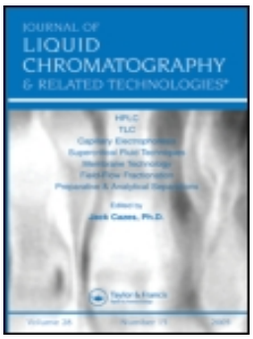

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STABILITY-INDICATING UPLC METHOD FOR DETERMINATION OF RAMELTEON AND THEIR DEGRADATION PRODUCTS IN ACTIVE PHARMACEUTICAL INGREDIENTS

I. Ugandar Reddy,[1,2] P. Nageswara Rao,[2] V. Ranga Reddy,[1] and K. V. V. Satyanarayana[2]

[1]*Dr. Reddy's Laboratories Ltd. Active Pharmaceutical Ingredients, IPDO, Hyderabad, India*
[2]*Department of Chemistry, National Institute of Technology, Warangal, India*

□ *A novel stability-indicating mass compatible gradient reverse phase ultra-performance liquid chromatographic (RP-UPLC) method was developed for the quantitative determination of purity of Ramelteon drug substance samples in the presence of its impurities and degradation products. The method was developed using Waters Acquity UPLC BEH SHIELD RP_{18} (100 mm × 2.1 mm, 1.7 μm) column with mobile phase containing a gradient mixture of solvents A and B. The eluted compounds were monitored at 230 nm, the run time was 10 min within which Ramelteon and its four impurities were well separated. Ramelteon was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal, and photolytic degradation. Ramelteon was found to degrade significantly in acidic and slightly in oxidative stress conditions and stable in base, hydrolytic, and photolytic degradation conditions. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision, and robustness.*

Keywords forced degradation, LC-MS, Ramelteon, stability-indicating, UPLC, validation

INTRODUCTION

Though high-performance liquid chromatography (HPLC) is a well-established reliable technique used in controlling the quality and consistency of active pharmaceutical ingredients (API's) and dosage forms, it is often a slow technique because of the complexity of some of the samples, it could still be improved.

Address correspondence to I. Ugandar Reddy, Dr. Reddy's Laboratories Ltd. Active Pharmaceutical Ingredients, IPDO, Bachupally, Hyderabad-500090 A.P., India. E-mail: ugandharri@drreddys.com or ugandar_reddy@yahoo.co.in

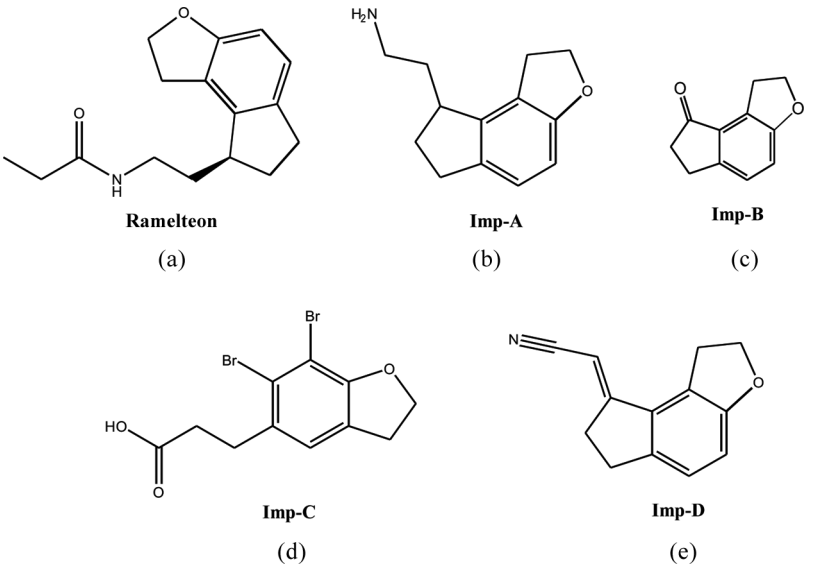

FIGURE 1 a: (S)-N-[2-(1,6,7,8-tetrahydro-2H-indeno-[5,4-b]furan-8-yl)ethyl] propionamide; b: 2-(1,6,7,8-Tetrahydro-2*H*-indeno[5,4-b]furan-8-yl)ethylamine; c: 1,2,6,7-tetrahydro-8H-indeno[5,4-b]furan-8-one; d: 3-(6,7-dibromo-2,3-dihydrobenzofuran-5-yl)propanoic acid; e: 2-(1,2,6,7-tetrahydro-8H-indeno[5,4-b]furan-8-ylidene)acetonitrile.

