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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR DETERMINATION OF BROMELAIN, TRYPSIN, RUTOSIDE AND DICLOFENAC IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Serine proteases are enzymes that cleave peptide bond in proteins in which serine serves as the nucleophilic amino acid at the active site. Tripsin is apancreatic serine protease with substrate specifically based upon positively charged lysine and arginine side chains. The selected dosage form trypsin, rutoside, bromelain contains natural pancreatic serine protease used to treat pain and inflammatory diseases. The objective of present research work is to develop a novel, simple, accurate and precise reversed phase High Performance Liquid Chromatography (HPLC) stability indicating method for rapid and simultaneous quantification of Bromelain, Trypsin, Rutoside and Diclofenac. Since this is a new combination of pharmaceutical dosage form and no HPLC methods were developed .There is a need to develop a chromatographic method which is cost effective. This chromatographic method is developed which can easily analyze mixture of compounds with less retention time and low solvent consumption. The chromatographic separation was achieved on Inertsil ODS (250x4.6mm, 5µ). Mobile phase contained a mixture of OPA buffer at pH 2.4 and Acetonitrile in the ratio of 50:50 v/v, flow rate 1.0 ml/min and UV detection at 257nm. The proposed method shows a good linearity in the concentration range of 22.5-135 µg/ml for Bromelain, 12-72µg/ml of Trypsin, 25-150µg/ml of Rutoside and 12.5-75 µg/ml for Diclofenac under optimised conditions. Precision and recovery study results are in between 98-102%. In the entire robustness conditions %RSD is below 2.0%. Degradation has minimum effect in stress condition and solutions are stable up to 24 hrs. This method is validated for different parameters. The study was determined according to the ICH Q2B guidelines. All the parameters of validation were found to be within the acceptance range of ICH guidelines. The developed chromatographic method can be used in routine analysis in pharmaceutical industry.

KEY WORDS: ***RP-HPLC, Bromelain, Trypsin, Rutoside, Diclofenac, Validation.***

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INTRODUCTION

Bromelain is an enzyme [1] extract derived from the stems [2]of pineapples [3], although it exists in all parts of the fresh plant and fruit. The extract has a history of folk medicine [4]use. As a culinary ingredient, it may be used as a meat tenderizer [5]. The term Bromelain may refer to either of two protease [6] enzymes [7] extracted from the plants of the familyBromeliaceae [8], or it may refer to a combination of those enzymes along with other compounds product in an extract. Trypsin is a serine protease [9] from the pancreatic acinarcells [10] superfamily, found in the digestive system[11] of many vertebrates [12], where it hydrolyzes proteins [13-14]. Trypsin is formed in the small intestine when its proenzyme form, the trypsinogen[15] produced by the pancreas [16] is activated. Trypsin cleaves peptide [17] chains mainly at the carboxyl side of the amino acids[18] lysine[19] or arginine[20], except when either is followed by proline[21]. It is used for numerous biotechnological processes. Rutin also called Rutoside, quercetin-3-O-rutinoside and sophorin is the glycoside[22] combining the flavonol[23] quercetin[24] and the disaccharide rutinose. It is a citrus flavonoid found in a wide variety of plants including citrus fruit. Diclofenac sold under the trade name Voltaren among others is a nonsteroidal anti-inflammatory drug (NSAID)[25] used to treat pain and inflammatory diseases such as gout. It is taken by mouth or applied to the skin. Improvements in pain typically occur within half an hour and last for as much as eight hours. It is also available in combination with misoprostol [26] in an effort to decrease stomach problems [27]. Till date there is no analytical based research work for drugs of Bromelain, Trypsin, Rutoside and Diclofenac for HPLC. So, we have developed stability indicating simultaneous estimation and degradation studies in bulk and pharmaceutical dosage form.

MATERIALS AND METHODS

Chemicals

Acetonitrile, Ortho Phosphoric Acid (OPA) and water (HPLC grade) were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Bromelain, Trypsin, Rutoside and Diclofenacas reference standards were procured from Spectrum Pharma research solutions pvt.ltd, Hyderabad.

Equipment

Waters alliance-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 were used.

