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FORCED DEGRADATION STUDIES DEVELOPMENT AND VALIDATION BY RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF COMBINATION DRUGS ELBASVIR AND GRAZOPREVIR IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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Abstract:
A Stability-indicating reverse phase – high performance liquid chromatography(RP-HPLC) method was developed and validated for the determination of Elbasvir and Grazoprevir in tablet dosage forms using C_{18} column Discovery(250x4.6 mm, 5 µ) with a mobile phase consisting of orthophosphoric acid and methanol (45:55% v/v). The pH was adjusted to 3.8 with dil. NaoH.The mobile phase was sonicated for 10min and filtered through a 0.45µm membrane filter at a flow rate of 1.0 ml/min.The Detection was carried out at 220nm and retention time of Grazoprevir was found to be 2.3min ,and retention time of Elbasvir was found to be 4.8min.Linearity for Grazoprevir was observed from 25-150 µg/ml,coefficient of determination R^2 was 0.999, with equation y=6488x+6259.Linearity for Elbasvir was observed from 12.5-75µg/ml ,coefficient of determination R^2 was 0.999,with equation y= 9099x+383.3.Elbasvir and Grazoprevir was subjected to stress conditions including acidic,alkaline,thermal,oxidation, photostability,neutral degradation,solution stability and the results showed that it was more sensitive towards acidic and alkaline degradation.The method was validated as per ICH guidelines.
Key words: *Elbasvir, Grazoprevir, RP-HPLC, stability indicating, validation*

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Please cite this article in press as Swapna.Goday et al, **Forced Degradation Studies Development and Validation by RP-HPLC Method for the Simultaneous Estimation of Combination Drugs Elbasvir and Grazoprevir in Bulk and Pharmaceutical Dosage Forms***, Indo Am. J. P. Sci, 2017; 4(08).*

INTRODUCTION:

Grazoprevir ,chemically known as Cyclopropanecarboxamide, N-[[[(1R,2R)-2-[5-(3-hydroxy-6-methoxy-2-quinoxalinyl)pentyl]cyclopropyl]oxy]carbonyl]-3-methyl-L-valyl-(4R)-4-hydroxy-L-prolyl-1-amino-N-(cyclopropylsulfonyl)-2-ethenyl-, cyclic (1→2)-ether, hydrate (1 :1) (1R,2S) [1] Fig.1.
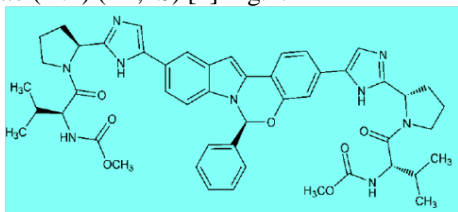

Fig 1.Molecular structure Grazoprevir
It is a second generation NS3/4a protease inhibitor approved for the treatment of hepatitis C virus (HCV). Elbasvir is chemically known as methyl N-[(2S)-1-[(2S)-2-[4-[(6S)-3-[2-[(2S)-1-[(2S)-2-(methoxycarbonylamino)-3-methylbutanoyl]pyrrolidin-2-yl]-4H-imidazol-4-yl]-6-phenyl-6H-indolo[1,2-c][1,3]benzoxazin-10-yl]-2H-imidazol-2-yl]pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate [2] Fig.2 .
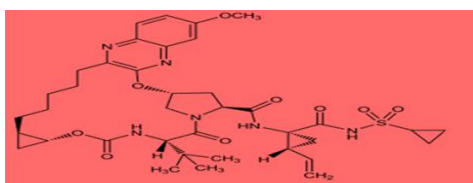

Fig.2. Molecular structure Elbasvir
It is an inhibitor of Hepatitis C Virus (HCV) non-structural protein 5A (NS5A) that is being developed for the treatment of HCV infection.The drug was approved by united states food and drugs administration(USFDA) in January 2016.[3].Hepatitis C is defined as inflammation of liver.HCV is caused by a virus transmitted through blood to blood contact.[4]Zepatier (elbasvir and grazoprevir) with or without ribavirin for the treatment of chronic hepatitis C virus (HCV) genotypes 1 and 4 infections in adult patients. Hepatitis C is a viral disease that causes inflammation of the liver that can lead to diminished liver function or liver failure . Zepatier with or without ribavirin was evaluated in clinical trails of 1,373 participants with chronic HCV genotype 1or 4 infections with and without cirrhosis.The studies were designed to measure whether a participants hepatitis c virus was no longer detected in the blood 12 weeks after finishing the treatment sustained virological response suggesting a participants

