approach, but after overcoming the various barriers, the prodrug should rapidly convert into active moiety after reaching the target site. The awareness that the onset, intensity and duration of drug action are greatly affected by the physicochemical properties of drug has promoted the emergence of various theoretical and predictive models for drug design and evaluation. The design of an efficient, stable, safe, acceptable and aesthetic way to target a drug to its site of action while overcoming various physical, chemical and social barriers is certainly an area where the utilization of the prodrug approach holds great potential.

Classification of Prodrugs

Prodrugs are categorized into four classes. They are given as follows; Carrier Linked Prodrugs

- Tripartite Prodrugs
- Mutual Prodrugs

Polymeric Prodrugs

Carrier linked prodrugs:

Various adverse physicochemical properties of drug can be tailored and side effects can be minimized by attaching a non- toxic carrier group or promoiety to form a new compound i.e., prodrug, from which the parent drug is regenerated *in vivo.* Common example is dipivalyl ester of epinephrine, which enhances the corneal absorption and inhibits the rapid metabolic destruction of epinephrine. In addition prodrug produces less cardiovascular side effects.

Tripartite Prodrugs:

Structures of most prodrugs are bipartite in nature in which parent drug is attached directly to promoiety. However in some cases bipartite prodrug may be unstable due to inherent nature of the drug-promoiety bond. This can be overcome by designing a tripartite prodrug, utilizing a spacer or connector group between the drug and promoiety. The spacer or connector group must be designed in such a way that the initial activation is followed by spontaneous cleavage of remaining drug spacer bond under physiological condition to release parent drug e.g. a model tripartite prodrug P–(N–(tert-butyloxy carbonyl) lysyl) amido) benzyloxy carbonyl)–P–nitro aniline has been designed in which N–tert butyloxy carbomyl lysine group is promoiety, P–amido benzyloxy carbonyl group is spacer group

and P–nitro aniline is the drug.

Mutual Prodrugs:

Mutual prodrugs are defined as two pharmacologically active agents joined together so that each acts as a promoiety for the other and vice versa. Benorylate is a common example of this category which is a prodrug of acetyl salicylic acid and paracetamol. Major advantage associated with this prodrug is in treatment of chronic inflammation at decreased dose and reduced risk of irritation.

Polymeric Prodrugs:

In this type, which is also known as macromolecular prodrug, the drug is dispersed or incorporated into the polymer (both naturally occurring and synthetically prepared) system without formation of covalent bond between drug and polymer. Example is p–phenylene diamine mustard is covalently attached to polyamine polymer backbone polyglutamic acid.

Functional Groups Amenable to Prodrug Design

Ideally, the design of an appropriate prodrug structure should be considered at the early stages of preclinical development, bearing in mind that prodrugs might alter the tissue distribution, efficacy and the toxicity of the parent drug.

Parent and prodrug: the absorption, distribution, metabolism, excretion (ADME) and pharmacokinetic properties need to be comprehensively understood.

Some of the most common functional groups that are amenable to prodrug design include carboxylic, hydroxyl, amine, phosphate/phosphonate and carbonyl groups. Prodrugs typically produced via the modification of these groups include esters, carbonates, carbamates, amides, phosphates and oximes.

However, other uncommon functional groups have also been investigated as potentially useful structures in prodrug design. For example, thiols react in a similar manner to alcohols and can be derivatized to thioethers and thioesters. Amines may be derivatized into imines and N-Mannich bases.

Paracetamol

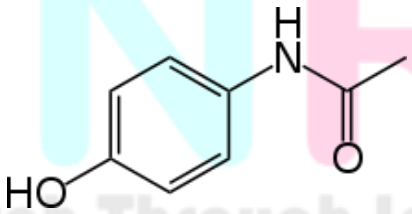

Fig. No. 1 Structure of Paracetamol

At a standard dose, paracetamol only slightly decreases body temperature; it is inferior to ibuprofen in that respect, and the benefits of its use for fever are unclear. Paracetamol may relieve pain in acute mild migraine but only slightly in episodic tension headache. However, the aspirin/paracetamol/caffeine combination helps with both conditions where the pain is mild and is recommended as a first-line treatment for them.

Aceclofenac

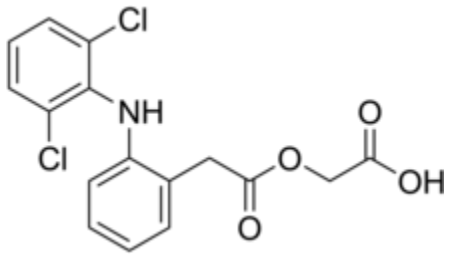

Fig. No. 2 Structure of Aceclofenac

Aceclofenac is a Nonsteroidal anti-inflammatory drug (NSAID) analog of diclofenac. It is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis and ankylosing spondylitis.

Materials & Methods:

Materials

Sr. No.	Name of Materials
1	Aceclofenac
2	Paracetamol
3	HPLC Grade Water
4	Potassium Dihydrogen phosphate
5	Di-potassium Hydrogen Phosphate
6	Methanol

Methods

Synthesis of Mutual Prodrug of Aceclofenac and Paracetamol

Aceclofenac (0.850 g, 4 mmol) and paracetamol (0.605 g, 4 mmol) were dissolved separately in dry pyridine (5 mL). The two solutions were mixed together under ice-cold conditions followed by dropwise addition of phosphorous oxychloride (0.5 mL). The contents were stirred for 4 h while maintaining the temperature below 5 °C. The reac- tion mixture was then decomposed by adding ice-cold water. A solid mass separated out which was filtered, washed with plenty of water and crystallized from metha- nol to give fine grey colored needles of AC-PR.

