ORIGINAL ARTICLE

Stability Indicating Analytical Method Development and Validation for the Estimation of Calcium Dobesilate in Bulk Drug Using RP-HPLC

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Abstract

Introduction: The aim of the study is to develop a simple, precise, accurate, linear, and rapid stability-indicating method for the separation and identification of degradation products and mass studies of calcium dobesilate (CAD) drugs used in the treatment of diabetic retinopathy and chronic kidney disease as per the International Conference on Harmonization's (ICH) guidelines. **Materials and Methods:** The technique makes use of an isocratic mode, an Enable C18 Kromasil (250 × 4.6 mm; 5µ) column, and a reverse phase column. The optimal parameters for the method are the mobile phase of acetonitrile (0.1%), OPA in water (20:80), a flow rate of 1 mL/min, and a UV detector with a detection wavelength of 310 nm. **Results and Discussion:** Degradation was carried out and CAD was sensitive to base conditions. Structure elucidation of the forced degradation products was observed using liquid chromatography-mass spectrometry. CAD was found to be stable under thermal, photolytic, acid, and peroxide conditions. Two degradation products were reported, but we did not get the m/z of degradation products of CAD. We report these degradation products as unknown impurities. The suggested procedure was validated in accordance with ICH Q2 (R1) standards. Accuracy studies were observed that the percentage recovery was found to be 98.0–102.0% and the linearity of the method was excellent over the range of 80–120 µg/mL. Relative standard deviations of 0.612% demonstrate the precision. The results showed that the limits of detection and quantitation were, respectively, 0.477 and 1.445 ug/mL. **Conclusion:** According to validation and degradation results, the method was found to have good stability, indicating its nature as well as being user-friendly.

Key words: Calcium dobesilate, forced degradation studies, HPLC, LC-MS, validation

INTRODUCTION

The molecular formula for calcium dobesilate (CAD) is $C_{12}H_{10}CaO_{10}S_2$. Dexium, dobesilate calcium, and calcium 2,5-dihydroxybenzenesulfonate are synonyms for CAD.[1,2] The IUPAC name of CAD is 2,5-dihydroxybenzene sulfonate [Figure 1].

Vasoprotective agents include CAD. It is dobesilic acid's calcium salt. It is a synthetic chemical that can lower the body's capillary permeability. The compound is a powder that is white and hygroscopic. It is completely insoluble in dichloromethane and poorly soluble in isopropyl alcohol, although it is highly soluble in water and readily soluble in alcohol.[3] The drug was recorded in the European Pharmacopoeia in 1997 and in the British Pharmacopoeia in 1998. It is currently used mainly in the treatment of microvascular-related diseases such as diabetic retinopathy and chronic kidney disease. As a medication for angioprotection, CAD is known to lessen hemorrhage and microcirculatory irregularities, as well as blood viscosity, platelet activity, and capillary permeability. Experiments *in vitro* strongly demonstrated that the antioxidant properties of CAD play an important role in microvascular injuries through its direct scavenging action by eliminating reactive oxygen species.[4] CAD is available in the following dosage forms: tablets, capsules, and ointments.[5]

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A thorough review of the literature reveals that CAD has been quantitatively estimated using RP-HPLC/UV techniques in a variety of matrixes, including human plasma and drug dosage forms.[6]

RP-HPLC methods are also reported for calcium dobesilate with other drugs in suppositories, ointments[7] and tablet dosage forms.[8,9]

As per our detailed literature survey as of date, there are no RP-HPLC methods reported for the CAD in APIs or bulk drugs, and no mass studies have been conducted on the degradation products of CAD. Thus, in accordance with the International Conference on Harmonization's (ICH) guidelines,[10] we present here a novel, straightforward, sensitive, quick, accurate, precise, and linear RP-HPLC isocratic method for estimating CAD in API.

MATERIAL AND METHODS

Experimental

Experimental work was performed on a UV-visible spectrophotometer, which was a double-beam UV-visible spectrophotometer model no. UV-550, Maker Jasco, equipped with Spectra Manager software. The HPLC system Quaternary Low-Pressure Gradient System Agilent, Model No. 1260 Infinity II, Software-Openlab EZ Chrome workstation, with a Rheodyne Loop Injector (7725 i) fitted with a 20 mL sample loop. Liquid chromatography–mass spectrometry studies were carried out on the Thermo Fisher Lab Discovery quantum max LC system (Thermo Fisher Ltd., India), comprised of a 410 auto-sampler and a 500 MS ion trap detector operated in the range of mass-to-charge ratios (m/z) of 50–2000. The whole system was operated using LC-Quan Quadra Pole technique chromatography software. For weighing Aczet CY224C with a range of 10 mg to 220 g, an analytical balance was used. For smooth dissolution, a bio-technic 13.5-l ultrasonicator was used.

