<u>Research Article</u>

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

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ABSTRACT

Objective: To develop a new, simple, precise, accurate, linear and rapid Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous quantitative estimation of Calcium dobesilate 500 mg and Troxerutin500 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 methanol:0.02M potassium dihydrogen orthophosphate buffer adjusted to pH 4 in the proportion of 50:50 v/v, flow rate of 0.8 ml/min and a detection wavelength of 210 nm using a UV detector.

Results: The developed method resulted in troxerutin eluting at 7.06 min and calcium dobesilate at 3.5 min. The method precision is exemplified by relative standard deviations of 0.7% for troxerutin and 0.71% for calcium dobesilate. The linearity of the method was over the range 62.5-250μg/ml for both the drugs. Accuracy studies revealed % mean recoveries between 98 and 102 during spiking experiments. The limit of detection was obtained as 3 ng/ml for Calcium dobesilate and 0.5μg/ml for Troxerutin, while the limit of quantitation was obtained as 8ng/ml for Calcium dobesilate and 1μg/ml for Troxerutin.

Conclusion: A new simple, precise, accurate, linear and rapid RP-HPLC method has been developed and validated for the simultaneous quantitative estimation of Calcium dobesilate and Troxerutin 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

Calcium dobesilate (Figure 1, calcium 2,5-dihydroxybenzenesulfonate) is a drug used for the treatment of diabetic retinopathy and chronic venous insufficiency. Calcium dobesilate shows anti-platelet and fibrinolytic activities by inhibiting platelet activation factor (PAF) and enhancing the release of tissue plasminogen activator (tPA) and acts selectively on the capillary walls regulating their physiological functions of resistance and permeability [1-3]. Troxerutin (Figure2, Trihydroxyethylrutin; 3',4',7-Tris[O-(2-hydroxyethyl)]rutin) is a flavonol, a hydroxyethylrutoside isolated from Sophorajaponica, the japanese pagoda tree. Troxerutin helps treat venous disorders such as varicose veins in a number of different ways. It helps to stabilize cell walls, making them less permeable, so that fluids are passed between cells and blood cells at normal levels. This drug is used as a vasoprotective agent prescribed for circulatory disorders. Troxerutin is used as an anti-clotting drug for the treatment of hemiplegia, aphasia, cardiac stem, arteriosclerosis etc.[4-5].
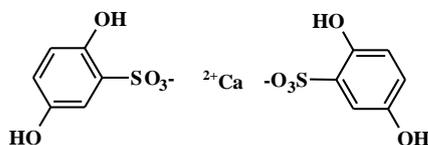

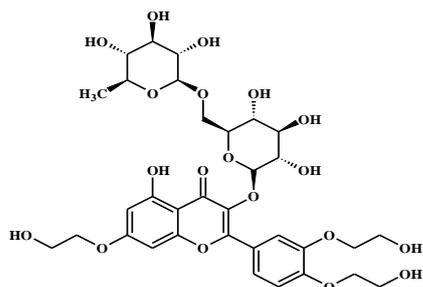**Fig. 1: Structure of Calcium dobesilate**

Fig. 2: Structure of Troxerutin

A detailed literature survey reveals that RP-HPLC/UV methods have been reported for the quantitative estimation of troxerutin[6-7] and calcium dobesilate[8] individually in various matrix such as human plasma and pharmaceutical dosage forms. RP-HPLC methods are also reported for troxerutin in combination with other drugs in rat urine, chicken plasma and food supplements[9-10] and similarly calcium dobesilate with other drugs in suppositories and in ointment[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. Hence we here report a new, 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 98% were obtained as gift samples from Chandra labs (Hyderabad, India) and tablet formulation [OXERUTECD (Brand name)] was procured from Apollo pharmacy, Hyderabad, India with labelled amount 500mg each of calcium dobesilate and troxerutin. Methanol(HPLC grade) was obtained from Sigma Aldrich (Hyderabad, India), Water (HPLC grade), Potassium dihydrogenorthophosphate (AR grade) and ortho 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-20ADpump, 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.
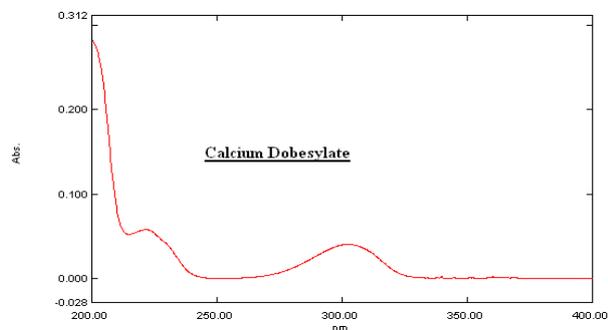
Method

Fig. 3: 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 methanol:potassium dihydrogen ortho phosphate buffer (20mM, pH adjusted to 4 using 30% v/v orthophosphoric acid) in ratio of 50:50, v/v. The mobile phase was set at a flow rate of 0.8 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 was 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 methanol and buffer in the ratio of 50:50, v/v and later sonicated for 10 minutes for the removal of air bubbles.

