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Simultaneous Determination of Imipenem and Cilastatin Impurities in Combination Product of Imipenem and Cilastatin Injection by RP-HPLC

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ABSTRACT

A simple, sensitive and robust RP-HPLC method for the determination &quantification of related substances of Imipenem and Cilastatin in Reference Standard as well as in marketed formulation. The chromatographic separation was achieved with gradient elution by using Waters X terra MS (C18, 250 mm×4.6mm, 5.0µm column) with mobile phase composition of solvent A (pH 7.30 phosphate buffer and acetonitrile in the ratio of 98:2 v/v) and solvent B (pH 2.80 phosphate buffer and acetonitrile in the ratio of 68:32 v/v) at a flow rate of 1.0 mL/min and Column oven temperature 35°C. The analytes eluted were detected and quantified at 210 nm using photodiode array (PDA) detector. The specificity of the method was investigated under different forced degradation conditions, including hydrolytic, oxidative, photolytic and thermal as recommended by ICH guidelines. Robustness against small modification in pH, column oven temperature, flow rate and percentage of the mobile phase composition was ascertained. The method was developed and quantified in a manner indicating the stability and the sensitivity of the drug substance.

Keywords: RP- HPLC, Imipenem, Cilastatin, Related substances, Method validation.

INTRODUCTION

Imipenem / Cilastatin [1] IUPAC name [(5R,6S)-6-[(1R)-1-hydroxyethyl]-3({2[(iminomethyl)amino] ethyl} thio)-7-oxo-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylicacid / (Z)-7-[(2R)-2-amino-3 hydroxy -3 oxopropyl] sulfanyl-2-{[(1S)-2,2dimethylcyclopropanecarbonyl] amino} hept-2-enoic acid] is a broad-spectrum beta-lactam antibiotic containing equal quantities of Imipenem (IMI) and Cilastatin (CIL). Imipenem [1, 2] is related to the penicillin/cephalosporin family of antibiotics but is classified as belonging to the carbapenem class. Imipemen (Fig.1) is an off-white, non-hygroscopic crystalline compound with a molecular weight of 317.37 amu. It is sparingly soluble in water, and slightly soluble in methanol. Its having chemical formula of $C_{12}H_{17}N_3O_4S.H_2O$. Cilastatin is a renal ehydropeptidase-I and leukotriene D4 dipeptidase inhibitor. Since the antibiotic, Imipenem,

hydrolised by dehydropeptidase-I, which resides in the brush border of the renal tubule, Cilastatin (Fig.2) is administered with Imipenem to increase its effectiveness. Cilastatin) is off white to yellowish-white, hygroscopic, amorphous compound with a molecular weight of 380.43 amu. It is having great solubility in water and in methanol. Its having chemical formula of $C_{12}H_{17}N_3O_4S.H_2O$.

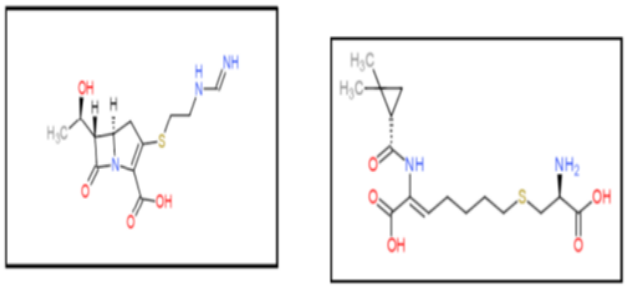


Figure.1 Chemical Structure of Imipenem and Cilastatin.

The literature survey revealed that none of the most recognized pharmacopoeias or any journals includes these drugs in combination for the simultaneous determination of organic impurities of Imipenem & Cilastatin and the information regarding the stability of these drugs is not available. So, it is essential to develop a liquid chromatographic procedure which will serve a reliable, accurate, sensitive and stability indicating RP-HPLC method for the simultaneous determination of related substances of Imipenem and Cilastatin in imipenem and cilastatin injection.

