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Identification, isolation, characterization and quantification of a new impurity in Rosuvastatin calcium tablet dosage form

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ABSTRACT

During the stability study of Rosuvastatin calcium (RSV) tablets, an unknown impurity was found at level of 0.8% by newly developed reverse phase HPLC method. The unknown impurity was identified by liquid chromatography–tandem mass spectrometry using electrospray ionization source and Q-trap mass analyzer (LC–ESI–QT/MS/MS). The drug product was subjected to stress to enhance the level of this impurity. The unknown was isolated by preparative liquid chromatography and characterized using the LC-MS, HRMS, NMR and IR spectral studies. Based on the spectroscopic data, the impurity was characterized as (3R,5S,E)-7-(4-(4-fluorophenyl)-6-isopropyl-2-(N-methylmethysulfonamido)pyrimidin-5-yl)-3,5-dihydroxy-N-methyl-N-((2S,3R,4R,5R)-2,3,4,5,6-pentahydroxyhexyl)hept-6-enamide (Imp-1). It is a novel impurity and not reported elsewhere. The newly developed method was validated as per ICH guidelines to demonstrate specificity, sensitivity, linearity, precision, accuracy and the stability-indicating nature.

KEYWORDS

Rosuvastatin calcium;
Novel impurity;
Stress studies;
Characterization,
Method development & validation;
HPLC.

INTRODUCTION

Rosuvastatin calcium (RSV) chemically described as Calcium bis [(3R,5S,6E)-7-(4-(4-fluorophenyl)-6-(1-methylethyl)-2-(ethyl(methylsulfonyl)amino)-5-pyrimidinyl)-3,5-dihydroxy-6-heptenoic acid] (Figure 1A). The drug is approved by United States Food and Drug Administration (USFDA) and marketed under the trade name of CRESTOR (Rosuvastatin calcium) tablets by AstraZeneca group of companies. RSV is an antilipemic agent that competitively inhibits hydroxyl methyl glutaryl-coenzyme A (HMG-CoA) reductase. HMG-CoA reducuase catalyzes the conversion of HMG-CoA to mevalonic acid, the rate-limiting step in cholesterol biosynthesis. Rosuvastatin belongs to a class of medications called statins. It is widely used in the treatment to reduce plasma cholesterol levels and prevent cardiovascular disease[1-3]. The literature survey revealed various spectrophotometric methods[4-7], HPLC methods for the quantification of RSV[8-10] and combined formulation dosage

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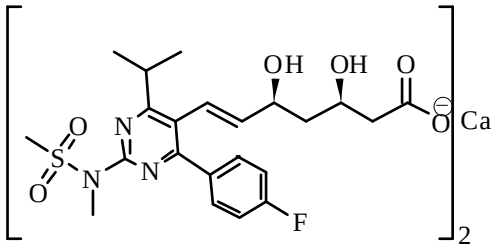
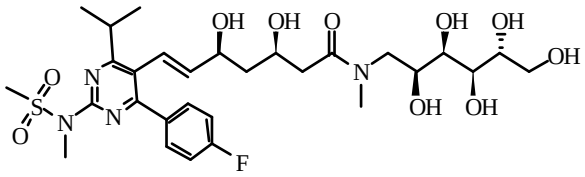
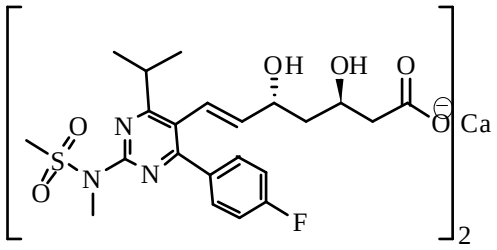
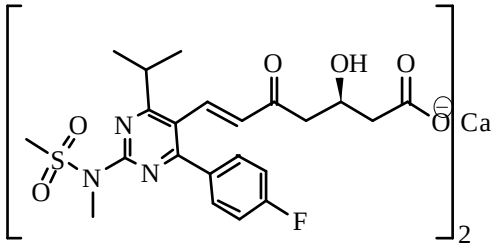
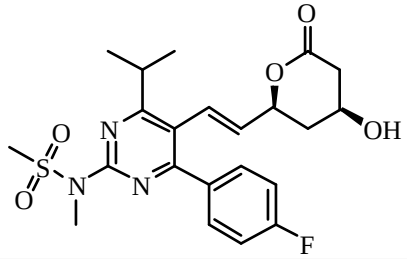
Figure	Name	Chemical Structure	Chemical name
1A)	RSV (Rosuvastatin calcium)		Calcium bis [(3R,5S,6E)-7-(4-(4-fluorophenyl)-6-(1-methylethyl)-2-(ethyl(methylsulfonyl)amino)-5-pyrimidinyl)-3,5-dihydroxy-6-heptenoic acid]
1B)	Imp-1(0.68 RRT Imp)		(3R,5S,6E)-7-(4-(4-fluorophenyl)-6-isoprpyl-2-(N-methylmethylsulfonamido)pyrimidin-5-yl)-3,5-dihydroxy-N-methyl-N-((2S,3R,4R,5R)-2,3,4,5,6-pentahydroxyhexyl)hept-6-enamide
1C)	Imp-A (Anti isomer)		Calcium bis[(3R,5R,6E)-7-[4-(4-fluorophenyl)-2-(N-methylmethanesulfonamido)-6-(propan-2-yl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid]
1D)	Imp-B (5- OXO Imp)		Calcium bis[(3R,6E)-7-(4-(4-fluorophenyl)-6-isopropyl-2-(N-methyl methyl sulfonamide)pyrimidin-5-yl)hydroxyl-5-oxoheptanoic acid]
1E)	Imp-C (Lactone)		N-(4-(4-fluorophenyl)-5-((E)-2-((2S,4R)-4-hydroxy-6-oxo-tetrahydro-2H-pyran-2-yl)vinyl)-6-isoprppylpyrimidin-2-yl)-N-methyl methane sulfonamide

Figure 1 : Chemical structure and name of RSV and impurities

forms[11–16] have been reported. UPLC method for the determination of related substances of RSV has been reported[17]. However, there is no information available for formation of this new impurity (Imp-1) in RSV drug product. This Imp-1 was detected in the drug product during stability stored sample analysis, which crossed the identification threshold. As per the stringent regulatory requirements recommended by the ICH and regulatory agencies, it is mandatory and important to identify and structurally characterize any impurity formed during production and stability testing, exceeding the identification threshold[18–20]. Various analytical instruments and advanced hyphenated techniques[21–24] are routinely used to carry out the impurity profile study. The drug product was subjected to stress to enhance the level of this impurity. The unknown impurity was isolated by preparative HPLC and subjected to LC/MS, LC-MS/MS, HRMS (high resolution mass spectrometry), NMR and IR spectral studies. Based on the spectral data, the unknown impurity was characterized as (3R,5S,E)-7-(4-(4-fluorophenyl)-6-isoprpyl-2-(N-

methyl methyl sulfonamido) pyrimidin-5-yl)-3,5-dihydroxy-N-methyl-N-((2S,3R,4R,5R)- 2,3,4,5,6-pentahy droxyhexyl)hept-6-enamide (Imp-1). To the best of our knowledge, this impurity has not been reported elsewhere. In the literature, there is no stability-indicating LC method available for the estimation of this impurity in pharmaceutical formulation. The present study describes the isolation and characterization of this Imp-1, as well as development and validation of a stability-indicating RP-HPLC method for the estimation of known impurities of RSV, namely Imp-1, Imp-A, Imp-B and Imp-C (Figure 1B -1E). Forced degradation studies were performed on RSV drug product to show the stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines[25, 26].

EXPERIMENTAL

Chemicals and reagents

RSV tablets and standards of impurities were supplied by Dr. Reddy's Laboratories Ltd., Hyderabad, India. HPLC grade methanol (MeOH), acetonitrile (ACN), analytical grade potassium dihydrogen phosphate (KH_2PO_4), ammonium acetate (CH_3COONH_4), formic acid (HCOOH), ortho phosphoric acid (H_3PO_4), Hydrochloric acid(HCl), sodium hydroxide(NaOH) and Hydrogen peroxide were purchased from Merck, Darmstadt, Germany. CD_3OD (Deuterated methanol) was obtained from Aldrich Chemical Co., USA. Deionized water was prepared using a Milli-Q plus water purification system from Millipore (Bedford, MA, USA).

Instrumentation and chromatographic conditions for HPLC

Samples were analyzed on a Waters alliance 2690 separation module equipped with 2487 UV detector (Waters Corporation, Milford, MA, USA) using an X-Bridge C18, (150 mm×4.6 mm, 3.5 µm, Waters Corporation, Milford, MA, USA). The mobile phase-A contains a mixture of 0.025M KH_2PO_4 buffer (pH 3.0 adjusted with H_3PO_4) and methanol in the ratio 80:20 (v/v). The mobile phase-B contains a mixture of buffer, ACN and methanol in the ratio 25:15:60 (v/v). The flow rate of mobile phase was 1.0 mL/min. The HPLC gradient program (time (min)/%B) was set as 0.01/40, 20/50, 45/90, 55/90, 56/40 and 60/40. The column temperature was maintained at 30ºC. The detection was monitored at wavelength 248 nm. The injection volume was set as 10.0µL. A mixture of water and ACN in the proportion of 30:70 (v/v) was used as a diluent.

