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Validated stability-indicating methods for determination of cilostazol in the presence of its degradation products according to the ICH guidelines

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Abstract

Sensitive and selective stability-indicating assay methods (SIAMs) are suggested for the determination of cilostazol (CIL) in the presence of its acid, alkaline and oxidative degradation products. Developing SIAMs is necessary to carry out any stability study. Stress testing of CIL was performed according to the International Conference on Harmonization (ICH) guidelines in order to validate the stability-indicating power of the analytical procedures. Stress testing showed that CIL underwent acid, alkaline and oxidative degradation; on the other hand, it showed stability towards photo- and thermal degradation. Two chromatographic SIAMs were developed, namely HPLC and HPTLC methods. The concentration range and the mean percentage recovery were 1.0–31.0 μg/ml and 99.96 ± 0.46 and 0.6–14.0 μg/spot and 99.88 ± 1.10 for HPLC and HPTLC methods, respectively. In addition, derivative spectrophotometric methods were developed in order to determine CIL in the presence of its acid degradation product; these were performed by using the third derivative spectra (^{3}D) and the first derivative of the ratio spectra (^{1}DD) methods. The linearity range and the mean percentage recovery were 2.0–34.0 μg/ml and 100.27 ± 1.20 for the (^{3}D) method, while they were 2.0–30.0 μg/ml and 99.94 ± 1.18 for the (^{1}DD) method. Also, two chemometric-assisted spectrophotometric methods, based on using partial least squares (PLS) and concentration residual augmented classical least squares method (CRACLS), for the determination of CIL were developed. Both methods were applied on zero order spectra of the mixtures of CIL and its acid degradation product, the mean percentage recovery was 100.03 ± 1.09 and 99.91 ± 1.27 for PLS and CRACLS, respectively. All methods were validated according to the International Conference on Harmonization (ICH) guidelines and applied on bulk powder and pharmaceutical formulations.

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Keywords: Cilostazol; Stability-indicating assays; Chromatographic methods; Derivative spectrophotometric methods; Chemometrics

1. Introduction

Cilostazol (CIL) 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl) butoxyl]-3,4-dihyro-2(1*H*)-quinolinone is a phosphodiestrase inhibitor with an antiplatelet and vasodilating activity used in the management of peripheral vascular diseases [1]. The drug is metabolized to at least 11 metabolites [2]. It was determined in the presence of some of its metabolites in liver microsomal solutions [3], and in human plasma using HPLC with gradient elution and by either UV [4,5] or MS [6] detection. Also, HPLC methods were reported for its determination in pharmaceutical formulation [7] and human plasma [8].

The International Conference on Harmonization (ICH) guidelines [9] requires performing stress testing of the drug substance, which can help identify the likely degradation products and can be useful in establishing degradation pathways and validating the stability-indicating power of the analytical procedures used. Moreover, validated stability-indicating method should be applied in the stability study [10]. Stability-indicating assay methods (SIAMs) can be specific one, which evaluates the drug in the presence of its degradation products, excipients and additives, or selective one which is able to measure the drug and all the degradation products in the presence of excipients and additives [11].

This work is aimed to develop several and alternative SIAMs for determination of CIL. To achieve this goal, stress testing study was performed according to the ICH recommendations. The developed methods included chromatographic methods

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(HPLC and HPTLC) used to resolve the drug from different degradation products obtained under acid, alkaline and oxidative stress testing. In addition, spectrophotometric methods namely derivative spectrophotometric using the third derivative (^{3}D) and the first derivative of the ratio spectra (^{1}DD) were developed for CIL determination in the presence of its acid degradation product. Chemometric-assisted spectrophotometric methods using partial least squares (PLS) and concentration residual augmented classical least squares (CRACLS) methods were also developed. These methods show the high capability of multivariate calibration methods for determination of spectrally interfering component mixtures. The choice of these two methods was based on that the PLS has excellent predictions for data sets even when some of the constituents have not been included in the calibration; whereas CRACLS, developed by Haaland and coworkers [12], retains the qualitative benefits of classical least squares (CLS), (which is based on Beer's Law to estimate absorptivity from a set of absorbances but requires all components of the measured sample to be known a prior) and maintains the flexibility of PLS modeling when spectrally active components are not explicitly included in the calibration [13]. These spectrally active components could be unidentified sources of spectral variation that are present in the calibration spectra. Reviewing the literature shows that the methods available are concerned with determination of CIL in presence of its metabolites in biological fluids; also an assay was reported for its determination in pharmaceutical formulation. Developing of SIAMs was not previously reported.

2. Experimental

2.1. Instrumentation

- For HPLC, a "LaChrom" HPLC instrument (HITACHI-MERCK, Germany), with an L-7150 pump, connected with an L-7455 photodiode array detector (DAD), was used for HPLC analysis. Injection was performed with a manual "Rheodyne" injector, model 7161 (Cotati, CA, USA) fitted with a 20-μl injector loop and a 100-μl "Hamilton" syringe. The instrument was connected to an IBM compatible PC, bundled with Merck-Hitachi, model D-7000, HPLC System Manager Data Station Software, an HP 800 inkjet printer, and an "Xterra™" RP_{18} (5 μm, 4.6 mm × 250 mm) column (Waters, Milford, MA, USA).
- For HPTLC, the plates used were (20 cm × 20 cm, ALUGRAM® Nano-SIL Silica Gel 60 F_{254} plates, Macherey Nagel, Germany) aluminum plates coated with 0.2-mm silica gel F_{254}, particle size 2–10 μm. The samples were applied to the plates using a "LINOMAT 5" HPTLC applicator (CAMAG, Switzerland). A "TLC SCANNER III" (CAMAG, Switzerland), was used for scanning in reflectance mode at 258.0 nm, scan speed of 20.0 mm/s, and track optimization of 7. Both instruments were connected to an IBM compatible PC, bundled with WinCats Software, version 1.4.2.
- For spectrophotometric methods, a double-beam "Shimadzu", model UV-1601, UV-Visible spectrophotometer (SHIMADZU, Japan) connected to an IBM compatible PC and an HP 800 inkjet printer. The bundled software was UVPC Personal Spectroscopy Software, version 3.7. The spectral bandwidth was 2 nm and wavelength-scanning speed was 2800 nm/min.
- Software for chemometric study: all computations were performed in Matlab for Windows™ version 6.5 Mathworks Inc., 2002 (MA, USA) with our own codes [13]. The (PLS) procedure was taken from PLS Toolbox 2.1, Eigenvector Research, Inc.
- A "Climacell 404 MMM" Medcenter Einrichtungen GmbH stability cabinet was used for stability studies.
- A "Soniclean 120T" sonicator (SONICLEAN, Australia) was used for extraction of drug from pharmaceutical formulation.
- A "Jenway 3505" pH-meter (Jenway, UK), equipped with combined glass electrode was used for pH adjustment.

