

Research Article

Validated Stability Indicating RP-HPLC Method for Simultaneous Estimation of Rutoside Trihydrate and Diclofenac Sodium.

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ABSTRACT

The study describes development and validation of stability indicating HPLC method for the fixed dose tablet formulation of rutoside trihydrate and diclofenac sodium. Chromatographic separation was achieved on Agilent C8 (150 X 4.6, 5µm) column with a mobile phase comprised of methanol: water: glacial acetic acid (80:20:0.5% v/v) flowing at a rate of 1 mL/min. Detection was carried out at 281nm using a PDA detector. The analysis can be completed in 10 minutes with retention times of rutoside trihydrate and diclofenac sodium being 2.6 and 5.3 min respectively. The linearity was obtained in the concentration range of 50-250 µg/mL for both drugs with correlation coefficients greater than 0.999. Both the drugs were exposed to acid, alkaline hydrolysis, oxidation, dry heat, and photolytic stress as per ICH guidelines. The method was validated for linearity, precision, accuracy and robustness. Validation data showed that the method is repeatable and selective for the estimation of rutoside trihydrate and diclofenac sodium.

KEYWORDS

HPLC, Rutoside Trihydrate, Diclofenac Sodium, Stability

1. INTRODUCTION

Rutoside Trihydrate is chemically 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxochromen-4-one; trihydrate. Also called as rutin, it is a flavonol possessing several kinds of therapeutic activities. In pharmaceutical preparations, the compound is also used as an anti-inflammatory agent in the treatment of major trauma, wound healing and in reducing healing time in the wound. It also reduces bleeding from wounds [1]. Diclofenac Sodium is chemically Sodium; 2-[2-(2, 6-dichloroanilino) phenyl] acetate and is used as a non-steroidal anti-inflammatory, antipyretic and analgesic agent [2]. Fixed dose formulation of these two drugs is a combination of phytonutrient and NSAID which helps in management of major trauma, wound healing and in reducing healing time in its unique two way action. Rutoside Trihydrate induces migration of neutrophils at the site of acute inflammation that helps in decreasing inflammation in colonic regions, also causes inhibition of vascular endothelial growth factor which helps to reduce oxidative damage and angiogenesis. Diclofenac Sodium is responsible for reducing inflammation and associated pain. Structures of both the drugs are shown in figure 1. Fixed dose formulation of rutoside trihydrate and diclofenac sodium often contain trypsin and bromelain to promote wound healing. To prevent hydrolysis of trypsin and bromelain in gastric acid, formulation is enteric coated. Marketed formulation includes tablets viz. Actiheal D, Enzoheal D, Enzomac Plus (Macleods), Fineheal D (Unichem) and Phytoflam (Cachet Pharma). Combination is useful in treatment of osteoarthritis [3].

Literature survey reveals that most of analytical methods are reported for estimation of rutoside trihydrate [4-10] and diclofenac sodium [11-18] either individually or in combination with other drugs. Being comparatively older drug, there are many more methods reported for diclofenac. Only UV spectrophotometric methods are available for simultaneous estimation of these drugs in combination [19-20]. There is neither assay nor stability indicating method is reported by HPLC. Therefore work was undertaken to develop and validate stability indicating RP-HPLC method for simultaneous estimation of rutoside and diclofenac sodium as per ICH guidelines [21-22].

2. MATERIALS AND METHODS

2.1. Reagents and materials

Gift samples of pure drug rutoside trihydrate and diclofenac sodium were obtained from Macleods Pharmaceuticals Pvt. Ltd., Daman along with certificate of analysis. HPLC grade Methanol was purchased from Merck. Other reagents like, glacial acetic acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide were procured from Loba Chemie Pvt. Ltd, Mumbai. HPLC grade water was prepared in house by double distillation. Tablet formulation Enzoheal-D was purchased from local market.

2.2. Instrumentation and chromatographic conditions

HPLC system (Shimadzu, Kyoto, Japan) comprising of binary pump (LC - 20AD), on line degasser, 20 μ L injection loop, Prominence diode array detector (SPD - M20A) and LC solution software was used.

