Method Development and Validation of Cabotegravir by Using HPLC and Characterization of its Degradants by LC-MS

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ABSTRACT:

The characterization and identification of the stressed breakdown products of cabetogravir (CBT) were accomplished by development and validation of a rapid, precise, and dependable isocratic LC-MS approach. According to the parameters established by the International conference on Harmonisation, the antiviral medication CBT was submitted to hydrolysis in acidic, alkaline, and neutral solutions as well as to oxidation, photolysis, and thermal stress. Under acid hydrolysis and oxidative stress conditions, the drug showed less degradation. But it remained stable in the presence of thermal, wet heat, photolysis stresses. Five different degradation products in total were identified, and the drug and these products were separated chromatographically on a Kinetex C18 column (250 4.6 mm, 5 m) using a mobile phase of 20 mM (pH 3), 70:30 v/v, methanol and phosphate buffer. By using LC-MS, the degradation products were identified, and their fragmentation paths have been proposed. With regard to specificity, linearity, accuracy, and precision, the LC-MS method was validated.

KEYWORDS: LC-MS, Fragmentation pathways, Oxidation, Validation, Characterization.

INTRODUCTION:

Cabotegravir is used to treat acquired immunodeficiency syndrome (AIDS). [1, 2] It is offered as an intramuscular injection and tablet form. [3, 4] the main purpose of the tablets is to gauge how well a person is responding to the treatment. [5, 6] Additionally, cabotegravir is the first antiretroviral medications to be formulated for injection and to have an extended half-life. [7]

In comparison to earlier non-nucleoside reverse transcriptase inhibitors like viramune, Cabotegravir, a second-generation inhibitor, has fewer side effects, is more potent, and has a longer half-life. [8] Although they are less frequent, rashes, insomnia, and headaches have been listed as some of the tablets' side effects in prior research. [9, 10] The objective of this study is to use LC-MS to investigate the Cabotegravir breakdown products. Cabotegravir's efficacy and safety throughout storage, delivery, and clinical usage depend on an understanding of its stability and breakdown processes. The purpose of this work is to characterise and identify the Cabotegravir degradation products in order to better understand its stability profile. [11, 12]

Structure of CBT:

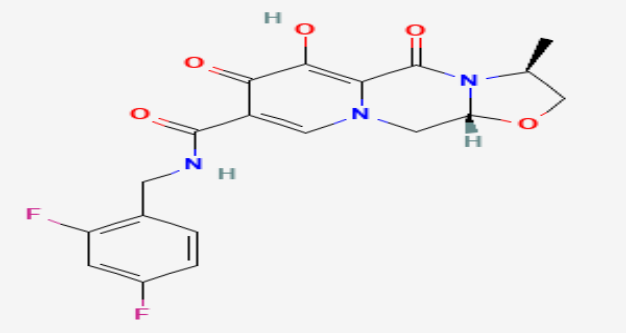

Fig No. : 1 Structure of CBT

Methods: Cabotegravir was obtained as a complimentary sample from Spectrum Laboratory Pvt. Ltd., located in Hyderabad. Analytical reagent quality solvents and other compounds used in the study were sourced from S.D. Fine Chemical and the Research Lab in Mumbai. For the analysis, water was utilized, which underwent filtration using a 0.45 µm syringe filter after being glass distilled using basic glass distillation equipment. The choice of high-quality reagents and proper filtration techniques ensured the reliability and accuracy of the experimental results.

Instrumentation : HPLC Waters Alliance LC (model 2695) monitored with empowering 2 data handling systems and fitted with kinetex C18 column (250 mm × 4.6 mm, 5 µm and a detector of photodiode array (model 2998) was used for this study.

LC-MS:

LC-MS conditions in the stress degradation study, HPLC was linked to a mass spectrophotometer with the splitter placed before the ESI source, allowing only 35% of eluent to enter. The following were the standard operating source conditions for Cabotegravir MS scans on positive ESI mode: the fragmented voltage was set at 80 V, the capillary at 3000 V, the skimmer at 60 V, nitrogen was used as a drying and nebulizing gas (45 psi), and highly filtered nitrogen gas was used as collision gas.

FTIR:

FTIR conditions FTIR was utilized to get insights concerning the presence of different functional groups like keto, Aldehydes, cyano alcohol, and amides present in the degradation samples**.** Infrared light is passed through a sample, and the interaction of the light with the sample's molecules is measured.

Buffer preparation

The addition of 1 litre of HPLC-grade water to 1 mL of formic acid, followed by filtration through a 0.45 μm membrane filter.

Mobile phase:

Preparation for the mobile phase Methanol and Phosphate buffer were thoroughly combined in a (70:30) ratio, and the liquid was then sonicated for five minutes before being filtered through a 0.22 m membrane filter. The HPLC analysis was performed on a reversed phase-HPLC system with isocratic elution mode, a kinetex C18 column (250 mm 4.6 mm, 5 m), a flow rate of 1 mL/min, and a photodiode array detector at 243 nm. The mobile phase used was a mixture of methanol and phosphate buffer (70:30). The mobile phase served as a diluent.

Standard solution preparation:

An accurately weighed 10 mg of Cabotegravir working standards into a 100 ml volumetric flask, add 70 ml of methanol, dissolve it, and makeup to the mark with methanol. This gave 100 μg/mL standard stock solutions for CBT.

