ORIGINAL ARTICLE

Quantification of Glecaprevir and Pibrentasvir with Deuterated Internal Standards in Spiked Human Plasma Samples by LC–ESI-MS/MS

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Abstract

A fixed oral dose combination of Glecaprevir and Pibrentasvir used for the treatment of patients with viral infections especially used as an NS5A inhibitor. Quantification of such drugs in human plasma somewhat help full. In this manuscript, the authors developed a simple, sensitive, and specific liquid chromatography – tandem mass spectrometry method was used for quantification of Glecaprevir and Pibrentasvir in human plasma using Glecaprevir-13C-d7 as internal standard. Agilent TC-C18, 4.6 × 75 mm, 3.5 µm, 80 Å column, 5 mM ammonium acetate: Acetonitrile (20: 80 v/v) mobile phase was used for chromatographic separation. MRM-positive mode was used to detect the Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 at m/z 838.87/337.26, 557.51/210.40, and 846.91/337.26, respectively. Liquid-liquid extraction was employed in the extraction of analytes and internal standard from human plasma. This method is validated over a linear concentration range of 50.0–10,000.0 pg/mL for Glecaprevir and Pibrentasvir with a correlation coefficient (r) of ≥0.9997. Both drugs were stable in plasma samples. The validated method was successfully.

Key words: Bioanalytical, glecaprevir, glecaprevir-13C-d7, human plasma, LC-ESI-MS/MS, pibrentasvir

INTRODUCTION

Infection with hepatitis C virus (HCV) genotype 3 is associated with higher rates of liver steatosis and achieving sustained virologic response quantifiably reverses its progression in those patients. GT3 has been shown to be an independent predictor of fibrosis progression and is associated with a higher incidence of hepatocellular carcinoma. Thus, effective HCV treatment options are critical for patients with HCV GT3 infection, particularly those with advanced liver disease and/or prior treatment experience. Glecaprevir is an antiviral agent and Hepatitis C virus NS3/4A protease inhibitor which directly targets the viral RNA replication.

Glecaprevir is chemically known as (3aR, 7S, 10S, 12R, 21E, and 24aR)-7-tert-butyl-N-{(1R, 2R)-2Â (difluoromethyl)-1-[(1-methylcyclopropane-1-sulfonyl) carbamoyl]cyclopropyl}-20, 20-difluoroÂ 5, 8-dioxo2, 3, 3a, 5, 6, 7, 8, 11, 12, 20, 23, 24a-dodecahydro-1H, 10H-9, 12 methanocyclopenta[28] trioxadiazacyclononadecino[11,12-b]quinoxaline-10Â carboxamidehydrate and corresponding molecular formula of C38H46F4N6O9S • xH2O (hydrate). It has a relative molecular mass 838.87 g/mol (anhydrate). Glecaprevir appears as a white to off-white crystalline powder. It shows pH dependent solubility in aqueous media, being practically insoluble at pH 2.1 and 5.1 and very slightly soluble at pH 6.6. Its pka values were found to be 4.0 and 11.7 and the partition coefficient (Log P) 2.5 at pH 7.4. Glecaprevir disrupts the intracellular processes of the viral life cycle through inhibiting the NS3/4A protease activity of cleaving downstream junctions of HCV polypeptide and proteolytic processing of mature structural proteins.[1-13]

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Received: 22-09-2022
Revised: 27-11-2022
Accepted: 04-12-2022

Pibrentasvir is chemically dimethyl ((2S,2â€™S,3R,3â€™R) ((2S,2â€™S)-(((2R,5R)-1-(3,5-difluoro-4-(4-(4-fluorophenyl)piperidin-1-yl)phenyl)pyrrolidine-2,5-diyl) bis(6-fluoro-1H-benzo[d]imidazole-5,2-diyl))bis(pyrrolidine-2,1-diyl))bis(3-methoxy-1-oxobutane-1,2-diyl)) dicarbamate-2 [Figure 1] corresponding to the molecular formula C57H65F5N10O8. It has a relative molecular mass of 1113.18 g/mol. Pibrentasvir active substance is a white to off-white to light yellow non-hygroscopic crystalline powder, practically insoluble in water, freely soluble in ethanol. It shows pH dependent solubility in aqueous media, being very slightly soluble at pH 1.1 and practically insoluble at and above pH 2.1. It also shows low passive permeability. Its pKa values were found to be 3.5, 4.1, and 11.6 and the partition coefficient (logP) is 7.5. It is a direct acting Antiviral agent and HCV NS5A inhibitor target the viral RNA replication and viron assembly. NS5A is a phosphoprotein that plays an essential role in replication, assembly and maturation of infectious viral proteins. The combination of Glecaprevir and Pibrentasvir seems to be effective option for treatment regardless of which genotype they have, and whether or not they have severe renal impairment or liver cirrhosis [Figure 2].[1-12]