Chromatographic conditions

Chromatographic separation was carried out in isocratic mode at room temperature using aODS C18 (250x4.6mm, 5µ) column. The mixture of 0.1% OPA: Acetonitrile 50:50 v/v at a flow rate 1.0 ml/min was used as a mobile phase. The injection volume was 20µl and eluents was monitored at 257nm using PDA detector. The run time was 10min.

Selection of wavelength

The absorption spectra of solution of each Bromelain, Trypsin, Rutoside and Diclofenac were scanned over the range 200-400nm by using PDA detector and the spectra were recorded. The spectrum was shown in Figure 1.

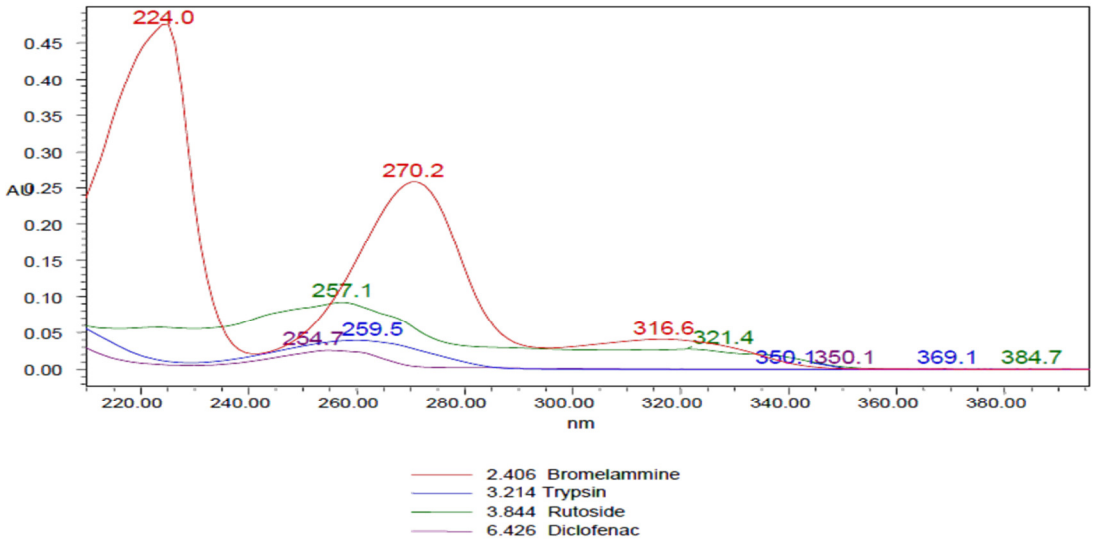

Figure 1

PDA Spectrum for Bromelain, Trypsin, Rutoside and Diclofenac

Preparation of Standard Solution

The standard solution was prepared by adding 9mg of Bromelain, 4.8mg of Trypsin, 10mg of Rutoside and 5mg of Diclofenac working standards were taken into a 10ml volumetric flask,7ml of methanol was added and the mixture was sonicated for 10min to dissolve the components, and then was made up to the volume with methanol and mixed. Further diluted 1ml of the above solution to 10ml volumetric flask with the diluent.

Preparation of Sample Solution

Accurately 10 tablets were weighed and crushed into fine powder equivalent weight of powder sample was transferred into a 100ml volumetric flask and 70ml of diluent was added and the mixture was sonicated for 30mins to dissolve the components and then diluted up to the mark with diluent. Further 1 ml of the above solution was diluted to 10mlwith the diluent and it was filtered through 0.45µ nylon syringe filter.

Validation Procedure [28]

The analytical method was validated as per ICH Q2(R1)[29] guidelines for the parameters like system suitability, specificity, accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantitation (LOQ) and forced degradation.

System Suitability

System suitability parameters were measured to verify the system performance. The parameters including USP plate count, USP tailing and % RSD are calculated and found to be within the limits.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other components (impurities, degradates or excepients), which may be expected to be present in the sample and standard solution. It was checked by examining the chromatograms of blank samples and samples spiked with Trypsin, Rutoside and Diclofenac.

Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. It was assessed by the recovery studies at three different concentration levels. In each level, a minimum of three injections were given and the amount of the drug present, percentage of recovery and related standard deviation were calculated.

Precision

Precision of an analytical method is the degree of agreement among individual test results. It was studied by analysis of multiple sampling of homogeneous sample. The precision of the present method was assessed in terms of repeatability, intra-day and inter-day variations. It was checked by analysing the samples at different time intervals of the same day as well as on different days.

