# DEVELOPMENT OF STABILITY INDICATING METHOD OF CLEVIDIPINE BUTYRATE

## 3.1. SELECTION OF DRUG

Clevidipine butyrate (CLEVI) is dihydropyridine calcium channel blocker [1]. It is approved by US, FDA in 2008 for the treatment of pre-operative and post-operative hypertension. It is marketed as CLEVIPREX injection by The Medicines Company, US. It is available as injectible emulsion when oral therapy is not desirable. Clevidipine has been included in British approved Names 2017 by British Pharmacopoeial Commission 2017 [2] .Till now it is not launched in India. At the time of undertaking the study, no data on stability was available in literature. During the conduct of study, a research paper has been published reporting the degradation data of Clevidipine butyrate [13]. The drug was therefore selected for developing HPLC and HPTLC based stability indicating assay method

## 3.2. DRUG PROFILE

### General Properties

*IUPAC name*: methyl 5-{[(butanoyloxy) methoxy] carbonyl}-4-(2, 3-dichlorophenyl)-2, 6-dimethyl-1, 4-dihydropyridine-3-carboxylate [3]

*Molecular Formula:* $C_{21}H_{23}Cl_2N_2O_6$ [4]

*Molecular Weight*: 456.316

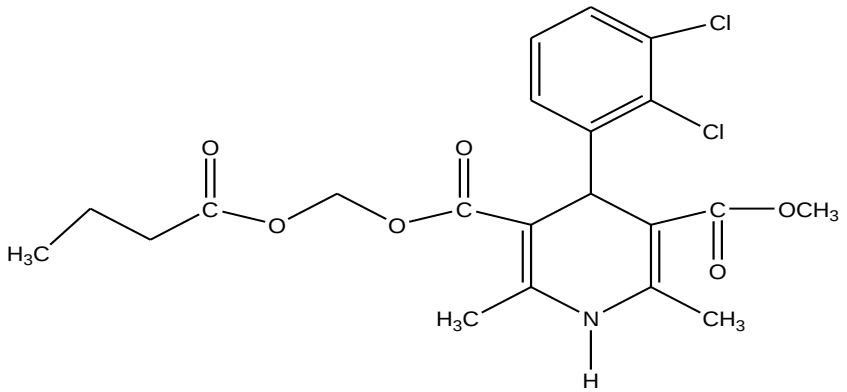


**Fig. 3. 1 - Structure of CLEVI**

*Chemical Structure:* racemic mixture

*Appearance:* White amorphous powder

*Melting point:* 135-137ºC

*Log P*: 4.98

*pKa*: Strongly Basic (5.31)

*Solubility*: practically insoluble in water, soluble in methanol and acetonitrile

*Drug Category*: calcium channel blocker

*Mechanism of action*: Clevidipine produces action by inhibition of transmembrane calcium influx through alpha 1 subunit of L-type voltage gated calcium channel. It causes vasodilatation of vascular smooth muscle with very little effect on myocardial contraction. It produces relaxation of thromboxane A2-induced contraction of arterial smooth muscle [5].

*Uses*: Antihypertensive

Marketed Formulation: Cleviprex injectible emulsion (The Medicines Company) contains 0.5 mg/mL of Clevidipine [6, 7]

As CLEVI formulation is not available in Indian market, synthetic mixture was prepared for degradation study and recovery study as per the formula mentioned in patent [14] and is shown in Table 3.1.

**Table 3. 1- Composition of CLEVI synthetic mixture**

| **Composition** | **% w/v** |
|---|---|
| Clevidipine | 0.05 |
| Soyabean oil | 20 |
| Glycerin | 2.25 |
| Disodium Edetate | 0.005 |
| Sodium citrate | 0.1 |
| Egg yolk phospholipid | 1.2 |

| | |
|---|---|
| Water for injection | Up to 100% |

### 3.3. LITERATURE REVIEW

- *Spectral analysis and structural elucidation of Clevidipine Butyrate by Huang L et.al.[8]*

CLEVI is a short acting dihydropyridine calcium channel blocker. The spectral data of CLEVI were reported by UV, IR, NMR ($^{1}$H NMR, $^{1}$H-$^{1}$H COSY, $^{13}$C NMR, DEPT, HMQC and HMBC) and MS data. The molecular structure of CLEVI was determined.

- *Separation of Clevidipine and enantiomers of dihydropyridine substituted acid using Supercritical Fluid Chromatography technique by Gyllenhaal et. al. [9]*

Enantiomers of dihydrosubstituted acid were separated on a 50 x 4.6 mm i.d. short Chiralpak AD column with 2-propanol modified carbon as the mobile phase. Enantiomers of dihydro substituted acid were obtained by alkaline hydrolysis and were extracted using dichloromethane. CLEVI was analysed in presence of enantiomers of dihydrosubstituted acid.

- *Determination of Clevidipine and its primary metabolite in rat plasma by a dispersive liquid-liquid micro extraction method by Zhou Y et.al.[10]*

Dispersive liquid –liquid micro extraction based on solidification of a floating organic droplet combined with HPLC with UV detection was developed for determination of CLEVI and its primary metabolite in Sprague-Dawley rat plasma samples. For extraction, plasma protein was precipitated by mixture of zinc sulphate solution and acetonitrile. The limits for quantification were 2.5 and 5.0 ng/mL for CLEVI and its primary metabolite. Linearity of CLEVI and its primary metabolite was obtained with coefficient of correlation above 0.9979. Precision and accuracy with intra and inter-assay coefficients of variation were less than 6.1% at all concentrations. The given pretreatment technique with HPLC was applied for determination of CLEVI and its primary metabolite in rat plasma samples.

- *Estimation of clevidipine in presence of its primary metabolite in dog plasma using liquid chromatography – tandem mass spectrometry and their pharmacokinetic studies by Zhou Y et. al. [11]*

LC-MS/MS method for determination of CLEVI and its primary metabolite H152/81 in dog plasma. Protein precipitation was performed with acetonitrile and Felodipine was used as internal standard. Separation was performed on XB C18 column (50 mm X 2.1mm, 3.5 µm). Buffer used in mobile phase was ammonium acetate buffer 20 mM pH 7.0. Mobile phase was composed of buffer and acetonitrile (60: 40) . Flow rate was maintained at 0.3 mL/min. Run time was 5.5 min. Mass spectrometric analysis was performed on triple quadruple mass spectrometer operated in the multiple reaction monitoring mode . The method was validated as per EMEA guidelines. The method was applied for pharmacokinetic study for determination of CLEVI and H152/81 in healthy beagle dogs.

- *Determination of clevidipine in presence of its primary metabolite in beagle plasma using LC-MS/MS by Chen T et. al. [12]*

LC-MS/MS method was developed for simultaneous determination of CLEVI and its primary metabolite desethylfelodipine in beagle plasma. Method was applied to bioequivalence study of CLEVI lipid emulsion injection in beagle dog. Nimodipine was used as internal standard. Method was developed on Synergi Polar-RP C 18 column with mobile phase composed of 0.1 % formic acid in acetonitrile and 10 mM ammonium acetate. Analytes were detected by multiple reaction monitoring mode with electron spray ionization. Linearity was plotted in the range of 1-200 ng/mL for CLEVI and 5-1000 ng/mL for desethylfelodipine with regression coefficient $r^2$ greater than 0.995.

- *RP-HPLC method for structural analysis and quantitative determination of Clevidipine butyrate and their impurities by Zhou F et.al. [13].*

Eleven degradation products and process-related compounds were analyzed by studies on the manufacturing process of CLEVI. MS, NMR techniques have been used for the structural characterization of three impurities. Advance RP-HPLC method was developed for separation and quantification of impurities. Four degradation products were observed under stress conditions. The method was developed as per ICH guidelines.

## 3.4. SECTION –A

## DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD

### 3.4.1. EXPERIMENTAL

#### 3.4.1.1. Chemicals and Reagents

- CLEVI bulk was purchased from Angene Chemical Ltd, China.
- Methanol and acetonitrile (HPLC grade) were procured from Rankem Pvt.Ltd. Mumbai.
- Potassium dihydrogen orthophosphate (AR grade) and ortho phosphoric acid were procured from Loba Chemie Pvt. Ltd. Mumbai.
- Hydrochloric acid (HCl), sodium hydroxide (NaOH), hydrogen peroxide ($H_2O_2$) were purchased from S.D. Fine Chemical Ltd. Mumbai.
- 0.22 µm Nylon 6,6 membrane filter , Ultipore® N,66® for filtration of mobile phase was procured from Pall Life Sciences ,USA.
- 0.45 µm Nylon 6,6 syringe filter for sample filtration was procured from Pall Life Sciences, USA.

#### 3.4.1.2. Equipments and Instruments

The instruments and equipments used throughout the research work are:

- **Precision analytical balance** (A X 120, by Shimadzu Corporation analytical and measuring Instruments division, Kyoto, Japan)
- **Ultrasonic bath** (Analab Scientific Instruments Pvt. Ltd., Vadodara)
- **pH Meter** (LabIndia Instruments Pvt. Ltd., Navi Mumbai)
- **Shimadzu UV-1700 double beam spectrophotometer** (Shimadzu, Japan) equipped with Shimadzu UV probe 2.10 software
- **Precision water/oil baths** attached with temperature controller for hydrolytic and oxidative degradation study
- **Photo stability chamber** (Thermolab Scientific Equipments Pvt. Ltd, Vadodara) containing light bank with four UV (OSRAM L73) and fluorescent (OSRAM L20) lamps, with specifications as per the guidelines by ICH Q1B for photolytic degradation study
- **High Performance Liquid Chromatography (HPLC)** : HPLC system comprised of Shimadzu (Shimadzu corporation, Kyoto, Japan) having

Shimadzu LC-20 AD pump, Shimadzu PDA-M20A diode Array Detector, Rheodyne 7725 injector valve with fixed loop at 20 µL. The software used for data acquisition and integration was LC solution software (Shimadzu Corporation, Kyoto, Japan).

### 3.4.1.3. Chromatographic conditions

Buffer used in the mobile phase was phosphate buffer (10 mM) which was prepared by adding 1.36 g of potassium dihydrogen orthophosphate ($KH_2PO_4$) to 1 L of double distilled water. The pH 3.0 with was adjusted with ortho phosphoric acid. The mobile phase consisted of mixture of 10 mM phosphate buffer (pH 3.0) and acetonitrile in the ratio of 40: 60. Analysis was performed at ambient temperature with detection wavelength at 239 nm and flow rate of 1mL/min. The injection volume was 20µL. Analysis was performed on Thermo Hypersil BDS C-18 column (250 x 4.6 mm i.d. x 5 µm particle size).

### 3.4.1.4. Preparation of Standard solution

CLEVI standard solution (1mg/mL) – 25 mg of CLEVI was weighed accurately and transferred to 25 mL volumetric flask, dissolved in methanol and volume was made up to the mark.

Working standard solutions were prepared in mobile phase to produce concentration in the range of 20- 120 µg/mL with respect to CLEVI.

### 3.4.1.5. Preparation of forced degradation sample

For forced degradation study, stock solution of CLEVI (1mg/mL) was prepared in methanol**.**

#### 3.4.1.5.1. Acid degradation

2.5 mL of CLEVI stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 1 M HCl. The solution was heated at 60ºC for 1 hour. The solution was neutralized with 1 mL of 1 M NaOH and volume was made up to 25 mL with mobile phase to make the concentration of 100µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

**3.4.1.5.2. Alkaline degradation**

2.5 mL of CLEVI stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 0.01 M NaOH. The solution was heated at 60ºC for 1 hour. The solution was neutralized with 1 mL of 0.01 M HCl and volume was made up to 25 mL with water to make the concentration of 100µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

**3.4.1.5.3. Oxidative degradation**

2.5 mL of CLEVI stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 3% hydrogen peroxide. The solution was kept at room temperature for 1 hour. The volume was made up to 25 mL with mobile phase to make the concentration of 100µg/mL. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter before injecting into HPLC system.

**3.4.1.5.4. Dry heat degradation**

For dry heat degradation, 25 mg of CLEVI was kept in oven at 80ºC for 11 days. The sample was transferred to 25 mL of volumetric flask, dissolved in methanol. From this, concentration of 100 µg/mL of solution was prepared and injected into HPLC system.

**3.4.1.5.5. Photolytic degradation**

For photolytic degradation, 25 mg of CLEVI was spread in 1 mm thickness and was exposed to 5383 Lux and 144 UV/$cm^2$ for 11 days. Volume was made up to 25 mL and from this, concentration of 100µg/mL was prepared and injected into HPLC system.

**3.4.1.6. HPLC method validation**

The developed method was validated as per ICH Q2B guideline.

For linearity, standard dilutions of CLEVI were prepared in the concentration ranging from 20 to 120µg/mL from CLEVI standard solution and were injected in triplicate. Linearity was determined by plotting peak area and concentration of solution. From the graph regression equation and regression coefficient was determined.

For precision, intra-day and inter-day precision were evaluated at six concentration levels (in triplicates). Peak areas corresponding to the concentration was calculated and % RSD was determined for intra-day and inter -day precision.

% Recovery was evaluated by standard addition method. Accuracy of method was confirmed by recovery study from synthetic mixture at 3 level of standard addition (50%, 100% and 150%). The concentrations (total concentration) for accuracy were 40, 60, 80, 100µg/mL. % recovery was evaluated at concentration of 40µg/mL. The concentrations were analysed in triplicates. % recovery and % RSD were calculated.

