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QUANTIFICATION OF TRACE LEVEL IMPURITIES IN ROSUVASTATIN CALCIUM AND IDENTIFICATION OF DEGRADATION IMPURITY BY MASS SPECTROSCOPY

V. Venkateswarlu*, K. Hussain Reddy and G. Ramireddy

Department of Chemistry, Sri Krishnadevaraya University, Anantapur 515055, A. P, India.

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***Corresponding Author**
V. Venkateswarlu
Department of Chemistry,
Sri Krishnadevaraya
University, Anantapur
515055, A. P, India.

ABSTRACT

A novel stability indicating reversed phase ultra performance liquid chromatographic method was developed for the quantification of Rosuvastatin calcium and its impurities in bulk samples. The chromatographic separation was achieved on Acquity BEH C-18 100mm x 2.1mm, 1.7um column using 0.1% TFA: Methanol with the flow rate of 0.3mL min-1. The stress degradation study including acid, base, H2O2, humidity, thermal and photolytic conditions were performed on tablets powder of Rosuvastatin calcium as per International Conference on Harmonization (ICH) guidelines to show the stability-indicating capability of the method. Major degradation was found in acid stress condition and the observed degradation impurity was identified using water Q-tof mass spectrometer. % Assay was calculated for the stressed samples against the qualified working standard. The developed method was validated with respect to specificity, LOD, LOQ, linearity, accuracy, precison and robustness as per ICH guidelines.

KEYWORDS: Rosuvastatin calcium, RP-UPLC stability indicating method, LC-MS.

INTRODUCTION

Rosuvastatin (RSV) is a synthetic lipid-lowering agent, chemically known as (3*R*,5*S*, 6*E*)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl) amino]-5-pyrimidinyl]-3,5-dihydroxy-6-heptenoicacid, calcium salt (2:1). It is used for the treatment of hyperlipidemia and is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis.[1] RSV calcium is an organic acid with pKa of 4.6 and very slightly soluble in aqueous solutions of pH 4.0 and below. Compared with other several

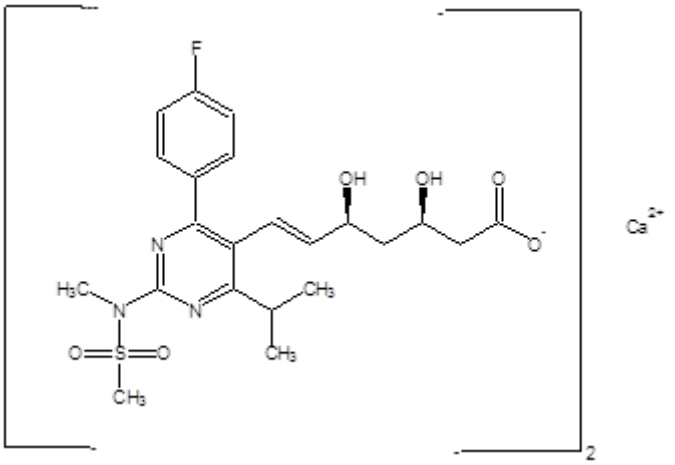
HMG-CoA inhibitors, RSV Ca does not appear to be metabolized significantly by cytochrome P4503A4[2] and therefore, may not possess the same potential for drug interaction as seen for some other statins, e.g. lovastatin and simvastatin.[3,4] It exhibits a high degree of specificity for uptake into the liver and is a potent in vitro and in vivo competitive inhibitor of HMG-CoA reductase.

Ultra performance liquid chromatography (UPLC) has been considered as a novel development in liquid chromatography. It is specially designed to withstand higher system pressures during chromatographic analysis so that it enables significant decrease in separation time and solvent consumption. The UPLC columns packed with 1.7 µm sized particles provides not only increased efficiency but also the ability to work at increased linear velocity without loss of efficiency, providing both resolution and speed. Using advantages of UPLC, various number of applications in different fields including pharmacy,[5] clinical analysis, pesticide analysis[6] and tetracyclines in human urine[7] have been reported.

