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International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.4, pp 1236-1244, Aug-Sept 2014

Determination of Specific proteolytic activity of Trypsin and Bromelain and simultaneous estimation of Rutoside Trihydrate, Diclofenac sodium, Trypsin and Bromelain in tablet formulation

S. S. Wani* & R. C. Mashru

Pharmacy Department, Faculty of Technology and Engineering, The M.S. University of Baroda, Kalabhavan, Vadodara-390001, Gujarat state, India

***Corres. author: supriya.wani90@gmail.com, rajshreemashru@yahoo.com, Tele: +91- 265 2434187; +91-9428977923;**

Abstract: Accurate and sensitive assay methods for quantitative determination of Rutoside trihydrate (rutin), Diclofenac sodium and proteolytic enzymes Trypsin and Bromelain in tablet formulation based on UV-spectrophotometric analysis were developed and validated. This four drug combination has anti-inflammatory properties and hence is used for treatment of edema and inflammations. Rutin and Diclofenac sodium were analyzed directly by UV-Vis spectrophotometer because Trypsin and Bromelain are UV-inactive and therefore they don't interfere in their analysis. Trypsin and Bromelain being proteolytic enzymes, whey protein was used as substrate for their analysis. Trypsin was inhibited by specific trypsin inhibitor Nα-p-tosyl-L-lysine chloromethyl Ketone (TLCK) and specific proteolytic activity of Bromelain was analysed using Whey protein. Bromelain was inactive towards N-a-benzoyl-DL-arginine p-nitroaniline (BApNA) therefore; BApNA was used as a specific substrate for analysis of Trypsin. BApNA was hydrolyzed to pNA by Trypsin which was further diazotized with N (1 Naphthyl) Ethylene Diamine Dihydochloride (NEDD) and absorbance was recorded at 545nm. Rutin and diclofenac were estimated simultaneously by three methods Simultaneous equation method (Vieordt's method) (Method A), First derivative Zero crossing Spectrophotometry (Method B) and Absorbance Correction method (Method C). All methods were validated as per ICH guidelines and can be adopted for the routine analysis of Trypsin, Bromelain, Rutoside trihydrate and Diclofenac sodium in tablet formulations.
Keywords: Trypsin, Bromelain, Rutoside Trihydrate, Diclofenac sodium, Whey Protein, BapNA.

Introduction and Experimental:

Trypsin:

Trypsin is a proteolytic enzyme obtained by the activation of trypsinogen extracted from the pancreas of healthy mammals. It is a member of mammalian "serine" protease family[1]. It hydrolyses protein hence it is generally used to digest proteins into peptides[2]. It improves humoral response & and prevents growth of pathogens by removing dead and necrotic tissue. It inhibits the C-reactive protein titres and prevents increase in serum acute phase proteins[3]. Thus, it has anti-inflammatory activity.

Bromelain:

Bromelain belongs to a group of protein digesting enzymes obtained commercially from the fruit or stem of pineapple[4]. "Bromelain" refers usually to the "stem bromelain" and is a member of cysteine proteinase

family. Bromelain exhibits various fibrinolytic, antiedematous, antithrombotic, and anti-inflammatory activities[5]. Bromelain is considerably absorbable in the body without losing its proteolytic activity and without producing any major side effects. Preferential cleavage site is the carbonyl end of lysine, alanine, tyrosine and glycine[6].

Rutoside Trihydrate:

Rutoside trihydrate also known as Rutin (3-[[6-O-(6-Deoxy-α-L-mannopyranosyl) β-D-glucopyranosyl] oxy]-2-(3, 4-dihydroxyphenyl)-5 7-dihydroxy-4H-1 benzopyran-4-one) is a flavonoid of the flavonol type[7]. It is found in many typical nutrimental plants such as buckwheat, apple and black tea. Rutin helps preventing hemorrhages and ruptures in the capillaries and connective tissues, and is therefore often used to treat chronic venous insufficiency, hemorrhages and epitaxis[8,9]. Similar to many flavonoid derivatives it also display a remarkable array of pharmacological and biological activities, such as antioxidant, antiinflammatory, anticarcinogenic, antithrombic and vasoprotective activities[10]. [Figure 1]