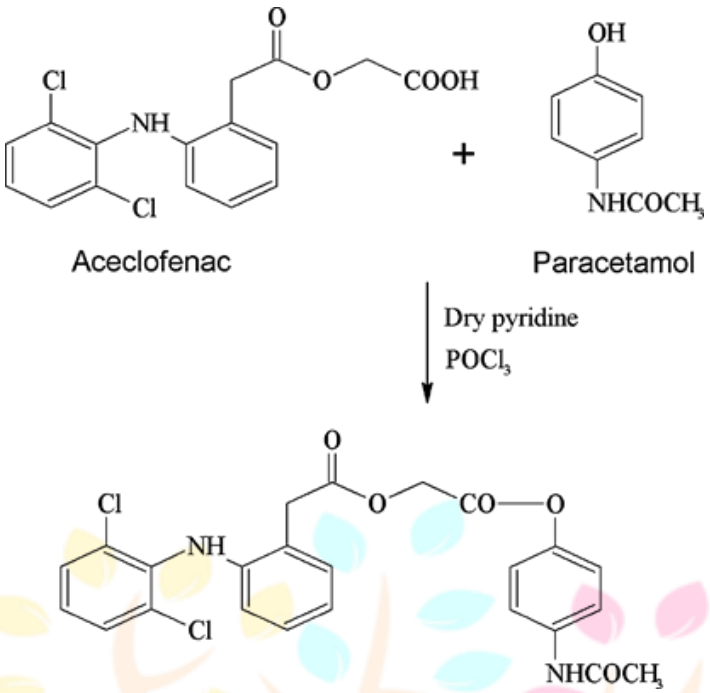

Figure 3: Scheme for Synthesis of Mutual prodrug of Aceclofenac and Paracetamol

Results and Discussion

Evaluation of synthesized Aceclofenac Paracetamol Mutual Prodrug Melting Point Determination

Melting point was determined by the use of digital melting point apparatus. The melting point of Aceclofenac Paracetamol Mutual Prodrug was found to be 138ºC as showed in as showed in figure 3.

Transform Infrared Spectroscopy (FT-IR)

Figure 4: FT-IR Spectra of Aceclofenac Paracetamol Mutual Prodrug

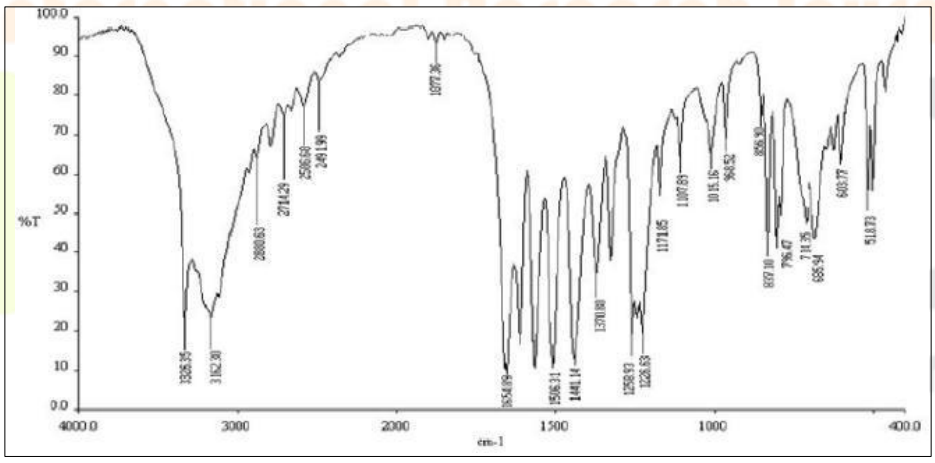

Table 3: Observed Group Frequencies by FT-IR

Name of Drug	Observed IR Frequencies	Functional group Present
Aceclofenac Paracetamol Mutual Prodrug	3225.14	N-H Primary and Secondary Amine and Amide
	3080.02	C-H, Alkane
	1638.10	C=O, amide
	1308.30	C–O, ester
	850.47	C–Cl
	745.62	C–N

Uv Method Development and Validation Aceclofenac and Paracetamol

Spectral Characteristics of Paracetamol and Aceclofenac

Absorbance maxima of Paracetamol and Aceclofenac were found to be on 243 nm and 273 nm respectively. The calibration curve of both the drugs was developed by using these maxima as fixed wavelength.