2.2. Materials and reagents

- Cilostazol was supplied by (Glenmark Pharmaceuticals Ltd., India) certified to have a purity of 100.50%; hydrochloric acid (Ubichem Limited, USA); ammonium acetate (Aldrich, Germany); acetonitrile, glacial acetic acid, sodium hydroxide, ethyl acetate, methanol, dimethyl sulfoxide (DMSO), hydrogen peroxide, and water (all were of HPLC grade) were purchased from E. Merck (Darmstadt, Germany).
- The commercial Pletaal® tablets (Batch no: 5B83PA1) used was manufactured by Egypt OTSUKA Pharmaceutical Co., S.A.E. under license of OTSUKA Pharmaceutical Co. Ltd., Japan, and labeled to contain 100 mg cilostazol per tablet.

2.3. Standard solutions

2.3.1. Standard solutions of the drug

Stock standard solutions of CIL having concentrations of 400 μg/ml (Stock 1) for HPLC method and 1000 μg/ml (Stock 2) for HPTLC and spectrophotometric methods were prepared by transferring separate portions 10 and 25 mg of CIL powder into 25-ml volumetric flasks, and dissolving in 1.0 ml glacial acetic acid; the volume was then made up to the mark with acetonitrile.

For the HPLC assay, a working standard solution having a concentration of 40 μg/ml was prepared from Stock 1 by appropriate dilution with acetonitrile.

For the spectrophotometric assay, a working standard solution having a concentration of 100 μg/ml was prepared from Stock 2 by appropriate dilution with deionized water.

2.3.2. Standard solutions of the degradation products

Solution of acid degradation product was prepared by dissolving 20 mg of CIL in 1.0 ml glacial acetic acid, then 20.0 ml of 2.0 M HCl were added, and the solution was refluxed. The degradation was followed by HPLC analysis, where complete degradation was achieved after 6 h of reflux. The solution was neutralized to pH 3.0 by a calculated volume of 2.0 M NaOH, then it was transferred into a 50-ml volumetric flask, and the volume was completed with water to obtain a concentration of 400 μg/ml.

Preparation of alkaline-induced degradation product was achieved by dissolving 20 mg of CIL in 1.0 ml DMSO, then 20.0 ml of 2.0 M NaOH were added, and the solution was refluxed for 2 h. The solution was neutralized to pH 10.0 by a calculated volume of 2.0 M acetic acid. The solution was resolved by HPTLC and HPLC after appropriate dilution with mobile phase.

Solution for oxidative degradation testing was prepared by dissolving 10 mg of CIL in 1.0 ml glacial acetic acid, 10.0 ml of 6% H_2O_2 were added and the solution was refluxed for 1.5 and 3 h at 100 °C. The solution was then aerated by carbon dioxide stream at 60 °C for 1 h to remove oxygen and decompose extra H_2O_2. The solution was resolved by HPTLC and HPLC after appropriate dilution with mobile phase.

For photo- and thermal degradation testing, three portions of CIL powder (50 mg each) were spread as thin films in three separate Petri dishes (5 mm diameter). The first portion was exposed to UV lamp, producing UVB radiation, at 15.0 cm distance for 6 h. The second portion was heated in an oven at 100 °C for 6 h; the third portion was left in the stability cabinet at 50 °C and 75% relative humidity for 45 days. From each dish, 10 mg were transferred into 10-ml volumetric flasks, dissolved in 1.0 ml acetic acid, and completed to the volume with acetonitrile to obtain concentrations of 1000 μg/ml. These solutions were used for HPTLC testing. For HPLC, the solutions were diluted by the mobile phase to obtain concentrations of 20 μg/ml. Samples were withdrawn from the third dish periodically at 7 days interval, and tested similarly.

2.4. Assay of the pharmaceutical formulation

Twenty of the Pletaal® tablets were accurately weighed and finely powdered. An accurately weighed amount of the powdered tablets equivalent to one tablet was transferred into a 100-ml volumetric flask, on which 5.0 ml glacial acetic acid were added. The sample was sonicated for 30 min, and then 75.0 ml acetonitrile were added. The sample was further sonicated for 10 min, completed to the volume with acetonitrile, and then filtered.

Solutions having the concentration of 20 μg/ml were prepared by appropriate dilutions with HPLC mobile phase and water and used for determination of the drug by the HPLC and spectrophotometric methods, respectively. For the HPTLC assay, 5 μl of the obtained solution were spotted and scanned. Each of the analyses was performed as under the respective described procedure mentioned in the following section.

