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Development and Validation of UPLC-ESI-MS/MS Technique for the Determination of 2-Isopropyl-4-(chloromethyl)thiazole in Ritonavir

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The objective of this work was to develop and validate a rapid, highly sensitive ultra performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-MS/MS) method for the quantification of 2-isopropyl-4-(chloromethyl)thiazole in ritonavir. Chromatographic conditions of this impurity were achieved on an AQUITY UPLC column HSS (high strength silica) T3 column (100 mm long, 2.1 mm internal diameter, 1.8 µm diameter) using a gradient elution with 0.1% formic acid in water and methanol at a flow rate of 0.3 mL/min. LCMS/MS was operated under the multiple reaction mode (MRM) using electrospray ionization technique in positive ion mode and the transitions of *m/z* 176.1[M+H]$^+$→140.1 for quantifier, 176.1[M+H]$^+$→71.0 for qualifier were used to measure the impurity, respectively. The total chromatographic run time was 10 min. Full validation of the analytical method was carried out, including its system precision, selectivity, linearity, accuracy, recovery, ruggedness, stability and robustness. A linear response function was achieved in the concentration range of 0.12-1.86 µg/g with r > 0.99. The detection limit and quantitation limit of this impurity were 0.06 and 0.12 µg/g, respectively. Consistent recoveries were obtained during intra- and inter-day precision experiments in validation ranged from 80-120%. The developed method could be helpful not only for quality control and also for risk management of potential genotoxicity of this impurity in ritonavir drug substance.

Keywords: UPLC-ESI-MS/MS, Potential genotoxic impurity, Method validation, Trace analysis.

INTRODUCTION

Ritonavir is an antiretroviral drug, from the protease inhibitor class used to treat HIV infection and acquired immune deficiency syndrome (AIDS). Antiretroviral therapy treats HIV by suppressing the virus the activity in the body. For most people who take them medications are very effective at keeping HIV under control. Treatment helps to improve quality of life and it can ensure that a person with HIV has a similar life expectancy to a person without the virus. The chemical name of ritonavir is 1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3- hydroxy-5-[(2*S*)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate. The drug marketed under the trade name of NORVIR (as tablets, capsules and oral solution formulations). Genotoxic impurities (GTIs) which are present in Pharmaceutical drug substances may induce genetic mutations, chromosomal breaks (rearrangements) and they have possible to cause cancer in human [1,2]. According to the European Medicines Agency (EMEA) and feedback from US Food and Drug Administration (USFDA) the projected use of a threshold of toxicological concern (TTC), it is established that genotoxic impurities will be limited to a daily dose of 1.0-1.5 g/day [3,4]. Consequently it is essential for process chemists to avoid such genotoxic impurities in the manufacturing process [5], though it would be difficult or impossible to eliminate potential genotoxic impurities (PGIs) completely from the synthetic route. Therefore, it is a great challenge to analyst to develop a suitable analytical method to quantify the impurity accurately and control their levels in drug substances.

According to ICH M7 guidelines [6], 2-isopropyl-4-(chloromethyl)thiazole is a potential genotoxic impurity (PGI) and

non-isolated intermediate in the route synthesis of starting material 2-isopropyl-4-[(*N*-methyl amino)methyl]thiazole, which was further steps to involved in the synthesis of *N*-[(N-methyl-*N*-[(2-isopropyl-4-thiazolyl)methyl]amino]carbonyl]-L-valine [MITAV], there after synthesis of final drug substance ritonavir shown in Fig. 1. 2-Isopropyl-4-(chloromethyl)thiazole toxicity is evaluated based on quantitative structure-activity relationships (QSARs), structure-activity relationships (SARs)