A literature survey revealed that HPLC[3-12] methods were reported for simultaneous estimation of Glecaprevir and Pibrentasvir in pharmaceutical dosage form but no method was developed for the estimation of these drugs in bio-fluids using LC-MS/MS using Glecaprevir-13C-d7 as internal standard. In the present investigation, a specific LC-MS/MS method was developed for the simultaneous estimation of Glecaprevir and Pibrentasvir in human plasma. It is important to develop the good bio analytical method with proper deuterated or analog-based internal standards in terms of matrix effect and reproducibility. Moreover, it should not consider the runtime always to minimize the analysis rather than reproducibility and stability for long analytical batches This paper reports the novel, sensitive, rapid, precise, and accurate method for the estimation of both Glecaprevir and Pibrentasvir in human plasma using LC-MS/MS.

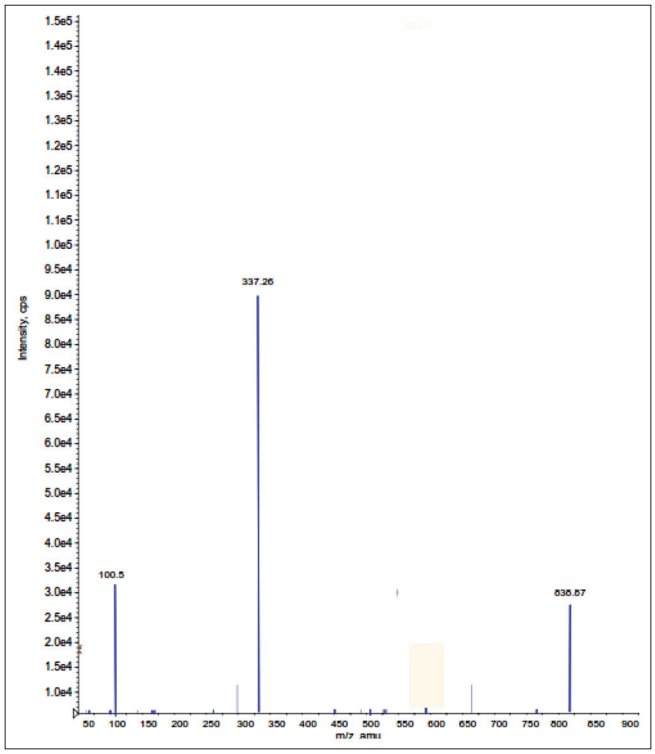

Figure 1: Parent and product ion mass spectrum of Glecaprevir

The main goal of the present study is to develop and validate[14-16] the novel simple, sensitive, selective, rapid, rugged, and reproducible analytical method for quantitative determination of Pibrentasvir and Glecaprevir in human plasma by LC-MS/MS with a small amount of sample volume. Moreover, it has to be developed simple extraction method, with a highly sensitive, good, and linear method with the small amount of plasma usage.

MATERIALS AND METHODS

Chemicals and reagents

The drugs Glecaprevir, Glecaprevir-13C-d7, and Pibrentasvir were procured as a gift samples from Symed labs, Hyderabad, India and Toronto research chemicals, Canada. Ethyl acetate, HPLC grade methanol, and acetonitrile were purchased from J.T. Baker USA. Sodium dihydrogen phosphate (NaH_2PO_4, reagent grade), Ammonium acetate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Human plasma was obtained from Doctors laboratories, Hyderabad, India. Ultra pure water from MilliQ-system (Millipore) was used through the study.

Instrumentation

The 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) was used in the present study. Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

Detection

The mass transitions were selected as Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 at m/z 838.87/337.26, 557.51/210.40, and 846.91/337.26, respectively.