Chemicals and reagents

CAD was purchased from provided by Thermo fisher Pvt. Ltd. (Aurangabad, India) from the local market. Methanol, water, and acetonitrile of HPLC grade were purchased from Merck (Mumbai, India). Ortho-phosphoric acid (AR grade) was obtained from Merck (Mumbai, India). Ultra-pure deionized water (HPLC grade) was obtained from Siddhi Lab.

Methods

Before method development is taken up, it is generally important to know various physicochemical properties like solubility, absorptivity, and wavelength maximum of the drug.[11]

Selection of wavelength

Water was used as a diluent for preparing the stock solution; the standard solution was prepared by Weighed 10 mg CAD and dissolved in 20 mL of water (500 PPM of CAD) out of that 20 ppm solution. The standard solution was scanned from 400 nm to 200 nm. CAD showed maximum absorbance at 300 and 220 nm. 300 nm is considered as an analytical wavelength for further determination [Figure 2].

Confirmation of solvent suitability for CAD

%RSD was not more than 2.0, and the correlation coefficient was found to be well within the acceptance limit (NLT=0.98). Hence, linearity meets acceptance criteria for water as a diluent.

Chromatographic conditions

The chromatographic separation was carried out on a reversed-phase Phenomenex; chrom-clone C18 column (250 mm × 4.6 mm i.d., 5 µm) using isocratic elution with a mobile phase consisted of acetonitrile: 0.1% OPA in water (10:90) pumped at a flow rate of 1.0 mL/min. Water was used as a diluent, CAD was eluted at 3.75 minutes with acceptable chromatography (asymmetry = 1.24 and theoretical plates = 12634). No hump was found near the main peak. The column oven temperature was kept at 35°C. For each sample,

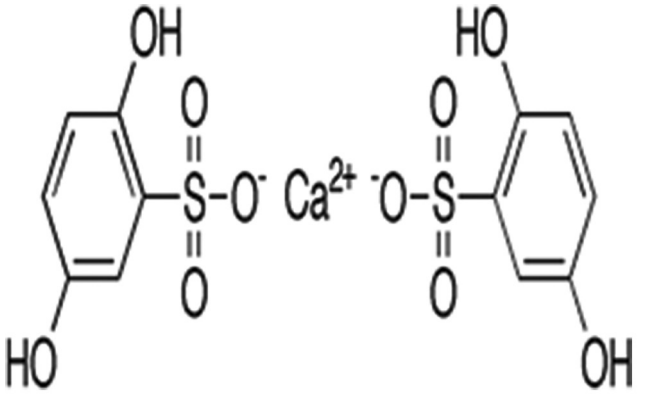

Figure 1: Structure of calcium dobesilate

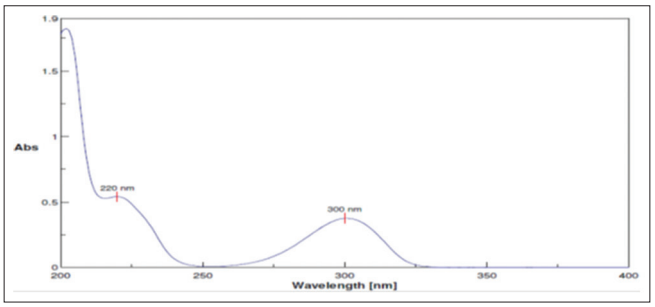

Figure 2: UV spectrum of calcium dobesilate

20 µL was injected. The wavelength of the detector was set at 321.0 nm. Ten trials were conducted, out of which the best acceptable chromatogram was selected for force degradation and validation [Figure 3].