Forced degradation of RSV drug product

Forced degradation studies were performed on the RSV drug product with the intention of determining the conditions responsible for the formation of the Imp-1 and stability indicating nature of the proposed method. 100 mg equivalent of RSV drug product was dissolved in 100mL volumetric flasks and subjected to forced degradation study under acid (0.1NHCl at 60°C for 2hrs), base (5N NaOH at 60°C,12hrs), neutral (water at 60°C for 30 minutes) and Oxidation (10.0% H_2O_2, 60°C for 30 minutes). The stressed samples of acid and base degradation were neutralized with 0.1N NaOH and 5N HCl respectively and made up to volume with diluent. The RSV drug product was placed in a thermally controlled oven at 105°C for 6hrs for thermal stress study. The RSV drug product was placed in the humidity chamber (90% RH at 25°C for 10days) for humidity degradation. Photolytic degradation was performed by exposing the drug to visible light and UV with minimum exposure of 1.2 million lux-hours and 200w-hr/m^2 respectively. The degradation samples were analyzed as per above described HPLC chromatographic condition. Peak purity test was carried out for the RSV peak by using PDA detector in all stressed samples. Placebo interference was evaluated by analysing the placebo prepared as per the test method.

Chromatographic conditions (preparative)

Preparative isolation work was performed on an Agilent 1200series preparative HPLC system which was equipped with an automated fraction collector and photodiode array detector and Chemstation software. A mixture of water and ACN in the ratio of 50:50 (v/v) used as diluent for sample preparation. Approximately 20 mg/mL of sample was prepared to load on to the column. Hyper Prep HS C18

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column (500mm×30mm, 10µm particle size) was employed. Mobile phase A was 0.01 ammonium acetate solution, pH adjusted to 3.0±0.1 with formic acid and mobile phase B was acetonitrile. Flow rate was 20 mL/min and UV detection was carried out at 248 nm. The gradient program was as follows: (time (min)/%B) was set as 0.01/40, 20/50, 21/100, 30/100, 30.1/40, 36/40. The impurity fractions were collected separately from several injections and pooled separately. The pooled fractions were concentrated by using rotavapour (Model: Heidolph Laboratory4002 control) under high vacuum. The

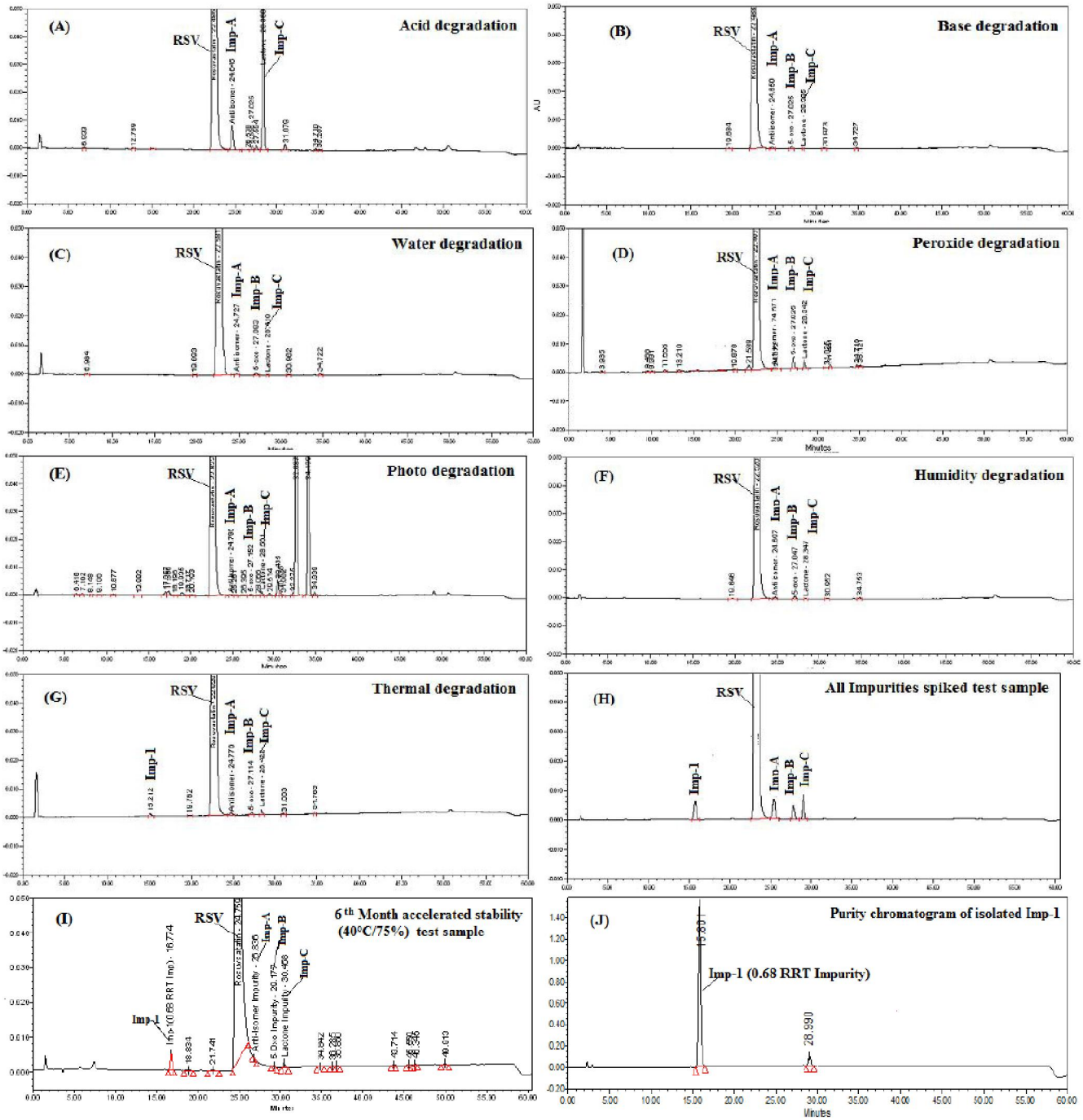

Figure 2 : Typical chromatograms of RSV under stress conditions: (A) acid hydrolysis, (B) base hydrolysis, (C) water hydrolysis (D) Peroxide degradation (E) photo degradation (F) Humidity degradation (G) Thermal degradation (H) All impurities spiked test sample (I) 6M 40°C/75% stability test sample (J) Preparative isolated Imp-1 purity chromatogram

aqueous solution was lyophilized to solidify the impurities.

LC-MS/MS conditions

LC–MS/MS system (Agilent 1200 series liquid chromatography coupled with Applied Bios stems 4000 Q Trap triple quadrupole mass spectrometer with Analyst 1.4 software, MDS SCIEX, USA) was used for identification of the Imp-1 formed during stability storage samples. X-Bridge C18 (150 x 4.6 mm, 3.5µm) column was used as stationary phase. Ammonium acetate (0.01M; pH-3.0 adjusted with formic acid) was used as buffer. Buffer and methanol in the ratio 80:20 (v/v) was used as mobile phase-A. Buffer, methanol and ACN in the ratio 25:15:60 (v/v) was used as mobile phase-B. The gradient program (time (min)/%B) was set as 0.01/40, 20/50, 45/90, 55/90, 56/40 and 60/40. Water and ACN in the ratio of 30:70(v/v) was used as diluent. The flow rate was kept 1.0 mL/min with injection volume 10µL. The detection was monitored at wavelength 248 nm. The analysis was performed in positive electro spray ionization mode. Ion Source voltage was 5000V. Source temperature was 450°C. GS1 and GS2 are optimized to 30 and 35 psi respectively. Curtain gas flow was 20 psi. For fragmentation (MS/MS) studies collision energy and declustering potential was set at 30eV and 30V, respectively.

UPLC-TOF-MS conditions (HRMS conditions)

The UPLC-TOF-MS system consisted of an ACQUITYTM Ultra Performance Liquid Chromatography (UPLC) system and a Micromass LCT Premier XE Mass Spectrometer (high sensitivity orthogonal time-of-flight instrument, Waters, Milford, USA) equipped with a lock mass sprayer, operating in either positive or negative ion mode. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine enkephalin was used as the lock mass. High resolution (W mode, FWHM 10500) positive polarity scan responses were collected from m/z 100 to 1000 at a rate of 1.0 s/scan. The chromatographic column used was an ACQUITY UPLC™ BEH shield RP18 (2.1 x 100 mm, 1.7 µm). Ammonium acetate (0.01M; pH-3.0 adjusted with formic acid) was used as buffer. Buffer and methanol in the ratio 80:20 (v/v) was used as mobile phase- A. Buffer, methanol and ACN in the ratio 25:15:60 (v/v) was used as mobile phase-B respectively. The gradient program (time (min) / %B) was set as 0/40, 2/50, 3/80, 5/90, 8/100, 8.1/40, and 10/40 with a flow rate of 0.4 mL/min and injection volume of 3.0 µL. The mobile phases were filtered through nylon 0.2 μm membrane filters and degassed. The column temperature was maintained at 30°C and the peaks were monitored at 248 nm. A mixture of water and ACN in the proportion of 30:70 (v/v) was used as diluent for sample preparation.