Chromatographic conditions

In the present study the stationary column used is Agilent TC-C 18, with dimensions of 4.6 x 75mm. The particle

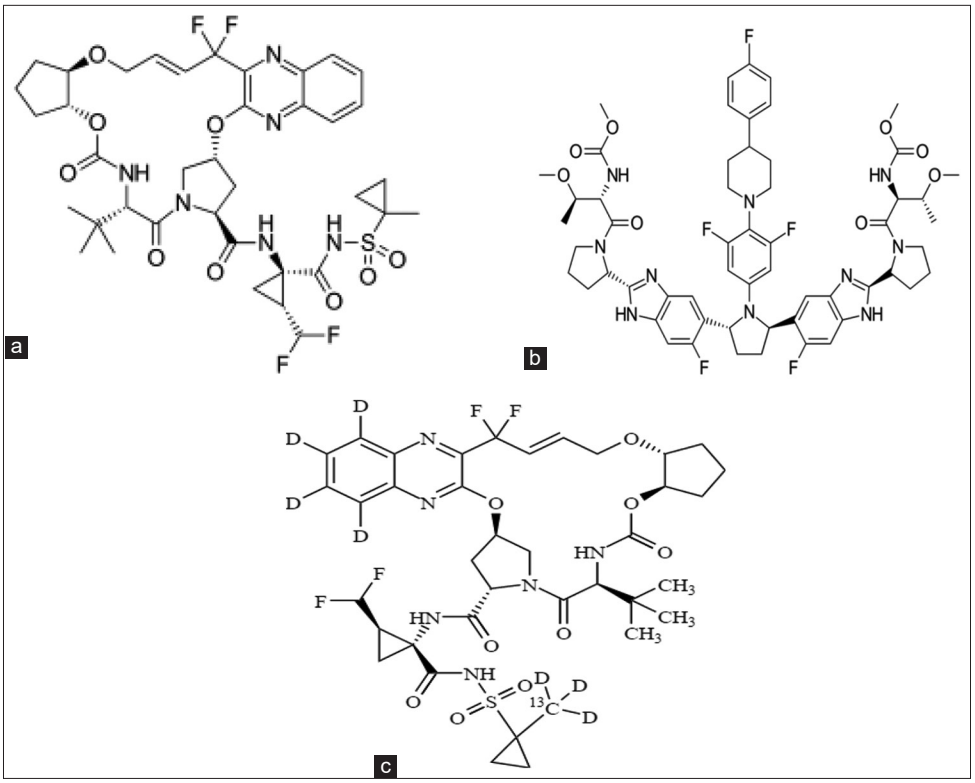

Figure 2: Chemical structures of (a) Glecaprevir, (b) Pibrentasvir, and (c) Glecaprevir-13C-d7

size of the column is 3.5µm and the pore size is 80 Å.The mobile phase is ammonium acetate : acetonitrile (20:80 v/v). The Ammonium acetate is prepared at the concentration of 5mM. The sample injected at the flow rate of 0.5ml/min. The column was placed at a temperature of 40°C. 20 µL of sample was injected into LC-MS/MS System. The analytes and internal standard were eluted at 1.61 min (Glecaprevir), 1.94 min (Glecaprevir-13C-d7), and 1.93 min (Pibrentasvir) with total runtime of 4 min for each injection.

Calibration standards and quality control samples

Standard stock solutions of Glecaprevir (100.0 µg/mL), Glecaprevir-13C-d7 (100.0 µg/mL), and Pibrentasvir (100.0 µg/mL) were prepared in methanol. From each stock solution, 100.0 ng/mL intermediate dilution was prepared in plasma. Aliquots of 100.0 ng/mL were used to spike blank human plasma to obtain calibration curve standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0, and 10000.0 pg/mL. Four levels of QC concentrations at 50.0, 150.0, 3000.0, and 7000.0 pg/mL (LLOQ, low quality control [LQC], medium quality control [MQC], and high quality control [HQC]) were prepared using the different plasma. Spiked calibration curve standards and quality control standards were stored at −30°C. Standard stock solutions of Glecaprevir-13C-d7 (100.0 µg/mL) were prepared in methanol and Glecaprevir-13C-d7 further diluted to 10.0 ng/mL (Spiked concentration of internal standard) using 50% methanol and stored in the refrigerator 2–8°C until analysis.

