EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 1
Issue: 2
Page No: 149-164
Year: 2014

A VALIDATED NEW GRADIENT STABILITY-INDICATING LC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CILOSTAZOL AND ASPIRIN IN BULK AND TABLET FORMULATION

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Article Received on 01/08/2014 **Article Revised on 22/08/2014** **Article Accepted on 15/09/2014**

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ABSTRACT

A simple, precise and gradient RP-HPLC method was developed and validated for the simultaneous estimation of Cilostazol (CIL) and Aspirin (ASP) in presence of their corresponding degradation products. The RP-HPLC method consisted of a Binary pumps (model Waters 515 HPLC pump) and auto sampler (model 717 plus) was used. Chromatographic separation was achieved with Nova-pack C18 (4.6mm x 250 mm, particle size 4µm) column using gradient mode of elution. The mobile phase comprises of Acetonitrile: Ammonium formate (10 mm) pH 3.5 (adjusted with Ortho Phosphoric acid) in gradient mode. The flow rate was 0.7 ml/min and column temperature maintained at 40ºC throughout separation. The eluent was monitored at 254 nm using photodiode array detector (Waters 2998). The retention times of CIL and ASP were 24.5 ± 0.33 min and 16.3 ± 0.19 min respectively. The method was validated in terms of specificity, accuracy, linearity, precision, limit of detection, limit of quantitation and robustness. Linearity for CIL and ASP was in the range of 5 to 100 µg/ml and 2 to 40 µg/ml, respectively and percentage recoveries of both analytes were in the range of 100 ± 1.5% and % RSD was <1.5. The stress testing of both the drugs individually and combined was carried out under acidic, alkaline, neutral, oxidation, photo-stability and dry heat conditions. ASP underwent extensive acid, base, neutral hydrolysis and oxidative degradation than photo and thermal degradation. On the

other hand, CIL was susceptible to acid, alkaline and oxidative degradation; while, it showed stability towards neutral hydrolysis, photo and thermal degradation. The degradation products were well resolved from the analyte peaks. Thus the proposed method was suitable for quantitative determination and stability study of CIL and ASP in pharmaceutical preparations and can be used in the quality control of bulk manufacturing and pharmaceutical dosage forms.

KEYWORDS: HPLC, Cilostazol, Aspirin, stability indicating method.

INTRODUCTION

Cilostazol (CIL), chemically 6-[4-(1-cyclohexyl- 1H -tetrazol-5-y1)-butoxy]-3,4-dihydro-2(1H)- quinolinone (Fig 1a), is a phosphodiestrase inhibitor with an antiplatelet and vasodilating activity used in the management of peripheral vascular diseases, approved by the US Food and Drug Administration (FDA) in 1999. It is used for inhibition of platelet aggregation and as a vasodilator [1, 2]. CIL is indicated for the treatment of intermittent claudication. It has been proved effective in significantly improving walking distances among claudicants[3-5].

CIL is official in USP[6], literature survey revealed that analytical methods like spectrophotometry, HPLC and LC/MS/MS for its determination in bulk and formulation are available[7,8]. Gradient HPLC procedure for its determination in the presence of some of its metabolites in liver microsomal solutions and in human plasma and stability indicating chromatographic methods like HPLC and HPTLC were reported for its determination in presence of degradation products[9-13].

Aspirin (ASP) is chemically 2-(acetyloxy) benzoic acid; Acetyl salicylic acid; salicylic acid acetate (Fig 1b)[14], it is often used as an analgesic, antipyretic, anti-inflammatory and an antiplatelet agent. ASP is official in IP, BP and USP[15-17]. Literature survey revealed that RP-HPLC stability indicating analytical methods for the determination of ASP in combination with other drugs or alone are reported[18,19].

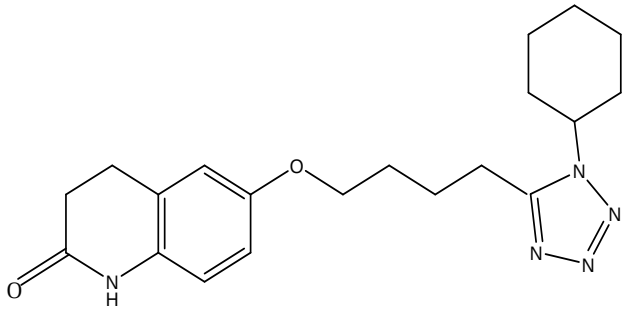

Fig 1a: Cilostazol

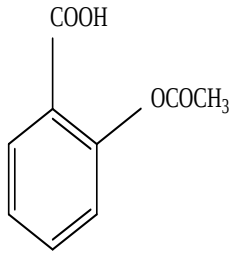

Fig 1b: Aspirin

According to literature survey, it has been revealed that there is only one UV spectrophotometric simultaneous method reported for estimation of the titled analytes[20].