NMR spectroscopy

Nuclear magnetic resonance (NMR) experiments (^{1}H and^{13}C) were performed using Bruker Avance 400MHz spectrometer in CD_3OD as solvent. ^{1}H NMR measurements were carried out at 400 MHz, while ^{13}C NMR experiments were performed at 100 MHz. The ^{1}H NMR spectra were recorded with 1 s pulse repetition time using 30æ% flip angle, whilst ^{13}C NMR spectra were recorded with power gated decoupling using 30æ% flip angle with repetition time of 2 s. Proton and carbon chemical shifts were reported on δ scale in ppm with respect to TMS (0.00ppm) and CD_3OD (δ 49.5 ppm) as internal standard, respectively. All spectra were recorded with sample spinning

FT-IR spectroscopy

IR spectra were recorded in solid state as KBr dispersion medium using Perkin-Elmer FT-IR spectrophotometer.

Preparation of solutions for method validation

Preparation of impurities stock and standard solutions

The individual stock solutions (100µg/mL) of Imp-1, Imp-A, Imp-B, Imp-C and RSV were prepared in the diluent. These solutions were prepared freshly and diluted further quantitatively to study the validation attributes. The specification limits considered for validation studies were 0.5% for Imp-1, Imp-A, Imp-B and Imp-C. Diluted standard solution of 2µg/mL was prepared from the RSV stock solu-

tion for the determination of the related compounds.

Preparation of sample solution

RSV tablets(n = 20) were weighed and averaged before being crushed into fine powder. Transferred an accurately weighed amount of tablet powder equivalent to 100 mg of RSV into a 100 mL volumetric flask, added 70 mL of diluent and sonicate for 30minutes with intermediate shaking, dilute to volume with diluent and mix well. This solution was centrifuged with 5000 RPM for 10 minutes.

RESULTS AND DISCUSSION

The new impurity (Imp-1) of RSV drug product was found at level of 0.8% under accelerated stability conditions [40°C /75% relative humidity (RH) for 6 months] with the relative retention time (RRT) of 0.68 in RP-HPLC (Figure 2I). The drug product was subjected to acid, base, water, peroxide, photolytic, humidity and thermal stress conditions to enhance the level of this impurity. The degradation samples were analyzed as per above HPLC chromatographic conditions. Under acid, base, water, peroxide, photo and humidity stressed conditions; the Imp-1 was not observed (Figure 2A-2F). In thermal stressed condition only the Imp-1 was formed up to 0.18 % (Figure 2G). Hence the thermal degradation route was chosen to enhance the impurity. The Imp-1 was enhanced up to 4.0 % by subjecting to heat at 105°C for 10 days (TABLE 1). This impurity was isolated by preparative HPLC and co-injected with RSV stability samples into HPLC to confirm the relative retention time. The same degradation sample and isolated impurity were subjected to LC–MS analysis using conditions as described in the LC/MS/MS section to identify the molecular mass of the impurity.

Isolation of Imp-1 (0.68 RRT impurity) by preparative HPLC

The thermal degraded sample was subjected to preparative chromatography to enable the isolation of the impurity. The purity of this solid was assessed to be more than 98.46% (Figure 2J) and potency of this Imp-1 conformed as 96.5%. This impurity was then characterized by spectroscopic techniques like LC/MS/MS, HRMS, NMR (^{1}H and ^{13}C) and FT-IR while the molecular weight was determined by mass spectrometry.

Identification of Imp-1 by LC/MS/MS

To identify of Imp-1 structure, The LC/MS/MS study was performed on accelerated stability sample of RSV drug product. In the course of the LC/MS studies the ESI mass spectrum of this impurity displayed a protonated molecule at m/z 659 $[M+H]^+$ in positive ion mode, which is 177 units more than that of Rosuvastatin molecular mass (Figure 3A). The mass data indicates formation of the condensed product of meglumine excipient with Rosuvastatin molecule. The positive HR-MS spectrum (Figure 3B) conforms protonated molecule at m/z 659.2752 corresponding to molecular formula $C_{29}H_{44}N_4O_{10}FS$.

TABLE 1 : Formation of Imp-1 at various stress conditions

S.NO	Stress Conditions	% formed Imp-1 (0.68RRT Impurity)
1	RSV tablets 6M (40°C/75%)	0.80
2	RSV drug product: Heated at 105°C, 1 Day	0.76
3	RSV drug product: Heated at 105°C, 2 Days	1.50
4	RSV drug product: Heated at 105°C, 10Days	4.03
5	RSV drug product: Water Sprinkled/Heated at 105°C, 1Day	0.56
6	RSV drug product: Water Sprinkled/Heated at 105°C, 2Days	1.34
7	RSV drug product and 30% H_2O_2 without heat(25°C)	ND
8	RSV drug substance (API) and Meglumine (1:1) in Compact tablet form at 105°C, 3 Days	0.06
9	RSV API and Meglumine in 5NHCl at 80°C, 12hrs	ND
10	Imp-C (Lactone) and Meglumine at 80°C,12hrs	0.23

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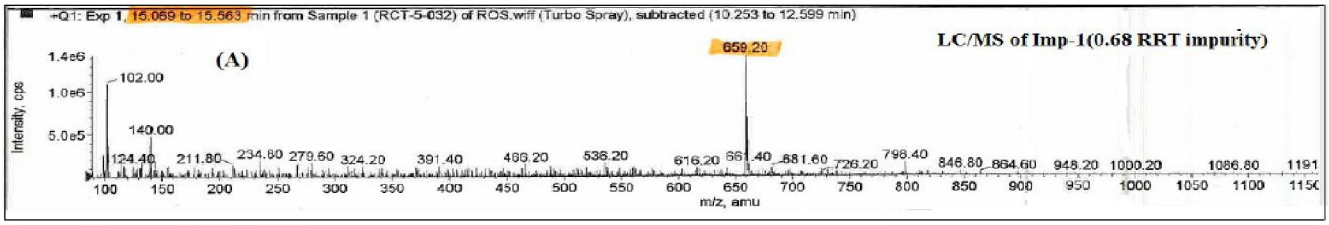
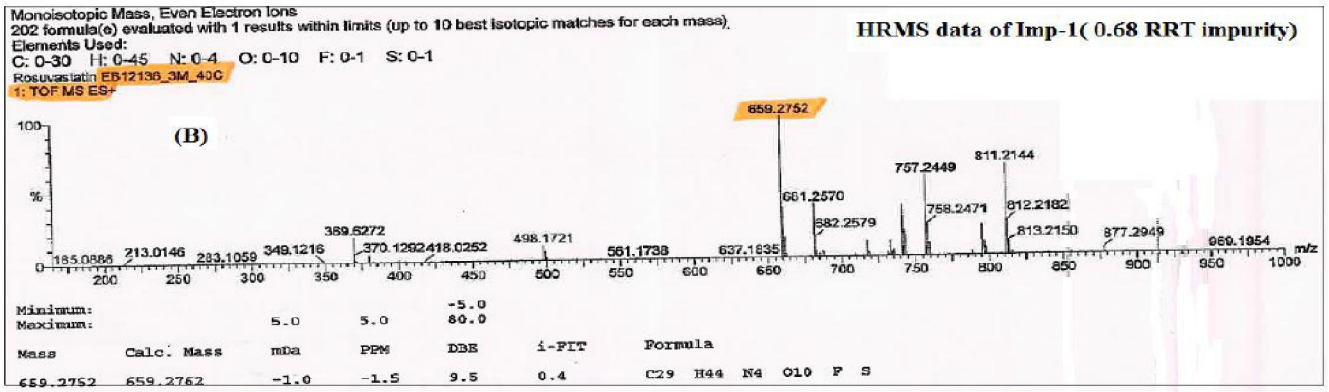
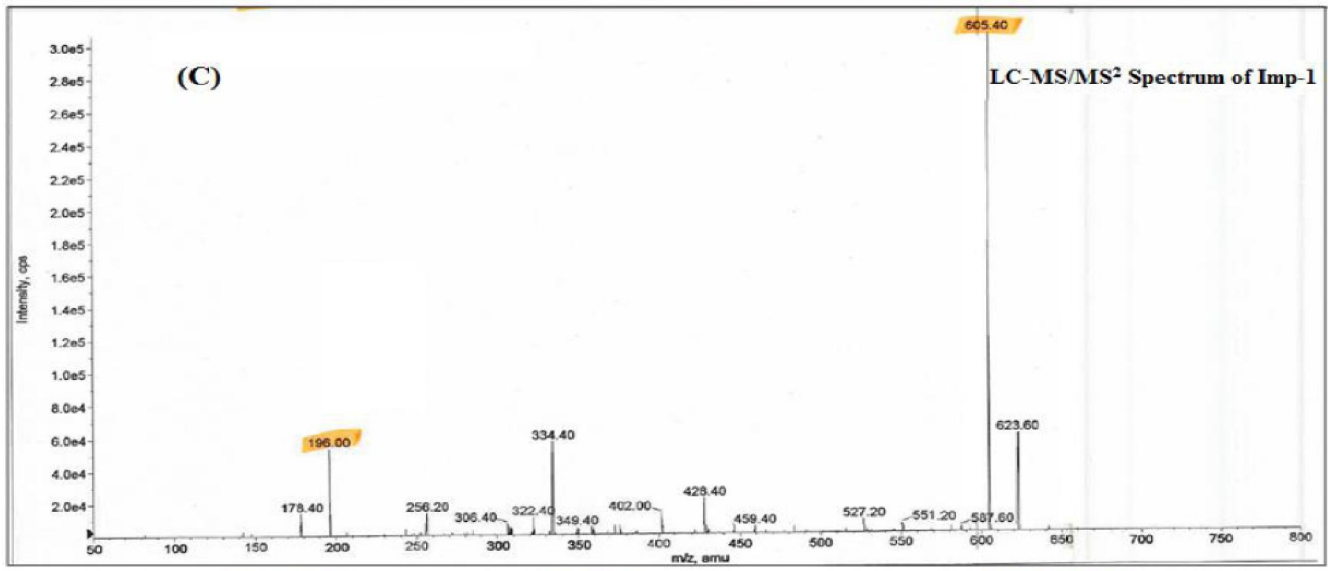
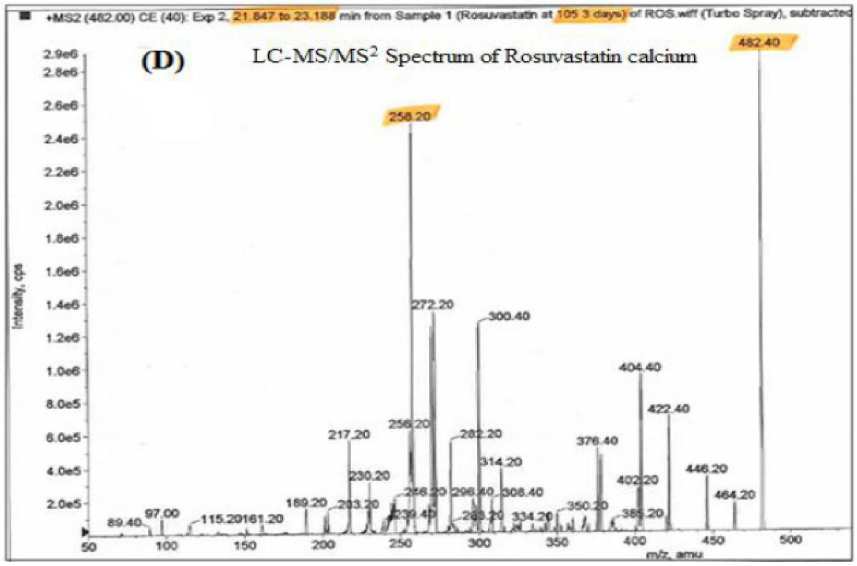
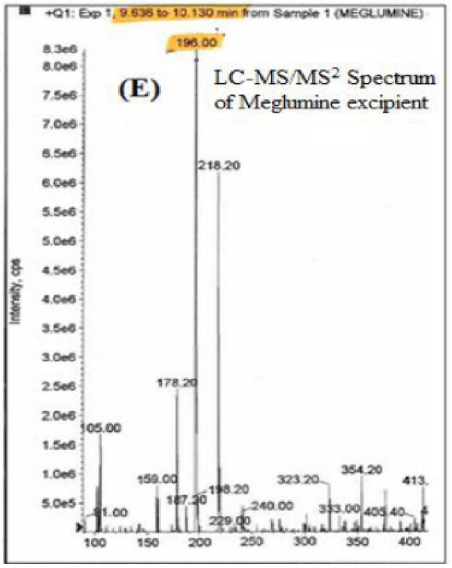

