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Research Article

Validation of Reverse Phase High-performance Liquid Chromatography Analytical Method for Osimertinib Mesylate and its Degradation Products for Osimertinib Mesylate Tablets

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ABSTRACT

The purpose of this study is to develop an accurate, simple, and fast reverse-phase high-performance liquid chromatographic (RP-HPLC) method and validate for analyzing organic impurities in osimertinib mesylate (OSM) tablets. The developed method separates and validated for chloro impurity, hydroxy impurity, methoxy impurity, des-acrylic acid impurity, N-oxide, OSM dimer and OSM. The separation of all these impurities were achieved on inert sustain C18 (4.6×250) mm, 3 µ column, The mobile phase consists of 0.1% formic acid with pH to 6.50 adjusted with ammonium hydroxide and acetonitrile delivered in gradient elution mode. The analytes were monitored at 268 nm. Stress studies were performed to evaluate the capability of stability-indicating of this method. The OSM was observed to be stable under thermal, photolytic and neutral conditions. However, it is labile under acidic, basic and oxidative conditions. All the degradants are well resolved form the principle peak OSM. All the major degradation products were isolated and characterized through UHPLC-IMS-Q-TOF-MS. The method was validated as per International Conference on Harmonization (ICH) guidelines, and the validation included specificity, limit of detection (LoD), limit of quantification (LoQ), linearity, accuracy, precision, robustness, and solution stability for isolated impurities and OSM. The method was developed for this drug product with the purpose of properly defining the quality specification of the drug product or drug substance. This method can be incorporated in the monographs of USP, EP/BP, IP etc.

Introduction

The OSM refer to Table 1 with IUPAC name: "N-(2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl) acrylamide" molecular formula: $C_{28}H_{33}N_7O_2$; formula weight: 499.61 Da and CAS No :1421373-65-0) appears as non-hygroscopic crystalline powder with tinted from white to brown.[1,2] More effective in "non-small cell lung cancer" (NSCLC).[3-6] The research has been made by Astra-Zeneca on OSM formulations and they developed Tagrisso™ tablets and secured the approval in November 2015 in USA, for therapy of patients with NSCLE.[7] It is 3rd era EGFR-TKIs, it is effective to altered response of tumor with treatment and it has less toxic side effects which do not affect patient's quality of life.[8]

In terms of safety and efficacy of the medicine in patient's standpoint the purity of the therapeutic drug is important, impurity exposure should be avoided or may be minimal.[9] If there is an additional impurity seen in product, the therapeutic index value may get altered.

Profiling of impurities is utmost important quality features for pharmaceutical preparations, ensuring that the patient receives a pure and safe medicine.[10] As a result, chromatography is critical in the separation of process/

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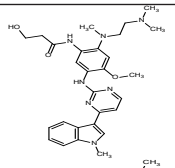
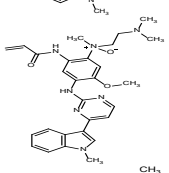
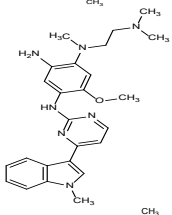
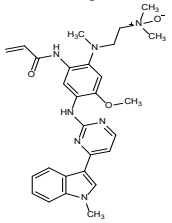
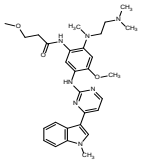
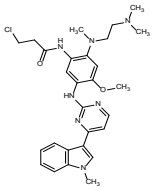
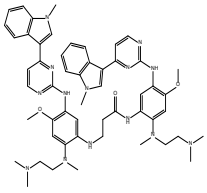
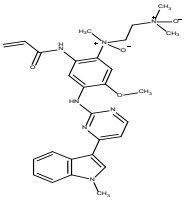
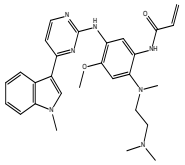
Table 1: Chemical structures and IUPAC names of OSM impurities

