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Development and Validation of a Reversed-Phase Liquid Chromatographic Method for Simultaneous Estimation of Paracetamol, Aceclofenac and Serritiopeptidase in Tablet Dosage Form

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Abstract

A Simple, sensitive, specific and economic chromatographic method was developed and validated for simultaneous estimation of Paracetamol, Aceclofenac and Serritiopeptidase in tablet dosage form. New method was based on the simultaneous estimation of drugs in a mixture without prior separation. Estimation was carried out using reverse phase column chromatographic technique with isocratic elution at 210 nm in Methanol: Buffer (pH 6.8): Acetonirile (5:4:1) mobile phase. The accuracy and precision of the proposed method were performed as per ICH guidelines and they lie within acceptable limit. Thus the proposed method can be successfully applied for simultaneous determination of Paracetamol, Aceclofenac and Serritiopeptidase respectively in routine analytical work.

Key-Words: Paracetamol, Aceclofenac, Serritiopeptidase, RP-HPLC, Simultaneous estimation

Introduction

Most of the pharmaceutical industries, are manufacturing multiple drug formulation to meet the market demand. It is a well known fact that a combination of drugs has a wider range to treat ailments as compared to the single drug component. The combination of Aceclofenac (ACE), Paracetamol (PCM) and Serratiopeptidase (SER) was introduced in to the market. Paracetamol N-(4-hydroxyphenyl) acetamide (**Fig 1A**) inhibits prostaglandin synthesis in the brain but hardly any in the periphery. Thus Paracetamol has central effect. Aceclofenac (2-[(2,6-dichlorophenyl) amine] phenyl acetoxy acetic acid (**Fig 1B**) has more of peripheral effect. Thus Aceclofenac and Paracetamol with different mechanisms of action this combination may be more effective than each drug used alone.

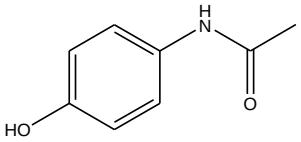

Fig 1 A: Paracetamol

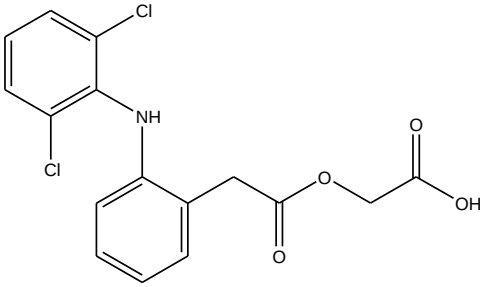

Fig 1 B: Aceclofenac

Serratiopeptidase is an enzyme derived from bacteria belonging to genus Serratia sp. Serratiopeptidase is a 'proteolytic' or protein digesting enzyme. Serratiopeptidase improves the spectrum of activity of NSAIDS by reducing the swelling, dissolving the dead and damaged tissue (such as blood clots, arterial plaque), increase circulation and help in the relief of inflammation. Also the mechanism of drugs is acting at different inflammatory mediators. i.e. Aceclofenac and Paracetamol on prostaglandins and Serratiopeptidase

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on histamine and bradykinin. Thus combination of Aceclofenac with Serratiopeptidase enhances the efficacy of Aceclofenac.

Several method were reported for estimation of Aceclofenac or its derivatives with combination of several drugs by using UV-spectrophotometry[1], using ion-pair HPLC[2], derivative spectrophotometry[3], Ion pair liquid chromatography[4], estimation in plasma and urine using HPLC[5-7], assay using RP-HPLC[8]. Many methods are available in literature for assay of paracetamol in diverse types of samples including pharmaceutical preparations. These methods are as diverse as a simple titrimetric method to HPCL and spectrophotometric methods.[9-17] Many UV[18,19] and HPLC[20-23] based methods have been reported for estimation of these drugs alone as well as in combination with other drugs in pharmaceutical dosage form.

There are very few methods reported for simultaneous analysis of drug component of multi-component formulation. Almost all pharmacopoeial methods available for the analysis of such formulation are applicable only after prior separation of drug components, hence, making them tedious and time consuming. There is likely to be loss of accuracy and precision due to extraction and/or separation. The Simultaneous analysis procedures avoid these time consuming extractions and separations, and are economical in the sense that use of expensive reagents is minimize. A successful attempt was made to develop accurate, precise and sensitive multi-component mode of analysis for estimation of both the drugs. The developed method is simple, rapid, selective, less expensive and less time consuming.