infection has been cured..[5]A bioanalytical method was developed forpharmacokinetic study of elbasvir in rat plasma[6]..Picogram quantification of elbasvir and grazoprevir in human plasma [7].stabiliy –indicating validatedRP-UPLCmethodfor simultaneous determination of elbasvir and grazoprevir in Bulk and pharmaceutical dosage form.[8]. According to ICH guidelines[8,9].our present work not only developed and validated the method but also a stability indicating study. It is required to demonstrate specificity of stability indicating methods and also provides insight degradation pathways and degradation products of drug substance and helps in structural elucidation of degradation products.[10] Stress studies should be performed in different pH solutions in the presence of oxygen,light,temperature to determine the stability of drug substance[11]. Purposeful degradation can be a useful tool to predict the stability of drug substance or drug product.[12].A stability indicating method accurately measures the active ingredients ,without the interference from degradation products ,process impurities.[13].The guidelines explicitily require conduct of forced degradation studies under a variety of conditions like PH,light ,oxidation,dry heat etc,and separation of drug from degradation products.[14].The intrinsic photostbility charecteristics of new drug substances and products should be evaluated to demonstrate that as appropriate light exposure do not affect in unacceptable changes. [15].various methods of literature involve determination of elbasvir and grazoprevir by UPLC/MS/MS[6], LC-ESI-MS/MS[7,]UPLC[8,].However no method is available for stability indicating RP-HPLC method in determination of grazoprevir and elbasvir in bulk and pharmaceutical dosage form.In the present work anew, economical, simple, precise stability indicating method is developed and validated iin accordance with ICH guidelines.

MATERIALS AND METHODS:
Chemicals
The regeants used in this work were Acetonitrile (HPLCgrade –Merck),orthophosphoric acid, ,HCl(AR),NaoH(AR),Hydrogenperoxide (85% w/v)(AR),which were procured from merck India.Distilled water (HPLCgrade)was obtained from Lichrosolv(Merck India).Pure drug was procured from standard Laboratories Hyderabad.Zepatier tablets were procured from local market Vijayawada.
Equipment
The instruments used in the study were electronic balance (sigma 200),digital pH meter (systronics),sonicator(cyberlabs),uv-visible

spectrophotometer (thermoscientific) HPLC (watersalliance,empower2 software) Detector (PDA)

Chromatographic conditions

HPLC instrument waters alliance with empower software ,PDA detector .sample name zepatier(Grazoprevir:Elbasvir), The mobile phase used was prepared 0.1M by mixing 6.8ml of orthophosphoric acid in 1000ml of purified water and pH adjusted to 3.8 with dil NaoH.Mobile phase composition phosphate buffer and methanol in the ratio of 45:55 %v/v respectively .Before use the mobile phase is filtered through 0.45µm memberane filter and degassed .Mobile phase flow rate was maintained at 1.0ml/min, eluents were monitored at 220nm.seperation was performed on column Discovery C18 250x4.6 mm, 5 µ ,at temperature 25°c.Injection volume 10µl,Run time of 8 minutes.The diluent used is a mixture of Water and methanol in the ratio of 50:50 v/v..The optimized chromatographic conditions are shown in Table 1.

Table 1: Optimized chromatographic conditions of Grazoprevir ;Elbasvir

Parameters	Conditions
Stationary phase(column)	Discovery C18 250x4.6 mm, 5 µ
Mobile phase	OrthoPhosphoricAcid :methanol(45:55)%v/vpH adjusted to 3.8
Flow rate(ml/min)	1.0
Run time(min)	8.0
Column temperature	Ambient temperature
Volume of injection(µl)	10
Detection wavelength(nm)	220
Retention time(min)	Grazoprevier 2.3,Elbasvir 4.8

Method Development

Selection and preparation of mobile phase

Various mobile phases containing water,methanol,acetonitrile, tetra hydrofuran in different ratios were tried by different columns ,flow rates.Good symmetrical peaks ,resolution and retention time was observed with mobile phase consisting oforthophosphoric acid:methanol in the ratio 45:55%v/v.with PH adjusted 3.8 with dil NaoH. Mobile phase was prepared by 6.8 ml of ortho phosphoric acid taken in a 1000ml of volumetric flask adds about 900ml of purified water added and degas to sonicate and finally make up the volume with water. pH adjusted to 3.8 with dil. NaoH .The mobile phase was sonicated for 10min and filtered through 0.45µm membrane filter.