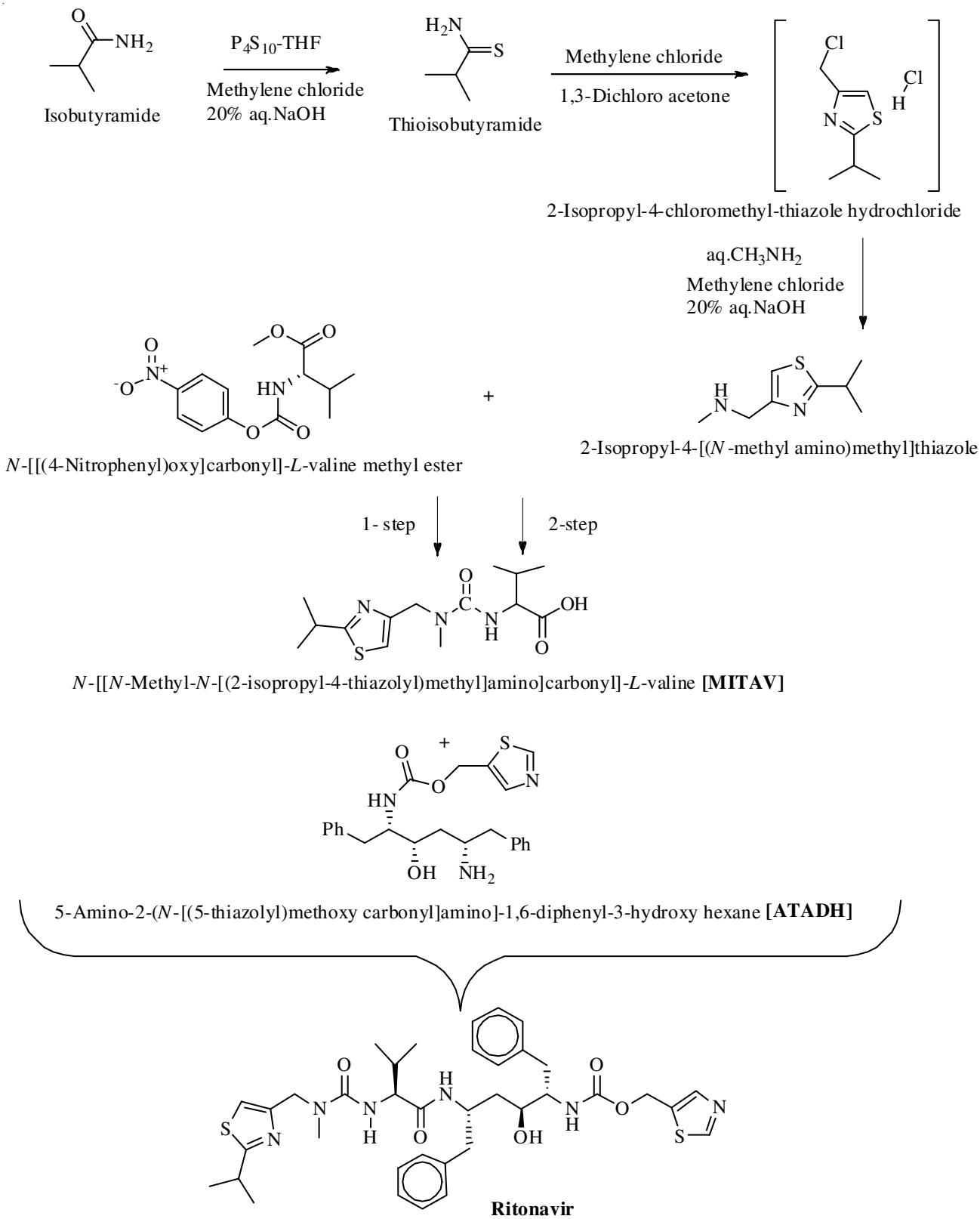

Fig. 1. Schematic representation of ritonavir synthesis and 2-isopropyl-4-(chloromethyl)thiazole (non-isolated impurity in the route synthesis of starting material 2-isopropyl-4-[(*N*-methyl amino)methyl]thiazole)

and/or expert systems by using Derek Nexus (a knowledge-based, expert decision support system for the prediction of toxicity) and Sarah Nexus (A statistical software tool for the prediction of mutagenicity) software applications. This data would ascertain that the regulatory bodies may be expected to control the levels of 2-isopropyl-4-(chloromethyl)thiazole to be 1.3 ppm in the drug substance (assuming TTC concept). A method capable of such a lower level of detection is great challenge for analytical method development for controlling this impurity.