+Q1: Exp 1, 15.069 to 15.563 min from Sample 1 (RCT-5-032) of ROS.wiff (Turbo Spray), subtracted (10.253 to 12.599 min)
LC/MS of Imp-1(0.68 RRT impurity)
(A)
659.20
102.00
140.00
Intensity, cps
m/z, amu


Monoisotopic Mass, Even Electron Ions
202 formula(e) evaluated with 1 results within limits (up to 10 best isotopic matches for each mass)
Elements Used:
C: 0-30 H: 0-45 N: 0-4 O: 0-10 F: 0-1 S: 0-1
Rosuvastatin EB12136_3M_40C
1: TOF MS ES+
HRMS data of Imp-1(0.68 RRT impurity)
(B)
659.2752
661.2570
757.2449
811.2144
Minimum: -5.0
Maximum: 5.0 5.0 80.0
Mass Calc. Mass mDa PPM DBE i-FIT Formula
659.2752 659.2762 -1.0 -1.5 9.5 0.4 C29 H44 N4 O10 F S


(C)
LC-MS/MS² Spectrum of Imp-1
605.40
623.60
334.40
196.00
428.40
Intensity, cps
m/z, amu


+MS2 (482.00) CE (40): Exp 2, 21.847 to 23.188 min from Sample 1 (Rosuvastatin at 105 3 days) of ROS.wiff (Turbo Spray), subtracted
(D)
LC-MS/MS² Spectrum of Rosuvastatin calcium
482.40
258.20
272.20
300.40
404.40
422.40
Intensity, cps
m/z, amu


+Q1: Exp 1, 9.636 to 10.130 min from Sample 1 (MEGLUMINE)
(E)
LC-MS/MS² Spectrum of Meglumine excipient
196.00
218.20
178.20
Intensity, cps

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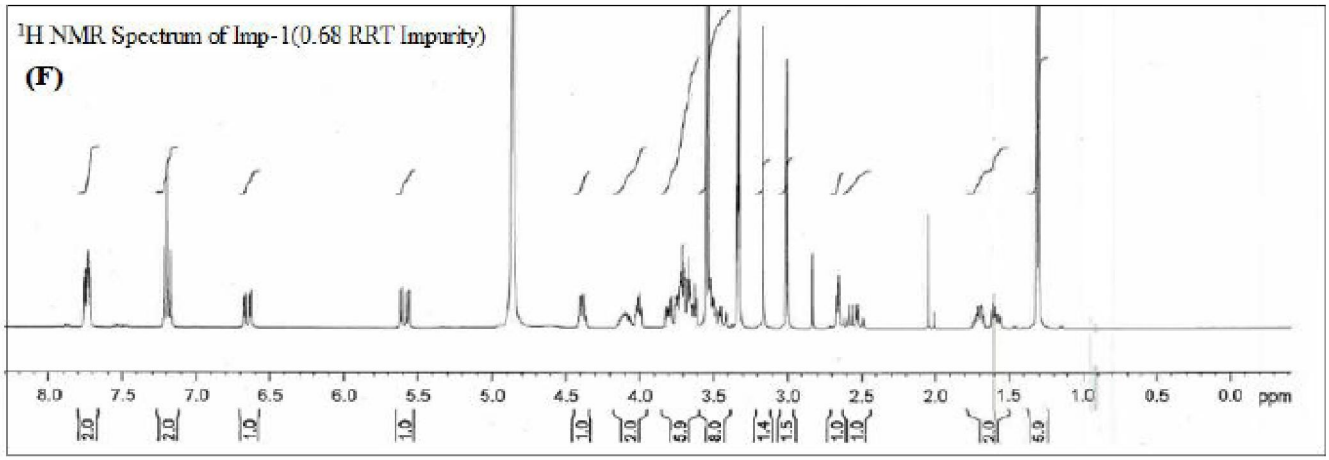
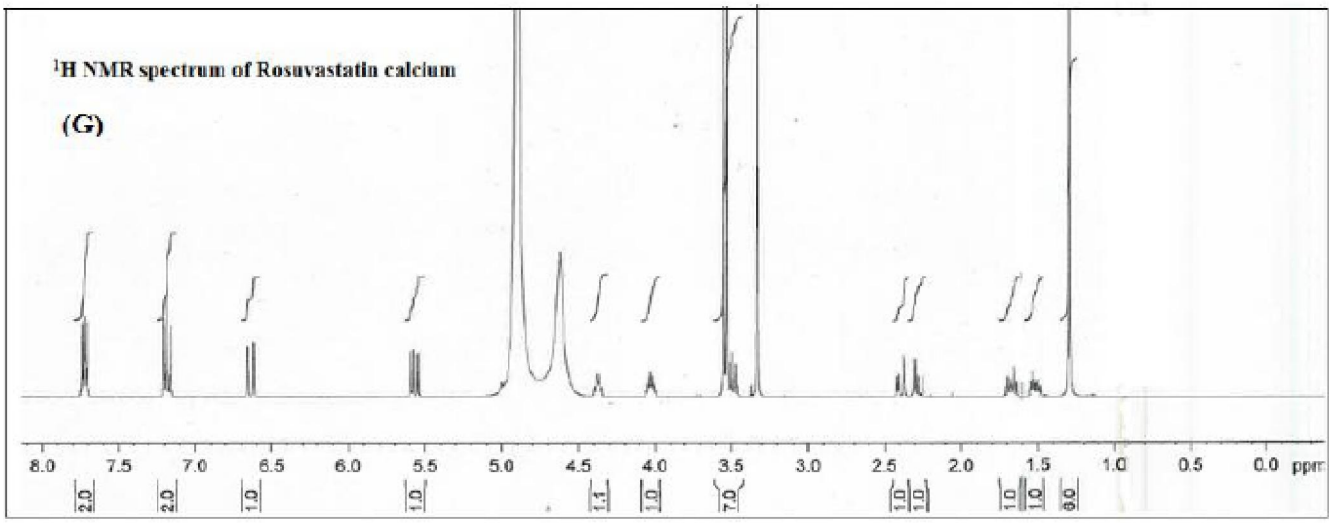
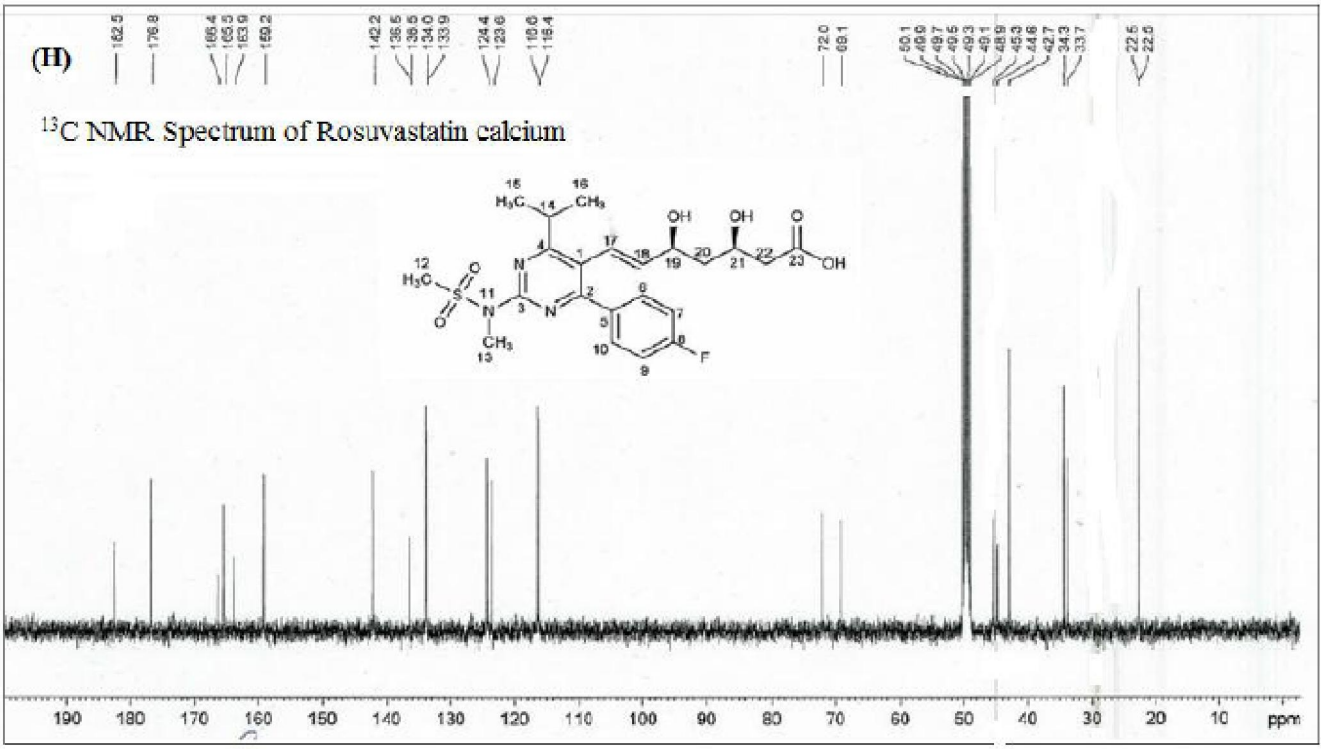

