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Research Article

VALIDATION OF LC-MS/MS METHOD FOR THE SIMULTANEOUS ESTIMATION OF ITRACONAZOLE AND ITS METABOLITE HYDROXY ITRACONAZOLE IN PLASMA

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ABSTRACT

Objective: To develop and validate a sensitive, selective and reproducible method for the estimation of Itraconazole (ITR) and its pharmacologically active metabolite Hydroxy Itraconazole (HITR) in human CPDA (Citrate Dextrose Phosphate Adenine) plasma, using Loratadine (L) as an internal standard (IS).

Methods: The drugs of interest and IS were extracted by solid phase extraction technique, using HLB cartridges (30mg/1cc). Chromatographic separation was achieved in 2.6 minutes on reverse phase Hypersil Gold (50×4.6) mm, 3µ column, using isocratic elution with methanol-buffer solution (2 Mm Ammonium acetate) in proportion of (90:10, v/v). Tandem mass spectrometer was used to detect the positive ions in the MRM (Multiple Reaction Monitoring) mode.

Results: The method was linear for ITR (1.08-403.28 ng/ml) and HITR (1.09-406.77 ng/ml). The coefficient of correlation (r) for the drug and the metabolite were better than 0.99 during the run of three precision and accuracy batch. The method was fully validated, complying FDA (Food and Drug Administration), EMEA (European Medicines Agency) guideline and recommendations of AAPS (American Association of Pharmaceutical Scientists) white papers demonstrating Selectivity, Sensitivity, Matrix factor, Precision and Accuracy, Linearity, Aqueous stability, Stability in biological matrix, Dilution Integrity, Reinjection Reproducibility, Recovery, Ruggedness and Extended Batch Verification.

Conclusion: The validated method is applicable for Bioavailability/ Bioequivalence and Pharmacokinetic studies.

Keywords: Itraconazole; Hydroxy Itraconazole; LC-MS/MS; Bioavailability, Bioequivalence.

INTRODUCTION

Itraconazole is a triazole antifungal agent with broad spectrum activity [1-4]. Itraconazole (R 51 211), (+ -)-ics-4[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl]-1-piperazinyl] phenyl]-2,4-dihydro-2(1-methylpropyl)-3H-1,2,4-triazol-3-one, is an orally active triazole antifungal agent which demonstrates broad spectrum activity against a number of fungal species including dermatophytes, *Malassesia furfur, Candida* species, *Aspergillus* species, and *Histoplasma capsulatum* var. capsulatum [5]. The mechanism of action of Itraconazole relates to the binding of fungal cytochrome P-450 with resultant inhibition of ergosterol synthesis, an essential element of membrane in propagating the growth of fungal and yeast colonies and perturbation of membrane bound enzyme function and membrane permeability [6].

Following oral absorption, it is extensively metabolized by side chain hydroxylation (by CYP3A4) to form hydroxyitraconazole. OH-ITZ, which is a major metabolite, is biologically active and its plasma concentration is twofold higher than parent at steady state [7]. Itraconazole is metabolized via CYP3A4 enzymatic system to form primarily three active metabolites viz, hydroxyitraconazole, Keto-itraconazole and N-desalkylitraconazole [8-10].

There are literature publishing method for the determination of Itraconazole and its hydroxy metabolite by HPLC, in serum [11-14], in plasma [15-20]. Generally, HPLC methods are less sensitive and not reliable particularly for the metabolites. Several papers were published for the determination of Itraconazole and hydroxy Itraconazole in human plasma using LC-MS/MS [21-34]. The published methods had several issues like; sensitivity, ULLOQ (Upper limit of quantitation) range were not sufficient, non applicability to human studies, metabolite were not estimated, single MS (Mass spectrometry) was used instead of MS/MS or some of the important parameters were not estimated or estimated differently.

Any method intended for the simultaneous estimation of Itraconazole and its metabolite for regulated bioanalysis, has to be performed as per the USFDA (United States Food and Drug Administration) [35] and EMEA (European Medicines Agency) [36] guidelines.

The objective of the study was to develop a method in CPDA plasma due to economical considerations and to establish all the parameters for a typical method validation. The validated method was sensitive, selective and complies with the regulatory postulates. The current understanding of the guidelines and best industry practice were also taken into consideration. The method is applicable for Bioavailability/Bioequivalence and pharmacokinetic studies.

MATERIALS AND METHODS

Reagents and Chemicals

Samples of ITR, HITR were procured from (Synfine Research Limited, India) and L was procured from (Arbro Pharmaceutical Limited, India). HPLC grade solvents like; acetonitrile, methanol and ammonia were supplied by (Renkem Limited, India). Purified water from Milli-Q (Millipore Advantage A10) instrument was used. Extraction and purification was performed on Oasis ® HLB 1cc (30 mg) extraction cartridges from (Waters, Ireland).