3. Results and discussion

CIL (Fig. 1a) was subjected to different stress conditions, as recommended by the ICH [9]. HPLC and HPTLC methods were developed for the separation of CIL from its degradation products. CIL showed degradation under stressed acid, alkaline and oxidative conditions, while it showed stability upon exposure to UVB source and dry heat. Also, monitoring of stability over 6 weeks at 50 °C and 70% relative humidity proved that the drug was stable during that period. Testing the drug stability

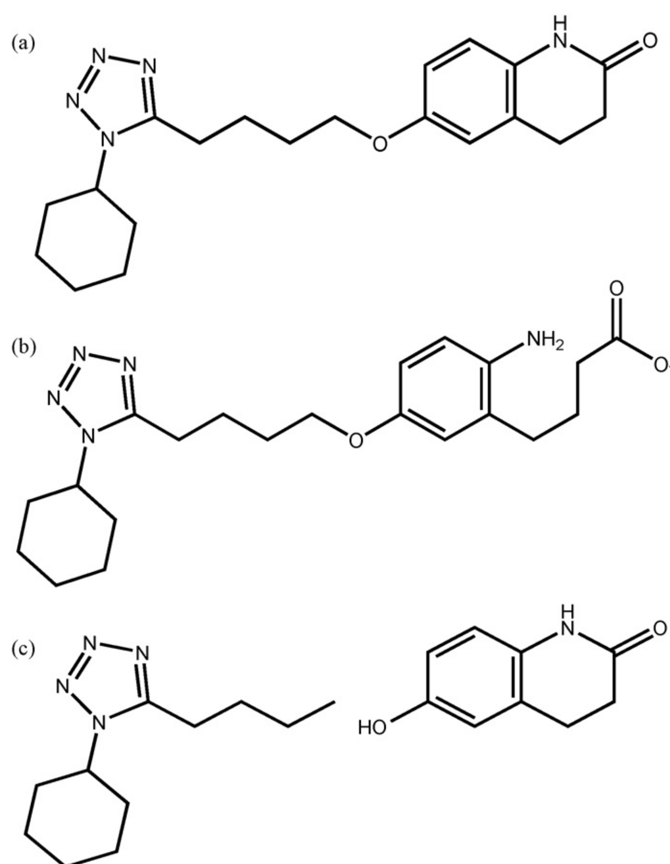

Fig. 1. (a) Cilostazol, (b) cilostazol proposed alkaline degradation product, and (c) cilostazol proposed acid degradation products.

was performed by both suggested HPLC and HPTLC methods, the chromatograms obtained are shown in Figs. 2–4. CIL chemical structure shows a cyclic amide group (δ-lactam) and an ether group, many amide and lactam containing drugs undergo alkaline degradation through hydrolysis with cleavage of the amide linkage [14] thus the probable alkaline degradation pathway for CIL will be the same, the proposed compound is shown in Fig. 1b. The spectrum obtained by DAD of the alkaline degradation product separated peak shows resemblance to that of the drug, this is may be due to that the degradation product retains the same chromophores as the intact drug, which assists the previous assumption. On the other hand, acid degradation probably takes place through cleavage of the ether linkage which is usually unstable in acid medium (Fig. 1c) this assumption is assisted by examining the spectrum obtained using DAD for the separated peak of the acid degraded sample which was completely different from that of the drug.

3.1. Optimization of the chromatographic conditions and construction of calibration curves

3.1.1. For HPLC method

Separation of CIL from its degradation products has been performed on "Xterra™" RP_{18} column. Different ratios of acetonitrile and ammonium acetate aqueous solution (pH 5; 0.02 M) were tried, the mobile phase was filtered using 0.45 μm Teflon®

