Pharmaceutical Methods 4 (2013) 33–42

Contents lists available at ScienceDirect

Pharmaceutical Methods

journal homepage: www.elsevier.com/locate/phme

Original article

Determination of eight isomers and related substance of Aprepitant using normal-phase and reverse-phase HPLC methods with mass spectrophotometric detection

Sukumar Sinha*, Vikas Parashar, Sudhir Pakhale, Kailas Mungase, Mohammad Amjed Khan, Swamy Reddy, S.K. Manirul Haque, Vinny Mehra

Analytical Research Lab, Glenmark Generics Ltd., Glenmark Research Centre, Navi Mumbai, Maharashtra 400709, India

ARTICLE INFO

Article history:
Received 28 August 2012
Accepted 12 October 2012
Available online 19 December 2013

Keywords:
Aprepitant
Chiral separation
Related substances
HPLC
Mass spectrophotometer

ABSTRACT

Introduction: Aprepitant is a chiral prodrug possessing three stereogenic centers and exists in eight stereochemical forms. It was aimed to estimate and validate Aprepitant using reverse phase HPLC.
Materials and methods: Chiral liquid chromatography with an ion-trap mass spectrophotometer was used to isolate all the isomers. The separation of the eight isomers was achieved on an amylase-based chiral column (Chiralpak ADH, 250 mm × 4.6 mm) using *n*-hexane/isopropyl alcohol/methanol/trifluoroacetic acid (970/40/4/0.5, vol/vol/vol/vol) as the mobile phase at a flow rate of 0.5 ml/min. A reverse-phase HPLC method was used to analyze Aprepitant and its related substances. The determination of Aprepitant and its related substances was developed by using Xterra RP – 18, 250 mm × 4.6 mm, 5 μm column.
Results and conclusion: These methods were validated according to the International Conference on Harmonization (ICH) guidelines. The limit of detection (LOD) and limit of quantitation (LOQ) for Aprepitant were found to be 0.14 μg/ml and 0.41 μg/ml, respectively, for chiral substance and 0.07 μg/ml and 0.21 μg/ml, respectively, for the related substance.

Aprepitant (APT) is a substance P (SP)/neurokinin 1 (NK_1) receptor antagonist and is chemically described as 5-[[(2R, 3S)-2-[(1R)-1-[3, 5-Bis (trifluoromethyl) phenyl] ethoxy] -3-(4-fluorophenyl)-4-morpholinyl] methyl]-1, 2-dihydro-3H-1,2,4-triazol-3-one (Fig. 1). It is a white to off-white crystalline solid with a molecular weight of 534.43 and an empirical formula of $C_{23}H_{21}F_7N_4O_3$. APT has recently been approved in combination with other agents for use as an effective treatment for preventing acute and delayed chemotherapy-induced nausea and vomiting (CINV).[1]This is the first FDA-approved drug for the treatment of CINV that persists for more than 24 h after chemotherapy treatment. It is a drug known to elicit activity against the human neurokinin-1 (NK_1) receptor.[2] This receptor is located at the brain stem nuclei of the dorsal vagal complex and is a crucial part of the regulation of emesis (vomiting), which is due to the receptor binding with substance P, a peptide neurotransmitter.[3–5]

Chiral separations can be achieved with HPLC through the following approaches: (a) direct separation of racemates to their corresponding enantiomers using chiral stationary phases (CSPs)[6]; (b) indirect separation of diastereoisomers, formed by the reaction of the enantiomers with a chiral derivatizing agent, using achiral stationary phases[7]; or (c) separation of chiral derivatives, formed by reaction with non-chiral derivatizating agents, using CSPs.[8–13] Direct methods based on CSPs are the preferred separation approaches because they are simple and rapid to apply at both the analytical and preparative scales.

Literature reviews reveal that very few analytical methods have been established for the estimation of Aprepitant in human plasma.[14–17] The following have been reported: an HPLC chromatographic reactor approach for investigating the hydrolytic stability of a pharmaceutical compound,[18] an estimation of APT in rhesus macaque plasma,[19] the characterization and quantization of APT drug substance polymorphs by attenuated total reflectance Fourier infrared spectroscopy,[20] an investigation into the stability of an extemporaneous oral liquid APT formulation,[21,22] and an estimation of APT capsules by RP-HPLC.[23]

Enantioseparation of chiral compounds containing multiple stereogenic centers poses some analytical challenges.[24] A complete

* Corresponding author. Analytical Research Lab, Glenmark Generics Ltd, Glenmark Research Centre, Plot A 607, TTC Industrial Area, MIDC, Mahape, Navi Mumbai, Maharashtra 400709, India. Tel.: +91 2267720000.
E-mail address: sukumars@glenmark-generics.com (S. Sinha).

2229-4708/$ – see front matter
http://dx.doi.org/10.1016/j.phme.2012.10.001

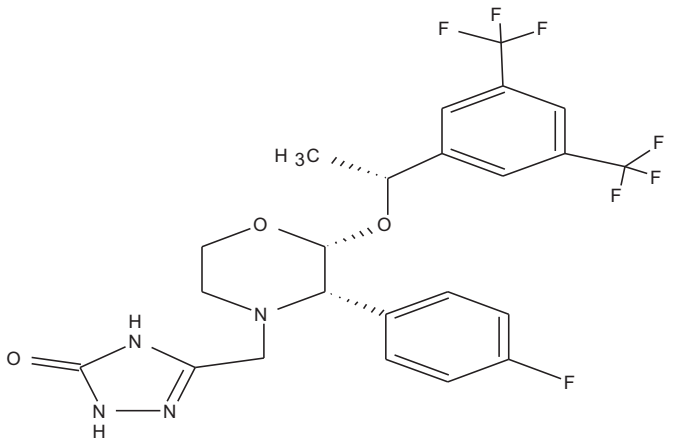

Fig. 1. The chemical structure of aprepitant.

resolution of all the potential stereoisomers of these compounds can only be achieved if the CSP used is highly discriminating for both enantiomers and diastereomers. This paper will highlight the challenges we faced in developing a chiral HPLC separation for APT, a novel chiral drug possessing three stereogenic centers that allow for eight stereoisomers (Table 1). The development of a comprehensive HPLC method was the proposed strategy for separating all eight isomers of Aprepitant. This strategy was based on the fact that CSP is highly effective in resolving enantiomers with complementary intermolecular interaction sites.[25] When the method development strategy was executed, several different CSPs, including an amylase-based CSP, were identified that possessed enantioselectivity for both enantiometric pairs (SR/RS and RR/SS) of APT. Furthermore, amylase-based CSPs showed the promise of simultaneously separating all eight isomers with the help of ion-trap mass spectrophotometry.

A relative response factor for all these impurities with respect to Aprepitant was determined for the quantitative determination of the known impurities, and other unknown impurities were determined using the diluted standard method. The determination of the related substances and degradation products in an API at a lower level of 0.10% or below often leads to a misleading interpretation of the results in the absence of a relative response factor. It has been observed in many cases that the related substance or the degradation product generated during the manufacturing of an API may not have the same UV response at the wavelength of determination in these tests. Therefore, there is a need to use either impurity standards or a relative response factor (RRF) for the quantitative determination of low levels of impurities and degradation products. Reverse-phase-HPLC methods were used for the qualitative and quantitative determination of Aprepitant. The column selection and the mobile-phase composition were found to play a vital role in the separation and sensitivity of the developed method. The proposed RP-HPLC method with UV detection has been validated using the ICH and USP[26,27] guidelines as a reference.

