NOVEL STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF CLOBAZAM AND ITS RELATED SUBSTANCES IN ORAL SUSPENSION

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ABSTRACT

A novel, sensitive, stability-indicating gradient RP-HPLC method has been developed for simultaneous estimation of clobazam and its related substances in oral suspension. The chromatographic separation of degradation products and matrix components was executed on a YMC Pack ODS-A column with gradient mode. The mobile phase composed of water and acetonitrile and flow rate was 1.0 mL min^{-1}, while 230 nm was wavelength of detection. The resolution greater than 2.0 between clobazam and the impurities was achieved. The forced degradation study was carried out as per ICH guidelines. The drug product was exposed to hydrolysis, oxidation, photolysis and thermal conditions to achieve degradant formation. Clobazam was degraded under acidic and basic hydrolytic conditions that produced impurity E. The specificity, linearity, limit of detection/quantification, accuracy, precision and robustness was validated as per ICH guidelines.

Keywords: Clobazam, stability indicating, RP-HPLC, suspension

INTRODUCTION

Clobazam (CLB) belongs to the benzodiazepine class of drugs with antiepileptic and anticonvulsant properties. Chemically, CLB is 7-chloro-1-methyl-5-phenyl-1*H*-1, 5 benzodiazepine-2, 4(3*H*, 5*H*)-dione (Fig. 1). It is an anti-epileptic drug with less sedative properties and is recommended in the treatment of seizures associated with Lennox–Gastaut syndrome in toddlers of 2 or more years of age[1,2]. It shows its action by binding to ϒ -aminobutyric acid (GABA)-A receptors[2]. The average half-life for CLB is 18 h (range, 10-30 h), but for its active metabolite i.e., *N*-desmethyl-CLB, it is 42 h (36-46 h)[3]. The five possible related compounds of CLB (degradants and process related) are desmethyl impurity A (7-chloro-5-phenyl-1, 5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione), deschloro impurity B(1-methyl-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione), monomethyl impurity C ((3*RS*)-7-chloro-1,3-dimethyl-5-phenyl-1,5-dihydro-3*H*-1,5 benzodiazepine-2,4-dione), impurity D (7-chloro-1,3,3 -trimethyl-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione) and impurity E (*N*-[4-chloro 2(phenylamino) phenyl]-*N*-methyl acetamide).

The official methods are available for quantitative determination of CLB and all related substance from tablet formulation only. Few HPLC methods for estimation of CLB and metabolite in human plasma have been published[4-8]. Also, CLB drug substance and its dosage forms quantitation by RP-HPLC is offered[9]. Further, basic hydrolytic degradants of CLB estimation by RP-HPLC method is described in literature[10].

Suspension is a complex system with preservatives and excipients like methyl paraben, propyl paraben, propylene glycol, citric acid monohydrate, etc. which may interfere in the analysis. It is essential to develop an analytical method for routine quality control of CLB in suspension that can also separate degradants. No method is reported for related substance of CLB in suspension. The present research offers a RP-HPLC method for the quantification of CLB in suspension. The stress studies under various conditions was executed as per ICH guidelines[11,12].

MATERIALS AND METHODS

Standards, chemicals and reagents

All standards and impurities were procured from Centaur Pharmaceuticals Pvt. Ltd., Mumbai, India. The

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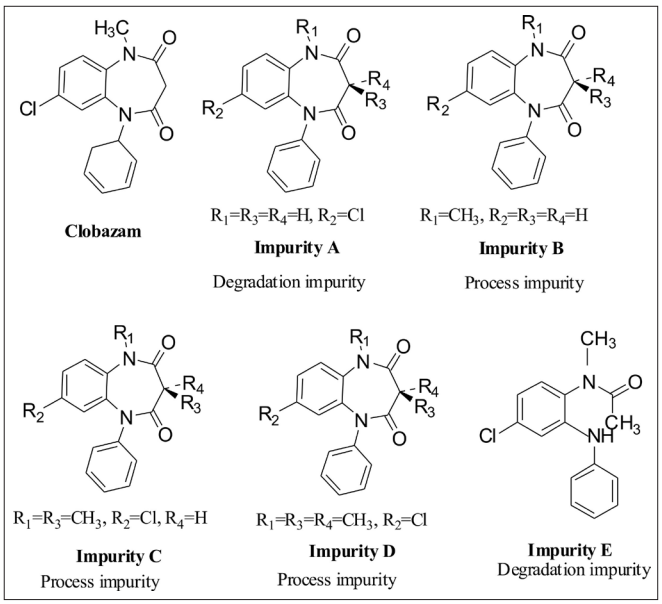

Fig. 1: Chemical structures of CLB and its impurities

CLB oral suspension 2.5 mg mL^{-1} developed by Callidus Research Lab Pvt. Ltd., Pune, India was used for analysis. The HPLC-grade acetonitrile (ACN) was procured from Merck. AR grade sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Rankem. ELGA water purification system was used to get HPLC grade water which was used throughout the studies.