Conventional analytical instruments in pharmaceutical industries such as HPLC and GC detection must be employed as the impurity standards in first attempt for genotoxic impurities [7,8]. But these have some drawbacks. Because in HPLC, GC response of impurities in trace level determination not always possible and not to be characterized on line, if that is new impurity. Hence accurate determinations of low ppm levels in above techniques are not enough. Spectrometric techniques are need for the trace level quantification. The literature survey revealed that some spectrometric methods and HPLC were developed for the determination of ritonavir with other substances such as lopinavir, nelfinavir, indinavir, saquinavir, etravirine, darunavir, *etc*., in dosage forms, plasma and degradation studies [9-18].

Ever since no method has been in the past period reported for the quantification of 2-isopropyl-4-(chloromethyl)thiazole in ritonavir. An attempt was made to develop a highly specific, selective and accurate UPLC MS/MS method, MRM mode was used for the quantification which gives better low ppm trace level analysis. The developed method is highly reproducible, necessary smaller time for analysis and validated as per International conference of Harmonization (ICH) guidelines [19].

EXPERIMENTAL

All chemicals and solvents were used in experiments procured from analytical grade. HPLC grade acetonitrile were purchased from Merck (Mumbai, India). Formic acid and methanol were obtained in their highest grade from S.D. Fine Chemicals Ltd., India. Related substances of ritonavir [ATADH (purity 97.7%), MITAV (purity 99.7%), ritonavir isoleucine analog (purity 98.4%), Ritonavir leucine analog (purity 93.5%), ritonavir oxidation impurity (purity 97.1%), ritonavir hydro peroxide (purity 96.8%), ritonavir acid/base byproduct (purity 95.7%), ritonavir base cyclization product (purity 98.7%), ritonavir acid hydrolysis product (purity 89.9%)], ritonavir drug substance (purity 99.5%) and 2-isopropyl-4-(chloromethyl)-thiazole (purity 97.3%) were received as a gift from Aurobindo Research Centre-II (a division of Aurobindo Pharma Limited, Hyderabad, India). High purity Milli-Q water was used with the help of Millipore Milli-Q plus purification system (Bedford, MA, USA).

Preparation of impurity stock and standard solutions: Impurity stock solution (0.31 mg/mL) was prepared by dissolving 2-isopropyl-4-(chloromethyl)thiazole in H_2O-CH_3CN (20:80, v/v). Preparation of stock standard solution of 0.0031 mg/mL was achieved on further dilution with H_2O-CH_3CN (20:80, v/v). Further this solution was diluted to 31.0 µg/g (with respect to sample concentration 10 mg/mL). Finally, desired concentration 1.24 µg/g of standard solution was prepared by diluting stock standard solution to 50 mL with H_2O-CH_3CN (20:80, v/v). Test sample of ritonavir drug substance was prepared by diluting 10 mg/mL in H_2O-CH_3CN (20:80, v/v). The standard solutions and test samples were optimized to achieve a preferred signal-to-noise ratio (S/N) and desired peak shape.

Operating parameters of UPLC ESI-MS/MS: Chromatographic experiments were performed on an Acquity UPLC system connected with MS/MS (both were Waters,USA). The analytical column used was Acquity UPLC column HSS T3 column (100 mm long, 2.1 mm internal diameter, 1.8 µm dia.). The gradient elution was achieved with mobile phase-A and mobile phase-B components.The mobile phase-A consists 0.1% formic acid in water and mobile phase-B consists methanol. The flow rate of the mobile phase was set at 0.3 mL/min and column temperature was maintained at 40 ºC. The gradient program was set as follows: time/% MP-B: 0.01/60, 3.0/60,3.5/90,7.0/90,7.5/60,10.0/60. Total run time was 10 min with the injection volume 10 µL.

The ESI-MS/MS system used was waters Xevo TQ-S triple quadrupole mass spectromer (USA) with eletrospray ionization (ESI) probe operated in both positive and negative polarities. The system control and data handling was done by Mass Lynx version 4.1 SCN950 software with target Lynx application manager. Typical operating conditions employed as follows: capillary: 2.5 kV; source temperature: 150 ºC; desolvation temperature: 500 ºC; cone gas flow: 150 L/h; desolvation gas flow,1000 L/h; nebulizer gas flow: 7.0 bar: dwell time: 0.163 s. Electrospray ionization in positive multiple reaction monitoring mode was used for the quantification of impurity at transition pair *m/z* 176.1 $[M+H]^+ \rightarrow$140.1 for quantifier, 176.1 $[M+H]^+ \rightarrow$ 71.0 for qualifier. The efficient separation between ritonavir and impurity in the shortest analysis time was set as the defined analytical target profile and to this purpose utilization of a switching valve allowed the flow to be sent to waste (4.5-9.0 min of time for standard and test sample solution), when ritonavir test sample peak was eluted.

