Low Level Quantification of Potential Genotoxic Impurity In Ertapenem Monosodium Drug Substance by HPLC

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ABSTRACT

A sensitive and rapid HPLC method developed and validated for the determination of potential genotoxic impurity namely *m*-aminobenzoic acid at trace level in Ertapenem monosodium by applying the concept of threshold of toxicological concern (TTC). The HPLC method was developed and optimized on Inertsil ODS-3V, 250 mm × 4.6 mm, 5μm column with oven temperature maintaining at 40°C. 0.02M Sodium Phosphate buffer pH 2.5 was chosen as mobile phase A and methanol was selected as mobile phase B in gradient reverse phase mode. Chromatographic parameters i.e flow rate: 1.0 ml/min, wavelength detection: 220 nm, injection volume: 10μl and run time: 20 min were applied in this methodology. Based on validation data, the method is found to be specific, sensitive, accurate and precise. The established limits of Limit of detection and Limit of quantification for this impurity are found to be 3.9 μg/g and 11.9 μg/g respectively. The average recovery obtained was 99.8% at four levels in twelve determinations for *m*-aminobenzoic acid in Ertapenem monosodium drug substance. This method can be used as good quality control tool for quantization of *m*-aminobenzoic acid at low level. The experimental results are discussed in detail in this research paper.

Keywords: Ertapenem monosodium, *m*-aminobenzoic acid, Genotoxicity, Validation & HPLC.

INTRODUCTION

Ertapenem monosodium is chemically known as [4*R*,5*S*,6*S*]-3-[[(3*S*,5*S*)-5-[[(3-carboxyphenyl)amino]carbonyl] -3-pyrrolidinyl]thio]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid sodium salt, its molecular formula is $C_{22}H_{24}N_3NaO_7S$ .and molecular weight is 497.50. Ertapenem (also known as MK-0826 and L-749,345) is a new long-half-life carbapenem with a broad spectrum of antimicrobial activity against both gram-positive and gram-negative bacteria[1-3]. It is effective as a once daily parenteral treatment of intra abdominal, skin, skin structure, community-acquired pneumonia, acute pelvic infections and urinary tract infections caused by susceptible bacteria[4]. The administration of a 1 g intravenous dose over 30min, peak plasma concentrations are approximately 150 mg /ml and the plasma half-life of ertapenem is about 4 hours[5]. The greater activity of ertapenem than of imipenem against the *Enterobacteriaceae* and the greater activity of imipenem against pseudomonads and gram-positive bacteria[6] Ertapenem is marketed under trade name INVANZ[7] and it is a sterile lyophilized powder formulation of ertapenem sodium. Each vial contains 1.046 g of ertapenem sodium, equivalent to 1g of ertapenem. The formulation also contains sodium bicarbonate and sodium hydroxide as stabilizers for the active substance INVANZ should be used only to treat or prevent infections that are proven or strongly suspected to be caused by susceptible bacteria. Few methods for the determination of the impurities either in bulk drugs or in pharmaceuticals have been reported. In the last few years, it was observed that an interest was increased for the identification and quantification of impurities in bulk drugs using new methodologies. For the determination of Ertapenem and its related substances, many methods are available in literature [8-12]. Chemical structure of Ertapenem monosodium is shown in Fig.1.
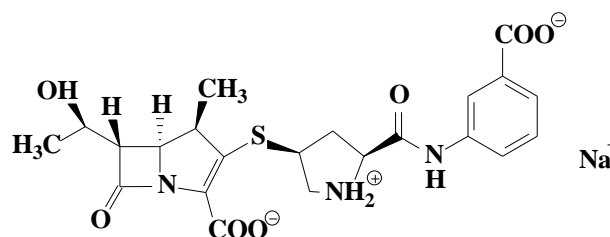

Figure 1: Chemical structure of Ertapenem monosodium

The risk of carryover into the drug substance should be assessed for identified impurities that are present in starting materials / intermediates and impurities that are reasonably expected by products in the route of synthesis from the starting material to the drug substance. (3-[[[(2S,4S)-4-mercapto-2-pyrrolidinyl]carbonyl] amino]benzoic acid) is one of the key raw materials in the

preparation of Ertapenem monosodium, in which *m*-aminobenzoic acid [MABA] is used as a reagent in the preparation of this raw material. As per regulatory agencies requirement, quantification of MABA is essential in Ertapenem monosodium drug substance with a limit of 120 µg/g calculated based on the maximum daily dose of Ertapenem monosodium drug substance by TTC approach considered as mutagenic/genotoxic and possibly carcinogenic to humans, according to ICH M7[13]. Antimutagenicity of three isomers of aminobenzoic acid in Salmonella typhimurium was reported in 1994[14]