Forced degradation studies

Forced degradation studies were carried out as per ICH guidelines[10] under different acid, base, oxidative, photolytic, and thermal stresses. Normally, for degradation, the concentration of API to inject on HPLC is selected at 0.1 mg/mL (100 ppm). Acid and base hydrolysis were investigated by dissolving a mass of 20 mg CAD in different concentrations of hydrochloric acid or sodium hydroxide (0.01N, 0.1N, 0.5N) and keeping the sample on the bench for 24 h. After 24 h, the reaction was neutralized by adding 2 mL of 5 N NaOH or 5 N HCl solutions. Volume made up to the mark with water. For oxidative studies, a mass of 20 mg CAD was transferred to a 20-mL volumetric flask. Added 15 mL of water and sonicated to dissolve the API completely. Added 2 mL of 30% hydrogen peroxide and kept the sample on the bench for 24 h. After 24 h, Volume made up to the mark with water. Further, it is diluted with 1 mL of stock solution to 10 mL with the mobile phase. For thermal stress testing Placed Sufficient amount of API was placed in a petri dish and covered with aluminum foil, and holes were made on the aluminum foil with pointed objects. It is then kept in a hot air oven at 60°C for 72 h. After 72 h, the sample was taken out and kept in the desiccator to reach R.T. The subject API was prepared as per sample preparation method. The treated API sample was transferred to a 20-mL volumetric flask. Added 15 mL of water and sonicated to dissolve the API completely. Volume made up to the mark with water. Further, it was diluted with 1 mL of stock solution to 10 mL with the mobile phase. Lastly, photostability was investigated by placed a sufficient amount of API in a petri dish and covered it with aluminum foil and made holes in the aluminum foil with a pointed object. Then it was kept in the sunlight for 7 days. After 7 days, the sample was taken out and kept on the benchtop to reach R.T. The subject API was prepared as per sample preparation method. The treated API sample was transferred to a 20-mL volumetric flask. Added 15 mL of water and sonicated to dissolve the API completely. Volume made up to the mark with water. In addition, 1 mL of the stock solution is diluted with 10 mL of mobile phase.

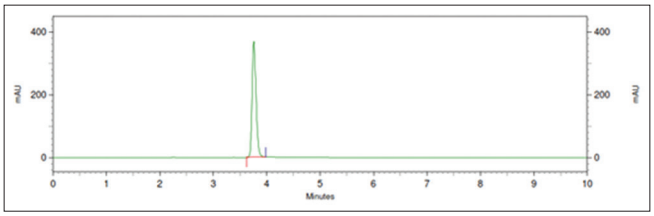

Figure 3: Optimized chromatogram of calcium dobesilate

RESULTS AND DISCUSSION

Degradation of CAD in various stress conditions

According to ICH guidelines,[10] CAD was exposed to various stress conditions, including acid, base hydrolysis, peroxide, thermal, and photolytic [Table 1]. For the acid stress condition, three trials were conducted in which CAD was treated with 5N HCl and kept for 24 h. The percentage degradation was 0.64%, 1.04%, and 0.37% [Figures 4-6]. For the peroxide stress condition, when the sample was treated with 30% hydrogen peroxide and kept for 24 h, the percentage degradation was 0.76%; in trial 2, the sample treated with 30% hydrogen peroxide and heated at 80°C for 24 h on a water bath; the percentage degradation was 1.25%; and in trial 3, the sample treated with 30% hydrogen peroxide and heated at 80°C for 48 h on a water bath; the percentage degradation was 0.95% [Figures 7-9]. For thermal stress conditions placed a sufficient amount of API in a petri dish and covered it with aluminum foil and made holes in the aluminum foil with a pointed object. Kept it in a hot air oven at 60°C for 72 h. The percentage degradation was 0.35% [Figure 10], and for the photolytic stress condition placed a sufficient amount of API in a petri dish, covered with aluminum foil, and made holes in the aluminum foil with a pointed object. Kept in the sunlight for 7 days, the percentage degradation was 0.51% [Figure 11], hence, up to 2% degradation. In general, it is not considered as degradation as it is variation due to a number of factors such as analysis, sample preparation, and instrument variation.[11] For the base stress condition, four trials were conducted: in trial 1, the API sample was treated with 5 N NaOH; percentage degradation was 100% [Figure 12]; in trial 2, the API sample was treated with 0.1 N NaOH; percentage degradation was 65.61% [Figure 13]; in trail 3, the API sample was treated with 0.4 mL of 0.1 N NaOH for 2 min at R.T; percentage degradation was 31.63% [Figure 14]; in trial 4, the API sample was treated with 0.4 mL of 0.01 N NaOH for 5 min at R.T.; percentage degradation was 9.97% observed [Figure 15]. At this condition, two degradation products were observed at R.T. 2.54 and 3.20 [Table 2a and b].

The percentage degradation was calculated by the formula:

$$\%\ Assay\ of\ \ \text{deg}raded\ sample = \frac{FD\ sample\ area}{Sample\ as\ such\ Area} \times \frac{Sample\ as\ such\ wt\ \ (mg)}{FD\ Sample\ wt\ \ (mg)} \times 100$$

Hence, CAD was susceptible to degradation under base stress conditions while stable to acid, peroxide, thermal, and photolytic stress conditions. All induced degradation products were subjected to mass spectral analysis for subsequent identification. Mass spectra for the base-induced degradation products showed when we injected FD samples on mass to check the m/z of the analyte and its degradation products. We did not get m/z of analyte and its degradation products