Chromatographic condition:

During the optimization process, various system suitability factors including theoretical plate count, resolution, and tailing were evaluated for different mobile phase combinations. Ultimately, a mobile phase consisting of a freshly prepared mixture of 70% methanol and 30% phosphate buffer was chosen. The separation was carried out using a flow rate of 1.0 ml/min. The detection wavelength for achieving a symmetric peak of Cabotegravir was set at 243 nm. It is worth mentioning that the ambient temperature was carefully maintained throughout the entire procedure to ensure consistent and accurate results.

Results:

UV absorption spectra of cabetogravir: UV absorption of 10 μg/mL solution of cabetogravir in methanol was recorded and 400-200 nm. λ_{max} of CBT in methanol was found to be 243 nm.

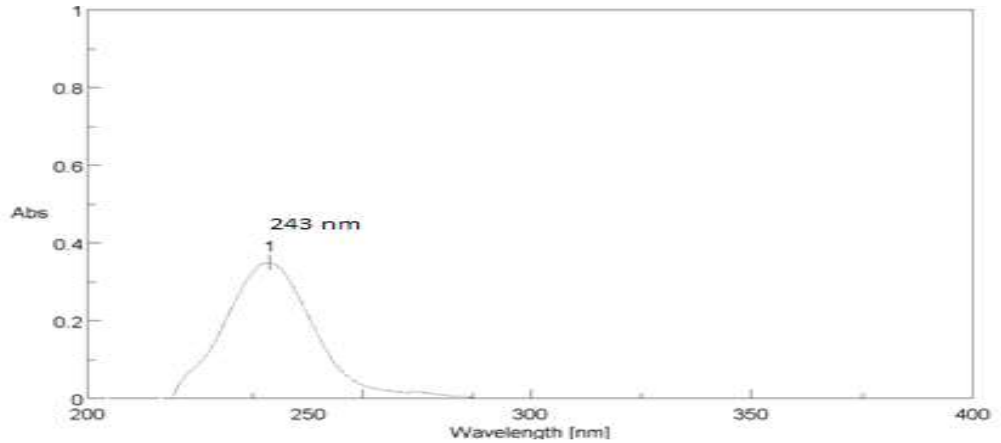

Fig.No.02. UV spectrum of Cabetogravir

To achieve the best chromatographic conditions, various columns such as C18, C8, as well as mobile phases, were tested. The best chromatographic separation occurred on kinetex C18 column (250 mm × 4.6 mm, 5 μm) with Methanol and Phosphate Buffer in (70:30) mobile phase at a flow rate of 1 ml/min and PDA detection at 243nm (Fig. 03).

Specificity: The HPLC chromatograms recorded for the blank solution and blank solution exposed to the degradation conditions showed no peaks at the retention time of CBT and also the representative chromatograms of stressed samples under various stress conditions showed that CBT was well resolved from its degradation products, indicating the specificity of the method. The HPLC chromatograms recorded for blank, placebo, standard and sample solution showed (Shown in Fig.No.03) that CBT peak was not affected by diluents and placebo.

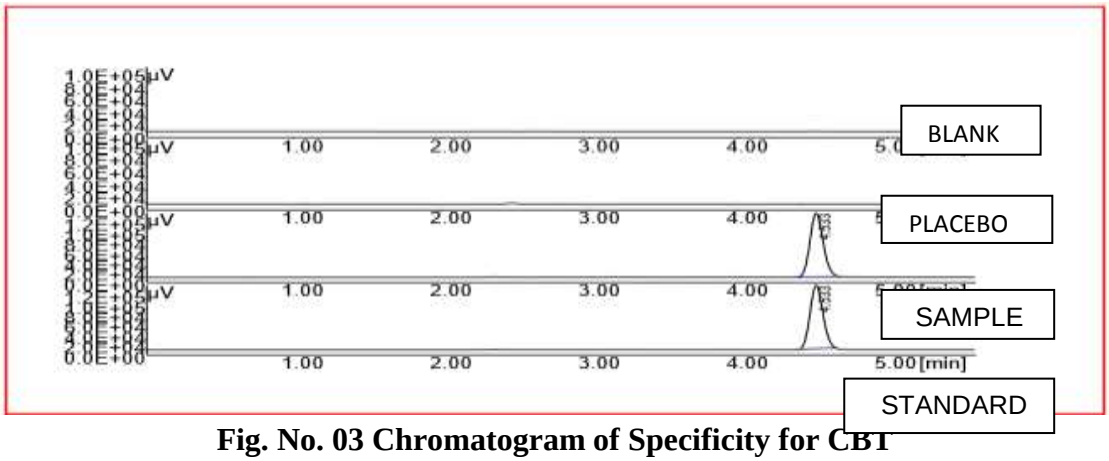

Fig. No. 03 Chromatogram of Specificity for CBT

Linearity:

The current application's linearity was determined by plotting a graph between concentration and corresponding peak area for Cabotegravir over concentration ranges of 2-12 µg/mL (Fig. 04) for cabetogravir correlation coefficient was found to be 0.9979. Summary of the linearity results shown in Table No. 01.