Limit of detection and limit of quantification was calculated on the basis of standard deviation of the intercept and slope of the calibration curve. LOD and LOQ were calculated using equation 3.3*(σ/S) and 10*(σ/S), where σ is the standard deviation of intercept and S is the slope of the calibration curve.

For robustness, factors like pH of buffer (2.8, 3.0 and 3.2), percentage of acetonitrile in the mobile phase (58, 60 and 62) and flow rate (0.9, 1.0, 1.1mL/min) were changed. Robustness of the method was evaluated at 20 µg/mL of concentration in triplicates.

The standard stock solution of CLEVI was prepared in methanol and kept for 24 hrs at room temperature. The stability of stock solution was determined.

Specificity of the method was evaluated by analysis of API as well as synthetic mixture along with degradation products and to check the method for interference of any peaks affecting the estimation of CLEVI.

System suitability tests were performed injecting six times of the concentration. The parameters retention time, asymmetry factor and theoretical plates were noted.

### 3.4.2. RESULTS

#### 3.4.2.1. Determination of suitable wavelength

CLEVI solution of 10µg/mL in methanol was prepared and was scanned in the UV region of 200-400 nm and the spectrum was recorded. CLEVI showed strong absorbance at 239 nm which was selected as the analytical wavelength (Fig. 3.2).

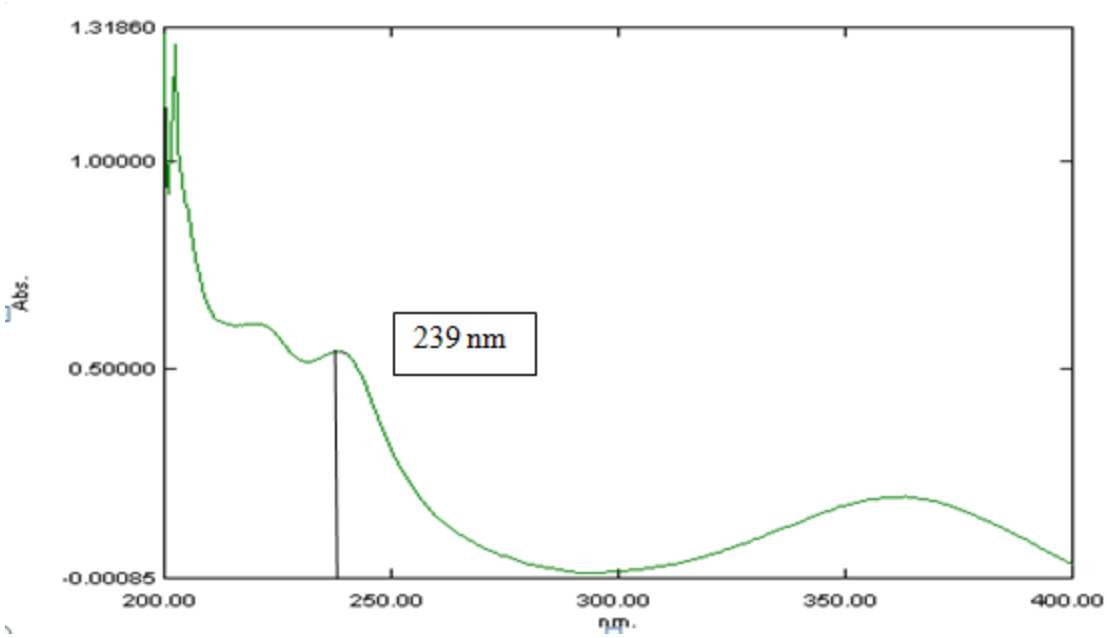


**Fig. 3. 2 – Zero order spectra of CLEVI (10µg/mL)**

### 3.4.2.2. Method optimisation and development

For optimisation of chromatographic conditions, effect of chromatographic parameters like pH of mobile phase, composition of mobile phase and flow rate were studied. The chromatograms were recorded and chromatographic parameters like theoretical plates, asymmetric factors were recorded. The results of the trials are shown in Table 3.2.

**Table 3. 2 – Optimisation of HPLC conditions**

| Mobile phase | Column | Rt (min) | Observation |
|---|---|---|---|
| Water : ACN (50 :50) | Thermo Hypersil BDS C-18 (250 x 4.6 , 5 µ) | --- | No peak up to 20 min |
| Water : ACN (40 :60) | Thermo Hypersil BDS C-18 (250 x 4.6 , 5 µ) | --- | No peak up to 20 min |
| Phosphate buffer pH 3.0 : | Thermo Hypersil BDS | 7.423 min | Sharp and Symmetrical |

| | | | |
|---|---|---|---|
| Acetonitrile : Methanol (30 : 65 : 5) | C-18 (250 x 4.6 , 5 µ) | | Peak |
| Phosphate buffer pH 3.0 : Acetonitrile (25 : 75) | Thermo Hypersil BDS C-18 (250 x 4.6 , 5 µ) | 5.417 min | Sharp and Symmetrical Peak |

Initially method was optimized with phosphate buffer pH 3 and acetonitrile in the ratio of 25: 75. Apart from quantification of CLEVI, degradation products estimation is also of importance development of stability indicating assay method. To resolve degradation products from CLEVI in development of stability indicating method, the method was modified. Phosphate buffer with acidic pH was found to be suitable for peak shape and retention of CLEVI, resolution of CLEVI and degradation product. The final optimised conditions for HPLC method of CLEVI are shown in Table 3.3 and optimised chromatogram of standard solution is shown in Fig.3.3.

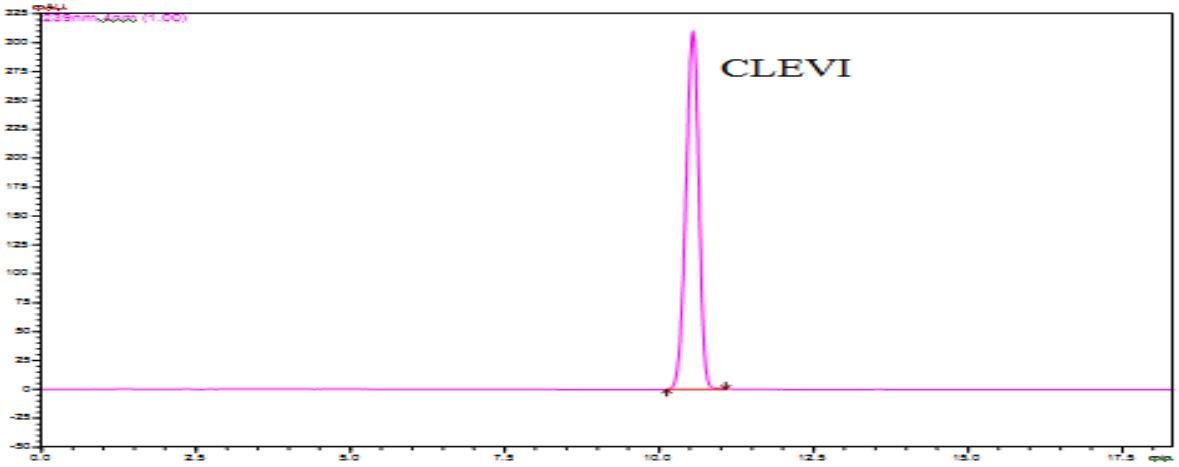


**Fig. 3. 3 – Chromatogram of standard solution of CLEVI (100 µg/mL)**

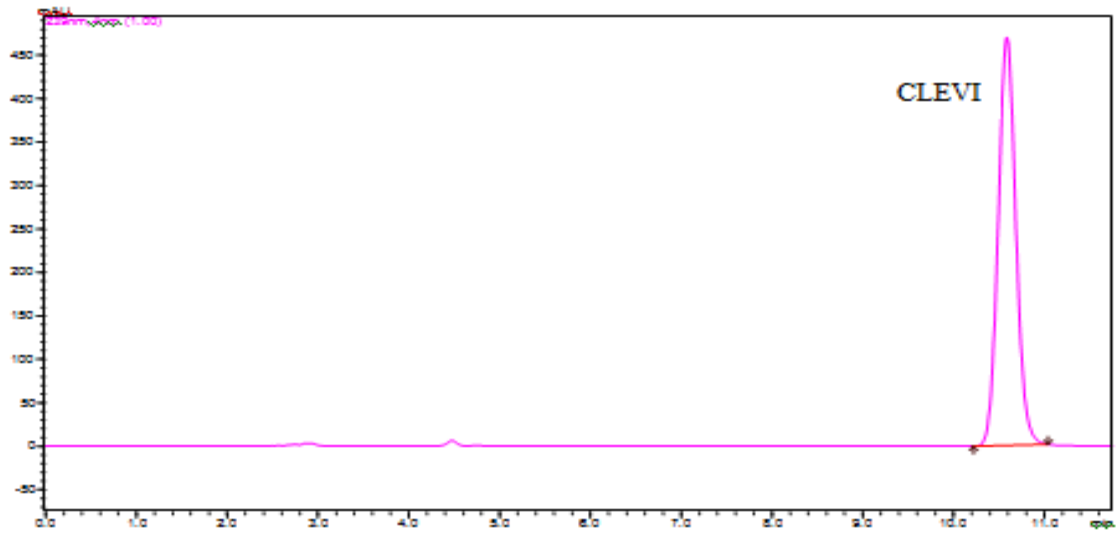


**Fig. 3. 4 - Chromatogram of synthetic mixture of CLEVI (100 µg/mL)**

**Table 3. 3 – Optimised HPLC parameters**

| Parameters | Optimised Value |
|---|---|
| Column | Thermo Hypersil BDS C-18 (250 x 4.6mm i.d. , 5µ particle size ) |
| Mobile phase | Phosphate buffer 10 mM pH 3.0 and acetonitrile (40 : 60) |
| Flow rate | 1.0 mL/min |
| Retention time | 10.65 ± 0.16 min |
| Detection wavelength | 239 nm |
| Needle wash | Mobile phase |
| Column temperature | Ambient |

### 3.4.2.3. Method validation using ICH Q2(R1) guideline

#### 3.4.2.3.1. *Linearity and range*

The calibration plotted for CLEVI was found to be linear in the range of 20-120µg/mL. The regression equation was found to be y= 44304x- 28213 with regression coefficient ($r^2$) of 0.999.The linearity data is shown in Table 3.4 and calibration curve is shown in Fig. 3.5.

**Table 3. 4 – Linearity data of CLEVI**

| Conc. (µg/mL) | Peak Area (Mean* ± %RSD) |
|---|---|
| 20 | 918254.66 ± 0.87 |
| 40 | 1671528.33± 0.58 |
| 60 | 2648847.00 ± 0.82 |
| 80 | 3506948.33 ± 0.65 |
| 100 | 4378131.33 ± 0.80 |
| 120 | 5240234.66 ± 0.91 |

*Average of three determinants

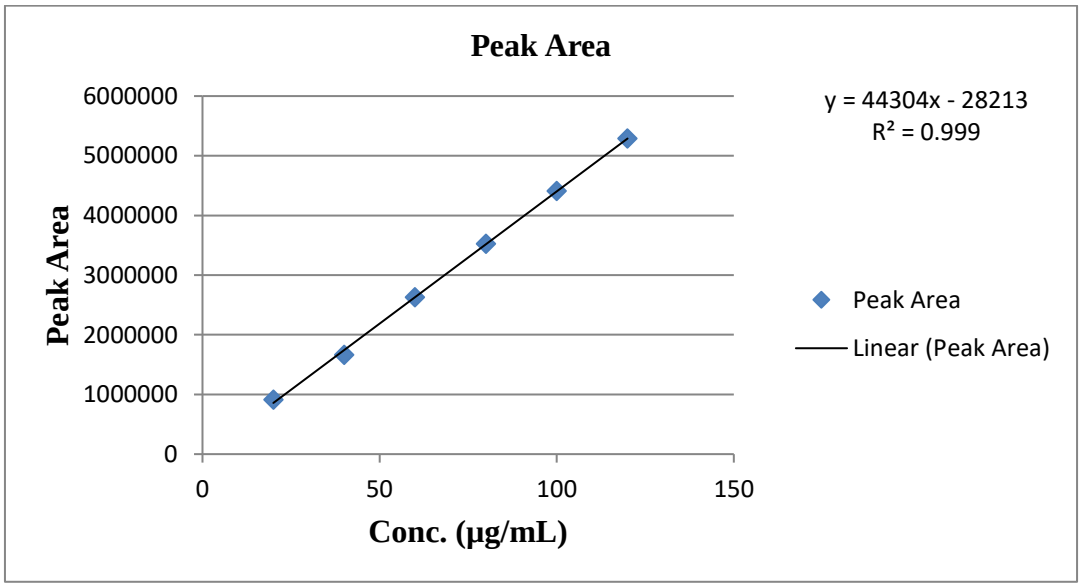


**Fig. 3. 5 – Calibration curve of CLEVI (Peak Area versus Conc. (µg/mL)**

### 3.4.2.3.2. *Precision*

Intra-day precision was performed by repeating the experiment three times in a day and inter-day precision was performed by repeating the experiments on three consecutive days. The average % RSD of intra-day and inter-day were found to be 0.78 and 0.88. The developed method was found to be precise (Table 3.5 and 3.6).