CIL and ASP are co-administered in patients with Intermittent Claudication (IC). This combination therapy significantly increases walking distance in claudicants and therefore has potential to be marketed as combined formulation in future[21-23]. Thus, validated stability indicating RP-HPLC assay method has been developed and applied for the estimation of CIL and ASP in bulk and in tablet formulation that was developed in house. The tablet contains a variable amount of active ingredients due to their recommended Pharmacological dose; i.e. 200 mg CIL and 80 mg ASP. This variable amount of ingredients in such a multi-drug formulation makes the process of routine analysis difficult. Moreover, the active compounds have very different polarity and, therefore chromatographic behavior.

To best of our knowledge, the methods described in the literature do not cover the stability indicating method for simultaneous analysis of selected two analytes. Thus, the objective of this research work was to develop validated stability indicating method for quantifying these two analytes which are present in variable concentrations in tablet dosage form. To achieve this goal, stress testing study was performed according to the International Conference on Harmonization (ICH) recommendations[24-26]. The developed HPLC method used to resolve the drug from different degradation products obtained under stress testing.

Thus, in the present investigation an attempt has been made to develop accurate, precise and selective RP-HPLC method for the simultaneous estimation of CIL and ASP in individual bulk drug samples and in combined dosage formulation. The proposed method was validated as per ICH guidelines.

EXPERIMENTAL

Instrumentation

Chromatographic separation was achieved using a HPLC system consisted of a binary pump (model Waters 515 HPLC pump), auto sampler (model 717 plus), column oven (model-Waters CHM), and PDA detector (Waters 2998). The column used was Nova-pack C18 (4.6mm x 250 mm, particle size 4µm) maintained at 40^0 C and PDA detector was used to acquire the data at 254nm. Data was integrated using Empower version 2 software. The peak purity was checked with the photodiode array detector.

Materials

CIL (% purity 99.88) was supplied as a gift sample by Glenmark Generics Ltd. and ASP (% purity 99.88) was supplied by Alta Lab Ltd. All chemicals and reagents used were of AR grade and all the solvents used were of HPLC grade. Acetonitrile (ACN) purchased from Merck Chemicals, Mumbai. OPA, tetrahydrofuran (THF) and Ammonium formate (AF) from Research Lab Fine Chemical Industries, Mumbai. In house tablet formulation that was developed in the research laboratory containing 200mg CIL and 80 mg ASP was used.

Methods

Chromatographic conditions: Gradient Procedure

Elution was carried out at a flow rate of 0.7 ml/ min with ACN as solvent A and AF (10 mM) as solvent B, pH 3.5, adjusted with OPA. Gradient used for elution with respect to time (and % ACN) was, at 5 min. (35%), 10 min. (65%), 15 min. (85%) and 25 min (35%). Each run was followed by a 5 min. wash with 35% A.

Preparation of solution

Standard Stock Solutions

The standard stock solutions containing 1000 µg/ml of each analytes were prepared separately in ACN. For this accurately weighed CIL and ASP, 25 mg each were transferred to separate 25 ml volumetric flasks containing 20 ml of ACN and volume was made up to the mark with ACN. These stock solutions were further diluted appropriately with solution containing ACN and AF (10 mM) in the ratio of 65:35 % v/v (pH 3.5, adjusted with OPA) hence forth called as diluent, to obtain solutions containing 100 µg/ml of each analyte separately. Mixed standard solutions were prepared from this solutions.

Sample Solution

To determine the content of CIL and ASP in conventional tablet, twenty tablets were weighed; their mean weight was determined and was finely powdered. The weight of the tablet triturate equivalent to 100 mg of CIL (40 mg of ASP) was transferred into a 100 ml volumetric flask containing 80 ml ACN and sonicated for 5 min to ensure complete dissolution of drugs. The extract was filtered, residue was washed with ACN and volume was made up to the mark by adding washings to the flask. 5 ml of this solution was further diluted to 50 µg/ml of CIL and 20 µg/ml of ASP with diluent and 20µL of the sample solution was injected after filtration through syringe filter (0.45 µ PALL Life Sciences) into HPLC system, under the conditions described above. Before sample analysis, system suitability test was performed by injecting the solution five times.

