Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 1, 2014

Research Article

REVERSE PHASE HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS QUANTITATIVE ESTIMATION OF TROXERUTIN AND CALCIUM DOBESILATE IN TABLETS

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Received: 04 Oct 2013, Revised and Accepted: 30 Oct 2013

ABSTRACT

Objective: To develop a simple, precise, accurate, linear and rapid Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous quantitative estimation of Troxerutin 500 mg and Calcium dobesilate 500 mg in tablets as per ICH guidelines.

Methods: The method uses reverse phase column, Enable C18G (250X4.6 mm; 5µ) column and an isocratic elution. Method optimized conditions include a mobile phase of acetonitrile:methanol:0.02M potassium dihydrogen orthophosphate buffer adjusted to pH 4 in the proportion of 25:10:65 v/v, flow rate of 0.5 ml/min and detection wavelength of 210 nm using a UV detector.

Results: The developed method resulted in calcium dobesilate eluting at 3.56 min and troxerutin at 6.69 min. The linearity of the method was excellent over the range 62.5-250 µg/ml for both the drugs. The precision is exemplified by relative standard deviations of 0.258% for calcium dobesilate and 0.215% for troxerutin. Accuracy studies revealed % mean recoveries during spiking experiments between 98 and 102. The limit of detection was obtained as 3 ng/mL for Calcium dobesilate and 0.5 µg/mL for Troxerutin, while the limit of quantitation was obtained as 10 ng/mL for Calcium dobesilate and 1µg/mL for Troxerutin.

Conclusion: A simple, precise, accurate, linear and rapid RP-HPLC method has been developed and validated for the simultaneous quantitative estimation of Troxerutin 500 mg and Calcium dobesilate 500 mg in tablets and hence it can be applicable in routine analysis of tablets in various pharmaceutical industries.

Keywords: RP-HPLC, Calcium dobesilate, Troxerutin, Validation.

INTRODUCTION

Troxerutin (**Figure 1,** Trihydroxyethylrutin; 3',4',7-Tris[O-(2-hydroxyethyl)]rutin) is a flavonol, a hydroxyethylrutoside isolated from Sophora japonica, the japanese pagoda tree. This drug is used as a vasoprotective agent prescribed for circulatory disorders [1]. Troxerutin is used as an anti-clotting drug for the treatment of hemiplegia, aphasia, cardiac stem, arteriosclerosis etc. [2]. Calcium dobesilate (**Figure 2,** calcium 2,5-dihydroxybenzenesulfonate) is a drug used for the treatment of diabetic retinopathy and chronic venous insufficiency. Calcium dobesilate acts selectively on the capillary walls regulating their physiological functions of resistance and permeability [3-5].

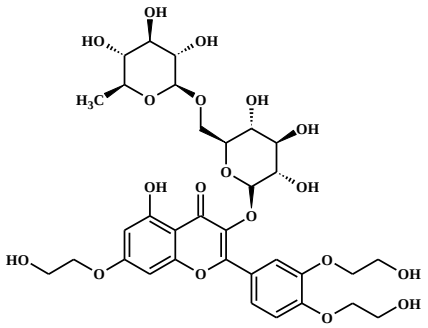

Fig. 1: Structure of Troxerutin

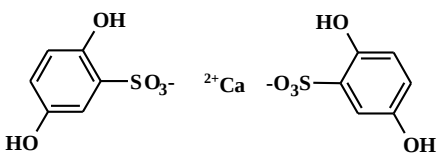

Fig. 2: Structure of Calcium dobesilate

A detailed literature survey reveals that RP-HPLC methods have been reported for the quantitative estimation of calcium dobesilate and troxerutin individually in various matrix such as human plasma, pharmaceutical dosage forms, bulk, rat urine, chicken plasma and food supplements. RP-HPLC methods are reported for troxerutin in combination with other drugs and similarly calcium dobesilate with other drugs [6-11]. As per our detailed literature survey as on date, there are no RP-HPLC methods reported for the simultaneous quantitative estimation of calcium dobesilate and troxerutin in any matrix either of pharmaceutical dosage forms, plasma, etc. In addition there exist no pharamcoepial methods available for analysis of individual drugs and even for combination of these two drugs. Hence we here report a simple, sensitive, rapid, precise, accurate and linear RP-HPLC isocratic method for the simultaneous quantitative estimation of calcium dobesilate and troxerutin in tablets as per ICH guidelines [12].

