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Development and Validation of Novel Analytical LC-MS Method for Simultaneous Quantification of Calcitonin Gene-Related Peptide Receptor Antagonists Ubrogepant and Atogepant in Human Plasma

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A simple, highly sensitive and selective liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was developed and validated for separation and simultaneous assay of approved calcitonin gene-related peptide receptor antagonists ubrogepant and atogepant in human plasma using frovatriptan as internal standards. Analytes were extracted using protein precipitation induced by acetonitrile followed by liquid-liquid extraction using dichloromethane. RP-HPLC analysis was carried using Xbridge C_{18} column (50 mm × 4.6 mm, 5 µm) with a simple isocratic mobile phase composed of 0.01% NH_3 in 2 mM ammonium formate at pH 6.4 and acetonitrile at 45:55 (v/v) as mobile phase at 0.4 mL/min flow rate. Detection was performed on a triple quadrupole mass spectrometer employing electrospray ionization technique, operating in multiple reaction monitoring (MRM), with the transitions of *m/z* 550→58, *m/z* 604→147, *m/z* and *m/z* 244→156 for ubrogepant, atogepant and frovatriptan, respectively, in the positive ion mode. The analysis was carried out within 5 min over a linear concentration range of 15-600 ng/mL for both ubrogepant and atogepant. The method was validated in accordance with the FDA guidelines for bioanalytical method and the results were within the acceptable limit for both the analytes. The method is useful for the analysis of ubrogepant and atogepant in human plasma and may also applicable for the pharmacokinetic study.

Keywords: Ubrogepant, Atogepant, LC-MS/MS, Human plasma, Stability studies.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a potent vasodilator and 37 amino acid neuropeptide that produced by neurons in both peripheral and central nervous systems and the increased levels of CGRP in the blood causes migraines, cluster headaches, *etc*. [1]. Migraine is a neurovascular disorder characterized by unilateral, throbbing headache along with vomiting, disability, nausea, phonophobia and photophobia that usually has 4-72 h duration [2]. In the treatment and prophylaxis of migraine, FDA approved various CGRP antagonists such as rimegepant, atogepant, ubrogepant, *etc*.

Ubrogepant (Fig. 1a) is the first approved CGRP antagonist prescribed for the immediate treatment of migraine in adults. It was not indicated for the preventive treatment migraine. Dry mouth, tiredness and nausea, tiredness and dry mouth are the possible side effects occurred while using the ubrogepant [3]. Atogepant (Fig. 1b) is an oral active CGRP antagonist drug prescribed in the treatment of migraines [4]. In comparison with other gepants, atogepant has high affinity at CGRP receptor with a Ki of 15-26 pM in humans. There is no considerable major treatment related side effects were reported for atogepant [5].

The literature survey for the available analytical methods for the estimation of ubrogepant and atogepant confirms that there is no analytical method reported for the estimation of these drugs in formulations as well as in biological samples. Hence the present work intended to develop a simple LC-MS method for the separation and estimation of ubrogepant and atogepant in biological samples. Another migraine drug frovatriptan was selected as internal standard in the drug.

EXPERIMENTAL

The analytical standards ubrogepant, atogepant and frovatriptan were procured from Allergan India Pvt. Ltd, Bangalore, Abbvie Biopharmaceuticals Private Ltd., Mumbai and Glenmark Pharmaceuticals Ltd, Secunderabad, India, respectively. HPLC