S. No.	*Name of Impurity*	*Chemical Structure*
1	**Hydroxy OSM Impurity (DP-1)** N-(2-((2-(dimethylamino)ethyl) (methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl)amino)phenyl)-3 hydroxypropanamide (Hydroxy OSM impurity).	
2	**N-oxide OSM Impurity (DP-2)** 2-acrylamido-N-(2-(dimethylamino)ethyl)-5-methoxy-N-methyl-4-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl)amino)aniline oxide	
3	**Des-acryl OSM Impurity (DP-3)** N1-(2-(dimethylamino) ethyl)-5-methoxy-N1-methyl-N4-(4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)benzene-1,2,4-triamine	
4	**N-oxide OSM Impurity (DP-4)** 2-((2-acrylamido-5-methoxy-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl) (methyl)amino)-N,N-dimethylethan-1-amine oxide	
5	**Methoxy OSM Impurity (DP-5)** N-(2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl)amino)phenyl)-3-methoxypropanamide	
6	**Chloro OSM Impurity (DP-6)** 3-chloro-N-(2-((2-(dimethylamino) ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)propenamide	
7	**OSM Dimer Impurity (DP-7)** N-(2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl)amino)phenyl)-3-((2-((2-(dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)amino)propenamide	
8	**N,N dioxide OSM Impurity (DP-8)** N1-(2-acrylamido-5-methoxy-4-((4-(1-methyl-1H-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-N1,N2,N2-trimethylethane-1,2-diamine dioxide	
9	**Osimertinib (OSM)** 2-Propenamide, N-[2-[[2-(dimethylamino) ethyl]methylamino]-4-methoxy-5-[[4-(1-methyl-1H-indol-3-yl)-2-pyrimidinyl]amino]phenyl]-	

degradation impurities; based on molecule chemistry a suitable column was chosen for analytical method development to determine the purity of OSM tablets. According to the various regulatory and ICH guidelines the stability testing is important to determine the potency of drug.[11] Which can also test the effect various external physical and chemical parameters.[12]

Self-life would be estimated for the formulations or API with the aid of data generated from stability intervals. As a result, drug stress study is vital for forecasting outcomes for analytical methods and quantification of impurities of OSM tablets post validation, which is outlined in this study. All parameters of HPLC were critically tuned for separation of all impurities with consistent results as the resolution and specificity are crucial during quantitative estimation of drug and impurities. Sometime, high concentrations of ion pairing reagents are required to achieve better separation; however there is a high possibility for column deterioration which increases the cost of analysis. As a result, developing a method with volatile buffers are recommended, since it is useful for further impurity identification by LC-MS and due to simple method, the cost of analysis would be optimum. By considering all these factors method has been developed.[13-16]

Validation of the method is critical for further quantification of impurities. This study discusses the complete validation of OSM and its impurities in tablet formulation.

The below listed impurities in Table-1 were isolated by prep HPLC and well characterized by different techniques, it is already discussed in detailed in our previously published literature.[17-19]

Only a few publications have been published on separation, isolation, and identification of OSM DPs using mass spectrometry,[17-19] and estimation of genotoxic chloro impurity for OSM drug material using a QDA-MS detector.[20] The estimation of OSM using techniques such as RP-HPLC,[21] quantitation and validation of OSM in plasma using HPLC-MS/MS.[22] A quantitation method development for pharmacokinetic investigation in rats using UPLC-TOF-MS[23] was published. However, there is no information available for simultaneous separation, identification, quantification, and validation of OSM and its degradants. Thus, a rapid and sensitive methodology for OSM impurities developed and validated further as per ICH refer Table 2 and 3 for chromatography.

Materials and Methods

Vital equipment like HPLC (Shimadzu and Agilent) furnished with mobile phase reservoir, degasser, auto-sampler with temperature controller, column heater, and detector with photodiode array detector. C18 (250×4.6) mm 3 µ column, filtration kit, filters for sample filtration, glass wares, mobile phase filtration assembly etc., were used all over the studies.

Chemicals and Solvents

OSM and isolated impurities were used for validation, viz. hydroxy OSM (DP-1), des-acryl OSM (DP-3), N-oxide OSM (DP-4), methoxy OSM (DP-5), chloro OSM (DP-6) and OSM dimer (DP-7).

Chemicals: Formic acid, ammonia solution, HCl, NaOH, H_2O_2 are of AR grade and were sourced from Merck

Solvents: Methanol, acetonitrile are HPLC grade were sourced from Merck.

Preparation of Mobile Phase

Buffer Preparation

Mobile Phase A: 1000 mL of 0.1% formic acid buffer prepared, and pH adjusted to 6.50 using ammonia solution, sonicate and filtered.

Mobile Phase-B: Acetonitrile

Diluent: Mobile Phase-A: acetonitrile (1:1*v/v*)

Preparation of Solutions

Impurity Stock Preparation (25 ppm)

Approximately weighed and transferred around 5 mg of each OSM impurity into a 200 mL volumetric flask, and approx 40 mL methanol added in same flask, further it was sonicated approximately for 5 minutes, and diluted with diluent.