^{1}H NMR Spectrum of Imp-1(0.68 RRT Impurity)
(F)
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm


^{1}H NMR spectrum of Rosuvastatin calcium
(G)
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm


(H)
^{13}C NMR Spectrum of Rosuvastatin calcium
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

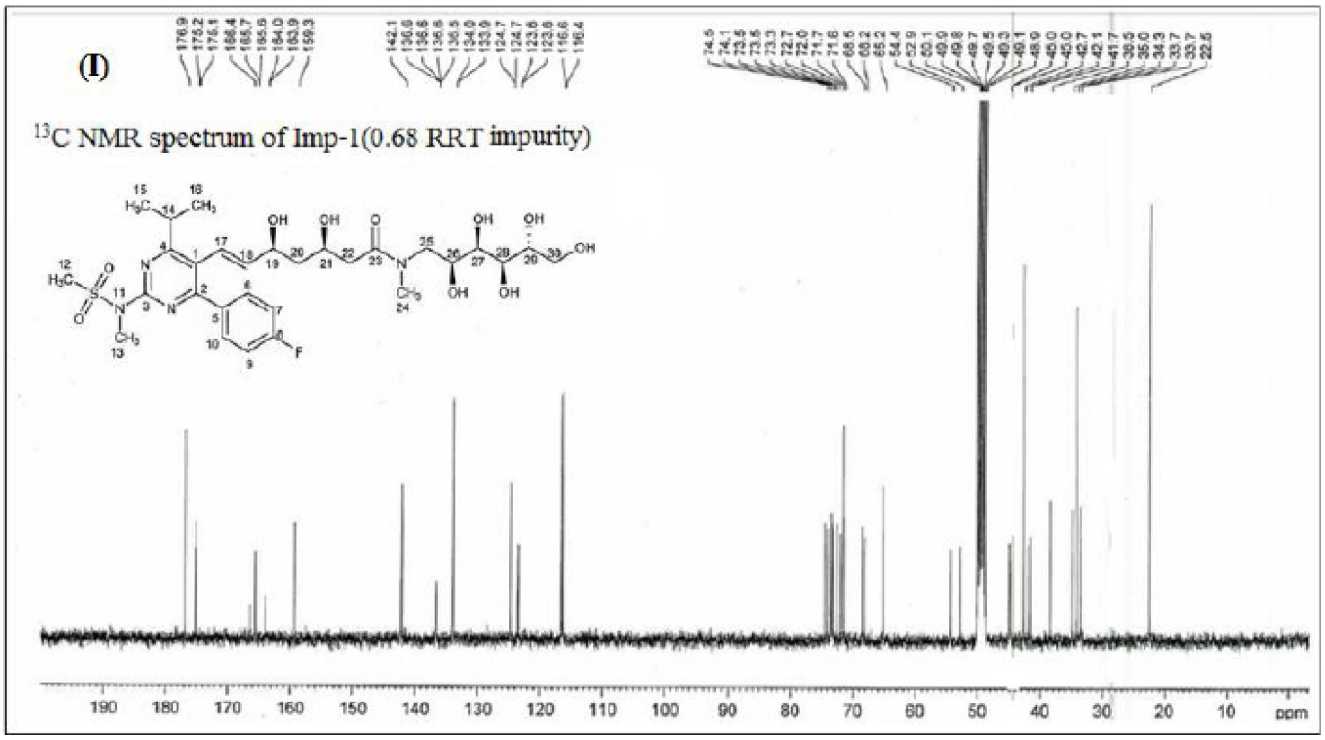


Figure 3 : LC-MS, HRMS, LC-MS/MS, NMR (^{1}H and ^{13}C) and HMBC Spectral data of Imp-1 and RSV (A) LC-MS spectrum of Imp-1 (B) HR-MS spectrum of Imp-1 (C) LC/MS/MS spectrum of Imp-1 (D) LC/MS/MS spectrum of RSV (E) LC/MS/MS spectrum of meglumine (F) ^{1}H NMR spectrum of Imp-1 (G) ^{1}H NMR spectrum of RSV (H) ^{13}C NMR spectrum of RSV (I) 13C NMR spectrum of Imp-1