Linearity and range

Linearity of an analytical method is its ability to obtain results directly proportional to the concentration of the analyte in the sample within a definite range. The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated.

LOD and LOQ

Lowest amount of analyte in a sample can be detected by LOD and while LOQ is the lowest amount of analyte in a sample that can be quantified can be determined with acceptable precision and accuracy. LOD and LOQ were separately determined based on the calibration curves. The LOD and LOQ for Trypsin, Rutoside and Diclofenac were determined by injecting progressively low concentrations of standard solutions using the developed RP-HPLC method. The LOD and LOQ were calculated as 3.3s/n and 10s/n respectively as per ICH guidelines, where s/n indicates signal-to-noise ratio.

Stress degradation

Stress degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. Stress degradation studies were performed as per ICH guidelines Q_1A (R_2).The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principle peaks shall pass. Forced degradation studies were performed by different types of stress conditions to obtain the degradation of about 20%.

Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study was performed by injecting standard solution into the HPLC system and altered chromatographic conditions such as flowrate (±0.2ml/min), wavelength (±5nm),

variation in PH (±0.5), organic content in the mobile phase (±2%). The separation factor, retention time and peak asymmetry were calculated by determining the effect of the modified parameters.

STATISTICAL ANALYSIS

The data obtained were analysed by Graph pad prism software version 8. The data is subjected to regression analysis to obtain the line of equation in linearity studies.

RESULTS AND DISCUSSION

Method Validation

In this method system suitability, linearity, precision, accuracy, LOD, LOQ, robustness, forced degradation and stability studies are validated for the selected drugs DTG, RLP drugs. The proposed method having standard solution and sample solution chromatograms are shown in Fig. 2 and 3.