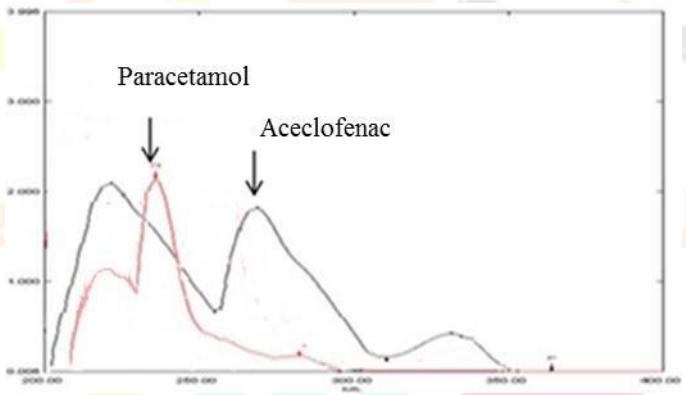

Figure 5: UV Spectrum of Paracetamol and Aceclofenac

Determination of Beer's law range for Paracetamol

The calibration curve of Paracetamol was performed and graph plotted concentration vs. absorbance. The absorbance values of different concentration were noted. The regression equation was found to be y = 0.0095x+0.0559, with R^2 value of 0.9992. The graph was found to be linear.

Table 4: Concentration range and respective absorbance of Paracetamol

Sr No.	Concentration (ppm)	Absorbance
1.	20	0.241
2.	40	0.434
3.	60	0.638
4.	80	0.823
5.	100	0.998

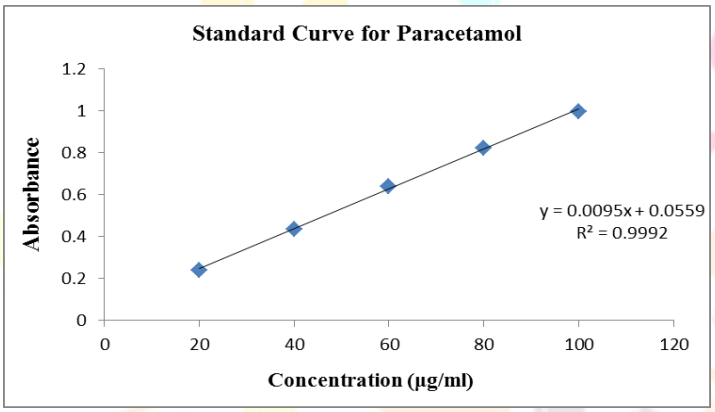

Figure 6: Standard Curve for Paracetamol

Determination of Beer's law range for Aceclofenac

Table 5: Concentration range and respective absorbance of Aceclofenac

Sr No.	Concentration (ppm)	Absorbance
1	5	0.191
2	10	0.356
3	15	0.554
4	20	0.716
5	25	0.886

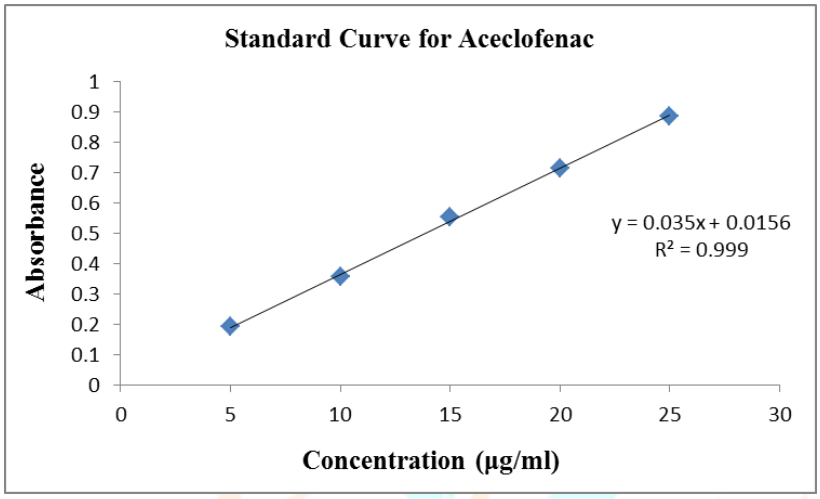

Figure 7: Standard Curve for Aceclofenac

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Form the results it was found that LOD & LOQ are in the sub-microgram level, which indicates the sensitivity of the method. (Table 6)

Table 6: Evaluation data for LOD & LOQ of Paracetamol & Aceclofenac

Paracetamol	
LOD	4.5 µg/ml
LOQ	13.5 µg/ml
Aceclofenac	
LOD	1.3 µg/ml
LOQ	3.9 µg/ml

Accuracy

Accuracy of the proposed UV method for Paracetamol and Aceclofenac was verified by conducting the recovery studies by using standard addition method. Standard drug concentration at three different percent levels was added to known amount of Paracetamol and Aceclofenac. The percent recovery of added standards was calculated (Table 7). The results showed better % mean recovery for respective percent levels. The % mean recovery values are closer to 100% showed high accuracy of the proposed UV analytical method.