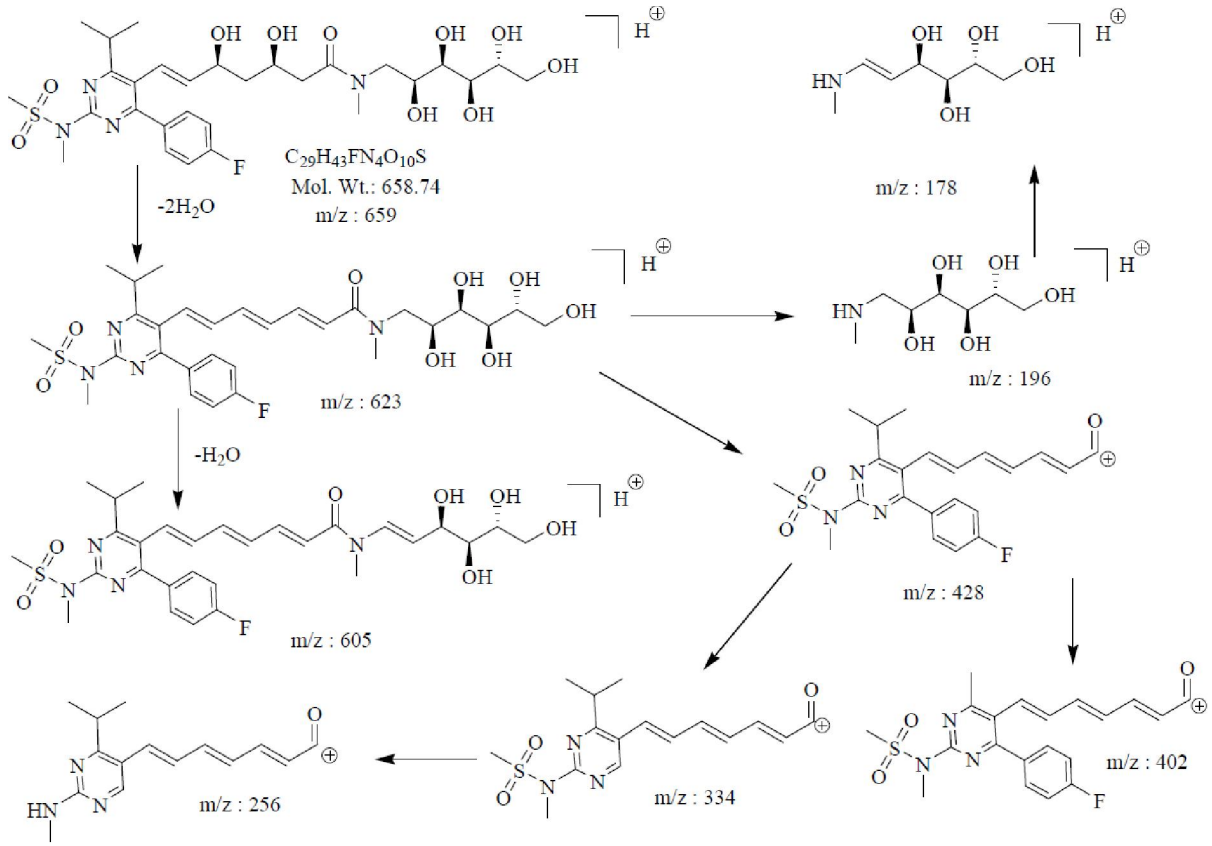


Figure 4A : LC-MS/MS fragmentation of Imp-1

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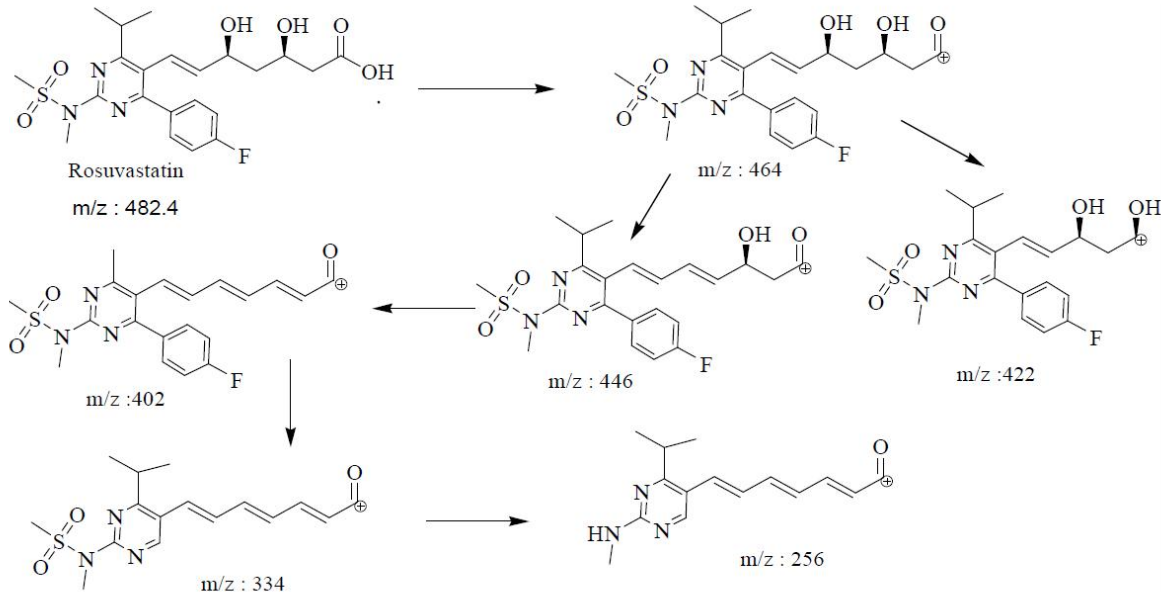

Figure 4B : LC-MS/MS fragmentation pathway of Rosuvastatin

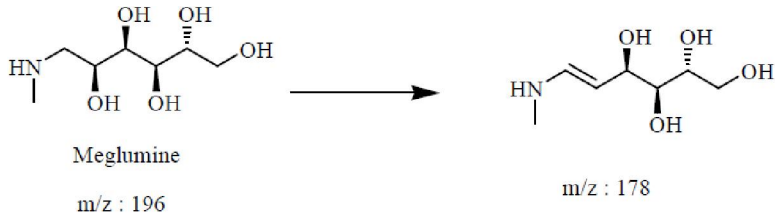

Figure 4C : LC-MS/MS fragmentation pathway of meglumin

Figure 4 : Plausible fragmentation pathway for (4A) Imp-1 (4B) Rosuvastatin (4C) meglumine

The LC/MS/MS analysis of Imp-1 produced fragments at m/z 623, 605, 428, 402, 334, 256,196 and 178 (Figure 3C& Figure 4A). The LC/MS/MS showed fragments at m/z 402, 334 and 256 matching with that of that of Rosuvastatin (Figure 3D&Figure 4B) and m/z 196, 178 similar to that of meglumine (Figure 3E& Figure 4C). The product ion of m/z 623 gave fragments at m/z 428 and m/z 196 (Figure 4A) matching with that of meglumine molecular ion and Rosuvastatin fragment ion. Based on LC/MS/MS analysis this impurity was suspected to be condensed product of meglumine excipient and Rosuvastatin molecule. A plausible fragmentation pattern showing the formation of these product ions is depicted in Figure (4A-4C).

Structural elucidation of Imp-1 by NMR and FT-IR

The NMR spectral data of RSV and Imp-1 were compared (TABLE 2). The ^{1}H NMR spectra of Imp-1 (Figure 3F) showed an additional signals at δ 3.10 ($N\text{-}CH_3$), 3.40-3.55 (4 –CH), 3.61-3.90 (5 OH) and 3.90-4.20 (2 $\text{-}CH_2$) when compare with RSV (Figure 3G). In ^{13}C NMR spectrum of RSV (Figure 3H) the signal corresponding to carboxylic acid (position 23) was observed at 182.5 ppm and when compared to Imp-1, this signal has been shifted to 176 ppm (Figure 3I). This could be due to the structural changes at carboxylic acid in RSV. Further, the structure of Imp-1 was confirmed by FT-IR spectral data. The absence of a characteristic strong absorption band at 1605cm”1 in comparison with RSV corresponding to C=O stretch supports the presence of amide moiety. This gives support to formation of amide bond between RSV and meglumine. Based on the above spectral data the structure of Imp-1 was characterized as (3R,5S,E)-7-(4-(4-fluorophenyl)-6-isoprpyl-2-(N-methyl methyl sulfonamido)pyrimidin-5-yl)-3,5-dihydroxy-N-methyl-N-((2S,3R,4R,5R)-2,3,4,5,6-pentahydroxyhexyl) hept-6-enamide.

Formation of impurity

Stability studies data indicates that the formation of this Imp-1 depends on the temperature. As shown in TABLE 1, the percent level of this impurity formed in RSV tablets stored at 105°C for 10 days was more than that of RSV tablets stored at 40±2°C/75±5%RH. Under thermal stress conditions amine functional group of meglumine reacts with carboxylic acid functional group of Rosuvastatin leading to formation of this Imp-1(Figure 5). During the excipient compatibility study also, this Imp-1 was not formed. This impurity is forming in tablet dosage form at accelerated stability condition only. The formation of this impurity was further verified by carrying out the experiments in the laboratory. RSV drug product and RSV drug substance (API) & meglumine were stressed under specific condition like dry heat, water-sprinkled heat, various types of oxidation etc. Sample analysis result exhibited that the dry heat and water-sprinkled sample have more escalated impurity. The experimental results are reported in TABLE 1. The probable mechanism for the formation of this impurity is given in Figure 5

Method development

The main objective of the chromatographic method development was to separate critical closely eluting compounds RSV and Imp-A, Imp-B and Imp-C with good resolution. The impurity mixture containing 1000 µg/mL of RSV and 5 µg/ml of each Imp-1, Imp-A, Imp-B and Imp-C was used for method development. A gradient elution method was employed using mobile phase-A (0.01 M KH_2PO_4