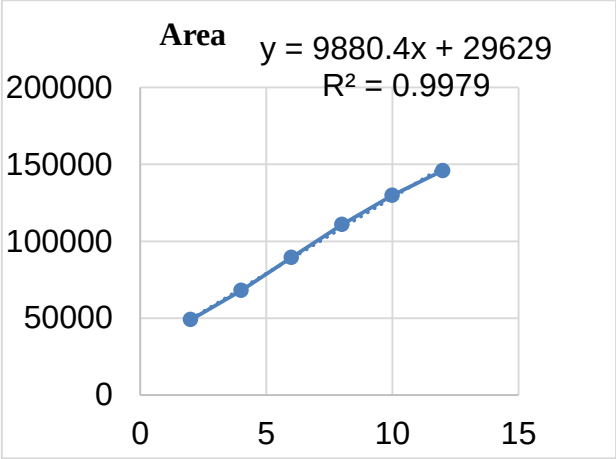

Fig. No. 4. Linearity curve of CBT

Table No. 1: Linearity data for CBT

Sr. No.	Concentration (µg/mL)	Area
1	2	48932
2	4	67920
3	6	89433

4	8	110782
5	10	129862
6	12	145822
Equation	y = 9880.4x + 29629	
Regression	0.9979	

Precision:

The intra-day precision was assessed by performing six analyses using standard stock solution containing analytes of interest. Similarly inter-day precision was assessed by performing replicate analysis using (8 mcg/mL) concentration of all the analytes under the same experimental conditions. And the %RSD was calculated. The % RSD was found <2%. These value depicted in Table No.02.

TableNo.02 Precision parameters of CBT

Sample	**Intraday**		**Interday**	
Injection	**Area**	**RSD (%)**	**Area**	**RSD (%)**
1	75040	0.75	75964	0.40
2	76511		76390.5	
3	76337		75626	
4	76578.3		76321	
5	76329.8		76392	
6	76231		76193	

Limit of Detection (LOD): The DL is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope, the detection limit was found to be 0.49 µg/ml.

Limit of Quantification (LOQ): The QL is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope, the quantification limit was found to be 1.47 µg/ml.

Robustness: flow rate has minimal impact on the retention time, while the mobile phase composition can slightly affect the retention time of the analyte. These findings are important for method development and optimization in liquid chromatography, as they can help determine the appropriate conditions for achieving desired retention times and separation of compounds of interest. The robustness value depicted in table no.03.

Table No.03: Robustness of CBT

Parameter	**Condition**	**Retention time**
Flow rate	0.9	4.6
	01 mL	4.7
	1.1	4.7
Mobile phase composition	Methanol: Phosphate buffer (68:22 % *v/v*)	4.7
	Methanol: Phosphate buffer (70:30 % *v/v*)	4.7
	Methanol: Phosphate buffer (72:28 % *v/v*)	4.6

Accuracy:

The result Accuracy studies on CBT for acid degradation and oxidation degradation. Cabotegravir were prepared in three concentration levels: 80%, 100%, and 120%. Recovery percentage was depicted in table no 04 for acid Degradation and Table no.05 for oxidation Degradation

Table No. 04: Accuracy studies on CBT for acid Degradation

Concentration added (µg/ml)	Calculated concentration (µg/ml)	S.D.	R.S.D. (%)	Recovery (%)
10+8 (18µg/ml) 80%	16.20992893	16954.71	1.445966083	90.05516072
	15.8939035			88.29946389
	16.39684229			91.0935683
10+10 (20µg/ml) 100%	18.06044022	9563.355	0.735048837	90.3022011
	17.96963745			90.3022011
	18.25062224			90.3022011
10+12 (22µg/ml) 120%	20.12881219	20878.36	1.444317962	91.06475436
	20.03424596			93.71600825
	20.61752182			91.49460085

Table No.05: Accuracy studies on CBT for oxidation Degradation

Concentration added (µg/ml)	Calculated concentration (µg/ml)	S.D.	R.S.D. (%)	Recovery (%)
10+8 (18µg/ml) 80%	17.46371488	22074.92	1.774845697	97.02063821
	17.38505713			96.5836507
	16.85515938			93.63977436
10+10 (20µg/ml) 100%	18.37899961	18066.58	1.353723741	91.89499805
	18.89984107			94.49920533
	18.5102558			92.55127898
10+12 (22µg/ml) 120%	20.79470417	21865.98	1.489477854	94.5213826
	20.2187753			91.9035241
	20.77821093			94.44641333

Degradation effects and its characterization:

Cabotegravir samples were subjected to a variety of stress degradation conditions to observe the drugs partial degradation. The stress studies revealed the conditions under which the drug

becomes unstable; these measures can be implemented during formulation to avoid potential instabilities. LC-MS and FTIR are used to characterize these degradation products.

Acid degradation:

Cabotegravir were studied in 0.1 N HCl and 60^0C reflux for 30 min. degradation product found at 2.85 min. the percent degradation was found to be 9.79 % degradation on acid hydrolysis depicted in in Fig No.05 and table No.06.