Preparation of standard stock solution

Accurately Weighed and transferred 5 mg& 10 mg of elbasvir and grazoprevir working Standards into a 10ml clean dry volumetric flask respectively, add 5ml of diluent, sonicated for 30 minutes and make up to the final volume with diluentsto get a concentration of100 µg/ml grazoprevir,50µg/ml elbasvir. From the above stock solutions, 1ml was pipette out in to a 10ml volumetric flask and then make up to the final volume with diluent.

Selection of wavelength

Ideal wavelength is the one that gives good response for the drugs that are to be detected. Overlay UV spectra of both the drugs showed that Grazoprevir and Elbasvir absorbed appreciably at 220 nm, so detection was carried out at 220 nm.Fig .3.A Representative chromatogram of Standard Grazoprevir of RT 2.3and RT of Elbasvir 4.8 are shown in Fig 4
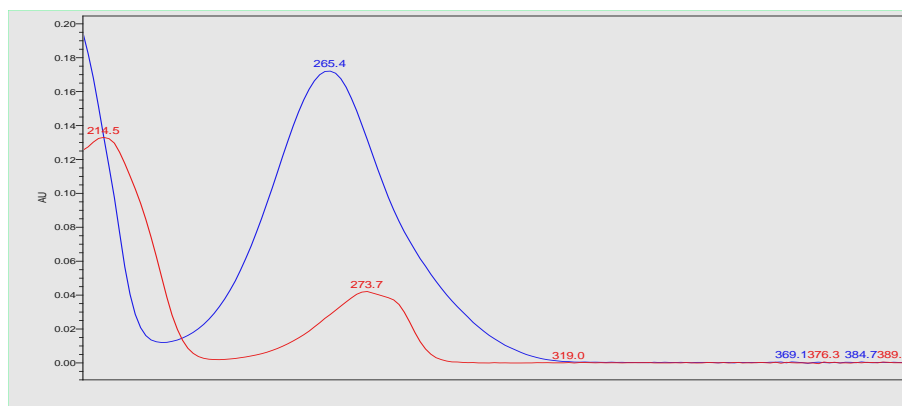

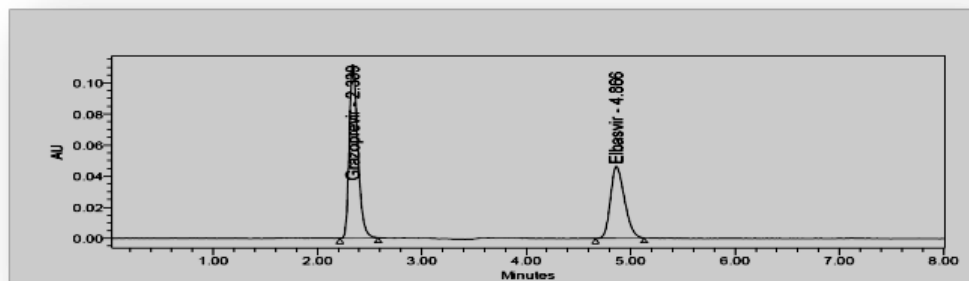**Fig 3: Overlay spectra of Elbasvir and Grazoprevir λmax 220nm**

Fig.4: A representative standard chromatogram of Grazoprevir RT 2.3and Elbasvir 4.8

Preparation of calibration curve

Calibration curve is prepared by appropriate aliquots of 0.25ml,0.5ml,0.75,1.0ml,1.25ml,1.5ml from working standard solution of Grazoprevir,and Elbasvir,in 10 ml volumetric flask and dilute upto mark with diluent to give a concentration range of 25-150 ppm for Grazoprevir and 12.5-75 ppm for Elbasvir.Triplicate injections of 10µlwere made and analyzed by chromatograph under the conditions as described above.Evaluationof the drug was performed and peak areas were recorded.calibration curve was constructed byplotting peak area on x axisand concentration on y axis.The calibration curve was evaluated by its coefficient of determination[R^2].