MATERIALS AND METHODS

Chemicals and Reagents

Analytically pure sample of calcium dobesilate and Troxerutin with purities greater than 99% were obtained as gift samples from Chandra labs (Hyderabad, India) and tablet formulation [OXERUTE CD (Brand name)] was procured from Apollo pharmacy, Hyderabad, India with labelled amount 500mg each of troxerutin and calcium dobesilate. Acetonitrile (HPLC grade) was obtained from Sigma Aldrich (Hyderabad, India), Water (HPLC grade), Potassium dihydrogen orthophosphate (AR grade) and phosphoric acid (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India). 0.45µm Nylon membrane filters were obtained from Spincotech private limited, Hyderabad, India.

Instrument

HPLC analysis was performed on Shimadzu LC-20AD Prominence Liquid Chromatograph comprising a LC-20AD pump, Shimadzu SPD-20A Prominence UV-Vis detector and Enable C18G reverse phase C18 column (250X4.6 mm, 5 micron particle size). A manually

operating Rheodyne injector with 20 µL sample loop was equipped with the HPLC system. The HPLC system was controlled with "Lab solutions lite" software. In addition, an electronic analytical weighing balance (0.1mg sensitivity, Shimadzu AY 220), digital pH meter (DELUX model 101), a sonicator (sonica, model 2200 MH) and UV-Visible Spectrophotometer (Shimadzu UV-1800 series, software-UV probe version 2.42) were used in this study.

Method

Selection of Wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of calcium dobesilate and troxerutin. Suitable wavelength selected was 210 nm (**Figures 3 and 4**).