Instrumentation

Thermo Fisher Scientific HPLC system with a PDA detector (DAD-3000RS) was used. The Chromeleon software (6.80 version) was used to monitor and process the output signal. Hydrolytic degradation was executed using a Bio-Technics India water bath. Photo stability study was performed in a photo stability chamber (Mack Pharmtech, India) while dry air oven (Bio-Technics, India) was used for the thermal stability studies. Humidity chamber of Mack Pharmtech, India was used.

Chromatographic conditions

The chromatographic separation was attained using YMC Pack ODS-A column (150 mm X 4.6 mm, 12 nm). Mobile phase A and B were degassed water and ACN respectively. The HPLC gradient mode was set as: time (min)/% solution B: 0/25, 25/25, 55/75, 63/75, 65/25 and 75/25 V/V with 1.0 mL min^{-1} flow rate. The column compartment temperature and sample oven temperature was 35 °C and 25 °C, respectively. The detection wavelength was 230 nm and 10 µL volume of injection was used. Diluent was water and ACN in 60:40 V/V ratio throughout the studies.

Standard stock solution preparation

About 20.62 mg of CLB standard was weighed, shifted into 50 mL volumetric flask containing 35 mL of diluent. The solution was sonicated to dissolve CLB completely and diluted up to mark to obtain standard stock I. Further 5.0 mL of standard stock I diluted to 100 mL (standard stock–II). An aliquot of 5.0 mL of stock-II diluted up to 100 mL, mixed well to get standard stock solution III. All the dilutions were carried out using diluent.

Preparation of sample solution

A sample solution of 500 µg mL^{-1} was obtained by transferring suspension containing 25 mg of CLB into a 50 mL volumetric flask containing 35 mL of diluent and sonicated for 30 min. The solution was diluted up to the mark after cooling with diluent. The solution was centrifuged for 10 minutes at 7000 rpm and filtered. It was filtered through 0.45 µm syringe filter prior analysis.

Impurity stock solution preparation

Weighed and transferred 1.005/ 2.337/ 2.098/ 2.015/ 1.990 mg of impurity A, B, C, D, and E into separate 20 mL flask. About 14 mL ACN added and sonicated to hasten dissolution. The solution was diluted up to the mark with

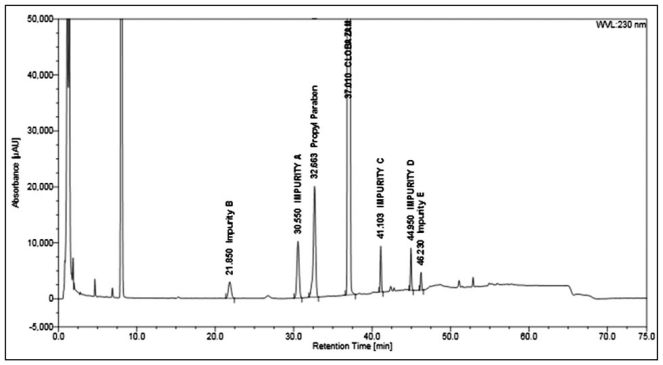

Fig. 2: Chromatogram showing separation of related substances, CLB and propyl paraben

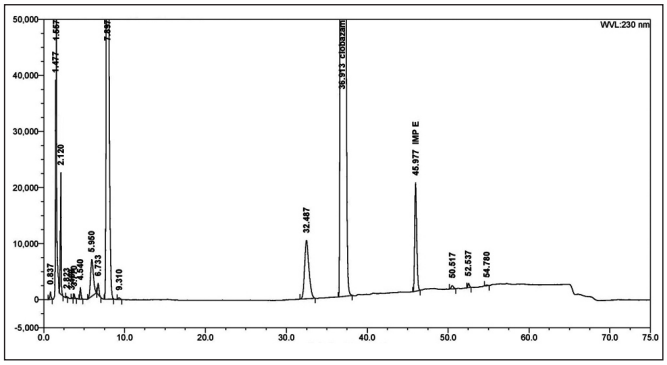

Fig. 3: Chromatogram produced after acid stress (5N HCl for 30 min at 80 °C)