Sample preparation

Liquid-liquid extraction was carried out to extract the drug and IS for this purpose 100 µL of respective concentration of plasma sample was taken into polypropylene tubes and mixed with 50 µL of internal standard (10.0 ng/mL). This was followed by addition of 100 µL of 5 mM NaH_2PO_4 solution and 3.0 mL of ethyl acetate and vortexed for approximately 10 min. Then, the samples were centrifuged at 4000 rpm for 10 min at 20°C. Further, the supernatant was transferred into labeled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then, the samples were reconstituted with the mobile phase and vortexed for 2 min. Finally, sample was transferred into auto sampler vials to inject into the LC-MS/MS.

Method validation[14-17]

Selectivity and specificity

Selectivity was performed by analyzed the human blank plasma samples from six different sources (donors) to test for interference at the retention times of analytes. The peak area of Glecaprevir and Pibrentasvir in blank samples should not be more than 20% of mean peak area of limit of quantification

(LOQ) of Glecaprevir and Pibrentasvir. Similarly, peak area of Glecaprevir-13C-d7 in a blank sample should not be more than 5% of mean peak area of LOQ of Glecaprevir-13C-d7.

Precision and accuracy

Precision and accuracy were determined by replicate analysis of quality control samples (n = 6) at LQC, MQC, and HQC levels. The % CV should be <15% and accuracy should be within 15% except LLOQ where it should be within 20%.

Matrix effect

The matrix effect due to plasma was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pre-treatment (Liquid-liquid extraction with ethyl acetate) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (% CV) of ≤15% was maintained.

Recovery

The extraction efficiencies of Glecaprevir, Pibrentasvir and Glecaprevir-13C-d7 were determined by analysis of six replicates at each quality control concentration level for Glecaprevir and Pibrentasvir and at one concentration for the internal standard Glecaprevir-13C-d7. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non-extracted standards (spiked into mobile phase).

Limit of detection (LOD) and LOQ

The LOD is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples.

The limit of LOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of Glecaprevir and Pibrentasvir.

Stability (freeze-thaw, auto sampler, room temperature, and Long-term)

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution.

Stability studies in plasma were performed at the LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % change is <15% as per US FDA guidelines.[17] The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 61 h. The stability of spiked human plasma samples stored at −30°C in autosampler (autosampler stability) was evaluated for 70.0 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the autosampler at 20°C for 70.0 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 20°C for 70 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at –30°C and thawed 3 times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze-thaw stability evaluation. For long-term stability evaluation, the concentrations obtained after 91 days were compared with initial concentrations.

RESULTS AND DISCUSSION

Method development and validation

The goal of this work is to develop and validate a simple, rapid, and sensitive assay method for the quantitative determination of Glecaprevir and Pibrentasvir from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tools in clinical pharmacokinetics for its selectivity, sensitivity, and reproducibility. The MS optimization was performed by direct infusion of solutions of Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 into the ESI source of the mass spectrometer. The vital parameters such as ionization type, temperature, voltage, gas parameters such as nebulizer and heater gases, compound parameters such as DP, EP, FP, CE, and CXP were optimized to obtain a better spray shape and ionization to form the respective productions from the protonated Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 molecules [Figures 1, 3, 4]. Chromatographic conditions, especially, composition of the mobile phase, selection of suitable column, were optimized through several trials to achieve the best resolution and increase the signal of analyte and internal standard. Different extraction methods such as solid phase extraction, liquid-liquid extraction, and precipitation methods were optimized for extraction of Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 from the plasma sample. A good separation and elution were achieved using 5 mM ammonium acetate: Acetonitrile (20: 80 v/v) as the mobile phase, at a flow-rate of 0.5 mL/min and injection volume of 20 µL. Liquid-liquid extraction was chosen to optimize the drug and internal standard. The analytes and internal standard were eluted