METHOD VALIDATION

The developed method was validated by evaluating linearity,precision, specificity, accuracy,limit of detection ,limit of quantification ,robustness, ruggedness, assay,forced degradation studies, solution stability.coefficient of variation and relative errors of less than 2% areconsidered acceptable except for limit of quantification for which these values were established at 2%.

Linearity

A stock solution of grazoprevir,elbasvir of 1000µg/ml was prepared with diluent. From it Standard calibration curve is prepared with Six calibrators over a concentration range of 25-150 µg/ml for Grazoprevir and 12.5-75 µg/ml for Elbasvir and injected into HPLC. The data of peak area versus drug concentration were treated by linear least square regression analysis. The standard curve is evaluated for linearity.

Accuracy

Inorder to determine the accuracy of method,recovery studies were planned and carried out by standard addition method at different levels of 50%,100%,150% and then comparing the differencee between the spiked value and actual found value.

Precision

The precision was ascertained from the peak area obtained by actual determination of six replicates of a fixed amount of drug 100µgl/ml for grazoprevir, 50µg/ml for elbasvir.The precision of the assay was also determined in terms of intra and inter - day variation in the peak area of the drug solution was calculated in terms of relative standard deviation (RSD).

Robustness

Robustness of the proposed method for grazoprevir and elbasvir was carried out by the slight variation in flow rate, mobile phase composition, column temperature the analytical parameter on retention time of drugs was examined.The method is found to be robust with no change in Retention time.

Ruggedness

The test solution is prepared as per test method and injected under variable conditons .Ruggedness of the method was studied by different analysts,

Detection limit and quantification limit

The limit of detection (LOD) and limit of quantification (LOQ) were established based on the calibration curve parameters according to the following formulas:

LOD=3.3 SD/slope

LOQ =10 SD/slope

Where σ is the standard deviation of y-intercept of regression line,and s is the slope of calibration curve.

Specificity

Specificity of a method can be defined as absence of any interference at retention times of peaks of interest, and was evaluated by observing the chromatograms of Blank samples and comparison with a placebo solution.

Assay

The weight equivalent to 1 tablet was transferred into a 10 ml volumetric flask, diluent added and sonicated for 30 min, further the volume made up with diluent

and filtered. From the filtered solution 1ml was pipette out into a 10 ml volumetric flask and made up to 10ml with diluent. Six injections of sample was injected, the average mean peak area responses, standard deviation, %relative standard deviation,% assay was calculated.

FORCED DEGRADATION STUDIES

The specificity of the method can be demonstrated through forced degradation studies conducted through the sample using acid, alkaline, oxidative, thermal, neutral, photostability, solution stability studies.

Hydrolytic degradation

Hydrolytic degradation was performed to force the the drug substance to degradation by exposure of the drug to acidic, basic,neutral conditions.The solubility of the drug substance was performed.solubility of about 1mg/ml of drug substance is recommend for hydrolytic degradation studies.

Oxidative degradation

Oxidative degradation promotes reaction between drug substance and molecular oxygen in pharmaceutical formulations.In the study peroxide mediated oxidation studies is induced.The reaction of the oxygen with the drug substance is processed in the oxidative degradation.

Photo stability studies

The photochemical stability of the drug was studied by exposing the UV Light by keeping the beaker in UV Chamber for 1hrs or 200 Watt hours/m^2 in photo stability chamber.

Thermal degradation studies

Thermal degradation is also called as dry heat degradation studies.The drug substance or solution is placed in the oven at 105°c for 6 hrs to study the dry heat stability studies.

Solution stability studies

It is often essential that sample and standard solutions be stable enough to allow for delays covering the instrument breakdown or overnight analysis.A minimum of 12hrs,18hrs, 24hrs, is recommend for chromatographic methods.

RESULTS AND DISCUSSION:

METHOD DEVELOPMENT

Chromatographic separation

A number of chromatographic conditions were investigated to optimize the method of grazoprevir and elbasvir in combination drug for the separation and quantification.The chromatographic conditions are shown in Table 1.