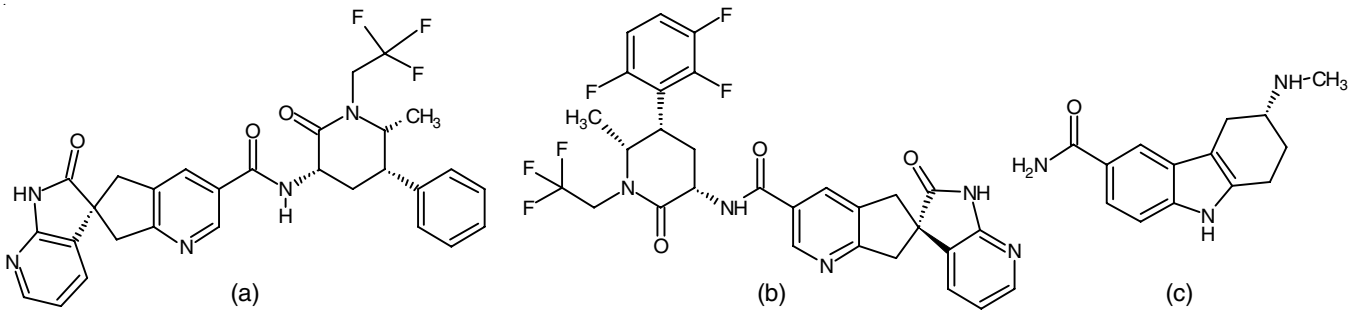

Fig. 1. Molecular structure of ubrogepant (a), atogepant (b) and frovatriptan internal standard (c)

grade methanol, acetonitrile and milli-Q water were purchased from Merck chemicals, Mumbai, India. Healthy human blood was procured from the diagnostic laboratory. The plasma from the whole blood was separated using Pasteur pipette after centrifugation. The experiment was performed on Waters (Japan) alliance 2695 LC-MS system coupled with triple quadrupole mass detector (Waters ZQ, LAA 1369). The system was equipped with auto-injector (0.1-1500 µL) and integration was carried on masslynx 4.2 (waters) software. The analytes was separated on Inertsil ODS C18 (4.6 mm × 100 mm, 5 µm) column.

Preparation of working standard solutions: The standard stock solution at a concentration of 1000 µg/mL of ubrogepant, atogepant and frovatriptan were prepared separately in a 25 mL volumetric flask. While preparing the standard stock solution, an accurately weighed 25 mg of standard drug was dissolved in 25 mL volumetric flask. Then the solution was diluted to get 1000 ng/mL stock solution. Then the solution was further diluted to get required concentrations for method development and validation study. The internal standard was diluted to 250 ng/mL concentration. The working standard solution and calibration curve dilutions were prepared by mixing equal volumes of selected concentration of both standards and 250 ng/mL internal standard stock solutions.

Preparation of spiked calibration curve standard solutions: The plasma spiked calibration curve dilutions were prepared by spiking 50 µL of selected concentration of ubrogepant, atogepant and 50 µL fixed concentration (250 ng/mL) of internal standard to the blank human plasma. Similarly, a blank without analytes and a zero sample that spiked with internal standard only was prepared. All the spiked plasma samples were treated as per the extraction protocol described below.

Extraction protocol: The extraction of drug from spiked plasma was carried by performing protein precipitation followed by liquid-liquid extraction. The protein precipitation was carried by adding 1 mL of acetonitrile solvent. Then the content was mixed for 2 min and the liquid-liquid extraction was performed using 3 mL of dichloromethane. It was, then mixed well for 5 min and then centrifuge at 4000 rpm for 5 min. The supernatant was separated carefully and the solvent was evaporated at 60 ºC. Then, the dried residue was reconstituted with 1 mL methanol and the solution was used for method development and validation study.

Method development: The LC-MS method for the separation and analysis of ubrogepant and atogepant was developed by optimizing various analytical method parameters. The analytical method parameters such as composition, pH, flow rate of mobile phase, type of stationary phase, mass operating conditions, *etc*., were studied. In the development of method, one parameter was changed and other parameters were kept constant. The method conditions that produce best chromatographic results with high system suitability was considered as suitable analytical conditions and the conditions were further studied for validation.

Method validation: The method validation guidelines given by FDA [6] were followed for the validation of method developed for the analysis of ubrogepant and atogepant using frovatriptan internal standard.

Selectivity: The interference of the endogenous compounds co-eluting with the analyte was evaluated in selectivity study. In this, the chromatographic results observed in the developed method for the unspiked (blank) plasma sample and spiked with the analytes at LLOQ level was compared and assess the selectivity of the method.