Validation study: The developed method for the determination of this potential genotoxic impurity in ritonavir was completely validated following US FDA and ICH guidelines.

Specificity: Specificity is the ability to assess the analyte unequivocally in presence of the components which are expected to present in sample matrix. The specificity of the developed method was determined by analyzing the ritonavir, impurity and related substances of ritonavir solutions. The solutions of ritonavir test sample, ritonavir spiked with related substances without impurity standard solutions at specification level and ritonavir spiked with impurity standard solutions at specification level prepared and injected for analysis. Further test sample of ritonavir, impurity stock and ritonavir with impurity stock injected in ms scan mode was tested for the interference. From the results, we observed that the related substances were not interfered at the retention time of impurity. The corresponding chromatogram is shown in Fig. 2. Hence, the specificity was conformed as the impurity peak was pure with ritonavir drug substance.

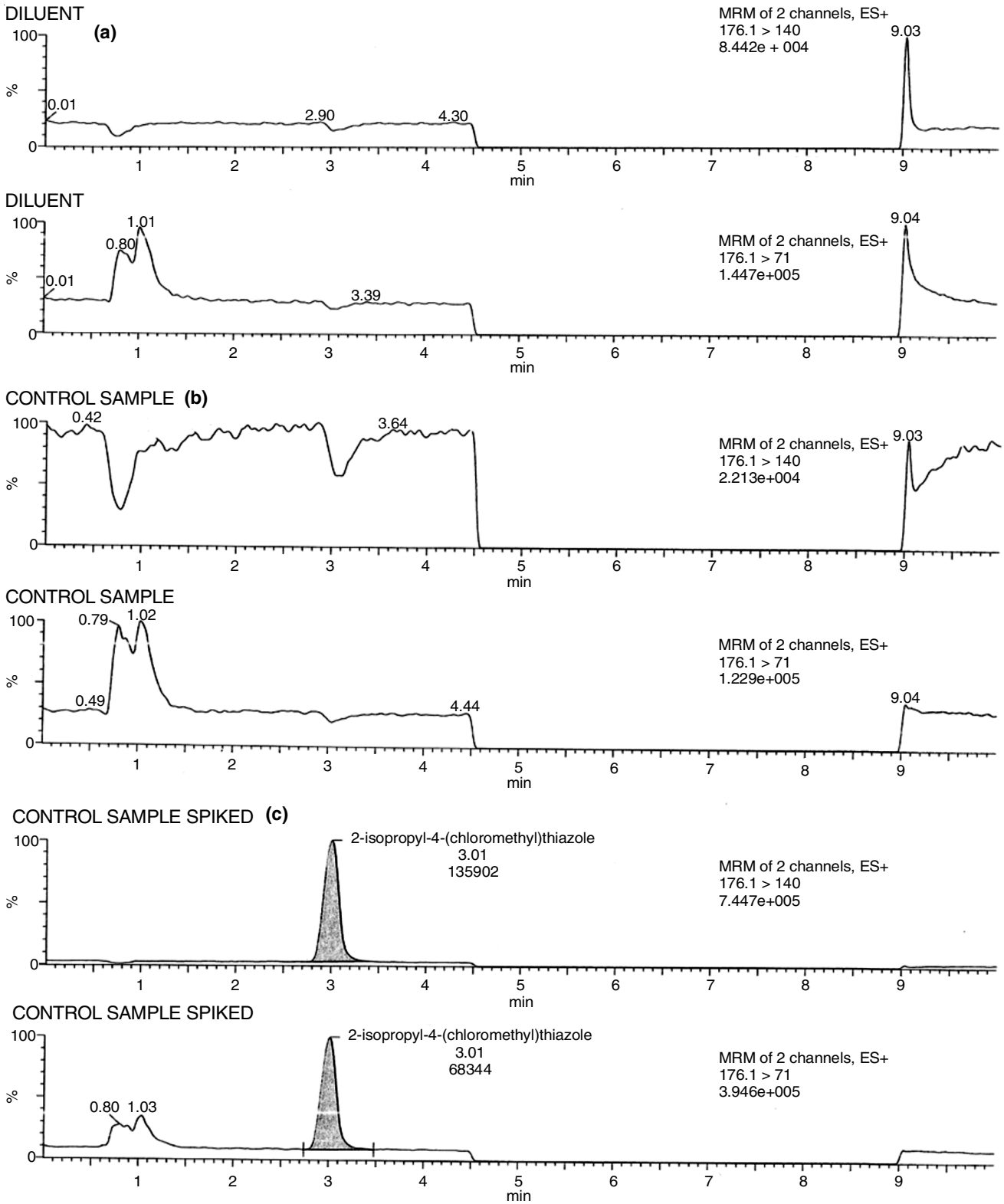