Table I: Results of method development trials

Trial no.	HPLC Condition	Remarks
1	Water: ACN (60:40 V/V)	i) All major placebo peaks and unknown peaks are eluted within 5 min. ii) The retention time for CLB peak was 10.4 min.
2	Water : ACN (70: 30 V/V)	i) The retention time for CLB peak was 30.8 min. ii) All known impurities were not eluted within given run time i.e. 60 min.
3	Water: ACN: Methanol (60:40:10 V/V/V)	i) The retention time for CLB peak was 17.9 min. ii) Run time was more. ii) One unknown unidentified peak eluted near to impurity A with poor resolution of 1.31.
4	Mobile Phase A- Water Mobile Phase B- ACN Time (min)/% Solution B: 0/30, 35/30, 40/85, 55/85, 60/30 and 70/30 V/V	i) The retention time for CLB peak was 30.8min. ii) Resolution between unknown peak and Impurity A peak was 4.47. iii) Baseline was not proper at the elution of Impurity C, D and E.
5	Mobile Phase A- Water Mobile Phase B- ACN Time (min)/% Solution B: 0/30, 25/30, 50/80, 58/80, 60/30 and 70/30 V/V	i) Small peak from blank eluting closely to impurity C with resolution of 1.66.
6	Mobile Phase A-Water Mobile Phase B- ACN Time (min)/% Solution B: 0/25, 25/25, 55/75, 63/75, 65/25 and 75/25 V/V.	i) No interfering peaks from blank and placebo. ii) Tailing factor of CLB was 1.04 Theoretical plates of CLB were 273443. iii) Resolution between CLB Impurity A and Propyl paraben was 4.24.

diluent and mixed well. Impurity mixture solution was prepared of each impurity at 0.2% concentration as that of CLB sample solution.

System suitability solution (SST) preparation

About 2.5 mg of Impurity A and 25 mg propyl paraben was transferred into 50 mL volumetric flask containing 35 mL diluent. The solution was sonicated and volume was made up to mark using diluent and mixed. This resulted in an impurity A solution of concentration 50 µg mL^{-1} while 500 µg mL^{-1} of propyl paraben solution.

Preparation of placebo solution

Following the same procedure as that of sample preparation, placebo was prepared. Instead of sample, placebo was weighed.

Forced degradation study of drug product

Forced degradation of CLB was executed to gain an insight about the stability profile of the drug and specificity of the method. The stress conditions for the studies included of light, heat, acid, base and aqueous hydrolysis, and oxidation.

METHOD VALIDATION

The developed method was validated for specificity, linearity, accuracy, precision, recovery, limit of detection (LOD), limit of quantification (LOQ) and robustness as per the ICH guideline.

Specificity

CLB formulation stress studies were executed to offer an indication of the stability profile of the drug and specificity

Table II: Chromatographic performance data

Compound	Rt (min)	RRT[a]	Theoretical Plates (USP)	Tailing factor	Resolution (USP)
Impurity B	21.850	0.59	17085	1.05	n.a
Impurity A	30.550	0.83	54454	1.00	14.59
Propyl paraben	32.663	0.88	68464	0.79	4.13
CLB	37.010	1.00	243780	1.06	10.88
Impurity C	41.103	1.11	396820	1.04	14.60
Impurity D	44.950	1.21	491365	1.04	14.87
Impurity E	46.230	1.25	484500	1.06	4.90

[a]*Relative retention time (RRT) was calculated against the retention time (Rt) of CLB*

Table III: System suitability parameters

Parameter	Chromatographic changes	System suitability				
		R_t of CLB in diluted standard solution	Resolution between Impurity A and Propyl Paraben in SST	Theoretical plates for CLB in diluted standard solution	Tailing for CLB in diluted standard solution	% RSD for diluted standard
Control	-	37.134	3.73	263036	1.03	2.16
Flow rate	- 0.20 mL min^{-1}	39.781	3.21	164929	0.93	0.49
	+ 0.20 mL min^{-1}	35.126	3.37	185967	1.08	0.25
Wavelength	- 5 nm	37.136	3.72	268337	1.03	2.15
	+ 5 nm	37.134	3.73	264258	1.03	1.76
Column Temperature	- 5 °C	38.869	3.00	237846	1.41	0.93
	+ 5 °C	37.777	2.27	225041	1.33	2.27

of the developed method. Deliberate forced degradation using heat (80°C for 48 h), photolytic (sunlight for ~1.2 million lux h, shorter and longer UV light, for ~200 Wh/m^3), acid (reflux with 5 N HCl at 80 °C for 30 min), base (72 h, reflux with 0.2 N NaOH at RT and at 80 °C with 0.5 N NaOH for 1h) and oxidation (reflux at 80 °C, 6 h, 30% H_2O_2) to assess the capacity of the projected method to resolve CLB from its degradation products. Sample was subjected to 75% relative humidity at 40 °C at 72 h for humidity stress.