**Table 3. 5 – Intraday Precision of CLEVI**

| | Peak Area | | | | |
|---|---|---|---|---|---|
| **Conc.(µg/mL)** | **Set 1** | **Set 2** | **Set 3** | **Mean** | **%RSD** |
| 20 | 912996 | 903845 | 918346 | 911729 | 0.80 |
| 40 | 1665859 | 1693745 | 1659364 | 1672989.33 | 1.09 |
| 60 | 2631258 | 2592643 | 2641846 | 2621915.66 | 0.98 |
| 80 | 3528108 | 3501734 | 3491745 | 3507195.66 | 0.53 |
| 100 | 4411056 | 4319354 | 4402845 | 4377751.66 | 1.15 |
| 120 | 5289040 | 5301745 | 5293745 | 5294843.33 | 0.12 |
| | | | Average | %RSD | 0.78 |

**Table 3. 6 – Interday Precision of CLEVI**

| | Peak Area | | | | |
|---|---|---|---|---|---|
| **Conc. (µg/mL)** | **Set 1** | **Set 2** | **Set 3** | **Mean** | **%RSD** |
| 20 | 912996 | 914311 | 917457 | 914921.33 | 0.25 |
| 40 | 1665859 | 1645881 | 1672845 | 1661528.33 | 0.84 |
| 60 | 2631258 | 2641818 | 2683465 | 2652180.33 | 1.04 |
| 80 | 3528108 | 3510106 | 3482631 | 3506948.33 | 0.65 |
| 100 | 4411056 | 4320700 | 4382638 | 4371464.66 | 1.05 |
| 120 | 5289040 | 5138235 | 5193429 | 5206901.33 | 1.46 |
| | | | Average | %RSD | 0.88 |

**3.4.2.3.3. *Accuracy***

Accuracy of method was determined by calculating % percent recovery of the analyte recovered. To the sample concentration of 40µg/mL, standard solution of CLEVI was added as 50%, 100% and 150% to give concentrations as 60, 80, 100 µg/mL. Recovery greater than 99% indicates the developed method was accurate (Table 3.7).

**Table 3. 7- Recovery study of CLEVI**

| Excess drug added to analyte (%) | Theoretical Content (µg/mL) | *Amount Found (µg/mL) | Recovery (%)± SD |
|---|---|---|---|
| 0 | 40 | 40.07 | 100.08 ± 0.28 |
| 50 | 60 | 60.00 | 99.68± 0.31 |
| 100 | 80 | 79.92 | 99.83 ± 0.38 |
| 150 | 100 | 99.94 | 99.72±0.12 |

*Average of three determinants

### 3.4.2.3.4. *Limit of detection and limit of quantification*

LOD and LOQ were found to be 0.65 and 1.97 µg/mL.

### 3.4.2.3.5. *Robustness*

For robustness study, slight changes were pH of buffer, % of organic in mobile phase and flow rate. The results were expressed as % RSD. % RSD less than 2 indicated that the developed method was robust (Table 3.8).

**Table 3. 8 – Robustness data of CLEVI**

| Factor | Level | Area | | Rt | | Asymmetry | | Theoretical Plates | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | %RSD | Mean | %RSD | Mean | %RSD | Mean | %RSD |
| pH | 2.8 | 1653811 | 1.25 | 10.53 | 0.06 | 0.93 | 0.34 | 11209.03 | 0.0002 |
| | 3 | 1656257 | 1.03 | 10.54 | 0.13 | 0.94 | 0.52 | 11339.13 | 0.003 |
| | 3.2 | 1652879 | 1.23 | 10.54 | 0.15 | 0.93 | 0.38 | 11340.14 | 0.001 |
| % Organic | 58 | 1645213 | 1.27 | 9.84 | 0.31 | 0.93 | 0.42 | 11157.1 | 0.13 |
| | 60 | 163405 | 1.29 | 10.5 | 0.15 | 0.93 | 0.32 | 11339.1 | 0.22 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 4 | | | | 7 | |
| | 62 | 1651930 | 1.08 | 11.06 | 0.22 | 0.93 | 0.38 | 11246.48 | 0.28 |
| Flow rate | 0.9 | 1668082 | 1.02 | 10.99 | 0.23 | 0.93 | 0.37 | 11172.8 | 0.37 |
| | 1 | 1640372 | 0.87 | 10.54 | 0.17 | 0.93 | 0.21 | 11339.17 | 0.22 |
| | 1.1 | 1638260 | 1.07 | 10.33 | 0.25 | 0.93 | 0.37 | 11327.81 | 0.46 |

Flow rate is the critical factor in the robustness method.

**3.4.2.3.6. *Stability in sample solutions***

Stock solution of CLEVI and stressed samples were prepared from standard stock solution and then stored at room temperature for 24 hrs. No additional peaks were observed which indicated stability of CLEVI sample solution.

**3.4.2.3.7. *Specificity***

The specificity was determined from the forced degradation studies as described in section 3.4.1.5. and 3.4.2.4. where Fig. 3.16 shows CLEVI peak is well separated from all degradation products formed during different stress conditions with sufficient resolution. In the forced degradation studies, for all degradation products, peak purity index was greater than single point threshold, ensures degradation peaks are pure and peaks are not co-eluting. The specificity study ensures selectivity of the developed method which is able to separate and quantify CLEVI in presence of degradation products. Peak purity data of CLEVI and degradation products are shown in Table 3.9.

**Table 3. 9- Peak purity data of CLEVI and its degradation products**

| S.No. | Peaks | Rt | Peak Purity Index | Single Point threshold | Resolution |
|---|---|---|---|---|---|
| 1 | CLEVI | 10.65 min | 1.0000 | 0.999999 | 9.163 |

| | | | | | |
|---|---|---|---|---|---|
| 2 | DP1 | 2.45 min | 0.998151 | 0.930635 | --- |
| 3 | DP2 | 6.74 min | 0.999968 | 0.999284 | 8.372 |
| 4 | DP3 | 11.44 min | 0.990859 | 0.954119 | 2.163 |
| 5 | DP4 | 4.14 min | 0.999998 | 0.999946 | 4.037 |

#### 3.4.2.3.8. *System Suitability Parameters*

System suitability tests were performed on freshly prepared solution with n=6 containing CLEVI. The results of system suitability parameters are shown in Table 3.10. Peak purity data of CLEVI is shown in Table 3.9 and peak purity curve is shown in Fig.3.6.

**Table 3. 10 – System suitability parameters of CLEVI**

| Parameters | Data Obtained |
|---|---|
| Retention Time (min ± SD) | 10.65 ± 0.16 |
| Tailing Factor ± SD | 0.93 ± 0.001 |
| Theoretical Plate ± SD | 11289.45 ± 8.45 |

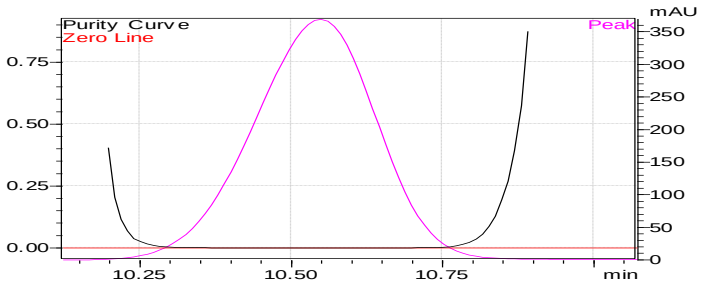


**Fig. 3. 6- Peak purity curve of CLEVI**

#### *3.4.2.4. Stress Degradation studies*

3.4.2.4.1. Acid degradation – Slight degradation (12.1%) was observed when CLEVI was subjected to 1 M HCl at 60ºC for 1 hr with the formation of three degradation products DP1, DP2and DP3 at retention time of 2.45 , 6.74 and 11.44 min respectively (Fig. 3.7).

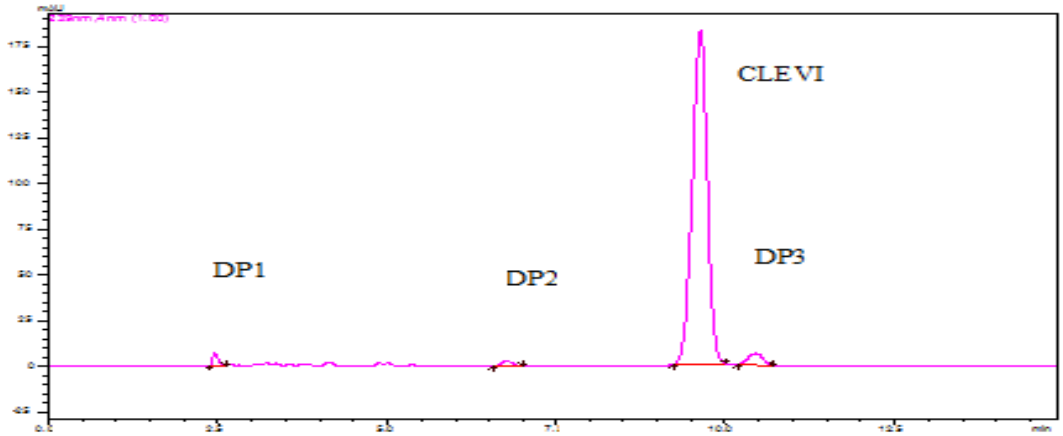


**Fig. 3. 7 – Chromatogram of acid degradation (API)**

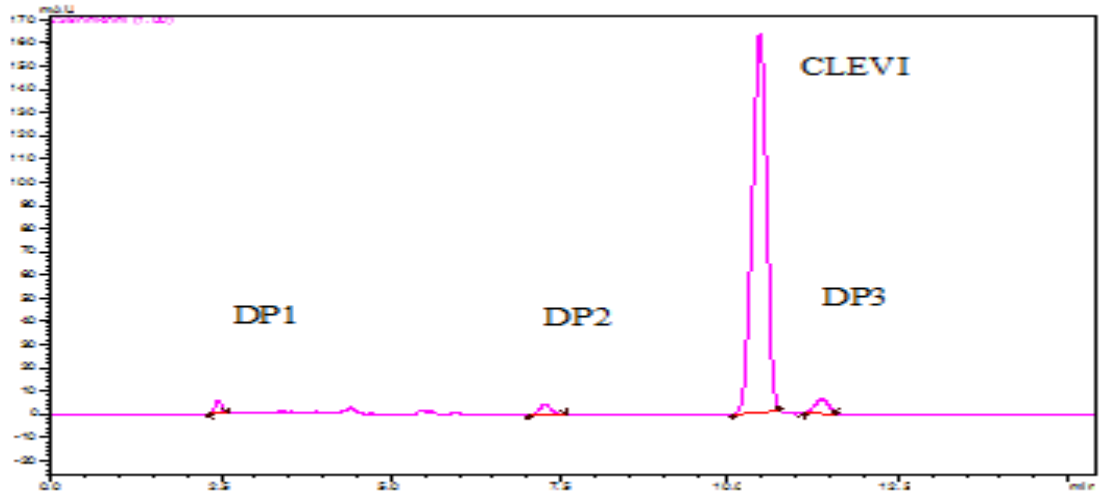


**Fig. 3. 8 – Chromatogram of acid degradation (synthetic mixture)**

3.4.2.4.2. Alkaline degradation – Significant degradation (40.2%) was observed CLEVI was subjected to 0.01 M NaOH at 60 ºC for 1 hr with the formation of two degradation products DP4, DP3 at retention time of 4.10 and 11.40 min respectively (Fig. 3.9).