Preparation of Spiked Test Sample (500 ppm OSM+1 ppm Impurity Mixture)

Weighed and transfer 320 mg of crushed powdered of OSM tablets (equivalent to 50 mg of OSM) sample into 100 mL

Table 2: Chromatographic conditions

Column	*Inersustain C18, (250 x 4.6) mm, 3.0 µ*
Elution mode	Gradient
Flow rate	1.0 mL/min
Detection wavelength	268 nm
Injection volume	10 µL
Column temperature	55°C
Analysis run time	60 minutes
Auto sampler temperature	Room temperature

Table 3: Gradient Program

Time	*Mobile phase A (%)*	*Mobile phase B (%)*
0.0	80	20
15	70	30
35	60	40
40	35	65
41	20	80
50	20	80
50.1	80	20
60	80	20

of flask, added 20 mL methanol, sonicated about 5 minutes then spiked 1-mL of impurity stock and then diluted with diluent for upto the mark. Filtered the solution through 0.45 micron filter and inject.

OSM Standard Stock Preparation (100 ppm)

Weighed approximately 5 mg standard of OSM and transferred into a 50 mL flask, added 10 mL methanol, sonicated about 5 minutes then diluted with diluent for upto the mark of volumetric flask.

System Suitability Preparation (5 ppm OSM +1 ppm Impurity Mixture)

Further dilute 1.25 mL of OSM stock solution into 25 mL volumetric flask and added 1mL of impurity mixture into 25 mL by micropipette and diluted with diluent.

Standard Solution Preparation

Dilute 1-mL stock of OSM (100 ppm) into 20 mL flask and then make the volume with diluent.

Preparation of Test Solution

Approximately weighed and transfer 320 mg crushed powdered of OSM tablets (equivalent to 50 mg of OSM) sample into 100 mL flask, then added 20 mL methanol and sonicated further approximately for 15 minutes then make up the volume with diluent. A 0.45 micron Polytetrafluoroethylene (PTFE) filter was used to filter the solution and resultant solution was injected on HPLC.

RESULTS AND DISCUSSION

Method Development and Optimization

Before initiation to development of liquid chromatography method, all the properties of the OSM were critically reviewed and found that RP-HPLC could be appropriate technique for OSM and its impurities in formulation. Different solvent compositions were used for development trials; later pH of water varied and mixing with various ratios of acids/bases were also tried. Different buffers were also experienced with various types of inorganic salts and during these trials, different characteristic of stationary phase combination was also evaluated.

Initially, published literature methods, were evaluated for all the impurities for separation however, all the forced degradation impurities were not resolved properly, therefore developed a new method to resolve all the degradants and further validated for intended use.

The separation of impurities from each other and OSM peak were optimized using mobile phase ratios. Physicochemical properties study showed OSM has good absorbance at 268 nm and found suitable for detection. To reduce the analysis time and for well eluted peaks of impurities; gradient elution and flow rate of 1-mL/min were optimized; the mobile phase consists of a mixture of Buffer: Acetonitrile in varied ratios with a gradient

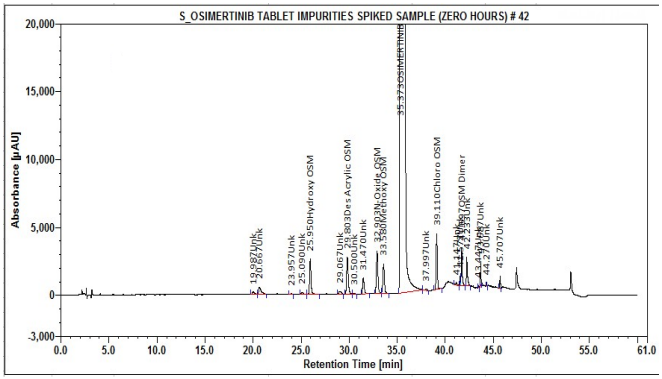


Fig. 1: Chromatogram for Test solution +Impurity spiked at LoQ. The novel developed method was validated in accordance with ICH guidelines.

Table 4: Results of system suitability study

Compound	*RT*	*RRT*	*Resolution*	*Tailing factor*	*%RSD (n=6)*
Hydroxy OSM	25.99	0.73	9.74	1.2	1.0
Des Acrylic OSM	29.87	0.83	14.3	1.3	1.2
N-Oxide OSM	32.90	0.92	10.5	1.1	0.6
Methoxy OSM	33.67	0.94	2.8	1.2	0.6
Chloro OSM	39.15	1.10	4.3	1.1	0.6
OSM Dimer	41.76	1.17	12.1	2.4	4.3
OSM	35.73	1	--	1.2	0.6

Limits: Resolution: NLT (not less than) 2.0, % RSD for six replicates: NMT (not more than) 5.0%, Tailing factor: NMT 2.0%

elution program. After using different columns for trials, inersustain C18 (250×4.6) mm, 3.0 µ column was found suitable. To retain the sharpness of the impurity peaks column heater was optimized to 55°C. These chromatographic parameters were found to be appropriate and optimized for conducting analysis, resulting in a good separation of all impurities and OSM. The retention times for OSM and six impurities (considered for validation) were eluted 41.76 and 25.99, 29.87, 32.90, 33.67, 39.15, 35.73 minutes, respectively.