Material and Methods

The present work was carried out on Analytical 3000 HPLC using Cosmosil $5C_{18}$-MS-II (4.61D*250mm) pack column. Standard gift sample of Paracetamol (PCM) and Aceclofenac (ACE) were procured from Cyano Pharma Pvt. Ltd. Indore and Serratiopeptidase (SER) procured from Plethico Pharmaceutical Ltd. Indore. Marketed formulation **Acifin-Plus** tablet containing Paracetamol 325mg; Aceclofenac 100mg and Seritiopeptidase 10mg was used as sample; purchased from local pharmacy Indore. Calibrated glassware's were used throughout the work.

Sampling Wavelength and Mobile Phase: On the basis of absorptivity of the drugs 210nm wavelength was considered as a sampling wavelength while methanol, buffer (pH 6.8) and acetonitrile in the ratio of 5:4:1 was selected as mobile phase on the basis of separation of drugs.

Standard Stock Solutions: Analysis was done by using standard stock solution of 200 µg/ml of each PCM, ACE and SER by dissolving 20 mg of standard PCM, standard ACE and SER separately in 100 ml volumetric flask with few mL of phosphate buffer pH 6.8 and methanol (1:1) and sonicated for 5min. Than volume was adjusted up to mark with same solvent. These stock solutions were diluted with mobile phase for subsequent use.

Chromatographic Conditions: A reverse phase column [Cosmosil $5C_{18}$-MS-II (4.61D*250mm) pack column], equilibrated with mobile phase [methanol: buffer (pH 6.8): acetonitrile (5:4:1)] was used. Mobile phase flow rate was maintained at 1ml/min and effluents were monitored at 210nm. The sample was injected using Hamilton syringe to 20 µL fixed loop injector and run time was 15 minutes.

System Suitability Parameters: System suitability tests are an integral part of chromatographic method. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared standard stock solution of PCM, ACE and SER. In addition to this standard deviation of PCM, ACE and SER standards were evaluated by injecting a mixed standard of PCM, ACE and SER (32.5, 10 and 1.0 µg/mL) as internal standard for six times at 20 min interval and the values were recorded.

Linearity of the Drugs: Linearity range of the drugs was determined through diluting the stock solution with mobile phase in concentration range from 0-100 µg/mL. Each solution was filtered using membrane filter (0.45µ) and these solutions were injected through Hamilton syringe one by one to the injector port. With flow rate of 1.0ml/min and peak area of each drug concentration were recorded. The retention time of PCM, ACE and SER are 2.964min, 10.452min and 2.603min respectively.

Validation of Proposed Method using In-house samples: The method was validated using in house mix samples. Various composition of in-house sample was prepared from standard drug sample. The prepared solutions were filtered through 0.22µm syringe filter. Each of the solution was injected in to the column. The area under curve (AUC) of all the drugs (PCM, ACE and SER) was determined through calibration curve.

Preparation of Sample Solution from Dosage Form: Twenty tablets were weighed and pulverized. The tablet powder equivalent to 20 mg of PCM was transferred to a 100 ml volumetric flask and few mL phosphate buffer pH 6.8 and methanol (1:1) was added and sonicated for 5 minutes. The volume was adjusted up to mark with phosphate buffer pH 6.8 and methanol (1:1). Then the sample solution kept in sonicater for 15

min and the solution was filtered through 0.45µm filter paper. Subsequently filtered solution was appropriately diluted with mobile phase for analysis.

Analysis of Commercial Formulation: With the optimized chromatographic conditions mentioned early, a steady base line was recorded. After stabilization of baseline, appropriately diluted sample solution (equivalent to 50 µg/ml of PCM) was run at an interval of 15 minutes and the peak areas were found and amount and percentage of the drug was calculated by regression equations of individual drug.

Validation of HPLC Method: The proposed RP-HPLC method was validated as per ICH guidelines.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ for PCM, ACE and SER were separately determined by based on calculating the signal-to-noise ratio (S/N is 3.3 for LOD and 10 for LOQ) and from the calibration curves the standard deviation of the intercepts and slope of the regression lines were used.

Precision: Precision study was performed to find out intraday and interday variations. The intraday and interday precision study of PCM, ACE and SER was carried out by estimating the correspondence response 3 times on the same day and on 3 different days for PCM, ACE and SER.