Most pharmaceutical drug substances have an acceptable intake of genotoxic that must be controlled to a daily dose of 1.5 g per person for long term treatment, which corresponds to a theoretical 1:100,000 carcinogenic risks. The goal of this research study is to develop a sensitive, selective, accurate, reproducible and simple method to analyze MABA in Ertapenem monosodium drug substance. Chemical structure of MABA is shown in Fig.2.
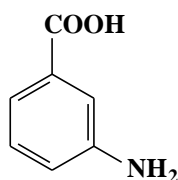

Figure 2: Chemical structure of MABA

For the sensitivity of impurity level, we have chosen HPLC technique. To the best of our knowledge, determination of MABA by HPLC in Ertapenem mono sodium drug substance has not been reported in literature till date. This paper describes the development, optimization of HPLC method for the determination of MABA and method validated accordance with ICH guidelines[15].

MATERIALS AND METHOD

Chemicals, reagents and samples

Ertapenem mono sodium drug substance, its related substances and MABA were procured from APL Research Centre-II (A division of Aurobindo Pharma Ltd., Hyderabad). Sodium dihydrogen phosphate monohydrate (Analytical grade), Orthophosphoric acid (GR grade), Methanol (HPLC grade) were procured from Merck and highly pure milli-Q water was obtained by using millipore purification system.

Instrumentation and Chromatographic conditions

Chromatographic separations were performed on HPLC (High Performance Liquid Chromatograph) system with Alliance –waters e2695 separation module with 2998 PDA detector and 2489 UV detector using Empower software. The mobile phase A was a prepared by

dissolving 2.7 g of Sodium dihydrogen phosphate monohydrate in 1000 ml of water. Adjust pH 2.5±0.05 with orthophosphoric acid. The mobile phase B was Methanol. The analysis was carried out on Inertsil ODS-3V, (250mm × 4.6 mm), 5 μm particle diameter column (Make: GL Sciences), maintained at temperature 40°C. Mobile phase was flushed through the column at a flow rate of 1.0 ml/min and pump was in gradient mode. The gradient program was as follows: Time (min)/ A (v/v): B(v/v); $T_{0.01}$/80:20, T_{10}/80:20, T_{12}/30:70,T_{20}/30:70,T_{22}/80:20, T_{22}/80:20. The run time for the standard was kept as 10 min with initial gradient ratio and the sample was 20 min. The injection volume was 10 μl and the analyte was monitored at 220 nm. A mixture of water and methanol in the ratio of 50:50 v/v was used as diluent. The retention time of MABA peak is at about 5 min.

Preparation of solutions

Standard solution (0.0006 *mg/ml*)

Accurately weigh and transfer about 30 mg of MABA reference standard into a 100 ml clean, dry volumetric flask, add 70 ml of diluent and sonicate to dissolve. Make up to volume with diluent. Dilute 5 ml of this solution to 100 ml with diluent. Further dilute 4 ml of this solution to 100ml with diluent.

Sample solution (5 *mg/ml*)

Accurately weigh and transfer about 50 mg of sample into a 10 ml clean, dry volumetric flask, add 7 ml of diluent and sonicate to dissolve. Make up to volume with diluent.

System suitability criteria

The column efficiency as determined from the MABA peak is not less   than 3500 USP plate count and USP tailing for the same peak is not more than 1.5 from MABA standard solution chromatogram.

RESULTS AND DISCUSSION

Method validation

The developed and optimized method was then validated for its specificity, linearity, LOD and LOQ, accuracy, stability of solutions and precision to demonstrate that the method is suitable for its intended use per regular sample analysis.
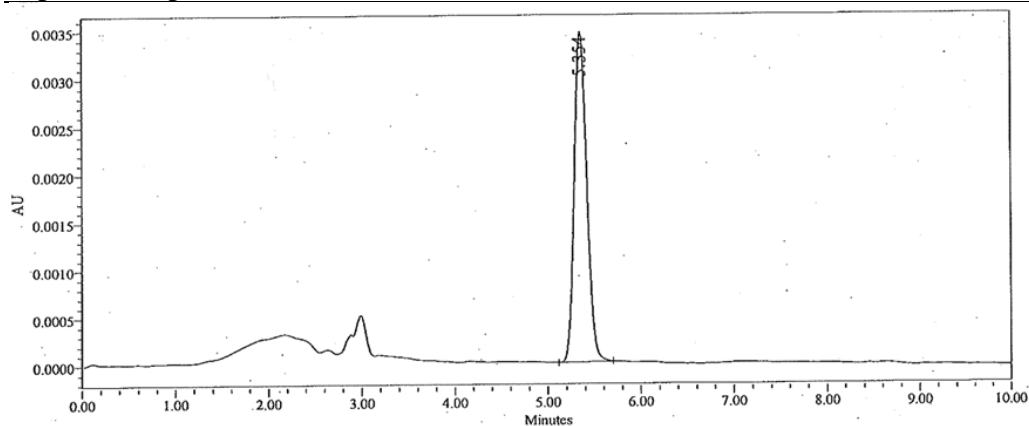
Specificity