Fig. 1 shows the chromatogram of test solution spiked with impurities on the optimized method.

Impurities in OSM tablets were successfully identified and quantified using this method.

System Suitability and Specificity

Six times, standard solution preparation was injected on the developed analytical methodology to test system suitability. The tailing factor, percent RSD, and theoretical plates were all measured. The spiked solution with all impurities does not show any interference. The specificity of the sample was also determined by forced degradation study, to prove the interference of degradation impurities with active and placebo. It is demonstrated that the testing

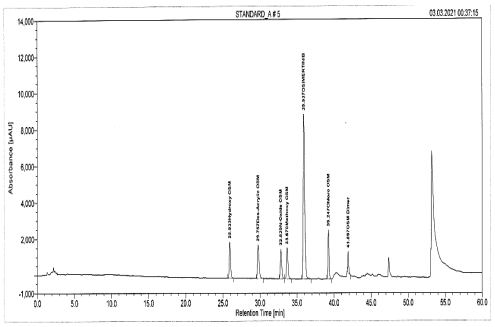


Fig. 2: Specificity chromatogram of Impurity spiked solution

procedure is unique, and it can be adopted for stability analysis. The findings of the system suitability study and FD dada were assembled and shown in Table 4 and 5. Fig. 2 presents chromatogram of impurity and OSM spiked in solution, which do not show any interference with placebo and any other impurity.

Forced Degradation

To demonstrate the method's specificity, degradation tests were carried out under the following conditions.

Acid Hydrolysis

Weighed and transfer approx 320 mg of crushed powdered of OSM tablets (corresponding to 50 mg of OSM) sample into 100 mL of volumetric flask, further added 20 mL methanol, and approx. 15 minutes sonicated. The 20 mL diluent was added in the same solution and swirl to mix, then 10 mL 1N HCl was added and refluxed upto 2 hours. Then the solution was cooled and neutralized with 10 mL 1N NaOH and make up the volume with diluent. Further filter the solution through 0.45 micron PTFE filter and use.

Base Hydrolysis

Weighed and transfer approx 320 mg of crushed powdered of OSM ablets (correspondent to 50 mg of OSM) sample into 100 mL of volumetric flask, further added 20 mL of methanol, sonicated about 15 minutes. The solution was then diluted with 20 mL diluent, swirl to mix, then 10 mL 1N NaOH was added and refluxed upto 2 hours. Then the solution was cooled and neutralized with 10 mL 1N HCl and make up the volume with diluent. Further filter the solution through 0.45 micron PTFE filter and use.

Oxidation

Weighed and transfer approx 320 mg of crushed powdered of OSM tablets (corresponding to 50 mg of OSM) sample into 100 mL flask, further 20 mL methanol added, approx. 15 minutes sonicated. Then added 20 mL diluent in same solution, swirl to mix properly, further, 10 mL 3% H_2O_2 (hydrogen peroxide) was added. The resultant sample heated at 80°C for 2 hours, Then the solution was cooled, make up the volume with diluent. Further filter the solution through 0.45 micron PTFE filter and use.

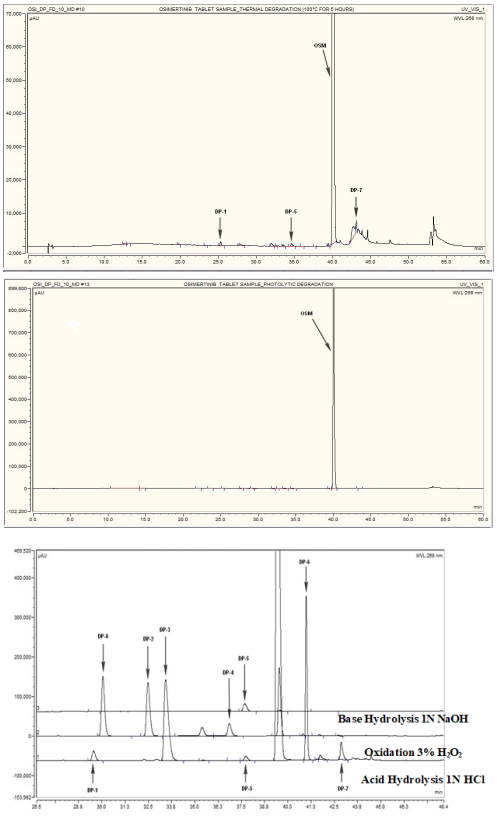


Fig. 3: a) Thermal exposure, sample exposed to 100°C for 5 days b) photo exposure, sample were kept under 1.2 lux hours for 7 days c) overlaid chromatogram of Base Hydrolysis, 10 mL 1N NaOH refluxed for 2 hours, Oxidation, 3% H_2O_2 heated at 80°C for 2 hours, Acid Hydrolysis, 10 mL 1N HCl refluxed for 2 hours to prove specificity.