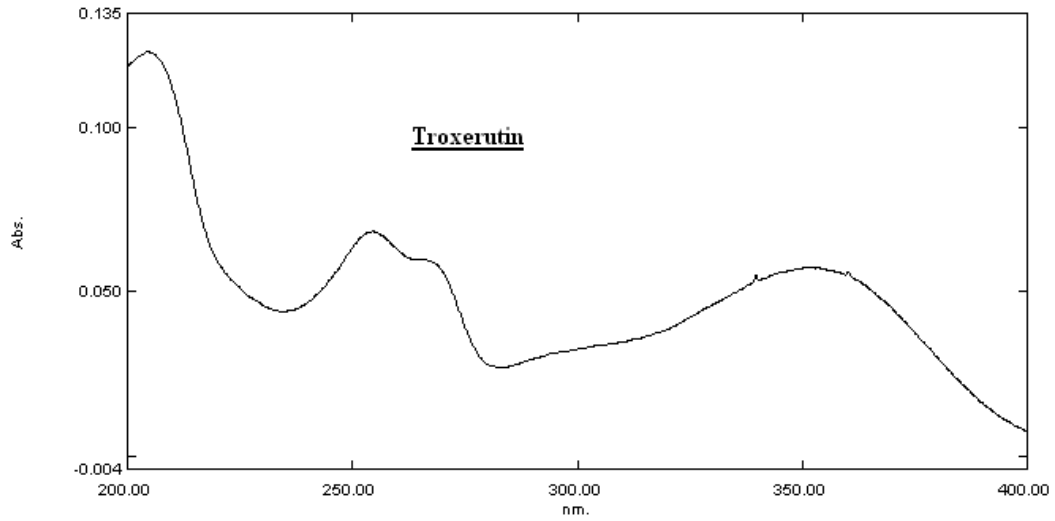

Fig. 3: UV spectrum of Troxerutin

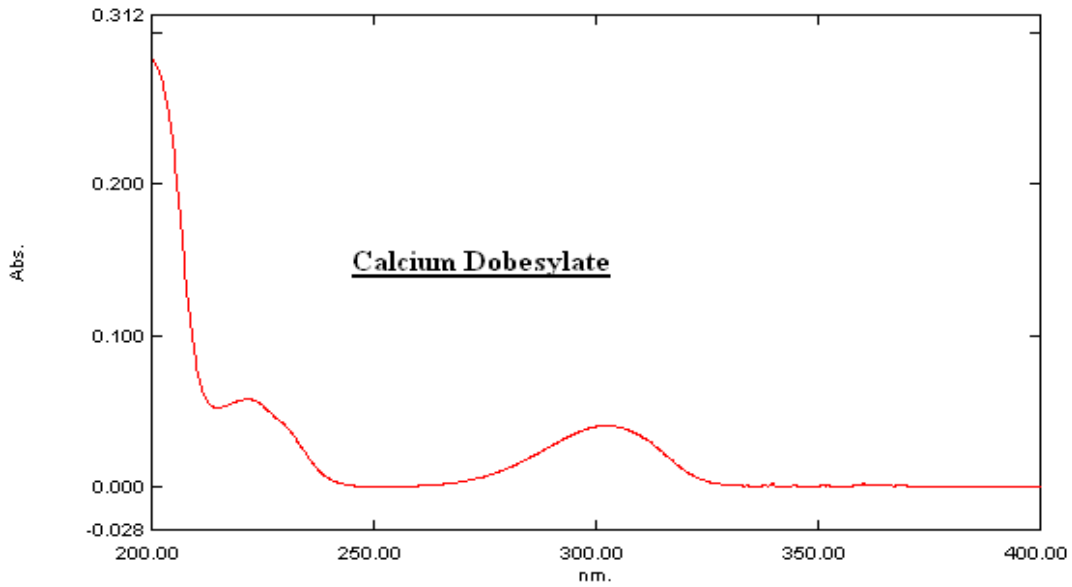

Fig. 4: UV spectrum of Calcium dobesilate

Chromatographic conditions

The separation of the drugs was achieved on a C18 column reverse phase (4.6 mm X 250 mm, 5 micron particle size). The mobile phase consists of a mixture of acetonitrile:methanol :potassium dihydrogen ortho phosphate buffer (20mM, pH adjusted to 4 using 30% v/v ortho phosphoric acid) in ratio of 25:10:65, v/v. The mobile phase was set at a flow rate of 0.5 ml/min and the volume injected was 20 µl for every injection. The detection wavelength was set at 210 nm.

Buffer Preparation

The buffer solution is prepared by weighing 2.736 g of potassium dihydrogen ortho phosphate (KH_2PO_4) and transferring to 1000 ml of HPLC grade water to get 20 mM buffer strength which was adjusted to pH 4 using 30% v/v ortho phosphoric acid and later the buffer was filtered through 0.45 µm nylon membrane filter.

Mobile phase Preparation

The mobile phase was prepared by mixing acetonitrile, methanol and buffer in the ratio of 25:10:65, v/v and later sonicated for 10 minutes for the removal of air bubbles.

Preparation of stock and working standard solution

50 mg of Calcium dobesilate and 50 mg of Troxerutin were accurately weighed and taken in 100 ml clean and dry volumetric flask containing 50 ml of diluent and then sonicated for 5 minutes to dissolve. Later the

solution was made up to the mark using the mobile phase. This is considered as standard stock solution, each drug concentration as 500µg/ml. 2.5 ml of the stock solution was pipetted out and made up to 10 ml to get a concentration of each drug 125 µg/ml, treated as working standard solution, 100% target concentration.

Preparation of stock and working sample solution

Sample solution containing both the drugs was prepared by dissolving tablet powder into diluent (mobile phase). Ten tablets were weighed separately and their average weights were determined. The average weight was taken from the ten tablets grinded in a pestle and mortar and then transferred to a 1000 ml volumetric flask containing 800 ml diluents. Sonication was done for five minutes and later the volume was made up to 1000 ml using mobile phase. Then the sample preparation was filtered through 0.45µ nylon membrane filter to get sample stock solution, each drug of 500µg/ml. 2.5 ml of the stock solution was pipetted out and made up to 10 ml to get a concentration of each drug 125 µg/ml, treated as working sample solution, 100% target concentration.

RESULTS AND DISCUSSION

Method Development

A Reverse phase HPLC method was developed keeping in mind the system suitability parameters i.e. resolution factor (Rf) between peaks, tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Calcium dobesilate at 3.56 min and Troxerutin at 6.69 min. **Figure 5** and **Figure 6** represent chromatograms of blank solution and mixture of standard solutions respectively. The total run time is 8 minutes with all system suitability parameters as ideal for the mixture of standard solutions. System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*Rt*), number of theoretical plates (*N*), peak resolution (Rs) and peak Tailing factor (T) were evaluated for six replicate injections of the standards at working concentration. The results given in **Table 1** were within acceptable limits.