Calibration curve

The correlation coefficient (R^2), slope, y-intercept for grazoprevir is 0.999,6488,6259 and for elbasvir is 0.999, 9099, 383.3 respectively.The calibration curve are shown in fig 5,6.
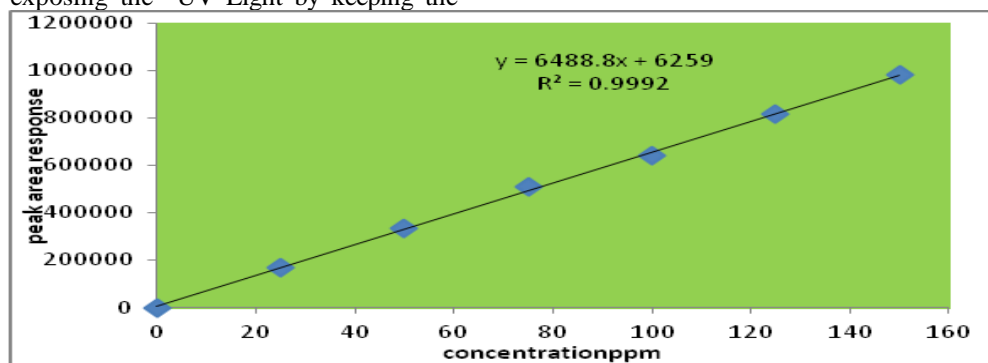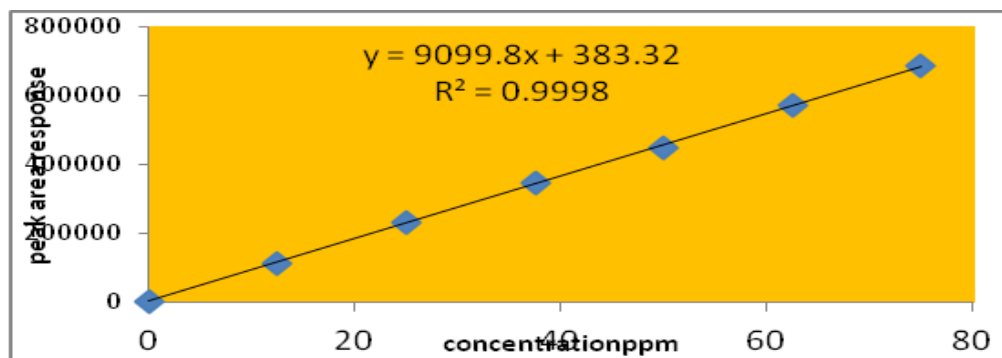

Fig .5.Calibration curve of Grazoprevir

Fig.6. Calibration curve of Elbasvir

METHOD VALIDATION
Linearity, accuracy, and precision
The correlation coefficient [R^2] for grazoprevir, elbasvir was 0.999.as shown in fig 3, 4.The accuracy of the method was indicated by the recovery.The results are shown in Table.2.
Intra and Inter day precision HPLC data are shown in Table 3,4.

Table 2: Accuracy of Grazoprevir and Elbasvir

S.No	Accuracy	Added amount Grazoprevir mg	Peak area	Amt recovered	%Recovery	Added amount Elbasvir mg	Peak area	Amount recovered	% recovery
1	50%	150.6113	983425	50.61128	101.22	74.73939	680437	24.73939	98.96
2	50%	150.5015	982713	50.50154	101.00	74.94084	682270	24.94084	99.76
3	50%	150.7018	984012	50.70176	101.40	75.05942	683349	24.94084	100.24
%RSD	0.2					0.1			
1	100%	198.5592	1294511	98.55919	98.56	99.06382	901765	49.06382	98.13
2	100%	198.9587	1297103	98.95869	98.96	99.41221	904935	49.41221	98.82
3	100%	198.8266	1296246	98.8266	98.83	99.63894	906998	49.63894	99.28
%RSD	0.3					0.1			
1	150%	251.408	1637394	151.408	100.94	126.0205	1147044	76.02052	101.36
2	150%	251.408	1637394	151.408	100.94	125.9944	1146806	75.99436	101.33
3	150%	247.9955	1615254	147.9955	98.66	125.7746	1144806	75.77456	101.03
%RSD	0.4					0.2			