Separations were carried out on Agilent C8 column (150 X 4.6 mm, 5 μ) as the stationary phase. The mobile phase consisting of methanol, water, and glacial acetic acid (80:20:0.5) was pumped

at a flow rate 1 mL/min. Overlain UV Spectra showed two isobestic points viz. at 281 nm and at 308 nm (Figure 2). Considering higher absorbance at 281 nm (over 308 nm), it was selected as analytical wavelength. Rutoside trihydrate and diclofenac sodium eluted at 2.6 and 5.3 min respectively. Total run time was 10 min. Standard chromatogram is shown in Figure 3.

2.3. Preparation of working standard solutions

Standard stock solutions of rutoside trihydrate and diclofenac sodium were prepared by separately dissolving 100 mg of each pure drug in 100 mL methanol to get concentration of 1000 µg/mL. From the respective standard stock solution, appropriate dilutions were made in methanol to prepare working solutions in the range of calibration curve.

2.4. Preparation of sample solution of Enzoheal-D tablet

Twenty tablets were weighed and average weight was noted. Quantity of powder equivalent to 100 mg of rutoside trihydrate (~50 mg of diclofenac sodium) was weighed and transferred into a 100mL volumetric flask, sufficient amount of methanol was added and sonicated for 10 min and volume was made up to the mark by methanol. This solution was filtered through a 0.45µm pore size membrane filter. From the above solution 1mL was removed and diluted up to 10 mL to get concentration of 100 µg/mL. Final dilution was injected into HPLC, peak areas were noted and the amount of drugs was calculated.

2.5. Forced degradation studies

Forced degradation studies were carried out to provide evidence on how the quality of drug varies under the influence of variety of environmental conditions like hydrolysis, oxidation, thermal and photolytic stress. Stress studies were carried out separately on individual drug as well as on their equimolar mixture.

2.6. Acid and alkaline hydrolysis

Samples were prepared by taking 1 mL of stock solution of each drug (1000 µg/mL) and 1 mL 1N HCl/NaOH in 10 mL volumetric flasks. Solutions were heated for 1 h at 80°C; samples were diluted about the mark, neutralized by using equal strength of alkali/acid. Finally volume was made up to the mark by using methanol and subjected for HPLC analysis.

2.7. Oxidation degradation

Oxidative degradation was carried out by using hydrogen peroxide. Samples were prepared by taking 1 mL of stock solution of each drug (1000 µg/mL) and 1 mL of 30% of hydrogen peroxide in 10 mL volumetric flasks. Solution was kept for 1 h at room temperature. After required exposure samples were diluted up to the mark by using methanol and subjected for HPLC analysis.

2.8. Thermal degradation

Solid drug samples sealed in glass ampoules were exposed to dry heat in hot air oven for 110°C for definite time intervals and similar controls were kept at room temperature. After 5 h separate solutions were prepared by dissolving samples exposed to thermal stress and control using methanol. The samples were further diluted with the help of methanol to get solution of 100 µg/mL and injected into stabilized HPLC system and chromatograms were recorded.

2.9. Photo degradation

Photo degradation of drug was carried out in solid phase. Drugs (10 mg) each was exposed to ICH recommended dose of light in photo stability chamber (overall illumination of not less than

1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter). After complete exposure solutions were prepared as explained for thermal degradation.

2.10. Method validation

To establish linearity and range, stock solution of drugs was further diluted with methanol to get drug concentration as 50, 100, 150, 200, 250 µg/mL. All the solutions were prepared in triplicate and injected into the HPLC. The peak areas were measured and linear regression coefficient was determined. Line equation generated in the form of $y = mx + c$ was noted and used for calculation of drug concentration in other validation parameters.

Accuracy of the method was determined by employing standard addition method. The standard of known concentration (80, 100 and 120 µg/mL) was spiked in degradants mixture (acid, alkali, oxidation, thermal and photo degradation) and percent recovery of the drug was calculated. Analysis was done in triplicate.