1. Experimental

1.1. Standards and solvents

Aprepitant, 5-[[2(R)-[1(S)-[3,5-bis(trifluoromethyl)phenyl] ethoxy]-3(S)-(4-fluorophenyl)-4 morpholinyl]methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one (Impurity A), hydrazinecarboxylic acid, 2-[2-[(2R,3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]-1-iminoethyl]-, methyl ester (Impurity B) and 5-[[2(R)-[1(R)-[3,5- bis(trifluoromethyl)phenyl] ethoxy]-3(S)- phenyl-4- morpholinyl]methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one (Impurity C) were used as inhouse reference standards (Glenmark Generics Ltd., Glenmark R & D Centre, MIDC, Mahape, Navi Mumbai, India). HPLC grade *n*-hexane, isopropyl alcohol (IPA), methanol, ethanol, and acetonitrile (ACN) were purchased from Rankem and GR-grade trifluoroacetic acid (TFA) and ortho-phosphoric acid (OPA) were purchased from Merck Specialties Chemicals Private Limited (Mumbai, India). Deionized water was purified with a TKA Ultrapure water system (Germany).

Table 1
The stereochemistry of all the possible isomers of Aprepitant.

Diastereomeric pair	Compound	Absolute configuration
I	I_a (Aprepitant)	$R_1R_2S_3$
	I_b (The enantiomer of Aprepitant)	$S_1S_2R_3$
II	II_a	$R_1S_2R_3$
	II_b	$S_1R_2S_3$
III	III_a	$R_1R_2R_3$
	III_b	$S_1S_2S_3$
IV	IV_a	$S_1R_2R_3$
	IV_b	$R_1S_2S_3$

1.2. Analytical mode high-performance liquid chromatography

A Waters (Milford, MA, USA) 2695 series HPLC system equipped with a 2695 series quaternary gradient pump, an autosampler with a cooler and a PDA system and a Shimadzu LC 2010 CHT (Japan) HPLC module equipped with a quaternary gradient pump, a column oven, an autosampler and a DAD system were used for the analysis and validation of the proposed method. The data were recording using Empower 2 and LC solution software for the Waters and Shimadzu systems, respectively.

1.3. Chromatographic conditions

1.3.1. Stereochemical purity

The analysis was conducted on an amylase-based Chiralpak AD-H (250 mm × 4.6 mm, 5 μm, Daicel Chemical Industries Limited, Japan) using a mobile phase consisting of *n*-hexane/isopropyl alcohol/methanol/trifluoroacetic acid (970/40/4/0.5, vol/vol/vol/vol) with a UV detector at a wavelength of 210 nm and a flow rate of 0.5 ml/min. The column was maintained at 35 °C throughout the analysis. A 20 μl sample of the concentrate was injected, and the chromatogram was recorded for 70 min.

1.3.2. The related substances

The mobile phase consisted of 0.1% OPA in water and acetonitrile. Analyses were performed with a gradient elution starting with 58/42 (vol/vol) for 44 min, then modified to 30/70 (vol/vol) for 10 min, and returned to 58/42 (vol/vol) after 55 min from the time of injection. The flow rate was 1.0 ml/min, and the total duration of the chromatographic run was 65 min. The Xterra RP – 18, 250 mm × 4.6 mm, 5 μm column was used for the determination of the related substances of Aprepitant.

1.4. Preparation of the reference, the test solutions and the system suitability solutions

1.4.1. Stereochemical purity

A diluent was prepared by mixing *n*-hexane and ethanol in a 95:05 ratio (vol/vol). Approximately 10.0 mg of the standard mixture of isomers was transferred into a 10 ml volumetric flask, and 5 ml of the diluent was added. After the isomers were dissolved, the flask was filled to the mark with the diluent. This

solution was labeled as Reference Solution A. A blank was prepared as a reference solution by mixing 10 ml of the diluent in a 10 ml volumetric flask. For the preparation of the test solution, approximately 125 mg of the Aprepitant sample was dissolved in 15 ml of ethanol in a 50 ml volumetric flask and subsequently filled to the mark with the diluent. A blank solution was prepared by mixing 15 ml of ethanol in a 50 ml volumetric flask and subsequently filling to volume with the diluent. For the preparation of the system suitability solution, approximately 2.5 mg of stereoisomer (Impurity A) was dissolved in 20 ml of ethanol in a 100 ml volumetric flask, and the flask was filled up to volume with diluents. This solution was labeled as Reference Solution B. Approximately 125 mg of Aprepitant in-house standard was dissolved in 10 ml of ethanol in a 50 ml volumetric flask. Subsequently, 7.5 ml of Reference Solution B was added, and the flask was filled to the mark with the diluent. Equal volumes of the blank solutions, the system suitability solution, Reference Solution A and the test solution were injected in duplicate, and all the peaks were integrated using a valley-to-valley integration. The results from all possible isomers are shown in Table 1.

1.4.2. The related substances

A diluent was prepared by mixing buffer and ACN in a ratio of 50:50 (vol/vol). Approximately 50 mg of the reference standard was dissolved in 15 ml of the diluent in a 25 ml volumetric flask, and the flask was subsequently filled to the mark with diluent. This solution was marked as Reference Solution A. 1 ml of Reference Solution A was transferred to a 200 ml volumetric flask, filled to the mark with diluent and mixed. This solution was marked as Reference Solution B.

For the preparation of the system suitability solution, approximately 5.0 mg each of Aprepitant impurity standards A, B and C were dissolved in 60 ml of diluent in a 100 ml volumetric flask, and 2.5 ml of Reference Solution A was added. The volume was subsequently filled up to the mark with diluent. This solution was labeled Reference Solution C. The test solution was prepared by weighing 50 mg of sample into a 25 ml volumetric flask. Approximately 15 ml of diluent was added, and the mixture was sonicated to ensure dissolution. The resulting mixture was subsequently filled to the mark with diluent and mixed.

Equal volumes of the blank solution, the system suitability solution (Reference C) and the six replicate injections of B were injected separately, the test solution was injected in duplicate and the chromatogram was recorded for all the injections. Impurity peaks could be eliminated by comparison to the blank. The peaks that were identified corresponded to impurities A, B and C from Reference Solution (c). All the known and unknown impurities were calculated against the area obtained from the replicate injections of Reference Solution A, and an RRF was applied for the calculation of the known impurities.

1.5. System suitability parameters

1.5.1. The stereochemical purity

The resolution between Aprepitant and its stereoisomer (Impurity A) should not be less than 1.0.

1.6. The related substances

In Reference Solution (C), the resolution between impurity C and Aprepitant should not be less than 2.5. The relative standard deviation of six replicate injections of reference solution B should not be greater than 5.0%.