Table 7: Evaluation data of Accuracy study of Paracetamol & Aceclofenac

	Paracetamol				
entration (%)	**rigin level (µg/ml)**	**mount added (µg/ml)**	**% Recovery**	**Mean % Recovery**	**% RSD**
80	20	16	99.76	100.28	0.457
80	20	16	100.45		
80	20	16	100.63		
100	60	60	101.27	100.60	0.698
100	60	60	100.67		
100	60	60	99.87		
120	100	120	100.57	100.71	0.162
120	100	120	100.89		
120	100	120	100.67		
	Aceclofenac				
entration (%)	**rigin level (µg/ml)**	**mount added (µg/ml)**	**% Recovery**	**Mean % Recovery**	**% RSD**
80	5	4	100.84	100.55	0.594
80	5	4	100.96		
80	5	4	99.87		
100	15	15	100.79	101.08	0776
100	15	15	101.98		
100	15	15	100.49		
120	25	30	100.98	100.19	0.948
120	25	30	101.47		
120	25	30	99.14		

Precision

Intra-day and inter-day precision study of drug were evaluated for the 20 µg/ml, 60 µg/ml and 100 µg/ml for Paracetamol and 5 µg/ml, 15 µg/ml and 25 µg/ml for Aceclofenac.. Absorbance mean, percent assay and percent RSD were calculated for the intra-day as well as inter-day precision study.

Table 8: Evaluation data for Intra-day and Inter-day study of Paracetamol

Intra-day	**Morning**			**Afternoon**			**Evening**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
20	0.241	99.57		0.247	100.52		0.248	101.57	
60	0.636	100.27		0.640	100.05		0.638	100.37	
100	0.995	100.45		0.990	99.67		0.989	100.21	
Inter-day	**Day 1**			**Day 2**			**Day 3**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
5	0.191	99.57		0.190	100.67				
15	0.556	100.24		0.558	100.89				
25	0.880	100.76		0.883	100.67				

Table 9: Evaluation data for Intra-day and Inter-day study of Aceclofenac

Intra-day	**Morning**			**Afternoon**			**Evening**		
Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
20	0.245	100.20	0.367	0.247	101.57	0.346	0.241	100.23	0.349
60	0.638	10048	0.846	0.640	100.63	0.796	0.647	100.57	0.785
100	0.987	100.94	0.756	0.990	99.67	0.763	0.991	100.96	0.719
Inter-day	**Day 1**			**Day 2**			**Day 3**		

Concentration Range (µg/ml)	Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
5	0.190	99.67	0.489	0.191	100.37	0.463	0.195	100.27	0.472
15	0.554	100.37	0.637	0.556	100.64	0.574	0.554	100.61	0.637
25	0.883	100.91	0.351	0.881	101.79	0.384	0.889	101.97	0.397

Ruggedness and Robustness

Ruggedness study of drug was carried out at the three different temperature levels. From the results it was found that the method was rugged showing the % RSD value less than 2% (Table 10).

Table 10: Evaluation data for Ruggedness of Paracetamol & Aceclofenac

Paracetamol			
Concentration (µg/ml)	**Temperature (°C)**	**Absorbance**	**% RSD**
60	25	0.639	0.467
60	37	0.640	0.438
60	60	0.639	0.410
Aceclofenac			
Concentration (µg/ml)	**Temperature (°C)**	**Absorbance**	**% RSD**
15	25	0.556	0.518
15	37	0.554	0.541
15	60	0.550	0.589

Robustness

Robustness study was evaluated by using three different solvent. The method was found to be robust as indicated by the % RSD values which are less than 2% (Table 11).

Table 11: Evaluation data for Robustness of Paracetamol & Aceclofenac

Paracetamol			
Concentration (µg/ml)	**Solvents**	**Absorbance**	**% RSD**
60	Ethanol	0.634	0.479
60	Methanol	0.638	0.524
Aceclofenac			
Concentration (µg/ml)	**Solvents**	**Absorbance**	**% RSD**
15	Ethanol	0.551	0.847
15	Methanol	0.553	0.961

HPLC Method Development and Validation

Table 12: Optimized condition for HPLC Method for Paracetamol & Aceclofenac

Sr. No.	**Parameter**	**Optimized condition**
1	Chromatograph	SHIMADZU LC 2010 AHT-HPLC
2..	Column	Hypersil C_{18} -ODS column (150mm x 4.6 mm)
3.	Mobile phase	Methanol: Buffer (85:15)
4.	Flow rate	1 ml/min
5.	Detection	UV at 276 nm
6.	Injection volume	20µl
7.	Temperature	Ambient