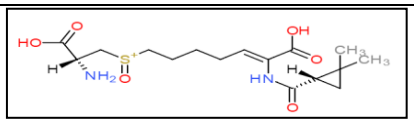
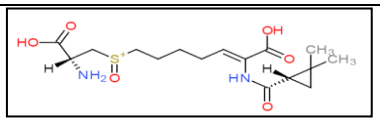
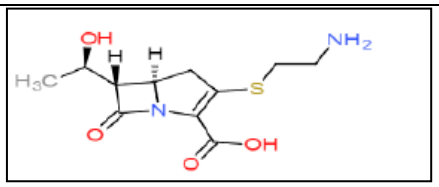
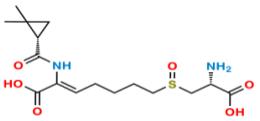
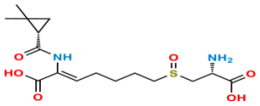
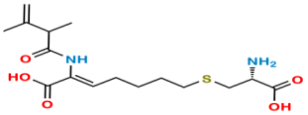
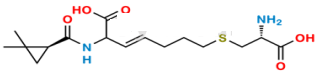
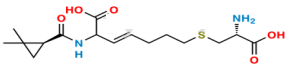
Present paper explains about method development and validation of brief description of organic impurities method for precise and accurate quantification of twelve potential impurities in IMI and CIL Injection as per **International Council for Harmonization** (ICH) recommendation. Degradation study was conducted for finished dosage form to identify degradation activities of the drug product or active pharmaceutical ingredient **(API)**. Forced degradation studies are a part of the analytical development approach and are also an integral constituent of validating analytical method that symbolizes the stability-indicating method and also detecting potential of impurities. This relates to the specificity section of the validation studies as recommended by ICH. To identify these factors analytical method should be stability-indicating and fully validated as per USP and ICH guidelines.

EXPERIMENTAL

Equipment and Chemicals

Drug Standards of pure Imipenem and Cilastatin active pharmaceutical ingredient (API) Samples and Organic impurities of both drugs has been obtained as gift samples received from Glenmark pharmaceuticals Ltd Mumbai. Analytical Grade Sodium dihydrogen phosphate, disodium hydrogen phosphate, ortho phosphoric acid and HPLC grade acetonitrile were procured from Merck chemicals

(Merck Limited, Mumbai, India). Ultrapure water (HPLC Grade) is prepared by using Millipore milli-Q water purification system. All the chemicals and reagents were used as such without purification and these prepared Solutions were filtered through 0.22-µm PVDF membrane filter from Millipore before usage. All the possible impurities that may raise from the IMI and CIL are mentioned below. Imipenem has the impurities of A1, A2 and B and rest all the impurities are for the Cilastatin.

S. No	Impurity Name	Structure
1	Impurity –A_1	
2	Impurity –A_2	
3	Impurity –B	
4	Impurity –C	
5	Impurity-D_1	
6	Impurity-D_2	
7	Impurity- E	
8	Impurity- F_1	
9	Impurity- F_2	

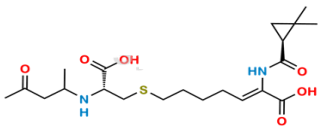
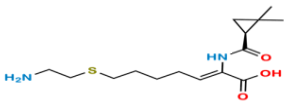
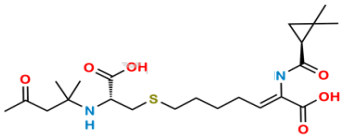
10	Impurity- G	
11	Impurity- H	
12	Impurity- I	

Table.1 Chemical Structure of Liquid Chromatography system.

The Liquid Chromatography system, used for method development and method validation was Waters-HPLC (Waters Corp., Milford, MA, USA) equipped with separation module consisting of binary gradient pump, thermostat column oven compartment, photo-diode array (PDA) detector, auto sampler, computer with Windows based Empower-3 method validation manager software. The output signal was monitored and processed using Empower-3 software. Column used for chromatography was Waters X terra MS (C_{18}, 250 mm×4.6mm, 5.0µm column) made by Waters Corp., Milford, MA, USA.