Instrumentation

The LC-MS/MS instrument consisted of liquid chromatography unit from Shimadzu, Japan (Model LC10), Binary pumps (LC 10ADp Series), Column Oven (CTO-10 ASVP), and Auto injector (SIL-10A) with System controller (SCL-10AP) tandem with triple quad API-4000 from MDS Sciex, Canada in positive ion mode. Analyst software 1.4.1 was used for data processing.

Chromatographic Conditions

Sample injection (10µL) was separated and chromatographed at a flow rate of 0.500 mL/minute on Hypersil Gold (50x4.6) mm, 3µ column, using methanol: buffer solution (90:10, v/v) as mobile phase. The buffer solution was 2 Mm ammonium acetate with 100 ml acetic acid in water. The column oven and auto sampler temperatures were maintained at 35 ± 2°C and 5 ± 1°C, respectively.

Mass Spectrometry

The Triple Quad API-4000 Mass Spectrometer was operated in positive ion mode. The gases i.e. GS1 (Nebulizer gas), GS2 (Heater gas) and curtain gas were optimised at 60.00, 68.00 and 11.00 while CAD gas (Collision Associated Dissociation) at 11.00 Psi. The Source temperature was regulated at 550^{0}C and Ionization spray potential (IS) at 5500 V. The different voltages which were optimized for desired results were Entering potential (EP-10V), Declustering potential [DP-100V], collision Energy [CE-50eV]. The Collision exit potential [CXP-10V]. The LLOQ was optimised with Unit: Low resolution in Q1 and Q3.

Preparation of stock solutions

The stock solution of ITR, HITR and L were prepared in acetonitrile to give the final concentration of approximately 1 mg/ml. The stock solutions were stored at refrigerated temperature of 2-8^{0}C until use. For CC/QC (Calibration Curve/Quality Control) spiking, stock solutions of ITR and HITR were further diluted using diluent solution acetonitrile: water: (50:50, v/v). IS stock dilution were diluted to 1000 ng/ml using diluent solution

Preparation of calibration standards and quality control samples in plasma

Different plasma lots were screened for any significant interference at the RT (Retention Time) of drug, metabolite or internal standard. The interference free batches were further subjected to matrix factor determination. Batches showing no significant interference and ion enhancement/suppression were pooled together for the spiking of CC/QC samples.

Calibration curve standards and quality control samples were prepared by spiking drug free pooled plasma. Stock dilution were prepared from the mother stock and spiked to obtain CC concentrations of 1.08, 2.17, 4.34, 54.31, 123.44, 246.87, 352.67, 403.28 ng/ml for ITR and 1.09, 2.19, 4.39, 54.87, 124.71, 249.41, 356.30, 406.77 ng/ml for HITR. Spiked QC samples were obtained at concentration for lower limit of quantitation quality control (LLOQQC= 1.08), lower quality control (LQC =3.20), middle quality control (MQC=202.51) and high quality control (HQC=316.42) for ITR and (LLOQQC=1.09), (LQC=3.24), (MQC=204.85) and (HQC= 320.08) for HITR. Spiked CC/QC samples were stored in -70^{0}C deep freezer, except 18 QC samples stored at -25^{0}C deep freezer for stability data generation at -25^{0}C. Six sensitivity samples were also spiked separately (1.08-ITR & 1.09-HITR ng/ml) and stored in -70^{0}C deep freezer, until analysis.

Sample Preparation

Aliquoted 200 µl of plasma sample, added 25 µL of IS (1000 ng/ml) and 50 µl of ammonia solution, samples were mixed by vortexing. Solid Phase Extraction Method (SPE) was used for sample cleanup and extraction. The simple step which followed is mentioned below;

Step 1: Conditioned the HLB cartridge (30 mg/1cc) with 1.0 mL methanol, 1.0 mL of Milli-Q water, Step 2: Loaded sample, Step 3: Washed the cartridges with 2.0 mL of Milli-Q water, Step 4: Eluted the sample with 1.0 mL acetonitrile, Step 5: Evaporated the eluate to dryness at 40°C under constant pressure in nitrogen evaporator, Step 6: Reconstituted the samples in 200 µL mobile phase and transferred into HPLC vials for analysis.

Validation Procedures

A full validation was performed in CPDA plasma according to USFDA and EMEA guidelines.

Selectivity

Seven CPDA plasma lots obtained from different sources were screened for significant interference at the RT of drug/metabolite and internal standard.

Matrix Factor (MF)

Six plasma batches, previously screened and found to be clean of any significant interference at the RT of drugs and IS were used for matrix factor experiment. The experiment was performed by 'post extraction spiking method'.

MF= Peak response in presence of matrix Ions/Peak response in absence of matrix Ions

Linearity

Eight non zero calibration standards were prepared having concentration of 1.08-403.28 ng/ml for ITR, also 1.09-406.77 ng/ml for HITR. The data from three precision and accuracy batches were subjected to goodness of fit analysis using 1/x and 1/x^2 weighing factor. Deviation from nominal concentration should be within ±20% for LLOQ and within ±15% for the other concentrations. At least 75% of non-zero standards should meet the criteria, including LLOQ and ULOQ standard. Linear coefficient of correlation (r) should be ≥0.98.