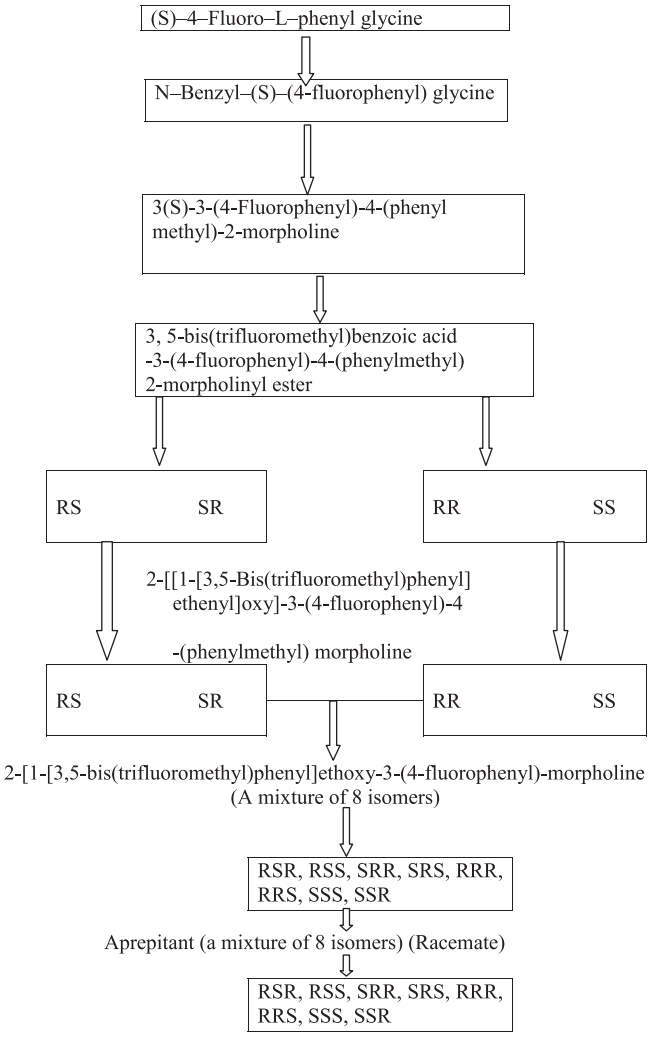

Fig. 2. A flow-diagram of the aprepitant racemate.

2. Results and discussion

2.1. The separation of the eight isomers of the aprepitant mixture

The current USP and European pharmacopoeia have not been able to separate all the possible isomers of Aprepitant until now. However, some researchers[28] have successfully separated two of the isomers of Aprepitant. Although there are eight possible isomers in synthetic mixtures of Aprepitant due to its three chiral centers (Fig. 2), the method developed herein initially focused on Aprepitant and the five isomers eluted immediately before it. Establishing preliminary chromatographic conditions to adequately resolve the first seven isomers could then be optimized to separate the remaining isomer. Depending on the mobile-phase conditions, the peaks merged with one another or with Aprepitant. Various chromatographic parameters were evaluated, including the column, the column temperature, the mobile-phase composition, and the diluent. The highlights of these results, including experimental parameters, are summarized in Table 2.

The preliminary mobile-phase conditions (Table 2) used in the study were unable to completely separate the eight isomers. Therefore, several different mobile phases were evaluated. As expected, the chromatographic separation and the elution order of the eight isomer peaks were different when different combinations of organic solvents were used for the mobile-phase. All eight peaks were

Table 2
Chromatographic parameters for the direct enantioseparation of the Aprepitant racemate.

Mobile phase composition	Peak-1	Peak-2	Peak-3	Peak-4	Peak-5	Peak-6	Peak-7	Peak-8	Observation
n-Hexane: IPA:MeOH:TFA (97:3:0.4:0.05)	32.6	38.9	42.6	44.7	46.7	49.1	59.4	64.9 Aprepitant	8 peaks
n-Hexane: IPA:MeOH:TFA (99:3:0.4:0.05) Change in *n*-Hexane	29.5	38.2	–	39.8	43.9	52.4	56.0	69.5 Aprepitant	7 peaks
n-Hexane:IPA:MeOH:TFA (95:3:0.4:0.05) Change in *n*-hexane	27.3	35.9	41.9	50.9 Merging with peak 5	55.0	–	–	70.0 Aprepitant	6 peaks
n-Hexane: IPA:MeOH:TFA (97:3:0.7:0.05) Change in MeOH	23.6	29.5	32.1 Merging	38.8	40.6	49.6 Aprepitant	–	–	6 peaks
n-Hexane: IPA:MeOH:TFA (97:3:1:0.05) Change in MeOH	22.3	26.7	29.2	32.4	34.9	37.1 Aprepitant	49.3	–	7 peaks
n-Hexane: IPA:MeOH:TFA (99:3:0.4:0.05) Change in *n*-hexane	29.5	38.2	–	39.8	43.9	52.4	56.0	69.5 Aprepitant	7 peaks
n-Hexane: IPA:MeOH:TFA (97:3:0.4:0.01) Change in TFA	33.6	36.6	41.4	44.9	47.3 Merging with Peak 6	48.4	63.1	65.4 Aprepitant	8 peaks
n-Hexane: IPA:MeOH: FA (97:3:0.4:0.04) Change in TFA	29.3	37.1	38.6 Merging with Peak 4	39.2	40.8	44.0	55.4	58.1 Aprepitant	8 peaks
n-Hexane: IPA:MeOH:TFA (97:2:0.4:0.05) Change in TFA	54.4	–	71.3	74.8	80.9	88.3	104.4	110.0 Aprepitant	7 peaks
n-Hexane: IPA:MeOH:TFA (97:4:0.4:0.05) Change in TFA	20.7	25.9	27.5	28.1	29.6	32.8	38.5	45.3 Aprepitant	8 peaks

separated using a Chiralpak AD-H, 250 mm × 4.6 mm column at 35 °C with an elution rate of 0.5 ml/min with *n*-hexane:IPA:MeOH:TFA (970:40:4:0.5, V/V/V/V) used as the mobile phase.

2.2. LC–MS/MS analysis

LC–MS/MS analysis was conducted using an LCQ DECA XP MAX ion-trap mass spectrophotometer (Thermo electron Corporation, USA, with Xcaliber software) coupled with a Shimadzu HPLC equipped with a UV–VIS detector, MS–ATVP pumps and LC Solutions software (used for data acquisition and data processing). The turbo ion-spray voltage was maintained at 5.0 KV and a temperature of 300 °C. Nitrogen was used as the nebulizer gas. LC–MS spectra were acquired from *m*/*z* 100 to 1000 in 0.1 amu steps with a 2.0 s dwell time. An Aprepitant sample was subjected to LC–MS/MS analysis. The analysis was conducted using a normal-phase column (chiralpak ADH, 250 mm × 4.6 mm, 5 μm). The mobile phase used was *n*-hexane:IPA:MeOH:TFA (970:40:4:0.5, vol/vol/vol/vol).

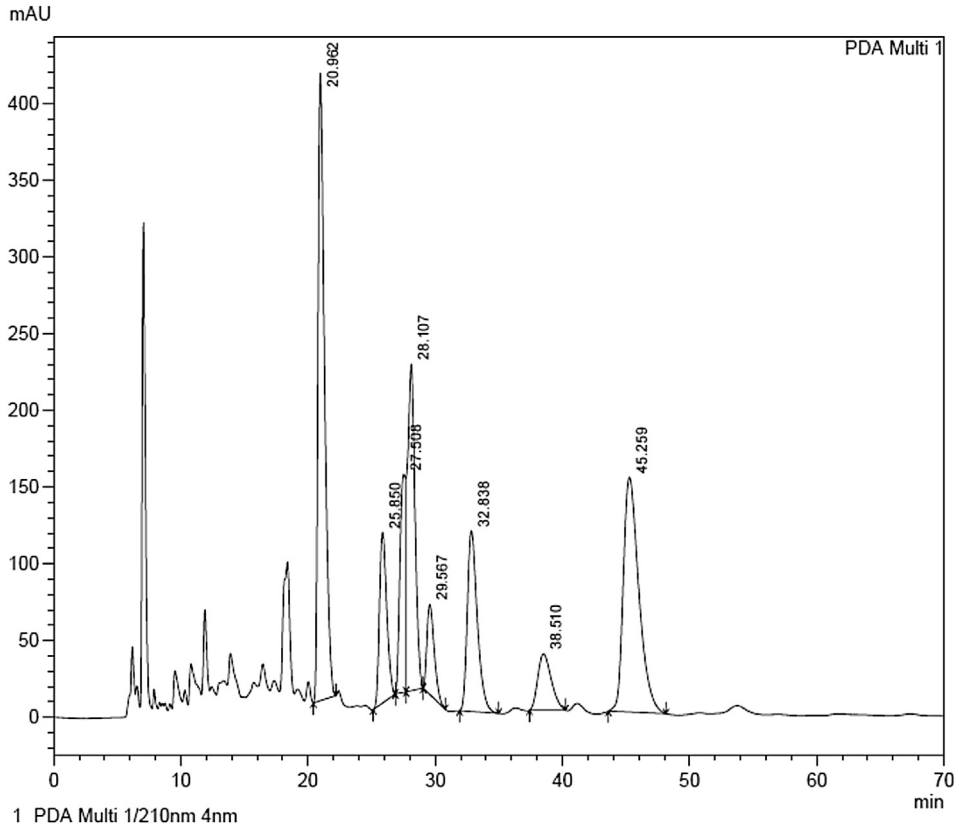

Fig. 3. The liquid chromatography spectra of aprepitant.