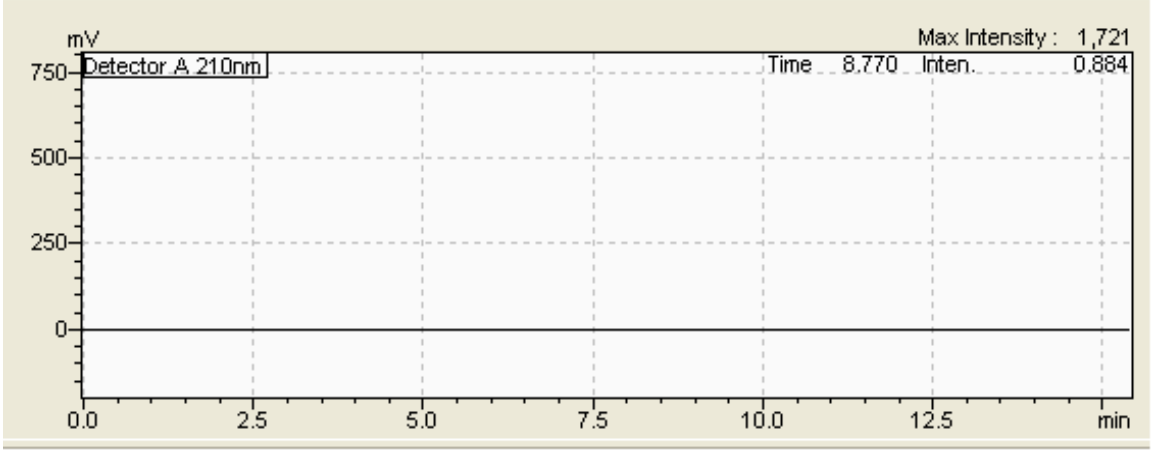

Fig. 5: Typical Chromatogram of Blank solution

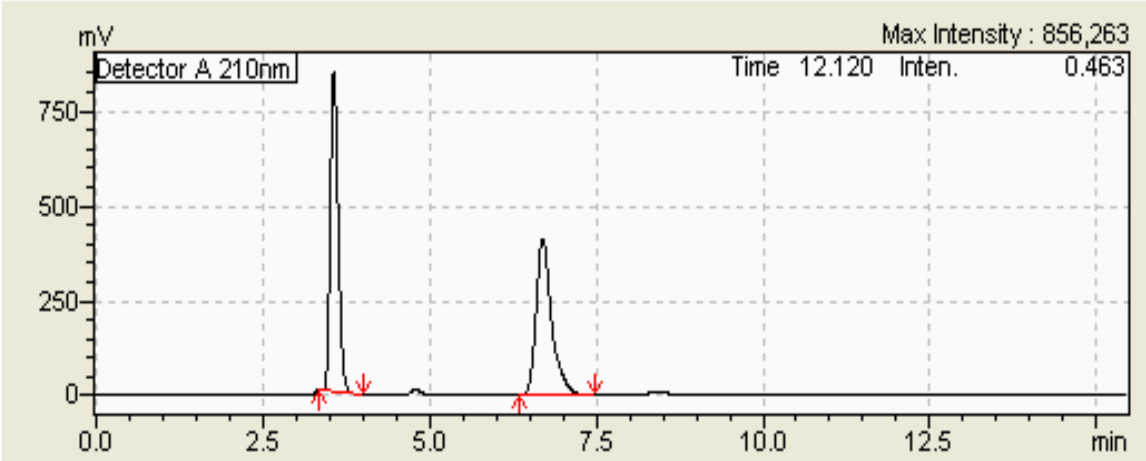

Fig. 6: Typical chromatogram of the mixture of Standard solution

Table 1: System suitability studies results.