Forced Degradation Studies

The study was intended to ensure the effective separation of CIL and ASP and their degradation peaks at the retention time of CIL and ASP. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method. The stock solutions were prepared with ACN that contains 500µg/ml and 200µg/ml of CIL and ASP, respectively. These solutions were subjected to stress degradation that was carried out in dark, to exclude the possible degradative effect of light except UV degradation. All samples were then diluted with diluent to give 50µg/ml and 20µg/ml of CIL and ASP, respectively, 20µl of sample solution was injected after filtration through syringe filter into HPLC system. The stress conditions are summarized below:

Acid, Base and Neutral Degradation: In the 2 ml of working standard, 1ml of 1N HCl / 1N NaOH / HPLC grade water was added and diluted to 8 ml with ACN, followed by heated at 50 ^{0}C for 2 hrs. The solution was cooled to RT, neutralized except for neutral hydrolysis and diluted up to 10 ml.

Peroxide Oxidation: The 2 ml of working standard was refluxed with 5 ml 6% H_2O_2 for 30 mins. The mixture was cooled at RT and the volume was made up to 10 ml.

Dry Heat Degradation and UV degradation: CIL and ASP 50 mg each were spread as thin film in two separate Petri dishes (50 mm diameter) in two sets. The first set of petri dish was heated in an oven at 100^{0}C for 5 h. while second set of petri dish was exposed to UV radiation 200Watt hrs/ m^2 for 28 h in UV chamber. From this 10 mg of powder was used to

prepared solution of 50µg/ml and 20µg/ml of CIL and ASP, respectively as per the procedure described under "Preparation of sample solution."

Method Validation

Validation of the optimized HPLC method was carried out with respect to the following parameters: Linearity, Precision, Accuracy, Selectivity, robustness, Limit of Detection (LOD), Limit of Quantitation (LOQ) and system suitability.

Specificity

The specificity of the HPLC method was evaluated by injecting the following solutions: solutions of placebo tablets, diluent, solutions of pure standards and sample tablet solutions. The mobile phase resolved both the drugs very efficiently. The specificity was determined under the selected stress conditions like acidic, basic, peroxide, thermal, dry heat and UV degradation.

Linearity and Range

Linearity of the method was established by injecting seven concentrations prepared from stock solutions and diluted as described in experimental section. Solutions containing analytes in the range 5 to 100 µg/ml and 2 to 40 µg/ml for CIL and ASP respectively, in five replicates were injected into the HPLC system keeping the injection volume 20µl and column temperature at 40^0C. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability was assessed by injecting six times 20µl of standard solution (50µg/ml of CIL and 20µg/ml and ASP) into HPLC system under stabilized chromatographic conditions. Further intra and inter-day variation for the determination of CIL and ASP was carried out at three different concentration levels (30, 40 and 50 µg/ml for CIL and 12, 16 and 20 µg/ml for ASP) of the drugs three times on the same day (intra-day) and three consecutive days (inter-day). The %RSD of the obtained assay values at three different concentration levels was calculated.

Limit of Detection and Limit of Quantitation

In order to determine detection and quantification limit, CIL and ASP concentrations in the lower part of the linear range of the calibration curve were used. Solutions containing analytes in the range 1.25 to 30 µg/ml and 0.5 to 12 µg/ml for CIL and ASP respectively were prepared and 20µl was injected into the HPLC system in triplicates. The LOD and LOQ were calculated as per International Conference on Harmonization guidelines (25), using equation: LOD=3.3 × N/B and LOQ=10×N/B, where, N is standard deviation of the peak areas of the drugs (n=3), taken as a measure of noise, and B is the slope of the corresponding calibration curve.

Accuracy

Accuracy of the method was carried out by applying the method to drug sample (CIL and ASP combination tablet) to which known amount of CIL and ASP standard solution corresponding to 50, 100 and 150 % of label claim had been added (Standard addition method), mixed and the powder was extracted and analyzed in optimized chromatographic conditions. Base level amount of CIL and ASP used for spiking were 20 µg/ml and 8 µg/ml respectively.