Fig. 2. (a) Diluent (b) Control sample of ritonavir (c) Control sample spiked with 2-isopropyl-4-(chloromethyl)thiazole at 1.24 µg/g level

Detection limit and quantification limit: The detection limit and quantification limit were injecting the standard stock solution in lower levels with respect to the drug substance concentration of 10 mg/mL and determining their S/N ratios. To evaluate LOD and LOQ values, their concentrations were reduced sequentially such that they yield S/N ratios > 2.0 and > 10.0, respectively. Each predicted concentrations were verified for precision by preparing the solution six times for analysis. The LOD and LOQ values calculated from S/N ratio were shown to be 0.06 and 0.12 µg/g, represented in Fig. 3, respectively. It is noted that the LOD value for impurity was below the required concentration limit (1.24 µg/g) for ritonavir.

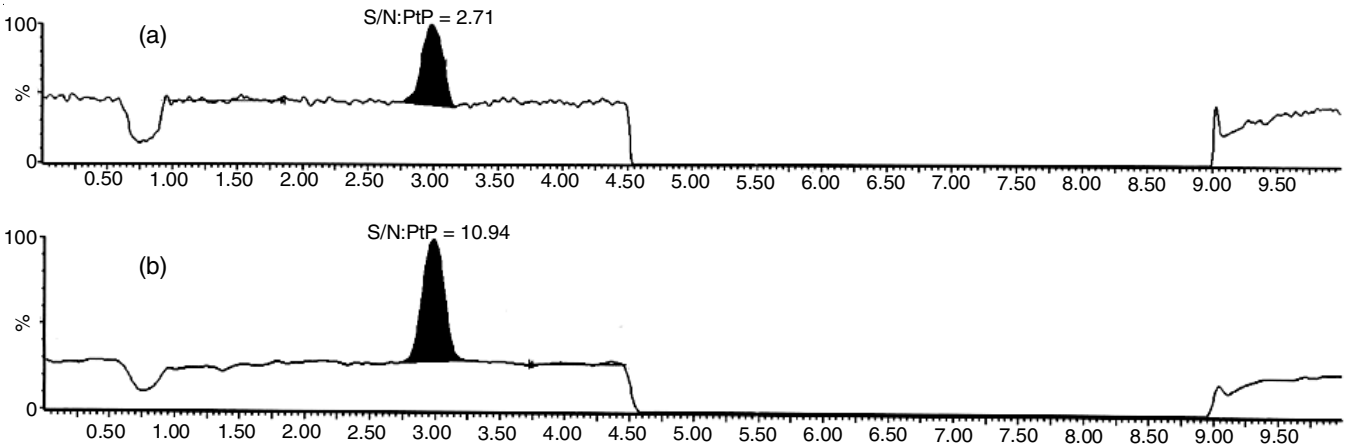

Fig. 3. (a) Signal to noise ratio of LOD solution 2.71 (b) Signal to noise ratio of LOQ solution 10.94

TABLE-1
INTRA-DAY AND INTER-DAY PRECISION AT 1.24 µg/g CONCENTRATION AND LINEARITY PLOT OF IMPURITY IN THE CONCENTRATION RANGE OF 0.12-1.87 µg/g LEVEL