Parameters*	Required Limits	Calcium dobesilate	Troxerutin
Retention time (min)	% RSD < 1%	3.561	6.699
Resolution factor (Rf)	Not less Than 2	9.934	
Number Of Theoretical plates (N)	Not less Than 2000	3891	4409
Tailing factor (T)	Not More Than 2	1.243	1.390

* Mean of six injections

In order to test the applicability of this method developed to a commercial formulation, 'Oxerute CD' was chromatographed at working concentration equivalent to standard working and it is shown in **Figure 7.** The sample peaks were identified by comparing the relative retention times with the standard drugs mixture, **Fig: 6**. System suitability parameters were ideal for the chromatographed sample. Integration of separated peak area was done and each drug was determined by using the peak area-concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the two drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

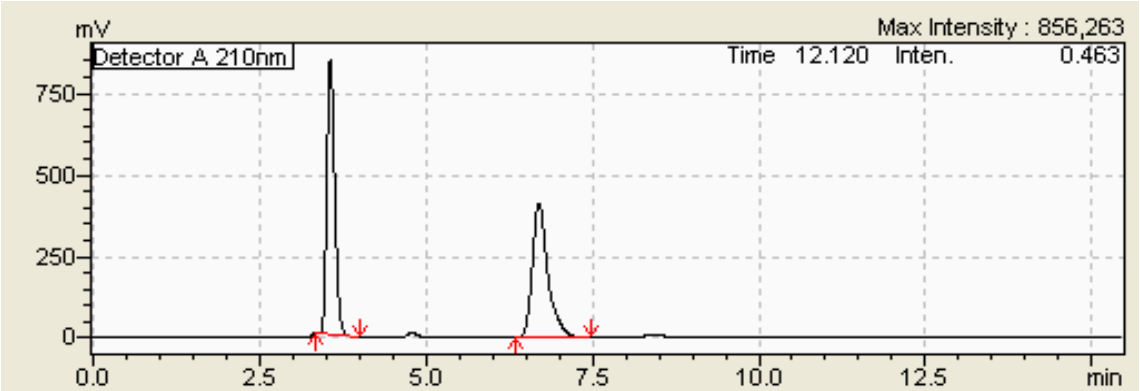

Fig. 7: Typical chromatogram of the sample (tablet).

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. The RP-HPLC method developed was validated according to International Conference on Harmonization guidelines [12] for validation of analytical procedures. The method was validated for the parameters in terms of system suitability, selectivity, linearity, accuracy, precision, ruggedness, robustness, limit of detection(LOD) and limit of quantitiation(LOQ).

Specificity

Figures 5-7 for blank, mixture of standard drug solution and sample chromatogram reveal that the peaks obtained in mixture of standard solution and sample solution at working concentrations are only because of the drugs as blank has no peaks at the retention times of Calcium dobesilate and Troxerutin. Accordingly it can be concluded that the method developed is said to be specific.

Precision

System precision

Six replicate injections of the mixture of standard solution at 100% target concentration showed % RSD (Relative Standard Deviation) less than 2 concerning peak areas for both the drugs, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in **Table 2**.

Method precision

Method precision was determined by performing assay of sample under the tests of (i) repeatability (Intra day precision) and (ii) Intermediate precision (Inter day precision) during 3 consecutive days, by three different analysts at working concentration.

Repeatability (Intra day precision)

Six consecutive injections of the sample at working concentration showed % RSD less than 2 concerning % assay for both the drugs which indicate the method developed is method precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results (**Table 3)**.

Ruggedness (Intermediate Precision / Inter day precision/)

Six consecutive injections of the sample solution at working concentration on three consecutive days by three different analysts, showed % RSD less than 2 for % assay for both the drugs within and between days, which indicate the method developed is inter day precise / rugged (**Table 4)**.

Linearity

Standard solutions of Calcium dobesilate and Troxerutin at different concentrations level (50%,75%,100%,125%,150%,175 and 200%) were prepared in triplicate. Calibration curves were constructed by plotting the concentrations level versus corresponding mean peak area. The results show an excellent correlation exists between mean peak area and concentrations level of drugs within the concentration range (62.5-250 µg/ml) and the results are given in **Tables 5, 6 and Figures 8, 9.** The correlation coefficients of Troxerutin and Calcium dobesilate are greater than 0.999, which meet the method validation acceptance criteria and hence the method is said to be linear in the range of 62.5-250 µg/ml.

Accuracy

Accuracy was determined by means of recovery experiments, by addition of active drug to preanalyzed sample at different spiked levels (50-150%). At each level, three determinations were performed and results obtained. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay. The amounts recovered, values of percent mean recovery were calculated as shown in **Tables 7 and 8.** The accepted limits of mean recovery are 98%-102% and all observed data are within the required range that indicates good recovery values and hence the accuracy of the method developed.