Precision

The repeatability was checked by analysis of test solution (500 µg mL^{-1} of CLB), spiked with the known impurities at specification level i.e. 0.50 % for Impurity A and 0.20 % for CLB Impurity B, C, D and E. The analysis was performed six times. The % RSD of individual impurity peak area was considered to establish precision.

Inter and intraday deviation and analysis by varied analysts was studied to govern intermediate precision of the said method. Intraday precision was assessed by 6-fold analysis of 500 µg mL^{-1} of CLB test, spiked with 0.50 % of Impurity A and 0.20% of Impurity B, C, D and E. The same procedure was repeated

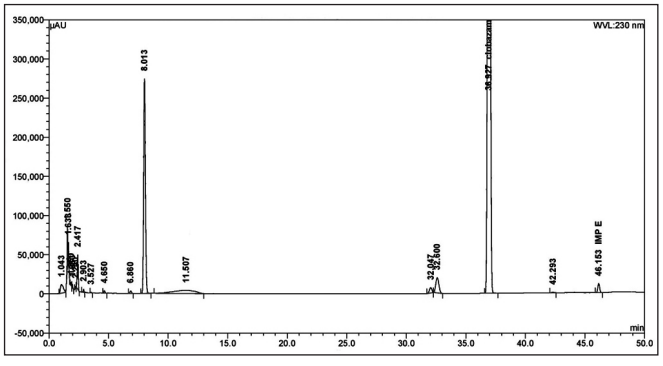

Fig. 4: Chromatogram produced after base stress (0.5 N NaOH for 1 h at 80 °C)

Table IV: Stress testing (forced degradation) data

Stress Condition	Peak purity match of CLB	%Net degradation					% Mass balance
		Impurity A	Impurity B	Impurity C	Impurity D	Impurity E	
Acid hydrolysis	1000	ND	ND	ND	ND	2.17	90.06
Base hydrolysis	1000	ND	ND	ND	ND	ND	96.27
Base hydrolysis	1000	ND	ND	ND	ND	1.45	87.02
Peroxide oxidation	1000	ND	0.08	ND	ND	ND	100.31
Photolytic[a]	1000	ND	ND	ND	ND	ND	101.26
Heat stress	1000	ND	ND	ND	ND	ND	99.62
Humidity stress	1000	ND	ND	ND	ND	ND	101.34

[a] *1.2 million lux hours\200 watt hours/square meter*

Table V: Regression and precision data

Parameter	CLB	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E
LOD (µg mL^{-1})	0.0307	0.0324	0.0290	0.0310	0.0321	0.0460
LOQ (µg mL^{-1})	0.1024	0.1081	0.0965	0.1035	0.1060	0.1530
Regression data						
Slope	94587.49	87713.75	79975.55	79994.71	70115.165	32475.007
Intercept	1849.6658	5727.4882	1554.3120	1185.0683	1371.6824	486.6037
Correlation coefficient RRF	0.9986 1.00	0.9988 0.93	0.9993 0.85	0.9992 0.85	0.9989 0.74	0.9993 0.34
Precision (%RSD)[a]	0.28	1.28	2.78	1.00	0.44	2.13
Intermediate Precision (%RSD)[a]	0.31	0.81	2.75	2.3	0.80	2.40
Precision at LOQ (%RSD)[a]	0.23	1.2	2.27	2.7	0.75	2.3

[a] *Calculations based on six determinations*

on three different days to study inter-day variation (n=18). Solutions were prepared by varied analysts on different days. The % RSD of individual impurity peak area was assessed.

Limit of detection and quantitation[13]

Analysis of series of CLB and related impurities standard solutions, was performed. LOD and LOQ for CLB and its six impurities were estimated at the amounts for which the signal-to-noise ratio was 3:1 and 10:1, respectively. Determination of precision at LOQ concentration and peak area % RSD was calculated for analytes.