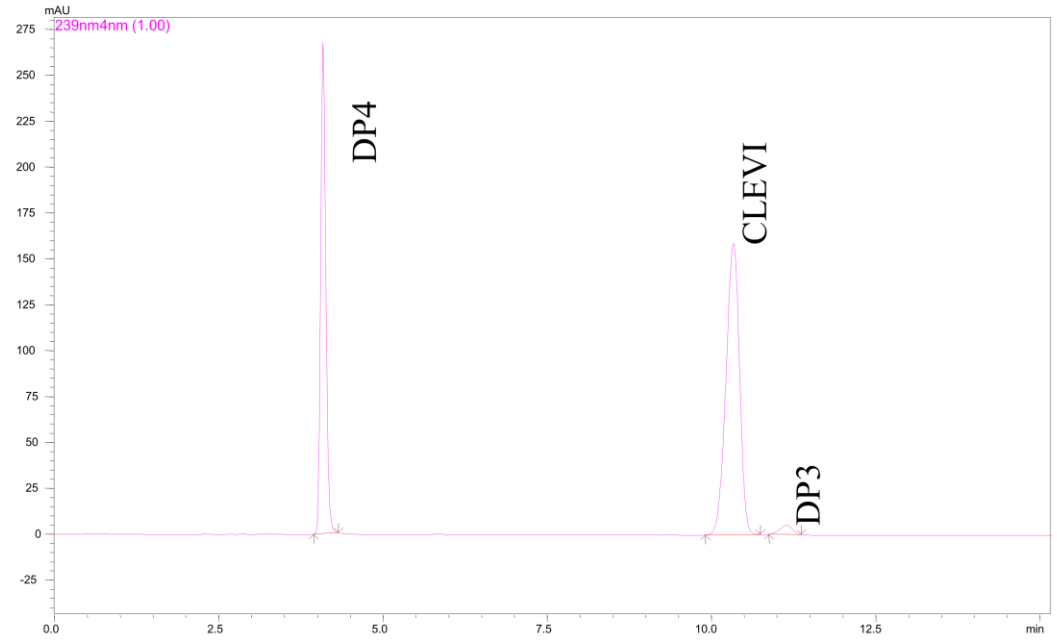


**Fig. 3. 9 – Chromatogram of alkaline degradation (API)**

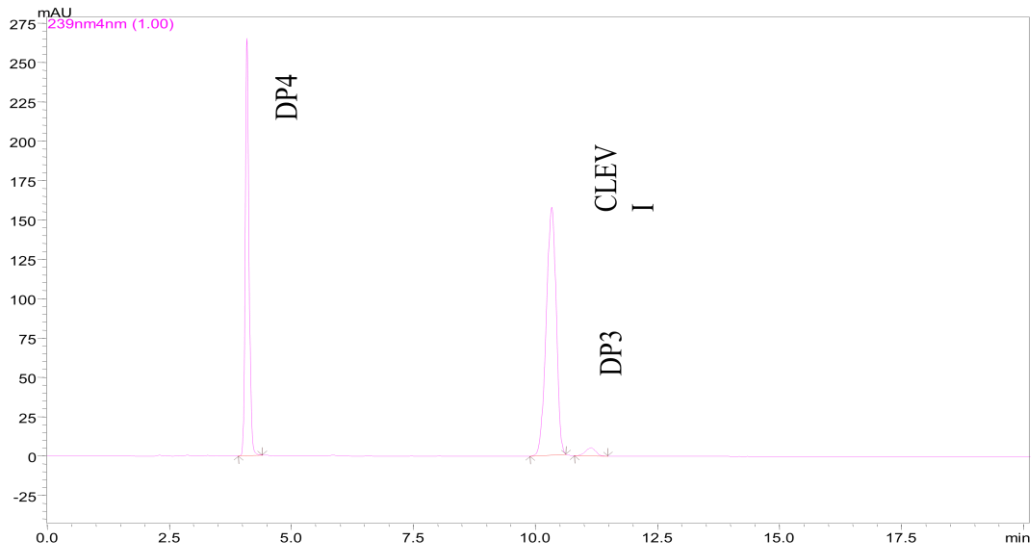


**Fig. 3. 10 – Chromatogram of alkaline degradation (synthetic mixture)**

3.4.2.4.3. Oxidative degradation- Slight degradation (5.7%) was observed when CLEVI was subjected to 3% hydrogen peroxide at room temperature for 1 hr with the formation of one degradation product DP4 at retention time of 4.14 min respectively (Fig. 3.11).

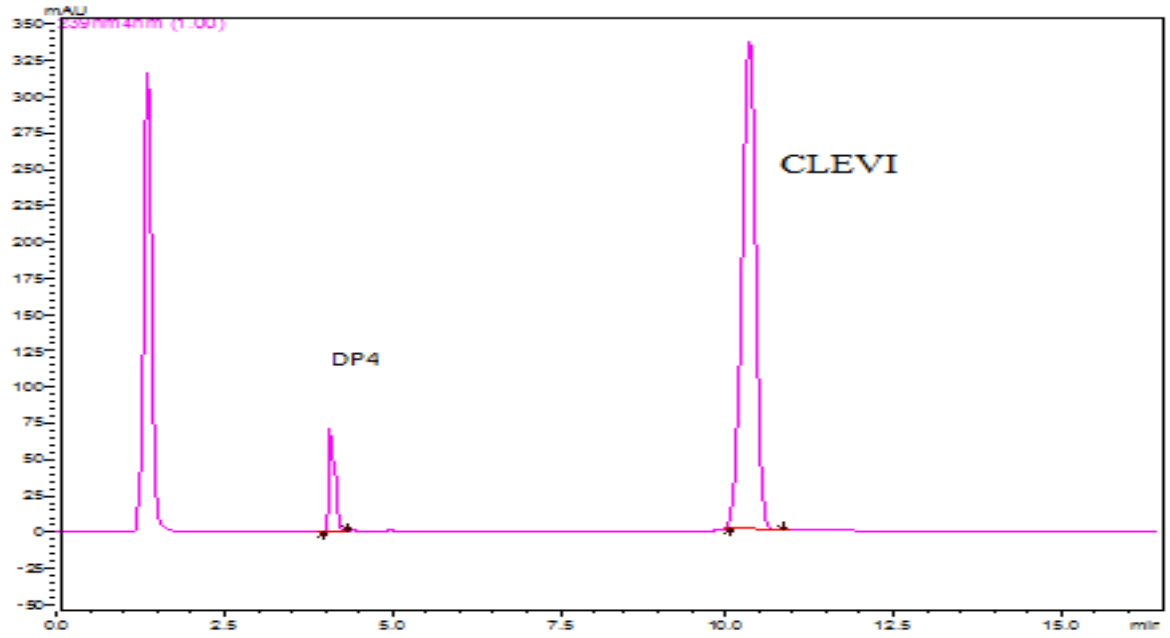


**Fig. 3. 11 –Chromatogram of oxidative degradation (API)**

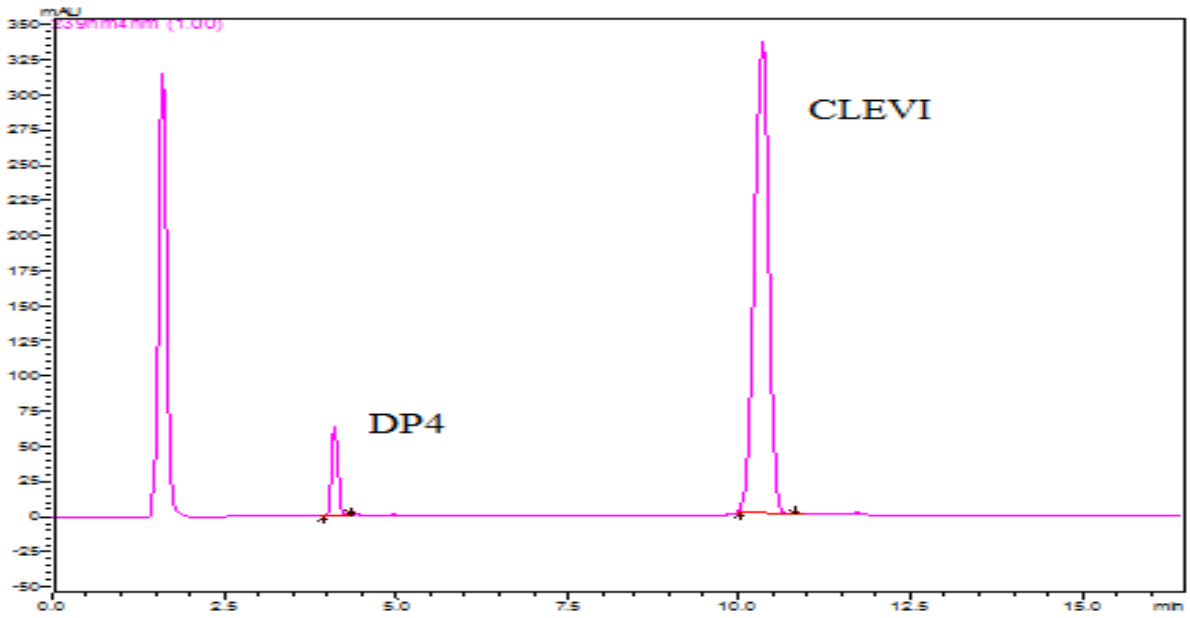


**Fig. 3. 12 – Chromatogram of oxidative degradation (synthetic mixture)**

3.4.2.4.4. Dry heat degradation -No degradation was observed when CLEVI was subjected to thermal degradation at 80ºC for 11 days (Fig. 3.13).

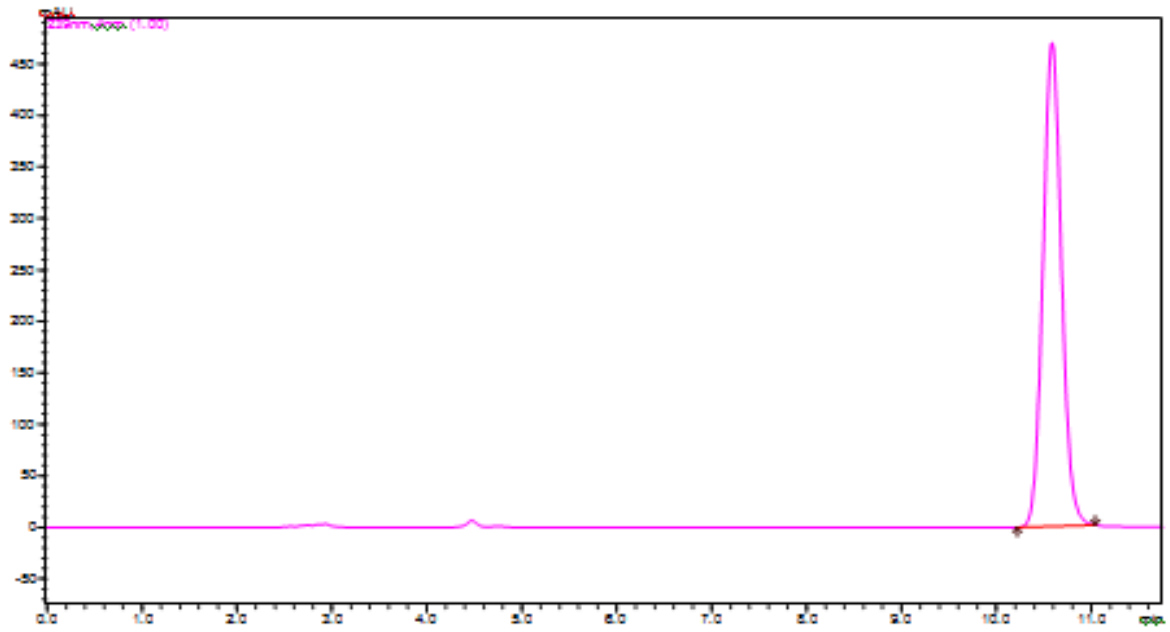

**Fig. 3. 13 –Chromatogram of thermal degradation (API)**

3.4.2.4.5. Photolytic degradation -Slight degradation (0.53%) was observed when CLEVI was subjected to photolytic condition for 11days with the formation of degradation product DP1 at 2.45 min (Fig. 3.14).