Water Hydrolysis

Weighed and transfer approx 320 mg of crushed powdered of OSM Tablets (corresponding to 50 mg of OSM) sample in 100 mL of flask, then 20 mL methanol was added, further approx. 15 minutes it was sonicated. Then added 20 mL diluent in same solution, swirl to mix, then 10 mL water was added and refluxed upto 2 hours. Then the solution was cooled and make up the volume with diluent. Further filter the solution through 0.45 micron PTFE filter and use.

Thermal exposure

Weighed and transfer approx 320 mg of crushed powdered of OSM Tablets (corresponding to 50 mg of OSM) sample into 100 mL of volumetric flask, then 20 mL methanol was added, further approx. 15 minutes it was sonicated. It was then diluted with 20 mL diluent, swirl to mix, further the sample was exposed to 100°C for 5 days. Then the solution was cooled and make up the volume with diluent. Further filter the solution through 0.45 micron PTFE filter and use.

Additionally, dry powder sample was also expose to 100°C for 5 days. Above same procedure followed for sample preparation.

Table 5: Results of forced degradation study (Specificity)

Analyte	*Degradation type (Degradation in %)*				
	Acid	*Alkali*	*Oxidative*	*Thermal*	*Photolytic*
Hydroxy OSM (DP-1)	1.365	2.877	ND	ND	0.027 (BLOQ)
N-Oxide OSM (DP-2)	ND	ND	29.382	ND	ND
Des Acrylic OSM (DP-3)	15.551	ND	ND	0.081 (BLOQ)	0.023 (BLOQ)
N-Oxide OSM (DP-4)	0.065 (BLOQ)	ND	7.392	0.101	0.111
Methoxy OSM (DP-5)	0.652	82.166	ND	ND	0.048 (BLOQ)
Chloro OSM (DP-6)	11.378	ND	0.502	0.02 (BLOQ)	0.084 (BLOQ)
OSM Dimer (DP-7)	1.822	ND	0.212	0.72	0.014 (BLOQ)
N,N Dioxide OSM (DP-8)	ND	ND	30.078	ND	ND

BLOQ: Below limit of quantification, **ND:** Not detected

Table 6: Results of limit of detection and quantification study

Peak Name	*Limit of Detection*			*Limit of Quantification*			
	In ppm	*In %*	*S/N ratio*	*In ppm*	*In %*	*S/N ratio*	*% RSD (n=3)*
Hydroxy OSM (DP-1)	0.101	0.024	17	0.42	0.10	67	3.7
Des Acrylic OSM (DP-3)	0.104	0.025	6	0.34	0.081	30	4.3
N-Oxide OSM (DP-4)	0.099	0.024	12	0.42	0.100	45	2.6
Methoxy OSM (DP-5)	0.100	0.024	15	0.37	0.088	58	2.5
Chloro OSM (DP-6)	0.103	0.024	21	0.49	0.117	83	0.9
OSM Dimer (DP-7)	0.098	0.023	20	0.40	0.095	47	2.0
OSM	0.100	0.024	12	0.38	0.090	47	3.2

Table 7: Results of linearity study of OSM Tablets and impurities

Compound Name	*Linear Conc. (ppm)*	*slope*	*Intercept*	*Correlation coefficient*	*Residual sum of square*	*RRF (for multiplication)*
Hydroxy OSM	0.42-1.56	22984.92	-1.582	1.000	0.999	1.21
Des Acrylic OSM	0.34-1.26	29278.62	-192.707	0.999	0.999	0.95
N-Oxide OSM	0.42-1.52	21916.685	70.799	1.000	0.999	1.26
Methoxy OSM	0.37-1.36	24083.537	7.441	1.000	0.999	1.15
Chloro OSM	0.49-1.61	20507.259	-70.975	1.000	0.999	1.36
*OSM Dimer	NA	NA	NA	NA	NA	NA
OSM	0.38-5.96	27765.311	468.978	0.999	0.999	1.00

* Note: OSM dimer is not stable in the diluent, thus linearity was failed and therefore not considered in above Table