Intra-day and Inter-day precision of the method was established by making triplicate injections of three samples at three different concentration levels, viz., 100, 150 and 250 µg/mL on the same day at the morning, afternoon and evening with interval not less than 5 h and on three consecutive days at fixed time. The values of percent relative standard deviations (%RSD) were calculated.

LOD was determined using formula of $3.3 \sigma / S$ and LOQ was determined using formula $10\sigma / S$ Where, 'σ' is Standard deviation of y- intercept and 'S' is average of slope in the regression equation of the calibration curve.

Robustness of the developed stability indicating method was checked by making small deliberate alterations in chromatographic parameters such as pH of buffer, flow rate, and detection wavelength. The pH of buffer was varied by (±) 0.25 of original pH, the flow rate was varied by (±) 0.1 mL/min of original flow rate and detection wavelength was varied by (±) 2 nm of original wavelength. Values % RSD of peak area under each altered condition was calculated.

3. RESULTS AND DISCUSSION

3.1. Forced degradation studies

Stress conditions were optimized to obtained degradation in the range of 5-30%. Both the drugs were found to be stable under photolytic, thermal degradation, hydrolytic stress and susceptible to oxidative degradation. Even when they are treated together percent degradation is much more as indicated in Table 1.

Diclofenac sodium generated one degradation product under acid hydrolysis. Chromatogram is shown in Figure 4. In rest all cases, drugs were seen to degrade in peak area but degradation products were not seen probably due to lack of chromophore.

3.2. Method validation

The specificity of the method was ascertained by peak purity profiling studies. It is clear from the chromatogram that developed method was selective to the drug as well as the degradation product. In the chromatogram absorbance of blanks i.e. HCl, NaOH, and H₂O₂ was negligible. The purity of peaks of degradation product and drug peaks in a mixture of stressed samples was established through PDA studies. The peak purity values indicate that drug peak and degradation

product was pure and free from eluting peaks. Moreover, resolution factor for the drug peak was > 2.0 from the nearest resolving peak, proved that method is suitable for simultaneous determination. Table 2 shows system suitability parameters and specificity indicators.

Good correlation coefficients ($r^2 = 0.9995$ for Rutoside Trihydrate and 0.9992 for Diclofenac Sodium) were obtained in tested concentration range 50 to 250 $\mu\text{g/mL}$. Excellent correlation exists between response factor and concentrations in the validation range. Calibration curves are depicted in figure 5.

The developed method was found to be precise as the RSD values for intra-day and inter-day precision studies were $< 2\%$ as recommended by ICH guidelines. For intra-day precision %RSD found to be 1.2 for rutoside trihydrate and 0.3 for diclofenac sodium. For inter-day precision %RSD found to be 1.5 for rutoside trihydrate and 0.2 for diclofenac sodium.

The LOD for rutoside trihydrate and diclofenac sodium were found to be 0.87 ng/band and 0.26 ng/band respectively while LOQ of rutoside trihydrate and diclofenac sodium were found to be 2.6 ng/band and 0.80 ng/band respectively.

Good recoveries were obtained at each concentration with mean recovery of 99.58% for rutoside trihydrate and 99.6% for diclofenac sodium.

Method was found to be robust as small variations in chromatographic parameters such as pH of buffer, flow rate, and detection wavelength did not affect the peak area determination. Results are quoted in Table 3.

3.3. Analysis of tablet formulation

The optimized RP-HPLC procedure was applied for the assay of drugs in the pharmaceutical formulation available in local market (Enzoheal-D tablet). The active ingredients were extracted with the methanol, and then dilution was made with methanol to reach concentration level within the linearity range. The active ingredients eluted at their specific retention time and no interfering peaks were observed from any of the excipients. The assay results revealed satisfactory accuracy and precision as indicated from amount found. The results are depicted in Table 1.4

4. CONCLUSION

This is the first HPLC method being reported for simultaneous estimation of rutoside trihydrate and diclofenac sodium from fixed dose combination. The method was able to chromatographically separate rutoside trihydrate and diclofenac sodium from each other and its degradation product; it can be used for determination of title drugs from bulk, formulation, and stability samples. Forced degradation studies were carried out at different stress conditions as per ICH requirements for hydrolysis, oxidation, thermal and photolytic degradation. Rutoside trihydrate and diclofenac Sodium both were found to be stable to photolytic degradation and unstable to acid/alkali hydrolysis, thermal degradation and highly susceptible to oxidative degradation.