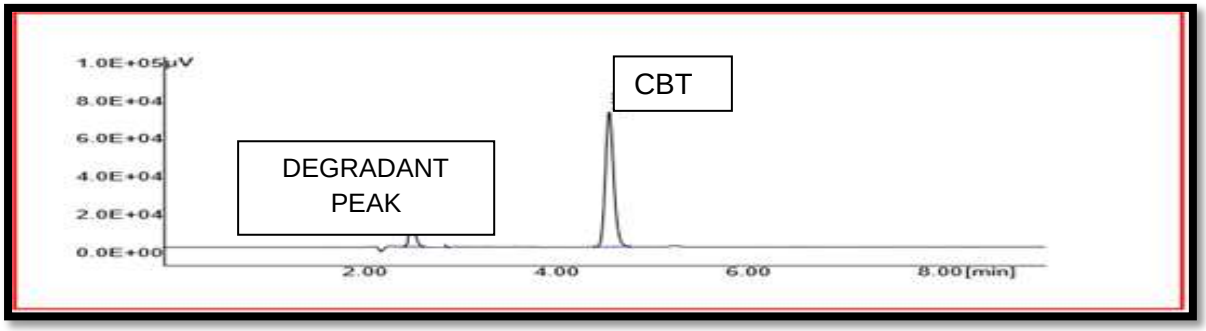

Fig.05. - Chromatogram of Acid treated of Cabetogravir (0.1 *N* HCL at 60°c for 30min.

Table No.06: Acid degradation results of cabetogravir

Sr	Stressed condition	Number of degradants form	RT of degradation product	RT of Degradation product	Area		% Degradation
					Unstressed	Stressed	
01	0.1 *N* HCl, 60^0**C** reflux for 30 min.	01	2.85	2.85	121047	109190	9.79

Alkali degradation:

Cabotegravir were stressed under in 0.1 N NaOH, 60^0C reflux for 15 min. there was three additional peaks at retention time 2.59 min. CBT in alkali stressed condition gave 18.22 % degradation product was formed on alkali hydrolysis depicted in Fig No.06 and table No.07

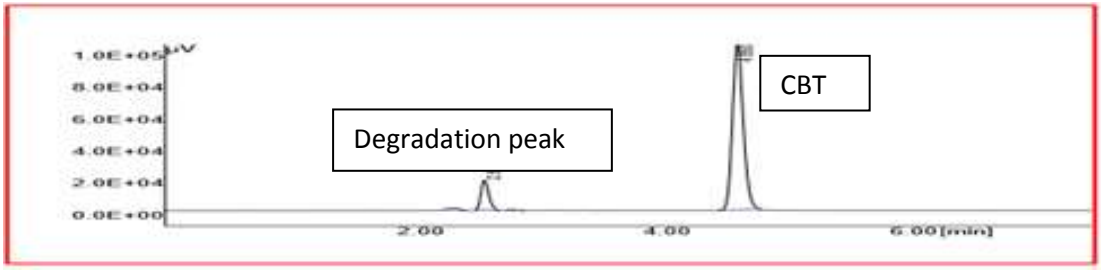

Fig. 06. Chromatogram of Alkali treated of Cabetogravir (0.1 *N* NaOH at 60°c for 15 min).

Table No. 07: Alkali degradation results of cabetogravir

Sr	Stressed condition	Number of degradants form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	0.1 *N* NaOH, 60^0C reflux for 15 min.	01	2.59	121047	98989	18.22

Oxidative degradation:

Cabotegravir were studied in 3% peroxide for 48 hrs.in dark conditions, and then no degradants peaks are formed. The stressed sample was heated for 5 minutes and then analysed after cooling, there was one additional peak at the retention time 2.05 min. oxidative degradation peak depicted in fig No.07& Table no.08

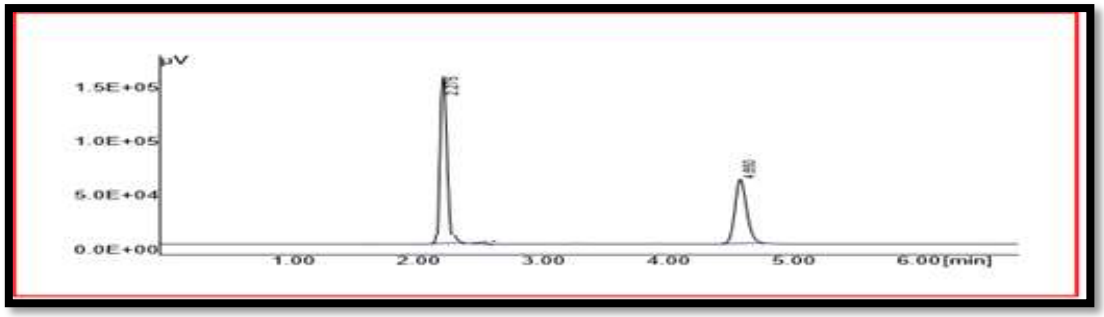

Fig. 07 - Chromatogram of oxidative degradation studies of CBT (3% H_2O_2 for 48hrs.

Table 08: oxidative degradation results of cabetogravir

Sr	Stressed condition	Number of degradant form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	03 % H_2O_2, for 48 HRS.	01	2.11	121047	78629	35.04

Wet heat degradation

When cabetogravir was subjected to wet degradation that is refluxed for 30 min with water at 60°C. When the stressed drug sample was analyzed, it was found that drug exhibits no degradation which was revealed from no additional peak. Further confirmation was done by

comparing the peak areas of stressed sample CBT and zero time samples, no difference in the chromatogram indicates, no degradation.