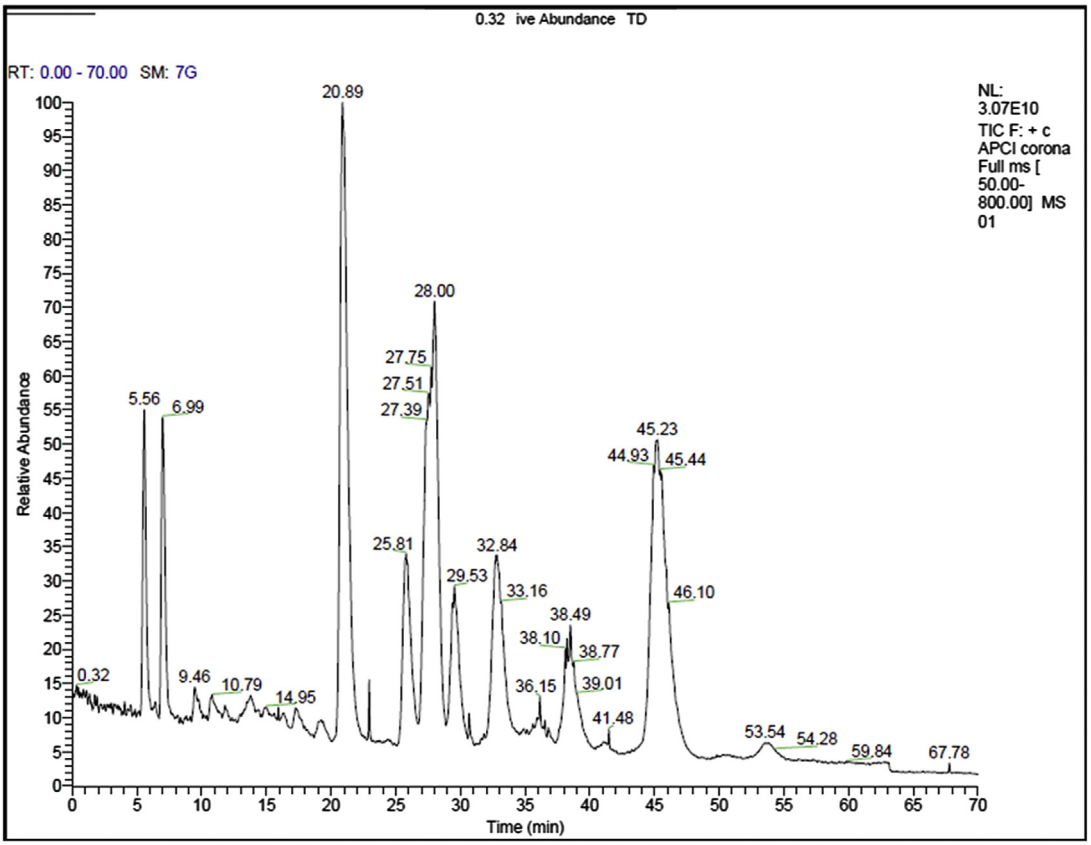

Fig. 4. The liquid chromatography-mass spectrophotometry spectra of the racemate of aprepitant.

Detection was conducted at a wavelength of 210 nm, and the flow rate was kept at 0.5 ml/min. An *n*-hexane and ethanol mixture in a ratio of 95:05 (vol/vol) was used as the diluent. The data acquisition time was 70 min. The eight isomers were separated from a stereochemically pure Aprepitant standard (Figs. 3 and 4). The masses of the detected peaks were 535.05 $[(M + H)^+]$ at RT 20.95, 535.06 $[(M + H)^+]$ at RT 27.51, 535.08 $[(M + H)^+]$ at RT 28.11, 537.07 $[(M + H)^+]$ at RT 29.56, 534.99 $[(M + H)^+]$ at RT 32.84, 535.05 $[(M + H)^+]$ at RT 38.62, and 535.07 $[(M + H)^+]$ at RT 45.45. The fragmentation patterns of the isomers and Aprepitant were also matched with each other (i.e., 277 D). Based on these mass values and fragmentation patterns, it was confirmed that the eight isomers were stereoisomers of Aprepitant.

2.3. Method validation

Method validation was performed to determine if the HPLC system was acceptable with respect to specificity, linearity of response, and precision and to determine the limit of detection.

2.4. System suitability

A system suitability test was performed to evaluate the chromatographic parameters (the capacity factor, the number of theoretical plates, the asymmetry of the peaks, the resolution between two consecutive peaks and the Relative Standard deviation of the five standards) before the validation runs.

2.5. Stereochemical purity

In reference solution (b), the resolution between Impurity A (a stereoisomer) and Aprepitant was greater than 1.0.

2.6. Related substance

The resolution between impurity C and the main peak was found to be 3.5. The relative standard deviation of six replicate injections of reference solution (b) was found to be 1.29.

2.7. Specificity (selectivity)

2.7.1. Stereochemical purity

The specificity was determined by separate and spiked injections of impurity C (an isomer) and Aprepitant to check their interference with the isomer and with the main peak. It was observed that the isomer did not interfere with the main peak. These results showed that the method was selective for estimating the stereochemical purity of Aprepitant. The diluent was injected into the column to check for interference from the diluent on the retention time of the main peak. It was observed that the diluent (*n*-hexane:ethanol = 95:05, vol/vol) did not interfere with the relative time of the main peak or the other streoisomers.

2.7.2. Related substances

The ability of the chromatographic system to resolve Aprepitant from its possible impurities was investigated. Impurity A, impurity B and impurity C were injected separately and spiked into the analyte to check their interference with the main peak. It was observed that impurities A, B and C did not interfere with the Aprepitant peak. This showed that the method was selective for the estimation of Aprepitant. To check the specificity of the method, the compound was subjected to forced degradation under sets of conditions including temperature, humidity, acid, base, and oxidation and photo degradation. After the study, the chromatograms were checked for the appearance of any extra peaks arising