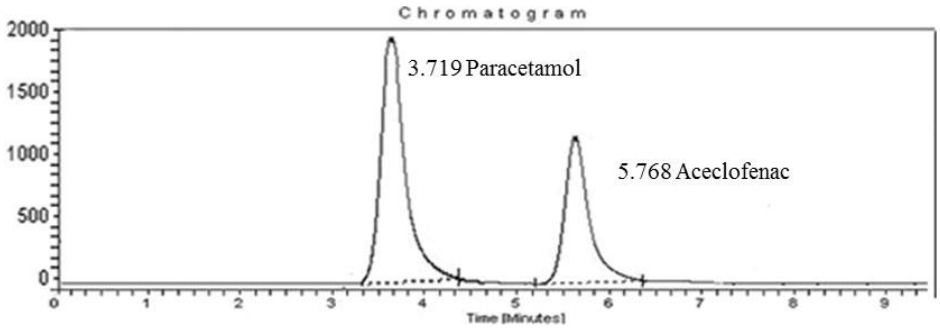

Figure 8: Standard Chromatogram of Paracetamol & Aceclofenac

Linearity Data of Paracetamol and Aceclofenac

Table 13: Linearity Data of Paracetamol

S. No.	Conc. in mcg/ml	Peak Area
1	200	3482.17
2	400	6845.18
3	600	9545.64
4	800	12812.24
5	1000	15629.54

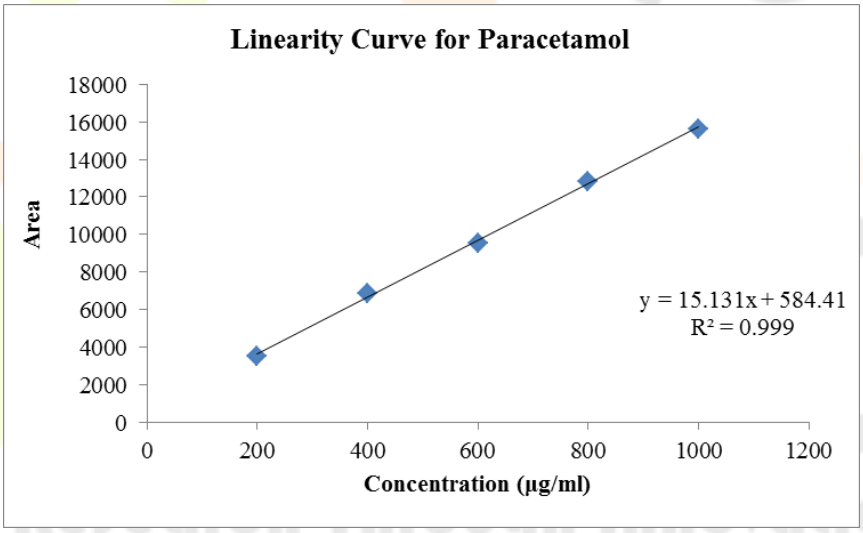

Figure 9: Linearity Curve for Paracetamol Table 14: Linearity Data of Aceclofenac

S. No.	Conc. in mcg/ml	Peak Area
1	50	1625.25
2	100	3256.78
3	150	4724.54
4	200	6235.98
5	250	7968.31

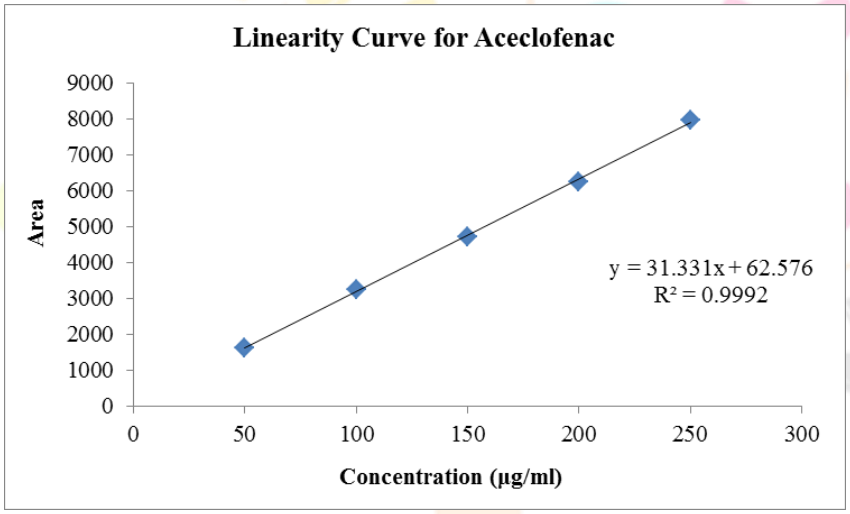

Figure 10: Linearity Curve for Aceclofenac

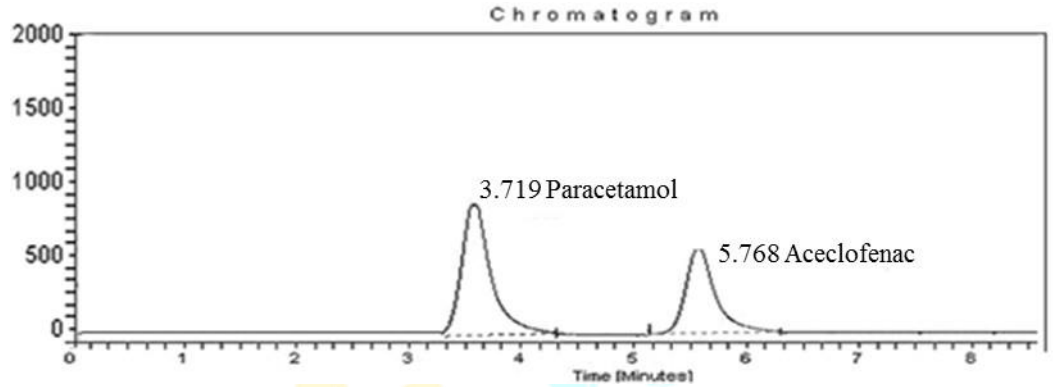

Figure 10: Chromatogram of Paracetamol and Aceclofenac standard mixture (1)