Response function

To check the response function, series of standard CLB and impurities solution over a range from 0.05 ppm to 1.5 ppm (upto 150 % of specification level i.e. 0.2%) for CLB and its impurities B, C, D and E and 0.05 ppm to 4.5 ppm for CLB Impurity A (up to 150 % of specification level i.e. 0.5 %) were prepared. For each impurity, the response factor was obtained from the linearity plots of concentration versus area response. Further, the response factor of individual impurity was correlated with the response factor of the CLB and the relative response factors (RRFs) were calculated. These RRF values were used to estimate each of the impurities existing in the sample against the CLB standard. From the linearity plot, the correlation coefficients, slopes and y-intercepts of the peak areas as well were stated.

Accuracy

The accurate analytical procedure expresses the closeness of agreement between the true value and the found value. Sample of CLB oral suspension was spiked with each of the impurities at four different concentration

Table VI: Evaluation of accuracy

Amount spiked[a]	% Recovery[b]				
	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E
LOQ	105.03	100.37	103.4	107.33	104.83
50%	98.5	103.73	101.47	106.1	103.83
100%	101.1	105.8	99.57	107.87	99.87
150%	101.77	-	-	-	-
200%	-	105	98.73	107.1	102.5
% RSD	2.78	2.93	1.96	0.85	2.13

[a]Amount of impurities B, C, D, E and CLB spiked with respect to 0.2% specification level and 0.5% for impurity A.[b] Mean recovery for three determinations.

Table VII: Robustness evaluation of the developed HPLC method

Parameter	Chromatographic changes	RRT of related compounds				
		Impurity B	Impurity A	Impurity C	Impurity D	Impurity E
Control	-	0.57	0.82	1.11	1.22	1.25
Flow rate	-0.20 mL min^{-1}	0.72	0.87	1.10	1.19	1.22
	+0.20 mL min^{-1}	0.52	0.76	1.12	1.23	1.27
Wavelength	-5 nm	0.57	0.82	1.11	1.22	1.25
	+5 nm	0.57	0.82	1.11	1.22	1.25
Column temperature	-5°C	0.72	0.87	1.09	1.19	1.22
	+5°C	0.64	0.85	1.10	1.20	1.24

levels: LOQ, 50%, 100%, and 150% of specification limit for Impurity A, B C, D and E. The % recovery of the impurities was then considered.

Robustness

To accomplish the robustness, the experimental factors of the developed were altered deliberately. The resolution between CLB and impurities was assessed. The flow rate of mobile phase was altered to 0.8 and 1.2 mL min^{-1} to study its effect on resolution. The effect of wavelength was studied at wavelengths of 225 and 235 nm (instead of 230 nm). Similarly, the column temperature was studied at varied conditions.

Solution stability

To estimate the solution stability of CLB and its impurities, the sample and reference standard were stored at room temperature for at least 48 h in a tightly closed volumetric flasks. The solutions were analysed during this period.

Filter equivalency

An impurity spiked sample (at 0.20% level for impurity B, C, D and E and 0.50% for Impurity A) was prepared. It was centrifuged and filtered through varied membrane filters such as Nylon (0.45 µm), PVDF (0.45 µm). The % area of individual impurity from respective filtered solution was compared against centrifuged sample solution.

RESULTS

Optimization of chromatographic conditions

The numerous experimental trials implemented to develop and optimize stability indicating HPLC method for related substances of CLB in its suspension dosage form are given in Table I. The basic purpose of the presented method was to meticulously resolve closely eluting impurities. Attempts were made using different mobile phase compositions with isocratic elution mode initially. Apparently, all these trials failed to separate CLB, impurities and degradation products as well. Hence, it was decided to try different gradient programs to optimize

the separation and to attain adequate system suitability parameters. The 230 nm wavelength was selected as the optimum sensitivity of CLB and its impurities was found at this wavelength. The impurity peaks and the CLB peaks were free from the solvent and excipients interference. Optimized method has given proper resolution, symmetric peak shape and an acceptable tailing factor (Fig. 2). The chromatogram of impurity spiked sample solution shows the adequate separation for all the known impurities and CLB. The interfering peaks from placebo at the retention time of the CLB and all the known impurities were absent. Similarly, all the known impurity peaks and CLB peak were found well separated from the placebo peaks. All the peaks of interest were eluted within 46 min. The extended runtime was given in order to wash out the carryover peaks from the column. The chromatographic performance data (Table II) proves the suitability of the developed method for its intended use.