Diclofenac Sodium was found susceptible to hydrolyse by acid probably due to conversion to its parent drug Diclofenac.

The developed method was validated as per ICH guidelines for accuracy, precision, specificity, linearity and robustness.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

Nil

7. REFERENCES

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Table 1. Degradation behavior of rutoside trihydrate and diclofenac sodium.

Degradation conditions	% Degradation		% Degradation	
	individually		in mixture	
	Rutoside Trihydrate	Diclofenac Sodium	Rutoside Trihydrate	Diclofenac Sodium
Acid hydrolysis (1 N HCl, 1 h, 80°C)	6.2%	6.3%	28.2%	32.5%
Base hydrolysis (1 N NaOH, 1h, 80°C)	9.8%	6.7%	25.3%	28%
Oxidation (30% H ₂ O ₂ , 1 h)	16.3%	17%	28.1%	31.6%
Dry heat (110°C, 5 h)	9%	5.8%	24%	10.1%
Photo stability (UV, 200 watt hrs/square meter, Florescence, 1.2 million Lux. hrs)	2.6%	0.2%	-	-

Table 2. System suitability parameters for rutoside trihydrate and diclofenac sodium.

Name of Degradant	Drug/ (A _s)	Asymmetry	No. of Theoretical plates (N)	Resolution	Capacity factor (k')	RRT	Peak purity
Rutoside Trihydrate		1.153	3607	0.000	0.000	2.6	0.9999
Diclofenac Sodium		1.134	2542	3.075	0.244	5.3	0.9999
Acid Deg.		1.057	7894	5.668	0.754	7.2	0.9999

Table 3. Robustness studies of rutoside Trihydrate and diclofenac Sodium

Parameter	value	RSD (%)	
		Rutoside Trihydrate	Diclofenac Sodium
Wavelength	279 nm	0.57	0.31
	281 nm		
	283 nm		
Flow rate	0.9 mL/min	1.36	0.26
	1 mL/min		
	1.1 mL/min		
pH	3.25	0.37	0.56
	3.5		
	3.75		

Table 4. Assay of tablet formulation.

Sr. No.	Label claim (mg/tablet)		Amount found (mg/tablet)		% of label claim	
	Rutoside Trihydrate	Diclofenac Sodium	Rutoside Trihydrate	Diclofenac Sodium	Rutoside Trihydrate	Diclofenac Sodium
1	100	50	101.1	50.1	101	100.2
2	100	50	100.18	49.81	100.18	99.62
3	100	50	99.52	48.95	99.52	97.9
4	100	50	98.90	50.15	98.9	100.3
5	100	50	99.84	49.59	99.84	98.98
6	100	50	100.25	50.02	100.2	100.04
		Average	99.96	49.67	99.96	99.50
		SD	0.74	0.55	0.74	0.92
		%RSD	1.34	0.89	1.34	1.07

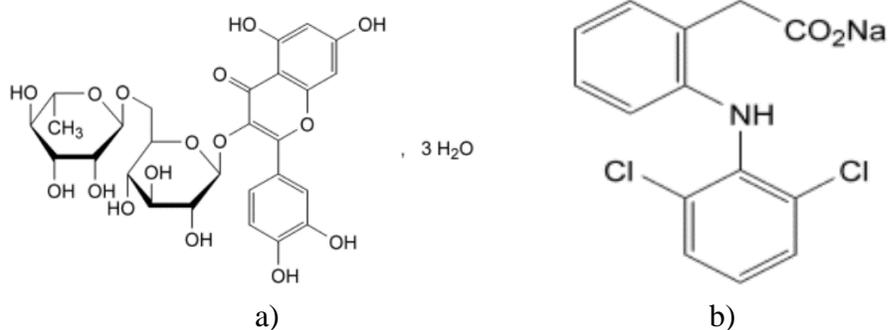


Fig. 1. Structure of Rutoside Trihydrate b) Diclofenac Sodium.