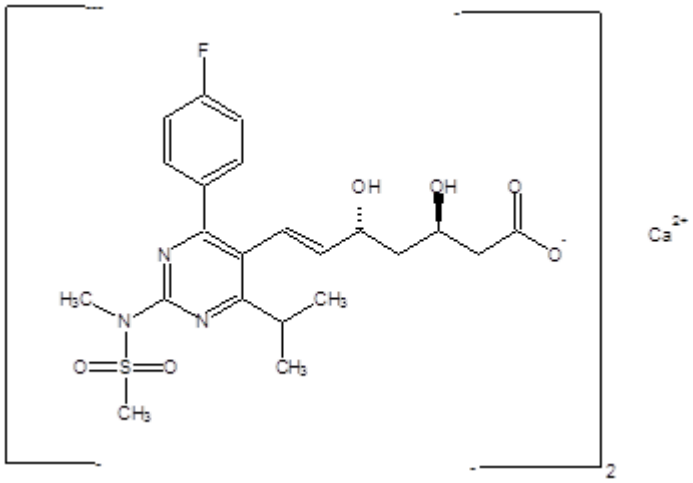
Different analytical methods were reported for determination of RSV Ca by capillary zone electrophoresis,[8] UV spectrophotometric determination[9,10] automated solid phase extraction using mass spectrometry[11] and ion pair liquid–liquid extraction using liquid chromatography (LC) with electrospray ionization tandem mass spectrometry.[12] Mehta et al.[13] reported a stability indicating assay method for the determination of RSV Ca in the presence of its various degradation products using LC. In their assay method, the run time has around 30-35 minutes for elution of all the degradation impurities and this method applicable for only RSV estimation but not for related substances. In the literature, there were no UPLC methods available for estimation of related substances of RSV and its assay during stability indicating analysis in pharmaceutical dosage forms.

The aim of the present work was the development of a stability indicating UPLC method for the determination of RSV and its related impurities in the pharmaceutical dosage forms. The developed method was validated as per ICH guidelines[14] to show the stability indicating capability of the method.

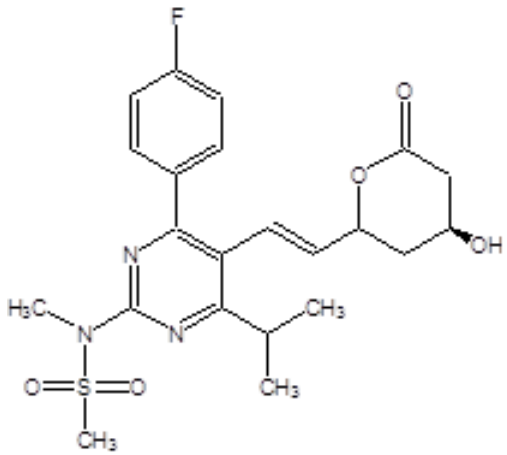
Structure of Rosuvastatin calcium



Structure of anti-isomer



Structure of Lactone



EXPERIMENTAL

Reagents and Chemicals

Reference standards of RSV calcium, anti-isomer and lactone impurities were gifted by Jubilant Organosys limited (Noida, UP, INDIA) with declared purities of 99.1 and 92.7 and 85.9%, respectively. Tablet formulations containing 5mg and 40 mg of RSV were obtained from Jubilant Organosys limited, Noida. Methanol (MeOH) of HPLC grade was received from J. T. Baker (NJ, USA). Potassium dihydrogen phosphate (KH_2PO_4) and trifluoroacetic acid was obtained from Qualigens fine chemicals (Mumbai, India). The 0.2 µm nylon filters used to filter the sample preparations were manufactured by Millipore Pvt. Ltd (Bangalore, India).

Chemical names for RSV and its related impurities

RSV: (3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]-5-pyrimidinyl]-3,5-dihydroxy-6-heptenoicacid.

Anti-isomer impurity: RSV was chemically named as (3*R*,5R)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]-3,5-dihydroxy-6(E)-heptenoic acid.

Lactone impurity: (E)-7-[4-(4-flurophenyl)-6-isopropyl-2-[methyl (methyl sulfonyl) amino] pyrimidine-5-yl]-(3R,5S),3-5-dihydroxy hept-6-enoic acid-(3,6) lactone.

N-{4-(4-Fluoro-phenyl)-5-[2-(4-hydroxy-6-oxo-tetrahydro-pyran-2-yl)-vinyl]-6-isopropyl-pyrimidin-2-yl}-N-methyl-methane sulfonamide.