Sensitivity: The sensitivity of the method was assessed by confirming detection limit (LOD) and quantification limit (LOQ) of both the analytes in the developed method. The LOD is the lowest concentration of sample the can detect in the method from background noise but not quantitated and LOQ is the lowest concentration of sample that can quantitated in the method. The LOD of analaytes in the developed method was evaluated by analysing the lowest concentration of analytes and the lowest concentration that produce acceptable system suitability with a signal to noise ratio of 3:1 was considered as LOD and LOQ was the lowest concentration that produces acceptable precision and accuracy. Experimentally, a signal to noise ratio of 10:1 was considered as acceptable.

Linearity: The linear calibration curve for both analytes was constructed from LOQ level to maximum concentration that fit in the linear calibration curve with high correlation coefficient. The individual calibration curves for both analytes was plotted by considering the concentration of analyte on *x*-axis and peak area response ratio of individual analyte to the internal standard on *y*-axis. The correlation coefficient of the calibration curve was calculated using simple linear regression analysis in the tested concentration range.

Accuracy and precision: The accuracy and precision was performed as intra-day and inter-day studies and was carried in three dissimilar concentrations such as low (LQC), middle (MQC) and high (HQC) concentrations in the linearity range.

The selected concentrations in the linearity range was analyzed in six replicates for both intra-day and inter-day precision studies. The results in the study were expressed as the % relative standard deviation and the % recovery for all the studied levels. The results concluded as acceptable when the variation results were within ± 15% in the three studied levels.

Recovery: The recovery of the method developed was evaluated by comparing the results observed during the analysis of analytes that were spiked with blank plasma which was exposed to the whole extraction procedure to the results observed for post-extracted plasma samples. The study was conducted at LQC, MQC and HQC levels in the calibration range. It can be considered that the recovery of the analytes must not be 100% but it is essential that the variability of the results in recovery must be reproducible, precise and consistent in different concentration ranges studied.

Matrix effects: The matrix effect of the method developed for the analysis of ubrogepant and atogepant was evaluated by investigating the effect of blank plasma on the results. In this, the blank plasma of six different batches was spiked with the investigated analytes at LQC and HQC levels and were analyzed in the developed method. The %RSD of the peak area response of both the analytes in the study was calculated and a %RSD of < 15% was confirms that the method having acceptable matrix effect.

Stability studies: The stability of ubrogepant and atogepant along with internal standard in human plasma was tested under various storage environments. Various stability studies such as short term, long term, auto sampler, freeze and thaw and dry extract stability were performed at LQC, MQC and HQC levels utilizing six replicates from each level. In short term stability, the defrosted samples was store in room temperature for 6 h and then analyzed in the developed method. In long term stability, the samples were stored in freezing temperature and was analyzed after 30 days of the incubation. In freeze thaw stability, the QC samples stability was investigated through four freeze-thaw cycles after being kept to freeze for 24 h. Samples were then thawed unassisted at room temperature for 2 h or even more then kept to freeze again at -86 ºC overnight for every freeze-thaw cycle. The consequences of infrequent delay of the sample injection in auto-sampler were evaluated in auto-sampler stability. The sample was analyzed after 24 h of incubation in an auto-sampler. The dry extract stability was assessed by incubating the dry residue at room temperature without reconstitution and was reconstituted after 24 h of incubation. The % recoveries and the % stability in all the studies were calculated in the studied concentration levels for both the analytes.

RESULTS AND DISCUSSION

In the development of suitable analytical conditions for the separation and analysis of ubrogepant and atogepant along with internal standard, considerable effort was accomplished to adjust the chromatographic conditions in order to achieve well resolved peaks with high peak area response, acceptable system suitability with no interference of endogenous compounds. In the selection of stationary phase, various types of stationary phases with different configurations were studied. In the selection of mobile phase, acetonitrile and methanol at various compositions was studied as organic modifier and 0.01% ammonia at various compositions and pH ranges was studied. Based on the results, it was identified that the presence of acetonitrile in the mobile phase produces best separation than the acetonitrile. The method development was concluded by achieving the best chromatographic results and separation was achieved using 0.01% ammonia in 2 mM ammonium formate at pH 6.4 and acetonitrile at 45:55 (v/v) as mobile phase at 0.4 mL/min flow rate of mobile phase that facilitates less consumption of mobile phase. Very nominal quantity of sample (2 µL) was separated on Xbridge C_{18} column (50 mm × 4.6 mm, 5 µm) at room temperature and the column eluents were monitored using UV and mass detector.