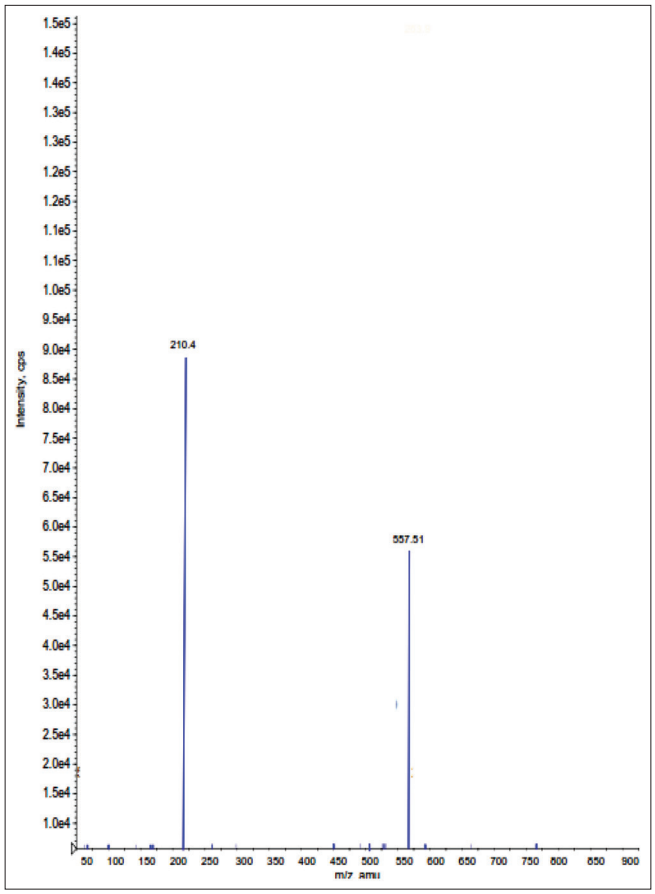

Figure 3: Parent and production mass spectrum of Pibrentasvir

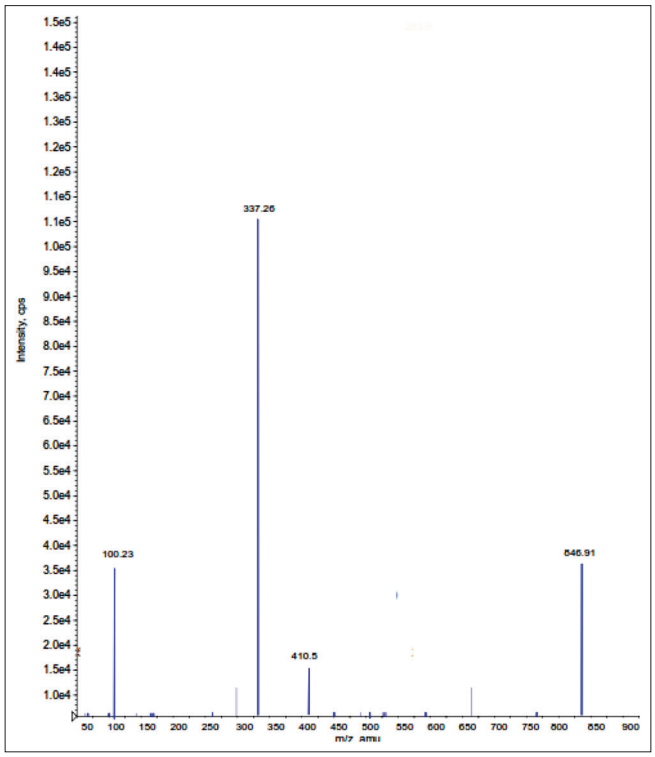

Figure 4: Parent and product ion mass spectrum of Glecaprevir-13C-d7

at 1.61 min (GLECAPREVIR), 1.94 min (Glecaprevir-13C-d7), and 1.93 min (PIBRENTASVIR) with total runtime of 4 min for each injection [Figures 5 and 6].

Linearity

Calibration curve was plotted as the peak area ratio (Glecaprevir/Glecaprevir-13C-d7) and Pibrentasvir/Glecaprevir-13C-d7) versus (Glecaprevir and Pibrentasvir) concentration. Calibration was found to be linear over the concentration range of 50.0–10,000.0 pg/mL. The correlation coefficient (r^2) was >0.9997 for all curves [Table 1].

Selectivity

The selectivity of the method assessed by comparing chromatograms of blank plasma. There were no significant endogenous peaks that were observed at respective retention time of Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7. The results indicate that the method exhibited both good specificity and selectivity [Figures 4 and 5].