Robustness

The robustness is the ability of a method to remain unaffected by small changes in conditions. To determine robustness of the method, experimental conditions were purposely altered, and chromatographic resolution between CIL and ASP was evaluated. A variation of ±1 unit in pH of the mobile phase, ±0.5 unit in flow rate, column temperature ± 2°C, different column suppliers and ± 1 unit in wavelength while other mobile phase components were held constant.

RESULTS AND DISCUSSION

Development and Optimization

The stability indicating method capable of eluting wide range of compounds of different polarity, with excellent efficiency and high resolution was thought to be developed. Based on literature survey and review of physico-chemical properties of analytes, chromatographic conditions were selected. Stationary phases (C18, C8), different mobile phases (containing buffers like ammonium acetate, phosphate, and formate) in different concentration (10 to 30 mM) with different pH (pH 3–5) and using organic modifier (ACN,THF 5 – 10 %) were tried in isocratic mode of chromatographic separation. At every time either the analytes and/or

degradation peaks were closed lead to poor resolution or peak shape, tailing factor were not optimum. The ACN: phosphate buffer gave promising separation of analytes, hence was selected for further optimization. Several trials were carried out with different chromatographic conditions like changes in ACN: buffer ratio, pH, temperature, flow rate to get sharp peak with optimum resolution. Although analyte peaks were well resolved, there was no change in the system suitability parameters of degradation peaks of analytes. Hence it was decided to apply gradient mode of elution. During optimization trials, it was found that 35% to 65% of ACN in the initial gradient mode gave sharp peak of ASP, while with further increase in ACN ratio to 85% gave optimally resolved CIL peak with good separation of degradation peaks of both the analytes. All the peaks were sharp with acceptable tailing factor < 1.3. Development studies revealed that ACN :Formate buffer pH 3.5 with OPA at a flow rate of 0.7 ml/min, and column maintained at 40^0 C was suitable conditions for a stability indicating method. Typical retention times of CIL and ASP were about 24.49 ± 0.33 min and 16.29 ± 0.19 min, respectively. Resolution between CIL and ASP founds to be more than 8. In optimized conditions CIL, ASP and their degradation products were well resolved. Chromatograms acquired using optimized conditions by injecting diluent, placebo, mixed standards, tablet formulation and system suitability standards. (Fig. 2)

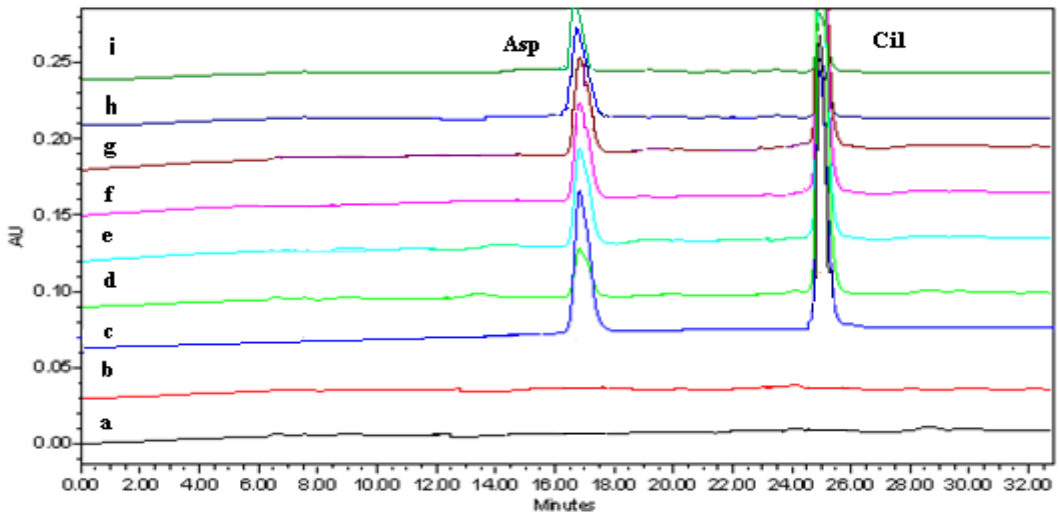

Fig. 2: Overlain chromatograms acquired with a-Mobile phase,b-placebo, c-mixed standard solution, d-tablet solution and e to i -System suitability injections at 20 µg/ml of ASP and 50 µg/ml of CIL.