Table 1: Forced degradation summary

Sample name	Treatment	Exposure condition	% Assay	% Degradation
API	Sample as such	NA	100.00	NA
	Thermal	60°C for 72 h	100.35	Nil
	Photolytic	Sunlight for 7 days	99.49	Nil
	Acid	2 mL of 5 N HCl for 24 h at R.T.	100.64	Nil
		2 mL of 5 N HCl heated at 80°C for 24 h	98.96	Nil
		2 mL of 5 N HCl heated at 80°C for 48 h	99.63	Nil
	Base	2 mL of 5 N NaOH for 24 h at R.T.	0	100
		0.4 mL of 0.1 N NaOH for 15 min at R.T.	34.39	65.61
		0.4 mL of 0.1 N NaOH for 2 min at R.T.	68.37	31.63
		0.4 mL of 0.01 N NaOH for 5 min at R.T.	90.03	9.97
	Peroxide	2 mL of 30% H2O2 for 24 h at R.T.	99.24	Nil
		2 mL of 30% H2O2 heated at 80°C for 24 h	98.75	Nil
		2 mL of 30% H2O2 heated at 80°C for 48 h	99.05	Nil

Up to 2% degradation. Generally it is not considered as degradation as it is variation due to number of factors like analyst, sample preparation, instrument variation etc.

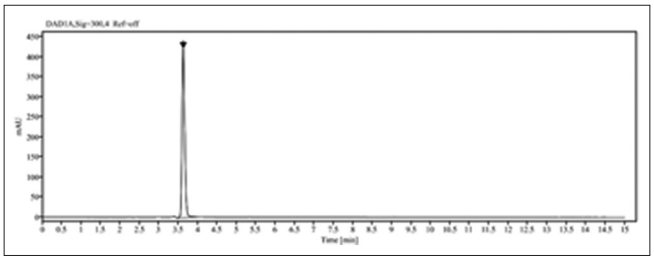

Figure 4: Acid stress condition Trial no.1 API sample treated with 2 mL of 5 N HCl for 24 h at R.T

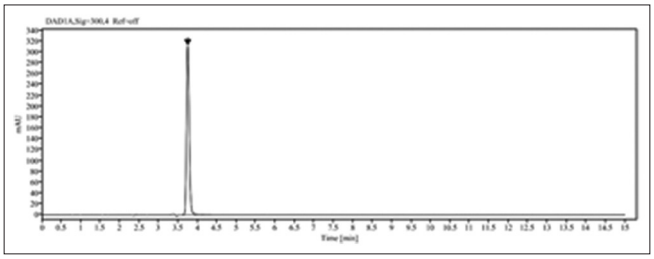

Figure 5: Acid stress condition trial no, 2 API sample treated with 2 mL of 5 N HCL treated at 80°C for 24 h

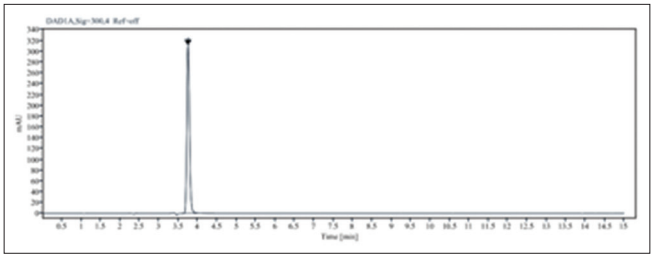

Figure 6: Acid stress condition trial no, 3 API sample treated with 2 mL of 5 N HCl treated at 80°C for 48 h

as analyte and its degradation products did not get ionized on mass (highly unionizable species) [Figure 16].

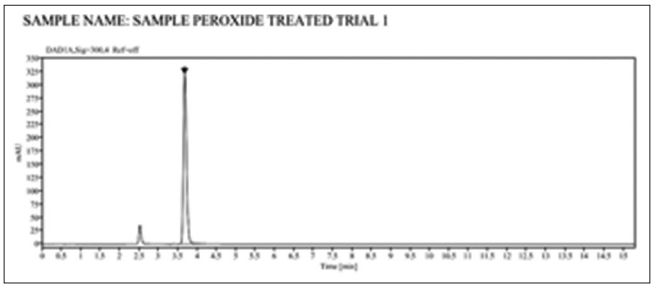

Figure 7: Peroxide stress condition Trial no. 1API treated with 2 mL of 30% H2O2 for 24 h at R.T

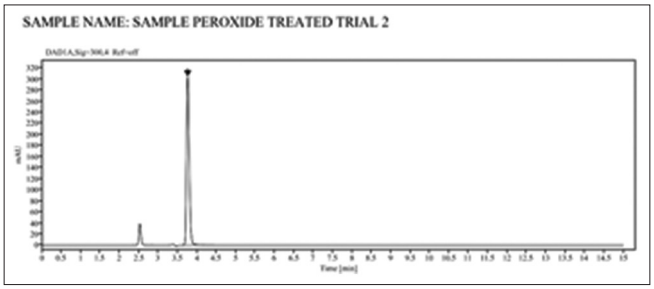

Figure 8: Peroxide stress condition trial no. 2 API treated with 2 mL of 30% H2O2 heated at 80°C for 24 h at R.T

Trial (1) When we injected 1000 ppm of CAD stock solution on mass, we did not get its m/z.