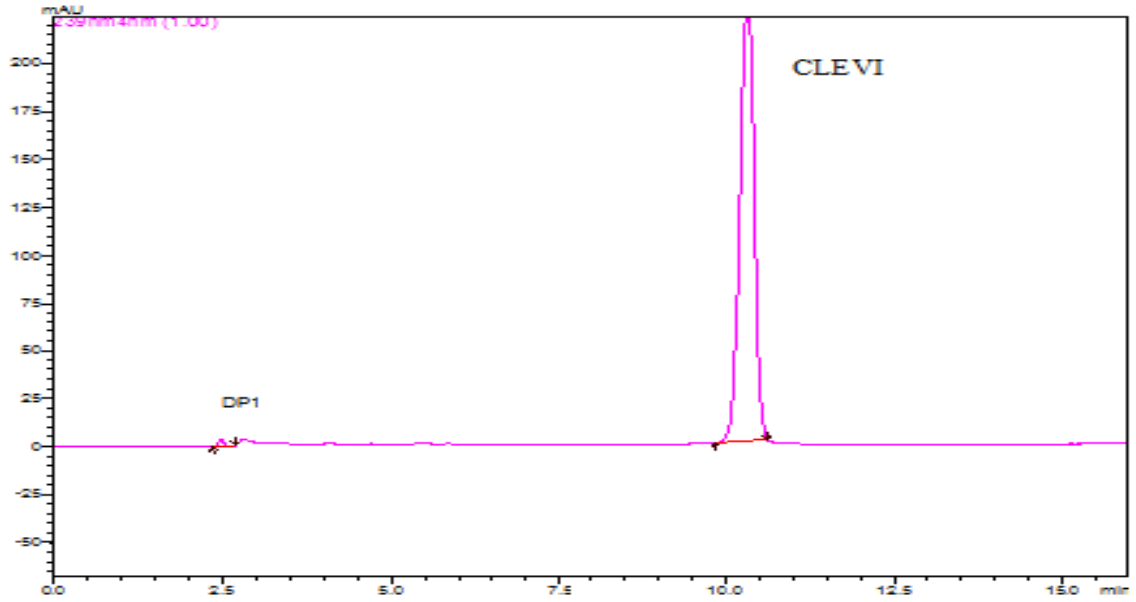


**Fig. 3. 14 – Chromatogram of photolytic degradation (API)**

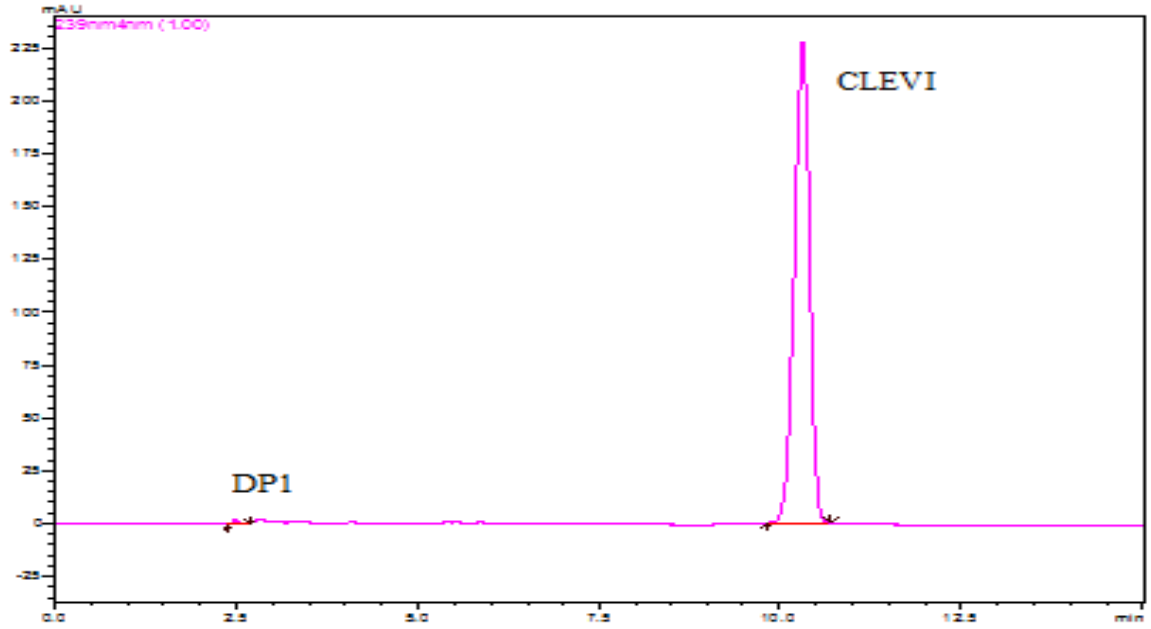


**Fig. 3. 15 –Chromatogram of photolytic degradation (synthetic mixture)**

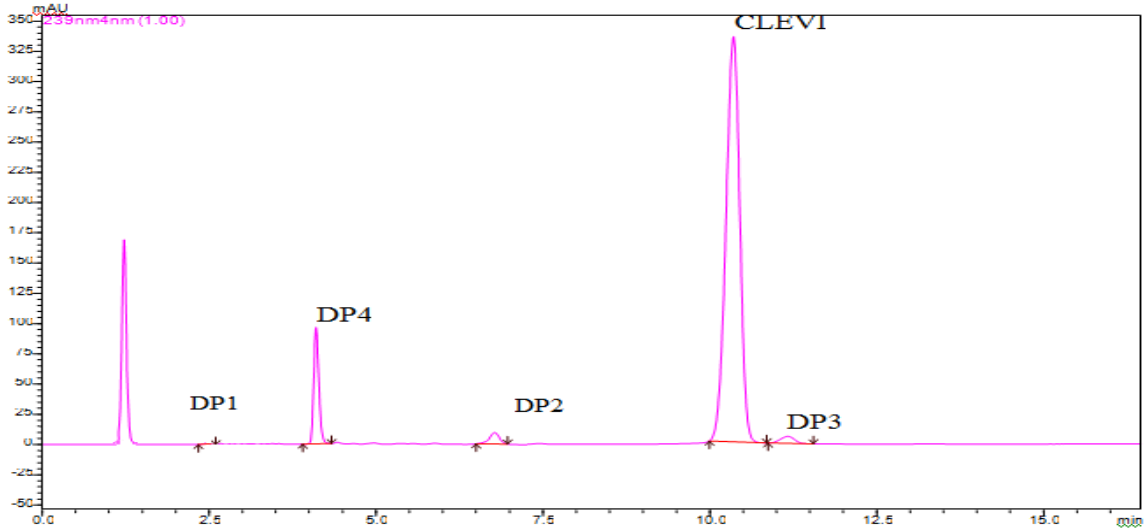


**Fig. 3. 16 - Chromatogram of combined degradation products of all stressors**

### *3.4.2.5. Applicability of the developed method for the analysis of formulation (laboratory mixture)*

Forced degradation study was performed on formulation containing CLEVI prepared in laboratory (synthetic mixture). The composition of synthetic mixture is shown in Table 3.1. The conditions were same as mentioned for API and were analyzed in the

same way as that of API. The degradation products were separated. Minor variation was observed in the degradation of API and formulation as shown in Table 3.11.

**Table 3. 11- Summary of forced degradation study of CLEVI**

| **Stressor** | **Conditions** | **RT of degradation products** | **%Degradation (API)** | **% Degradation (Synthetic mixture)** |
|---|---|---|---|---|
| Acidic | 1 M HCl 60℃ for 1 hr | 2.45(DP1)<br>6.74 (DP2)<br>11.44 (DP3) | 12.1% | 10.5% |
| Alkaline | 0.01 M NaOH 60ºC for 1 hr | 4.10 (DP4)<br>11.40 (DP3) | 40.2% | 38.7% |
| Oxidation | 3% $H_2O_2$ RT for 1 hr | 4.14 (DP4) | 5.7% | 4.2% |
| Thermal | Dry at 80℃ for 11 days | --- | --- | --- |
| Photolytic | 5382 Lux and 144 $UVcm^{-2}$ for 11 days | 2.45 (DP1) | 0.53% | 0.47% |

### 3.4.3. DISCUSSIONS

Maximum absorption wavelength of 239 nm was selected as detection wavelength after scanning CLEVI in the range from 200- 400 nm. Various trials were taken for optimisation. With mobile phase ratio of water and acetonitrile in different ratio, CLEVI was not eluting so buffer was added to the mobile phase. CLEVI has pKa value 5.31, phosphate buffer 10 mM pH 3.0 was found to be suitable. Among organic modifier, sharp symmetrical peak was obtained with acetonitrile. Initially well resolved chromatogram was obtained with phosphate buffer pH 3 and acetonitrile in

the ratio of 25: 75. During forced degradation study, DP3 in acidic and alkaline conditions was co-eluting with CLEVI and DP4 in alkaline and oxidative was not eluting. For separation of DP3 from CLEVI, buffer was added in the mobile phase and finally method was optimized for separation of DP3 and elution of DP4 with phosphate buffer 10 mM pH 3 and acetonitrile in the ratio of 40 : 60. Significant degradation was observed in alkaline condition with the formation of two degradation products in alkaline condition whereas only slight degradation of CLEVI was observed in acidic, oxidative and photolytic conditions. CLEVI was found to be stable in thermal condition. The results of degradation studies are summarized in Table 3.11. There is difference in percentage degradation of CLEVI and synthetic mixture may be attributed to the fact that excipients present might be suppressing the degradation. The developed method was validated as per ICH guidelines with respect to linearity, precision, accuracy, limits of detection and robustness so that CLEVI can be estimated in presence of its degradation products. Good correlation was obtained between peak area and concentration of CLEVI in the range of 20µg/mL to 120 µg/mL with regression coefficient $r^2$ 0.999. % RSD for intra-day and inter-day precision was less than 2%. % Recovery was found to be in the range of 99.68-99.8. % RSD for robustness studies was less than 2%. This indicated that the developed method is precise, accurate and robust to small changes in the experimental conditions.

### 3.4.4. Summary of forced degradation studies

A simple, precise and sensitive stability indicating analytical method has been developed for determination of CLEVI in API and synthetic mixture. The method was validated as per ICH guidelines. The results of forced degradation studies are shown in Table 3.11. The combined chromatogram of CLEVI along with degradation products are shown in Fig 3.15. Degradation products are well resolved from CLEVI and so the method is stability indicating. CLEVI is susceptible to alkaline condition while slight degradation is observed in acidic, oxidative and photolytic condition. CLEVI is stable in thermal degradation. The reported HPLC [13] method shows degradation in acidic, alkaline, oxidative, thermal and photolytic conditions. Comparison of reported method and our developed HPLC method is shown in Table 3.12 which suggests that slight change in degradation conditions can alter the number

of degradation products. Compared to the reported method, the developed HPLC method is simple, accurate, precise and isocratic elution with shorter run time . Statistical comparison was not done with the reported HPLC method [13] since in reported validation parameter % recovery was not performed for determination of CLEVI.

**Table 3. 12 – Comparison of reported method and developed HPLC method**

| Degradation conditions | Reported Method [13] | Developed method |
|---|---|---|
| Acidic | 1 M HCl 80˚c for 30 min<br>1.7 % degradation, 2 DPs | 1 M HCl 60˚c for 1 hr,<br>12.1% % degradation, 3 DPs |
| Alkaline | 1 M NaOH 80˚C for 30 min, 3% degradation , 3 DPs | 0.01M NaOH 60˚C for 1hr,40.2 % degradation, 2 DPs |
| Oxidative | 0.3% $H_2O_2$ RT 30 min, 1.3% degradation , 1DP | 3% $H_2O_2$ RT 1 hr, 5.7% degradation , 1DP |
| Thermal | 105˚C for 8 days<br>1.3% degradation , 1 DP | Dry at 80℃ for 11 days, No degradation |
| Photolytic | 5 days, 1.7% , 1DP | 11 days , 0.53% , 1 DP |
| Other information | 7 process related impurities | ---- |

## 3.5. SECTION –B

## DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD

### 3.5.1. EXPERIMENTAL

#### 3.5.1.1. Chemicals and Reagents

- HPLC grade Methanol (MeOH), Toluene and Ethyl acetate were purchased from S.D. Fine Chemicals Pvt. Ltd. Mumbai.
- Pre-coated Silica Gel $60F_{254}$ aluminum plates (20 X 20 cm, 100mm thickness) were purchased from E. Merck , Darmstadt, Germany
- Hydrochloric acid (HCl), sodium hydroxide (NaOH), hydrogen peroxide $H_2O_2$) were purchased from S.D. Fine Chemical Ltd. Mumbai.
- 0.45 µm Nylon 6,6 syringe filter for sample filtration was procured from Pall Life Sciences, USA.

#### 3.5.1.2. Equipments and Instruments

HPTLC – Camag HPTLC system (Switzerland) comprised of CAMAG Linomat 5 automatic sample applicator, CAMAG TLC Scanner 4, CAMAG twin trough chamber (20 x 10 cm), CAMAG winCATS version 1.4.6. software, Hamilton 100 µL syringe (Linomat syringe 659.0014, Hamilton Bonaduz Schweig, Camag).

#### 3.5.1.3. Chromatographic conditions

Chromatographic method was developed on pre-coated silica gel aluminum plate 60 $F_{254}$ (20 x 20 cm), 100µm thickness. Before use, the pates were pre-washed with methanol and dried in the current of dry air and activated at 120˚C for 5 min. Mobile phase used was toluene and ethyl acetate in the ratio of 8:2. Chromatographic chamber was saturated with mobile phase for 20 minutes. Samples were filtered through 0.45 µ Nylon 6, 6 syringe filter. Samples were applied to TLC plates as bands 6 mm using Linomat 5 sample applicator under stream of nitrogen gas with application rate 150 mL/s. Development distance was 80 mm. The wavelength scanning was done at 370 nm with slit dimension of 6 x 0.3 mm micro, data resolution 100 µm/step, optical filter second order, filter factor (Savitsky Golay 7) with scanning speed 20 mm/sec. Deuterium was the source of radiation with emission of continuous

UV spectrum between 200-400nm.After the development, TLC plates were dried in a current of dry air with air dryer.

**3.5.1.4. Preparation of Standard solution**

10 mg of CLEVI was accurately weighed and transferred to 10 mL volumetric flask and dissolved and volume was made up to 10 mL with methanol. From the standard solution, 1, 2, 3, 4, 5 and 6 µL of solutions were applied in the form of bands to the HPTLC plate in the concentration ranging from 1000-6000 ng/band.

**3.5.1.5. Preparation of forced degradation sample**

For forced degradation study, stock solution of CLEVI was prepared in concentration of 10 mg/mL in methanol.

**3.5.1.5.1. Acid degradation**

To 2.5 mL of CLEVI stock solution in 25 mL of volumetric flask, 1 mL of 1M HCl was added. The solution was heated at 60ºC for 1 hr. The solution was neutralized with 1 M NaOH and volume was made up to the mark with methanol. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter. The resulting solution, 5 µL (5000 ng/band) was applied to the TLC plate and chromatogram was run as mentioned above.

**3.5.1.5.2. Alkaline degradation**

To 2.5 mL of CLEVI stock solution in 25 mL of volumetric flask, 1 mL of 0.01 M NaOH was added. The solution was heated at 60ºC for 1 hr. The solution was neutralized with 0.01 M HCl and volume was made up to the mark with methanol .The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter. 5 µL of solution (5000 ng/band) was applied to the TLC plate and chromatogram was run as mentioned above.

**3.5.1.5.3. Oxidative degradation**

2.5 mL of CLEVI stock solution was transferred to 25 mL of volumetric flask, to this was added 1 mL of 3% hydrogen peroxide. The solution was kept at room temperature for 1 hr. The volume was made up to the mark with methanol. The solution was filtered through 0.45 µ Nylon 6, 6 syringe filter. 5 µL of solution (5000 ng/band) was applied to the TLC plate and chromatogram was run as mentioned above.

**3.5.1.5.4. Dry heat degradation**

For dry heat degradation, 25 mg of CLEVI was kept in oven at 80ºC for 11 days. The sample was transferred to 25 mL of volumetric flask, dissolved in methanol. 5 µL of solution (5000 ng/band) was applied to the TLC plate and chromatogram was run as mentioned above.