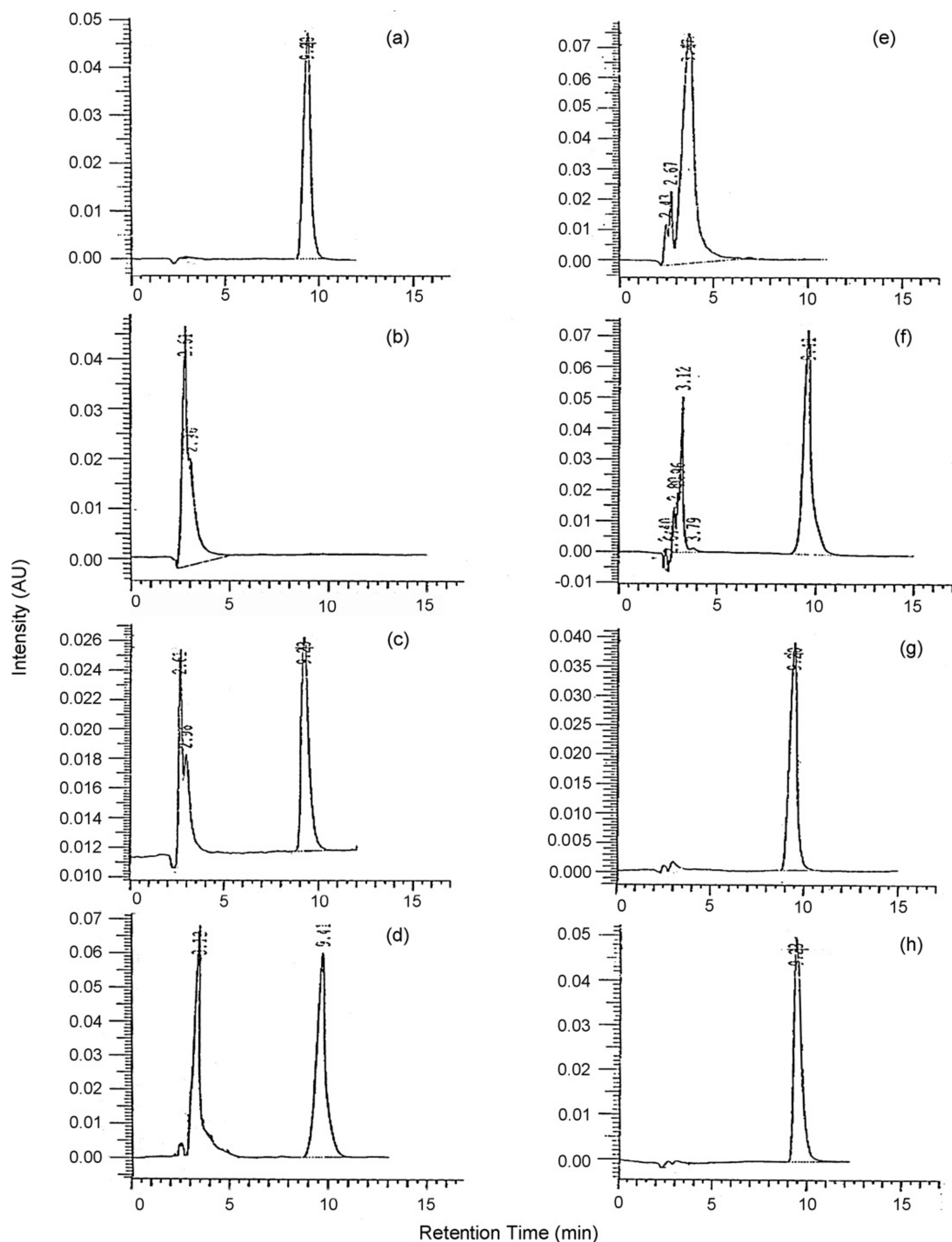

Fig. 2. HPLC chromatograms of (a) cilostazol 24 μg/ml, (b) acid degradation product of cilostazol 24 μg/ml, (c) mixture solution containing 8 μg/ml from acid degradation product and 12 μg/ml from cilostazol, (d) alkaline-induced degraded cilostazol 30 μg/ml, (e) oxidatively degraded cilostazol 24 μg/ml (reflux for 3 h), (f) oxidatively degraded cilostazol 24 μg/ml (reflux for 1.5 h), (g) thermally degraded cilostazol 20 μg/ml, and (h) photo-degraded cilostazol 24 μg/ml.

membrane filter (Millipore, Millford, MA, USA), and degassed by sonication prior to use. The flow rate was 1.0 ml/min. Increasing the acetonitrile ratio was accompanied by decrease in retention time (R_t) of different components; however the separation was still achieved. In order to ensure complete separation and high resolution (R_s) values, the chosen ratio was acetonitrile and ammonium acetate (40:55, v/v). The detection was performed at 254.0 nm, where the maximum sensitivity was observed. The specificity of the method is illustrated in Fig. 2 and the average retention time of CIL for 10 replicates was 9.31 ± 0.10 min. Construction of calibration curve was performed by transferring aliquots of CIL stock and working standard solutions into a series of 10-ml volumetric flasks and diluting to volume with the mobile phase to obtain solutions in the concentration range of 1–31 μg/ml. A 20 μl volume from each solution was injected in triplicate; chromatographic separation was run under the previously mentioned conditions. All determinations were performed at ambient temperature; the

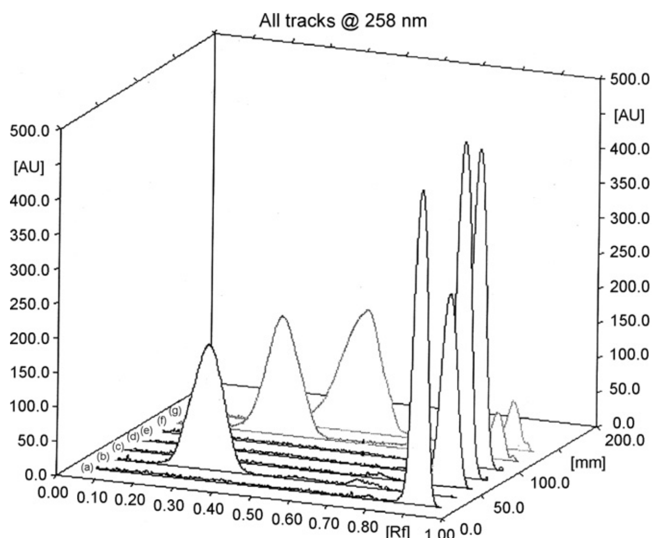

Fig. 3. Three-dimensional HPTLC chromatograms of (a) cilostazol 10 μg/spot, (b) acid degradation products of cilostazol 8 μg/spot, (c) cilostazol 5 μg/spot, (d) thermally degraded cilostazol 10 μg/spot, (e) photo-degraded cilostazol 10 μg/spot, (f) mixture containing cilostazol 1 μg/spot and its acid degradation product 8 μg/spot, and (g) alkaline-induced degraded cilostazol 8 μg/spot.

average peak area obtained for each concentration was plotted *versus* concentration.

3.1.2. For HPTLC method

Experimental conditions, such as mobile phase composition, track optimization, scan speed, and wavelength of detection were optimized to provide accurate and reproducible results. The chosen wavelength was 258.0 nm, while the optimum mobile phase was methanol–ethyl acetate–water (95:5:1.5, v/v/v). The plates were primarily washed by developing with the mobile phase and then activated for 10 min by heating in an oven at 100 °C before use. The best results were obtained when spotting was in the form of 6.0 mm bands at 5.0 mm intervals using the "CAMAG LINOMAT 5" applicator under a stream of nitrogen. The chromatographic tank was saturated with the mobile phase for 15 min prior use. The plates were developed using the above-mentioned solvent (over a distance of 15.0 cm) in an ascending manner, air-dried and scanned at $\lambda = 258.0$ nm. For detection of the degraded samples under different stressed conditions, volumes from each of standard CIL and degradation product solutions of different concentrations within the quantitation range were spotted manually with the aid of a 25-μl "Hamilton" syringe as separate compact spots 20 mm apart and 20 mm from the bottom of the plates. The retention factor (R_f) value of CIL was 0.82 while that of different degradation products was between 0.16 and 0.53 as indicated in Figs. 3 and 4, representing the specificity of the method. For construction of calibration curve, duplicate volumes in the range of 0.6–14 μl from CIL stock standard solution (1000 μg/ml) were applied with the aid of a 100-μl "CAMAG" syringe. The standard and sample calibration graph was drawn by plotting the average peak areas *versus* concentration.