Chromatographic conditions:

Xterra MS C_{18} column (250 X 4.6mm, 5µm particle size) was used as a stationary phase maintained at oven of 35°C. The mobile phase involved a variable composition of solvent A solution containing p^H 7.30 phosphate buffer and acetonitrile in the ratio of 98:2 v/v and solvent B p^H 2.80 phosphate buffer and acetonitrile in the ratio of 68:32 v/v delivered at a flow rate of 1.0 mL/min. The optimum wavelength selected was 210 nm which represents the wavelength of maximum response for all impurities in order to permit simultaneous determination of related impurities of IMI and CIL.

The forced degradation (stressed) samples were analyzed using a PDA detector covering the range of 200 -400nm. Diluent finalised was 0.09% w/v NaCl in saline water for the dilutions of the drug substances and for their impurities.

S. No	Impurity Name	Retention Times
1	Impurity –A_1	3.230
2	Impurity –A_2	3.562
3	Impurity –B	7.821
4	Impurity –C	10.593
5	Impurity-D_1	38.690
6	Impurity-D_2	39.561
7	Impurity- E	52.31
8	Impurity- F_1	56.235
9	Impurity- F_2	57.021
10	Impurity- G	69.268
11	Impurity- H	71.230
12	Impurity- I	73.269

Table.2 Individual Impurities Retention Times.

Time (min)	Mobile phase A (%v/v)	Mobile phase B (%v/v)
0	100	0
15	100	0
25	90	10
45	75	25
60	60	40
70	40	60
80	28	72
85	0	100
95	0	100
98	100	0
100	100	0

Table.2 Gradient program.

Preparation of standard solution

Standard stock solutions containing about IMI (1 mg/mL) and CLI (1 mg/mL) were prepared in the 0.09% w/v NaCl in water. Sequential dilutions were made with diluent to obtain a concentration of 2 µg/mL each IMI and CLI

Preparation of sample solution

Reconstituted Imipenem and Cilastatin 250 mg with 10mL of diluents, a stock solution of Imipenem and Cilastatin (2.5 mg/mL) was prepared by using diluent. The stock solution diluted accordingly to give solution containing 500 µg/ mL as sample solution

Procedure for method validation

The developed method was subjected to validation for different parameters such as Specificity,
Forced degradation, Precision, Sensitivity (LOD -Limit of detection and LOQ-Limit of Quantification), Linearity, Range, Accuracy and Robustness as recommended by ICH.

Specificity and stress studies

To assess the specificity of the developed method for IMI and CIL Injection, diluent, placebo& sample solutions were prepared and injected. Also, further sequence of injections was performed by preparing and injecting the solutions of Impurity $–A_1$, Impurity $–A_2$, Impurity –B, Impurity –C, Impurity-D_1, Impurity-D_2, Impurity- E, Impurity- F_1, Impurity- F_2, Impurity- G, Impurity- H and Impurity- I of at a level of 0.05% to 0.1% test concentration. Concentrations of impurities were fixed based on nature and response of the impurities.

Forced Degradation Studies

Forced degradation studies of IMI and CIL Injection under different stress conditions Acid hydrolysis (0.025 Molar HCl / Room Temperature / 15 min), Base hydrolysis (0.025 Molar NaoH / Room Temperature / 15 min), Oxidation (0.05 % H2O2 / Room Temperature / 30 min), Thermal (90°C /48 hours), Humidity (90 % Relative Humidity / 25°C / 5 days) and Photolytic (white fluorescent light 10 K Lux for 120 hours UV light of 200 watt Hr/m^2 for 5 days) were performed to quantifies the potential interference's of degradation products.

Method precision

The precision of the method was investigated by analyzing six individual preparations of IMI and CIL Injection spiked with above specified levels of all organic impurities. The percentage RSD for % w/w of each impurity is calculated.

Sensitivity of method (Limit of detection and Limit of Quantitation)

For the establishment of LOD and LOQ values, test solutions were prepared in series from 1 to
150 % level of respective impurity specification level by making dilutions to the impurity stock solution to the required levels. Linearity curves were drawn by impurity concentration (on X-Axis) Vs individual impurity area (On Y-Axis). From these curves, LOD and LOQ were predicted from the formulae $3.3\sigma/S$ and $10\sigma/S$ respectively where σ is the standard deviation of the response and S is the slope of the linearity

curve. Precision was performed at predicted LOD and LOQ levels and finalized the LOD and LOQ concentration values.