Injection ID	Impurity Peak area		Points	Linearity Concentration (µg/g)		Impurity Peak area
	Intra-day	Inter-day		Nominal	Obtained	
1	137748	142032	1	0.12	0.12	12056
2	134774	141783	2	0.31	0.31	32919
3	124022	137220	3	0.62	0.61	66143
4	134064	134584	4	0.94	0.95	104830
5	134239	135153	5	1.25	1.28	141300
6	138033	140135	6	1.56	1.48	163666
SD	5105.38	3289.40	7	1.87	1.92	212666
%RSD	3.8	2.4	Slope	111561		
			Intercept	-1504.44		
			Regression [r]	0.9991		

Linearity: By multiple reaction monitoring (MRM), the linearity of 2-isopropyl-4-(chloromethyl)thiazole was explained satisfactory with a six point calibration graph range of LOQ-150%. The slope, intercept and correlation coefficient values were derived from linear regression analysis and the data is represented in Table-1. The linearity experiment revealed that the mass spectrometric responses were proportional to their concentrations up to 150% of the estimated level. It reveals that the mass spectrometric responses were proportional to their concentrations within the range of 0.12-1.86 µg/g. The regression (r) for impurity was 0.9991. The typical mass spectra and linearity graph of impurity shown in Fig. 4.

Precision: The precision of the method was verified by repeatability, method precision and intermediate precision. Repeatability was tested by injecting six individual preparations of test sample ritonavir 10 mg/mL spiked with 1.24 µg/g of the impurity. The same experiment was done on next day to evaluate intermediate precision. The developed method was found to be precise as the % RSD value for intra- and inter-day precision overall are less than 3.0. This concludes that the method was very much precise for the impurity and the results summarized in Table-2.

Recovery studies: The recovery studies by the standard addition method were performed to evaluate accuracy and

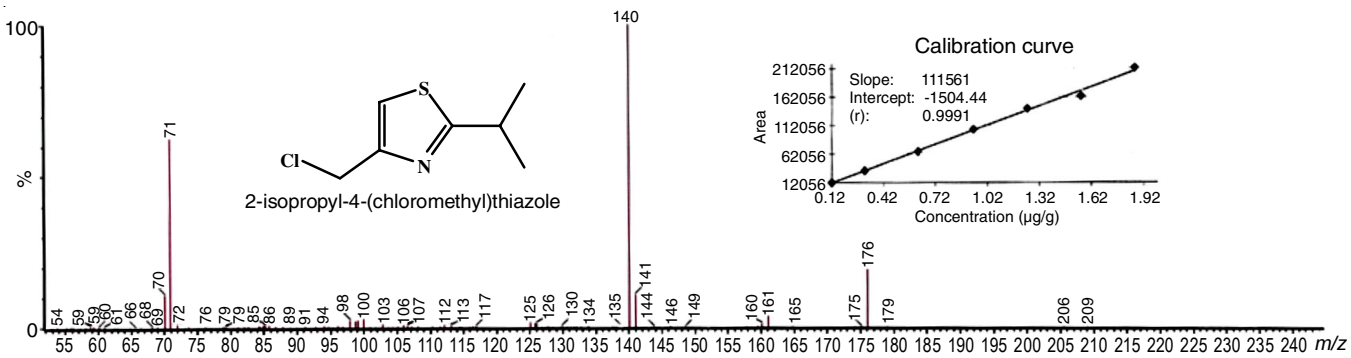

Fig. 4. Mass spectra and linearity plot of 2-isopropyl-4-(chloromethyl)thiazole impurity

TABLE-2
METHOD PRECISION AND INTERMEDIATE PRECISION OF THE IMPURITY AT 1.24 µg/g CONCENTRATION

Injection ID	Method precision and accuracy			Intermediate precision and accuracy		
	Concentration (µg/g)		Accuracy (%)	Concentration (µg/g)		Accuracy (%)
	Nominal	Observed		Nominal	Observed	
1	1.250	1.271	101.7	1.250	1.264	101.1
2	1.250	1.233	98.7	1.250	1.220	97.6
3	1.250	1.243	99.5	1.250	1.279	102.3
4	1.250	1.208	96.6	1.250	1.253	100.3
5	1.250	1.196	95.7	1.250	1.296	103.6
6	1.250	1.222	97.7	1.250	1.257	100.6
	Mean	1.230	98.3	Mean	1.260	100.9
	SD	0.02	2.15	SD	0.03	2.03
	%RSD	2.0	2.2	%RSD	2.2	2.0