Ultra-performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 μm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity, and speed of analysis. Owing to its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis. In the present work, this technology has been applied to the method development and validation study of related substance determination of Ramelteon bulk drug.

The Ramelteon is an orally active hypnotic chemically designated as (S)-N-(2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-b]furan-8-yl)ethyl)propionamide and contains one chiral center (Figure 1a). Ramelteon is highly selective melatonin MT(1)/MT(2) receptor agonist used for sleep disorders.[1] Very few methods appeared in the literature for the determination of enantiomeric purity of Ramelteon on high-performance liquid chromatography (HPLC)[2] but not on the related substances.

To the best of our knowledge, none of the currently available analytical methods can separate and quantify all the known related compounds and degradation impurities of Ramelteon API. Furthermore, there is no stability-indicating HPLC/UPLC method reported in the literature that

can adequately separate and accurately quantify Ramelteon API. It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination of Ramelteon. We intend to opt for a faster chromatographic technique UPLC for the current study. An attempt was made to determine whether UPLC can reduce analysis times without compromising the resolution and sensitivity.

Hence, a reproducible stability-indicating RP UPLC method was developed for the quantitative determination of Ramelteon and its four impurities namely Imp-A, B, C, and D (Figure 1b–e).This method was successfully validated according to the International Conference on Harmonization (ICH) guidelines (Validation of Analytical Procedures: Test and Methodology Q2).[3]

EXPERIMENTAL

Materials and Reagents

Active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories Limited, IPDO, Hyderabad, India. The HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany and analytical grade Trifluoroacetic acid 99%, extra pure was purchased from ACROS ORGANICS, Geel, Belgium. Water was prepared by using Millipore Milli-Q Integral 5 water purification system.

Chromatographic Conditions and Equipment

LC was carried out on a Waters Acquity UPLC with photodiode array detector. The output signal was monitored and processed using Empower software. The chromatographic column used was Waters Acquity UPLC BEH SHIELD RP_{18} 100 mm, 2.1 mm, and 1.7 µm particle size. The separation was achieved with a gradient method. The solvent A contains 0.1% Trifluoroacetic acid; and the solvent B contains a mixture of water and acetonitrile in the ratio 20:80 (v/v), respectively.

The flow rate of mobile phase was 0.3 mL/min. The UPLC gradient program (T/%B) was set as 0.01/40, 8.0/90, 9.0/90, 9.01/40, and 10.0/40. The column temperature was maintained at 35°C and the detection was monitored at wavelength 230 nm. The injection volume was 1.0 µL.

LC-MS/MS Conditions

LC–MS/MS system (Agilent 1200 series liquid chromatograph coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrometer

with Analyst 1.4 software, MDS SCIEX,USA) was used for the unknown compounds formed during forced degradation studies. Develosil ODS MG-5, 250 × 4.6 mm, 5 µm column (Nomura Chemical Co, Japan) was used as stationary phase. The 0.1% Trifluoroacetic acid (ACROS ORGANICS, Geel, Belgium) was used as buffer. The 100% buffer was used as solvent A and buffer and acetonitrile in the ratio 15:85, v/v was used as solvent B. The gradient program (T/%B) was set as 0.01/35, 20/70, 40/80, 45/95, 64/95, 65/35, and 70/35. Mixture of acetonitrile and Solvent A in the ratio 1:9, v/v was used as diluent. The flow rate was 1.0 mL/min. The analysis was performed in positive electro spray positive ionization mode. Ion Source voltage was 5000 V. Source temperature was 450°C. GS1 and GS2 are optimized to 30 and 35 psi, respectively. Curtain gas flow was 20 psi.

Preparation of Standard Solutions

A stock solution of Ramelteon (200 µg/mL) was prepared by dissolving an appropriate amount of drug in Acetonitrile:Solvent A 10:90 (v/v), respectively. Working solutions containing 0.2 µg/mL were prepared from this stock solution for determination of related substances. A mixed stock solution (20 µg/mL) of the impurities (denoted Imp-A to Imp-D) was also prepared in diluent.