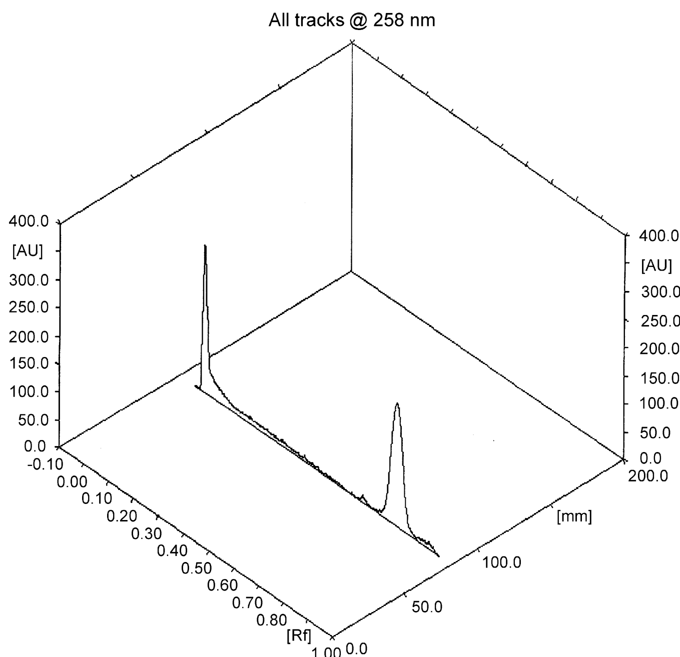

Fig. 4. Three-dimensional HPTLC chromatogram of oxidative degradation product of cilostazol 10 μg/spot of a sample refluxed with H_2O_2 for 1.5 h.

3.2. Assay parameters and construction of calibration curves for spectrophotometric methods

3.2.1. Third derivative (^3D) UV-spectrophotometric method

The UV-spectra of CIL and its acid degradation product (I) showed overlapping as shown in Fig. 5a, which would not permit zero order determination of CIL in the presence of (I), thus derivative spectrophotometric methods were applied. The ^3D method permitted selective determination of CIL in the presence of (I) as shown in Fig. 5b. CIL could be determined at 275.0 nm, where zero-crossing point for (I) is indicated. The assay parameters namely $\Delta\lambda$, scaling factor, and smoothing were optimized. The best results obtained when using $\Delta\lambda = 8$ nm using 400 as scaling factor. For construction of calibration curve, aliquots of CIL working standard solution 100 μg/ml were transferred in a series of 10-ml volumetric flasks, and diluted to volume with water to obtain solutions in the concentration range of 2–34 μg/ml. The absorption spectra of the solutions were scanned in the range of 200.0–350.0 nm. The ^3D curves were computed under the previously mentioned conditions. The calibration was constructed by plotting values of the ^3D amplitude at 275.0 nm *versus* concentrations and the regression equations were computed.

3.2.2. First derivative of ratio spectra (^1DD) method

The main advantage of the derivative of the ratio spectra method might be the chance of taking measurement in correspondence to peaks and that the whole spectrum of interfering substance is cancelled, thus the wavelength selection for calibration is not critical. In trials to chose the best wavelength, different divisor concentrations were tried namely 3, 6, 9, 12, and 15 μg/ml of (I). The best results were obtained when using the spectrum of 9 μg/ml of (I) as divisor (Fig. 5c). The wavelength 255.0 nm was chosen as the analytical λ, where

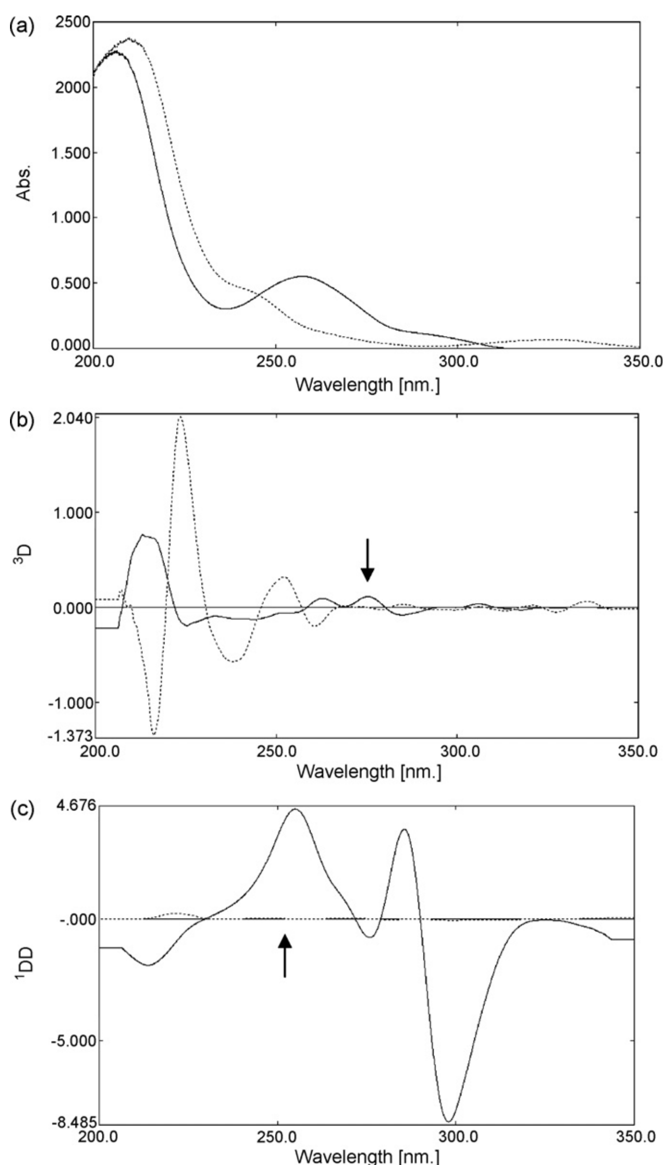

Fig. 5. (a) Zero order absorption (^{0}D) spectra of cilostazol (14 μg/ml) (—) and its acid degradation product (6 μg/ml) (- - -). (b) Third derivative (^{3}D) spectra of cilostazol (10 μg/ml) (—) and its acid degradation product (9 μg/ml) (- - -). (c) First derivative of the ratio spectra of cilostazol (10 μg/ml) (—) and its acid degradation product (9 μg/ml) (- - -).

no noise was observed from the divisor. Both first and second derivatives of ratio spectra methods were tried; ^{1}DD gave more accurate results. Construction of calibration curve was performed by transferring aliquots of CIL working standard solution 100 μg/ml in a series of 10-ml volumetric flasks, and diluting to volume with water to obtain solutions in the concentration range of 2–30 μg/ml. The absorption spectra of the solutions were scanned in the range of 200.0–350.0 nm. The absorption spectrum of acid degradation product (I) solution having the concentration of 9 μg/ml was scanned and stored in the instrument PC. The UV-spectra of CIL standard solutions were divided by the spectrum of (I) solution. The first derivative of the ratio spectra were computed at $\Delta\lambda = 8$ nm using 50 as scaling factor. The amplitudes were recorded at 255.0 nm. The calibration curve was constructed by plotting values ^{1}DD *versus* concentrations, and the regression equations were computed.