Specificity of the method is its ability to detect and separate all the impurities present in the drug substance. Specificity of the method is demonstrated in terms of spectral as well as peak purity data of the drug and its impurities present in the drug. Peak passed the peak purity test. The solution of diluent, Ertapenem monosodium drug substance spiked with MABA (control sample)

and Ertapenem monosodium drug substance spiked with all known related substances including MABA (spiked sample) were injected to confirm any co-elution with MABA peak from any known related substances. Peak purity for MABA was established by using waters Empower software and found to be passed (Purity angle should be less than purity threshold). Moreover, No peak is observed at the retention time of MABA peak in the diluent chromatogram and all related substances are well separated from MABA peak. Hence, this method is specific and selective. Typical HPLC chromatograms of standard solution, Ertapenem monosodium spiked with MABA and Ertapenem monosodium spiked with all known related substances including MABA are shown in the Figure 3. The specificity experiments data is given in Table 1. Based on this experimental data, the peak purity data of MABA from control sample and spiked sample indicated that the peaks were homogeneous and have no co-eluting peaks. Hence, it can be concluded that, there is no interference due to listed known related substances for the determination of MABA content in Ertapenem monosodium drug substance.

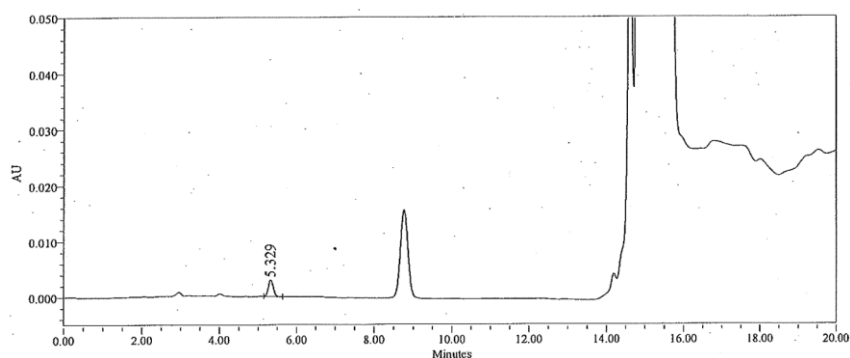**Table 1: Specificity data**

Sample	MABA Retention Time (min)	Peak Purity	
		Purity angle	Purity threshold
Control sample	5.314	0.105	0.343
Spiked sample	5.329	0.575	1.420

Standard solution
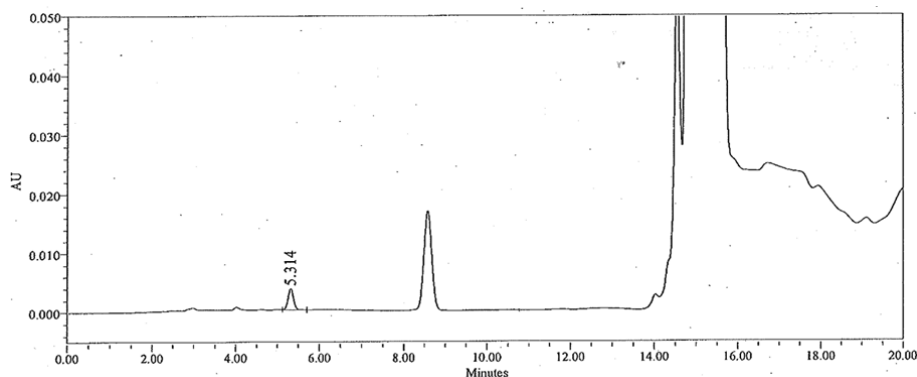
Control sample (spiked with MABA)

Spiked sample (spiked with MABA and known related substances)

Figure 3: Typical HPLC chromatograms of specificity experiment

LOD and LOQ

The sensitivity for detection can be demonstrated by determining the limit of detection (LOD) and limit of quantitition (LOQ). LOD/LOQ values of MABA were determined from based on response of analyte. The predicted concentrations of LOD and LOQ for MABA were verified for precision by preparing the solutions containing MABA at about predicted concentrations. Each of these solutions six times injected into the HPLC.