Recovery Studies: Accuracy of analytical method was evaluated by fortifying tablet samples through recovery studies which was carried out by spiking the pre-analyzed sample of tablets with different known concentration of standard PCM, ACE and SER. Concentration of resulting solution was determined through develop analytical method. Precision for assay was determined by repeatability, interday, intraday precision for drugs (each in three replicate).

Robustness: The robustness study was done by making small changes in the optimized method parameters like ±1nm change in wavelength and ±0.1ml/min change in flow rate. There was no significant impact on the retention time and tailing factor.

Results and Discussion

In order to develop simultaneous estimation of three components under isocratic conditions, the mixture of methanol, acetonitrile with buffer in different ratios were assayed as the mobile phase. A mixture of buffer, methanol, and acetonitrile in different ratios were also tried for the assay of combined dosage forms. Finally a mixture of methanol, buffer (pH 6.8), acetonitrile in ratio of 5:4:1, proved to be the effective mixture than the other mixture used for the separation. Then the flow rates were tested includes 0.5, 0.8, 1.0, 1.5 and 2.0 ml, among these flow rates 1.0mL was selected for the assay because better resolution of the peaks were observed. System suitability test was applied to freshly prepare stock solution of PCM, ACE and SER to check the parameters like tailing factors, resolution, theoretical plates, relative standard deviation as shown in **Table 1.** The retention times observed were 2.603, 2.964 and 10.452 min for PCM, ACE and SER respectively (**Fig 2-4**).

Table 1: Various system suitability data of Paracetemol, Aceclofenac and Seritiopeptidase

Parameters	Data Obtained		
	SER	PCM	ACE
Retention Time (t_R in sec)	156.0	178.1	624.9
Peak Width (W in sec)	14.2	15.0	34.6
Theoretical Plates	1928.3	2258.1	5216.0
HETP (in mm)	0.0778	0.0664	0.0288
Tailing factor	0.98	1.05	1.07
Capacity Factor(k')	0.159	0.320	3.654
Resolution	-	2.011	22.985
Relative Standard Deviation	0.060	0.011	0.104
LOD	0.001	0.089	0.100
LOQ	0.003	0.270	0.302