EQUIPMENTS

Analysis was performed on Acquity UPLC^{TM} system (Waters, Milford, USA) consists of a binary solvent manager, a sample manager and a photodiode array (PDA) detector. Cintex digital water bath was used for hydrolysis studies. Photostability studies were carried out in a photostability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India). Intermediate precision was performed on different Acquity UPLC^{TM} system (Waters, Milford, USA) consists of a binary solvent manager, a sample manager and a tunable ultraviolet (TUV) detector.

Chromatographic conditions

The mobile phase was 0.1% trifluoroacetic acid in water and methanol in the ratio of 50:50 using isocratic elution. The detector was set at sampling rate of 20 points s^{-1} and filter time constant of 0.2 s. System control, data collection and data processing were accomplished using Waters Empower chromatography data software. The analytical column used was 100 x 2.1 mm, 1.7 µm acquity UPLC BEH C-18 column (Waters, Mainfold, USA). The optimized conditions were as follows: injection volume: 2.0 µL, flow rate: 0.3 mL min^{-1} at a column temperature of 40 °C and a sample cooler temperature of 10 °C, detection wavelength 240 nm. Under these conditions the system back pressure was about 7200 psi. Diluent was 50:50 of water and methanol.

UPLC-MS conditions

To identify the degradant formed in the acid degradation sample, LC-MS analysis was performed using Waters Acquity UPLC^{TM} system (Waters, Milford, USA) consists of a binary solvent manager, a sample manager and a photodiode array (PDA) detector connected to Q-ToF mass spectrometer. Mobile phase composed of 0.1% TFA and MeOH in the ratio of 50:50 with the flow rate of 0.3 mL/min. The analytical column used was 100 mm x 2.1 mm, 1.7 µm acquity BEH C-18 column (Waters, Milford, USA). The analysis was performed using electrospray ionization in positive mode conditions and operating parameters as follows: sample cone set to 30 volts, capillary at 3500 volts and extraction cone at 1.0 volts. Source temperature set at 100^{o}C and desolvation temperature at 250^{o}C.

Preparation of standard stock solutions

The individual standard stock solutions of RSV, anti-isomer and lactone impurities were prepared by dissolving standard substance of respective compounds in diluent to obtain a concentration of 500, 50 and 50 µg mL^{-1}, respectively. 5 ml of standard stock solution of RSV was diluted to 50 ml with diluent and used as assay standard. 5 mL of this solution assay standard solution was further diluted to 50 mL with the diluent and used as standard to quantify the impurities.

Preparation of system suitability solution

The system suitability solution was prepared at the concentrations of 500, 5 and 5 µg mL^{-1} respectively for RSV, anti-isomer and lactone impurities and was used to measure the resolution between anti-isomer and RSV.

Preparation of sample solution

Taken 50 mg of RSV into a 100 mL volumetric flask, added 70 mL of the diluent and sonicated for 30 min. Kept the solution aside to attain room temperature and made up to the volume with the diluent. This solution used as sample solution for related substances (RS). 5 ml of this RS solution was diluted to 50 ml with diluent and filtered and used as assay sample solution.

System suitability

System suitability parameters were measured so as to verify the system performance. Injected system suitability solution and diluted standard solution of RSV at six replicate injections and the important characteristics including peak resolution, tailing factor and theoretical plates were measured.

Specificity

Forced degradation studies were performed to demonstrate selectivity and stability indicating capability of the proposed method. The sample was exposed to acidic (1N HCl, 3 hours), alkaline (1N NaOH, 70^{o}C, 2hours), strong oxidizing (5% H_2O_2, 70◦C, 2 hours), thermal (105^{o}C, 7 days), humidity condition (90% RH, 25^{o}C, 7 days) and photolytic (1.2 million lux hours, 200 wh/Sqm^2, 7days) degradation conditions. Samples were withdrawn at appropriate times and subjected to UPLC analysis after suitable dilution (500 ppm) to evaluate the ability of the proposed method to separate RSV from its degradation products. Photodiode array detector was employed to check and to ensure the homogeneity and purity of RSV peak in all the stressed sample solutions.