3.3. Chemometric methods; partial least squares (PLS) and concentration residual augmented classical least squares (CRACLS) methods

The analysis of a mixture composed of CIL and its acid degradation product (I) by conventional spectrophotometry is not feasible, because of the overlapping of the broad absorption spectral bands as shown in Fig. 5a. Therefore, chemometrics was applied.

In this work, two multivariate calibration methods namely the partial least squares (PLS) and the concentration residual augmented classical least squares (CRACLS) were performed on experimental data obtained from UV-spectra of mixtures composed of CIL and (I) for their resolution. The developed methods were applied for the analysis of CIL in presence of (I). A training (calibration) set was designed with, 16 synthetic mixtures with different concentration ratios of CIL and its acid degradation product (I), containing intact drug in the range of 8–14 μg/ml and (I) in the range of 0.5–4 μg/ml, solutions were prepared by mixing different aliquots of CIL working standard solutions in 10-ml volumetric flasks, then the volumes were completed with water. Table 1 shows the concentrations of the prepared mixtures solutions. The UV-spectra of the prepared solutions were recorded over the range 200–350 nm. The data points of the spectra were exposed to Matlab version 6.5. Multivariate calibration models were applied to these mixtures to calculate the concentration of CIL in the presence of (I). The training set of the two components was designed to give symmetric and orthogonal distribution in order to allow determination of CIL in different concentrations accurately. To test the model prediction ability, validation set was composed of another six samples was prepared. Upon optimization of data handling, it was found that the best results were obtained when the spectra were dig-

Table 1
Concentration of cilostazol and its acid degradation product in the training set for PLS and CRACLS chemometric methods

Mixture no.	Concentration (μg/ml)	
	Cilostazol	Acid degradation product
1	8.0	0.5
2	8.0	1.0
3	8.0	2.0
4	8.0	4.0
5	10.0	0.5
6	10.0	1.0
7	10.0	2.0
8	10.0	4.0
9	12.0	0.5
10	12.0	1.0
11	12.0	2.0
12	12.0	4.0
13	14.0	0.5
14	14.0	1.0
15	14.0	2.0
16	14.0	4.0

Table 2
Specificity of the proposed PLS and CRACLS chemometric methods for the determination of cilostazol in the validation set of solution of cilostazol and its acid degradation product (I)

Mixture no.	Concentration (μg/ml)		Recovery% of cilostazol	
	Cilostazol	(I)	PLS	CRACLS
1	6.0	0.5	99.50	99.06
2	8.0	1.0	100.75	99.39
3	12.0	4.0	99.58	101.01
4	10.0	2.0	99.80	99.08
5	4.0	4.0	99.25	99.76
6	14.0	4.0	99.00	99.12
Mean			99.65	99.57
R.S.D.%			0.61	0.75

itized each at 0.1 nm in the range of 220–350 nm, where 1301 experimental points were used in the calculations. In order to determine the correct number of latent variables to be used for modeling of the data, a cross-validation with random subset selection procedure [15] was performed for all the samples in the training set. The 16 calibration samples were randomly divided into four sets; three sets were used in building the model while the fourth set was predicted by the model. This procedure was iterated five times, and the average root mean squares error of cross-validation (RMSECV) was calculated. The RMSECV was calculated in the same manner each time a new latent variable was added to the model. This method involves the comparison between RMSECV of all models with that of the model yielding the minimum RMSECV (V*), and the selection of the model with the smallest number of variables such that the RMSECV for the selected model was not significantly greater than that from the model yielding V* [16,17]. Two latent variables were found optimum for the mean centered data. The first latent variable models the sum of the concentrations of both CIL and (I) while the second latent variable models the difference between them. It can be observed that sample (1) represents the minimum sum of concentrations while sample (16) represents the maximum sum of them. Likewise, sample (4) represents the minimum difference between concentrations while sample (13) represents the maximum difference between them. To test the prediction ability of the PLS and CRACLS chemometric methods, the model was challenged with the spectra of a validation set, made of six samples different than those of the training set. The mean recovery percent of CIL in the validation set was 99.65 ± 0.61 and 99.57 ± 0.75 for PLS and CRACLS, respectively (Table 2). The predicted concentrations of CIL in each sample of the validation set were compared with their known concentrations, and the root mean square error of prediction (RMSEP) was calculated. The RMSEP was used as a diagnostic test for examining the errors in predicted concentrations. It indicates both precision and accuracy of predictions [18], as shown in Table 3. Also a linear relationship with a slope approaches one when plotting the predicted concentration against the true ones indicates the precision of the methods (Table 3). CRACLS models were built for CIL, where the estimated pure components spectra that resulted by including CIL concentration and augmenting 2 times, as shown in Fig. 6. The produced models

Table 3
Assay validation sheet of the proposed PLS and CRACLS chemometric methods for the determination of cilostazol

Parameters	Method	
	PLS	CRACLS
Linearity	2.0–34.0 μg/ml	
Accuracy[a]	100.03 ± 1.09	99.91 ± 1.27
Precision		
Repeatability[a]	99.60 ± 1.40	99.63 ± 1.42
Intermediate precision[a]	99.75 ± 1.32	100.14 ± 1.62
Slope	0.9901	0.9917
Intercept	0.0537	0.0347
RMSEP	0.0746	0.0822
"r"[b]	0.9999	0.9998

[a] Mean ± S.D. (n = 9).
[b] "r" parameter was calculated for the correlation between predicted and true sample concentrations for the validation set.

were used for analysis of CIL in pharmaceutical formulation. The results were compared with the suggested HPLC method, where no significant difference for both accuracy and precision was observed as indicated by t-test and F-test (Table 8).