Figure 1: Structure of rutoside trihydrate

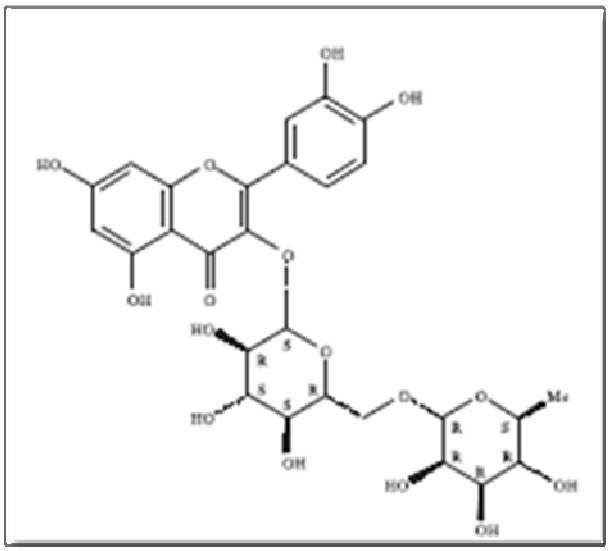
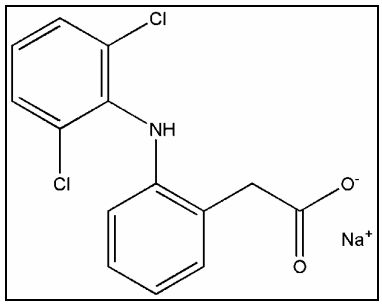


Figure 2: Sructure of Diclofenac sodium



Diclofenac sodium:

Diclofenac Sodium (sodium 2-[(2,6-dichlorophenyl)-amino]phenylacetate) is a non-steroidal anti-inflammatory drug used as an anti-inflammatory, antipyretic, and analgesic. It acts by inhibiting of prostaglandin synthesis by inhibition of cyclooxygenase (COX). [Figure 2]

So far, a number of analytical techniques have been described for determination of trypsin, bromelain, rutin and diclofenac sodium. However, no method is described for simultaneous estimation of rutin and diclofenac with both proteolytic enzymes in any dosage form.

This study describes accurate and precise methods for determination of trypsin, bromelain, rutoside trihydrate and diclofenac sodium in oral dosage forms. The method has been validated with respect to linearity range, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy[11]. The proposed methods have been applied to the analysis of all the three drugs in commercially available pharmaceutical preparations Enzomac Plus (Trypsin 48mg, Bromelain 90 mg, Rutoside Trihydate 100mg, Diclofenac sodium 50mg).

Material and Methods:

Apparatus:

A Shimadzu model 1700 double beam UV-Visible spectrophotometer connected with computer and also with spectral width of 1 nm, wavelength accuracy of ± 0.1 nm and a pair of 10 mm matched quartz cell was used to measure absorbance of all the solutions. Spectra were automatically obtained by UV-Probe system

software (Ver.2.34). The samples were weighed on electronic analytical balance (A×120, shimadzu). pH meter (Lab india), Sonicator (SelecXT543), Centrifuge (REMI).

Reagents and chemicals:

Rutin, Trypsin, Trichloroacetic acid, Tris base Buffer, p-nitroaniline, DMSO, Phosphate buffer standards were purchased from Loba Chemie. BApNA and TLCK were purchased from Sigma Aldrich. Methanol (AR grade) was purchased from Rankem. Bromelain was obtained as a gift sample from Meteoric Life Sciences, Ahmedabad. Whey Protein was obtained as a gift sample from Pruthvi's Food Pvt.Ltd. Tablet formulation (Enzomac) was purchased from local market. All the chemicals used were of analytical grade.

Preparation of Reagents:

BApNA solution- 5mg/ml BApNA solution was prepared by first dissolving BApNA in DMSO and making up the volume with tris buffer. **Whey protein:** 50mg/ml whey protein solution was prepared by dissolving Whey protein in 0.2M phosphate buffer.

General Procedures:

Method for simultaneous analysis of Trypsin and Bromelain: (Determination of total proteolytic activity):

Whey protein was used as a substrate for determining total proteolytic activity[12].

2ml of Whey protein was taken and 1ml of enzyme solution was added and incubated at 37°c for 20minutes. 10%v/v TCA solution was added and kept at room temperature for 15minutes[13,14]. Solutions were centrifuged at 8000rpm for 10min. supernatant was collected and 1% w/v SLS solution was added. Solutions were scanned from 200-400nm and 229nm was selected as analytical wavelength.