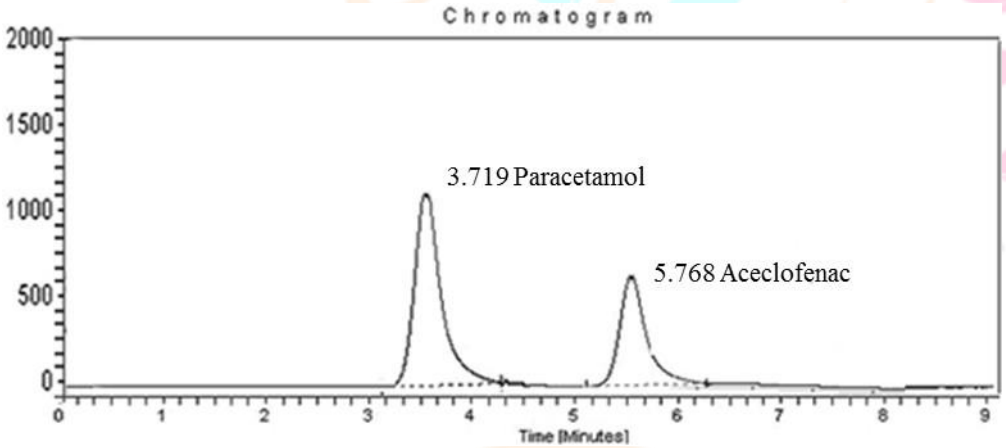

Figure 11: Chromatogram of Paracetamol and Aceclofenac standard mixture (2)

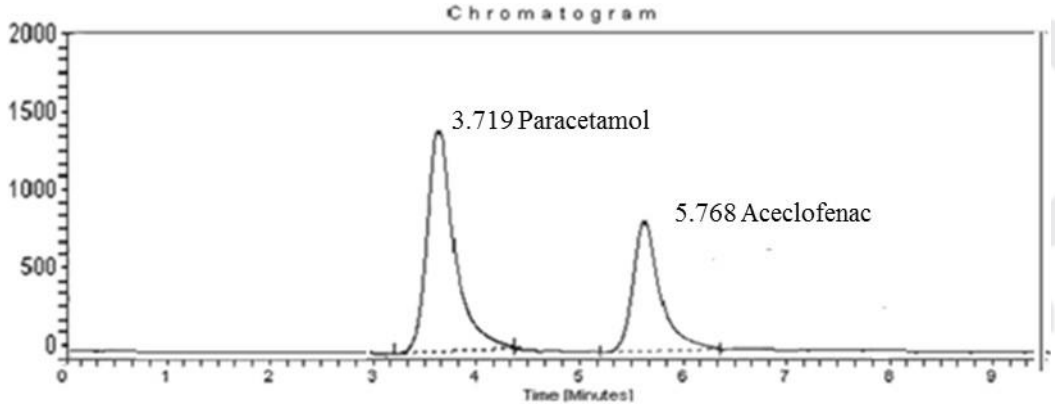

Figure 12: Chromatogram of Paracetamol and Aceclofenac standard mixture (3)

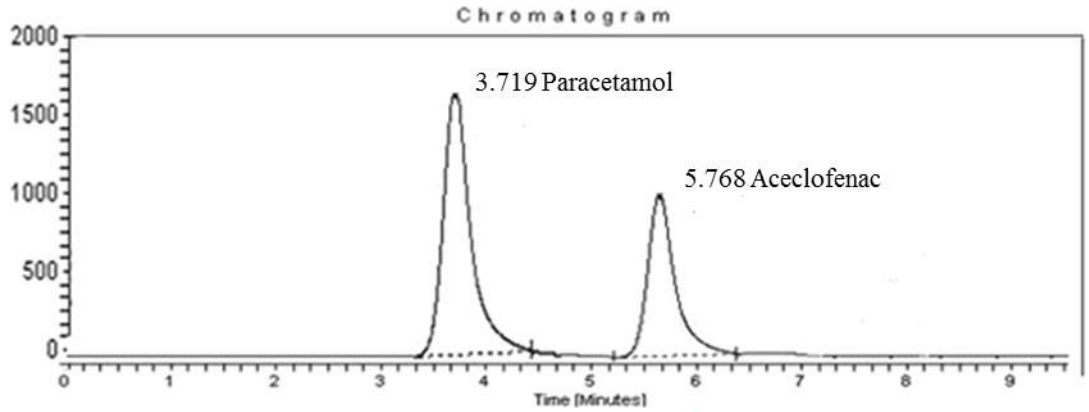

Figure 13: Chromatogram of Paracetamol and Aceclofenac standard mixture (4)

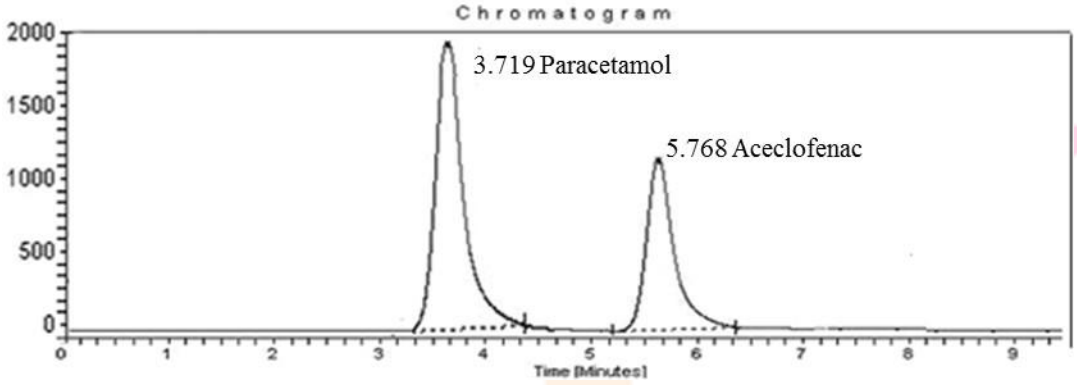

Figure 14: Chromatogram of Paracetamol and Aceclofenac standard mixture (5)

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For Paracetamol LOD and LOQ were found to be 0.294µg/ml and 0.178µg/ml respectively. For Aceclofenac LOD and LOQ were found to be 0.0213µg/ml and 0.052µg/ml respectively. These values indicate that the method is suitable for the determination of the lower concentration and confirms that proposed method is sensitive for the determination.