Trial (2) When we injected 5000ppm of CAD stock solution on mass, we did get m/z at 419.47.

Reporting of degradation products

As we did not get the m/z of degradation products of CAD, we report these degradation products as unknown impurities.

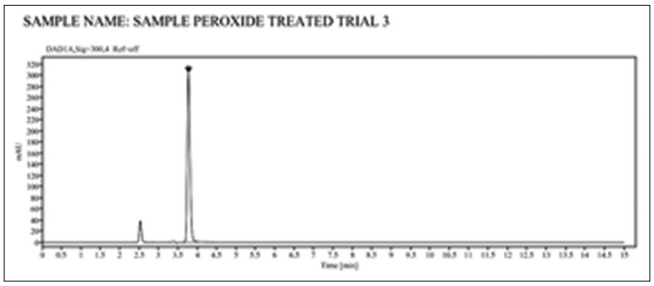

Figure 9: Peroxide stress condition Trial no. 3API treated with 2 mL of 30% H2O2 heated at80°C for 48 h at R.T

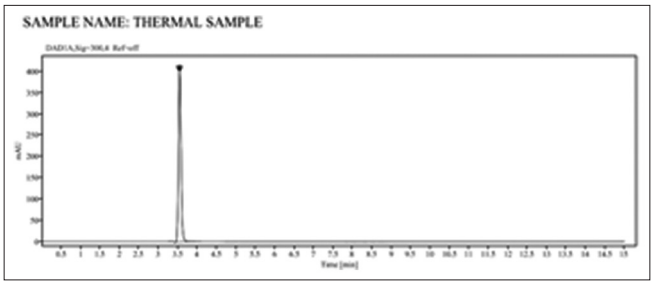

Figure 10: Thermal Stress condition

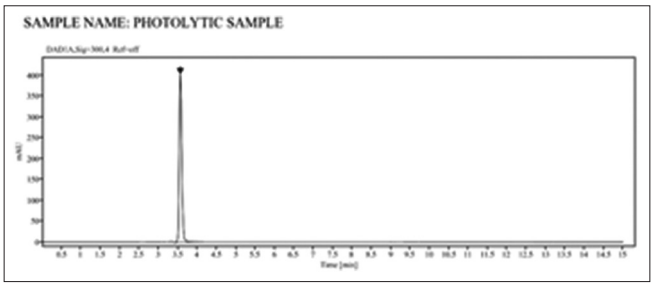

Figure 11: Photolytic stress condition

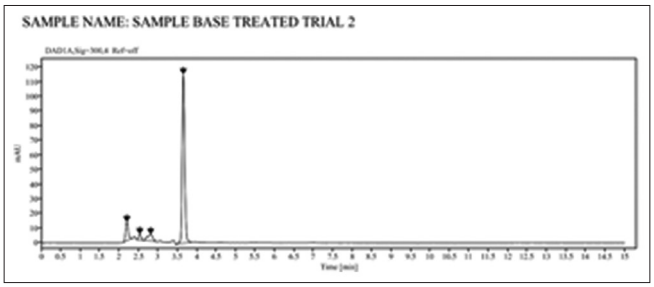

Figure 12: Base stress condition Trial no. 1 API treated with 2 mL of 5 N NaOH for 24 h at R.T

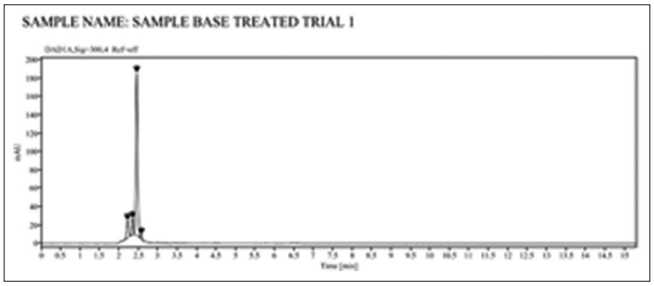

Figure 13: Base stress condition trial no. 2 API treated with 0.4 mL of 0.1 N NaOH for 15 min at R.T