Specificity

Overlain chromatograms acquired with a-Mobile phase, b-placebo, c-mixed standard solution, d- tablet solution and e to i -System suitability injections at 20 µg/ml of ASP and 50 µg/ml of CIL (Fig 2). No peak was observed in the diluent and placebo chromatograms, indicating an absence of interference from the excepients commonly used in tablet formulations. Additionally well-resolved peaks were observed in the standard mixture chromatograms. The system suitability parameters for the investigated compounds were within the acceptable range (Table 1). Under selected stress conditions, degraded product peaks were well resolved from analyte peaks. Further there was no co-elution of degraded products with analyte peaks as assessed by peak purity study. The Peak angle value was always less than peak threshold value which indicate homogenous peak and absence of co-elution of degraded products. (Table 6)

Linearity

The Linearity range for both analytes was found to be 5 to 100 µg/ml and 2 to 40 µg/ml for CIL and ASP respectively The regression equation for CIL and ASP were found to be y = 59868x - 1266 and y = 6025x + 259.2 respectively with correlation coefficient of determination, (r^2) 0.999 for both the analytes. (Table 1).

LOD and LOQ

The LOD and LOQ were found to be 0.37 and 1.14 µg/ml for CIL, and 0.25 and 0.78 µg/ml for ASP. The calculated values were assessed practically as described above. Low values of LOD and LOQ indicates adequate sensitivity of the method (Table 1).

Table 1: Regression Characteristics and System Suitability parameters of proposed RP-HPLC method

Parameter↓ / Analytes (t_R) →	**ASP**	**CIL**
Concentration range (µg/ml)	2 to 40	5 to 100
Retention time (min)	16.34	24.39
Regression equation (Y= b × Concentration ± a) data		
Intercept	259.2	-1266
Slope	6025	59868
Correlation coefficient (r^2)	0.999	0.999
Method sensitivity (µg/ml)		
Limit of Detection	0.25	0.37
Limit of Quantitation	0.78	1.14
System suitability Test (SST) Parameter		
Peak Area, % RSD	121389.8, 0.81	3006128, 0.85
No of theoretical plates (% RSD)	15505.73, 2.12	30138.89, 1.72
USP Tailing Factor (±SD)	1.823 ± 0.064	1.266 ± 0.037
USP resolution(R) and Capacity Factor (k)	1.5 (k)	8.18 (R)
Typical Peak Purity data		
Peak Angle	0.145	0.191
Peak threshold	0.257	0.215

Precision

The RSD in precision studies was found to be 0.21 – 0.48% (Intra-day) and 0.44 – 0.79% (Inter-day) indicating that the method is precise (Table 2).

Table 2 : Intra-day and Inter-day Precision Studies (n=3)

Drug	**Conc.**	**Intra-day**		**Inter-day**	
		Mean peak area*, (±SD)	**% RSD**	**Mean peak area*,** (±SD)	**% RSD**
CIL	30 µg/ml	1781984 ± 8645.6	0.485	1740650 ± 8202.7	0.471
	40µg/ml	2504527 ± 7941.1	0.317	2498159 ± 11100.2	0.444
	50µg/ml	3059992 ± 10423.6	0.341	3116076 ± 24824.0	0.797
ASP	12 µg/ml	78183 ± 166.7	0.213	77772.67 ± 593.5	0.763
	16µg/ml	97184.67 ± 212.1	0.218	97643.33 ± 480.1	0.492
	20µg/ml	128025.7 ± 500.2	0.391	122303.7 ± 591.9	0.484

*mean of three determinations.

Accuracy (Recovery Studies) and Analysis of the Commercial Formulation

Results of recovery studies were in the range of 98 to 102% (Table 3) show that formulation excepients do not have effect on recovery of analytes. The % RSD of analysis of tablet formulation was found to be less than 2 % (Table 4).

Table 3 : Accuracy–Recovery study by standard-addition method.

Recovery	Base Level Amount (µg/ml)		Amount Spiked (µg/ml)		% Mean Recovery, % RSD (n=3)	
Level	CIL	ASP	CIL	ASP	CIL	ASP
50%	20	8	10	4	98.95, 0.91	99.99, 0.14
100%	20	8	20	8	101.64, 0.77	101.55, 0.73
150%	20	8	30	12	100.38, 0.86	100.82, 0.91

Table 4 : Assay of Tablet formulation

Formulation	Labeled Claim (mg)		Amount found (n=3)		% Estimated, % RDS	
	CIL	ASP	CIL	ASP	CIL	ASP
F1	200	80	200.56	80.78	100.28, 1.04	100.97, 1.39
F2	200	80	200.95	79.92	100.47, 1.13	99.91, 0.91

Robustness

Small deliberate alteration in chromatographic conditions did not cause significant change in the relative retention times between peaks for the investigated compounds (Table 5) indicates that the HPLC method was robust to minor changes in the experimental conditions. The % RSD of resolution was less than 2% indicate that the developed method was robust.