In the developed method conditions, the chromatogram observed for blank (unspiked) doesn't show any peak throughout the run time whereas the spiked standard chromatogram shows peaks at a retention time of 1.02 min, 2.92 min and 3.75 min, respectively for ubrogepant, atogepant and internal standard. The peak area response and the elution time of the individual analysis results of each analyte was observed to be same as the results observed on combined spiked sample chromatogram. The chromatogram of both individual analysis as well as the combined analysis doesn't shows any interference of endogenous plasma components confirms that the method was specific for the analysis of analytes in the study. Fig. 2 shows the chromatograms observed during the analysis of unspiked sample, spiked with atogepant, ubrogepant, frovatriptan internal standard and combination of both standards as well as internal standard spiked sample.

The column eluents were simultaneously detected using mass detector for the qualitative analysis of drug using the possible mass fragments of the analytes. The multiple reaction monitoring (MRM) mode was used for the mass spectral analysis of column eluents in the study. The analytes were monitored in both positive (+ESI) and negative (–ESI) modes. The results observed in both modes during the method development study confirm that the +ESI mode shows high intense signals with significantly less noise for the analytes in the study. Due to this, in the method validation study, the eluents was monitored in +ESI mode only.

In the full scan Q1 mass spectra of shows predominant protonated $[M+H]^+$ parent ions at *m/z* of 604, 550 and 244 for atogepant, ubrogepant and frovatriptan, respectively. The characteristic most abundant fragment ions found in the product ion mass spectrum were 326, 315 and 227, respectively for atogepant, ubrogepant and frovatriptan. The mass spectral parameters are summarized in Table-1 and full scan mass spectra observed for the analyst in the study are shown in Fig. 3.

The detection limit of the developed method was confirmed as 4.55 ng/mL for both ubrogepant and atogepant whereas the quantification limit was confirmed as 15 ng/mL. This confirms that the method was sensitive and can detect till at very less concentration of 4.55 ng/mL. The calibration dilution was prepared from the LOQ concentration to a very high concentration range and based on the regression analysis, an accurate and