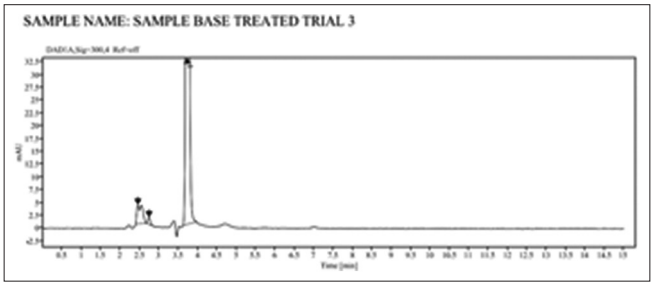

Figure 14: Base stress condition trial no. 3API treated with 0.4 mL of 0.1 N NaOH for 2 min at R.T

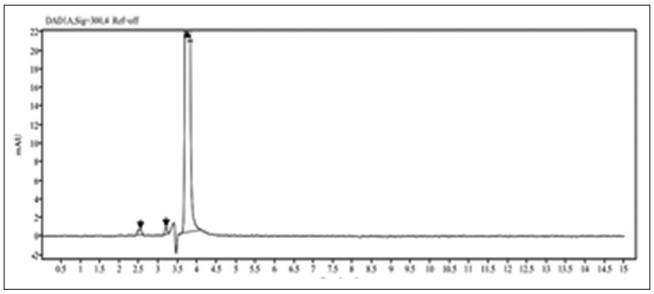

Figure 15: Base stress condition trial no. 4API treated with 0.4 mL of 0.01 N NaOH for 5 min at R.T

HPLC chromatograms of acid stress conditions

Calcium dobesilate was treated with 5N HCl and kept for 24 h percentage degradation was 0.64%, 1.04% and 0.37% [Figures 4-6].

HPLC chromatograms of peroxide stress conditions

When sample treated with 30% hydrogen peroxide and kept for 24 h, heated at 80°C for 24 h and heated at 80°C for 48 h on water bath percentage degradation was 0.76%, 1.25% and 0.95% [Figures 7-9].

HPLC chromatograms of thermal stress conditions

Kept it is in hot air oven at 60ºC for 72 h. Percentage degradation was 0.35% [Figure 10].

HPLC chromatograms of photolytic stress conditions

API in petri dish and covered with aluminium foil and made holes on aluminium foil with pointed object Kept in sun light for 7 days percentage degradation was 0.51% [Figure 11]. Generally, up to 2% degradation is not considered as degradation.

HPLC chromatograms of base stress conditions

Under the influence of 0.01N NaOH for 5 min at R.T. the drug show degradation.

Table 2a: Degradation product summary

Condition	Optimized trial no.	Treatment	% Assay	% Degradation	Analytes	RT	M/Z
Base treated	Trial no. 4	0.4 mL of 0.01 N NaOH (2% of total volume) B.T. for 5 minutes	90.03	9.97	Calcium Dobesilate	3.74	419.47
					DP-1	2.54	Unknown
					DP-2	3.20	Unknown

Table 2b: Degradation product summary

Sample Name	RT	RRT	Area	Purity
Base DP_1	2.54	0.68	21	986
Base DP_2	3.20	0.86	10	992
API	3.74	1.00	1334	999
		Sum	1365	