Table 5 : Result of robustness study (n=3)

Parameter (Limit)	Level	Resolution (± SD)	% Assay, % RSD
Flow Rate , (± 0.02 ml/min)	(–) 0.68	8.18 ± 0.22	98.89, 0.76
	(+) 0.72	8.36 ± 0.41	100.87, 0.94
pH of Mobile Phase (± 0.1)	(–) 3.4	8.05 ± 0.25	100.2, 1.4
	(+) 3.6	8.30 ± 0.27	101.79, 1.36
Column	CI[a]	8.17 ± 0.35	99.38, 0.62
	CI[b]	8.08 ± 0.22	100.87, 0.94
Wavelength (± 1 nm)	(–) 249	8.15 ± 0.25	101.2, 1.2
	(+) 251	8.12 ± 0.29	100.15, 0.82
Column Temp. (± 2 ^{O}C)	(–) 38	8.05 ±0.35	101.23, 1.26
	(+) 42	8.17 ± 0.32	98.94, 1.23

[a]*Nova Pack C18,* [b] *Kromasil C18 columns.*

Specificity and Forced Degradation

During the forced degradation experiments it was observed that ASP was more sensitive towards acid, alkaline, neutral and oxidative degradation than photo and thermal degradation. On the other hand, CIL was susceptible to acid, alkaline and oxidative degradation; while, it

showed stability towards neutral hydrolysis, photo and thermal degradation. Overall, ASP was found to be more sensitive towards degradation conditions than CIL. Under the optimized chromatographic conditions degradation products of both analytes were well separated and there was no degradation peak eluting at the retention time of either analyte. (Fig.3 - Fig.8) Chromatographic peak purity data was obtained from the spectral analysis report. Purity angle values were less than purity threshold values are indicative of a homogeneous peak thus established the specificity of the assay. The percentage degradation of CIL and ASP when subjected to selected stress conditions, were calculated by comparing the peak area of CIL and ASP before and after treatment (Table 6).