Stress Studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities.[4] The specificity of the developed LC method for Ramelteon was carried out in the presence of its four impurities. Stress studies were performed at an initial concentration 200 µg/mL of Ramelteon drug substance to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress condition of UV light (254 nm), heat (60°C), acid (1 N HCl at 60°C), base (0.5 N NaOH at 60°C), hydrolytic (60°C), and oxidation (3.0% H_2O_2 at 60°C) to evaluate the ability of the proposed method to separate Ramelteon from its degradation products. For heat and light studies, the study period was 10 d; whereas for hydrolytic and base studies, the period was 24 hr; for acid studies, the period was 4 hr; and for oxidation studies, the period was 24 hr.

The purity of peaks obtained from stressed samples was checked by use of the PDA detector. The purity angle was within the purity threshold limit obtained in all stressed samples and demonstrates the analyte peak homogeneity.

METHOD VALIDATION

The described method has been extensively validated for related substances by UPLC determination.[3]

Precision

The repeatability of the related-substance method was checked by a six-fold analysis of 200 µg/mL Ramelteon spiked with 0.15% of each of the four impurities (Figure 2a). The RSD (%) of peak area was calculated for each impurity.

Inter- and intra-day variation and analyst variation was studied to determine intermediate precision of the proposed method. Intra-day precision was determined by six-fold analysis of 200 µg/mL Ramelteon spiked with 0.15% of each of the four impurities. The same protocol was followed for two different days to study inter-day variation ($n = 6$). Different analysts prepared different solutions on different days. The RSD (%) of peak area was calculated for each impurity.

Limit of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ for Ramelteon and its impurities were determined at a signal-to noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six ($n = 6$) individual preparations and calculating the RSD (%) of the area for each impurity.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found. For impurities, recovery was determined in triplicate for 0.075, 0.15, and 0.225% of the analyte concentration (200 µg/mL) on drug substance and recovery of the impurities was calculated.

Linearity of Response

Detector response linearity for all four impurities and Ramelteon was assessed by injecting six separately prepared solutions covering the range 25% to 150% (0.0375, 0.075, 0.1125, 0.15, 0.1875, 0.225) of the normal sample concentration (200 µg/mL). The correlation coefficients, slopes, and Y-intercepts of the calibration curve were determined.