TABLE 2 : Comparative NMR assignment for RSV and Imp-1

Position	RSV			Imp-1		
	^1H	δ (ppm) /J (Hz)	^{13}C	^1H	δ (ppm)/J (Hz)	^{13}C
1	-	-	116.4	-	-	116.6
2	-	-	165.5	-	-	165.7
3	-	-	166.4	-	-	166.5
4	-	-	176.8	-	-	175.2
5	-	-	136.5	-	-	136.6
6, 10	2H	7.76 (d) / 7.5	134.0, 136.5	2H	7.72 (d) / 7.5	134.0, 136.6
7, 9	2H	7.20 (d) / 8.0	123.6, 124.4	2H	7.25 (d) / 8.0	123.7, 124.7
8	-	-	163.9	-	-	164.0
11	-	-	-	-	-	-
12	3H	3.56 (s)	44.6	3H	3.45 (s)	45.0
13	3H	3.35 (s)	33.7	3H	3.32 (s)	33.7
14	1H	3.90-4.12 (m)	34.3	1H	4.43-4.50 (m)	34.3
15, 16	6H	1.32 (d) / 6.8	22.5	6H	1.30 (d) / 6.8	22.5
17	1H	6.65 (d) / 15.0	124.3	1H	7.67 (d) / 15.1	124.7
18	1H	5.60 (dd) / 6.0, 15.0	142.2	1H	5.61 (dd) / 6.2, 15.1	142.2
19, 21	2H	3.40-3.60 (m)	69.1, 72.3	4H	3.65-3.78 (m)	68.5, 71.6
19, 21	2H (OH)	4.0-4.5 (m)	-	2H (OH)	4.40-4.60 (m)	-
20	2H	1.55-1.71 (m)	45.3	2H	1.52-1.78 (m)	41.8
22	2H	2.25-2.42 (m)	42.7	2H	2.50-2.70 (m)	42.7
23	-	-	182.5	-	-	176.9
24	-	-	-	3H	3.10 (s)	35.0
25	-	-	-	2H	3.50-3.55 (m)	52.9
26,27,28, 29	-	-	-	4H (OH)	3.61-3.82 (m)	-
27, 28, 29				3H (CH)	3.40-3.50 (m)	72.7, 73.5, 74.6
30	-	-	-	2H (CH)	3.90-4.20 (m)	65.2
30	-	-	-	1H (OH)	3.81-3.90 (m)	-

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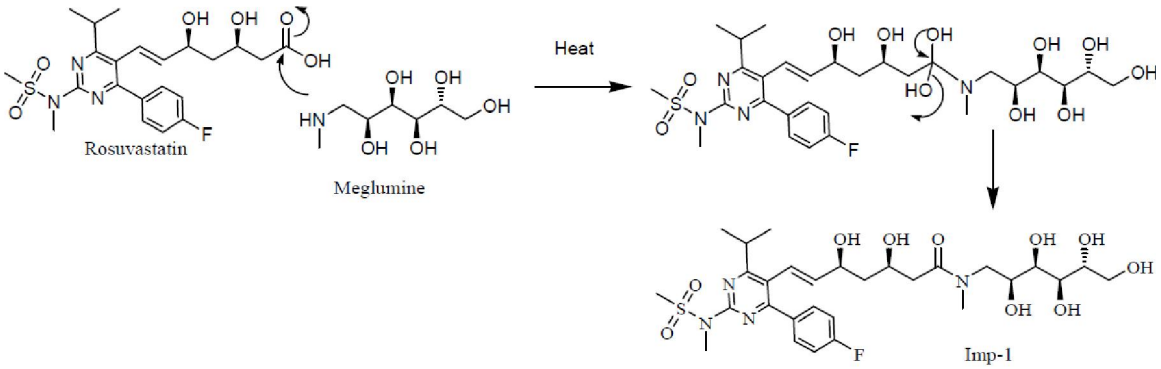

Figure 5 : Plausible reaction pathway for the formation of Imp-1

TABLE 3 : System suitability data

S. No	Impurity Name	RT(in min)	RRT	USP Resolution	USP tailing factor	USP Plate Count	%RSD[a] (n=6)
1	Imp-1	16.816	0.68	NA	1.1	16504	-
2	RSV	24.669	-	14.9	1.1	36831	0.6
3	Imp-A	26.766	1.09	3.9	1.1	19686	-
4	Imp-B	29.057	1.18	4.5	1.0	25813	-
5	Imp-C	30.330	1.23	2.8	1.0	24144	-

n: Number of determinations, RSD: Relative standard deviation, a: RSV diluted standard (2 µg/mL)

buffer, pH 6.0) and ACN as mobile phase-B, Waters X terra RP-18 (150 mm X 4.6 mm, 5µm) column with flow rate 1.0 mL/min on HPLC equipped with photo diode array detector. Resolution between RSV & Imp-A and Imp-B &Imp-C was less than 1.5 and poor impurity peak shapes were observed. To increase the resolution and good peak shapes, many attempts were made with modified mobile phase-A (buffer (0.025M KH_2PO_4, pH 3.0 adjusted with H_3PO_4) and methanol in the ratio 80:20 (v/v)) and mobile phase-B (mixture of buffer, ACN and methanol in the ratio 25:15:60 (v/v) using X-Bridge C18, (150 mm×4.6 mm, 3.5 µm). On the optimization of gradient program, RSV and all four impurity peaks were well resolved from each other and degradation products. Based on these experiments, the final optimized conditions are described below.

Waters X-Bridge C18 (150 mm x 4.6 mm) 3.5 µm particle size column was used as stationary phase. The mobile phase-A consisted of 0.025M KH_2PO_4 buffer (pH 3.0 adjusted with H_3PO_4) and methanol in the ratio 80:20 (v/v) and The mobile phase-B contained a mixture of buffer, ACN and methanol in the ratio 25:15:60 (v/v), respectively. The gradient program (time (min)/%B) was set as 0.01/40, 20/50, 45/90, 55/90, 56/40 and 60/40. The flow rate of the mobile phase was set at 1.0 mL/min. The column temperature was maintained at 30° C and the eluted related compounds were monitored at the wavelength of 248 nm. The sample injection volume was 10 µl. A mixture of water and ACN in the proportion of 30:70 (v/v) was used as a diluent.

Using these chromatographic parameters, efficient separation was achieved for all four impurities and RSV with resolution more than 2.0. Typical blend chromatogram of RSV and its impurities is shown in Figure 2H. Placebo interference was also verified and found that no interference was observed at the retention time of any of the impurities and RSV.

Method validation

The proposed method was validated according to the ICH guidelines[27] for its, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, solution stabilityand robustness. Validation study was carried out for the analysis of Imp-1, Imp-A, Imp-B, and Imp-C. Moreover, relative response factors (RRF) for the impurities of RSV was also determined.

System suitability

System suitability shall be checked for the con-

formance of suitability and reproducibility of the chromatographic system for analysis. The system suitability was evaluated on the basis of resolution, RSD (%) of the peak are, USP tailing factor and USP plate count for the RSV peak from the standard solution. (TABLE 3).

Specificity

Specificity is the ability of the method to unequivocally assess the analyte response in the presence of its potential impurities. Placebo interference was evaluated by analysing the placebo prepared as per the test method. No peak due to the placebo was detected at the retention time of RSV and its impurities. Significant degradation was observed under acid hydrolysis, peroxide degradation, photo degradation and slight degradation was observed under base hydrolysis, water degradation, humidity degradation and thermal degradation. All the forced degradation samples were analysed using a PDA detector to ensure the homogeneity and purity of the RSV peak. The results from the peak purity assessment revealed that the purity angle was less than the purity threshold in all of the stressed samples. The mass balance (% assay + % sum of all impurities) results were calculated and found to be more than 97.0% (TABLE 4). The purity of RSV was unaffected by the presence of its impurities, degradation products, and other excipients (placebo) and thus confirms the stability-indicating power of the method.

Sensitivity

The LOD and LOQ values for all impurities were determined by injecting a series of diluted solutions with known concentration to obtain S/N ratio values of 3 and 10, respectively. For LOQ, the signal to noise ratio's for all the impurities were ranging from 9.7 to 10.4. Precision study was performed at the LOQ level by injecting six individual preparations of RSV and impurities and calculated the %RSD for the areas of each peak. The RSDs were found to be between 1.9 to 5.1%. Accuracy at LOQ level was verified by injecting three individual preparations of RSV spiked with impurities at LOQ level. The results were shown in TABLE 5.

Precision

The repeatability and ruggedness of the method was performed by six individual determinations of RSV (1000 µg/mL) by spiking with impurities at the specification level. The ruggedness was determined by repeating the same experiment on two different days by different analysts using different equipment. The % RSD was calculated for each impurity (TABLE 5). These results confirmed the high precision.