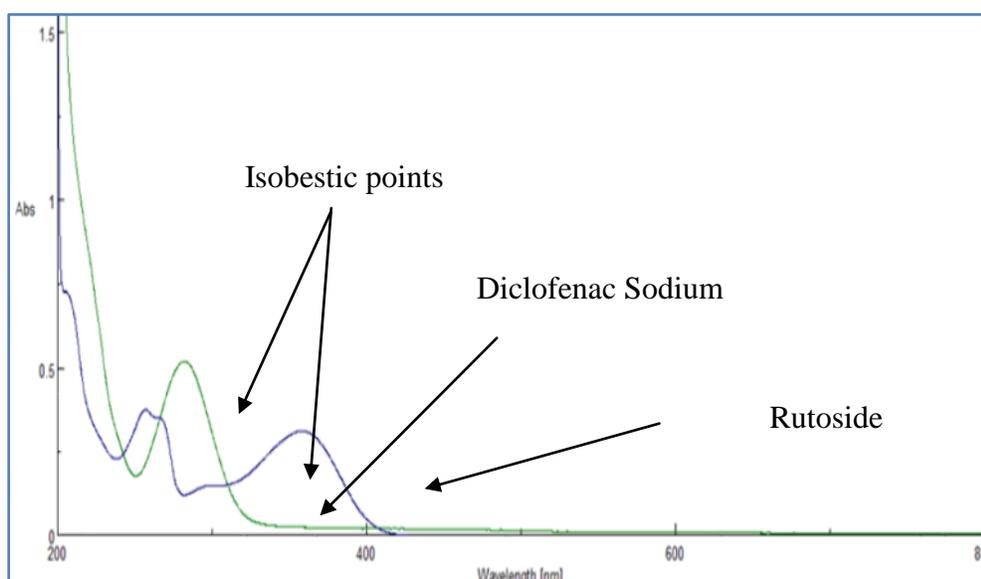


Fig. 2. Overlain UV spectrum of rutoside trihydrate and diclofenac sodium.

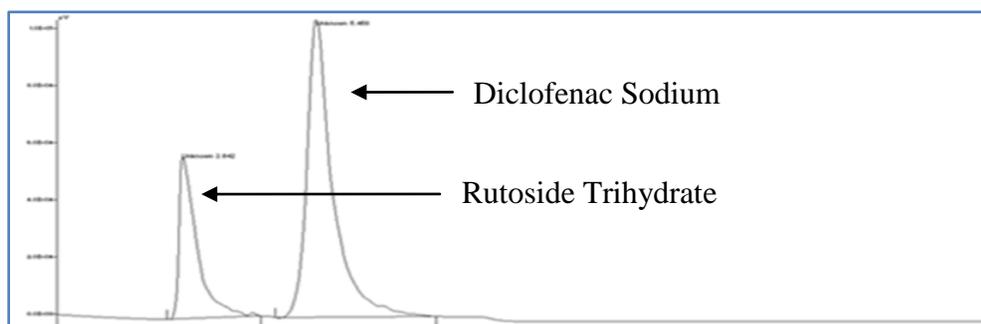


Fig. 3. Representative chromatogram of rutoside trihydrate and diclofenac sodium.

