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Research Article

High Pressure Liquid Chromatography Identification and Quantification of Morpholine Residual Content in Cobicistat After Derivatization

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ABSTRACT

A derivatization HPLC procedure has been developed to identify and enumerate morpholine (MPE) in cobicistat (CCT) pharmaceutical active substances. MPE react with acetonitrile solution of 1-Naphthyl isothiocyanate to produce stable MPE derivative. The MPE derivative produced was identified and enumerated by HPLC based procedure. The linearity range of MPE was 0.3002675–1.201070 µg/mL with good squared correlation (0.9995), and limits of detection and quantification were 0.1000 µg/mL and 0.3001 µg/mL, respectively. Low (50% of specification quantity), medium (100% of specification quantity), and high (150% of specification quantity) concentrations of MPE were added in CCT samples to evaluate recovery rate. The added recovery rate ranged from 97.9% to 100.4%. The precision value was 0.79% relative standard deviation. The developed procedure also has good selectivity and robustness. This quantitative procedure for MPE can successfully be implemented to analyse the MPE residual quantities in CCT pharmaceutical active substances.

Introduction

Cobicistat (CCT), a blocker of CYP3A (cytochrome P450 3A) enzyme, is marketed as Tybost tablet (previously GS-9350) and suggested for the management of human immunodeficiency viral infections.[1,2] Though it has no anti-human immunodeficiency viral function, CCT serves as a pharmacokinetic booster by suppressing isoforms of the CYP3A. Consequently, CCT improves plasma quantities of co-administered agents (darunavir/atazanavir) metabolized via CYP3A enzyme.[3] Raising plasma quantities of darunavir/atazanavir without raising dose enables improved patient results and a reduced profile of side effects.

As it is a cyclic amine, more polar and more cost-effective, morpholine (MPE) is widely utilized as a solvent in pharmaceutical industry and research laboratories for the making of various pharmaceutical active substances.[4-6] MPE residues can subsequently be found in the pharmaceutical active substances or their finished components. MPE induces irritation of skin, eye and digestive system tract and could be absorbed into body through contacts via skin, ingestion, and inhalation.[7] It is therefore of utmost importance to develop a fast and efficient method for the identification and quantitation of MPE in pharmaceutical active substances and in their finished components.

MPE residues was quantified in juice, peel and pulp of fruits like apple, citrus, orange, strawberries, grapes and soy nutraceuticals using GC-MS,[8-10] UPLC-MS[11] and LC-MS/MS.[12] Quantification of residues of MPE in ibuprofen using GC-MS was also reported.[8] MPE is a raw material used in the n-2 stage synthesis of CCT. Quantification of residues of MPE in CCT pharmaceutical active substances was not reported yet. The aim in this work was to quantify MPE residues in CCT pharmaceutical

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active substances using HPLC approach. For this, an HPLC procedure was developed and validated.

Materials and Methods

Apparatus and Conditions

Waters Alliance (2695 Module) HPLC device equipped with UV (2487 Module) detector, PDA (2489 module) and Empower software was utilized. MPE residues analysis in CCT was performed on ACE C18-AR (3.0 µm id, 4.6 mm × 150 mm) using gradient mode mobile phase of 0.1% orthophosphoric acid (Phase A) and acetonitrile (Phase B). The gradient line-up was: 0 min (72% Phase A and 28% Phase B), 10 min (67% Phase A and 33% Phase B), 13 min (67% Phase A and 33% Phase B), 15 min (25% Phase A and 75% Phase B), 20 min (25% Phase A and 75% Phase B), 21 min (72% Phase A and 28% Phase B) and 25 min (72% Phase A and 28% Phase B). 0.45 µm filter membrane was used for filtering of mobile phase and samples earlier to use. The flow rate, column temperature and sample volume were tuned at 0.8 mL/min, 50°C and 10 µL, respectively. Detection and evaluation of MPE residues was completed at detector tuned at 220 nm.