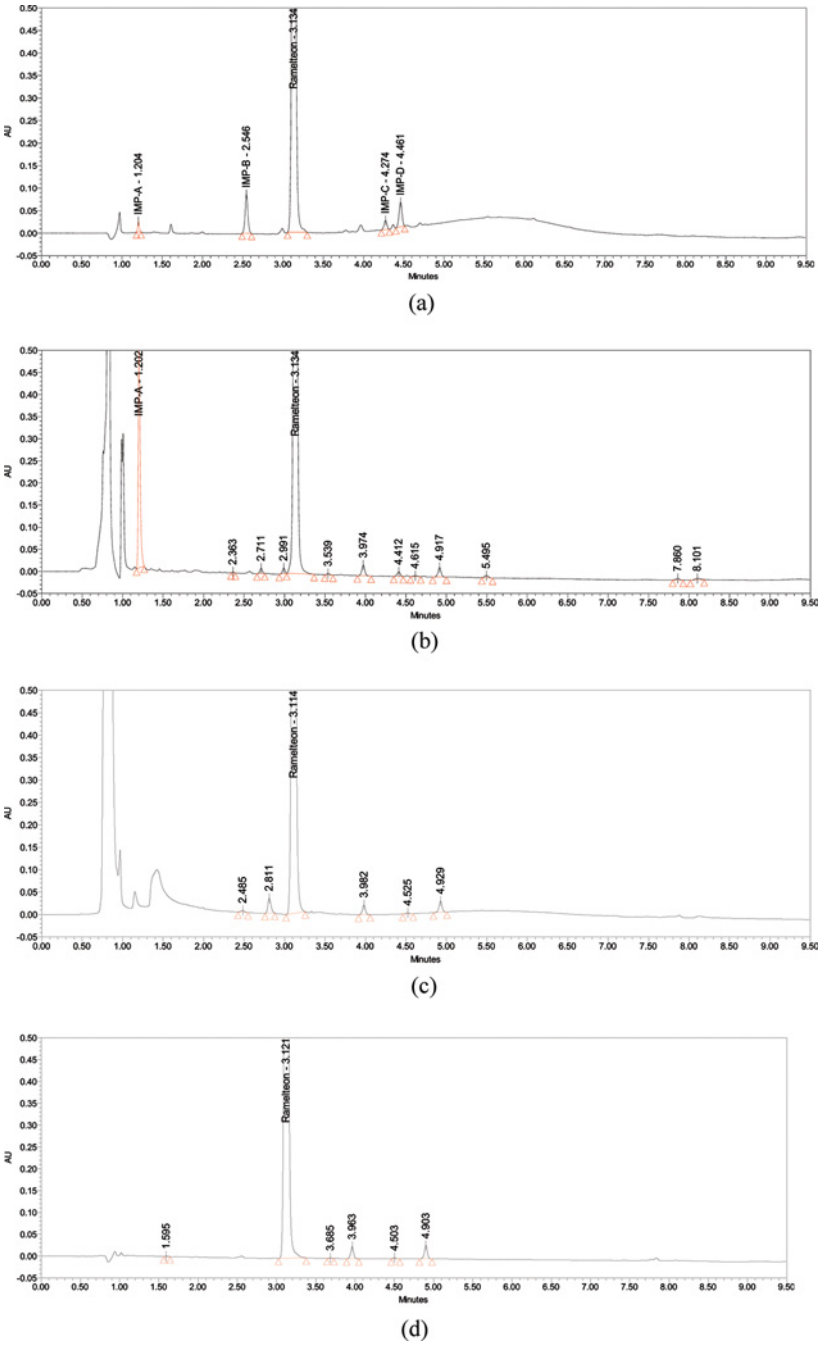

FIGURE 2 a: Impurities spiked chromatogram; b: Acid degradation chromatogram; c: Base degradation chromatogram; d: Water degradation chromatogram; e: Peroxide degradation chromatogram; f: Thermal degradation chromatogram; g: Photo degradation chromatogram. (Color figure available online.)

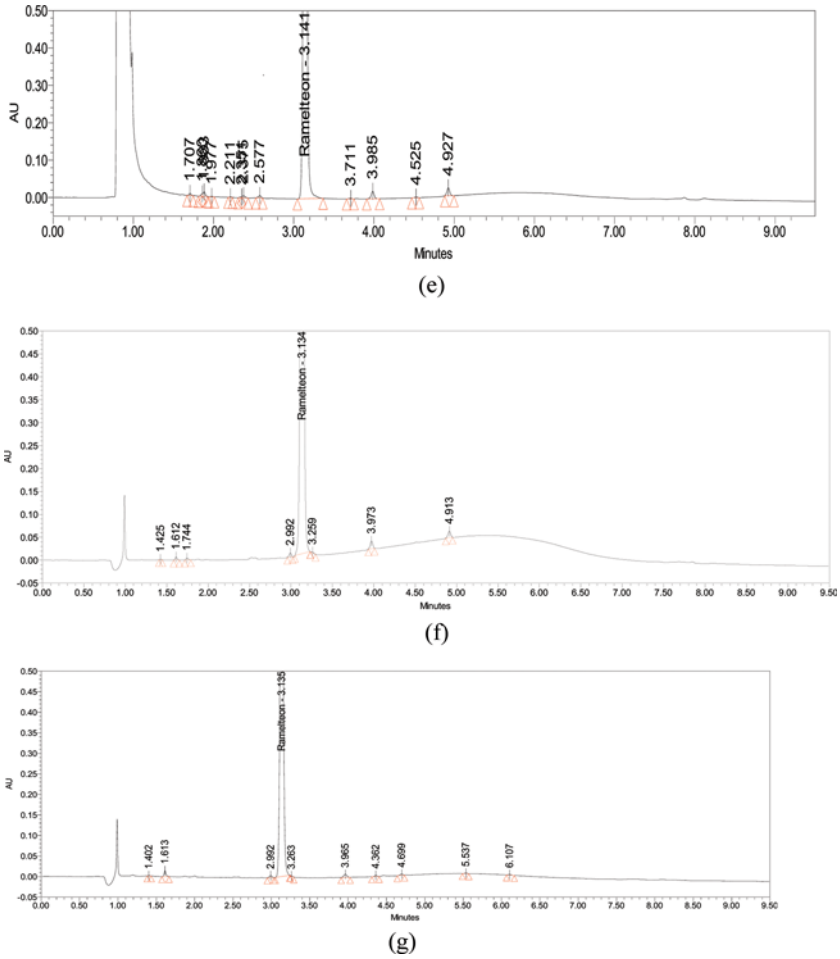

FIGURE 2 Continued.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

To determine the robustness of the method the experimental conditions were deliberately changed. The resolution of Ramelteon and the four impurities were evaluated. The mobile phase flow rate was 0.30 mL/min; to study the effect of flow rate on resolution it was changed to 0.27 and 0.33 mL/min. The effect of column temperature was studied at 30°C and 40°C.