Precision

a) System Precision

The system precision was performed by measuring the peak response for standard drugs solutions in six replicates. Peak responses, mean, standard deviation and % relative standard deviation (%RSD) for Paracetamol & Aceclofenac was found to be 0.495 and 0.520 %. The results are shown in table 15 and were found well within the acceptable criteria.

Table 15: System Precision Data of Paracetamol & Aceclofenac

Sr. No.	Peak areas of Paracetamol	Peak areas of Aceclofenac
1.	3482.45	1630.67
2.	6837.34	3251.89
3.	9541.61	4728.45
4.	12815.41	6242.57
5.	15631.32	7971.23
Mean	**9661.62**	**4764.96**
SD (±)	**4789.48**	**2478.81**
RSD (%)	**0.495**	**0.520**
Acceptance criteria	**% RSD should not be more than 2**	

b) Method Precision

The method precision was performed by measuring the peak response for sample solutions in six replicates. The % assay for Paracetamol and Aceclofenac in six samples was calculated. The results of % assay and % RSD are shown in table 16. The chromatograms for the method precision are shown in fig ure16-21.

Table 16: Method Precision Data of Paracetamol & Aceclofenac

ımple No.	% Assay of Paracetamol (w/w)	% Assay of Aceclofenac (w/w)
1.	100.57	99.67
2.	99.65	100.20
3.	99.37	101.48
4.	100.41	101.42
5.	101.24	99.74
6.	100.48	100.55
Mean	**100.28**	**100.55**

SD (±)	**0.676**	**0.795**
RSD (%)	**0.674**	**0.791**
cceptan ce criteria	**% RSD should not be more than 2**	