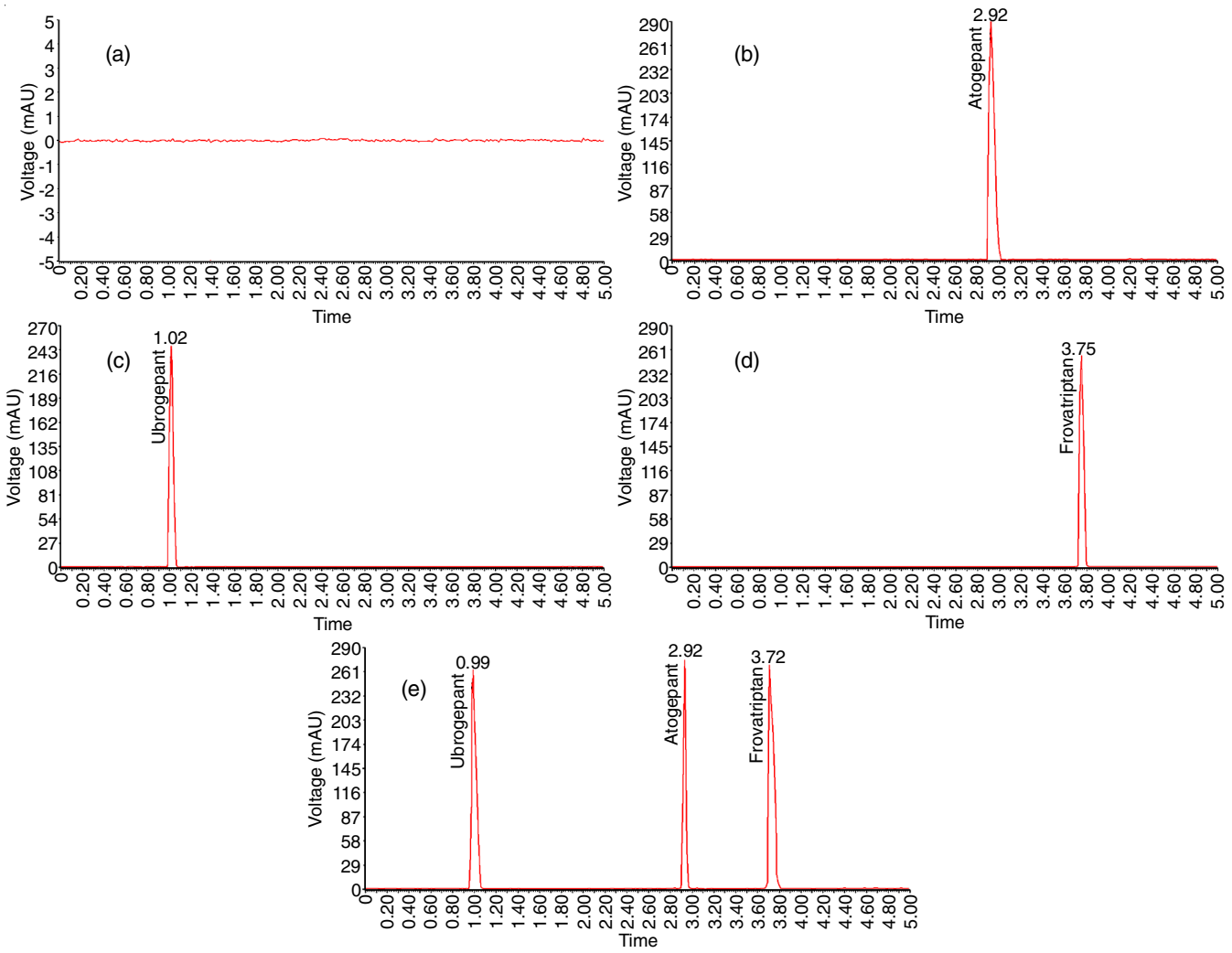

Fig. 2. LC chromatograms obtained in the optimized conditions [(a) unspiked plasma sample; (b) spiked with atogepant; (c) spiked with ubrogepant; (d) spiked with internal standard (frovatriptan); (e) spiked with both analytes and internal standard]

TABLE-1
LC-MS/MS PARAMETERS SELECTED FOR THE QUANTIFICATION OF ATOGEPANT AND UBROGEPANT USING FROVATRIPTAN AS INTERNAL STANDARD (IS)

Parameter	Ubrogepant	Atogepant	Frovatriptan
Precursor ion (*m/z*)	550	604	244
Product ion (*m/z*)	315	326	227
Declustering potential (v)	29	45	37
Entrance potential (v)	12	9	11
Collision energy (v)	19	34	34
Cell exit potential (v)	11	21	19

high correlate calibration curve was obtained in the concentration range of 15 to 600 ng/mL for both analytes with regression equation of $y = 0.0038x + 0.0958$ ($R^2 = 0.9992$) and $y = 0.0042x + 0.088$ ($R^2 = 0.9998$) for ubrogepant and atogepant respectively. The results of the linearity (Table-2) confirmed that the method has broad and sensitive calibration curve.

The repeatability and reproducibility of the method developed for the analysis of ubrogepant and atogepant using LC-MS was evaluated by performing precision study and the study was conducted in HQC, MQC and LQC levels in the calibration

TABLE-2
LINEARITY RESULTS OBSERVED IN THE DEVELOPED METHOD

Concentration (ng/mL)	Peak area of ubrogepant	Peak area of atogepant	Peak area of IS	Area ratio of ubrogepant and IS	Area ratio of atogepant and IS
15	32632.8	34656.0	231456.8	0.141	0.150
50	68157.4	70338.4	231991.3	0.294	0.303
75	90353.9	95594.5	231215.8	0.391	0.413
100	108595.2	113373.3	231525.8	0.469	0.490
200	198352.8	212039.1	231447.1	0.857	0.916
400	389157.5	412896.1	231636.5	1.680	1.783
600	548575.9	598747.6	231002.4	2.375	2.592

IS = Internal standard (frovatriptan)