Linearity and range

Linearity curves were drawn from the finalized LOQ value to 150 % of the impurity specification level. The correlation coefficient, slope and Y-intercept of the Linearity plots are calculated for each individual impurity. The range of the analytical method as demonstrated from LOQ to 150 % of each individual impurity specification levels.

Accuracy

To assess accuracy, sample solutions were prepared by spiking the impurity stock solutions at LOQ level, 50 %, 100 % and 150 % of the analyte concentration. The % w/w of recoveries for all the impurities was calculated.

Stability of solutions

In order to prove the stability of both standard and sample solutions, these solutions were prepared freshly and injected immediately followed by injecting at periodical intervals by maintaining the solutions at room temperature (~25°C) and refrigerator temperature (~ 6°C) conditions.

Robustness of the method

To check the method robustness, experimental parameters are deliberately changed and the impact of the variation was studied for each impurity. To study the impact of flow rate, ± 0.1 mL/min (±10 %) unit was changed. The effect of column oven temperature (± 5°C) is checked. For variation in gradient program ± 1%, the composition of Mobile phase-B was changed ± 2% absolute. pH of buffer for Mobile phase –B by ± 0.2 units. For wavelength variability, ± 5 nm was varied from the working wavelength. In all the robustness variations, only one parameter was modified by keeping all remaining conditions unchanged.

RESULTS AND DISCUSSION

Validation of stability indicating method Specificity and stress studies

During Specificity experiment, it was observed that diluent and Placebo do not show any interference at the retention times of peaks (Figure a & b). Based on stress studies on IMI and CLI Injection subjected to various stress conditions, it was observed that IMI and CLI Injection is suspectable to degradation under Acid, Base and Thermal degradation conditions (Table. 4), while it is found stable to remaining degradation conditions employed. Further, the evaluation of Peak purity of IMI and CLI peaks from the analysis of every stress condition sample showed that these are homogeneous and have no co-eluting peaks. Evidencing the ability of the method to assess unequivocally the analyte of interest in the presence of potential interference. Baseline resolution was achieved for all components.