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is concluded that the method is robust as it is found that the % RSD is less than 2 concerning % assay despite deliberate variations done concerning flow rate (± 0.2), pH (± 0.2) and % organic phase (± 5%).

Sensitivity

The sensitivity of measurement of Calcium dobesilate and Troxerutin by use of the proposed method was estimated in terms of the limit of quantitation(LOQ) and the limit of detection (LOD).The limit of detection (LOD) was obtained as 3 ng/ml for Calcium dobesilate and 0.5 µg/ml for Troxerutin. The limit of quantitation (LOQ) was obtained as 10 ng/ml for Calcium dobesilate and 1µg/ml for Troxerutin.

Table 2: System precision results

Injection number (n)	Troxerutin		Calcium dobesilate	
	Rt	Peak Area	Rt	Peak Area
1	3.562	8001223	6.691	9860074
2	3.563	8021325	6.691	9849584
3	3.572	8031452	6.685	9864785
4	3.564	8029635	6.695	9825689
5	3.561	7988458	6.673	9885984
6	3.579	8022694	6.691	9896598
Average		**8027464.5**		**9863785.667**
S.D.		**18602.5**		**25444.974**
% R.S.D.		**0.23**		**0.257**

Table 3: Intra day precision results

n	Troxerutin	Calcium dobesilate
	% Assay	% Assay
1	99.9	99.94
2	100.15	99.84
3	100.28	99.99
4	100.26	99.59
5	99.74	100.21
6	100.17	100.31
Average	**100.08**	**99.98**
S.D.	**0.216**	**0.258**
% R.S.D.	**0.215**	**0.258**

Table 4: Inter day precision results.

n	% Assay Troxerutin			% Assay Calcium dobesilate		
	Day 1	Day 2	Day 3	Day 1	Day2	Day 3
1	99.9	99.15	99.18	99.94	99.20	98.85
2	100.15	99.82	99.35	99.84	99.03	99.06
3	100.28	99.01	99.02	99.99	100	99.95
4	100.26	101.3	99.75	99.59	100.5	100..6
5	99.74	99.50	101	100.2	99.8	101.5
6	100.17	99.44	99.89	100.3	99.3	100.6
Average	**100.08**	**99.7**	**99.69**	**99.98**	**99.64**	**99.99**
S.D.	**0.216**	**0.83**	**0.72**	**0.258**	**0.56**	**1.1**
% R.S.D.	**0.215**	**0.83**	**0.72**	**0.258**	**0.56**	**1.1**

Table 5: Calibration data for Calcium dobesilate and Troxerutin

% Level	% Concentration (µg/ml)	Peak Area	
		Troxerutin	Calcium dobesilate
50	62.5	4065947	4089625
75	93.75	6069842	6073297
100	125	8008745	8025357
125	156.25	10058736	9865242
150	187.5	12052551	11568745
175	218.75	13923456	13468745
200	250	15893956	14995956

Table 6: Linearity of the chromatography system

Drugs	Linearity range (µg/ml)	R^2	Slope	Intercept
Calcium dobesilate	62.5 – 250	0.9991	76085.01	239447.02
Troxerutin	62.5 – 250	0.9998	79423.223	72239.07

Table 7: Results of Accuracy studies for Calcium dobesilate

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Mean recovery
50	62.5	62.76	100.42
100	125	125.3	100.24
150	187.5	185.43	98.9