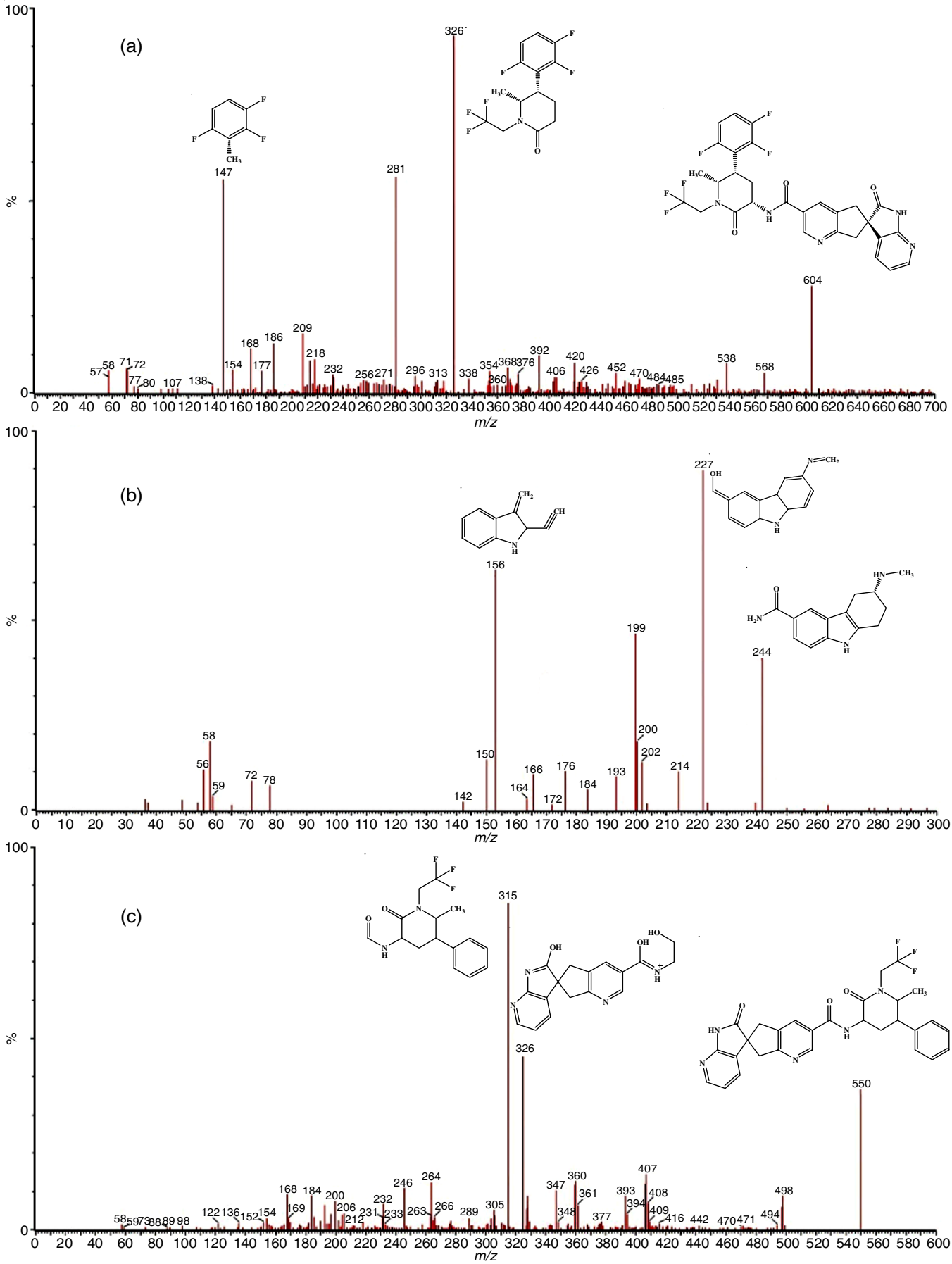

Fig. 3. Full scan mass spectra observed in the optimized conditions [(a) atogepant; (b) frovatriptan internal standard; (c) ubrogepant]

range for both analytes. The % accuracy was observed to be in the range of 99.59-100.34% in intra-day precision and 96.65-97.53% in inter-day precision for ubrogepant. The % accuracy of atogepant was observed in the range of 99.69-102.05% in intra-day precision, 96.36-99.00% in inter-day precision. The % accuracy were in acceptable limit and the % RSD of the repeated analysis results was observed to be within the acceptable limit of less than 2 for both ubrogepant and atogepant (Table-3). Based on the results it can be concluded that the method was precise and accurate.