TABLE-3
EVALUATION OF RECOVERY AT THREE DIFFERENT CONCENTRATIONS OF THE IMPURITY

Recovery level	Sample area	Standard area	Spiked area	Concentration (µg/g)		Recovery (%)
				Nominal	Observed	
0.12 µg/g	0	11982	12277	0.120	0.117	97.4
	0	12905	12911	0.120	0.124	102.4
	0	12926	12242	0.120	0.117	97.1
1.24 µg/g	0	137335	136502	1.250	1.283	102.4
	0	133812	127024	1.250	1.194	95.2
	0	128947	134307	1.250	1.262	100.7
1.86 µg/g	0	203317	203862	1.870	1.855	97.7
	0	208909	210524	1.870	1.916	100.9
	0	213688	192786	1.870	1.754	92.4

selectivity, accordingly the accuracy of the method was resolute in triplicate at LOQ, 1.24 µg/g and 1.86 µg/g in test sample. Then the percentage recoveries were calculated. The recoveries at all the three concentrations were satisfactory. Therefore, accuracy of the method in the range LOQ-150% was confirmed by the recovery data shown in Table-3. Excellent recovery values of impurity (92.4-102.4%) were obtained. The method has been used successfully for the determination of five different batches of ritonavir API (active pharma ingredient) samples, but the concentration of impurity was found below the detection level, also there was no interference of sample matrix and related substances of ritonavir shown in Table-4.

TABLE-4
ANALYSIS IN FIVE DIFFERENT BATCHES OF RITONAVIR DRUG SUBSTANCE, LOQ AND LOD CONCENTRATIONS

No. of samples	Concentration (µg/g)		Impurity
	LOQ	LOD	
Sample-1	0.12	0.06	Not detected
Sample-2	0.12	0.06	Not detected
Sample-3	0.12	0.06	Not detected
Sample-4	0.12	0.06	Not detected
Sample-5	0.12	0.06	Not detected

Robustness: Robustness of the method was determined by making changes in experimental conditions were intentionally altered including mobile phase flow rate, source cleaning after system suitability and column oven temperature. The actual flow rate of the mobile phase was 0.3 mL/min and same was altered by ±10% *i.e.* 0.27 mL (low flow) and 0.33 mL (high flow). The source cleaning before and after the effect on system suitability, no deliberate changes in %RSD values obtained in standard precision experiment and below the %RSD 5.0. The effect of column oven temperature on chloromatographic resolution was also studied at 38 and 42 ºC (altered by 2 ºC units). No significant change in the chromatographic system, which indicated that method, was robust in the specified range.

Stability studies: To evaluate stability study, standard solution and sample solution spiked with impurity at 1.24 µg/g level were prepared as per test method and analyzed in triplicate injections each, at least after 4 h by keeping the solutions at 25 ± 2 ºC. The percentage difference after 29 h standard, spiked sample areas obtained at initial and specified time interval were 1.8 and 4.0%, respectively. No significant change in the amount of impurity observed during the study, which is shown in Table-5. It concludes that standard solution and sample solution was stable up to 29 h at 25 ± 2 ºC.

RESULTS AND DISCUSSION

Optimization of mass spectrometer conditions: The main objective of method development was to achieve trace level, simple, efficient and rapid determination of 2-isopropyl-4-(chloromethyl)thiazole in ritonavir drug substance to facilitate the method for routine use in quality control laboratory. Due to the combination of a unique product ion and elimination of back ground noise, MRM (multiple reaction monitoring) results found consistently low limits of detection even for complex matrices comparatively with SIM (selected ion monitoring). This mode permits significant enhancement of selectivity and sensitivity for screening of impurity quantification. Optimization of mass spectrometric conditions of 2-isopropyl-4-(chloro-