Chemicals

Acetonitrile (Rankem India Ltd), orthophosphoric acid (Rankem India Ltd), 1-Naphthyl isothiocyanate (Sigma Aldrich) and Water (Milli-Q system), were utilized. CCT and MPE were obtained from Mylan laboratories limited and Sigma Aldrich, respectively.

Derivatization Reagent

Solution of derivatization reagent (DR) was made by dissolving 1-Naphthyl isothiocyanate in 50 mL of acetonitrile.

Preparation of Diluent

Weighed and transferred about 0.44 g of dipotassium hydrogen phosphate (Rankem India Ltd) accurately in 500 mL flask, added 250 mL water. Mixed well and dissolved through sonication. Further bring the volume with acetonitrile up to mark.

Solutions

Blank

Transferred 1.0 mL of DR to a 10 mL volumetric flask, dilute to 10 mL volume with diluent (acetonitrile and water – 50:50 v/v ratio) and closed using glass stopper. Sealed the flask securely with teflon, and held for 90 min at 60 °C. Once 90 mins have been done allow the solution to be cooled at room temperature and injected for evaluation.

Stock MPE Solution

Primary stock MPE solution was made at concentration value of 1000 µg/mL in diluent. Secondary stock MPE solution was made from dilution of primary stock MPE (1000 µg/mL mg/mL) at concentration value of 10 µg/mL with diluent.

CCT Sample Solution

CCT sample solution was made at concentration value of 2000 µg/mL in diluent.

Derivatization of Standard MPE Solution

Transferred 1.0-mL of DR and 1-mL of secondary stock MPE solution (10 µg/mL) to a 10 mL volumetric flask, dilute to 10 mL volume with diluent (acetonitrile and water – 50:50 v/v ratio) and closed using glass stopper. Sealed the flask securely with teflon, and held for 90 min at 60°C. The the solution was cooled at room temperature and injected for evaluation.

Derivatization of CCT Sample Solution

Transferred 1.0 mL of DR and precisely weighed 20 mg of CCT sample to a 10 mL volumetric flask, dilute to 10 mL volume with diluent (acetonitrile and water – 50:50 v/v ratio) and closed using glass stopper. Sealed the flask tightly with teflon and placed at 60°C for 90 minutes. After that, the solution is cooled at room temperature and injected for testing.

Analysis of MPE Residue in CCT Sample

Injected diluent (acetonitrile and water – 50:50 *v/v* ratio, one injection), derivatized MPE solution (1.0 µg/mL, six injections) and derivatized CCT sample solution (2000 µg/mL, two injections) into the ACE C18-AR column and anlyze the applying conditions given in segment "Apparatus and conditions". The MPE peak areas from derivatized MPE solution and derivatized CCT sample solution were then employed to gauge MPE content in CCT samples.

Results and Discussion

Reaction between MPE and 1-Naphthyl Isothiocyanate

For the identification and assessment of various amines the reaction with 1-naphthylisothiocyanate was used. Primary and secondary amines easily and quantitatively interact with1-naphthylisothiocyanate to produce stable derivatives of thiourea (Fig. 1). These derivatives exhibit high sensitivity. Therefore, they can be used for analysis through HPLC and UV detection.

Method Optimization

The optimization of MPE residue assay method in CCT sample was conducted by altering the type of elution;

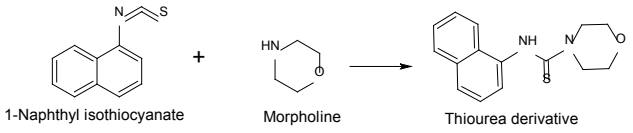

Fig. 1: Formation of thiourea derivative

ratio of 0.1% orthophosphoric acid (Phase A) and acetonitrile (Phase B); flow rate, column temperature and wavelength. With the collection of conditions given in the "Apparatus and Conditions" section, the best conditions (good peak design shape, higher response, and adequate plate counts) were obtained. Hence, the same set of conditions were employed for MPE residue assay in CCT sample.