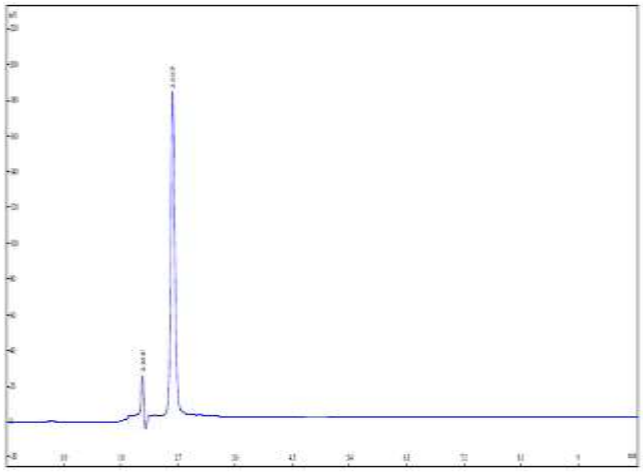

Fig 2: Typical chromatogram of SER

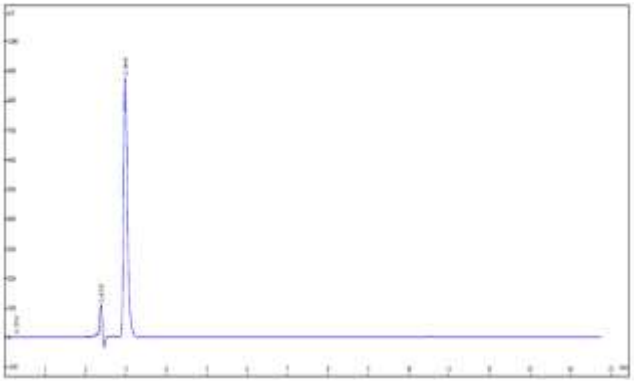

Fig 3: Typical chromatogram of Paracetamol

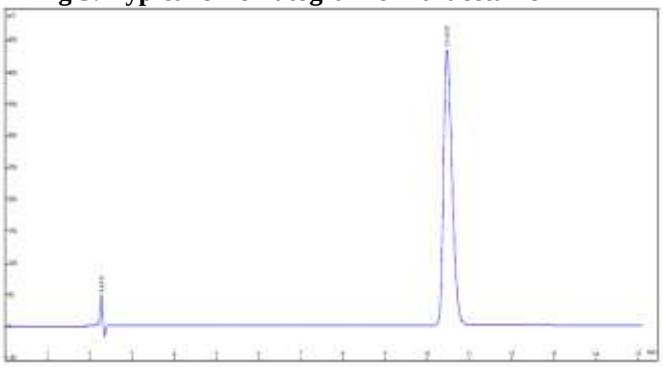

Fig 4: Typical chromatogram of Aceclofenac

The linearity for detector response was observed in the concentration range of 1-70 µg/mL for PCM, ACE and SER showed in concentration range 1-50 µg/mL and the correlation coefficient (r) for calibration curve was found to be 0.9979, 0.9989 and 0.9976 for PCM, ACE and SER respectively **(Fig 5-7 and Table 2)**.

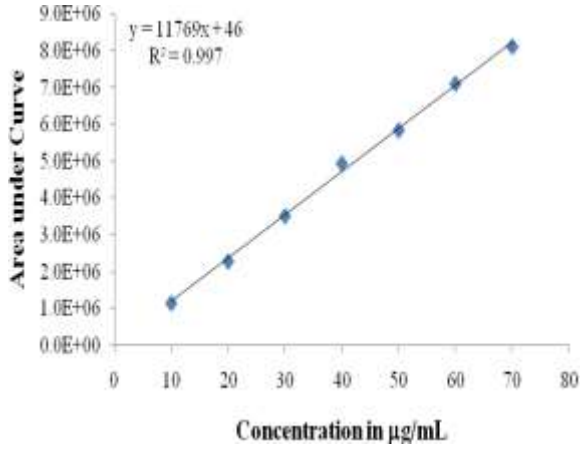

Fig.5: Calibration curve of Paracetamol

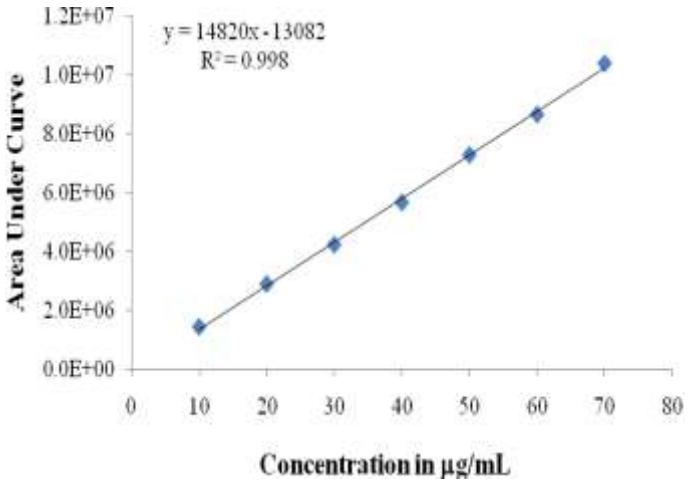

Fig.6: Calibration curve of Aceclofenac

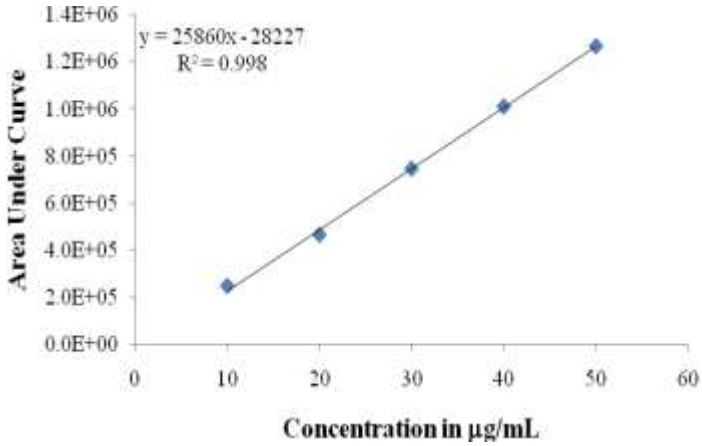

Fig.7: Calibration curve of Seritiopeptidase

Table 2: Linearity range, slope and intercept of Paracetemol, Aceclofenac and Seritiopeptidase

Parameters	PCM	ACE	SER
Linearity range (µg/ml)	00-70	00-70	00-50
Slope (m)	117694.8	148199.7	25860.33
Intercept (b)	658.8683	143379	28226.67

Develop method was check for laboratory samples and result of PCM, ACE and SER were found to be 99.71, 98.70 and 99.50 percentage respectively. Optimized Chromatographic Conditions for proposed method are shown in **Table 3**. System and method precision data are shown in **Table 4**.