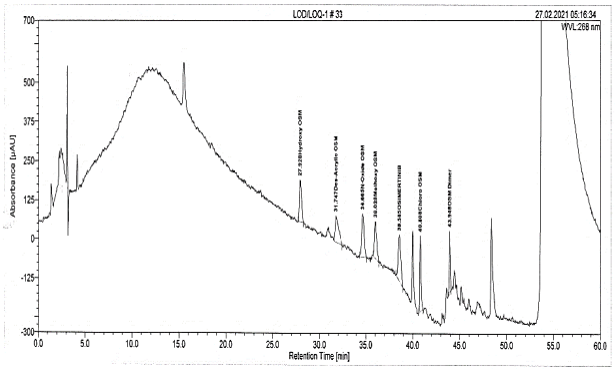


Fig. 4: limit of detection chromatogram for spiked impurities

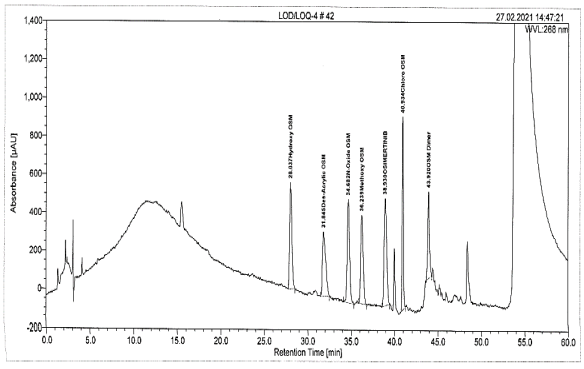


Fig. 5: Chromatogram of limit of quantification for spiked impurities

Table 8: Summary Table for method precision

S. No	*Impurity Name*	*%RSD*
1	Hydroxy OSM (DP-1)	3.1
2	Des Acrylic OSM (DP-3)	4.4
3	N-Oxide OSM (DP-4)	2.5
4	Methoxy OSM (DP-5)	3.5
5	Chloro OSM (DP-6)	3.9
6	*OSM Dimer (DP-7)	11.5
7	OSM	3.7

*Note: dimer is not stable in solution (diluent) form therefore the %RSD is not in limit

Table 9: Results of accuracy (recovery study) of impurities in OSM tablets

Level	*Hydroxy OSM*	*Des Acrylic OSM*	*N-Oxide OSM*	*Methoxy OSM*	*Chloro OSM*
	Recovery%				
	114.08	116.39	113.18	110.48	124.68
LOQ	114.47	118.81	110.53	113.67	126.30
	117.17	118.57	109.03	115.53	120.74
Mean	115.24	117.92	110.91	113.23	123.91
	101.68	103.67	81.43	105.78	106.24
50%	101.87	106.08	85.58	108.04	111.06
	106.43	110.32	87.03	114.33	114.28
Mean	103.33	106.69	84.68	109.39	110.53
	91.36	101.37	94.01	98.24	104.68
100%	89.94	99.12	91.24	95.86	103.29
	91.02	99.22	91.31	97.17	103.57
Mean	90.77	99.90	92.19	97.09	103.85
	100.38	98.79	96.70	105.89	104.39
150%	99.69	98.09	97.27	105.32	101.99
	100.23	104.92	96.89	107.26	103.28
Mean	100.10	100.60	96.95	106.16	103.22

The % recovery for the accuracy levels and %RSD for % recoveries are within the acceptance criteria. Hence the method is accurate

Photo Exposure

Weighed and transfer approx 320 mg of crushed powdered of OSM Tablets (corresponding to 50 mg of OSM) sample in to 100 mL of volumetric flask, then 20 mL methanol