Linearity

Linearity test solutions of RS were prepared from the impurities' stock solution at five different concentration levels from LOQ to 150% of the specification (LOQ, 50%, 75%, 100%, 120% and 150%) to impurities' level. The calibration curves were constructed by plotting the peak areas of impurity versus its corresponding concentrations. The slope, Y-intercept and correlation coefficient of the calibration curve were calculated for all impurities.

For assay linearity, the solutions were prepared from RSV stock solution at five different concentration levels ranging from 50-150% of assay sample concentration.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of the impurities were determined by using the signal to noise approach as defined in International Conference on Harmonization (ICH) guidelines.[14] Increasingly dilute solution of each drug and impurity was injected into the chromatograph and signal to noise was calculated at each concentration.

Precision

System precision

System precision was performed by injecting six replicate injections of the system suitability solution and calculated the % RSD for each impurity.

Method precision

Method precision was carried out by injecting the six different preparations of the system suitability solution and calculated the % RSD for each impurity.

Method precision for assay was executed by injecting six independent assay preparations of RSV sample against qualified working standard and calculated the % RSD.

Accuracy

To confirm the accuracy of the proposed method, recovery experiments were carried out. Injected the sample spiked with the impurities at LOQ level in triplicate. Sample of RSV spiked with known concentration of impurities at 50%, 100% and 150% of specification level in triplicate. The samples were analyzed by the proposed method and calculated the amount of recovery of impurities. The mean percentage recovery of individual impurity was calculated by external standard method.

Prepared triplicate samples of RSV at three different concentration levels i.e. 50%, 100%, and 150% to the test concentration and injected. The percentage recoveries were calculated.

Robustness

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was studied by testing influence of small changes in mobile phase composition (2% absolute change), change in column temperature (± 5 ^{o}C) and the flow rate (±0.1 mL min^{-1}).

Stability of sample preparation

Stability of sample solution was established by storage of sample solution at ambient temperature (25 °C) for 24 hours. Sample solution was re-analyzed up to 24 hours with the time interval of 3hours and calculated the results and compared against fresh sample.

RESULTS AND DISCUSSION

Method development and optimization

During the literature survey of the RSV, there were no methods available for estimation of related substances of Rosuvastatin in its drug product. The main criterion of the present work is the development of a successful UPLC method for the determination of RSV and impurities in tables during stability analysis. During the course of method development, initial analysis was carried out using a solution containing 500 µg mL^{-1} of RSV, 0.5 µg mL^{-1} of anti-isomer and 0.5 µg mL^{-1} of lactone impurity and trials were performed by reversed phase UPLC on a water Acquity BEH column, 50 mm x 2.1 with 1.7 µm particle size with a mobile phase, 0.01 M potassium dihydrogen phosphate of pH 3.0 and methanol in the ratio of 40:60. It was observed that the lactone impurity was well separated from the main peak but the anti-isomer was co-eluted with RSV. To separate the anti-isomer impurity decreased the organic ratio from 60 to 55% and observed that anti-isomer impurity was poorly separated. For better separation, increased the column length from 50 mm to 100 mm. Anti-isomer was separated from the RSV using 100 mm column but peak tailing was observed for RSV. For getting the proper peak shape, replaced the potassium buffer with 0.1% TFA and carryout the analysis. Anti-isomer is clearly separated from the main peak with a resolution of 3.2 and lactone having the resolution of 7.5 within a total run time of 7.5 minutes with isocratic elution. But in the specificity study of RSV, especially in acid degradation, some non-polar degradants are formed. To elute these impurities, different isocratic and gradient trials were performed. But in one of the trial having the mobile phase composition of buffer (0.1% TFA): methanol (50:50), all the known impurities and unknown degradants were separated with proper peak shape with a resolution of not less than 1.5. The detection wavelength of 240 nm was selected because at this wavelength all the degradation impurities show similar response. A chromatogram shows the separation of RSV and its known impurities was presented in Fig. 1 and the chromatographic data was presented in Table 1. The RSV and its placebo were treated with different degradation conditions including acid, base, peroxide, photolytic, humidity and thermal degradations and the respective typical chromatograms were shown in Figs. 2.