*Mean of three replicates

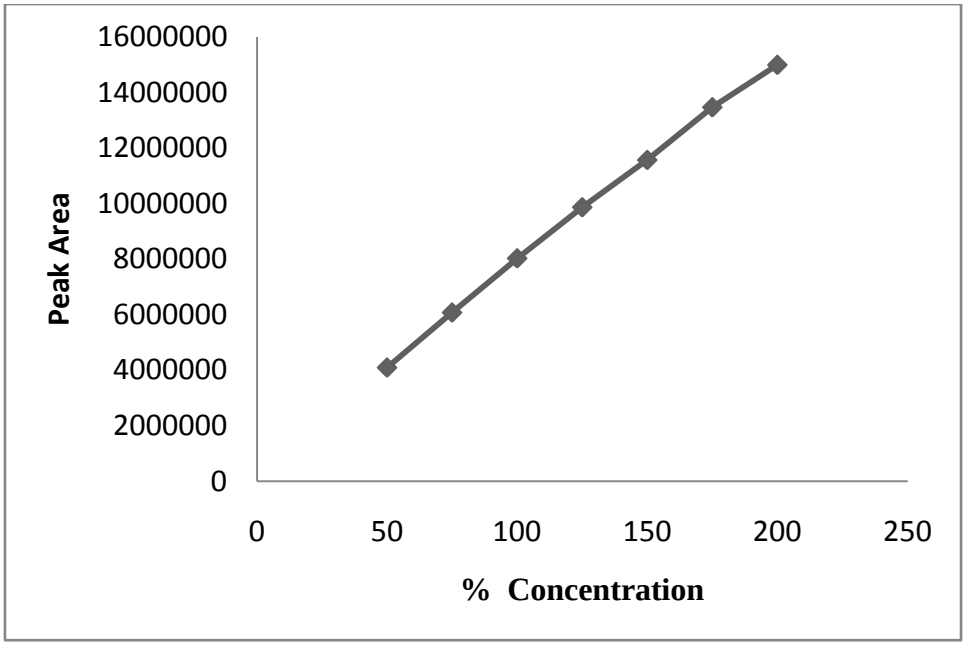

Fig. 8: Calibration curve for Calcium dobesilate.

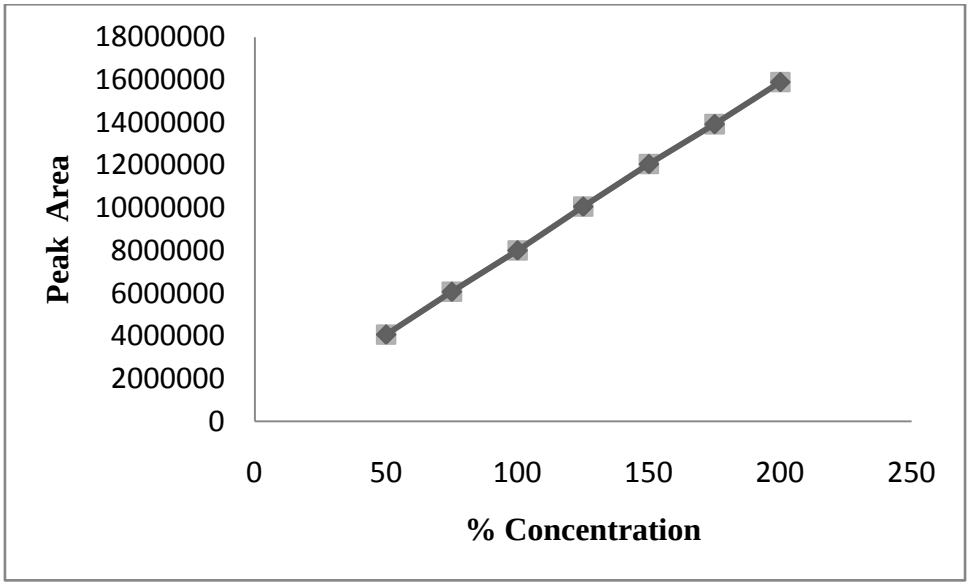

Fig. 9: Calibration curve for Troxerutin.

Table 8: Results of Accuracy studies for Troxerutin

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Mean recovery
50	62.5	63.36	101.38
100	125	125.1	100.08
150	187.5	191.25	101

*Mean of three replicates

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, ruggedness, robustness, limit of detection and limit of quantitiation, for the simultaneous quantitative estimation of calcium dobesilate and troxerutin in tablets. A good linear relationship was observed for both the drugs between concentration ranges of 62.5 and 250 µg/ml. The correlation coefficients were greater than 0.999 for both the drugs. The inter day and intraday precision results were good enough to say that the method developed is precise and reproducible. Accuracy studies revealed that mean recoveries after spiking experiments were between 98 and 102%, an indicative of accurate method. Accordingly it can be concluded that the developed reverse phase isocratic HPLC method is accurate, precise, linear, rugged and robust and therefore the

method can be used for the routine analysis of calcium dobesilate and troxerutin in tablets.

ACKNOWLEDGEMENT

The authors would like to thank the management of Vijaya college of pharmacy (VJYH), Hyderabad, for providing the necessary facilities to carry out of this research work. The authors are grateful to Biophore pharma and Chandra labs, Hyderabad for providing gift samples of Calcium dobesilate and Troxerutin.

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