Precision and accuracy

Precision and accuracy for this method were controlled by calculating the within-run and between-run variations at three concentrations (150.0, 3000.0, and 7000.0 pg/mL) of QC samples in six replicates. As shown in Table 2, the within-run precision and accuracy were between 1.4–4.3 and 84.8–106.4% for Glecaprevir, 1.4–4.3 and 84.8–106.4% for Pibrentasvir. Similarly, the between-run precision and accuracy were between 1.1–4.3 and 84.8–106.4% for Glecaprevir, 1.1–3.8 and 99.4–1207.1% for Pibrentasvir. These results indicate the adequate reliability and reproducibility of this method within the analytical range [Table 2].

Matrix effect

The ion suppression/enhancement in the signal at MQC level was found %CV 1.27 for Glecaprevir and %CV 1.20 for Pibrentasvir, respectively. These results indicating that there is no effect on ion suppression and ion enhancement.

Recovery

The extraction recoveries of Glecaprevir were determined at three different concentrations 150.0, 3000.0, and 7000.0 pg/mL were found to be 99.6 ± 3.53, 88.2 ± 2.7 and 97.60 ± 4.7%., Similarly, extraction recoveries of Pibrentasvir were determined at three different concentrations 150.0, 3000.0, and 7000.0 pg/mL were found to be 95.5 ± 9.7, 92.6 ± 10.21 and 92.3 ± 4.7%. The overall average recoveries