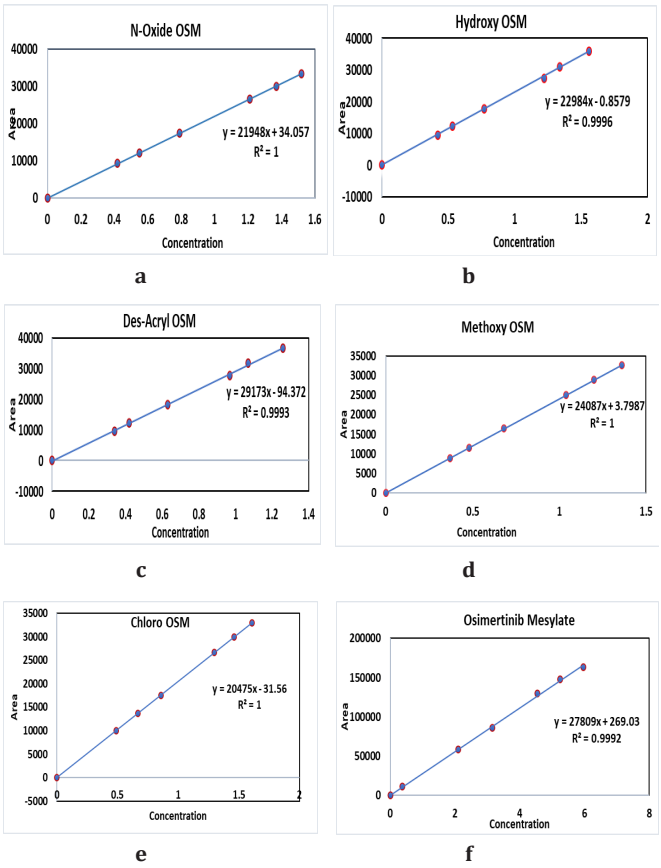


Fig. 6: linearity plots for a) N-Oxide OSM, b) Hydroxy OSM, c) Des-Acryl OSM, d) Methoxy OSM, e) Chloro OSM, f) Osimertinib Mesylate to determine RRF and regression coefficient.

was added, further approx. 15 minutes it was sonicated. It was then diluted with 20 mL diluent, swirl to mix, the sample were kept under 1.2 lux hours for 7 days for photolytic degradation. Same procedure was repeated for dry powder. Then the solution was cooled and make up the volume with diluent. Further filter the solution through 0.45 micron PTFE filter and use. The chromatograms are shown in Fig. 3. The summary of the degradation shown in Table 5.

All parameters of system suitability observed within ICH criteria, typical chromatogram of impurity spiked solution. The forced degradation study proves there is no interference of any other impurity with each other and OSM which evident that the method is specific.

Table 10: Summary Table of solution stability

Impurity Names \ *Intervals*	*initial*	*21 hours*	*26 hours*	*38 hours*	*43 hours*	*Average (% relative difference)*
Hydroxy OSM	0.25	0.04	0.06	0.06	0.02	0.05
Des Acrylic OSM	0.24	0.00	0.08	0.11	0.12	0.08
N-Oxide OSM	0.35	0.00	0.01	0.02	0.09	0.03
Methoxy OSM	0.22	0.03	0.06	0.06	0.05	0.05
Chloro OSM	0.38	0.04	0.08	0.02	0.18	0.01
*OSM Dimer	0.13	NA	NA	NA	NA	NA

*Dimer is not stable in the diluent, therefore its not shown in extended timepoints

Table 11: Robustness results of HPLC method

Impurity Name	*Precision (%RSD, N=6)*	*Temp (- 2) % relative difference n=3*	*Temp (+2) % relative difference n=3*	*Flow (+0.1 mL) % relative difference n=3*	*Flow (-0.1 mL) % relative difference n=3*	*pH (+0.2) % relative difference n=3*	*pH (-0.2) % relative difference n=3*
Hydroxy OSM	3.1	2.0	3.1	0.4	2.2	0.7	0.0
Des Acrylic OSM	4.4	0.3	0.1	3.6	4.5	3.4	24.5
N-Oxide OSM	2.5	1.8	3.9	2.2	0.0	0.0	0.1
Methoxy OSM	3.5	0.8	2.6	0.0	0.5	0.0	0.0
Chloro OSM	3.9	0.8	0.6	2.0	0.0	2.2	3.4
*OSM Dimer	11.5	312.5	9.2	3152.2	1420.4	66.1	10296.1
OSM	3.7	0.6	0.6	0.5	2.4	2.6	0.6

*Dimer is not stable in the diluent, therefore, not considered in the robustness

Limit of Detection and Quantification

To determine LoD and LoQ, various concentrations of OSM and its impurities mixed solutions were prepared and evaluated. The area and response of the chromatogram was determined. The LoD and LoQ were determined by plotting the graph of areas Vs concentration of peak. Table 6 represents the data and Figs 4 and 5 are corresponding to limit of detection and quantification respectively for spiked impurities.

The s/n ratio is well within limit. Typical limit of detection level chromatogram as depicted in Fig. 4 and for Limit of quantification refer to Fig. 5. The %RSD for retention time and area are NMT 2.0% and NMT 5.0%, respectively. Also signal to noise ratio is well within limit.

Linearity

Linearity was plotted for series of spiked impurities and OSM solutions from LoQ to 150% of specified level of impurity and injected on HPLC. The mean peak area response was determined from the chromatograms. Further the areas were utilized to plot the linearity in comparison with concentration. Further the regression technique was used to calculate the intercept and correlation coefficient (r). The r value is observed well within the acceptable limit 0.999.

Table 7 shows the linearity data, and Fig. 6 shows the linearity plot of impurities and OSM.

The correlation coefficient is observed NLT 0.999, The % RSD for sum of the peak area response is observed within limit NMT 2.0% and %RSD for retention time is observed within NMT 2.0%.