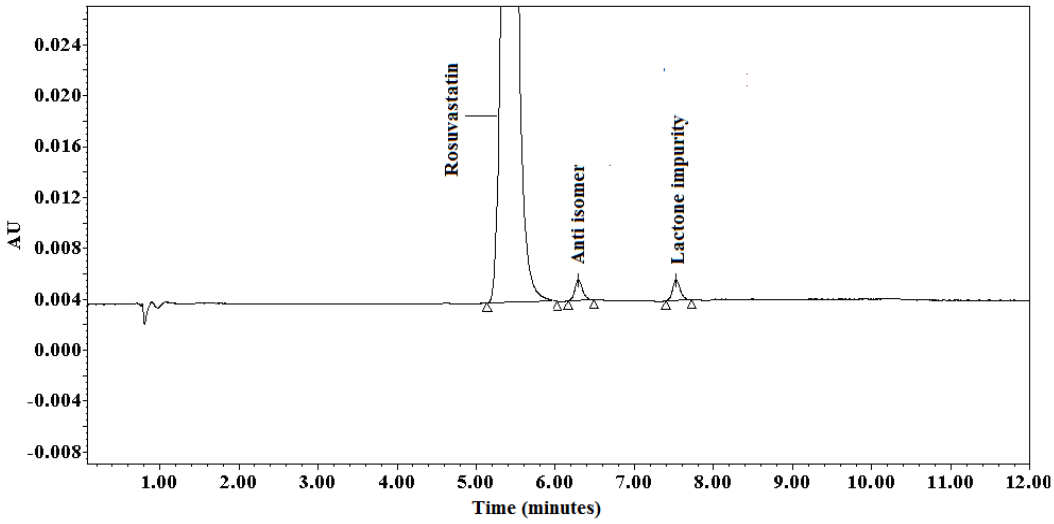


Fig. 1: Typical chromatogram of RSV and its related impurities.

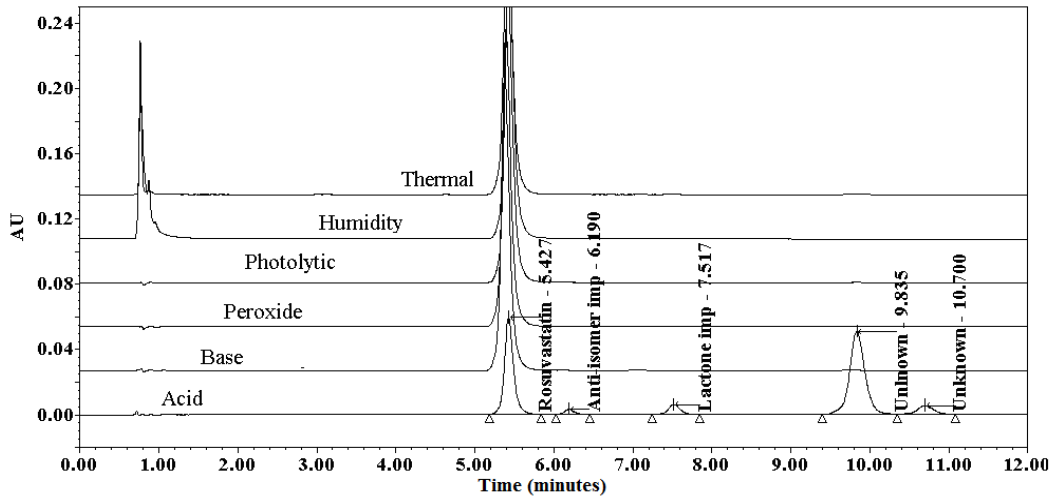


Fig. 2: Typical chromatograms of RSV forced degradation samples.

Analytical parameters and validation

After satisfactory development of method, RSV bulk sample was subjected to method validation as per ICH guidelines.[14] The method has validated to demonstrate that is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics including specificity, accuracy, precision, robustness, LOD, LOQ, linearity and stability.

System suitability

The main purpose to perform system suitability was to check the resolution between RSV and anti-isomer and is found to be greater than 2.5. The percentage RSD for area of six replicate

injections of diluted standard was found to be below 0.24, the tailing factor was less than 1 and theoretical plates are around 13000. Results were summarized in Table-1.

Table 1: Chromatographic performance data.

Compound	RT (min)	RRT[a,b]	Resolution[b,c]	Tailing factor[b]	Theoretical plates[b]	RRF[b,d]
RSV	5.41	--	--	1.16	13000	--
Anti-isomer	6.17	1.14	3.11	0.92	11000	0.94
Lactone	7.41	1.37	8.38	1.12	10500	0.91

[a]Relative retention times (RRT) are calculated against the retention time of RSV.