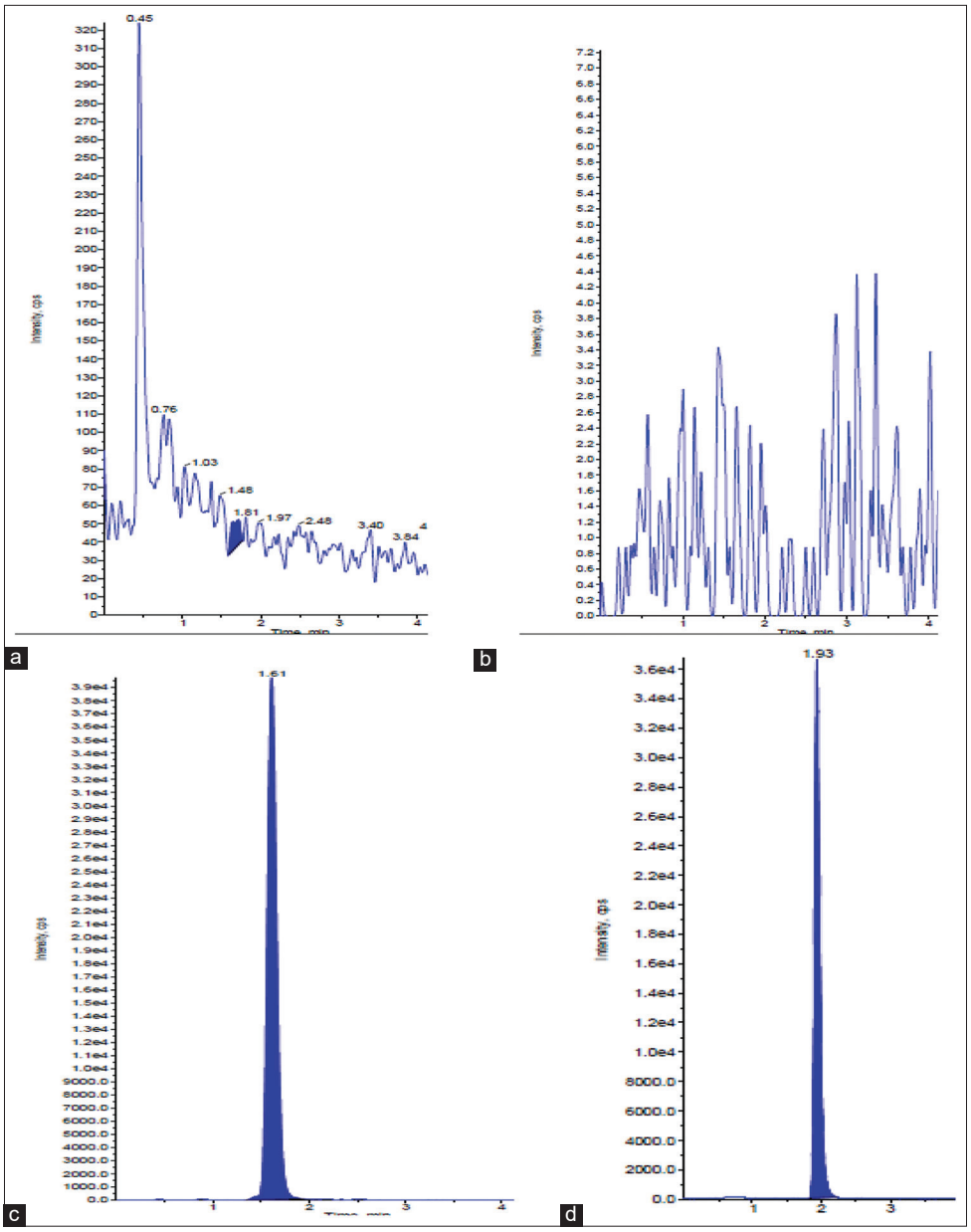

Figure 5: Typical MRM chromatograms. (a and b) Blank chromatograms of Glecaprevir, Glecaprevir-13C-d7; (c and d) Glecaprevir in plasma spiked with Glecaprevir-13C-d7

Table 1: Calibration curve details of Glecaprevir and Pibrentasvir

Spiked plasma concentration (pg/mL)	Concentration measured (mean) (pg/mL), (*n*=5)	Precision (CV %) (*n*=5)	Concentration measured (mean) (pg/mL), (*n*=5)	Precision (CV %) (*n*=5)
	Glecaprevir		Pibrentasvir	
50.0	51.0±1.3	2.5	51.2±1.0	2.0
100.0	96.6±4.7	4.9	95.8±3.4	3.5
500.0	498.4±24.7	5.0	495.1±26.3	5.3
1000.0	1000.0±17.1	1.7	1010.5±28.3	2.8
2000.0	2013.0±74.6	3.7	2019.1±70.0	3.5
4000.0	4008.4±206.6	5.2	4067.1±224.9	5.5
6000.0	5956.5±190.7	3.2	5628.5±735.7	13.1
8000.0	7952.2±165.6	2.1	8162.8±191.2	2.3
10000.0	10317.1±487.6	4.7	10440.0±521.5	5.0

Table 2: Precision and accuracy

Glecaprevir						
Spiked plasma concentration (pg/mL)	**Within-run (*n*=6)**			**Between-run (*n*=30)**		
	Concentration measured (pg/mL) (mean±S.D.)	**Precision (CV %)**	**Accuracy %**	**Concentration measured (pg/mL) (mean±S.D.)**	**Precision (CV %)**	**Accuracy %**
50.0	51.4±2.3	4.5	102.7	55.5±4.1	7.4	110.6
150.0	152.9±1.4	2.2	105.5	151.9±1.7	1.6	102.2
3000.0	3103.8±102.0	3.3	103.2	3133.0±108.2	3.5	104.3
7000.0	7197.1±89.9	1.2	91.7	7178.7±275.5	1.8	103.9
Pibrentasvir						
50.0	42.4±1.0	2.4	84.8	49.9±6.9	3.8	99.4
150.0	152.6±2.3	1.4	106.4	151.5±1.6	1.2	101.6
3000.0	3072.4±132.6	4.3	102.4	3216.1±162.6	5.1	107.1
7000.0	7160.1±105.8	1.4	98.0	7174.3±123.9	1.1	102.1

Table 3: Stability of Glecaprevir and Pibrentasvir in spiked human plasma samples

Stability experiments	**Storage condition**	**Spiked plasma concentration (pg/mL)**	**Concentration measured (*n*=6) Mean±SD**	**CV (%) (*n*=6)**	**Accuracy (%)**
Glecaprevir					
Bench top (Room temperature)	RT 61 h	150.0	148.3±8.1	5.5	98.9
		7000.0	6728.3±206.3	3.1	81.5
Processed (extracted sample)	Autosampler 70 h	150.0	162.3±2.4	1.5	108.2
		7000.0	7536.7±294.5	3.9	90.4
Freeze and Thaw stability	–30°C Cycle-3	150.0	156.5±4.0	2.5	104.3
		7000.0	7381.7±173.4	2.3	90.4
Long-term stability	–30°C, 91 days	50.0	160.3±13.2	8.2	106.9
		7000.0	7450.0±229.1	3.1	90.5
Pibrentasvir					
Bench top (Room temperature)	RT 61 h	150.0	156.3±8.7	5.6	104.2
		7000.0	7411.7±213.7	2.9	92.6
Processed (extracted sample)	Autosampler 70 h	150.0	161.7±4.9	3.0	107.8
		7000.0	7675.0±473.5	6.2	95.9
Freeze and thaw stability	–30°C Cycle-3	150.0	159.7±7.6	4.7	106.4
		7000.0	7540.0±323.0	4.3	94.3
Long-term stability	–30°C, 91 days	50.0	159.0±6.3	4.0	106.0
		7000.0	7608.3±297.2	3.9	95.1

of Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 were found to be 95.1 ± 6.1, 93.5 ± 1.8, and 96.0 ± 2.8%. Recoveries of the analyte and IS were consistent, precise, and reproducible.