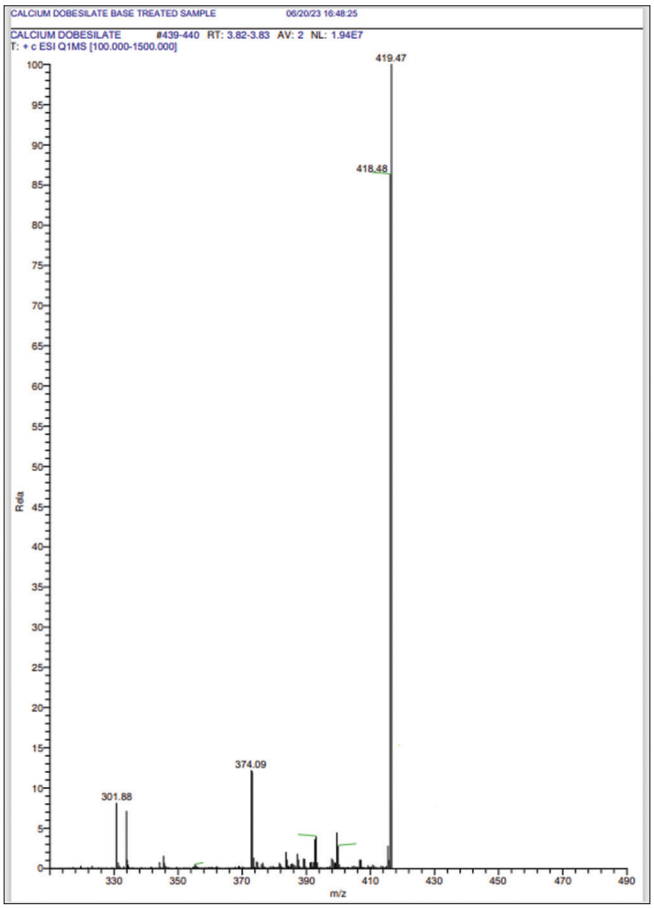

Figure 16: Mass spectra of FD sample

Analysis of FD samples on mass to check m/z

The API and its degradation products did not ionize on mass, so we were unable to obtain the m/z of the analyte and its degradation products (highly unionizable species) [Figure 16].

Method validation

The ICH (Q2) R1 criteria[10] for the validation of analytical techniques were followed in the development of the RP-HPLC method. System appropriateness, linearity, accuracy, precision, robustness, ruggedness, robustness, limit of detection (LOD), and limit of quantitation (LOQ) were the parameters.

The chromatographic conditions maintained during validation were carried out on a reversed-phase Phenomenex chrom-clone C18 column (250 mm × 4.6 mm i.d., 5 µm) using isocratic elution with a mobile phase consisting of acetonitrile. 0.1% OPA in water (10:90) pumped at a flow rate of 1.0 mL/min. Water was used as a diluent. The column oven temperature was kept at 35°C. For each sample, 20 µL was injected. The wavelength of the detector was set at 321.0 nm.

System suitability

Weighed 20.0 mg of CAD API and transferred it to a 20-mL volumetric flask. Further diluted 2 mL of stock solution to 20 mL with mobile phase (about 100 ppm of CAD).

The % RSD for the area of 5 replicates of the standard solution was not more than 2.0, the theoretical plate was not <2000, and the asymmetry was not <2.0, hence the system suitability passed the acceptance criteria [Table 3].

Solution stability

Standard solution initially at 12 h and 24 h; percent absolute difference calculated with respect to the initial area.

The % absolute difference was not more than 2.0; hence, the standard solution was found stable for 24 h, and hence, the prepared solution can be used up to 24 h [Table 4].

Specificity

The standard solution passed its peak purity after the blank and standard solutions were injected. Blank is not having interference at the R.T. of CAD. Hence, the developed chromatographic method passed the criteria for specificity. In accordance with ICH criteria,[10] it was determined that the established procedure was considered specific.

Linearity

5 levels were prepared, ranging from 80% to 120% of working concentration. Each level is injected in triplicate. The linearity graph was plotted by concentration level versus mean peak area and calculated intercept, slope, and regression coefficient. The findings, which are displayed in [Table 5 and Figure 17], demonstrate a strong association

Table 3: System suitability observation summary

S. No.	Standard solution	Area	Asymmetry	Theoretical plates
1	Standard_1	31471290	1.25	12667
2	Standard_2	31452501	1.24	12661
3	Standard_3	31497517	1.25	12654
4	Standard_4	31430060	1.25	12673
5	Standard_5	31469824	1.24	12648
Mean		31464238	1.25	12661
STD Dev		24971.5778		
% RSD		0.079		

Table 4: Solution stability of calcium dobesilate observation summary

Standard solution		
Time point	**Area**	**% Absolute difference**
Initial	31514073	NA
12 h	31460192	0.17
24 h	31419125	0.30

Table 5: Linearity range of calcium dobesilate observation summary

Level	Conc (µg/mL)	Area (mean)	SD	% RSD
80%	80	25374223	14459.603	0.057
90%	90	28392076	20334.983	0.072
100%	100	31457710	23022.950	0.073
110%	110	34620470	16191.802	0.047
120%	120	37743470	22062.994	0.058

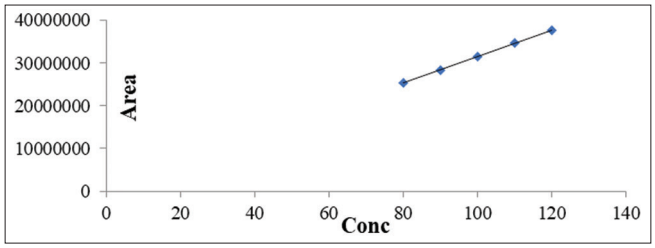

Figure 17: Calcium dobesilate linearity

between mean peak area and drug concentrations within the concentration range of 80–120 µg/mL. The correlation coefficients of CAD were < 0.9996, the intercept 550701.8, and the slope was 309668.88. Because the correlation the coefficients are above 0.98, the method is considered linear in the 80–120 µg/mL range and meets the acceptance criteria for method validation.