Solution Stability and Mobile Phase Stability

Ramelteon solutions (spiked) prepared in diluent were injected at 0 hr, 24 hr, and 48 hr of time intervals, calculated the impurity content (Imp-A to Imp-D) and checked the consistency in the % area of the principal peak at each interval. Mobile phase prepared was kept constant during the study period.

The mobile phase stability was demonstrated by injecting the freshly prepared solution of Ramelteon and its impurities at different time intervals (0 hr, 24 hr, and 48 hr).

RESULTS AND DISCUSSION

Method Development and Optimization

From the literature it was found that the pKa of the molecule is −0.84. Due to the lower pKa of this molecule it was decided to adopt 0.1% Trifluoroacetic acid as solvent A. The blend containing 200 µg/mL of Ramelteon and 2 µg/mL of each impurity (four) was prepared in the mixture of Acetonitrile and solvent A (1:9, v/v). Ramelteon spiked solutions were subjected to separation by reverse-phase LC on a Waters Acquity BEH C18, 50 × 2.1 mm, 1.7 µm column with 0.1% of Trifluoroacetic acid as solvent A and Acetonitrile:water (80:20, v/v) as solvent B. Flow rate was set at 0.3 mL/min. The UPLC gradient program (T/%B) was set as 0.01/40, 8.0/90, 9.0/90, 9.01/40, and 10.0/40. Column temperature was maintained at 35°C (Trial-1). In this trial one of the unknown impurity closely eluting with Imp-A and other unknown impurity with Imp-C (Resolution <1.5). Efforts were made to separate these closely eluting pair of compounds. In order to increase the resolution between these pairs of compounds, buffer concentration was increased from 60 to 90 in the initial gradient step. With this increased buffer composition the retention time of Ramelteon was increased but Imp-A and its adjacent peak was co-eluting. Efforts were made to separate the pairs of compounds on Waters Acquity BEH C18, 100 × 2.1 mm, 1.7 µm column. The chromatographic conditions of Trial-1 were employed in this trial. With the increase in column length Imp-A and its adjacent peak were separated (Resolution >2) but the resolution between Imp-C and its adjacent peak was not improved. Various trials were made by changing the gradient compositions but none of the trial could serve the purpose. It was decided to change the column chemistry and Acquity UPLC BEH SHIELD RP_{18} 100 mm, 2.1 mm, and 1.7 µm column was used with the conditions mentioned in trial 1. It was found that all the peaks were separated with a resolution greater than 2.

System suitability parameters were evaluated for Ramelteon and its four impurities. Tailing factor for all four impurities and Ramelteon was found

less than 1.2. USP Resolution of Ramelteon and four potential impurities was greater than 2.0 for all pairs of compounds.

Validation of the Method

Precision

The RSD (%) of peak area for the four impurities namely Imp-A, Imp-B, Imp-C, and Imp-D in the study of the repeatability is shown in Table 1. RSD (%) results of Ramelteon and its impurities for intermediate precision (intra- and inter-day repeatability) are within 4.0%. These results confirmed that the method was highly precise.

Limits of Detection and Quantification

The determined limit of detection, limit of quantification, values for Ramelteon and its four impurities are reported in Table 1.

Accuracy

The percentage recovery of four impurities of Ramelteon in bulk drug samples ranged from 87.0% to 104.9%.

Linearity

For all four impurities and Ramelteon, linear calibration curve was obtained ranging from 0.0375% to 0.225% (25%, 50%, 75%, 100%, 125%, and 150%). The correlation coefficient obtained was greater than 0.999 (Table 1). The results indicate excellent linearity.

Robustness

In all the deliberate varied chromatographic conditions (flow rate and column temperature), all analyte peaks were adequately resolved and elution orders remained unchanged.

Stability in Solution and in the Mobile Phase

No significant changes in the amounts of the four impurities were observed during solution stability and mobile phase experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable for up to 48 hr during determination of related substances.