Dry heat degradation

In dry heat drug sample was kept for 1 hr at 80 °C. When the stressed sample was analyzed, no degradation was found and hence it was decided to extend the heating time for 2hrs, 3hrs, 4hrs and 5hrs with increased temperature of 100°C.There was no degradation found when the stressed sample was analyzed. Hence, CBT was found stable in Dry heat conditions. Degradation depicted in fig No.08& Table no.09

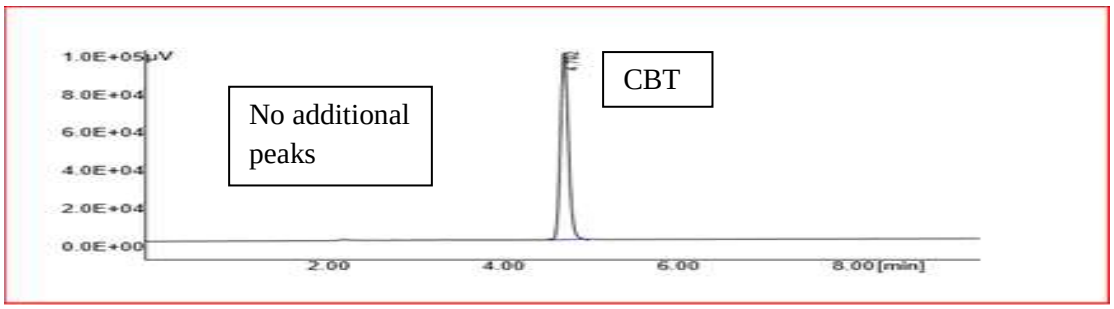

Fig. 08 - Chromatogram of Dry Heat degradation studies of CBT (1 hr for 80^{0}C)

Table No. 09: Dry Heat degradation results of cabetogravir

Sr	Stressed condition	Number of degradant form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	100^{0}C, for 01 Hrs.	00	NA	121047	121036	NA

Photolytic degradation:

For the first trial of photolytic degradation, the sample was exposed to Sun light for 8 Hrs and the exposed sample was analyzed, and no degradants peaks are formed. After that, the exposed sample was exposed time for 24 hrs. and 48 hrs. No degradants peaks were formed depicted in fig No.09& Table no.10.

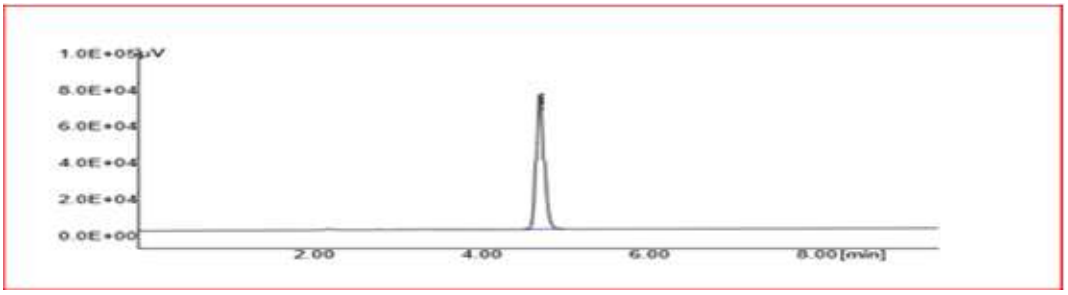

Fig. 09 - Chromatogram of Photolytic degradation studies of CBT (8 hrs.)

Table No.10: Photolytic degradation results of cabetogravir

Sr	Stressed condition	Number of degradant form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	Sunlight 48 HRS.	00	NA	121047	121009	NA

Analysis of formulation: The chromatograms of the drug samples extracted did not show any change in the retention time (Shown in Fig.10). There was no interference from excipients, which are commonly present in the tablets. The drug content was found to be 101.21% with a % RSD of 0.0069% as depicted in Table No. 11. Therefore it was concluded that, degradation of CBT had not found.

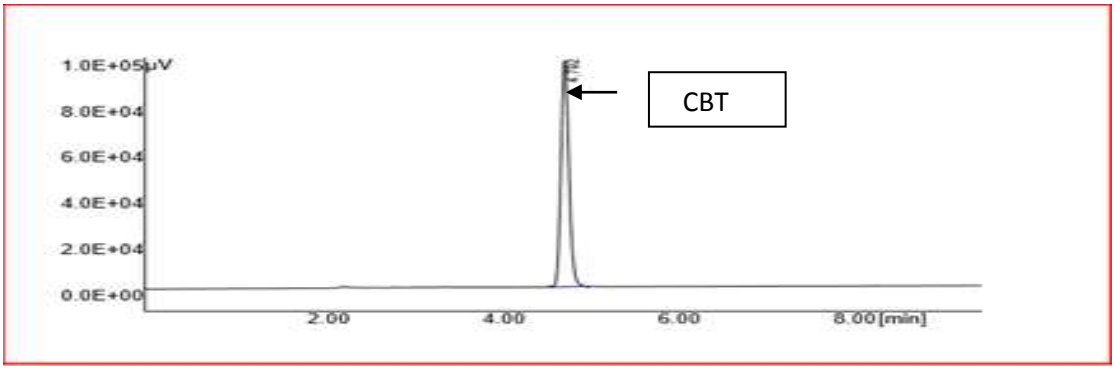

Fig.10. Chromatogram of formulation of CBT

Table No. 11: Analysis of formulation for CBT

Amount per tablet(mg)	Amount Found (mg)	(%) Found	Average (%)	±SD	%RSD
120	120.09	100.43	101.20	0.70	0.0069
120	123.46	101.38			
120	121.03	101.81			