Determination of specific proteolytic activity of Bromelain:

Specific proteolytic activity was determined by inhibiting Trypsin by TLCK[15]. 5mg/ml of TLCK solution was prepared in distill water. 5ml of this solution was added to 500µg/ml of total proteolytic enzyme concentration and incubated at 37°C for 10 min[16]. Whey protein was added after 10 min. and further procedure was carried out in a similar manner for hydrolysis of whey protein.

Method for Analysis Of Specific Proteolytic Activity Of Trypsin:

Analysis of trysin by hydrolysis of substrate BApNA:

0.6ml of enzyme solution prepared in distill water was taken and 3ml of 50mM tris buffer with 0.6 ml of distill water was added[17]. Solution was shaken well. 0.6 ml of freshly prepared BApNA solution was added and incubated at 35-40°c for 30 min. Reaction was stopped by adding 2ml of 3N HCL[18]. BApNA was hydrolyzed to pNA which was analyzed in following manner[19,20]. 1ml of each diluted solution containing pNA was taken and 4ml of 0.1% sodium nitrite solution was added. This was kept in icebath for 10 min. Then 4ml of 0.5% ammonium sulfamate was added, shaken well and again kept in icebath for 5 min. Finally, 1ml of 1% NEDD dye was added and the solution was kept at room temperature for 15 min. The absorbance of resulting solution was measured at its analytical wavelength 545nm. A calibration curve as concentration vs. absorbance was constructed using standard pNA solutions to study the Beer-Lambert's Law and regression equation.

Simultaneous analysis of Rutin and Diclofenac sodium:

Preparation of stock solution:

Stock Solutions of 1mg/ml in methanol of pure sample of Rutin and Diclofenac sodium were prepared freshly respectively. The standard solutions were diluted appropriately with the methanol to get a working standard solution of 100 ppm Rutin[21] and Diclofenac sodium respectively. All reagents were tested for stability in solution and during the actual analysis. The behavior of the analytes remained unchanged up to about 24 hr from their preparation at the room temperature. Both the drugs were found to be stable during each kind of experimental measurements. Each measurement was done at room temperature. The solutions of standard Rutin and Diclofenac sodium were prepared according to their ratio in Enzomac Plus tablets in the range of 5.0-30.0

µg/ml and 2.5-15.0 µg/ml respectively. The absorption spectra of the solutions of Rutin and Diclofenac sodium were scanned in the range of 200 nm to 400 nm and were stored in the memory of the instrument.

Method A: Simultaneous Equation Method (Vierodt's Method) for simultaneous analysis of Rutin and Diclofenac sodium

If a sample contains two absorbing drugs (X and Y) each of this absorbs at λmax of other. It may possible to determine both drugs by the technique of simultaneous equations (Vierodt's method) provided that certain criteria apply. Two equations are constructed based upon the fact that at λ1 and λ2 the absorbance of the mixture is the sum of the individual absorbance of X and Y[22,23]. Absorptivity of Rutin and Diclo were calculated at both the wavelengths. The concentrations of Rutin and DICLO can be calculated from following equations·

C_x (rutin) = (A2 ay1 – A1 ay2) / (ax2 ay1 – ax1 ay2)..... (1)

C_y (Diclo) = (A1 ax2 – A2 ax1) / (ax2 ay1 – ax1 ay2)..... (2)

Where; C_x & C_y are concentrations of Rutin and Diclo respectively in gm/100 ml in the sample solution. A1 & A2 are the absorbance of the mixture at 257 nm & 282.50 nm respectively; aX1 and aX2 = Absorptivity of Rutin at 257 nm and 282.5 nm; aY1 and aY2 = Absorptivity of Diclo at 257 nm and 282.50 nm**.** [Figure 3]

Method B: Zero Crossing First Derivative Spectrophotometry for simultaneous analysis of Rutin and Diclofenac sodium:

The recorded data of Rutin and Diclo were transformed to second derivative with Δλ = 8nm and scaling factor 50 and Zero crossing point of both the drugs were determined[22,23]. The ZCP of rutin was 258.70nm on this wavelength absorbance of Diclo is determined while ZCP of Diclo is250.70nm on which absorbance of Rutin is determined. [Figure 4]

Figure 3: Zero order Overlain spectra of rutin (5-30µg/mL Blue) and diclo(2.5-15µg/mL Red)