Accuracy

This is frequently referred to as trueness.[10] Recovery tests were used to measure accuracy, adding active medication to pre-analyzed samples at various spike amounts (50–150%). Three decisions were made, and outcomes were obtained at each stage. The percentage of the analyte recovered by the assay was used to compute the accuracy based on the test findings. Recovery was performed at three levels. 80%, 100%, and 120% levels are prepared. Practical were carried out in triplicate. Take a clean and dried 9 volumetric flask of 20 mL. Weigh the CAD API as per the accuracy level and transfer it into the same 20-mL volumetric flask. Add 15 mL of water and sonicate it for 10 min with intermittent shaking. Make the volume up to the mark with water. Further, dilute 2 mL to 20 mL with the mobile phase.

The amounts recovered and values of percent mean recovery were calculated as shown in [Table 6]. The accepted limits of mean recovery are 98–102%, and percent recovery was found to be well within the acceptance range at all three levels. Consequently, positive outcomes were attained.

LOD and LOQ

The LOD and LOQ were determined based on standard deviation of intercept and slope of the calibration curve [Table 7] by following equation

LOD = 3.3× Sigma/Slope so, LOD=0.447 µg/mL

LOQ = 10× Sigma/Slope so, LOQ=1.445 µg/mL

CORR. COEF. -0.99996, INTERCEPT-550701.8, SLOPE-309668.88

Precision (repeatability)

Precision performed by preparing 6 test sample, it was observed that % assay within 98–102% and % RSD not more than 2. Hence, precision passes the criteria, no variation found by preparing six different samples. Results are good reproducible [Table 8].

Intermediate precision

Six samples were prepared, and it was observed that the % assay was within 98% to 102% and the percent RSD was not

Table 6: Amount of recovery shown in the table observation summary

Level (%)	Area	Recovered conc	Added conc	% Recovery	Mean recovery	% RSD
80	25339510	80.534	81.500	98.81	99.56	0.900
	25309147	80.438	80.000	100.55		
	25154780	79.947	80.500	99.31		
100	31297081	99.469	100.500	98.97	99.27	0.441
	31392870	99.773	100.000	99.77		
	31480990	100.053	101.000	99.06		
120	37736981	119.936	120.500	99.53	99.27	0.394
	37710704	119.853	120.500	99.46		
	37620790	119.567	121.000	98.82		

Table 7: LOD and LOQ

Conc (PPM)	Practical area	Theoretical area	Residual
80.00	25374223	25324212	50011
90.00	28392076	28420901	−28825
100.00	31457710	31517590	−59880
110.00	34620470	34614279	6191
120.00	37743470	37710967	32503
		SD (SIGMA)	44755.92

Table 8: Repeatability data for calcium dobesilate

Sample	Area	% Assay
Sample 1	31469175	99.03
Sample 2	31597041	98.45
Sample 3	31417964	99.36
Sample 4	31499740	100.11
Sample 5	31467975	98.53
Sample 6	31490710	99.09
Mean		99.10
SD		0.606622
% RSD		0.612

Table 9: Intermediate precision data for calcium dobesilate

Sample	Area	% Assay
Sample 1	31394682	99.28
Sample 2	31364710	99.19
Sample 3	31496403	98.62
Sample 4	31390940	98.78
Sample 5	31357169	99.66
Sample 6	31368175	98.71
Mean		99.04
SD		0.4043
% RSD		0.408
Precision plus intermediate precision	Mean	99.068
	SD	0.4923
	% RSD	0.497

more than 2. Hence, precision passes the criteria; no variation was found by preparing six different samples. Results are good reproducible [Table 9].

Robustness

Robustness shows the reliability of an analysis with respect to deliberate variations in method parameters, including scanning wavelength (±2 nm), flow rate (±10%), and column temperature (±2°C). It was concluded that the method is robust as chromatography was not compromised by changes in wavelength, flow rate, and column oven temperature. Good %RSD values, theoretical plates not <2000, and asymmetry not more than 2.0.

CONCLUSION

In accordance with ICH criteria for accuracy, precision, linearity, specificity, and system appropriateness, an RP-HPLC technique for estimating CAD was developed and validated. The outcomes met the requirements for acceptance. The proposed method was applied for the determination of CAD in bulk drugs. Hence, the proposed method was found to be satisfactory and could be used for the routine analysis of CAD in any dosage form or pre-formulation studies.

DATA AVAILABILITY STATEMENT

This article contains all of the data generated or analyzed during this investigation.

ETHICAL APPROVAL

Because, in the present study, animals were not used as a result of this, ethical approval was not required.

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