Confirmation of degradation product: The degradation product was exposed to LC-MS studies. Below given are the mass spectra of CBT (Fig No.11) and Degradation product. CBT and the acidic depicted in fig.No.12.and alkaline degradation product were separated by LC-MS technique depicted in Fig.No.13

Mass Spectra of CBT:

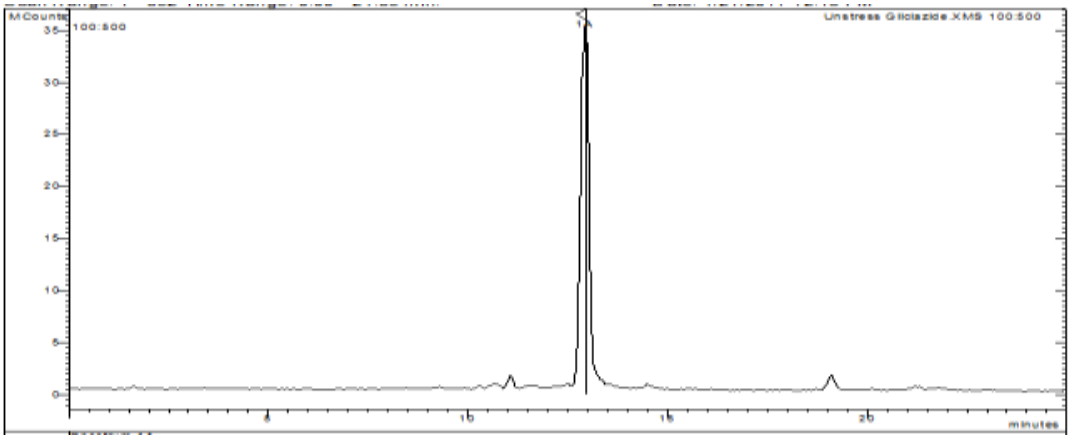

Fig.No. 11 Mass spectra of CBT

Acid degradation product characterization:

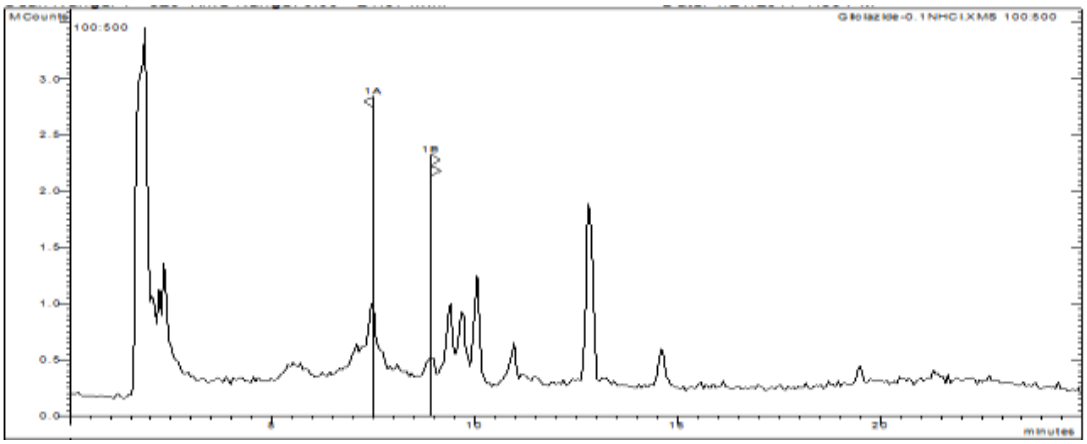

Fig.No. 12 MSD Chromatogram of CBT and stressed CBT (0.1N HCl 100°C for 30 min)

Alkali degradation product characterization:

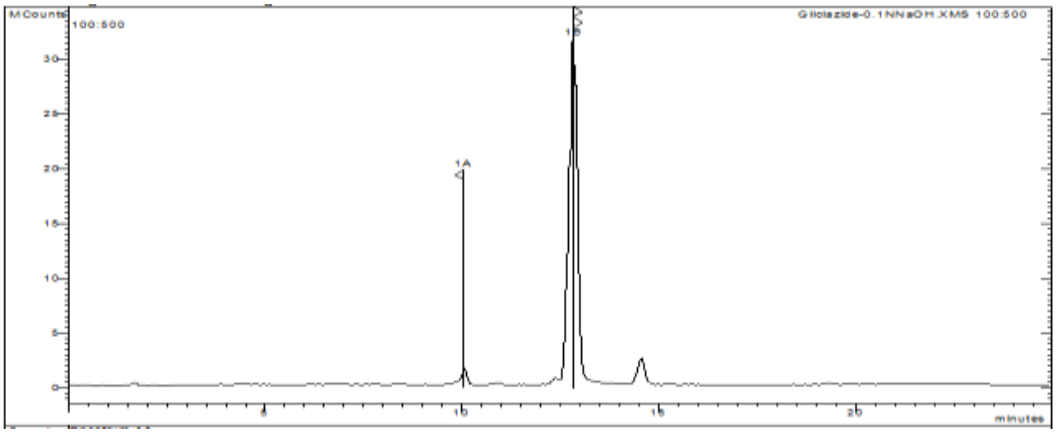

Fig.13. MSD Chromatogram of CBT and Stressed CBT (0.1N NaOH 100°C for 30 min)