[b]Average of five determinations.

[c]Resolutions are calculated between two adjacent peaks.

[d]Relative response factor was calculated using slope method.

Forced degradation study results

The degradation study revealed that RSV is very sensitive to acid hydrolysis rather than other degradation conditions. The major degradation products of acid hydrolysis were lactone and an unknown impurity with RTs of 7.5 and 8.5 min, respectively. The degradation study shows that RSV Ca is considerably stable under neutral and basic conditions. The forced degradation results were shown in Table 2. The degradation of RSV in thermal, humidity and photolytic was observed to be less. Further, spectra of the unknown impurities formed in the degradation of RSV were similar to that of individual standards eluting at respective retention times. Also spectra of known impurities in degraded tablets sample were similar to its respective impurity standard substance, indicating that there was no co-elution of unknown degradation peak at retention time of respective known and unknown impurities. Spectral purity of RSV and its impurities in the chromatogram of all exposed samples found spectrally pure. The max plot of chromatograms of degradation sample was also checked to ensure that no degradation peak is missed due to use of wavelength of 240 nm.

For assay, forced degradation study was performed by injecting the above degradation samples at assay concentration levels i.e. 50 ppm and calculated the assay percentage against the reference standard. Mass balance was calculated for all the degradation samples by summing all the peaks found in the sample analysis (area of RSV + area of all the degradants formed in the degradation) and found to be about 98%.

Table 2: Forced degradation results.

Degradation conditions	% Assay for RSV	% Anti-isomer impurity	% Lactone impurity	% Major degradation product	Mass balance
Acid treatment (1N HCl, 3 hrs at room temperature)	62.7	0.59	7.12	30.02	98.6
Base treatment (1N NaOH, 70 ºC, 2 hrs)	98.3	0.02	0.03	0.11	98.1
H_2O_2 treatment (5% H_2O_2, 70 °C, 2 hrs)	97.5	0.02	0.05	0.34	98.9
Thermal-105 °C, 7 days	98.7	0.02	0.04	0.55	98.3
Humidity-90% RH, 25 °C, 7 days	99.1	0.03	0.03	0.17	99.0
Photolytic-1.2 m lux hours, 200 Whrs/sqmt, 7 days	99.5	0.01	0.03	0.15	99.3

LOD and LOQ

The concentration with signal to noise ratio of at least 3 was taken as LOD and concentration with signal to noise ratio of at least 10 was taken as LOQ, which meets the criteria defined by ICH guidances.[14] The LOD and LOQ results of RSV and known impurities were presented in Table 3.

Linearity

To demonstrate the linearity of detector response for impurity RSV, anti-isomer impurity and lactone impurity, injected the solutions with concentrations ranging from LOQ level to 150% of the target concentration of RSV and its impurities. Plot a graph to peak area versus the concentration. Summarize the results in the Table 3 given below.

For the assay method, the linearity study was executed by injecting the RSV at five different concentration levels i.e. 50%, 70%, 100%, 125% and 150% to the target sample concentration and calculated the correlation co-efficient and found to be greater than 0.999 shows that magnificent correlation between the analyte concentration and the peak area.

Table 3: LOD, LOQ, linearity and precision results.

Parameter	RSV	Anti-isomer impurity	Lactone impurity
LOD (µg mL^{-1})	0.005	0.007	0.008
S/N ratio	2.7	3.1	3.2
LOQ (µg mL^{-1})	0.023	0.020	0.025
S/N ratio	9.8	10.2	10.6
Regression statistics			
Slope (b)	155378	187029	184535
Intercept (a)	2736.2	-3722.5	-1020.2
Correlation coefficient	0.9996	0.9995	0.9998
Method precision (% RSD)[a]	1.12	1.35	1.54
Intermediate precision (% RSD)[b]	0.96	0.78	0.93

Linearity range is LOQ-150% with respect to 0.5 mg/ml of RSV for related impurities; linearity range is 50–150% with respect to 0.1 mg/ml of RSV for assay.