3.4. Methods validation

ICH guidelines [10] for method validation were followed for HPLC, HPTLC, ^{3}D and ^{1}DD methods. All validation parameters are shown in Table 4.

3.4.1. Linearity

3.4.1.1. For HPLC method. A linear correlations were obtained between peak area and concentration in a range of 1–31 μg/ml the correlation coefficient r = 0.9999.

3.4.1.2. For HPTLC method. The linear regression between CIL concentrations and peak areas of the spots was investigated and the correlation coefficient (r) was found to be 0.998 for

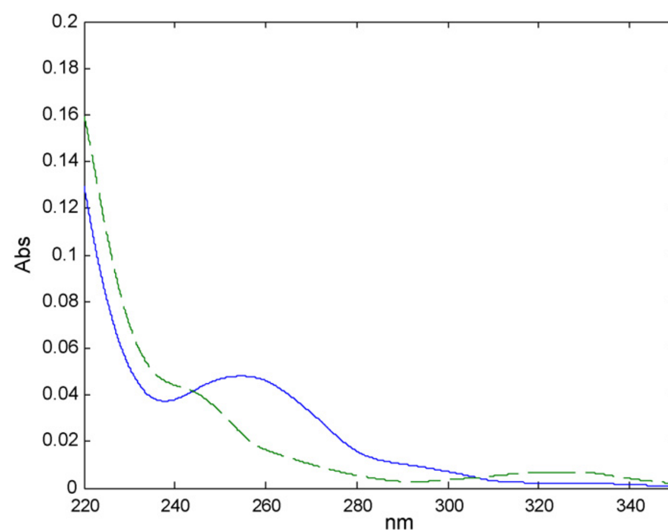

Fig. 6. UV-spectra of cilostazol (—) and its acid degradation product (- - -) as estimated by CRACLS chemometric method.

Table 4
Validation report of the proposed HPLC, HPTLC and spectrophotometric methods for the determination of cilostazol

Parameters	Method			
	HPLC	HPTLC	^{3}D	^{1}DD
Linearity	1.0–31.0 μg/ml	0.6–14.0 μg/spot	2.0–34.0 μg/ml	2.0–30.0 μg/ml
Intercept (a)[a]	4.09×10^3	3.44×10^3	-2.60×10^{-3}	-41.30×10^{-3}
Slope (b)[a]	27.65×10^3	–	11.50×10^{-3}	444.80×10^{-3}
S.E.[b]	0.09×10^3	–	0.10×10^{-3}	2.00×10^{-3}
Coefficient 1 (b_1)[c]	–	6.16×10^3	–	–
Coefficient 2 (b_2)[c]	–	-0.23×10^3	–	–
Correlation coefficient (r)	0.9999	0.9999	0.9998	0.9998
Accuracy[d]	99.96 ± 0.46	99.88 ± 1.10	100.27 ± 1.20	99.94 ± 1.18
Precision				
Repeatability[d]	100.10 ± 0.98	100.46 ± 1.19	99.79 ± 1.20	100.10 ± 1.11
Intermediate precision[d]	99.98 ± 0.69	100.49 ± 1.08	100.01 ± 0.69	99.85 ± 1.39
LOD	0.24 μg/ml	0.14 μg/spot	0.12 μg/ml	0.16 μg/ml
LOQ	0.72 μg/ml	0.43 μg/spot	0.36 μg/ml	0.50 μg/ml

[a] Regression equation = “$A = a + bc$” for HPLC, ^{3}D, and ^{1}DD methods; where “A” = area for HPLC, ^{3}D amplitude for ^{3}D, and ^{1}DD amplitude for ^{1}DD methods, “c” = the concentration (μg/ml).
[b] Standard error of the slope.
[c] Regression equation = “$A = a + b_1c + b_2c^2$” for HPTLC; where “A” = the area and “c” = the concentration (μg/spot).
[d] Mean ± S.D. (n = 9).

11 points in the concentration range of 0.6–14 μg/spot. Thus, the second order polynomial fit was found to be more suitable. Moreover, the ICH guidelines [10] mentioned that for some analytical procedures which do not demonstrate linearity, the analytical response should be described by an appropriate function of the concentration of an analyte sample. The regression equation showed an r-value of 0.9999 in the same concentration range, and the residual plot showed a much more even scatter than that of linear model, indicating good correlation. The regression equations for both methods are shown in Table 4.

3.4.1.3. For ^{3}D and ^{1}DD methods. The linear regression data for the calibration curves showed good linear relationship over the concentration range of 2–34 and 2–30 μg/ml for ^{3}D and ^{1}DD methods, respectively, with $r = 0.9998$ (Table 4).

3.4.2. Accuracy

The accuracy of the four methods were tested by analyzing freshly prepared solutions of CIL in triplicate at concentrations of 9, 12, and 15 μg/ml for HPLC; 1, 4, and 7 μg/spot for HPTLC;

Table 5
Statistical comparison between the proposed methods using ANOVA test

Parameter	Method					
	HPLC	HPTLC	^{3}D	^{1}DD	PLS	CRACLS
Accuracy						
n	9					
Mean	99.96	99.88	100.27	99.94	100.03	99.91
V (variance)	0.21	1.21	1.46	1.40	1.22	1.60
Degree of freedom	5					
F	0.155					
F-critical	2.408					
Repeatability						
n	9					
Mean	100.10	100.46	99.79	100.10	99.59	99.62
V (variance)	0.96	1.45	1.42	1.24	1.98	2.04
Degree of freedom	5					
F	0.665					
F-critical	2.408					
Intermediate precision						
n	9					
Mean	99.98	100.49	100.01	99.85	99.75	100.14
V (variance)	0.48	1.18	0.49	1.94	1.79	2.66
Degree of freedom	5					
F	0.428					
F-critical	2.408					

Table 6
Results of the laboratory prepared mixtures for cilostazol with its acid degradation product by the proposed HPLC and spectrophotometric methods

Sample no.	(%) of degradate	Recovery (%) of CIL		
		HPLC	^{3}D	^{1}DD
1	10	99.28	99.82	98.88
2	20	99.42	99.45	99.39
3	30	101.48	100.69	100.61
4	50	99.60	100.65	100.20
5	70	100.23	100.36	99.31
6	80	100.13	99.76	99.79
7	90	–	100.86	101.47
Mean		100.02	100.22	99.95
R.S.D.%		0.81	0.54	0.89

4, 14, and 24 for both ^{3}D and ^{1}DD methods. The recovery percent and standard deviations (S.D.) revealed excellent accuracy (Table 4). The four suggested methods were statistically compared upon using the analysis of variance (ANOVA) test. The data showed that there is no significant difference between them (Table 5).