Table 3: Intraday precision(Repeatability) of Grazoprevirand Elbasvi

Injection	Peak area response Grazoprevir	USP plate count	USPTailing	Peakarea response Elbasvir	USPplate count	USPresolution
	634659	4131	1.28	445857	5707	12.2
	635560	4119	1.28	447714	5664	12.2
	632603	4023	1.28	448092	5667	12.2
	652874	4027	1.28	442561	5930	12.4
	648255	4033	1.29	441280	5960	12.4
	651562	3916	1.29	448149	5999	12.3
	642586			445609		
	9278.1			3004.7		
	1.4			0.7		

Table 4: Interday precision (Ruggedness) of Grazoprevirand Elbasvir

Injection	Peak area response Grazoprevir	USP plate count	USPTailing	Peakarea response Elbasvir	USPplate count	USPresolution
1	636569	4143	1.28	451413	5718	12.2
2	639074	4128	1.28	450350	5675	12.2
3	635981	4034	1.28	451765	5678	12.2
4	637585	4038	1.28	447778	5940	12.4
5	638420	4042	1.29	452374	5980	12.4
6	639869	4920	1.29	450336	5989	12.3
AVG	637916			450669		
SD	1488.4			1626.8		
%RSD	0.2			0.4		

Assay

The assay was performed to study the average mean peak area responses, standard deviation, and % relative standard deviation, % assay and amount was calculated data is shown in Table .5.

Table 5: Assay of Grazoprevir and Elbasvir

DRUG	LABELED AMOUNT(mg)	AMOUNT PRESENT(mg)	% ASSAY
Elbasvir	50	50.4	100.82
Grazoprevir	100	99.5	99.58

Table 6: Forced degradation studies

sno	Stress conditions	Peak area Grazoprevir	% Degraded	Purity Angle	Purity Threshold	Peak area Elbasvir	% Degraded	Purity Angle	Purity Threshold
1	CONTROL	644626	0.00	0.097	1.091	441557	0.00	0.098	1.121
2	ACID	614464	4.77	3.727	3.803	421366	4.67	0.198	0.401
3	ALKALI	626105	2.97	0.487	0.492	429318	2.87	0.134	0.333
4	PEROXIDE	634161	1.72	0.627	0.798	434648	1.66	0.137	0.339
5	THERMAL	640680	0.71	0.835	0.855	439231	0.63	0.138	0.339
6	PHOTOSTABILTY	641089	0.65	0.604	0.672	438139	0.87	0.129	0.332
7	NEUTRAL DEGRADATION	640824	0.69	0.716	0.779	439304	0.61	0.170	0.379

Robustness

In the robustness study the influence of small, deliberate variations of the analytical parameter on retention time of drugs was examined.The following three factors were selected for change: flow rate of mobile phase (1ml ± 0.1ml/min), mobile phase composition (45:55± 5%), column tempertaure (25°c± 10°c) .There is influence on retention times of the drug and RSD NMT 2% in modified condition.

Stability of Solution

Solution stability studies were performed to determine the stability of the samples the stability of the solution was above 24hrs.

Forced degradation studies

To detect a decrease in the amount of the active pharmaceutical ingredient (API) present due to the degradation.

Purity angle is less than Purity Threshold. The results are shown in Table.6.Degradation of Elbasvir and Grazoprevir was more in acidic,alkali,peroxide conditions it was less in thermal,photolytic,neutral degradation.The degradation products produced did not interfere with elbaasvir,grazoprevir,Hence the proposed method can consequently be regarded as stability –indicating.

CONCLUSION:

A simple and sensitive stability-indicating RP-HPLC method was explored for the simultaneous determination of elbasvir and grazoprevir in pure form and in commercially available tablet dosage forms. The method was validated as per ICH guidelines. Forced degradation studies were also conducted using different stresscondition as per ICH guidelines. The method proved the selectivity, precision, accuracy and mobile phase used to provide simple and economic application. The method was capable to resolve the peak of selected drugs from stress degradation products. Consequently, the stability indicates the power of the method can be assessed. Therefore, the method was found to be suitable for the routine quality control analysis of elbasvir and grazoprevir simultaneously in

laboratories with no interference from the excipients or the stress degradation products

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