The extraction efficiency of both ubrogepant and atogepant in the developed method was evaluated by performing recovery studies. In this, the % accuracy the spiked sample was calculated by comparing the peak area ratio of spiked sample with the aqueous calibration curve in the same concentration level in the calibration range. The analysis was performed in HQC, MQC and LQC levels in the calibration curve range. The % recovery was observed in the range of 96.25-102.58% for ubrogepant and 95.21-103.06% for atogepant in the developed method. The results confirm that the method shows high extraction efficiency and hence the method was accurate and recoverable.

The impact of sample dilution on the accuracy and precision of the developed method was evaluated in dilution integrity study. The 2 factor higher concentration than HQC level was prepared and diluted to the HQC level prior to the analysis. The accuracy in the dilution integrity study was observed to be 98.14% and 97.39% for ubrogepant and atogepant, respectively confirm that the method was accurate and precise.

The short term and long term stability was performed to evaluate the stability of analytes in different storage time intervals. Freeze thaw stability was studied to evaluate the stability of analytes after three freeze thaw cycles and is necessary to avoid repeated access to deep freezer within shorter intervals. The stability of analytes that was stored in auto sampler was evaluated in auto sampler stability. In all the stability studies, the analytes at low, medium, high quality control samples were analyzed and the % stability was calculated by comparing the results observed in each stability study for both the analytes with the corresponding standard calibration curve. The stability studies such as short term, long term, freeze-thaw, auto-sampler and dry extract stability showed that the mean% nominal values of the analytes were within ±15% of the predicted concentrations for the analytes at their LQC, HQC and LQC levels. Thestability studies results (Table-4) confirmed that the stabilities were acceptable limits and proved the good stability of ubrogepant and atogepant proved that the method was stable.

The solid-phase extraction and liquid-liquid extraction were extensively utilized for the extraction of analytes from biological complexes. In the solid phase extraction method,

TABLE-3
INTRA-DAY AND INTER-DAY PRECISION AND ACCURACY RESULTS FOR UBROGEPANT AND ATOGEPANT IN THE DEVELOPED METHOD

Analyte	QC level	Intra-day precision (n = 6)			Inter-day precision (n = 6)		
		Conc. found (ng/mL)	Accuracy (%)	RSD (%)	Conc. found (ng/mL)	Accuracy (%)	RSD (%)
Ubrogepant	HQC (600 ng/mL)	597.6	99.59	0.41	579.9	96.65	0.94
	MQC (100 ng/mL)	100.34	100.34	1.10	96.98	96.98	1.75
	LQC (15 ng/mL)	14.94	99.61	0.95	14.63	97.53	1.08
Atogepant	HQC (600 ng/mL)	598.2	99.69	0.25	578.2	96.36	1.46
	MQC (100 ng/mL)	100.94	100.94	1.36	97.96	97.96	1.80
	LQC (15 ng/mL)	15.31	102.05	0.61	14.85	99.00	1.27