Precision

System precision

Prepare comparison standard solution as per test method, inject six times into the HPLC system. Calculate the RSD for the peak area of RSV from six replicate injections and tabulated the results in the Table 3. Low value of RSD indicates that the method was precise. Precision was calculated by injecting six replicate injections of the standard solution at assay concentration level and the % RSD for assay was tabulated in the Table 3.

Method precision

Determine the precision of test method by injecting six samples prepared by spiking test preparation with RSV Impurities blend solution to get 0.1% of each impurity on RSV tablet powder or on tablets prepared as per manufacturing formula. Calculate RSD of % of RSV, anti-isomer and lactone impurity and the results presented were in the Table 3.

For assay, method precision was calculated by injecting six preparations of the sample and the results were tabulated in the Table 3.

Accuracy

In related substances method, obtained % recovery for anti-isomer, was ranged from 97.3 to 100.2 and for lactone between 97.8 to 99.8. The obtained % recovery for RSV tablets was between 99.1 to 100.8 for assay. The results were tabulated in the Table 4.

Table 4: Accuracy results.

Amount added	% Recovery range for triplicate injections		
	RSV[a]	Anti-isomer impurity[b]	Lactone impurity[b]
LOQ	99.1–100.3	98.3–100.2	97.8–99.4
50%	99.3–99.8	97.3–99.7	98.2–99.5
100%	99.7–100.8	98.6–99.4	98.7–99.4
150%	99.6–100.5	99.1–99.6	99.2–99.8

[a] % Recovery obtained from assay

[b] % Recovery obtained from related substances

Robustness

In all the robust conditions the resolution between RSV and anti isomer was found to be greater than 2.0 and the resolution between anti isomer and lactone impurity was not less than

5.0. Peak shape for all the impurities and RSV found to be good in all the conditions and the assay variability was within ± 2.0% and the discrepancy in the estimation of RSV impurities was within ± 7.0%. Thus, the method was found to be robust with respect to variability in above all conditions.

Stability of sample solution

In the solution stability of RSV at 12 hrs lactone impurity was found to be degraded and anti-isomer found to be stable up to 24 hrs. Therefore, prepared sample solutions were used upto 12hrs only.

Identification of the unknown major degradant formed in the acid degradation

LC-MS analysis was performed for the acid stress sample for the identification of unknown major degradant using waters Q-ToF mass spectrometer with the same mobile phase using in UPLC. In LC-MS analysis, RSV showing the molecular ion at *m/z* 482 and the unknown degradant shows the molecular ion at *m/z* 496 which is 14 mass units higher than the RSV and was matched with the methylene group (-CH_2). MS/MS study was performed for both RSV and unknown impurity using product ion mode by applying collision energy of about 20 eV and the corresponding MS/MS spectra were shown in Fig.3(a) and Fig.3(b). In the MS/MS study of the unknown impurity, the major fragments observed were *m/z* 460, 418 and 258. The major fragments observed for RSV were *m/z* 464, 446, 272 and 258. The possible way to form the unknown impurity is the esterification of the acid group present in the RSV and was shown in Fig. 4.

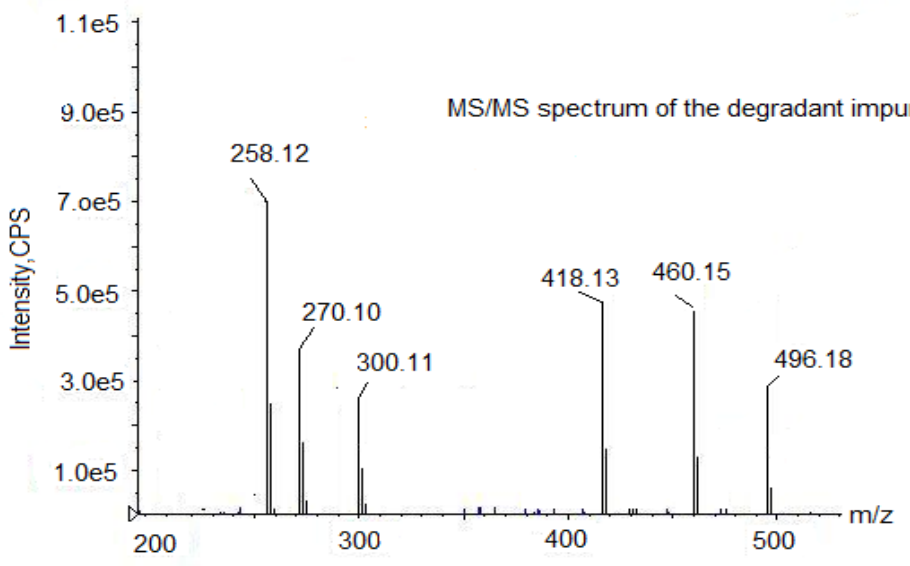


Fig. 3(a): MS/MS spectra of degradation impurity.