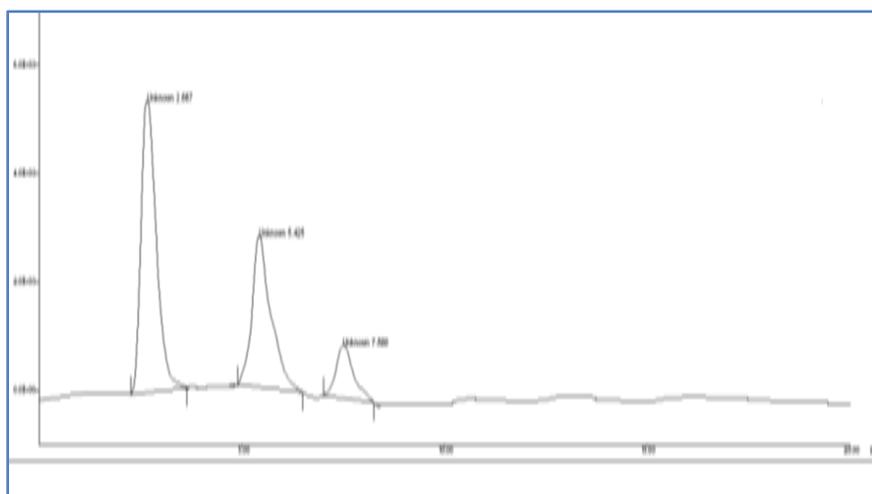


Fig. 4. Chromatogram after exposure of drugs to acid hydrolysis.

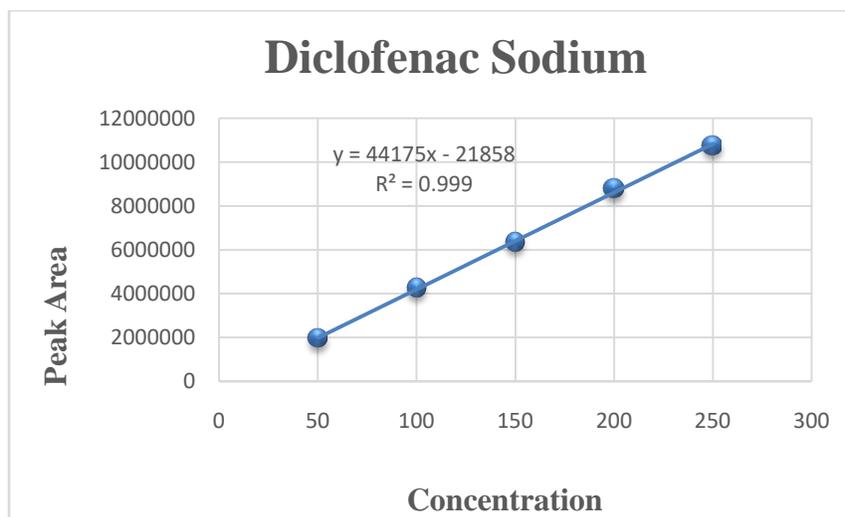
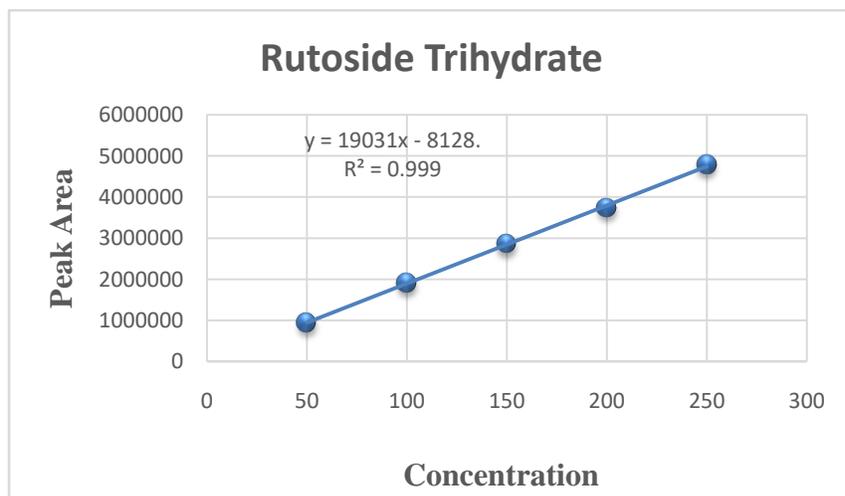


Fig. 5. Calibration curves for rutoside trihydrate and diclofenac sodium.