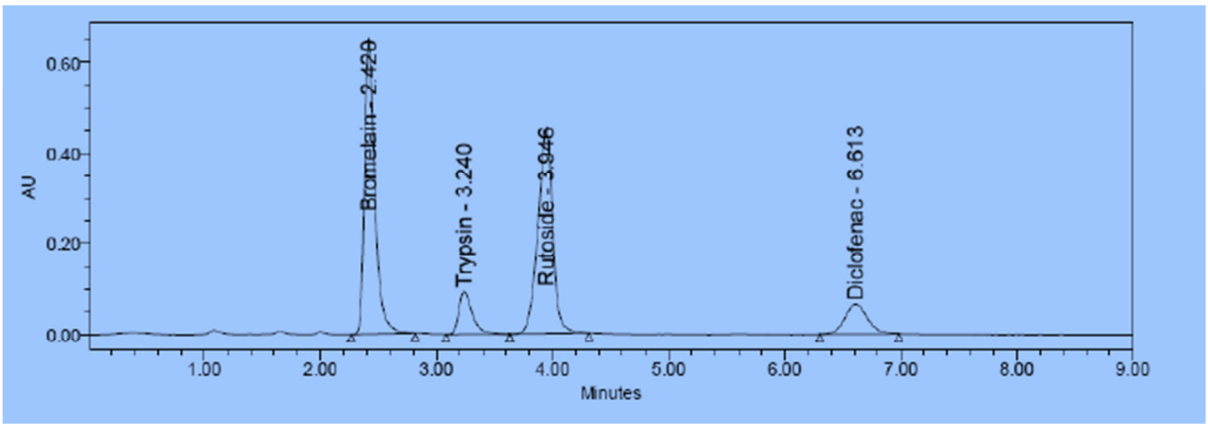

Figure2
Standard Chromatogram

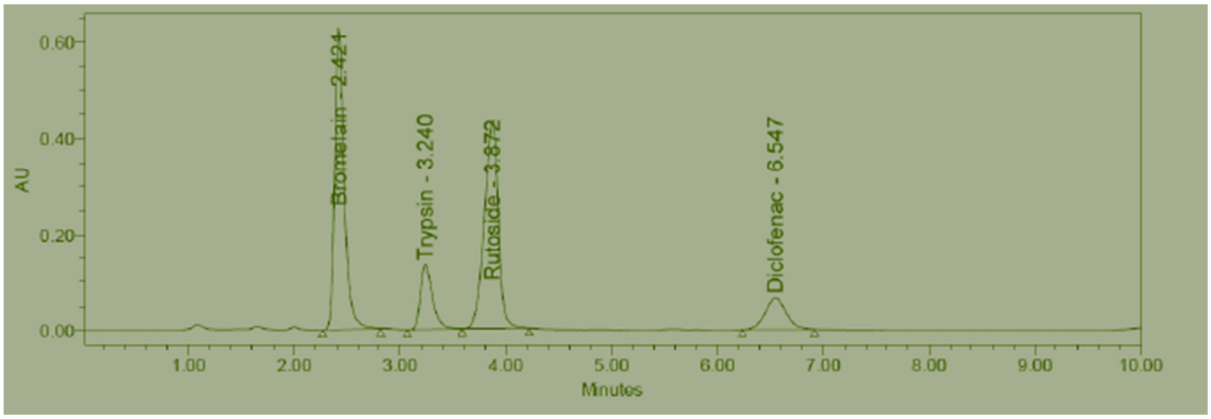

Figure 3
Sample Chromatogram

System suitability

The HPLC system was stabilized for 60min to get a stable base line. Six replicate injections of the standard solution containing 90µg/ml of Bromelain, 48µg/ml of Trypsin, 100µg/ml of Rutoside and 50µg/ml of Diclofenac were assessed to check the system suitability. The number of theoretical plate count for Bromelain, Trypsin, Rutoside and Diclofenac were 3772, 4195, 4836 and 5672 respectively. Tailing factor for Bromelain, Trypsin, Rutoside and Diclofenac were 1.40, 1.51, 0.91 and 1.10 respectively. All these parameters were found to be with limit.

Linearity

Linearity of the method was evaluated by preparing a standard solution containing 90µg/ml of Bromelain, 48µg/ml of Trypsin, 100µg/ml of Rutoside and 50µg/ml of Diclofenac (100% of targeted level of the assay concentration). Sequential dilutions were performed to the given solutions at 25, 50, 75, 100, 125 and 150% of the target concentrations. These were injected and the peak areas were used to plot calibration curves against the concentration. The results are shown in Table 1.

Table 1
Linearity Study Results

Analyte	Linearity Range	Equation of calibration curve	Correlation coefficient
Bromelain	22.5-135.0 µg/ml	Y=43411x+25373	0.999
Trypsin	12.0-72.0 µg/ml	Y=15549x+5323	0.999
Rutoside	25.0-150.0 µg/ml	Y=39106x+27354	0.999
iclofenac	12.5-75.0 µg/ml	Y=17437x+1035	0.999

The equation of calibration curve and correlation coefficient is shown in figure 4 linearity plot for bromelain, Figure 5 linearity plot for trypsin, figure 6 linearity plot for rutoside, figure 7 linearity plot for diclofenac. The correlation coefficient values of these analytes were 0.999.