TABLE 4 : Summary of forced degradation results

Degradation condition	% Assay	Mass balance (%Assay + % deg.products)	RS by HPLC % degradation	Remarks/observation
Acid hydrolysis (0.1NHCl, 60°C,2hrs)	93.5	99.5	6.04	Imp-A and Imp-C degradation products were formed
Base hydrolysis (5N NaOH, 60°C,12hrs)	97.6	97.8	0.25	No significant degradation observed
Water hydrolysis (60°C,30 min)	98.5	98.8	0.29	No significant degradation observed
Oxidation (10.0% H_2O_2, 60°C,30min)	96.5	97.9	1.45	Imp-A, Imp-B, Imp-C and low level unknown degradation products were formed
Photo degradation: Exposed to 200 watt hour/m^2 and 1.2 million lux hour in Photo stability chamber for 16hours	85.2	97.2	12.0	Two major unknown degradation degradation products were formed.
Thermal degradation (105°C,6hrs)	98.1	98.6	0.46	Low level Imp-1, Imp-A, Imp-B, and Imp-C degradation products were formed
Humidity at 90% RH (25°C,7Days)	98.4	98.7	0.25	No significant degradation observed

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TABLE 5 : Summary of method validation for RSV and its impurities

Validation Parameter	Imp-1	Imp-A	Imp-B	Imp-C	RSV
Specifications (%)	0.5	0.5	0.5	0.5	-
RRF	0.65	0.94	0.61	1.03	-
LOD (%)	0.01	0.01	0.01	0.01	0.01
LOQ (%)	0.03	0.03	0.03	0.02	0.03
LOQ Accuracy	103.2	99.1	101.2	98.8	102.5
%RSD (n=6) [a]	1.9	3.9	5.1	4.6	2.3
%RSD (n=6) [b]	0.6	0.6	0.5	0.7	-
%RSD (n=6) [c]	1.1	0.8	1.8	2.1	-
Accuracy at 50% (n=3)	95.6	105.2	102.1	101.1	-
Accuracy at 100% (n=3)	96.1	104.8	105.1	102.1	-
Accuracy at 150% (n=3)	94.9	104.1	107.1	101.8	-
		Regression equation(y)			
Correlation	0.999	0.9999	0.9998	1.00000	0.9999
Slope(b)	16361.895	21913.77	14368.61	24450.54	23842.5
Intercept(a)	626.869	553.4708	338.6483	233.5764	179.7495
% Y-intercept	1.9	0.51	0.49	0.19	0.15

[a] LOQ Precision [b] Repeatability [c] ruggedness(Intermediate precision)

TABLE 6 : Results of robustness study

Parameter/variation	Relative retention time			
	Imp-1	Imp-A	Imp-B	Imp-C
As such conditions	0.68	1.08	1.18	1.23
Organic solvent in mobile phase -A				
a) 90% of Methanol	0.69	1.08	1.16	1.21
b) 110% of Methanol	0.67	1.1	1.22	1.29
Organic solvent in mobile phase -B				
a) 90% of Methanol	0.68	1.08	1.16	1.21
b) 110% of Methanol	0.68	1.09	1.19	1.24
Organic solvent in mobile phase -B				
a) 90% of ACN	0.68	1.09	1.18	1.23
b) 110% of ACN	0.68	1.11	1.23	1.30
Mobile phase pH				
a) pH :2.8	0.66	1.11	1.24	1.3
b) pH 3.2	0.69	1.07	1.16	1.21
Flow rate (mL/min)				
a) 0.8 ml/min	0.72	1.06	1.13	1.18
b) 1.2 ml/min	0.67	1.1	1.21	1.27
Column Temp. (?C)				
a) 25°C	0.70	1.07	1.15	1.19
b) 35° C	0.68	1.09	1.19	1.25

Linearity

Linearity test solutions were prepared from impurity stock solution at six different concentration levels ranging from LOQ to 150% of the specification level (i.e. LOQ, 1.0, 3.0, 5.0, 6.0, 7.5µg/mL for Imp-1, Imp-A, Imp-B, Imp-C and RSV). The calibration curve was drawn by plotting impurity area

versus the concentration. The correlation coefficient obtained was greater than 0.999 for all the impurities (TABLE 5). The result showed an excellent linear response for RSV and its impurities.

Accuracy

Accuracy of the method was evaluated by spiking with known amounts of impurities to the placebo-based solution of test sample (1000 µg/mL) at the level of 50%, 100% and 150% of specification in triplicate. The percent recoveries were calculated for related substances and those are ranging from 94.9% to 107.1% (TABLE 5).

Solution stability and mobile phase stability

The solution stability and mobile phase stabilities at 25°C temperature were evaluated by injecting the test solutions spiked with impurities daily for up to 5 days. Prepared mobile phase was kept constant during the study period. No significant changes in the amounts of impurities were observed during the study. These results confirmed that sample solution and mobile phase were stable up to 5 days at ambient temperature.

Robustness

To determine the robustness of the method, experimental conditions were deliberately altered. The factors chosen for this study, which were the critical sources of variability in the operating procedures such as flow rate (1.0 ± 0.2 mL/min), mobile phase pH (3.0 ± 0.2), mobile phase composition (mobile phase A; ± 10% ACN, mobile phase B; ± 10% ACN and 10% Methanol) and column oven temperature (30 ± 5°C) were identified. Resolution between RSV and impurities was greater than 2.0and there was no significant change in relative retention time for impurities in spiked sample illustrating the robustness of the method(TABLE 6).

CONCLUSION

In this study, a new impurity of RSV drug product was found during the analysis of accelerated stability storage samples by HPLC method. The impurity was identified by LC/MS/MS analysis and plausible structure was proposed. Stress studies were performed to enhance the impurity. Stress results exhibited that the dry heat has more escalated the impurity. The impurity was isolated by a preparative HPLC method and characterized by LC/MS, HRMS, NMR and IR spectroscopic techniques. Based on the spectral data, the molecular formula of this impurity was deduced as $C_{29}H_{44}N_4O_{10}$ FS and the corresponding structure was characterized as 3R,5S,E)-7-(4-(4-fluorophenyl)-6-isoprpyl-2-(N-methylmethylsulfonamido)pyrimidin-5-yl)-3,5-dihydroxy-N-methyl-N-((2S,3R,4R,5R)- 2,3,4,5,6-pentahydroxyhexyl)hept-6-enamide. The proposed method was validated as per the ICH guidelines and found to be specific, precise, accurate, linear and stability indicating. Thus, the method can be used for quality control analysis of pharmaceutical dosage forms.

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COMPETING INTERESTS

The authors declare no conflict of interest

REFERENCES

[1] Drugs.com, Rosuvastatin calcium.http://www.drugs.com/ppa/rosuvastatin-calcium.html
[2] Rxlist, Rosuvastatin calcium.http://www.rxlist.com/crestor-drug.htm
[3] Drug Bank, Rosuvastatin calcium.http://www.drugbank.ca/drugs/DB01098.
[4] B.Patel, A.Jadav, H.Solanki, S.Parmar, V.Parmar, C.Anadikumari; Int.J.Pharm.Rev.Res., **2,** 1-6 **(2013)**.
[5] A.Afroz, T.Haque, Talukder.M.U.Md, S.M.A.Islam; Asian J.Pharm.Ana., **1,** 74-78 **(2011)**.
[6] G.K.Anuradha, S.D.Vishal; Int J Pharm.Pharm.Sci., **2**, 131-138 **(2010)**.
[7] Ramadan.A.A, Mandil.H, Alshelhawi.N; Int J Pharm Pharm Sci., **6**, 579-585**(2014)**.

Full Paper

[8] Krishnaiah.Ch, Vishnu Murthy.M, Durga Prasad.B.J, Satyanarayana, Kumar.R, Mukkanti.K; Anal Chem : Ind J., **8**, 277-283**(2009)**.
[9] Chakraborty.A.K, Mishra.S.R, Sahoo.H.B; Int J, Anal.Bioanal.Chem., **1**, 89-101**(2011)**.
[10] Turabi.Z.M, Khatatbeh.O.A; Int.J.Pharm.Sci.Drug.Res., **6**, 154-159**(2014)**.
[11] Tajane.D, Raurale.A.M, Bharande.P.D, Mali.A.N, Gadkari.A.V, Bhosale.V.R; j.chem.pharm.res., **4**, 2789-2794**(2012)**.
[12] Vijay.G.D, Pintu.P.B, Bhavin.M.P, Sailesh.S.A; Int.res.J.pharm., **3**, 173-175 **(2012)**
[13] Ankireddy.S, Venkateswararao.P, Sudhakarbabu.AMS, Pramod.N; Int.J.Bio.Pharm.Res., **3**, 935-941**(2012)**.
[14] Beludari M I, Prakash K V, Mohan G K.; Int.j.chem.Ana.Sci., **4**, 205-209**(2013)**.
[15] Sharma.T, C.SI Sudam, Sankar.D.G; Int J Chem tech Res., **6**, 1115-1123**(2014)**.G.S.Devika, Sudhakar.M, Venkateshwararao.J; Int.J.Bio.Pharm.Res., **3**, 311-315**(2011)**.
[16] Trivedi.H.K, Patel.M C; Scipharm., **80**, 393-406 **(2012)**.
[17] ICH Q3A(R2) Impurities in New Drug substances, **(2006)**
[18] ICH Q3B (R2), Impurities in New Drug Products, **(2006)**
[19] U.S.Food and Drug Administration Drug Stability Guidelines, February, **(1987)**.
[20] S.Ahuja, K.M.Alsante; Eur.J.Pharma.Biopharma., **65**, 257-258 **(2007)**.
[21] S.W.Baertschi; Trends.ana.chem., **25**, 758–767 **(2006)**
[22] Olsen A.Bernard, Castle C.Bryan, Myers P.David; Trends.ana.chem., **25**, 796–805 **(2006)**.
[23] Krishnaiah.Ch, R.A.Raghupathi, Kumar.R, K.Mukkanti; J.Pharm.Biomed.Anal., **53**, 483-489**(2010)**.
[24] ICH Q1A (R2), Stability testing of new drug substances and products, **(2003)**
[25] ICH Q1B, Photo stability testing of new drug substances and products, **(1996)**
[26] ICH Q2 (R1), Validation of analytical procedures: Text and methodology, **(2005)**.