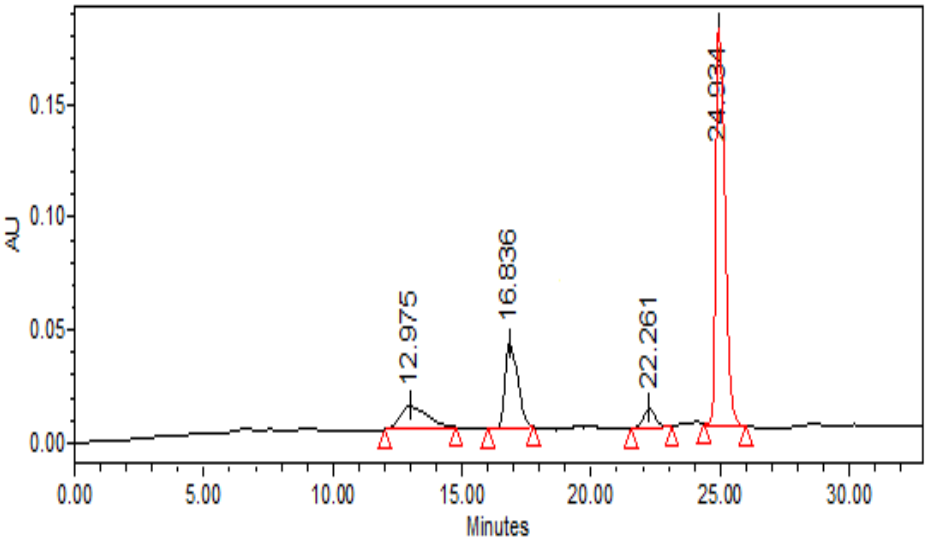

Fig 3: Chromatogram: Acid degradation product: CIL and ASP

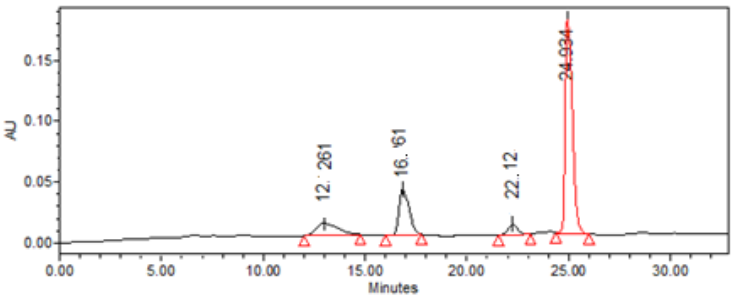

Fig 4: Chromatogram: Base degradation product, CIL and ASP

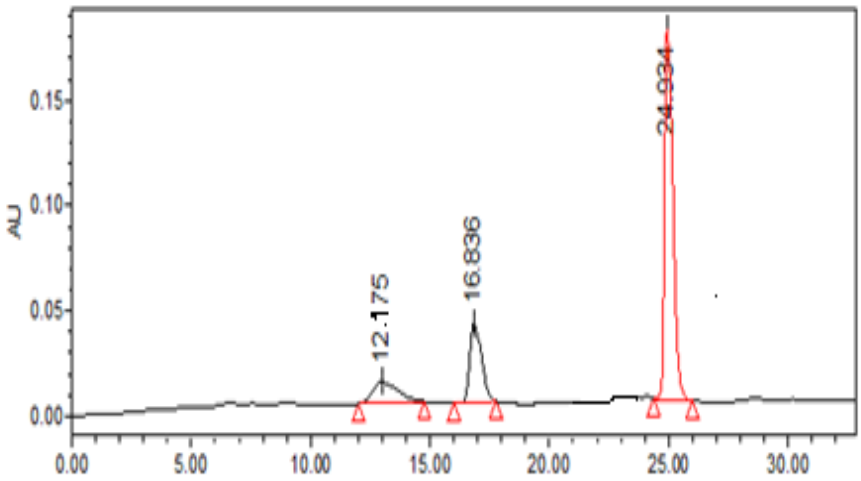

Fig 5: Chromatogram: Neutral Hydrolysis product: CIL and ASP

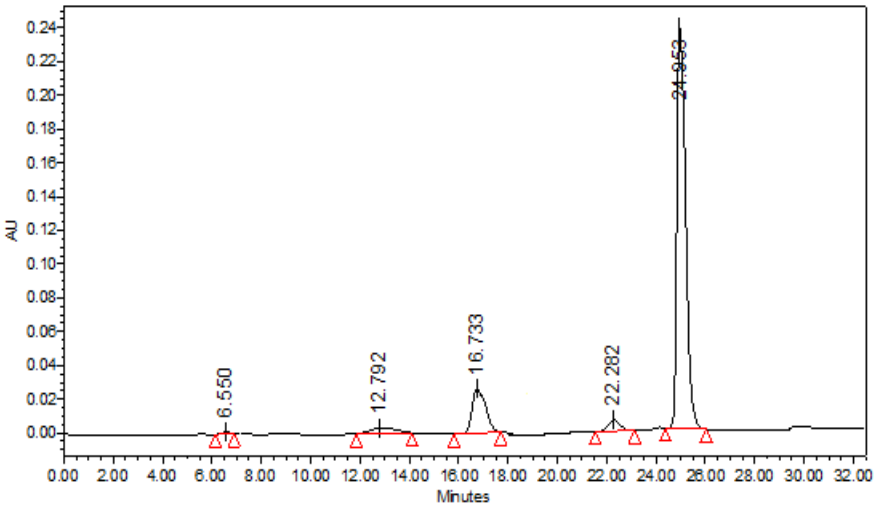

Fig 6: Chromatogram: Oxidation degradation product: CIL and ASP

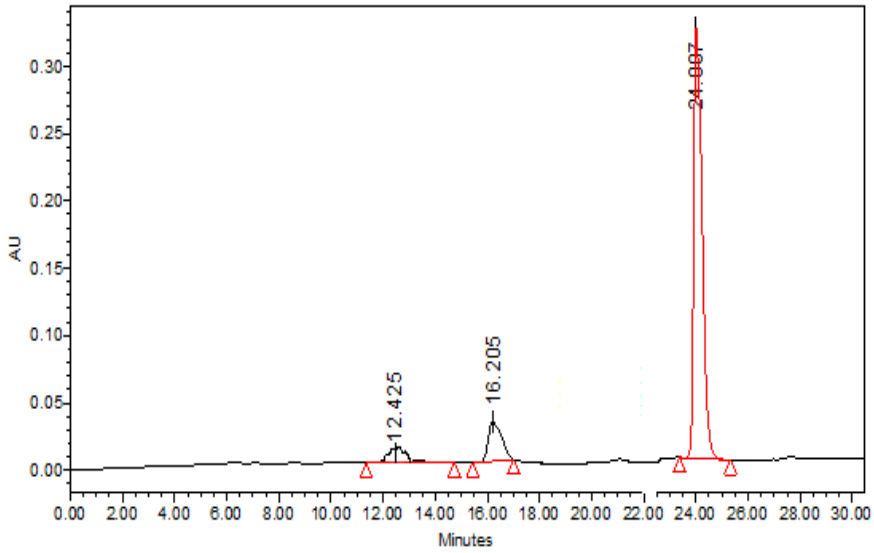

Fig.7: Chromatogram: Thermal degradation product: CIL and ASP

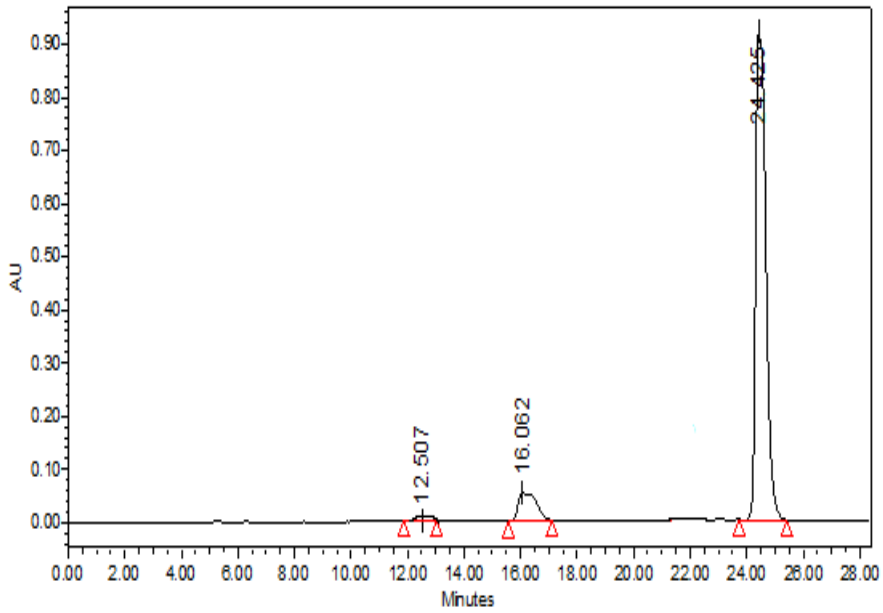

Fig 8 : Chromatogram: UV degradation product: CIL and ASP

Table 6: Summary of Forced Degradation Study for CIL and ASP

Analyte→ Stress condition ↓	Cilostazol			Aspirin		
	t_R of Degraded Product	% Recovery	Peak* angle, Threshold	t_R of Degraded Product	% Recovery	Peak * angle, Threshold
Acid (1 N HCL, 2 h. , 50 ^{0} C)	22.26	97.78	0.156, 0.271	12.97	90.89	0.181, 0.242
Base(1 N NaOH, 2 h/ mins,50 ^{0} C)	22.12	98.53	0.245, 0.356	12.26	80.42	0.246, 0.384
Neutral (reflux on water bath, 2 h)	___	____	0.189, 0.372	12.17	88.20	0.182, 0.243
Hydrogen peroxide 6% (reflux, 30 mins)	22.28	95.62	0.177, 0.302	12.79	85.82	0.149, 0.221
Dry Heat (100^{0}C, 5 h)	___	99.84	0.256, 0.312	12.42	95.32	0.183, 0.264
UV Photostability 200Watt hrs/ m^2 for 26 hrs	___	99.53	0.167, 0.335	12.50	97.73	0.172, 0.318

*t_R = Retention time, * Peak angle value less than peak threshold indicate homogenous peak*

CONCLUSION

HPLC method was developed and validated as per ICH guidelines. The method showed linear response in stated range and was accurate and precise. Statistical analysis proves that

the method is suitable for simultaneous quantitative analysis of CIL and ASP in bulk drugs and formulations without any interference from the excepients and degradation. Thus this method can be successfully employed for the analysis of stability samples of CIL and ASP.

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