TABLE-5
SOLUTION STABILITY DATA OF IMPURITY IN DILUENT

Name	Temperature about 25 ± 2 °C duration after 29 h					
	Standard area		Difference (%)	Spiked sample area		Difference (%)
	Control	Stability		Control	Stability	
Impurity	136069	138798	1.8	143652	133928	4.0
	135812	138526		135719	135885	
	136348	138318		139577	132215	
Mean	136076	138547		139649	134009	
SD	268.08	240.71		3966.99	1836.35	
%RSD	0.2	0.2		2.8	1.4	

methyl)thiazole stock solution was prepared in acetonitrile and water (80:20 v/v) and diluted to get a final concentration of about 3.35 µg/g. This solution was used to generate MRM transitions through the automated MRM method development software (Intellistart) of MassLynx. Impurity was subjected to run in Q1 scan modes (both positive and negative) and ionized well in positive mode rather than negative. Impurity in scan mode 176[M+H]$^+$ with chloro pattern and gives major daughter fragments were 71,126,140. MRM tuning results of impurity better response observed in ESI mode than APCI mode and stable, intense fragments 71 and 140 were used for quantitation (71 as qualifier and 140 as quantifier).The ion source parameters were optimized to get proper impurity optimum response.

Selection of UPLC column: Several attempts were made with different C18 UPLC columns *viz*. Inertsil ODS-3 (50 mm × 2.1 mm, 2.0 µm) and Acquity UPLC BEH C18 column (100 mm × 2.1 mm and 1.7 µm) using isocratic and gradient elution, but in all the above conditions the separation of the related substances of ritonavir drug sunstance with analyte impurity was not satisfactory. Because Inertsil ODS-3, BEH C18 columns were found, not to suitable as the response of analyte and related substance peaks were not resolved among themselves and from drug substance peak. But, on Acquity UPLC column HSS T3 column (100 mm long, 2.1 mm internal diameter, 1.8 µm dia.), the related substances were well resolved from the analyte and response of analyte peak was also found good.

Optimization of chromatographic conditions: Chromatographic conditions of impurity and ritonavir drug substance were optimized an AQUITY UPLC column HSS (high strength silica) T3 column (100 mm long, 2.1 mm internal diameter, 1.8 µm dia.) started with isocratic flow rate 0.25 mL/min [0.1% formic acid in water (mobile phase-A)-aceonitrile (mobile phase-B) (60:40 v/v)] and injection volume 5.0 µL. Column oven temperature was 35 ºC. Standard solution was 1.25 µg/g (sample concentration 1.0 mg/mL) injected to optimized tuning UPLC-ESI-MS/MS system. The response of impurity peak was very low and not better resolution with the ritonavir drug substance at this chromatographic system. To overcome this ratio of mobile phase was changed to 40:60 v/v (mobile phase-A and B) gradient elutions (time (min)/% mobile phase-B) 0.01/60, 3.0/60,3.5/90,7.0/90,7.5/60,10.0/60 with flow rate 0.3 mL/min and column oven temperature was 40 ºC. Enabled separation of impurity and drug substance with good peak shape was observed. For response of impurity, optimized the sample concentration to 10 mg/mL.

Optimization of sample preparation: Sample preparation is an important part of the pharmaceutical impurity analysis, because the sample matrix effects in trace levels causes loss of abnormal recovery, sensitivity and analyte reproducibility. Different diluents were evaluated with respect to chromatographic concert, solubility of both impurity and drug substance were good in acetonitrile and water. Premix of acetonitrile and water in different ratios were evaluated. Impurity and ritonavir better solubility was observed in acetonitrile and water in ratio of (80:20 v/v) at 1.0 mg /mL and 10.0 mg/mL. Moreover good response and symmetric good peak shape of impurity, drug substance were found in acetonitrile:water (80:20 v/v), hence chosen as a diluent for quantification. Good recoveries (92.4-102.4%) were obtained for impurity in this diluent.

Conclusion

A simple, selective and standard and spiked solutions stability indicating UPLC-ESI-MS/MS method has been developed for the quantitative determination of 2-isopropyl-4-(chloromethyl)thiazole in ritonavir. Good results were obtained from validation experiments of the method and found to be specific, precise, linear, accurate and robust. The detection levels are low *i.e*. 0.06 µg/g and can be used for routine quality control analysis of production samples and pharmaceutical dosage forms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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