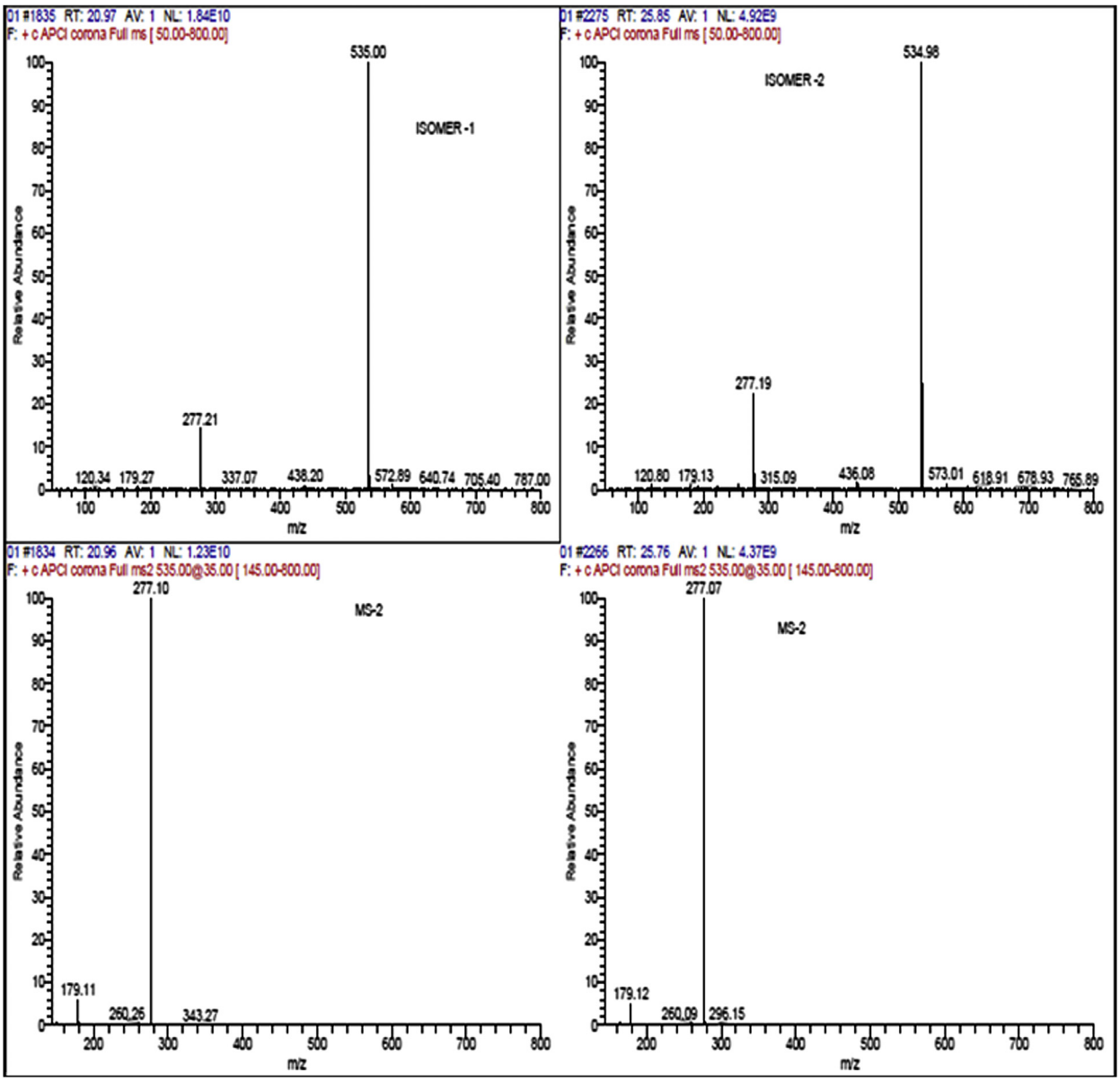

Fig. 4. (*continued*).

from the degradation of the analyte under the stress conditions. When peaks were found, their respective retention times were recorded. The purity of the main peak was recorded. The data from the degradation studies revealed that the degradation products were well separated from Aprepitant and its known related substances. The peak purity (defined as when the purity angle is less than the purity threshold) of Aprepitant indicated that it was spectrally pure. The data from forced degradation studies are given in Table 3.

2.8. Linearity

2.8.1. Stereochemical purity

Solutions of lower concentrations of Aprepitant were prepared, and each concentration was injected on the same day. The data generated were analyzed by linear regression analysis to calculate the slope, the intercept and the correlation coefficient. Linearity graphs were plotted. The method was linear over the range of 1.25 μg/ml to 3.75 μg/ml for Aprepitant (i.e., 50%–150%) with a correlation coefficient of 0.996.

2.9. Related substance

The data were subjected to statistical analysis using a linear regression model, and the standard deviation of the slope and the intercept were calculated. The method was linear over a range of 1.0 μg/l–3 μg/l (i.e., 50%–150%) for Aprepitant and 1.5 μg/l–4.5 μg/l (i.e., 50%–150%) for impurities A, B and C. All correlation coefficients were greater than 0.99.

2.10. Accuracy and precision

2.10.1. Stereochemical purity

The precision was calculated by estimating the stereochemical purity of six samples on the same day. The %RSD for the stereochemical purity of the six samples was calculated and found to be

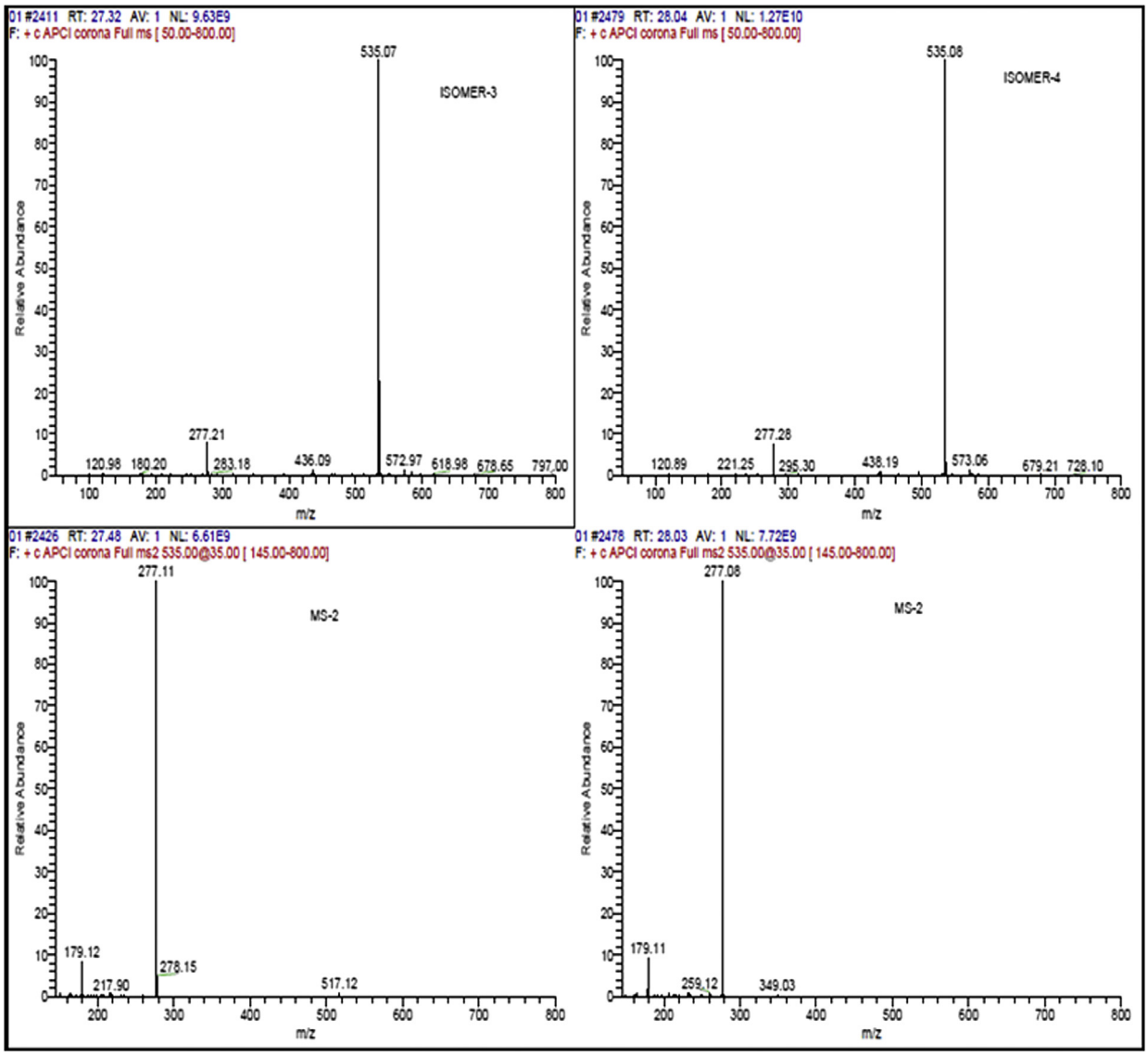

Fig. 4. (*continued*).

well within the derived limits (the acceptance criteria is that the % RSD should not be more than 10%).