The concentration and corresponding peak area of OSM and impurities have a strong linear relationship which evident the developed novel method is linear. The individual plots of linearity shown in Fig. 6.

Determination of RRF for Impurities

RRF= Slope of OSM linearity curve/slope of impurity curve.

Precision

According to precision is the degree of reproducibility of an analytical process under normal conditions. The developed analytical method precision was determined by method precision, system precision, and intermediate precision.

System Precision

The system precision was determined by producing an optimized OSM standard solution and evaluating it in six replicates using liquid chromatography. The area response %RSD was calculated and found to be 0.60%, which was within the 5.0% acceptability requirement.

Method Precision

Repeatability was carried out with six separate sample preparations and injected on newly developed HPLC method. The impurities percentage and RSD were determined and found to be within the acceptability standards, which indicates the established testing procedure is precise.

Intermediate Precision (Ruggedness)

The experiment was repeated with a different instrument, column, and day by using six preparations differently, to demonstrate the method's reproducibility. The impurities percentage and RSD were determined and found to be within the acceptability standards, which indicates the established testing procedure is rugged.

Accuracy Study (Recovery Study)

In the accuracy study known amounts of all impurity concentrations such as LoQ, 50, 100, and 150%, respectively was prepared in triplicate and utilized to test the analytical method's accuracy.

The area of individual impurity in the OSM tablets was determined before and after the impurities was added. The recovery for the LoQ level was in the range of 110.91 to 123.91%, which is well within the acceptance criteria of 70–130%, and for 50, 100 and 150% was observed in range of 84.68–110.53%, which is well within the range of 80–120%. These observations were evident that the developed method is suitable for quantitation. The results tabulated in Table 9.

The % Recovery can be calculated as per below formula.

$$\% \text{ Recovery} = \frac{\text{amount found}}{\text{mount added}} \times 100$$

Stability of Analytical Solution

To test the solution's stability, an impurity spiked solution in OSM tablets was kept at room temperature for an extended period. Sample was injected at regular intervals on HPLC for up to 43 hours. Each time, the area was measured and compared to the initial injection area. The difference between the initial and up to 43 hours was found to be between 0 and 0.18%. The %relative difference for solution stability for area response is determined for all intervals. Table 10 summarizes the findings for solution stability.

When the initial analysis results were compared to the analysis results after each time interval, it was discovered that they were both well within the predetermined limit for the solution stability study. The appropriateness of the system and the sample solution are both stable for up to 43 hours.

Robustness

The robustness of novel analytical method was checked by deliberate changes in chromatographic conditions, as well as tracking the influence on system suitability parameters optimal chromatographic parameters were changed to achieve this. The standard and test solutions were examined under different situations. After the response was read, the chromatograms were recorded. Table 11 summarizes the findings of the observed system suitability parameters requirements, with values falling within the NMT 25% acceptance threshold for concentrations ranging from 0.26 to 1.0%.

Conclusion

Identification and quantitation of impurities in drug substance and drug product by chromatography is made mandatory by various country regulatory authorities due to the precision in reproducing the results. The validated analytical method has an important application in drug quality control for maintain the purity. As per ICH guidance the above said method has been validated. The results summarized in Table 4 and 5 for specificity revealed that blank and its impurities had no effect on OSM. The LoD and LoQ results were observed in the range of 0.1 ppm for LoD and 0.34 to 0.49 ppm for LoQ for all impurities and OSM drug. The proposed methodology yielded the lowest LoD and LoQ values (refer Table 6), indicating the sensitive nature of the developed.

The concentration and corresponding peak regions of OSM and impurities were found to have a good linear relationship (correlation coefficient-0.9990). The linearity ranged from 0.37 to 1.61 ppm (Table 7). The suggested method's accuracy (recovery) of all impurities was investigated by evaluating different known concentration spiked in test product. For recovery were estimated impurities by comparing the area of an OSM sample before and after impurities were added, and the findings ranged from 84.68 to 123.91% (Table 9). The values were found to be within the acceptance requirements, demonstrating the method's accuracy.

The precision of the developed novel method was determined observed in a range of 2.5 to 11.5% RSD. The higher value 11.5% is observed only for dimer due to instability in diluent. However, other impurities are well below the limit of 5% RSD refer Table 8. The results met the acceptance requirements, indicating that the proposed procedure is of a precise nature.

HPLC parameters were purposefully changed for method robustness. The system suitability criterion limits were not significantly changed by the deliberate changes in the method, evident the method is robust.

The above study shows that the method is suitable for quantitation of impurities in OSM tablets and drug substance. It is simple to implement, sensitive, robust, and precise. The newly established method is a superior, cost-effective solution and that can be easily used in quality control labs. It can be incorporated in the various monograph for identification and quantification of related substances of OSM.

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