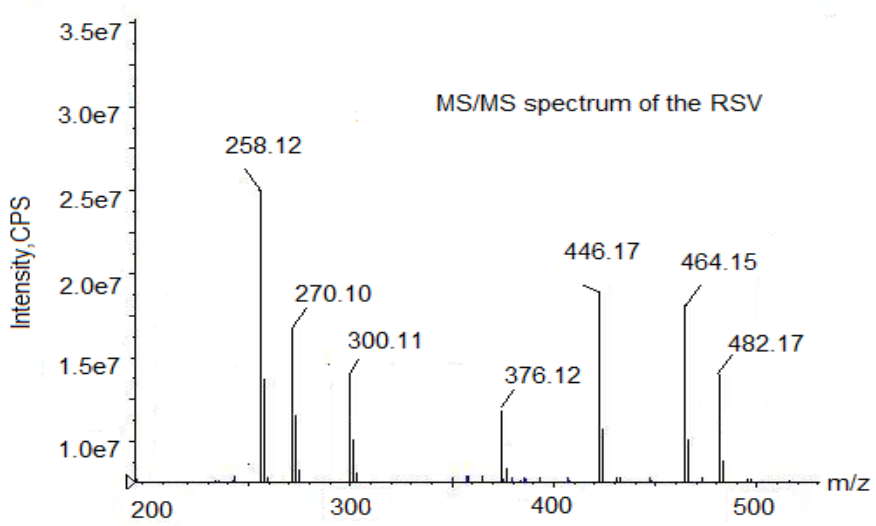


Fig. 3(b): MS/MS spectra of RSV.

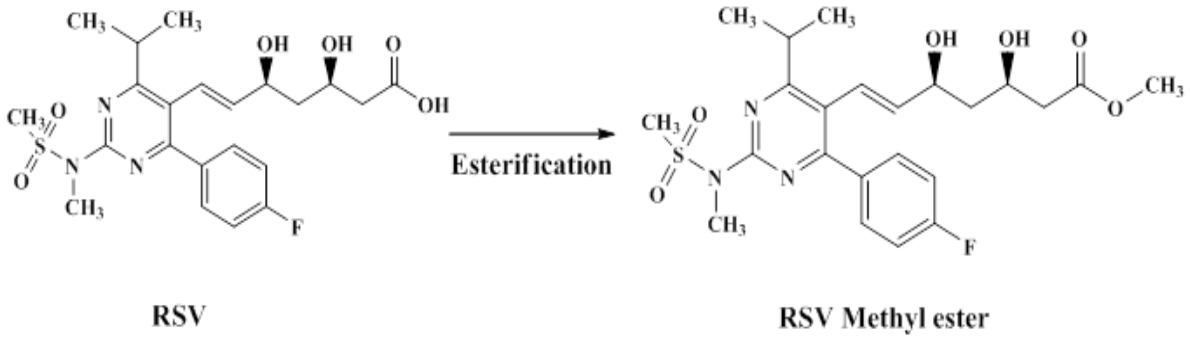


Fig. 4: Formation of RSV methyl ester (major acid degradant).

CONCLUSION

So that the method can be able to determine the RSV and its known impurities in presence of other degradation impurities with accurate, reproducible, robust, stability indicating and straight forward enough for routine use in quality control laboratory.

A simple isocratic RP-UPLC method was developed for the quantification of RSV as well as its impurities in its pharmaceutical bulk samples. Degradation behaviour of the RSV was studied under varies stress conditions. Major degradants formed in acid stress condition was identified using Q-tof mass spectrometer and named as RSV methyl ester. The mobile phase is mass compatible and this method can also be used in LC-MS. All the process impurities and the degradation impurities were well separated from the RSV revealed the stability indicating capability of the method. It can be used for the quantification of RSV and its impurities in routine bulk samples analysis.

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