3.4.3. Repeatability and reproducibility

The intra- and interday precision was evaluated by assaying freshly prepared solutions in triplicate on the same day and on three successive days, respectively using the four suggested methods. Table 4 shows the precision and ruggedness of the methods; also Table 5 shows that there was no significant difference between their reproducibility as analyzed by ANOVA test. The methods are suitable for quality control of CIL.

3.4.4. Specificity

The specificity of the HPLC and HPTLC methods was illustrated by the complete separation of CIL from different degradation products, as shown in Figs. 2–4. The R_s values from alkaline, acidic, and oxidative degradation products were always above 2, while the selectivity factor (α) was more than 1, which ensured complete separation of CIL from its degradation products. Furthermore, CIL was determined in solutions containing different amounts of its acid degradation product (I) by the HPLC method. The Recovery% and R.S.D.% proved the specificity of the method (Table 6). The asymmetry factors were 1.45 and 1.10 for the HPLC and HPTLC methods, respectively, which revealed linear isotherm peak elution without tailing. Peak information are given in Table 7.

For the ^{3}D and ^{1}DD methods, solutions of laboratory prepared mixtures of CIL and (I) were analyzed, where excellent recovery of CIL revealed the high specificity of both methods (Table 6).

For chemometric methods, the specificity is tested by using the validation set as previously mentioned and the results are shown in Table 2.

3.4.5. Robustness and system suitability of the HPLC method

Separation of CIL from its acid degradation products (I) was performed on two HPLC instruments, namely "LaChrom" and "Shimadzu". For system suitability testing, solution mixtures of CIL and (I) were separated under different conditions by using different pH values ranging from 4.50 to 5.50, different flow rates (0.90–1.10 ml/min), and different acetonitrile–ammonium acetate aqueous solution (pH 5; 0.02 M) ratios of (40:55, v/v), (45:55, v/v), and (40:60, v/v) as the mobile phase. The R_t values of the separated peaks using the mentioned pH range did not change, while changing the flow rate and mobile phase was accompanied by slight decrease or increase of R_t of all peaks. However, the calculated R_s values were always above 2, ensuring complete separation.

3.4.6. Limit of detection (LOD) and limit of quantitation (LOQ)

The ICH guidelines [10] for calculation of LOD and LOQ were followed. For HPLC, ^{3}D and ^{1}DD methods, the calculation was based on the standard deviation of the response and the slope using the calibration curve, while for HPTLC, the signal-to-noise method was used (Table 4).

3.5. Assay of pharmaceutical formulation

All of the six proposed methods were applied for the determination of CIL in commercial tablets. The results shown in Table 8 were satisfactory and with good agreement with the labeled amount.

To assess the validity of the six proposed methods, the standard addition technique was applied by adding amounts of CIL

Table 7
Peak information for the separated cilostazol and its degradation product[a]

Parameters	Cilostazol		Acid degradation products		Main alkaline degradation product		Main oxidative degradation product	
	HPLC	HPTLC	HPLC	HPTLC	HPLC	HPTLC	HPLC	HPTLC
R[b]	9.41	0.82	2.96, 2.61	0.30	3.09	0.50	3.12	0.10
Purity	0.9999	–	0.8890, 0.9629	–	0.975	–	–	–
Asymmetry factor	1.45	1.10	–	1.14	–	2.00	–	–
R_s (relative to cilostazol)	–	–	12.05	3.27	11.38	1.97	14.29	10.80
Selectivity factor (relative to cilostazol)	–	–	3.30	16.60	3.02	4.27	3.02	36.00

[a] Peak information for HPLC was obtained from the instrument software, while for HPTLC, it was calculated from the measured peak width and the retention factor (R_f) values of the obtained chromatograms.

[b] "R" is the retention time (R_t) in min for the HPLC method, and the R_f for the HPTLC method.

Table 8
Determination of cilostazol in pharmaceutical formulation[a] using the proposed HPLC, HPTLC, spectrophotometric and chemometric methods and application of standard addition technique

Parameters	Method					
	HPLC	HPTLC	^{3}D	^{1}DD	PLS	CRACLS
Mean found (mg per tablet) ± S.D.[b]	100.31 ± 0.65	100.56 ± 1.14	99.62 ± 0.59	99.29 ± 0.80	98.89 ± 0.61	100.26 ± 0.95
R.S.D.%	0.64	1.13	0.59	0.80	0.61	0.94
Recovery (%) ± S.D. of the standard addition technique[c]	100.05 ± 1.05	99.99 ± 1.44	100.98 ± 0.87	99.28 ± 0.77	100.60 ± 1.27	101.28 ± 1.32
R.S.D.%	1.05	1.44	0.86	0.77	1.26	1.30

[a] Pletaal® tablets (Batch no: 5B83PA1) (labeled to contain 100 mg cilostazol per tablet).
[b] Mean of four determinations.
[c] Mean of six determinations (two determinations each for 50, 100, 150% level of claim labeled amount).

in 50, 100, and 150% levels to the labeled claim. The results revealed accuracy of the methods and that there were no interference from tablet excipients (Table 8).

4. Conclusion

The proposed methods are precise, specific, accurate and stability-indicating ones. CIL can be determined in bulk powder, pharmaceutical formulation, as well as in the presence of all its degradation products by HPLC and HPTLC methods, while spectrophotometric methods are used for CIL determination in the presence of its acid degradation product. The CRACLS has prediction abilities comparable to PLS and yet has better qualitative characteristics than PLS. ICH guidelines were followed throughout the study for method validation and stress testing, and the suggested methods can be applied for quality control and routine analysis.

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