**3.5.1.5.5. Photolytic degradation**

For photolytic degradation, 25 mg of CLEVI was spread in 1 mm thickness and was exposed to 5383 Lux and 144 UV/$cm^2$ for 11 days. Volume was made up to 25 mL and 5 µL of solution (5000 ng/band) was applied to the TLC plate and chromatogram was run as mentioned above

**3.5.1.6. HPTLC method validation**

The developed method was validated as per ICH Q2B guideline.

For linearity, standard dilutions of CLEVI were prepared in the concentration ranging from 1000 to 6000 ng/spot from CLEVI standard solution and were spotted on a TLC plate in triplicate. Linearity was determined by plotting peak area and concentration of solution. From the graph regression equation and regression coefficient was determined.

For precision, intra-day and inter-day precision were evaluated at six concentration levels (in triplicates). Peak areas corresponding to the concentration was calculated and % RSD was determined for intra-day and inter -day precision.

% Recovery was evaluated by standard addition method. Accuracy of method was confirmed by recovery study from laboratory mixture at 3 level of standard addition (50%, 100% and 150%). Accuracy of method was evaluated at concentration of 2500ng/band. The concentrations (total concentration) for accuracy were 2500, 3750, 5000, 6250 ng/band. The concentrations were analysed in triplicates. % recovery and % RSD were calculated.

Limit of detection and limit of quantitation was calculated on the basis of standard deviation of the intercept and slope of the calibration curve. LOD and LOQ were calculated using equation 3.3*(σ/S) and 10*(σ/S), where σ is the standard deviation of intercept and S is the slope of the calibration curve.

For robustness, mobile phase composition (7.8, 8.0, 8.2), saturation of mobile phase (17, 20, 23 min), development distance (7.5, 8.0, 8.5 cm), wavelength (368, 370, 372nm) were changed. Robustness of the method was evaluated at 3000 ng/band of concentration in triplicates.

The standard stock solution of CLEVI was prepared in methanol and kept for 24 hours at room temperature. The stability of stock solution was determined.

Specificity of the method was evaluated by analysis of API as well as synthetic mixture along with degradation products and to check the method for interference of any peaks affecting the estimation of CLEVI.

### 3.5.2. RESULTS

#### 3.5.2.1. Determination of suitable wavelength

Solution of CLEVI of 1000 ng/band was prepared in methanol and scanned in the UV region of 200-400 nm and spectrum was recorded. CLEVI showed maximum absorbance at 370 nm which was selected as the analytical wavelength (Fig. 3.17)

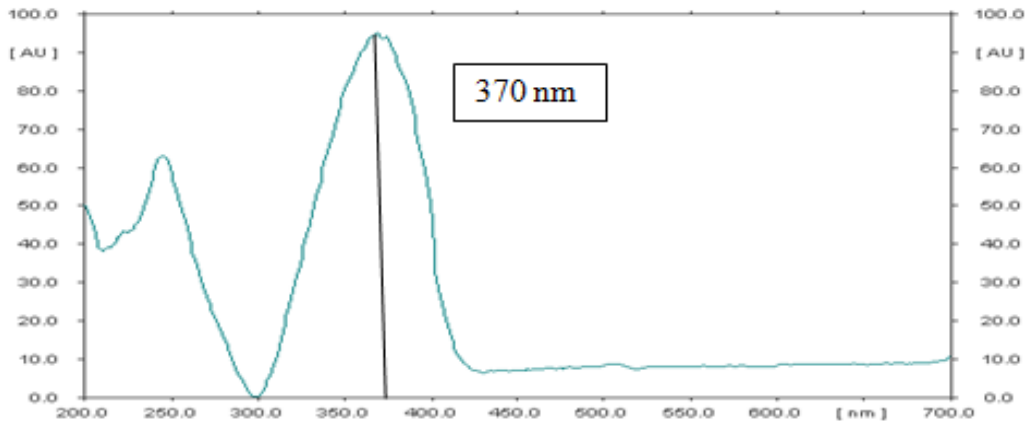


**Fig. 3. 17 – UV spectra of CLEVI**

#### 3.5.2.2. Method optimisation and development

For optimisation of chromatographic conditions, trials were taken with acetonitrile and methanol and with formic acid, CLEVI was eluted fast. Finally method was optimised with toluene and ethyl acetate in the ratio of 8:2. The results of trials and optimized conditions are shown in Table 3.13 and 3.14.

**Table 3. 13 - Optimisation of HPTLC conditions**

| Mobile Phase | Ratio (% v/v) | $R_f$ value |
|---|---|---|
| Acetonitrile : Methanol | 5:5 | Fast elution |
| Acetonitrile: Methanol: F.A. | 5 : 5 : 0.5 | Fast elution |
| Toluene : Ethyl acetate | 8: 2 | 0.49 |

**Table 3. 14 – Optimised HPTLC parameters**

| Parameters | Optimised Value |
|---|---|
| Stationary phase | Precoated Silica gel 250 $F_{254}$ HPTLC plate |
| Mobile phase ratio | Ethyl acetate : Toluene ( 2: 8) |
| Wavelength | 370 nm |
| Chamber saturation time | 20 min |
| Developmental distance | 8 cm |
| Slit dimension | 6 X 0.3 mm, micro |
| $R_f$ value | 0.49 |

The optimized densitogram is shown in Fig. 3.18.

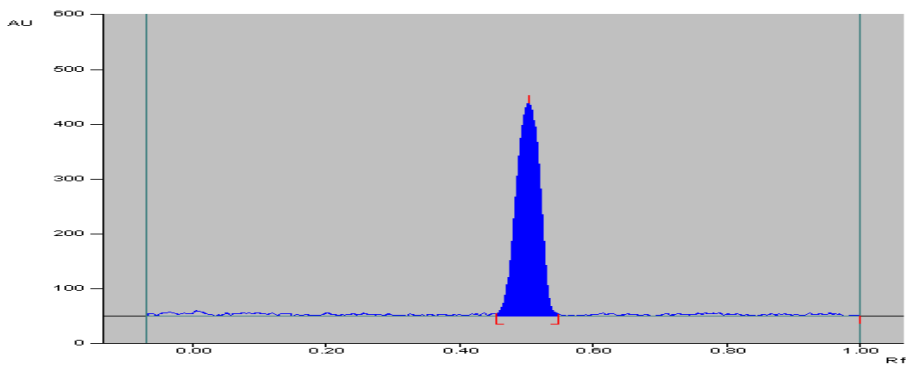


**Fig. 3. 18 - Densitogram of CLEVI standard**

### 3.5.2.3. Method validation using ICH Q2(R1) guideline

#### 3.5.2.3.1. *Linearity and range*

The calibration plotted for CLEVI was found to be linear in the range of 1000-6000 ng/band (Fig. 3.19). The regression equation was found to be y= 2.004x+1974 with regression coefficient ($r^2$) of 0.999.The linearity data is shown in Table 3.15 and calibration curve is shown in Fig. 3.20.

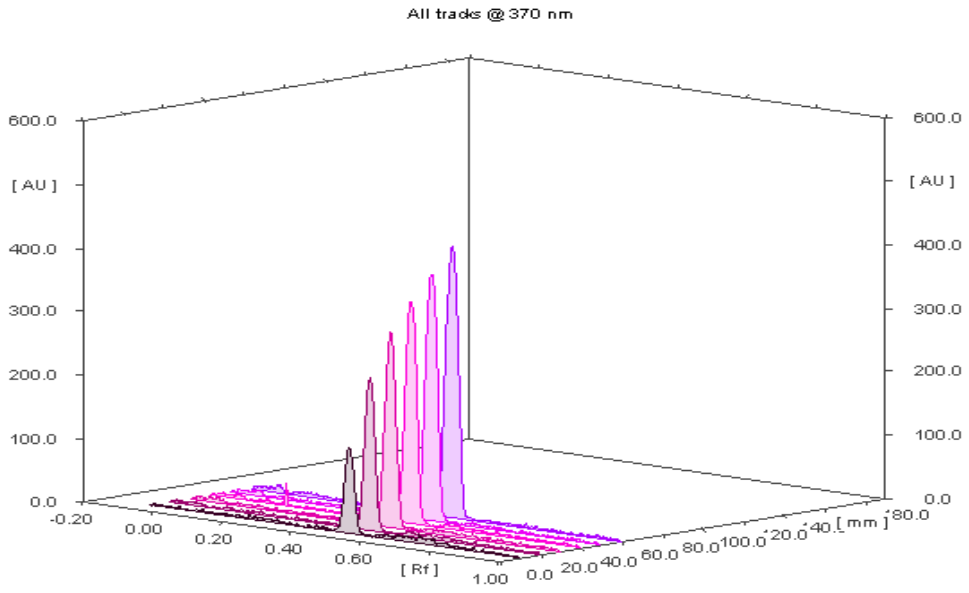


**Fig. 3. 19 - 3 D densitogram of linearity band of CLEVI**

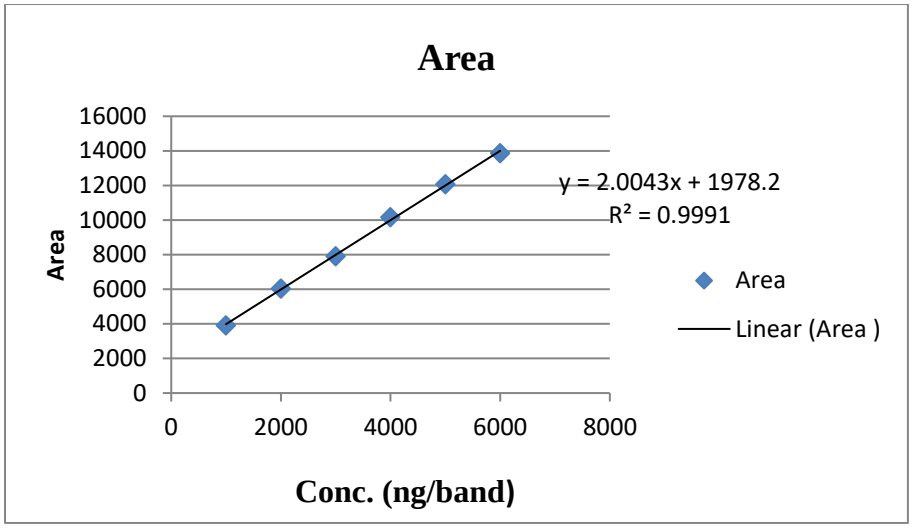


**Fig. 3. 20 - Calibration curve of CLEVI (Area versus concentration ng/band)**

**Table 3. 15- Linearity data of CLEVI**

| Conc. (ng/band) | Area (Mean* ±%RSD |
|---|---|
| 1000 | 4036.86 ±1.32 |
| 2000 | 6224.5±1.25 |
| 3000 | 8130.73 ±1.36 |
| 4000 | 10110.03 ±0.89 |
| 5000 | 12038.7 ±1.24 |
| 6000 | 13729.1±1.16 |

***Average of three determinants**

**3.5.2.3.2. *Precision***

Intra-day precision was performed by repeating the experiment three times in a day and inter-day precision was performed by repeating the experiments on three consecutive days. The average %RSD of intra-day and inter-day were found to be 1.16 and 1.33. The developed method was found to be precise (Table 3.16 and Table 3.17).

**Table 3. 16 – Intraday precision of CLEVI**

| Conc.(ng/band) | Area | | | | |
|---|---|---|---|---|---|
| | Set 1 | Set 2 | Set 3 | Mean | %RSD |
| 1000 | 4040.7 | 3963.5 | 3976.4 | 3993.53 | 1.03 |
| 2000 | 6153.4 | 6036.3 | 6183.8 | 6124.5 | 1.27 |
| 3000 | 8104.2 | 8002.4 | 8184.6 | 8097.07 | 1.12 |
| 4000 | 10006.3 | 10167.5 | 9956.3 | 10043.4 | 1.09 |
| 5000 | 11877.1 | 12205.4 | 12013.6 | 12032 | 1.37 |
| 6000 | 13557.7 | 13273.4 | 13356.2 | 13395.8 | 1.09 |
| | | | Average % RSD | | 1.16 |

**Table 3. 17 - Interday precision of CLEVI**

| Conc. (ng/band) | Area | | | | |
|---|---|---|---|---|---|
| | Set 1 | Set 2 | Set 3 | Average | %RSD |
| 1000 | 4040.7 | 3975.5 | 3938.8 | 3985 | 1.29 |
| 2000 | 6193.4 | 6046.3 | 6085.5 | 6108.4 | 1.24 |
| 3000 | 8314.2 | 8258.4 | 8103.5 | 8225.36 | 1.32 |
| 4000 | 10006.3 | 9813.6 | 9983.2 | 9934.36 | 1.05 |
| 5000 | 12377.1 | 12763.2 | 12496.3 | 12545.5 | 1.57 |
| 6000 | 13557.7 | 13173.2 | 13245.6 | 13325.5 | 1.53 |
| | | | Average % RSD | | 1.33 |

**3.5.2.3.3.** ***Accuracy***

Accuracy of method was determined by calculating % percent recovery of the analyte recovered. To the sample solution of 2500 ng/band, standard solution of CLEVI was added as 50%, 100% and 150% to give concentrations as 3750, 5000 and 6250 ng/band. The developed method was accurate (Table 3.18).