Table 3: Optimized chromatographic condition for proposed method

S. No.	Parameter	Condition
1	Mobile Phase	Methanol: Buffer (6.8 pH):Acetonirile (5:4:1)
2	Column	Cosmosil $5C_{18}$-MS-II (4.61D*250mm)
3	Flow rate	1 mL/min
4	Detection(λ_{max})	210 nm
5	Injection volume	20µL
6	Temperature	Ambient
7	Retention time PCM	2.964 minutes
8	Retention time ACE	10.452 minutes
9	Retention time SER	2.603 minutes
10	Run time	15 minutes

Table 4: System and method precision data of Paracetemol, Aceclofenac and Seritiopeptidase

Parameters	System Precision Data			Method Precision Data		
	PCM	ACE	SER	PCM	ACE	SER
Replicate-1	3511903	422958	746558	7573917	2780205	49261
Replicate-2	3514876	422899	745528	7572568	2812411	49885
Replicate-3	3513921	422918	745987	7574312	2798412	51023
Replicate-4	3510299	422882	746856	7605655	2819213	49780
Replicate-5	3515955	422845	746858	7617912	2818865	49750
Replicate-6	3512121	422829	747756	7609580	2818831	49824
Mean	3513179	422888	746591	7592324	2807990	49921
Standard Deviation	2106.110	473.102	773.901	20898.709	15779.976	584.779
% RSD	0.060	0.011	0.104	0.275	0.562	1.171

The percentage of PCM, ACE and SER in commercial formulations were found to be 99.43, 101.10 and 100.33 respectively. The chromatogram of formulation is shown in **figure 8**. The results for the drugs assay showed good agreement with label claims. Percentage recovery studies in pre-analyzed sample of tablets for 80, 100 and 120 percent of individual drugs were found to be within the range of 98.0-102.0 percent indicating accuracy of the method (**Table 5**).

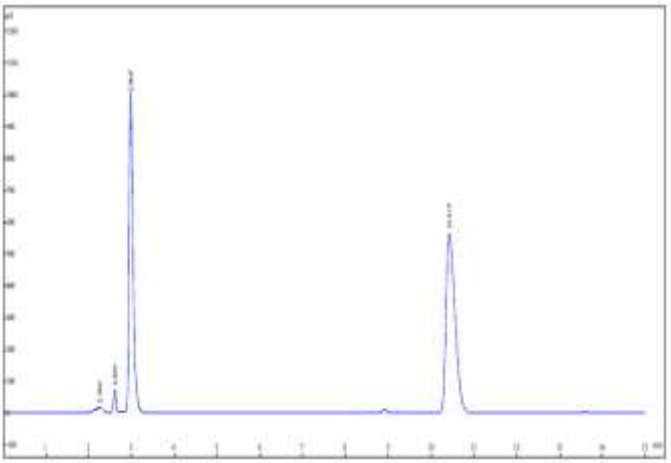

Fig 8: Typical chromatogram of formulation of PCM, ACE & SER

Table 5: Assay and recovery study data of Paracetemol, Aceclofenac and Seritiopeptidase in tablet formulation

Parameters		PCM	ACE	SER
Assay	**Mean % Assay**	99.43	101.10	100.33
	Standard Deviation	0.89	1.39	0.86
	% RSD	0.897	1.374	0.859
Recovery Study	**Mean % Recovery**	100.467	100.623	100.353
	Standard Deviation	1.394	0.773	0.809
	% RSD	1.388	0.768	0.806

The Robustness of the method evaluated by changing the chromatographic condition and results were examined. The percentage RSD was below 2.0%, Showed robustness of the method.

Conclusion

New RP-HPLC method had been developed for simultaneous estimation of PCM, ACE and SER in tablet formulation. It was shown that the method was linear, accurate, precise, reproducible, economical, selective and specific. It produces symmetric peak shapes, good resolution, and reasonable retention times for the drugs. The method was fully validated and showing satisfactory data for all tested validation parameters. Hence, it can be concluded that the

developed RP-HPLC method is accurate, precise, rapid and selective and can be employed successfully for the estimation of Paracetemol, Aceclofenac and Seritiopeptidase in pharmaceutical dosage forms.

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