2.10.2. Related substances

The accuracy of the method was determined by spiking the known impurities at 50%, 100% and 150% w/w of their specified limits. The accuracy of impurities A, B and C was within the prescribed range of 90%–110%. The system precision was calculated by six replicate injections of reference solution (b). The %RSD of the six replicate injections of reference solution (b) was found to 1.29% for Aprepitant.

The precision of the method was calculated by estimating the amount of the related substances in three control samples and six spiked samples on the same day. The %RSD for the known, any other unknown individual impurity and total impurity of six samples was calculated and found to be well within the desired limits.

2.11. Limit of detection (LOD) and limit of quantitation (LOQ)

2.11.1. Stereochemical purity

The limit of detection was determined from the linearity experiment where lower concentrations of Aprepitant were analyzed. The LOD concentration was found to be 0.14 μg/ml, i.e., 0.01% w/w of the test concentration for Aprepitant. The %RSD for six replicate injections of Aprepitant was found to be 9.44%. The signal-to-noise ratio was above 3. The LOQ concentration was found to be 0.41 μg/ml, i.e., 0.02% w/w of the test concentration for Aprepitant. The RSD for six replicate injections of Aprepitant was found to be 2.35%, and the signal-to-noise ratio was above 10.

2.11.2. Related substances

The LOD and LOQ represent the concentration of the analyte that would yield a signal-to-noise ratio of 3 for the LOD and 10 for the LOQ, respectively. The LOD and the LOQ were determined by measuring the magnitude of the analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until S/N ratio of 3 for the LOD and 10 for the LOQ was achieved. The LOD concentration was found to be 0.003% for Aprepitant, impurity B and impurity C and was 0.004% for impurity A. The %RSD for six replicate injections of impurities A, B, C and Aprepitant were found to be 4.84%, 7.68%, 7.71% and 20.13%, respectively. The LOQ concentration was found to be 0.01% for Aprepitant and impurities A, B and impurity C. The %

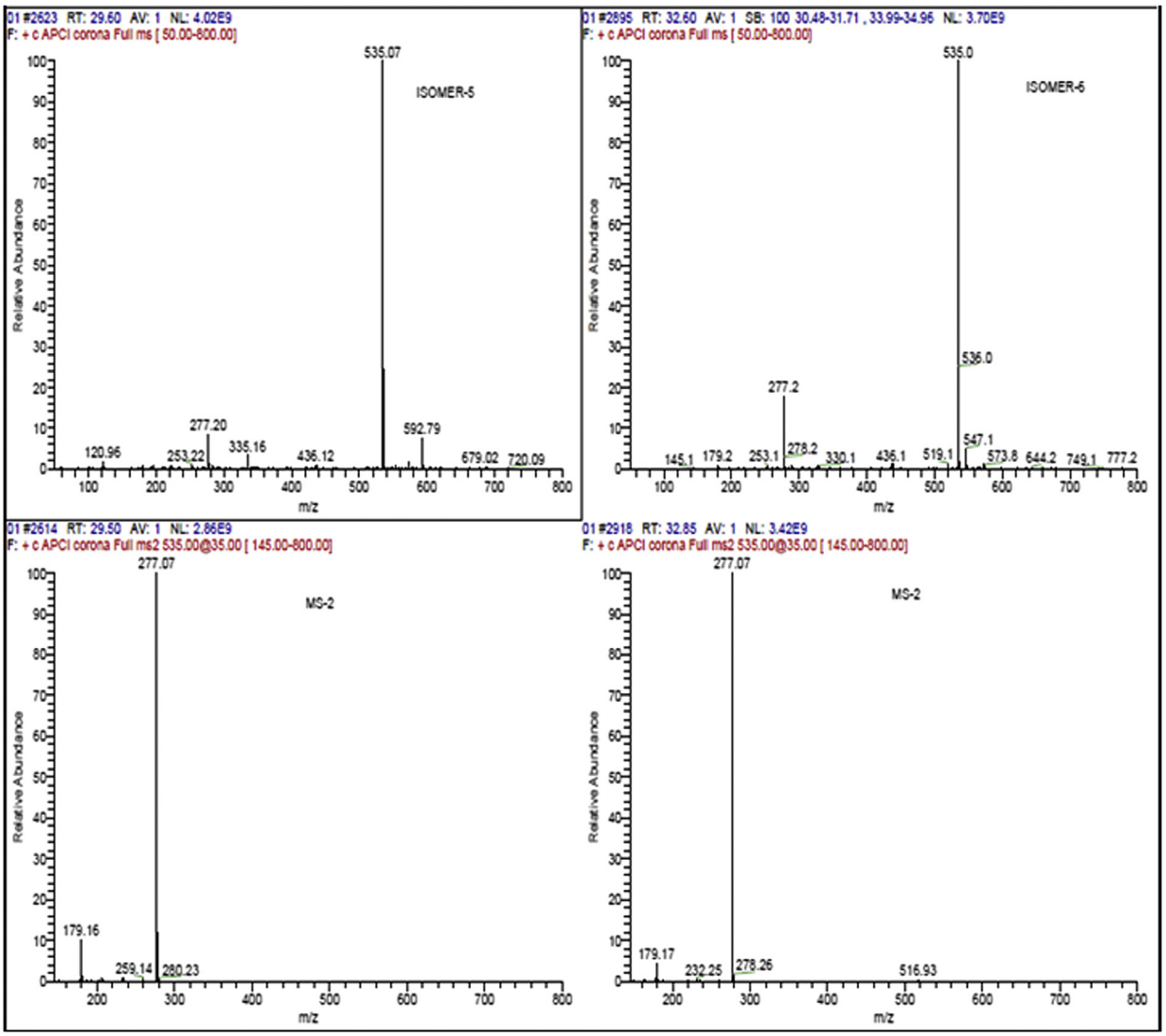

Fig. 4. (*continued*).

RSD for six replicate injections of impurities A, B and C and Aprepitant were found to be 3.68%, 2.30%, 2.63% and 3.42%.

2.12. The ruggedness

2.12.1. The stereochemical purity

The ruggedness was calculated by estimating the stereochemical purity of six samples of two different analytes using different HPLC columns on different instruments on two different days. The %RSD of the stereochemical purity from these twelve samples was estimated.

2.12.2. Related substance

The %RSD for the known impurity, any other unknown individual impurity and total impurity from twelve samples (six control samples and six spiked samples) was determined.