Preparation of stock and working standard solution

50mg of Calcium dobesilate and 50mg of Troxerutin were accurately weighed and taken in 100ml 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 125µg/ml, treated as working standard solution, 100% target concentration.
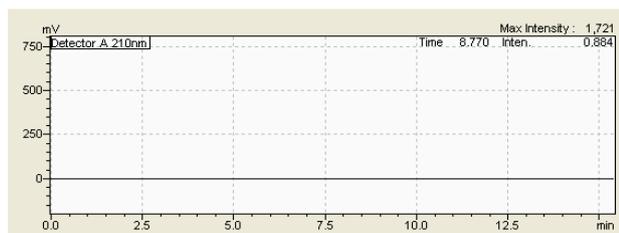
Selection of Wavelength

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**).
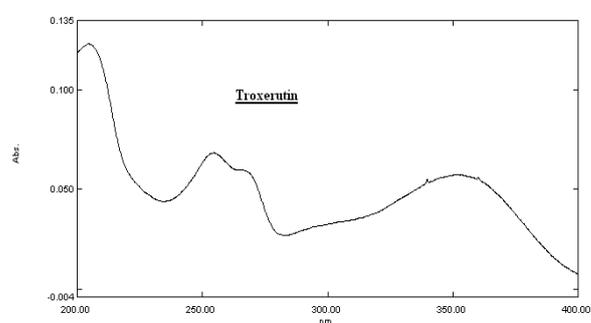

Fig. 4: UV spectrum of Troxerutin

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 1000ml 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 125µg/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 7.06 min. **Figure 5** and **Figure 6** represent chromatograms of blank solution and mixture of standard solutions respectively. The total run time is 10 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.
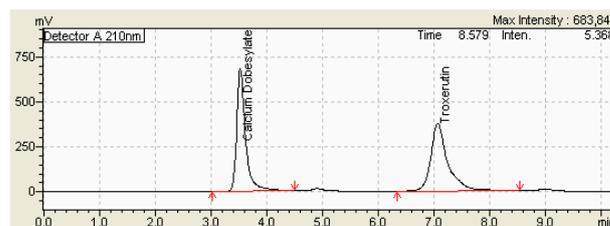

Fig. 5: Typical Chromatogram of Blank solution

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

Table 1: System suitability studies results.

Parameters*	Required Limits	Calcium dobesilate	Troxerutin
Retention time (min)	% RSD < 1	3.56	7.06
Resolution factor (Rf)	Not less Than 2	9.385	
Number Of Theoretical plates (N)	Not less Than 2000	2690	3410
Tailing factor(T)	Not More Than 2	1.550	1.428

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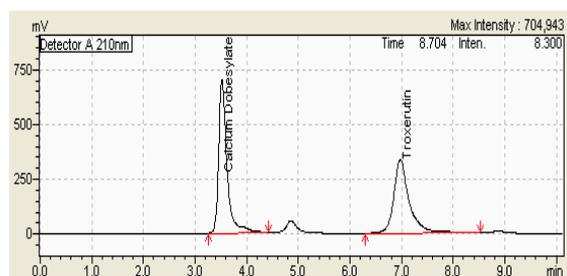
Fig. 7: Typical chromatogram of the sample (tablet).

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. 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, specificity, 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**.