**Table 3. 18 – Recovery study of CLEVI**

| Excess drug added to analyte (%) | Theoretical Content (ng/band) | *Amount Found (ng/band) | Recovery (%±SD) |
|---|---|---|---|
| 0 | 2500 | 2495.33 | 99.81 ± 0.08 |
| 50 | 3750 | 3737.73 | 99.03 ± 0.14 |
| 100 | 5000 | 4988.46 | 99.54 ±0.06 |
| 150 | 6250 | 6240.80 | 99.57 ±0.04 |

*Average of three determinations

**3.5.2.3.4.** ***Limit of detection and limit of quantification***

LOD and LOQ were found to be 196.75 and 596.23 ng/band respectively.

**3.5.2.3.5.** ***Robustness***

For robustness study, slight changes mobile phase composition, saturation of mobile phase, development distance and wavelength. The results were expressed as % RSD. % RSD less than 2 indicated that the developed method was robust (Table 3.19).

**Table 3. 19 – Robustness data of CLEVI**

| Factor | Level | $R_f$ | | Area | |
|---|---|---|---|---|---|
| | | Mean | %RSD | Mean | %RSD |
| Mobile Phase Composition | 7.8 : 2.2 | 0.47 | 1.21 | 8085.6 | 1.37 |
| | 8:02 | 0.49 | 1.17 | 8184.37 | 1.16 |
| | 8.2 : 1.8 | 0.52 | 1.10 | 8023.27 | 0.94 |
| Saturation of mobile phase (min) | 17 | 0.48 | 1.19 | 8052.4 | 1.23 |
| | 20 | 0.49 | 1.17 | 8184.37 | 1.16 |
| | 23 | 0.49 | 1.17 | 7911.83 | 0.82 |
| Development distance (cm) | 7.5 | 0.46 | 1.23 | 8054.97 | 1.25 |
| | 8 | 0.49 | 1.17 | 8184.37 | 1.16 |
| | 8.5 | 0.52 | 1.10 | 7939.53 | 1.33 |
| Wavelength | 368 | 0.48 | 1.19 | 8075.27 | 0.76 |
| | 370 | 0.49 | 1.17 | 8184.37 | 1.16 |
| | 372 | 0.49 | 1.17 | 8022.02 | 0.78 |

None of the factors are affecting the robustness of the method.

### *3.5.2.4. Stress Degradation studies*

3.5.2.4.1. Acid degradation – Slight degradation (22.5%) was observed when CLEVI was subjected to 1 M HCl at 60ºC for 1 hr with the formation of two degradation products DP1 and DP2 at $R_f$ of 0.01 and 0.02 respectively (Fig. 3.21).

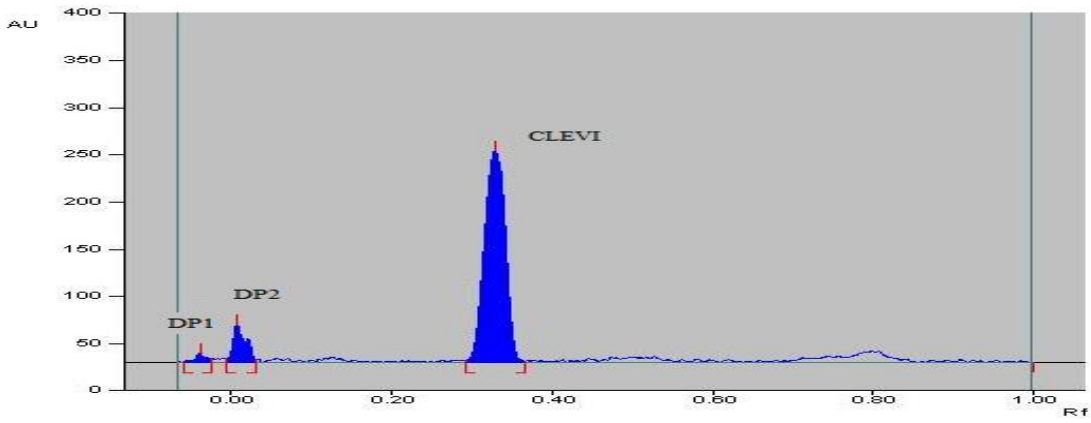


**Fig. 3. 21 - Densitogram of acid degradation (API)**

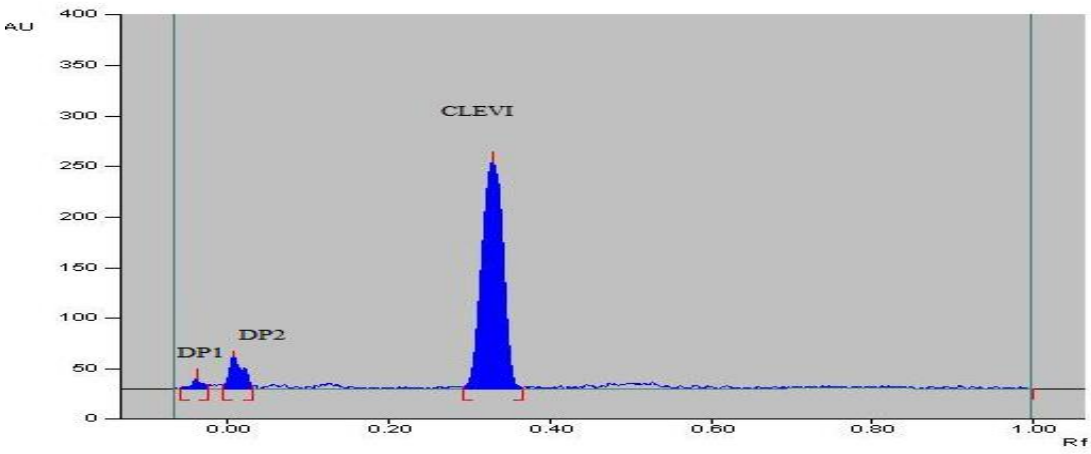


**Fig. 3. 22 - Densitogram of acid degradation (synthetic mixture)**

3.5.2.4.2. Alkaline degradation -Significant degradation(45%) was observed CLEVI was subjected to 0.01 M NaOH at 60 ºC for 1 hr with the formation of degradation product DP3 at $R_f$ of 0.27 (Fig.3.23).

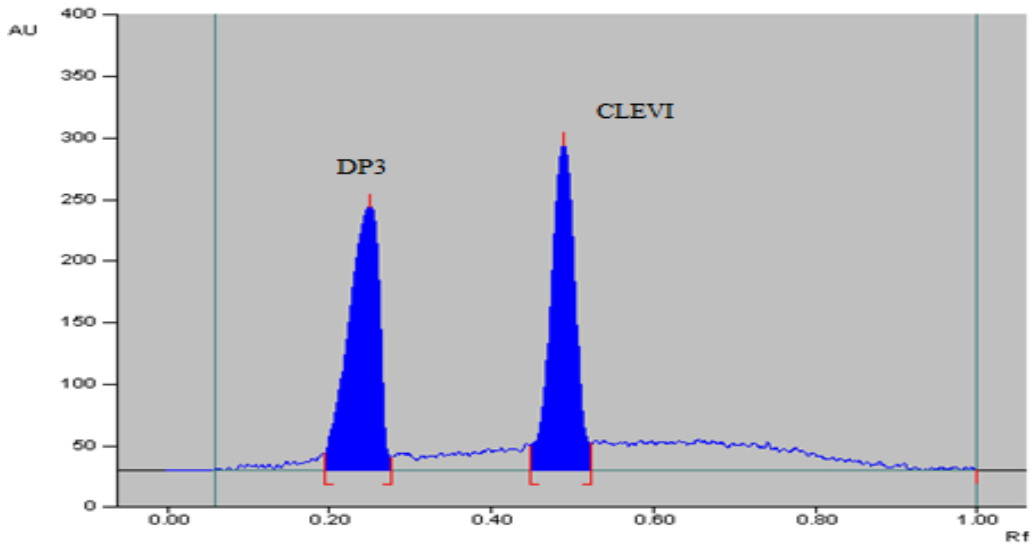


**Fig. 3. 23 - Densitogram of alkaline degradation (API)**

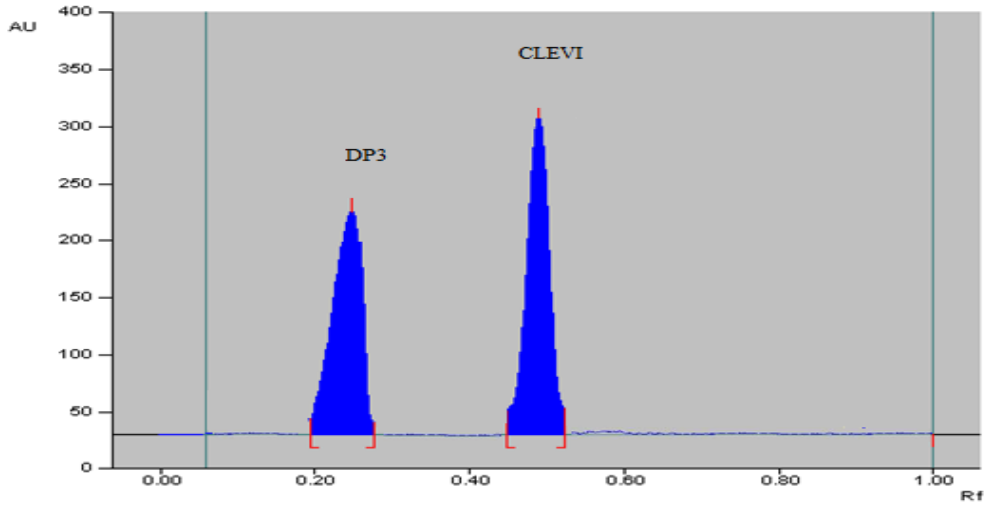


**Fig. 3. 24 -Densitogram of alkaline degradation (synthetic mixture)**

3.5.2.4.3. Oxidative degradation- Slight degradation (8.5%) was observed when CLEVI was subjected to 3% hydrogen peroxide at room temperature for 1 hr with the formation of degradation product DP3 at $R_f$ of 0.27 respectively (Fig. 3.25).

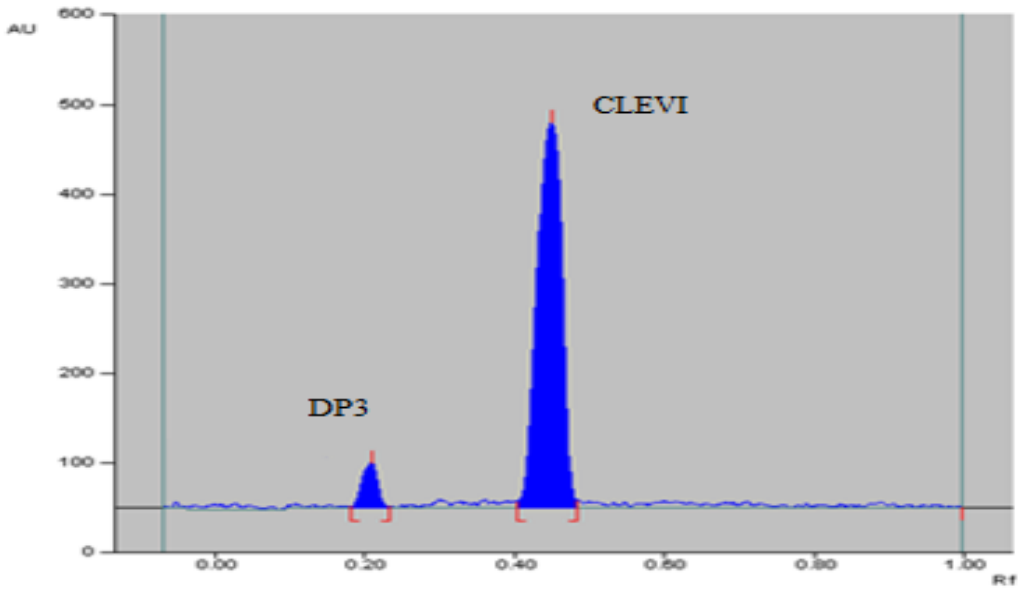


**Fig. 3. 25 -Densitogram of oxidative degradation (API)**

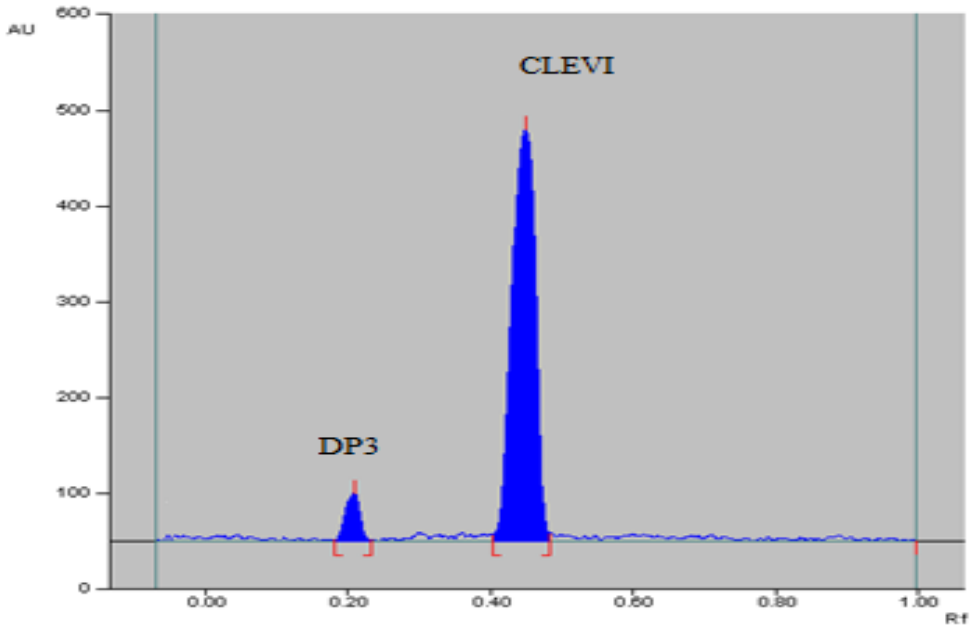


**Fig. 3. 26 - Densitogram of oxidative degradation (synthetic mixture)**

3.5.2.4.4. Dry heat degradation -No degradation was observed when CLEVI was subjected to thermal degradation at 80ºC for 11 days (Fig. 3.27).