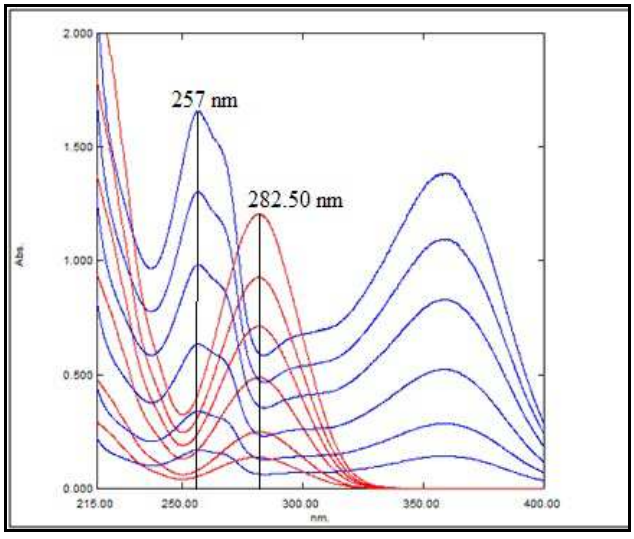
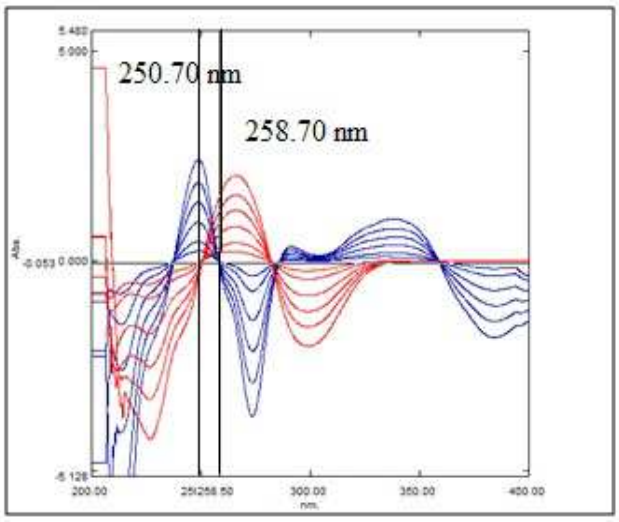


Figure 4: First order overlain spectra of Rutin (5-30µg/mL Blue) and Diclo (2.5-15µg/mL Red)



Method C: Absorbance Correction Method for Simulataneous analysis of Rutin and Diclofenac sodium:

Major parameter that affects absorbance correction method is selection of analytical wavelength. Absorbance of rutin is estimated directly at 360 nm, because Diclo does not show any absorbance at 360 nm so it doesnot interfere in the analysis of rutin. Diclo is showing maximum absorbance at 282.50 nm. Rutin is also showing considerable absorbance at 282.50 nm, so corrected absorbance for Diclo at 282.50 nm is calculated using the following formula[22]:

$C_{(rutin)} = A1 / aX1$

$C_{(Diclo)} = A2 - C \times aX2 / aY2$

Where; $C_{(rutin)}$ & $C_{(Diclo)}$ are concentrations of Rutin and Diclofenac sodium respectively in gm/100 ml in the sample solution.

A1 & A2 are the absorbance of the mixture at 360.0 nm & 282.50 nm respectively; aX1 and aX2 = Absorptivity of rutin at 360.0 nm and 282.50 nm; aY2 = Absorptivity of Diclofenac sodium at 282.50 nm.

Analysis of Marketed formulation:

Validity of the proposed methods was tested for pharmaceutical preparation by assaying Enzomac Plus tablets (labeled to contaion 100mg of rutoside trihydrate, 50mg of Diclofenac sodium and proteolytic activity of Bromelain and Trypsin not less than 1095 FIP units).

ANOVA:

Statistical analysis was performed to assess the effect of three methods in simultaneous estimation of Quercetin and Ascorbic acid using one-way analysis of variance ($P < 0.05$) (Sigma Stat version 2.03; Systat Software Inc., San Jose, CA, USA). [Table 3 & 4]

Validation of Developed Methods:

Validation of all the methods was carried out according to ICH guidelines Q2B.