Validation

Validation was done for the developed procedure conferring to ICH guiding principles[13] and US Pharmacopoeia guiding principles.[14]

Selectivity

To measure selectivity, the standard MPE solution (1.0 µg/mL), blank solution, CCE sample solution, MPE spiked CCT solution (1.0 µg/mL) were analysed. The chromatogram of standard MPE solution (1.0 µg/mL) displayed a peak in the period retention of 11.968 (Fig. 2a). The chromatogram of MPE spiked CCT solution (Fig. 2d) displayed a peak in period retention of (12.820 min) similar to standard MPE solution (Fig. 2a). The constituents of the blank (Fig. 2b) and CCT samples (Fig. 2c) don't really interfere with the evaluation, so no peak was identified in the MPE area of the main peak. The chromatogram peaks indicated the method's selectivity as per ICH guiding principles.

Limit of Detection and Limit of Quantitation

LOD was measured as concentration that gives a value of 3:1 signal to noise ratio following ICH guiding principles. LOQ was measured as concentration that gives a value of 10:1 signal to noise ratio following ICH guiding principles. The LOD was found as 0.1000 µg/mL and LOQ as 0.3001 µg/mL for MPE.

Linearity

Measurement of linearity, six standard MPE solutions covering different concentrations of MPE (0.3002675, 0.4003566, 0.6005349, 0.8007132, 1.00892, and 1.201070 µg/mL) were analyzed. The standard MPE curve of calibration was drawn between the peak response area of the MPE and its value of concentrations. Furthermore, linearity was measured by using calibration data regression analysis as well as the coefficient of correlation. The approach was linear over a concentration's spectrum (0.3002675–1.201070 µg/mL) with a coefficient of correlation of 0.9998 and a coefficient of squared correlation of 0.9995. The straight-line approximation from the experimental findings was: y = 378325051.9833x -1554.8356. The regression statistics were displayed in Table 1.

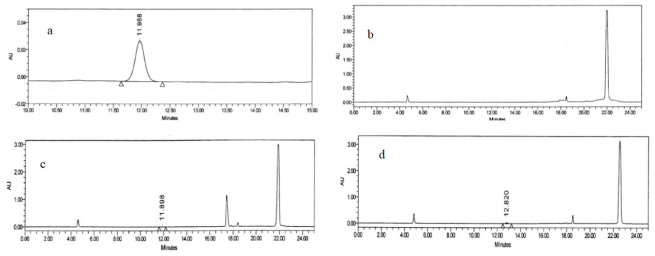

Fig. 2: [a] Standard MPE chromatogram [b] Blank chromatogram [c] CCT sample chromatogram [d] MPE spiked CCT sample chromatogram

Precision

To measure precision (system), standard MPE solution (1.0 µg/mL) was evaluated six times. The relative standard deviation value for determined MPE peak areas were calculated from the ratio of standard deviation to mean MPE peak areas determined and broached as percentage (Table 2). The value was fewer than 1.0% and the precision for this system was approved as per ICH guiding principles.

To measure precision (method), MPE spiked CCT sample solution (1.0 µg/mL) was evaluated six times. The relative standard deviation value for determined MPE quantities were calculated from the ratio of standard deviation to mean MPE quantity determined and broached as percentage (Table 2). The value was fewer than 1.0% and the precision for this method was approved as per ICH guiding principles.

Accuracy

To measure accuracy, analyzed MPE spiked CCT sample solution three times at three concentrations (0.48 µg/mL - equal to 50% of specification concentration, 0.97 µg/mL - equal to 100% of specification concentration, and 1.1 µg/mL - equal to 120% of specification concentration). Accuracy was conveyed as MPE recovery percentage (Table 3). The recovery percent of MPE in CCT sample ranged from 97.9% to 100.4%, revealed the accuracy of this method according to ICH guiding principles.