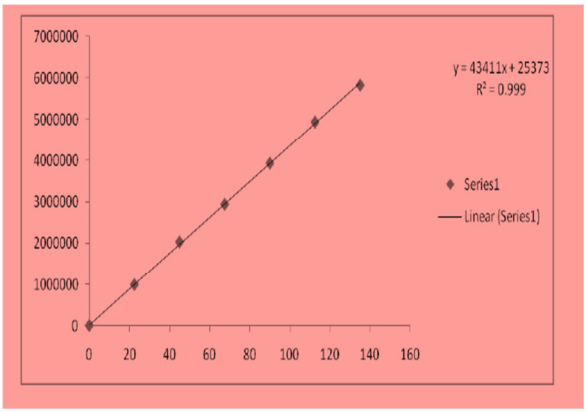

Figure 4
Linearity plot for Bromelain

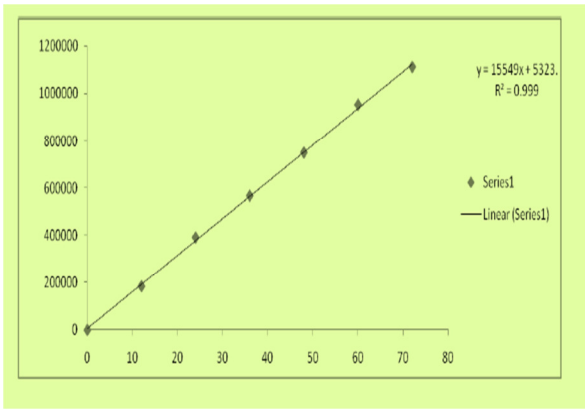

Figure 5
Linearity plot for Trypsin

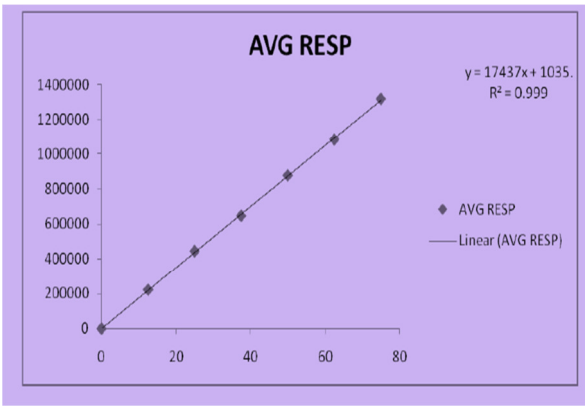

Figure 6
Linearity plot for Rutoside

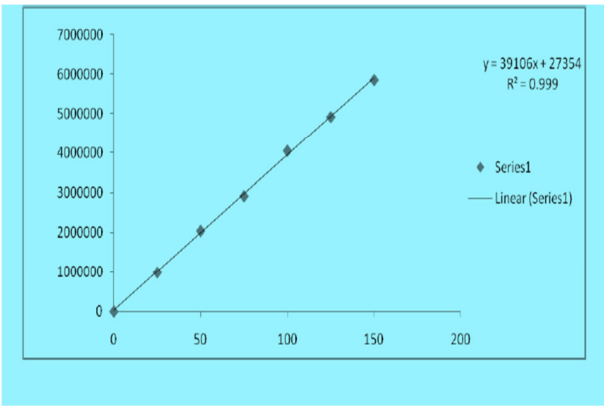

Figure 7
Linearity plot for Diclofenac

Limit of detection and quantification

Limit of detection and quantification minimum concentration level at which the analyte can be reliably detected, quantified by using the standard formulas (3.3 times σ/s and 10 times σ/s for LOD and LOQ respectively). LOD values for Bromelain, Trypsin, Rutoside and Diclofenac were 0.09µg/ml, 0.09µg/ml, 0.23µg/ml and 0.02µg/ml. LOQ values for Bromelain, Trypsin, Rutoside and Diclofenac were 0.27µg/ml, 0.27µg/ml, 0.70µg/ml and 0.06µg/ml.

Precision[30]

Method precision was investigated by the analysis of six separately prepared samples of the same batch. From these six separate samples, solution was injected and the peak areas obtained used to calculate mean and percentage RSD values. The present method was found to be precise as % RSD of the less than 2.0%.The results are given in Table 2.

Table 2
Method Precision

Analyte	Amount present	% RSD
Bromelain	90 mg	0.6
Trypsin	48 mg	0.5
Rutoside	100 mg	0.3
Diclofenac	50 mg	0.3

Accuracy
Accuracy was determined by recovery studies which were carried out in three different concentration levels (50%, 100% and 150%). APIs with concentration 45,90 and 135µg/ml of Bromelain; 24,48 and 72µg/ml of Trypsin; 50,100,150µg/ml of Rutoside; 25, 50 and 75µg/ml of Diclofenac were prepared. As per the test method the test solution was injected tothree preparations each spike level and the assay was performed. The percentage recovery values were found to be in the range of 98-101%. The results are given in Table 3.

Table 3
Accuracy (recovery) study

% of Target Conc.	Bromelain (% Recovery)	Trypsin (%Recovery)	Rutoside (% Recovery)	Diclofenac (% Recovery)
50	99.64	99.11	99.09	98.15
100	99.32	100.94	100.46	98.63
150	99.77	99.27	100.74	98.57
Mean (% Recovery)	99.58	99.77	100.10	98.78

Ruggedness
Ruggedness of the method was studied and the study showed that chromatographic conditions did not significantly change when different HPLC system, analyst and column was used to study the deliberate changes. The value of percentage of RSD was below 2%, which exhibits the ruggedness of the developed method.