2.13. Robustness

2.13.1. Stereochemical purity

To determine the robustness of the developed method, the experimental conditions were deliberately altered, and the stereochemical purity of Aprepitant was determined. The following modifications to the chromatographic conditions were evaluated.

Change in Column Temperature: The normal experimental condition for column temperature was 35 °C. The effect of a change in column temperature was studied for a variation of ±5 °C from the temperature used in the actual method, i.e., at 30 °C and 40 °C. Change in wavelength: In the actual method, the detector wavelength was set at 210 nm. A change of ±2 nm from the wavelength used for the actual method was studied. Change in flow rate: Samples were analyzed at a flow rate of 0.4 ml/min and 0.6 ml/min. Change in mobile-phase composition: The mobile-phase compositions were changed from the actual method as follows: *n*-hexane and IPA (±2 ml), methanol (±0.4 ml) and TFA (±0.45 ml). The robustness studies showed that the method was robust and not affected by small changes in the experimental conditions.

2.13.2. Related substances

To evaluate the robustness of the method, the influence of small and premeditated alternations of the analytical parameters on the quantification of the related substances and their selectivities were studied. The parameters selected were flow rate (±0.2 ml/min), column oven temperature (±5 °C), wavelength (±2 nm), column (same make, different lot number) and concentration of the buffer.

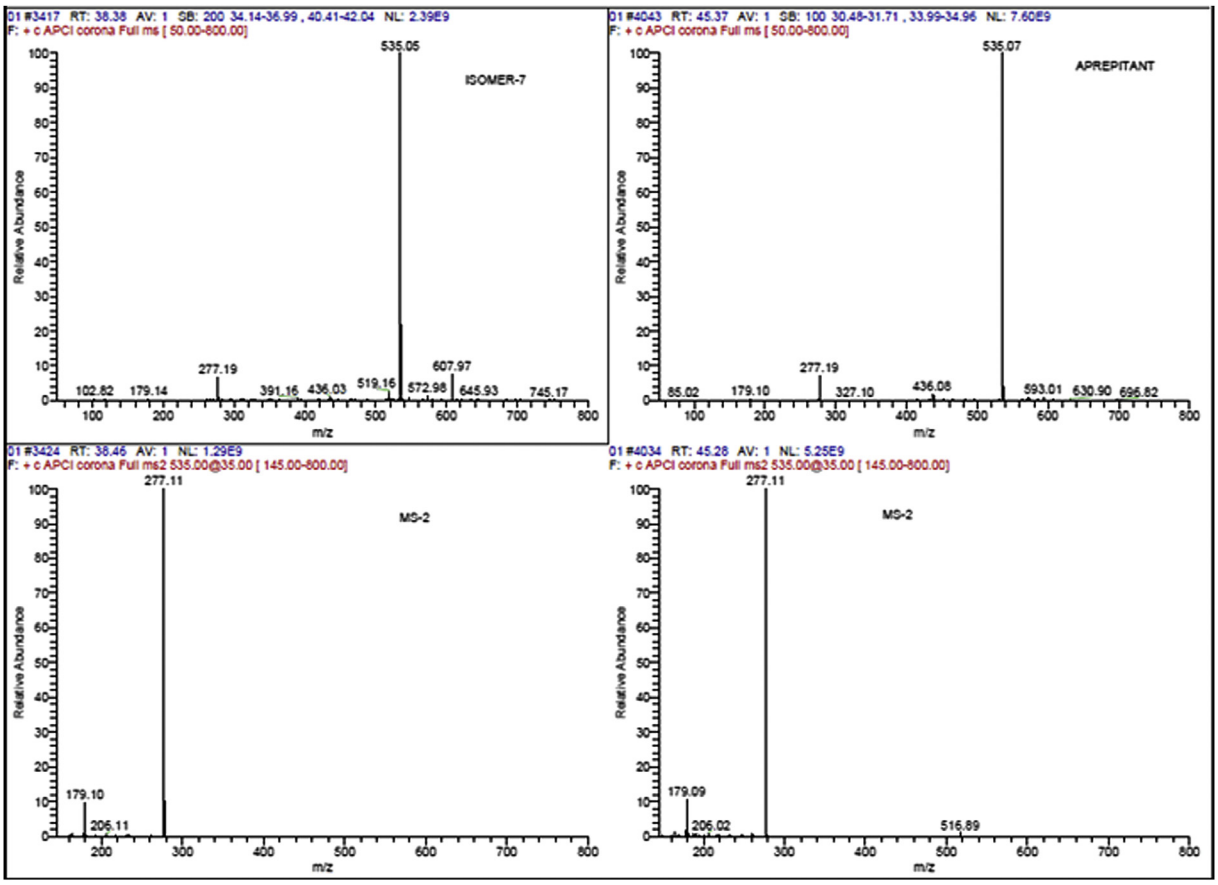

Fig. 4. (*continued*).

The %RSD for each related substance was evaluated. The studies indicated no effect on the determination of the related substances and their selectivities. Therefore, the test method was robust for quantification of the related substances.

2.14. Solution stability

The stability of a drug is a function of its storage conditions and the chemical properties of the drug and its impurities. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data are required to show that the concentration and purity of the analyte in the sample at the time of analysis corresponds to the concentration and purity of analyte at the time of sampling. The stability study of the drug should be conducted at the temperatures, for example, room temperature and refrigerator conditions that will be experienced over the period needed to process a batch of study samples. The study revealed that the percentage of

Table 3
The specificity studies of the related substances of Aprepitant.

Sr. No.	Test	Conditions	Appearance of an extra peak	Purity angle	Purity threshold	Remarks
1.	Acid Degradation 2 N HCl					
I.	Zero Hours	Sample – 2 N HCl – 0 HR	–	0.256	0.389	No degradation observed
II.	Twelve Hours	Sample – 2 N HCl – 12 HR	–	0.291	0.372	No degradation observed
III.	Twenty four Hours	Sample – 2 N HCl – 24 HR	13.852 min (0.21%)	0.249	0.385	No major degradation observed
2.	Base Degradation 2N NaOH					
I.	Zero Hours	Sample-2N NaOH- 0 HR	–	0.291	0.415	No degradation observed
II.	Twelve Hours	Sample – 2N NaOH-12 HR	–	0.272	0.381	No degradation observed
III.	Twenty Four Hours	Sample – 2N NaOH-24 HR	–	0.286	0.387	No major degradation observed
3.	Oxidative degradation 30% H_2O_2	Sample – 30% H_2O_2–0 HR	3.964 min (21.09%) 4.936 min (0.33%)	0.116	0.344	Approximately 21% degradation observed
		Sample – 30% H_2O_2 – 12 HR	3.951 min (28.43%) 4.922 min (0.45%)	0.095	0.318	Approximately 29% degradation observed
		Sample – 30% H_2O_2 – 24 HR	3.960 min (29.67%) 4.935 min (0.36%)	0.112	0.332	Approximately 30% degradation observed
4.	The effect of temperature	Sample – Heat	–	0.231	0.406	No degradation observed
5.	The effect of humidity	Sample – Humidity	–	0.217	0.394	No degradation observed
6.	The effect of light	Sample – Heat	–	0.250	0.394	No degradation observed

stereoisomer in Aprepitant was stable in the diluent solution for 72 h. The sample solutions were stored for 48 h, and the percentage of individual impurity, total impurity and peak purity was calculated by using the developed method. It was found that there was no difference in the percentage impurity and peak purity of Aprepitant.