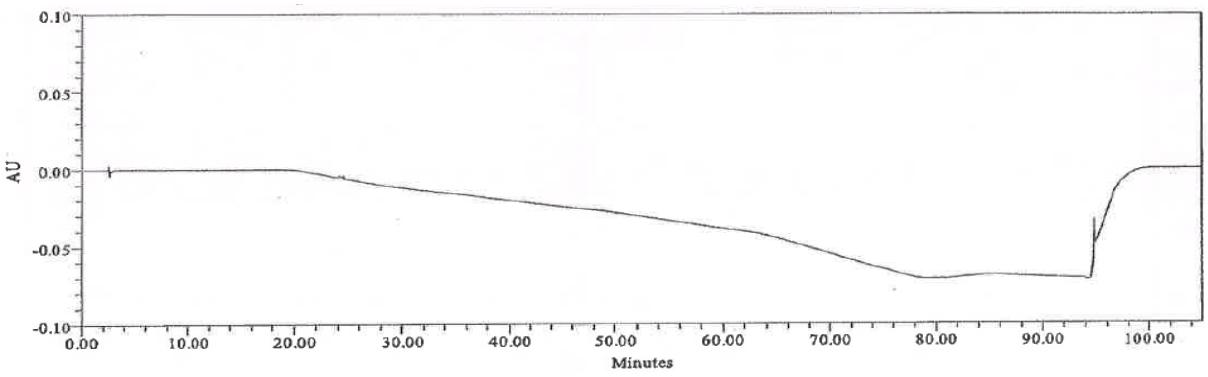


Figure.2 Typical chromatogram of Blank solution

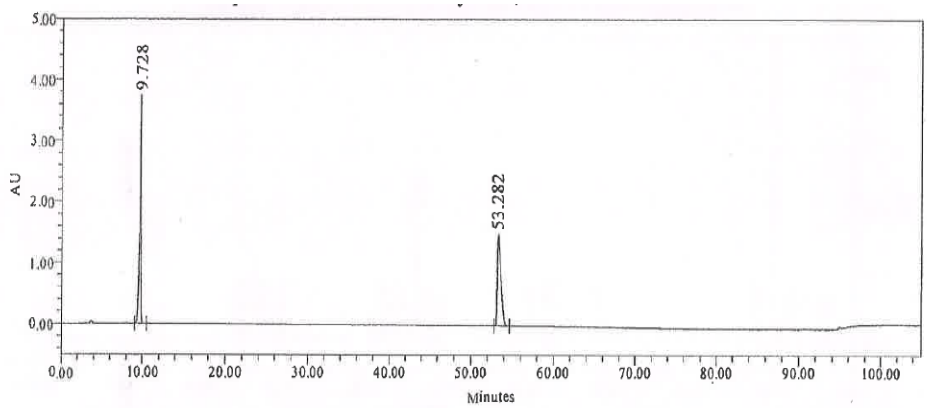


Figure.3 Typical chromatogram of Standard solution.

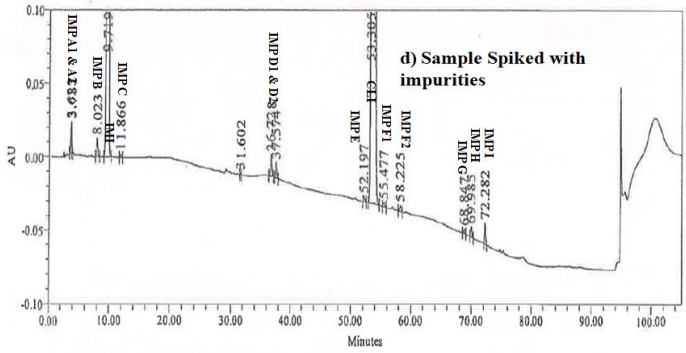


Figure.4 Typical chromatogram of Sample spiked with Impurities solution

Stress condition and Time	% AY of IMI	% Deg	% AY of CLI	% Deg	Mass balance With respect to IMI	Mass balance With respect to CIL
Acid hydrolysis	94.9	5.1	98.9	1.1	100.0	100
Alkali hydrolysis	84.2	15.8	98.1	1.9	99.4	99.4
Oxidation	96.6	3.4	95.8	4.2	99.2	98.4
Thermal degradation	98.9	1.1	95.6	4.4	100.0	97.0
Photolytic degradation	100	0	99.7	0.3	100	100

Precision

The percentage RSD of % w/w of Impurity-A1&A2, Impurity-B, IMI, Impurity-C, Impurity-D1&D2, Impurity-E , Impurity-F1 & F2, Impurity- G, Impurity- H Impurity- I and CLI is 5.3, 0.8, 1.9 , 1.2, 1.3, 2.0 1.3, 2.1,1.3,1.1 and 1.5 respectively confirming the good precision of the developed method. The % RSD obtained in intermediate precision study for the same impurities is 3.4, 0.6, 1.0 , 0.8, 1.6, 1.7, 1.3 , 3.5,4.3,1.3 and 1.0 respectively confirming the intermediate precision (ruggedness) of the method.

Sensitivity (Limit of detection and Limit of quantification)

The LOD values for Impurity-A1&A2, Impurity-B, Impurity-C, Impurity-D1&D2,Impurity-E , Impurity-F1 & F2, Impurity- G, Impurity- H and Impurity- I is 0.040%,0.031%,0.038% , 0.049%, 0.015%, 0.062% , 0.033%,0.052%respectively (of analyte concentration. 0.2 µg/mL and 0.75 µg/mL for IMI and CLI respectively). The LOQ values for the same impurities is 0.078 %,0.068% ,0.071%, 0.1%,0.028% , 0.109%, 0.082%, 0.009% respectively of analyte concentration, i.e. 0.4 µg/mL and 1.5 µg/mL for IMI and CLI respectively.

Linearity and range

Calibration curves were drawn between peak area and concentration of impurities using least square

regression analysis. The curves found to have satisfactory correlation coefficient values of greater than 0.990 for the range of concentrations used. Linearity curves were considered from the levels of LOQ to 150 %. The results indicate that, there is very good correlation attained between response and concentration for all the impurity peaks of IMI and CLI.