LOD and LOQ

The LOQ signal-to-noise (S/N) values found for six injections of Glecaprevir and Pibrentasvir at LOQ concentration were 31.95 and 40.23.

Stability (freeze-thaw, auto sampler, room temperature, and long-term)

Stock solution stability was performed to check stability of Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 in stock solutions prepared in methanol and stored at 2–8°C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 25 days. The % change for Glecaprevir, Pibrentasvir, and Glecaprevir-13C-d7 were −0.02%, 0.01%, and 0.02%, respectively, indicates that stock solutions were stable at least for 25 days.

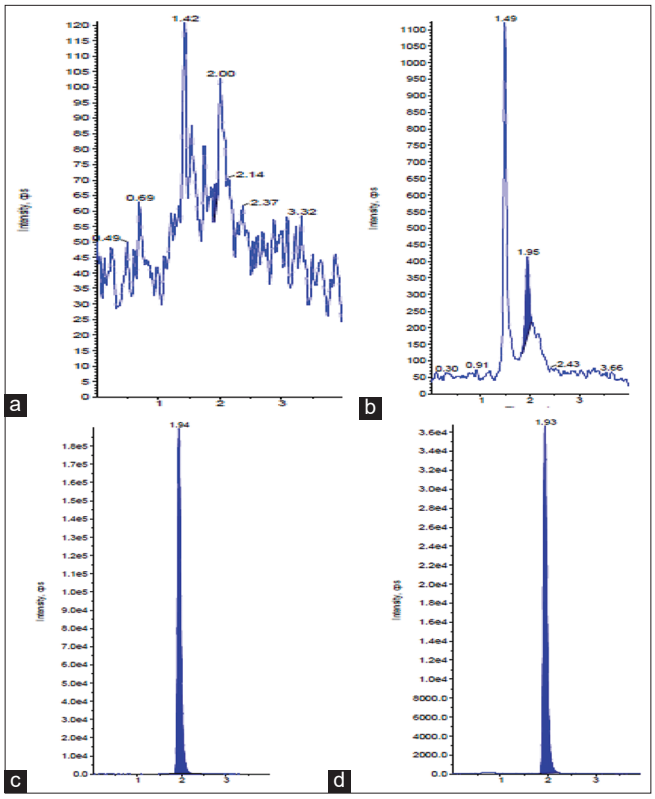

Figure 6: Typical MRM chromatograms. (a and b) Blank chromatograms of Pibrentasvir and Glecaprevir-13C-d7; (c and d) pibrentasvir in plasma spiked with Glecaprevir-13C-d7

Room temperature and autosampler stability for Glecaprevir and Pibrentasvir were investigated at LQC and HQC levels. The results revealed that Glecaprevir and Pibrentasvir were stable in plasma for at least 60 h at room temperature, and 70 h in an auto sampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Glecaprevir and Pibrentasvir at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Glecaprevir and Pibrentasvir were stable in a matrix up to 91 days at a storage temperature of −30°C. The results obtained from all these stability studies are tabulated in Table 3. Precision (%CV) is <5 for room temperature, long-term, Freeze thaw, and auto sampler stability.

CONCLUSION

The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other described methods in previously. Quantification of Glecaprevir and Pibrentasvir were compared with respective isotope labeled internal standards. Extraction of analyte and IS was achieved using LLE. Linearity range, column, mobile phase, flow rate, injection volume, and plasma usage volume for analysis were improved. Hence, this method has significant advantages over previously reported methods in-terms of selectivity, sensitivity, linearity, and reproducibility.

ACKNOWLEDGMENTS

The authors wish to thank the support received from Azidus clinical research laboratories ltd, Chennai, India, for providing literature survey and to carry out this research work.

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Source of Support: Nil. **Conflicts of Interest:** None declared.