Results from Forced Degradation Studies

All forced degradation samples were analyzed at an initial concentration 200 µg/mL of Ramelteon with UPLC conditions mentioned in conditions using PDA detector to ensure the homogeneity and purity of Ramelteon peak. Degradation was not observed when Ramelteon was subjected to

TABLE 1 Results of Validation

Regression and Precision Data

Parameter	Ramelteon	Imp-A	Imp-B	Imp-C	Imp-D
LOD (μg/ml)	0.018	0.022	0.014	0.025	0.021
LOQ (μg/ml)	0.05	0.07	0.04	0.08	0.06
Regression equation (y)					
Slope (b)	36722962.90	40380190.48	219550144.90	65856677.25	125571724.90
Intercept (a)	182.87	−75.80	−441.87	1168.20	1368.90
Correlation coefficient	0.9993	0.9990	0.9997	0.9991	0.9994
Y-intercept at 100% level	1.82%	−0.64%	−0.60%	5.06%	3.12%
Precision (% RSD)[a]	–	3.90	0.87	1.41	0.81
Intermediate precision (% RSD)#	–	1.57	0.77	1.10	1.06

Linearity range is 25%–150% with respect to 0.2 mg/mL of Ramelteon for impurities.

[a] n = six determinations using 0.15% solution for impurities.

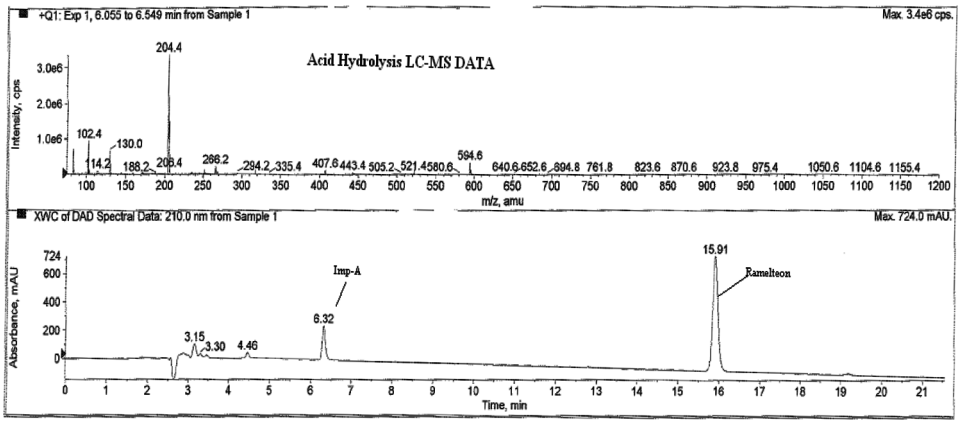

FIGURE 3 LC-MS spectra of acid hydrolysis of Ramelteon.

TABLE 2 Summary of Forced Degradation Results

Degradation Condition	Time	RS by UPLC % Degradation	Remarks/Observation
HCl-1N 60°C (Acid hydrolysis)	4 hr	12.6%	Impurity-A degradation product were formed
NaOH-0.5 N 60°C (Base hydrolysis)	24 hr	0.3%	No significant degradation observed
Water hydrolysis (60°C)	24 hr	0.2%	No significant degradation observed
Oxidation by H2O2-3.0% 60°C	24 hr	1.5%	Degradation observed
Thermal (60°C) solid	10 d	0.2%	No degradation observed
UV at 254 nm	10 d	0.2%	No degradation observed

hydrolytic (Figure 2d), base (Figure 2c), light (Figure 2g), and heat (Figure 2f) conditions. Slight degradation was observed when the drug was subjected to oxidative hydrolysis (Figure 2e) (3.0% H_2O_2 at 60°C for 24 hr) and significant degradation was observed in acid (1 N HCl at 60°C for 4 hr). Acid degradation was leading to the formation of Imp-A (Figure 2b). This was confirmed by co-injecting Imp-A standard to these degraded samples and by LC-MS/MS analysis. The m/z of the impurity was 204 which corresponded to Imp-A (Figure 3). Peak-Purity test results obtained by use of the PDA confirmed the Ramelteon peak obtained from all the degradation conditions was found to be homogenous and pure. Results from force degradation studies are presented in Table 2.

CONCLUSION

The rapid gradient RP-UPLC method developed for quantitative analysis of Ramelteon and related substances in bulk drugs is precise, accurate, linear, robust, and specific. Satisfactory results were obtained from

validation of the method. The retention time (3.0 min) enables rapid determination of the drug. This method exhibited an excellent performance in terms of sensitivity and speed. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples of Ramelteon.

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