Name of the Impurity	Trend line equation	Range (µg/mL)	Correlation coefficient	Intercept	Residual sum of squares
Impurity - A1&A2	Y=16392X+2111	1.521-34.216	0.9998	2111	2999
Impurity - B	Y=6512X-690	1.202-36.221	0.9999	-690	293
Impurity - C	Y=4063X+277	2.301-9.075	0.9995	277	363
Imipenem	Y=19863X+2751	0.403-36.272	0.9999	2751	2565
Impurity - D1&D2	Y=19433X-1025	1.509-23.933	0.9999	-1025	2058
Impurity - E	Y=19678X-946	1.202-4.525	0.9994	-946	974
Cilastatin	Y=22292X-3287	1.563-30.262	0.9998	-3287	3843
Impurity - F1&F2	Y=16952X-2914	1.910-8.999	0.9990	-2914	2207
Impurity - H	Y=26919X-839	0.499-4.491	0.9998	-839	758

Linearity Data

Accuracy

Accuracy of the method established by preparing three spiked samples at LOQ, 50 %, 100 % and 150 % levels of the specification level of the impurities. The recovery results obtained were satisfactory in the levels of 90 to 110 % with the % RSD below 5.0% which indicates that the developed method is able to recover the impurities from the sample matrix.

Sample spiked level	Impurity-D1 & D2		%Recovery	Impurity -F1 & F2		%Recovery
	Amount added (%w/w)	Amount Recovered (%w/w)		Amount added (%w/w)	Amount Recovered (%w/w)	
LOQ sample-1	0.0750	0.0754	97.9	0.0929	0.0848	91.3
LOQ sample-2	0.0750	0.0706	94.1	0.0929	0.0947	101.9
LOQ sample-3	0.0750	0.0715	95.3	0.0929	0.0908	97.7
50% sample-1	0.404	0.402	99.5	0.150	0.151	100.7
50% sample-2	0.404	0.413	102.2	0.150	0.148	98.7
50% sample-3	0.404	0.382	94.6	0.150	0.153	102.0
100% sample-1	0.807	0.751	93.1	0.301	0.276	91.7

100% sample-2	0.807	0.735	91.1	0.301	0.304	101.0
100% sample-3	0.807	0.786	97.4	0.301	0.283	94.0
150% sample-1	1.211	1.146	94.6	0.451	0.411	91.1
150% sample-2	1.211	1.143	94.4	0.451	0.412	91.4
150% sample-3	1.211	1.146	94.6	0.451	0.423	93.8

Solution Stability

No significant changes are observed in the area of Impurity-A1&A2, Impurity-B, Impurity-C, Impurity-D1&D2,Impurity-E , Impurity-F1 & F2, Impurity- G, Impurity- H and Impurity- I during solution stability experiment at room temperature and refrigerator temperature. The data confirms that standard and sample solutions were stable up to 15 hours and 30 hours at room temperature and refrigerator temperature respectively.

Robustness

Close observation of analysis results for deliberately changed chromatographic conditions Flow rate, column oven temperature, wave length and change of organic component in gradient programme revealed that the separation between all the impurities of both IMI and CLI is consistently maintained in all the variations. Also, there is no significant change observed in the relative retention times of the main analytes and their corresponding impurities illustrating the robustness of the method.

CONCLUSION

A stability study was performed and an efficient RP - HPLC method for the quantification of related substances of Imipenem and Cilastatin in injection (250mg/vial) was developed and validated. The results of the stress testing of the drug, undertaken according to the ICH guidelines, revealed that the degradation products were formed in hydrolytic (acid and base) conditions. Validation experiments provided proof that the HPLC analytical method is linear in the proposed working range as well as accurate, precise (repeatability and intermediate precision levels) and specific, being able to separate the main drug from its degradation products. The proposed method was also found to be robust with respect to flow rate, column oven temperature and composition of mobile phase. Due to these characteristics, the method has stability indicating properties being fit for its intended purpose; it may find application for the routine analysis of the related substances of Imipenem and Cilastatin injection (250mg/vial).

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