Accuracy:

For studying the accuracy of the proposed methods, and for checking the interference from excipients used in the dosage forms, recovery experiments were carried out by the standard addition method. This study was evaluated through the percentage of recovery of known amounts of rutin, diclofenac sodium, bromelain and trypsin added to solutions of the commercial product. The analyzed samples were spiked with extra 50, 100 and 150 % of standard rutin, diclofenac sodium, Bromelain and trypsin solution. Accuracy was calculated from the following equation: [(spiked concentration−mean concentration)/ spiked concentration] ×100 [Table 2]

Precision:

Intra-day precision and inter-day precision for the developed methods were measured in terms of % R.S.D. The experiments were repeated three times a day for intra-day precision and on 3 different days for inter-day precision. The concentration values for both intra-day precision and inter-day precision were calculated three times separately and % R.S.D. were calculated. [Table 1]

Limit Of Detection (LOD) And Limit Of Quantitation (LOQ):

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the 3s/m and 10s/m criterions, respectively, where s is the standard deviation of intercept (n =6) of the sample and m is the slope of the corresponding calibration curve. [Table 1]

Table 1: Summary of Validation parameters by developed methods:

	Trypsin	**Bromelain**	**Simultaneous determination of Rutin and Diclofenac sodium**					
Parameter	By Hydrolysis of BApNA	By Hydrolysis of Whey Protein	Method A		Method B		Method C	
			Rutin	Diclo	Rutin	Diclo	Rutin	Diclo
Analytical wavelength	545nm	229nm	257	282.50	258.70	250.70	360	282.50
Beer's range (µg/mL)	100-600	200-800	5-30	2.5-15	5-30	2.5-15	5-30	2.5-15
Slope	0.024223	0.013	0.059	0.084	0.083	0.029	0.037	0.084
Intercept	0.999	0.998	0.999	0.999	0.998	0.998	0.999	0.999
Correlation Coefficient	y = 0.024x - 0.004	y = 0.013x + 0.049	y = 0.059x + 0.024	y = 0.084x + 0.050	y = 0.083x - 0.004	y = 0.029x + 0.022	y = 0.037x + 0.005	y = 0.084x + 0.050
Intraday precision (%RSD)	1.2295	1.364	0.8461	1.0909	1..2615	1.2862	1.2520	1.0909
Interday precision (%RSD)	1.5133	1.769	1.0221	1.4207	1.5140	1.3698	1.5570	1.4207
LOD(µg/ml)	6.477	3.76	0.5062	0.3026	0.7041	0.4060	0.6671	0.3025
LOQ(µg/ml)	19.66	8.56	1.5342	0.9172	2.1337	1.2303	2.0216	0.9166

Table 2: Result of Recovery studies of Trypsin, Bromelain, rutin and Diclofenac sodium by Developed Methods

Method	***% Spiking***	***C Actual* µg/Ml**		***C Added* µg/Ml**		***Recovery* (Mean)* % ± Sd**	
Trypsin	50	200		100		99.85±1.35	
	100	200		200		100.21±1.87	
	150	200		300		101.21±1.32	
Bromelain	50	100		50		99.24±1.21	
	100	100		100		101.65±2.87	
	150	100		150		102.23±1.46	
		Rutin	Diclo	Rutin	Diclo	Rutin	Diclo
A	50	10	5	5	2.5	98.92±1.73	100.24±0.89
	100	10	5	10	5	102.47±2.02	102.33±1.89
	150	10	5	15	7.5	100.44±1.29	101.64±1.10
B	50	10	5	5	2.5	99.91±1.48	98.42±1.34
	100	10	5	10	5	99.67±1.16	101.99±0.62
	150	10	5	15	7.5	98.72±1.19	102.92±1.0
C	50	10	5	5	2.5	101.79±0.36	102.53±1.07
	100	10	5	10	5	101.82±0.67	101.04±1.69
	150	10	5	15	7.5	102.63±1.49	100.02±1.55

Table 3: Result of Statistical analysis of Rutoside Trihydrate:

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	8.218492	3	2.739497	2.090426	**0.202943**	4.757063
Columns	9.362817	2	4.681408	3.572238	0.095109	5.143253
Error	7.862983	6	1.310497			
Total	25.44429	11				

Table 4: Result of statistical analysis of Diclofenac sodium

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	5.586292	3	1.862097	1.964381	**0.2208**	4.757063
Columns	2.613017	2	1.306508	1.378274	0.321703	5.143253
Error	5.687583	6	0.947931			
Total	13.88689	11				

Results and Discussion:

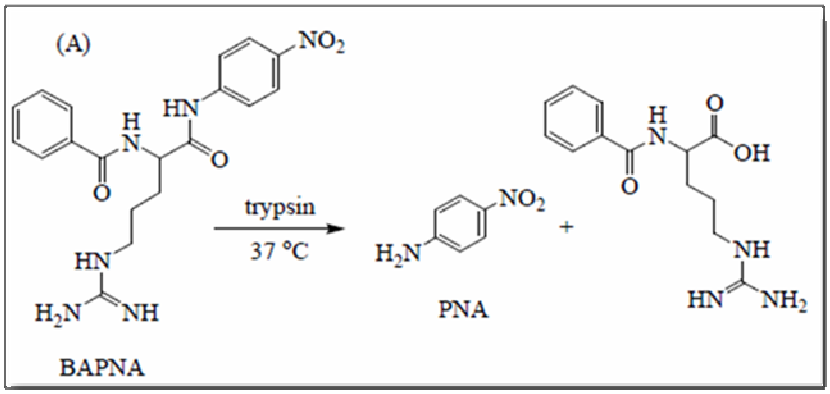
Analysis of Bromelain:

Trysin was inhibited by specific trypsin inhibitor TLCK as it inhibits serine which is end amino acid of trypsin. Bromelain is therefore unaffected by TLCK so bromelain remaining in the solution is determined by hydrolysis of whey protein. Whey protein is hydrolyzed to amino acids, a-lactalbumin and b-lactoglobulin which are analyzed directly by UV-Spectrophotometer.

Analysis of Trypsin:

Trypsin hydrolyses BApNA to pNA at alkaline pH. Arginine is not a preferential cleavage site for Bromelain so it doesn't hydrolyze BApNA. The reaction is inhibited by acididfying the solution by addition of 3N HCL. pNA formed is the diazotized by NEDD to give a colored product which is then determined by UV-Vis spectrophotometer at 545nm. [Figure 5]

Figure 5: Reaction Mechanism of Hydrolysis of BapNA by Trypsin



Determination of Proteolytic activity:

Proteolytic activity was determined in terms of FIP units. 1 FIP unit of enzyme is the amount of enzyme that hydrolyzes protein under the standard conditions into not acid-precipitable peptides at an initial rate such that there is liberated per minute an amount of peptides which gives the same absorbance as 1 °mole of that peptide.

1 Ph.Eur. Unit = 1BP Unit = 1FIP Unit ~ 62.5 USP Units

1FIP unit = 1 U/g

For Bromelain:

5.0 FIP units/mg ≈ 2500 GDU/g ≈ 1560 CDU/mg,

On the basis of these conversions FIP units in tablet was calculated.

Simultaneous analysis of quercetin and ascorbic acid:

Method A:

In zero order overlay spectra both the drugs absorbs at each other's analytical wavelength, therefore simultaneous equation 1 and 2 were utilized for simultaneous estimation of Rutin and Diclo in Enzomac Plus tablets.

Method B:

In contrast to zero-order spectra, First derivative spectra show more resolution in terms of zero crossing points. At 258.70nm, Rutin has zero crossing point and diclo can be determined. At 250.70 nm, Diclo has zero crossing point and Rutin can be determined. The individual spectra of both the drugs were superimposed to obtain the best suitable wavelength in order to develop this method.

Method C:

In this method, absorbance of Rutin is estimated directly at the wavelength where interference by other drug i.e. Diclo is negligible. For the estimation of Diclo, corrected absorbance is calculated which is free from the interference by Rutin.

Validation was carried out according to ICH guideline[22]. [Table 1] exhibits results of marketed formulation, summary of various validation parameters, [Table 2] exhibits results of accuracy studies for all methods respectively and [Table 3 and 4] exhibits results of statistical analysis.

Results of Marketed formulations:

The content of Rutin and diclo based on mean value of three determinations was found out to be 100.61± 0.485% w/w and 99.85± 0.297% w/w respectively. Total proteolytic activity was found to be 1101 FIP units. Specific proteolytic activity of Bromelain was 974 FIP units while that of Trypsin was found out to be 127 FIP units**.**

Conclusion:

The proposed methods are simple, accurate, rapid and selective for routine analysis of Rutin, Diclofenac sodium, Bromelain and Trypsin in tablet formulations. The methods are more selective than reported spectrophotometric methods and are free from interferences from the common excipients. The statistical parameters and the recovery data reveal good accuracy and precision of the methods. The developed methods can be used as general methods for determination of proteolytic activity of proteolytic enzyme in any dosage form or formulation.

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