Table 2: LOD/LOQ and Linearity experiments data

LOD & LOQ		
LOD	3.9 µg/g	2.1 (% RSD)
LOQ	11.9 µg/g	0.6 (% RSD)
Linearity		
Slope	49541	
Intercept	2	
Residual sum of squares	15078	
Correlation coefficent	0.9999	

Linearity

Linearity of the method was checked by preparing solutions at nine concentration levels from LOQ to 150% of specification level (120µg/g) by prepared using MABA standard solution and each solution was injected into HPLC. Linearity was established by using concentration (µg/ml) on X-axis, area on Y-axis and calculated statistical values like slope, intercept, residual sum of squares and correlation coefficient. The linearity, LOD and LOQ experiments data is shown in Table 2.

Accuracy

Accuracy of the method was performed by recovery experiments using standard addition technique. Sample solutions were prepared in triplicate by spiking MABA at levels of LOQ, 50%, 100% and 150% of specification limit as per test method and injected each solution into HPLC as

per methodology and the percentage recoveries were calculated. The fully validated recovery results are shown in Table 3.

Table 3: Accuracy data

LOQ level

% Level / Sample ID	Amount Added (µg/g)		Amount Found (µg/g)			% Recovery
LOQ Level Sample - 1	12.04		11.12			92.6
LOQ Level Sample - 2	12.04		11.38			94.5
LOQ Level Sample - 3	12.06		11.91			98.8
Statistical Analysis						
Mean 95.3	SD	3.18	% RSD	3.3	95% Confidence Interval (±)	7.9

(50% to 150% level)

Concentration / Sample ID	Amount Added (µg/g)	Amount Found (µg/g)	% Recovery	Statistical Analysis	
50% Level Sample 1	56.67	57.85	102.1	Mean	102.2
50% Level Sample 2	57.10	58.16	101.9	SD	0.36
50% Level Sample 3	56.66	58.15	102.6	% RSD	0.4
100% Level Sample 1	114.16	113.07	99.0	Mean	99.2
100% Level Sample 2	114.53	113.68	99.3	SD	0.15
100% Level Sample 3	113.60	112.70	99.2	% RSD	0.2
150% Level Sample 1	169.19	178.11	105.3	Mean	102.5
150% Level Sample 2	172.72	168.65	97.6	SD	4.28
150% Level Sample 3	171.48	179.51	104.7	% RSD	4.2
Overall Statistical Analysis					
Mean 101.3	SD	2.68	% RSD	2.6	95% Confidence Interval (±) 2.1

Precision

System precision was demonstrated by preparing the standard solution of MABA as per methodology and analyzed by injecting six replicates. Method precision experiments demonstrated by preparing six sample solutions individually using a single batch of Ertapenem monosodium drug substance spiked with MABA at specification level and determined the MABA content by HPLC. Achieved results like %RSD and 95% confidence interval for six determinations are summarized in Table 4.

Table 4: Precision experiments data

Precision	Injection ID	MABA area	Statistical Analysis	
	1	28257		
	2	28554		
	3	28796	Mean	28910
	4	29093	SD	459

| | | | 5 | 29303 | % RSD | 1.6 |
| | | | 6 | 29456 | 95% Confidence Interval (±) | 482 |

Method Precision	Sample	MABA (µg/g)	Statistical Analysis	
	1	139		
	2	142		
	3	142	Mean	140
	4	138	SD	1.8
	5	138	% RSD	1.3
	6	140	95% Confidence Interval (±)	1.9

Intermediate Precision	Sample	MABA (µg/g)	Statistical Analysis		
	1	133			
	2	133		For ruggedness	overall
	3	134	Mean	133	136
	4	132	SD	0.6	3.8
	5	133	% RSD	0.5	2.8
	6	133	95% Confidence Interval (±)	0.6	2.4

Solution stability

For the determination of stability of the standard and sample solutions, standard solution and sample solution spiked with MABA at specification level were prepared as per methodology and analyzed initially and at different time intervals by keeping the solution at room temperature (25°±2°C) and refrigerator condition (~ 5°±3°C). The % difference in the peak area obtained at initial and after 15 hours time interval was found to be less than 4.9 for standard solution at room temperature (25°±2°C). The results concluded that the standard solution is stable for at least 15 hours at 25°±2°C. But the sample solution area was continuously increasing at this temperature, but stable at refrigerator condition. The % difference obtained was 0.2. Based on data, it was concluded that the sample solution is stable at least for 15 hours at refrigerator condition (~ 5°±3°C).

Standard		MABA area	% Difference
(at 25°±2°C)	Initial	32421	4.9
	After 15 hours	30828	
Sample	Initial	34667	47.8
(at 25°±2°C)	After 15 hours	51252	
Sample	Initial	37869	0.2
(~ 5°±3°C)	After 15 hours	37928	

CONCLUSION

The HPLC chromatography method was developed, optimized and validated for the determination of MABA content in Ertapenem monosodium drug substance and the results of various validation

parameters proved that the method is specific, sensitive, precise and accurate and the method can be introduced into routine testing.

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