Robustness
Robustness of the method was established and %RSD was found to be less than 2%. Slight variations were done in the optimised method parameters like flow rate (±10%), Organic content in mobile phase (±5%), Temperature of the column variation (±5°C). The results are given in Table 4.

Table 4
Robustness Results

Drug Name	Flow Plus (1.1ml/min)	Flow Minus (0.9ml/min)	Org Plus (A55+45)	Org Minus (A45+55)	Temp. Plus (+35°C)	Temp. Minus (-25°C)
	% RSD					
Bromelain	0.1	0.5	0.2	1.2	0.1	0.5
Trypsin	0.0	1.5	0.7	1.9	0.0	1.5
Rutoside	0.2	0.0	0.1	1.5	0.2	0.0
Diclofenac	0.7	0.2	0.2	1.2	0.7	0.2

Forced Degradation
Forced degradation conditions such as acidic, basic, oxidation, thermal, UV and Water stress were attempted as per ICH Q1A (R2). The effects of assay on their results are shown below Table 5.

Table 5
Forced degradation results

Degradation	Bromelain (% of Degradation)	Trypsin (% of Degradation)	Rutoside (% of Degradation)	Diclofenac (% of Degradation)
Acid	4.90	4.88	4.71	4.86
Alkali	2.93	2.82	2.56	2.91
Peroxide	1.84	1.91	1.69	1.92
Thermal	0.66	0.87	0.58	0.88
UV	0.70	0.86	0.64	0.79
Water	0.82	0.89	0.59	0.77

According to the literature review there is no HPLC analytical method reported for Bromelain, Trypsin, Rutoside and Diclofenac. Only one uv-visible spectrophotometer method was reported in the literature for Bromelain, Trypsin, Rutoside, Wani and Mashru, 2014. The aim of the present study was to develop a new HPLC method for rapid and simultaneous quantification of Bromelain, Trypsin, Rutoside and Diclofenac, and its validation as well as application to stability study. The present HPLC method was developed with the trials of different mobile phases such as methanol and water system, methanol and phosphate buffer system or acetonitrile and phosphate buffer system respectively. The acetonitrile and phosphate buffer system produced the optimized separation capacity (Fig. 2) using Inertsil ODS (250x4.6mm, 5µ) column. The developed method was validated as per ICH guidelines (Table 1-5).The validation parameters such as specificity, precision (% RSD), linearity (R2 as 0.9998), accuracy (%RSD), ruggedness and robustness, system suitability results met the requirements and fulfilled the ICH guidelines for Bromelain, Trypsin, Rutoside and Diclofenac[28]. The optimized developed HPLC method was fast, accurate, precise and reproducible. The validation parameters tallied nicely with ICH guidelines. The linearity study in this proposed work showed R2 of 0.999 and Wani and Mashru, 2014, the linearity of bromalein was R2of 0.997. The specificity, ruggedness and robustness were comparable toICH guidelines. Precision and recovery study results are in between 98-102%, and Wani and Mashru for uv-visible method the recovery was 98-101%. According to ICH guidelines for stability study[28-29]the optimized method was performed and results were strictly within the pre-specified limits. The stability results were within the intended limits. The assay test of tablets showed comparable values with ICH within the range of 99to100 % for all the tablet formulations. In addition, there was no degradation in the samples collected from the oxidative degradation study of the tablets (Table 5). So, the tablets were free from oxidative degradation during the period.

CONCLUSION

This method described the quantification of Bromelain, Trypsin, Rutoside and Diclofenac in bulk and pharmaceutical formulation as per ICH guidelines. The developed method was found to be accurate, precise, linear and reliable. The advantage lies in the simplicity of sample preparation and the less expensive agents were used. In addition four compounds are eluted within 10mins. The developed HPLC chromatographic conditions make certain that sufficient resolution and the precise quantification of the compounds. Overview of analytical experimental result indicates that the precision and reproducibility data are satisfactory. The developed chromatographic method can be practically applied for routine analysis in drug research.

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AUTHORS CONTRIBUTION STATEMENT

Professor Dr. SK. Abdul Rahaman guided me in conducting this research study. Professor Dr. A. Prameela Rani evaluated the results and reviewed the manuscript.

CONFLICT OF INTEREST

Conflict of interest declared none.

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