Table 1: Regression statistics data for MPE linearity

Parameter	*Value*	
	Regression statistics	
Slope	378325051.9833	
Intercept	-1554.8356	
Multiple R	0.9998	
R square	0.9995	
	ANOVA	
Parameter	df value	SS value
Regression	1	87225971863.2991
Residual	4	42389065.5342
Total	5	87268360928.8333
	Confidence intervals	
Parameter	Lower 95%	Upper 95%
Intercept	-10642.5102	7532.8390
X variable	366747194.2488	389902909.7177

Table 2: MPE precision in this method

Sample injection	*MPE taken (mg/mL)*	*MPE quantified (mg/mL)*	*Mean MPE quantified (mg/mL)*	*RSD (%)*
		Precision (method)		
1	0.969386	0.949405	0.957807	0.710
2	0.965180	0.950801		
3	0.977911	0.966513		
4	0.974578	0.962706		
5	0.967045	0.956418		
6	0.967980	0.961001		
		Precision (system)		
Sample injection	MPE Taken (mg/mL)	MPE peak area	Mean MPE peak area	RSD (%)
1	0.969386	394201	398935	0.834
2	0.965180	397846		
3	0.977911	400254		
4	0.974578	396501		
5	0.967045	402277		
6	0.967980	402536		

Table 3: MPE recovery in this method

Added level (%)	*MPE added (mg/mL)*	*MPE quantified (mg/mL)*	*MPE recovered (%)*
50	0.485870	0.479571	98.7
50	0.487764	0.482856	99.0
50	0.487764	0.483161	99.1
100	0.969386	0.949405	97.9
100	0.965180	0.950801	98.5
100	0.977911	0.966513	98.8
150	1.158216	1.152762	99.5
150	1.171775	1.174227	100.2
150	1.164391	1.168850	100.4

Table 4: MPE robustness in this method

Parameter	*Retention period (min)*	*Concentration added (µg/mL)*	*Concentration found (µg/mL)*	*Recovery (%)*
Optimal conditions	11.900	0.969386	0.949405	97.9
Flow rate variation – 0.6 mL/min	16.188	0.987653	1.080457	109.4
Flow rate variation – 1.0 mL/min	10.705	0.981853	1.021948	104.1
Oven temperature variation – 48°C	13.071	0.981853	1.033022	105.2
Oven temperature variation – 52°C	12.531	0.981853	1.048305	106.8
Wavelength variation – 218 nm	12.803	0.987653	1.052358	106.6
Wavelength variation – 222 nm	12.757	0.987653	1.042851	105.6

Robustness

To measure robustness, analyzed standard MPE solution (0.001 mg/mL) with slightly modified conditions of procedure like flow rate (0.8 ± 0.2 mL/min), oven temperature (50 ± 2°C) and wavelength for quantification (220 ± 2 nm). Robustness was conveyed as variation of MPE quantity determined and retention period of MPE in chosen conditions and in slightly modified conditions (Table 4). Significant changes were not noticed in MPE quantity determined and retention period of MPE, revealed the robustness of this method according to ICH guiding principles.

Table 5: MPE robustness in this method

Parameter	*Peak response area RSD (%)*	*Tailing factor*	*Plate counts*
LOD	2.1	1.0	22973
LOQ	2.0	1.0	23159
Selectivity	0.4	1.0	24079
Precision, accuracy, and robustness (actual conditions)	0.9	1.0	23800
Robustness modified conditions	0.3	1.0	28616

System Suitability

To verify the efficiency of the instrument, system suitability testing was performed prior each validation factor was assessed. To measure system efficiency, standard MPE solution (1.0 µg/mL) was evaluated six times. The relative standard deviation percentage for the peak response area of MPE, tailing factor for MPE peak and plate counts for MPE peak were documented and the values obtained for this method were acceptable as per ICH guiding principles (Table 5).

Batch Analysis

This assay was built and validated for real-world industrial setting to facilitate quality control of CCT samples. We analyzed, employing the described method, three batch samples of CCT for MPE residues. The MPE residues level was below detection limits.

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