3. Conclusions

Reverse- and normal- phase HPLC methods for the determination of stereochemical purity, assay and related substances of Aprepitant were developed. The methods were then validated according to a strategy based on the accuracy profiles. Good performance with respect to specificity, precision and accuracy were obtained. The limit of quantification (0.02%), the limit of detection (0.01%) and the analysis time make suitable for rapid quality control of the enantiometric purity of Aprepitant. The method was sensitive and suitable for determination of all eight isomers in comparison to existing methods. The RP-HPLC method described in this investigation proved to be an ideal tool for the low-level detection of all the impurities in Aprepitant. Method validation data demonstrated that the developed method was sensitive and accurate for the estimation of substances related to Aprepitant and robust to minor variations in the chromatographic parameters. The developed method presented involves conventional and versatile HPLC–UV detection and is rapid, economical and simple. The specificity and stability, which indicate the capability of the method, were established through forced degradation studies. Hence, the proposed HPLC methods with UV detection can be used conveniently in pharmaceutical analysis for stability monitoring and routine quality control samples.

Conflicts of interest

All authors have none to declare.

Acknowledgments

The authors wish to thank Mr. Glenn Saldanha, Chairman and MD, Glenmark Pharmaceuticals Limited, Dr. Shekhar Bhirud, Head API Operations, Glenmark Generics Limited and the management of the Glenmark Generics Limited for their invariable encouragement.

References

1. Dando TM, Perry CM. Aprepitant: a review of its use in the prevention of chemotherapy-induced nausea and vomiting. *Drugs*. 2004;64:777–794.
2. Rupniak NM, Tattersall FD, Williams AR, et al. In vitro and in vivo predictors of the anti-emetic activity of tachykinin NK1 receptor antagonists. *Eur J Pharmacol*. 1997;326:201–209.
3. Chawla SP, Grunberg SM, Gralla RJ, et al. Establishing the dose of the oral NK1 antagonist aprepitant for the prevention of chemotherapy-induced nausea and vomiting. *Cancer*. 2003;97:2290–2300.
4. Warr DG, Grunberg SM, Gralla RJ, et al. The oral NK_1 antagonist aprepitant for the prevention of acute and delayed chemotherapy-induced nausea and vomiting: pooled data from 2 randomised, double-blind, placebo controlled trials. *Eur J Cancer*. 2005;41:1278–1285.
5. Keller M, Montgomery S, Ball W, et al. Lack of efficacy of the substance p (neurokinin1 receptor) antagonist aprepitant in the treatment of major depressive disorder. *Biol Psychiatry*. 2006;59:216–223.
6. Subramanian G. *Chiral Separation Techniques—a Practical Approach*. 2nd ed. Weinheim: Wiley-VCH; 2001.
7. Toyóoka T. Resolution of chiral drugs by liquid chromatography based upon diastereomer formation with chiral derivatization reagents. *J Biochem Biophys Methods*. 2002;54:25–56.
8. Chen S, Pawlowska M, Armstrong DW. HPLC enantioseparation of di-and tripeptides on cyclodextrin bonded stationary phases after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). *J Liq Chromatogr*. 1994;17:483–497.
9. Czerwenka C, Lämmerhofer M, Lindner W. Structure-enantioselectivity relationships for the study of chiral recognition in peptide enantiomer separation on cinchona alkaloid-based chiral stationary phases by HPLC: Influence of the N-terminal protecting group. *J Sep Sci*. 2003;26:1499–1508.
10. Lee KA, Yeo S, Kim KH, Lee W, Kang JS. Enantioseparation of *N*-fluorenylmethoxycarbonyl α-amino acids on polysaccharide-derived chiral stationary phases by reverse mode liquid chromatography. *J Pharm Biomed Anal*. 2008;46:914–919.
11. Kraml CM, Zhou D, Byrne N, McConnell O. Enhanced chromatographic resolution of amine enantiomers as carbobenzyloxy derivatives in high-performance liquid chromatography and supercritical fluid chromatography. *J Chromatogr A*. 2005;1100:108–115.
12. Patti A, Pedotti S, Sanfilippo C. Chiral HPLC analysis of milnacipran and its FMOC-derivative on cellulose-based stationary phases. *Chirality*. 2008;20:63–68.
13. Hsieh C, Wang S, Kwan AL, Wu HL. Fluorescent high-performance liquid chromatographic analysis of vigabatrin enantiomers after derivatizing with naproxen acyl chloride. *J Chromatogr A*. 2008;1178:166–170.
14. Chavez-Eng CM, Constanzer ML, Matuszewski BK. Simultaneous determination of Aprepitant and two metabolites in human plasma by high-performance liquid chromatography with tandem mass spectrometric detection. *J Pharm Biomed Anal*. 2004;35:1213–1229.
15. Constanzer ML, Chavez-Eng CM, Dru J, Kline WF, Matuszewski BK. Determination of a novel substance P inhibitor in human plasma by high-performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometric detection using single and triple quadrupole detectors. *J Chromatogr B*. 2004;807:243–250.
16. Kline WF, Woolf EJ, Matuszewski BK. Assessment of the in-vivo stereochemical integrity of aprepitant based on the analysis of human plasma samples via high-performance liquid chromatography with mass spectrometric detection. *Biomed Chromatogr*. 2005;19:513–517.
17. Huskey SEW, Dean BJ, Bakthiar R, Sanchez RI. Brain penetration of aprepitant, a substance P receptor antagonist, in ferrets. *Drug Metab Dispose*. 2003;31:785–791.
18. Peter JS, Ahmad A, Yan W. An HPLC chromatographic reactor approach for investigating the hydrolytic stability of a pharmaceutical compound. *J Pharm Biomed Anal*. 2006;41:883–890.
19. Di W, Dustin JP, Xianguo Z, Steven D, Jeffrey SB. A sensitive and rapid liquid chromatography-tandem mass spectrometry method for the quantification of the novel neurokinin-1 receptor antagonist aprepitant in rhesus macaque plasma, and cerebral spinal fluid, and human plasma with application in translational NeuroAIDs research. *J Pharm Biomed Anal*. 2009;49:739–745.
20. Roy H, George X, Yadon W, et al. Characterization and quantitation of aprepitant drug substance polymorphs by attenuated total reflectance Fourier transform infrared spectroscopy. *Anal Chem*. 2003;75:605–611.
21. Lee LD, Karen WL, Scott EW. *Support Car Cane*. 2009;17:701–706.
22. Sreekanth N, Bahlul AZ, Babu CR, Mukkanti K. A validated stability indicating RP-HPLC method for the determination of aprepitant in bulk and pharmaceutical dosage forms. *Recent Resear Sci Tech*. 2011;3:16–24.
23. Kiran VK, Appala NR, Begum SH, Seshagiri JVLNR, Satyanarayana T. The estimation of aprepitant in capsules dosage forms by RP-HPLC. *Resear J Pharm Tech*. 2009;2:412–414.
24. Aboul-Enein HY. High-performance liquid chromatographic enantioseparation of drugs containing multiple chiral centers on polysaccharide-type chiral stationary phases. *J Chromatogr A*. 2001;906:185–193.
25. Lammerhofer M, Lindner W. Quinine and quinidine derivatives as chiral selectors I. Brush type chiral stationary phases for high-performance liquid chromatography based on cinchonan carbamates and their application as chiral anion exchangers. *J Chromatogr A*. 1996;741:33–48.
26. ICH. *Validation of Analytical Procedures*. Q2 (R1), Step 5. 2005.
27. USP32 NF27*US Pharmacopeial Convention*. 2009:733.
28. Radhakrishnanand P, Rao DVS, Surendranath KV, Subrahmanyam D. A validated chiral LC method for the separation and quantification of (S, R, S)-Enantiomer and (R, R, R)-Isomer of aprepitant. *Chromatogr*. 2008;68:669–673.