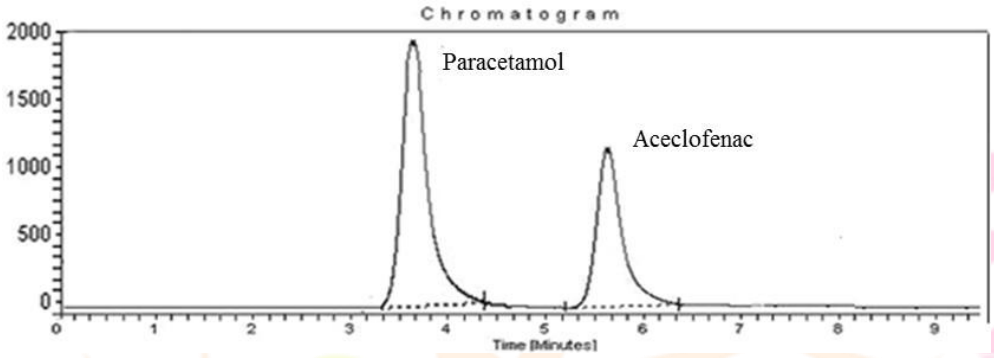

Figure 15: Chromatogram of Method precision 1

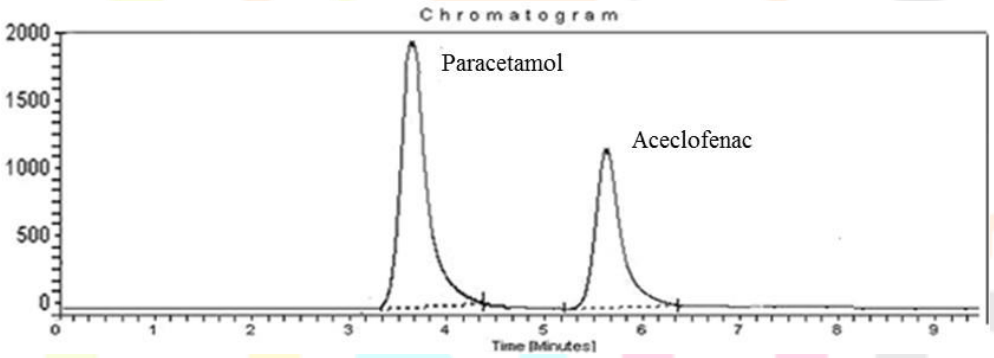

Figure 16: Chromatogram of Method precision 2

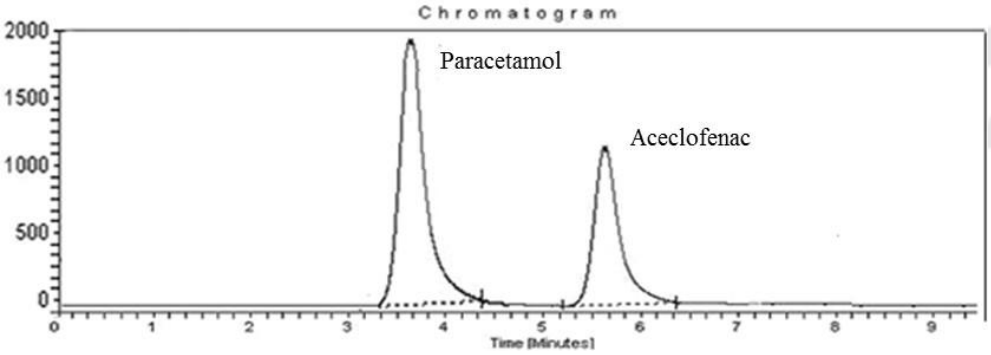

Figure 17: Chromatogram of Method precision 3

Table 17: Precision data of Paracetamol & Aceclofenac

Paracetamol	
Precision	**Area**
Precision-1	15630
Precision -2	15645
Precision -3	15634
Precision -4	15684
Precision -5	15603
Precision -6	15628
Mean	**15637.33**
Standard Deviation (SD)	**26.710**
%RSD	**0.170**
Aceclofenac	
Precision	**Area**
Precision-1	7965
Precision -2	7935
Precision -3	7921
Precision -4	7937
Precision -5	7962
Precision -6	7947
Mean	**7944.50**
Standard Deviation (SD)	**16.920**
%RSD	**0.212**

c) Intraday and Inter-day Precision

The % RSD in intraday precision for Paracetamol (200, 600, 1000 µg/ml) was found to be 0.881, 0.318, 0.183% and for Aceclofenac (50, 150, 250 µg/ml) was found to be 1.684, 0.452, 0.216 % respectively. In inter-day precision % RSD for Paracetamol (200, 600, 1000 µg/ml) was found to be 0.834, 0.141, 0.220% and for Aceclofenac (50, 150, 250 µg/ml) was found to be 1.933, 0.804, 0.251% respectively. % RSD in intraday and inter-day studies were found well within the acceptable limits. The results obtained are mentioned in the table 18, 19**.**

Table 18: Intraday Precision data of Paracetamol & Aceclofenac

	Paracetamol					**Aceclofenac**				
Sr. no.	**Conc.** (µg/ml)	**Area**	**mean peak area**	**SD(±)**	**%RSD**	**Conc.** (µg/ml)	**Area**	**anpeak area**	**SD(±)**	**%RSD**
1	200	3482 3426 3476	3461.33	30.746	0.881	50	1625 1627 1674	1642	27.730	1.684
2	600	9545 9524 9584	9551	30.446	0.318	150	4724 4763 4728	4738.31	21.455	0.452
3	1000	15629 15634 15681	15648	28.687	0.183	250	7968 7934 7956	7952.64	17.243	0.216

Table 19: Inter-day Precision data of Paracetamol & Aceclofenac

		Paracetamol					**Aceclofenac**				
Sr. no.	Day	Conc. (µg/ml)	Peak Area	Mean Peak Area	SD(±)	%RSD	Conc. (µg/ml)	Peak Area	Mean Peak Area	SD(±)	%RSD
1	Day 1	200	3480	3462.36	28.884	0.834	50	1698	1661.64	32.129	1.933
	Day 2		3429					1637			
	Day 3		3478					1650			
2	Day 1	600	9548	9547	13.527	0.141	150	4721	4765	38.314	0.804
	Day 2		9560					4783			
	Day 3		9533					4791			
3	Day 1	1000	15647	15659	34.597	0.220	250	7965	7963.62	20.033	0.251
	Day 2		15632					7983			
	Day 3		15698					7943			

Accuracy (Recovery Study)

The accuracy of the assay method was evaluated by standard addition method in triplicate at 100% level of the labeled claim and the percentage recovery was calculated. The mean % recovery was found to be 100.31 % & 100.35 % for Paracetamol & Aceclofenac respectively.

Ruggedness

The ruggedness parameter was determined by analyzing the different concentration at different temperature. The results were showed in table.

Table 21: Data of Ruggedness for Paracetamol & Aceclofenac

Paracetamol				
Change in Parameters	**Area of Standard**	**Mean**	**SD**	**%RSD**
25°C	9566 9547 9503	9538.67	32.316	**0.338**
37°C	9514 9532 9541	9529	13.747	**0.144**
60 °C	9501 9523 9512	9512	11	**0.115**
Aceclofenac				
Change in Parameters	**Area of Standard**	**Mean**	**SD**	**%RSD**
25°C	4766 4728 4723	4739	23.515	**0.496**
37°C	4765 4743 4756	4754.62	11.060	**0.232**
60 °C	4726 4703 4722	4717	12.288	**0.260**

Conclusion

The suggested methods are easy to use, quick to complete, verified, and effective for routine simultaneous estimate of Aceclofenac and Paracetamol. The ICH guidelines were followed in the validation of the approach. The two approaches' respective capabilities compliment one another. Therefore, they can be thought of as straightforward, accurate, and sensitive techniques for estimating the combined dosage forms of Aceclofenac and Paracetamol. The accuracy, precision, robustness, ruggedness, and percentage RSD values fall between 0.8 and 2.

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