TABLE-4
RESULTS OBSERVED IN VARIOUS STABILITY STUDIES CONDUCTED FOR UBROGEPANT AND ATOGEPANT IN THE DEVELOPED METHOD

Test	QC level	Ubrogepant			Atogepant		
		Conc. found (ng/mL)	Stability (%)	RSD (%)	Conc. found (ng/mL)	Stability (%)	RSD (%)
Short term stability	HQC (600 ng/mL)	594.1	99.02	0.34	595.8	99.29	0.55
	MQC (100 ng/mL)	99.76	99.76	1.10	100.36	100.36	1.27
	LQC (15 ng/mL)	14.84	98.94	1.28	15.09	100.58	0.56
Long term stability	HQC (600 ng/mL)	591.4	98.57	0.42	592.3	98.72	0.80
	MQC (100 ng/mL)	99.26	99.26	0.96	99.78	99.78	1.20
	LQC (15 ng/mL)	14.63	97.52	1.33	14.86	99.07	0.83
Freeze–thaw stability	HQC (600 ng/mL)	577.5	96.25	1.30	582.4	97.07	0.60
	MQC (100 ng/mL)	95.94	95.94	1.64	96.83	96.83	1.57
	LQC (15 ng/mL)	14.38	95.85	1.57	14.63	97.53	0.81
Auto-sampler stability	HQC (600 ng/mL)	594.1	99.02	0.34	595.5	99.24	0.43
	MQC (100 ng/mL)	95.93	95.93	1.99	96.75	96.75	1.77
	LQC (15 ng/mL)	14.62	97.48	1.17	14.84	98.93	0.69
Dry extract stability	HQC (600 ng/mL)	567.8	94.64	1.58	572.7	95.44	1.25
	MQC (100 ng/mL)	95.84	95.84	1.64	96.19	96.19	1.82
	LQC (15 ng/mL)	14.60	97.34	1.22	14.79	98.60	1.00

the extraction of highly polar compounds was very difficult and this technique treated as expensive whereas the liquid-liquid extraction was treated as simple and inexpensive. Hence liquid-liquid extraction technique was used for the extraction of ubrogepant and atogepant along with internal standard from spiked human plasma. Prior to the liquid-liquid extraction, the protein precipitation was performed using acetonitrile solvent and various organic solvents were studied for the extraction of analytes from the plasma matrix. In each solvent used for the extraction of analytes, the solvent that produces high chromatographic response was selected as suitable solvent for the extraction of analytes. Based on the results, it was observed that dichloromethane shows high peak area response of ubrogepant, atogepant and internal standard with significantly less noise and no chromatographic interference. Hence, dichloromethane was selected as suitable solvent for the extraction of analytes from plasma matrix. The extracted sample was utilized in the method development and validation study using LC-MS.

The chromatographic conditions were progressively optimized for the development of suitable analytical conditions for the analysis of ubrogepant and atogepant using frovatriptan as internal standard. The method development was concluded by achieving the best chromatographic results and separation was achieved using 0.01% NH_3 in 2 mM ammonium formate at pH 6.4 and acetonitrile at 45:55 (v/v) as mobile phase at 0.4 mL/min flow rate of mobile phase that facilitates less consumption of mobile phase. Very nominal quantity of sample (2 µL) was separated on Xbridge C_{18} column (50 mm × 4.6 mm, 5 µm) at room temperature and the column eluents were monitored using UV and mass detector. The mass spectral analysis was performed using mass detector in MRM positive ion mode. The mass spectra shows clear mass fragmentation corresponding to the analytes. The analysis was completed within shortest run time of 5 min and the analytes were detected at a retention time of 1.02, 2.92 and 3.75 min, respectively for ubrogepant, atogepant and internal standard. There is no interference of the impurities were detected throughout the run time of the chromatogram as well as clear mass fragmentation pattern was identified for individual analytes in the developed method proved that the method was specific with no matrix effect.

The method shows very sensitive detection limit of 4.55 ng/mL for both the analytes with quantification limit of 15 ng/mL. The method shows calibration curve linear in the concentration range of 15 ng/mL (LOQ) to 600 ng/mL with correlation coefficient of more than 0.999 for both the analytes. The method was validated and the results observed were within the acceptable limit confirms that the method was enough suitable for the analysis of ubrogepant and atogepant in biological samples.

Conclusion

The present study described a simple and stable HPLC-MS/MS method for the separation and simultaneous quantification of recently approved calcitonin gene-related peptide receptor antagonist drugs ubrogepant and atogepant in the spiked human plasma. The method utilizes a simple protein precipitation followed by liquid-liquid extraction of analytes from the spiked plasma matrix. The method was observed to be sensitive that can detect the analytes up to 4.55 ng/mL concentration and shows high accurate fit linear calibration curve in the concentration range of LOQ (15 ng/mL) to 600 ng/mL. The method was validated and reported to be valid in all the parameters and based on the results achieved in the study, it can be concluded that the method was suitable for the separation and simultaneous analysis of ubrogepant and atogepant and may be applicable for the pharmacokinetic profiling of the studied drugs.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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