Degraded Probable CBT:

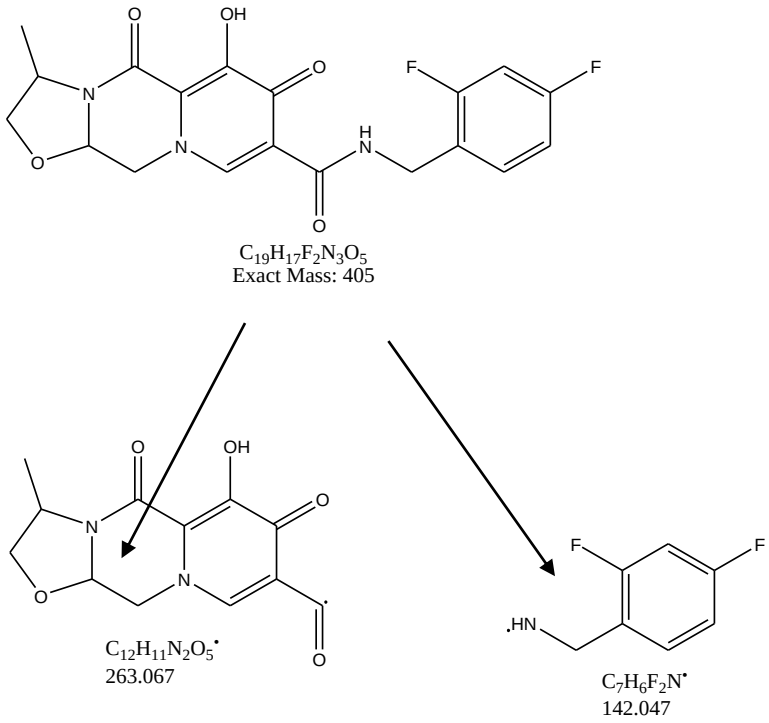

Fig No.14.Probable fragments obtained

Characterization of degradation products:

By ^{1}H NMR Spectroscopy: Characterization of acid degradation product was done by ^{1}H NMR spectroscopy. The compound was found soluble in water. The NMR spectrum of acid degradation product is shown below in Fig 15.NMR data interpretation shown in table no.12.

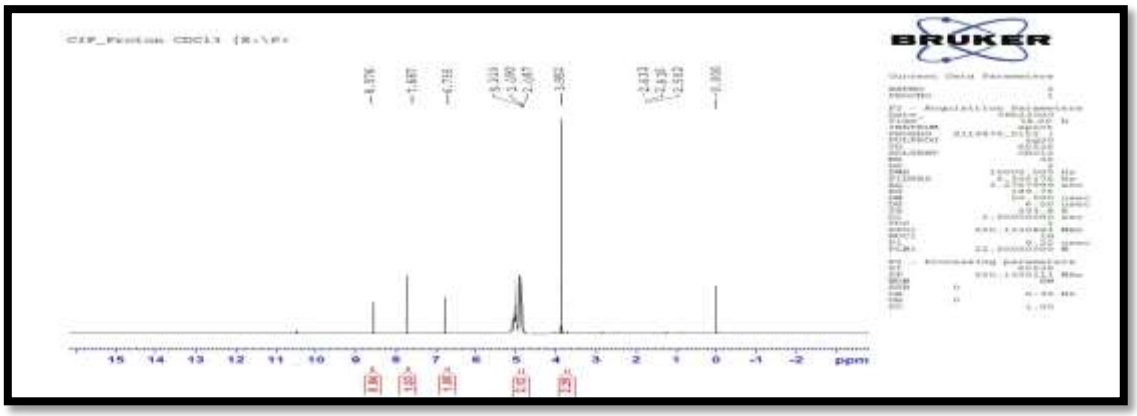

Fig. No. 15 1H NMR spectrum of acid degradation product

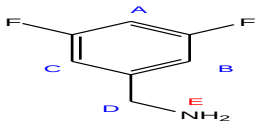

Table 12: Data interpretation of NMR [400 MHz ∂, ppm water]

∂ ppm	Signal	No. of Hydrogens	Group
6.7	singlet	1 H	Aromatic
7.6	singlet	1 H	Aromatic
8.5	singlet	1 H	Aromatic
3.9	singlet	2H	$-CH_2$ (Benzylic)
5.1	singlet	2H	$-NH_2$

Discussion:

For evaluating the stability of cabotegravir in this investigation, we created a precise and sensitive high-performance liquid chromatography (HPLC) method [13–15].