Table 2: System precision results

Injection number (n)	Troxerutin		Calcium dobesilate	
	Rt	Peak Area	Rt	Peak Area
1	3.56	8001223	7.06	8030074
2	3.56	8101325	7.05	8039584
3	3.57	8101452	7.08	8134785
4	3.56	8089635	7.06	8045689
5	3.56	8008458	7.09	8165984
6	3.57	8142694	7.06	8046598
Average		**8074131.16**		**8077119**
S.D.		**56666.97**		**56903.09**
% R.S.D.		**0.7**		**0.71**

Table 3: Intra day precision results

n	Troxerutin	Calcium dobesilate
	% Assay	% Assay
1	98.37	99.36
2	99.36	98.56
3	98.45	99.14
4	99.54	98.48
5	98.35	99.78
6	100.54	100.23
Average	99.1	99.258
S.D.	0.877	0.68
% R.S.D.	0.88	0.68

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 (50,75,100,125,150,175,200 and 250) were prepared in triplicate. Calibration curves were constructed by plotting the concentrations level versus corresponding mean peak area. The results show an excellent correlation between mean peak area and concentrations level of both the 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 for both the drugs.

Table 4: Inter day precision results.

n	% Assay Troxerutin			% Assay Calcium dobesilate		
	Day 1	Day 2	Day 3	Day 1	Day2	Day 3
1	100.37	98.22	98.12	98.37	99.4	98.37
2	99.36	100.1	99.24	99.52	100.2	99.36
3	99.45	99.23	99.42	98.31	100.5	100.1
4	98.54	99.32	99.12	100.4	98.65	101.2
5	99.35	98.35	98.24	100.5	99.22	98.53
6	100.54	100.8	99.5	100.1	100.5	99.89
Average	**99.6**	**99.33**	**98.83**	**99.53**	**99.74**	**99.57**
S.D.	**0.74**	**0.99**	**0.5**	**0.98**	**0.76**	**1.06**
% R.S.D.	**0.74**	**0.99**	**0.5**	**0.98**	**0.76**	**1.06**

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

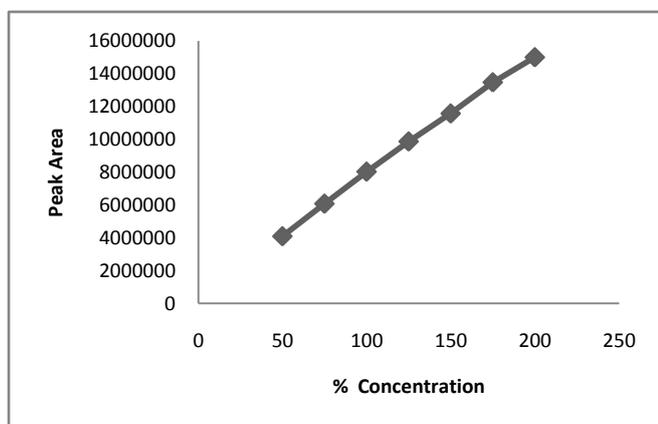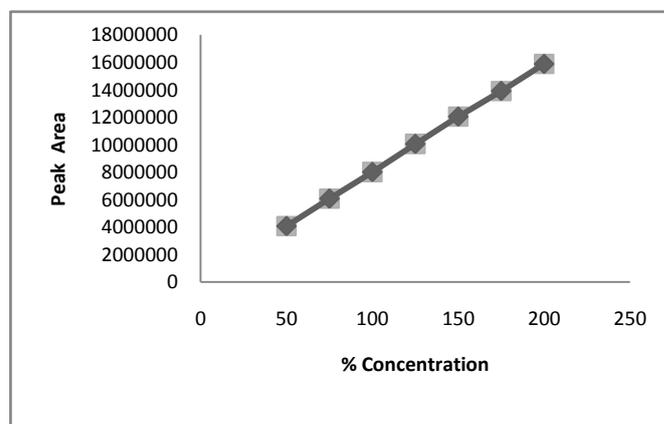| Drugs	Linearity range (µg/ml)	R^2	Slope	Intercept
Calcium dobesilate	62.5 – 250	0.9991	76085.01	239447.02
Troxerutin	62.5 – 250	0.9998	79423.223	72239.07

Fig.8: Calibration curve for Calcium dobesilate

Fig.9: Calibration curve for Troxerutin

Table 7: Results of Accuracy studies for Calcium dobesilate

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	62.5	61.7	98.72
100	125	124.1	99.28
150	187.5	186.2	99.3

*Mean of three replicates

Table 8: Results of Accuracy studies for Troxerutin

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	62.5	62.3	99.68
100	125	124.1	99.28
150	187.5	188.2	100.37

*Mean of three replicates

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 3ng/ml for Calcium dobesilate and 0.5 µg/ml for Troxerutin. The limit of quantitation (LOQ) was obtained as 8ng/ml for Calcium dobesilate and 1µg/ml for Troxerutin.

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

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