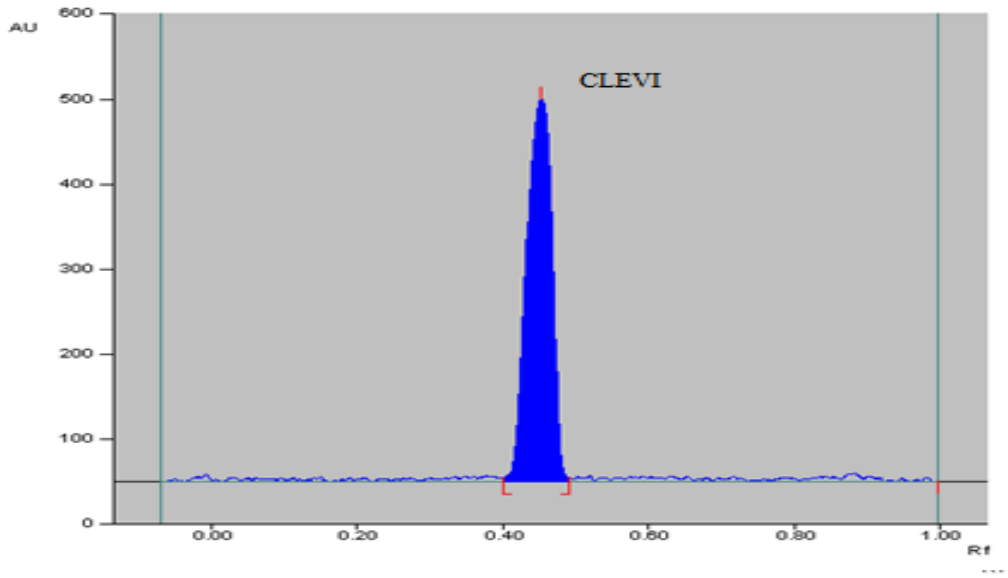


**Fig. 3. 27 – Densitogram of thermal degradation**

3.5.2.4.5. Photolytic degradation -Slight degradation (0.6%) was observed when CLEVI was subjected to photolytic condition for 11days with the formation of degradation product DP1 at $R_f$ of 0.01 (Fig.3.28).

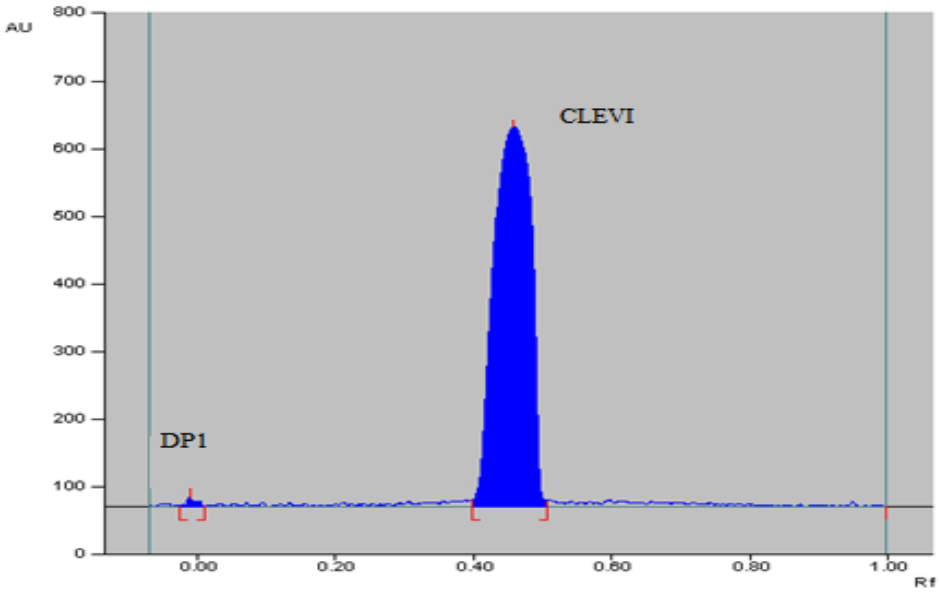


**Fig. 3. 28 - Densitogram of photolytic degradation (API)**

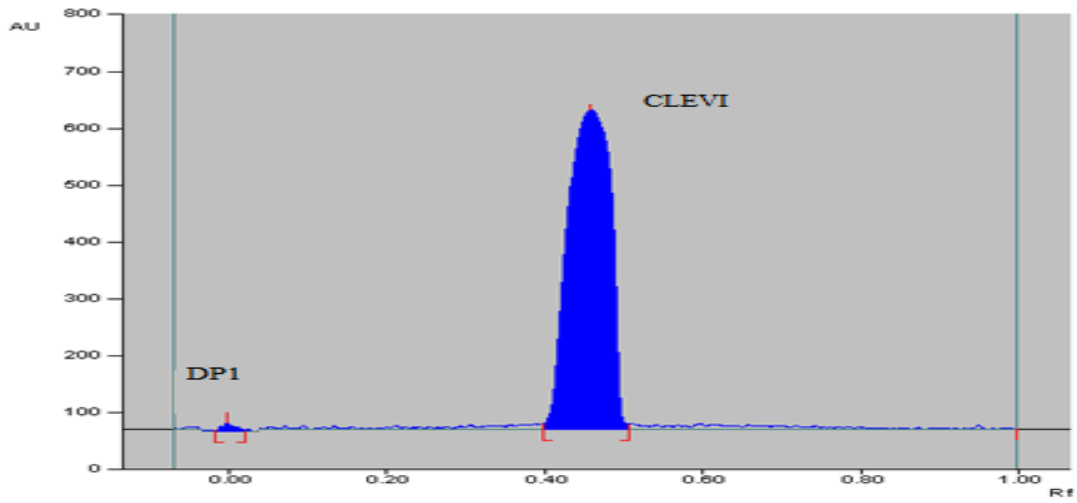


**Fig. 3. 29 - Densitogram of photolytic degradation (synthetic mixture)**

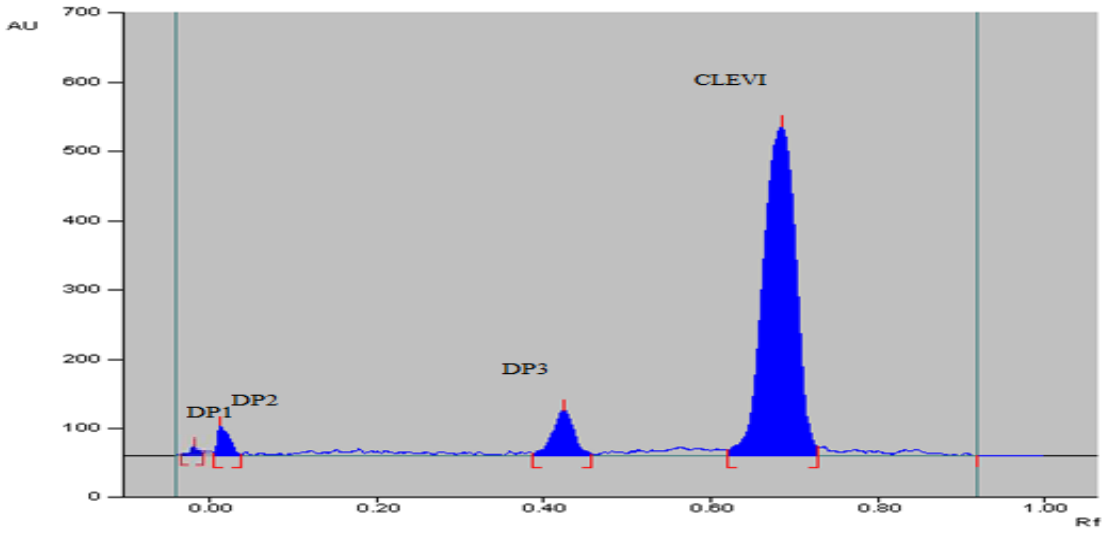


**Fig. 3. 30 - Densitogram of mixture of degradation products of all stressors**

### *3.5.2.5. Applicability of the developed method for the analysis of formulation (synthetic mixture)*

Forced degradation study was performed on formulation containing CLEVI prepared in laboratory (synthetic mixture). The conditions were same as mentioned for API and were analyzed in the same way as that of API. The degradation products were separated. Minor variation was observed in the degradation of API and formulation as shown in Table 3.20.

**Table 3. 20 – Summary of forced degradation study of CLEVI**

| Condition | API | | Synthetic mixture | |
|---|---|---|---|---|
| Stress condition | $R_f$ of degraded product | % Degradation | $R_f$ of degraded product | % Degradation |
| 1 M HCl 60ºC for 1 hr | 0.01, 0.02 | 22.5% | 0.01, 0.02 | 19.5% |
| 0.01 M NaOH 60ºC for 1 hr | 0.27 | 45% | 0.27 | 42% |
| 3% $H_2O_2$ RT for 1 hr | 0.27 | 8.5% | 0.27 | 6.3% |
| Dry heat 80ºC for 8 days | --- | No degradation | --- | No degradation |
| Photolytic | 0.01 | 0.6% | 0.01 | 0.6% |

**3.5.2.6. Comparison of HPLC and HPTLC methods for CLEVI**

Based on the above results, stability indicating HPLC and HPTLC methods were developed for quantitative determination of CLEVI in presence of degradation products. Both the methods are accurate and selective methods. HPLC method has the advantage of more reproducibility, providing peak purity data for degradation products and higher resolution. HPTLC method is simple, highly sensitive and uses minimal volume of solvent for developing system.

Developed HPLC and HPTLC SIAM methods for CLEVI were statistically compared for the significant difference between them. The statistical test t-test was applied to statistically to compare these two analytical methods at 95% confidence interval level

($p< 0.05$). Since t calculated < t tabulated within 95% confidence, the means of two methods are statistically similar (Table 3.21).

*Parameters for t-test*

Degrees of freedom: 10

Null hypothesis ($H_0$): There is no significant difference between two analytical methods

Alternate hypothesis: There is significant difference between two methods

**Table 3. 21- t-test for comparison of HPLC and HPTLC methods**

| t-test : Two sample assuming equal variances | | |
|---|---|---|
| Parameters | HPLC | HPTLC |
| Mean | 99.8 | 99.63 |
| Variance | 0.00925 | 0.0380 |
| Observations | 6 | 6 |
| df | 5 | 5 |
| t- Tabulated | 2.2281 | |
| t- Calculated | 1.8775 | |
| t-Tabulated > t- Calculated | Yes | |
| Alternate hypothesis | Fail | |

As there is no significant difference between the two analytical methods null hypothesis passes. Both the methods can be used for determination of CLEVI.

### 3.5.3. DISCUSSIONS

Maximum absorption wavelength of 370 nm was selected as detection wavelength by scanning in the range from 200- 400 nm. Various trials were optimized for determination CLEVI. With mobile phase using polar solvents acetonitrile and methanol in different ratios CLEVI was eluting fast. Method was developed with toluene and ethyl acetate in the ratio of 8:2. Significant degradation was observed in alkaline condition, with formation of one degradation product. Slight degradation was observed in acidic condition with formation of two degradation products, in oxidative condition and in photolytic condition with the formation of one degradation product. CLEVI was found to be stable in thermal condition. The developed was validated as per ICH guideline with respect to linearity, precision, accuracy, limits of detection and robustness. Good correlation was obtained between peak area and concentration of CLEVI in the range of 1000 ng/band to 6000 ng/band with regression coefficient $r^2$ 0.999. % RSD for intra-day and inter-day precision was less than 2%. % Recovery was found to be in the range of 99.03-99.57. % RSD for robustness studies was less than 2%. This indicated that the developed method is precise, accurate and robust to small changes in the experimental conditions.

## 3.6. CONCLUSION

Stability indicating method was developed for determination of Clevidipine by HPLC and HPTLC method. Significant degradation of CLEVI was observed in alkaline condition and slight degradation was observed in acidic, oxidative and photolytic condition. The developed methods by HPLC and HPTLC were validated as per ICH guidelines. Both the methods are simple, accurate and sensitive which are applicable for determination of CLEVI and degradation products of CLEVI.

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