Experimental breakdown tests were carried out in accordance with International Council for Harmonisation (ICH) recommendations, and the breakdown products that resulted were identified using liquid chromatography-mass spectrometry (LC-MS) and Fourier-transform infrared spectroscopy (FTIR) [16–20]. It is remarkable that there haven't been many studies in recent years on how to determine cabotegravir using HPLC. Determining a novel HPLC method capable of reliably analysing cabotegravir and detecting its breakdown products using supplementary LC-MS and FTIR methods was the main goal of our research.

CONCLUSION:

In accordance with ICH recommendations, a validated HPLC method was created for cabetogravir drug estimation. All validation parameters, such as system suitability, method precision, accuracy, LOD, and LOQ, Robustness showed satisfactory results for the method. Shorter run times, lower costs, accessibility, excellent sensitivity, reliability, and reproducibility are just a few benefits of this established approach. Under various stress conditions, including acid, base, oxidation, reduction, photolytic, and thermal stress, and the degradation behaviour of the drugs was examined. The drugs were discovered to be unstable when exposed to acid, alkali, and oxidative circumstances, but they were stable when exposed to reduction, thermal, and photolytic conditions. LC-MS and FTIR tests were carried out to more fully characterise the degradation products.

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CONFLICT OF INTEREST:

The author declares no conflict of interest for the present manuscript.

REFERENCES

1. De Cock KM, Jafe HW, Curran JW (2012) The evolving epidemiology of HIV/AIDS. AIDS 26(10):1205–1213.
2. Blankson JN (2010) Control of HIV-1 replication in elite suppressors. Discov Med 9(46):261–266 .
3. Sisson H (2015) Aspirating during the intramuscular injection procedure: a systematic literature review. J Clin Nurs 24(18):2368–2375 .
4. Şanlialp Zeyrek A, Takmak Ş, Kurban NK, Arslan S (2019) Systematic review and meta-analysis: physical-procedural interventions used to reduce pain during intramuscular injections in adults. J Adv Nurs 75(12):3346–3361.
5. De Clercq E (1998) the role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. Antivir Res 38(3):153–179 .
6. Das K, Sarafanos SG, Clark AD Jr, Boyer PL, Hughes SH, Arnold E (2007) Crystal structures of clinically relevant Lys103Asn/Tyr181Cys double mutant HIV-1 reverse transcriptase in complexes with ATP and nonnucleoside inhibitor HBY 097. J Mol Biol 365(1):77–89.
7. Stellbrink HJ (2007) Antiviral drugs in the treatment of AIDS: what is in the pipeline? Eur J Med Res 12(9):483–495.
8. 8.Goebel F, Yakovlev A, Pozniak AL, Vinogradova E, Boogaerts G, Hoetelmans R, de Béthune MP, Peeters M, Woodfall B (2006) Short-term antiviral activity of TMC278—a novel NNRTI—in treatment-naive HIV1-infected subjects. AIDS. 20(13):1721–6.
9. Wilson JF (2008) In the clinic. Insomnia. Ann Intern Med 14891:ITC13-1-ITC13-16.
10. Drake CL, Roehrs T, Roth T (2003) Insomnia causes, consequences, and therapeutics: an overview. Depress Anxiety 18(4):163–176.
11. ICH (2003) Q1A (R2) Stability testing of new drug substances and products. https://www.ich.org/fleadmin/Public_Web_Site/ICH_Products/ Guidelines/Quality/Q 1A_R2/Step4/Q1A_R2_Guideline.pdf.
12. ICH guidelines, Q1A (R2): Stability Testing of New Drug Substances and Products (revision2), International Conference on Harmonization. Available from: ⟨http://www.fda.gov/downloads Regulatory Information/guidance/ucm128204.pdf⟩, 2003.
13. Beckett AH, Stenlake JB (2001) Practical Pharmaceutical Chemistry Part-II. UV-visible Spectrophotometry, CBS Publishers and Distributors, New Delhi, pp. 285-297.
14. Sethi PD High Performance Liquid Chromatography. Quantitative Analysis of Pharmaceutical Formulations; CBS Publishers and Distributors, New Delhi, pp. 14-15, and pp. 116-120.
15. S. K. PATRO, ''Development and Validation of High Performance Liquid Chromatographic Method for Determination of Lamivudine from Pharmaceutical Preparation," E-Journal of Chemistry, 2010, 7(1),pp. 117-122.
16. R .N. Rao," LCMS/MS Structural characteracterisation of stress degradation products including development of stability indicating assay Darunair: Anti HIV Drugs,

Journal of Pharmaceutical and Biomedical analysis 2014, (89), 23-33.

17. Vejendla et al. "Method development and validation for Cabotegravir and Rilpivirine by using HPLC and its degradants are characterized by LCMS and FTIR,"Future J Pharm Sci ,2021, 7 pp. 1-18.
18. Hemlata M.Nimje et al "Method Development and Force Degradation Study for Daclatasvir Using LC-MS/MS, Advances in Science and Engineering Technology",2020, pp.1-6.
19. Benedito Roberto de,"Chemo metrics Approaches in Forced Degradation Studies of Pharmaceutical Drugs",MDPI, 2019, 24,pp 1-24.
20. Prashant S. Devrukhakar "A stability-indicating LC–MS/MS method for zidovudine: Identification, characterization and toxicity prediction of two major acid degradation products", Journal of Pharmaceutical Analysis, (2017), .07, pp231-236.