Column used in all trials was YMC Pack ODS A (150 x 4.6 mm, 3 µm,12nm). The flow rate of mobile phase was 1 mL min^{-1}

Setting of system suitability criteria

Impurity A and propyl paraben showed close retention time while the developing the method. So, it was necessary to critically attempt resolution in between them till the stability testing of formulation. Therefore, in this system suitability, impurity A and propyl paraben resolution was checked meticulously (Table III).

METHOD VALIDATION

The developed method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and LOD, LOQ, solution stability and robustness.

Specificity

Specificity of the analytical method is the ability to measure the response of analyte in the presence of its potential degradants. The specificity of the method was tested by injecting solution containing excipients devoid of drug substances. There was no chromatographic interference from excipients at the retention time of the CLB. Further, all the impurity peaks were eluted and resolved. Peak purity was verified by confirming homogeneous spectral data of CLB.

Forced degradation study

Sample concentration of 500 µg mL^{-1} of CLB with LC conditions mentioned in the chromatographic conditions were executed for analysis. The use of a PDA detector ensured the homogeneity and purity of CLB and its impurities peak area. Substantial degradation of CLB sample was detected in acid hydrolytic condition (5 N HCL, 30 min, 80 °C) including base hydrolysis condition (0.5 N NaOH, 1 h, 80 °C). The chromatograms are given in Fig. 3 and 4, respectively. Under both conditions, there was rise in the concentration of impurity E. Mild degradation was observed in oxidative condition (30% H_2O_2 for 80 °C at 6 h). All the stress samples were evaluated for peak purity test by using PDA (Table IV). The result confirms peak purity data of CLB peak and known impurity peaks in every degradation sample is homogeneous and devoid of any co-eluting peaks thus, demonstrating the stability indicating potential and specificity as well.

Precision

The results of intermediate precision are presented in Table V. The % RSD of AUC should be NMT 5.0 % and NMT 10.0 % for CLB and its impurities respectively for intra- and inter-day repeatability. The optimum results established the precision of the developed method.

LOD and LOQ

The LOD, LOQ and precision at LOQ for CLB and its impurities are presented in the Table V. The lower values of LOQ confirmed that the method was highly sensitive.

Response function

The response of the CLB and its impurities were found to be linear over the concentration range of 0.05 ppm to 1.5 ppm for CLB, Impurity B, Impurity C, Impurity D and Impurity E and 0.05 ppm to 4.5 ppm for CLB Impurity A. The correlation coefficient for CLB and all the known impurities are greater than 0.99 for studied concentration range for each of the component. These RRF values, correlation coefficients, slopes and y-intercepts of the peak areas prove the linearity of the proposed method (Table V).

Accuracy

Recovery of CLB and its impurities present in pharmaceutical dosage forms were ranged from 85% to 115%. The % RSD for mean recovery is less than 10.0% for all the known impurities (Table VI). Therefore; the HPLC method for the related substances of CLB is accurate.

Robustness

The ability of the analytical method to remain unaffected even after minute but deliberate alterations in method conditions indicate robustness. It proves the reliability of the method during routine application.

Under all applied deliberate varied experimental parameters (flow rate, column temperature and wavelength), each of the analyte was satisfactorily resolved and elution orders stayed unaffected, as seen in Table VII.

Solution stability

The amount of CLB and its impurities remained constant without any significant changes, as mentioned in Table VII. The results from solution stability experiment confirmed that the standard solutions were stable up to 52 h at room temperature.

Filter equivalency

The percent area difference with centrifuged sample for each impurity from each filter is not more than 10.0 % for 0.45µm Nylon and PVDF filters. Therefore, these filters are suitable for filtration of sample solution.

DISCUSSION

The results from the method development and validation ensure that the proposed method is best fit for the analysis of the CLB and its impurities. All the peaks of the interest were eluting in 46 min. To remove any inactive components that might have retained on the column; prolonged runtime was set. The results from the forced degradation study proved the stability-indicating capability of the method. The drug is stable at all conditions except acid and base stress. Under acid and basic stress, there was generation of impurity E. The lower LOD and LOQ values indicate higher sensitivity of the method. The results from the method validation study ascertain that the proposed analytical method is precise, accurate, linear and specific.

CONCLUSION

The validated stability indicating RP-HPLC method was developed for quantitative analysis of CLB and its related substances present in its oral suspension. It is sensitive, precise, accurate, linear, robust and specific. It can be used for routine analysis of production samples and to check the stability of CLB in suspension.

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