Accuracy and Precision

The precision of the assay was measured by the percent coefficient of variation (% CV) for the concentration at LLOQQC, LQC, MQC and HQC level. Six replicates at each QC level, in a single analytical run constitute one PA (Precision and Accuracy) batch. Three batches were run to calculate the Intra and Inter Batch precision and accuracy. The batches run on two different days provide the Inter batch estimate. Deviation from nominal concentration should be within ±20% at LLOQQC and within ±15 % for the other concentrations. A minimum of 67% of the total QC and 50% at each concentration level should pass the % nominal criteria. The % CV at each concentration level should be ≤ 15%, except LLOQQC (≤ 20%).

Sensitivity

Sensitivity of the method is defined as the lowest concentration of analyte that can be measured with acceptable limit of precision and accuracy. Five LLOQ samples were run and back calculated against a calibration curve. The % nominal and % CV should be within ±20% and <20%, respectively.

Carry over

Two processed blank samples were injected following ULOQ of each calibration curve. The permissible interference at the RT of analyte should be <20% of LLOQ, while area response at the RT of IS should be <5 % of mean IS area in the calibration curve.

Stability

Stability of analyte in stock solution and stability of analyte in plasma under different conditions were both investigated.

Stability of mother stock in refrigerator (2-8^{0}C), at room temperature and stock dilution stability at room temperature were compared with fresh solutions. Six replicates each from stability and comparison stock were injected to compare their response. The percent change should be within ±7 %.

Quality control samples for bench top stability (BT) at room temperature, freeze thaw stability (FT) after three successive freeze & thaw cycles, long term stability (LT) in -25^{0}C & -70^{0}C deep freezers and autosampler stability (AI) at 5^{0}C were back calculated against freshly prepared calibration curve. 67% of the total QC, including minimum 50% at each concentration (Low & High) should be within ±15% of the nominal values. The % CV should be ≤15%.

% Change = [(Mean response of comparison samples – Mean corrected response of stability samples)/ Mean response of comparison samples] x 100

Correction Factor = Concentration of comparison dilution/ Concentration of Stability Dilution

Corrected Response = Mean Stability dilution response x correction factor

Reinjection Reproducibility

A Precision and accuracy batch was reinjected after a period of storage into the autosampler to assess the reinjectibility of the samples. The batch should follow the acceptance criteria of a precision and accuracy batch.

Ruggedness

To demonstrate the method can withstand minor changes in reagents/solutions and chromatographic condition, ruggedness of method was investigated. A precision and accuracy batch was processed with fresh reagent and solutions. The analytical column was replaced with an identical column, but different serial number. The batch should follow the acceptance criteria of a precision and accuracy batch.

Recovery

There should not be significant difference between the recoveries at different concentration level. A mixture of drug and metabolite, representing 100% extraction at low, middle and high concentrations were prepared and serve as non-extracted ‘comparison samples’. 6 QC samples each at LQC, MQC and HQC level were extracted as per the method. They serve as the extracted ‘recovery samples’. Inject 6 replicates of the ‘comparison’ samples and 6 replicates of the ‘recovery samples’ at each concentration level. Calculate the percent recovery as follows;

% Recovery = [Mean peak area response of recovery samples/ Mean Peak area response of comparison sample] × 100

Comparison sample were prepared by taking 10 µL drugs (5% Spiking) + 25 µL (IS dilution) + 165 µL mobile phase to obtain an equivalent of extracted concentration.

The % recovery, mean % recovery and % CV among mean % recoveries at low, medium and high levels were calculated. % recovery for (IS) was calculated from the data of 18 comparison and recovery samples. The % CV among % recoveries at Low, Middle and High QC should be <20%.

Dilution Integrity (DI)

The drug free matrix was spiked to obtain the plasma concentration higher than calibration range (2019.13 & 2066.64 ng/ml) for ITR and HITR, respectively. Six replicates of dilution integrity QC samples were diluted ten times (DI-10) with similar blank matrix and processed as per the method. The samples were back calculated against a calibration curve by applying dilution factor of (DI-10). The % nominal and precision should be within ±15% and <15%, respectively.

Extended Batch Verification

Extended batch verification is performed to establish the analytical batch size for the method. It further demonstrates the ruggedness of the method under the given experimental conditions.

Six batches of test samples (QC interspersed between blank samples), processed and analyzed against a calibration curve. Evaluate the results for each batch separately. At least 67% of the QC samples including minimum 50% at each concentration level should be within ±15% of nominal values.

RESULTS AND DISCUSSION

Mass Spectrometry

For scanning an aqueous mixture of intermediate dilution was prepared containing drugs (ITR & HITR) and IS. During the Q1 +ve scan, the parent ions selected were [(705.20-ITR), (721.30-HITR) & (383.10-IS)]. The most abundant product ions were paired with parent ions and optimised in MRM mode. The transition optimised were (705.20----392.10 a.m.u) ITR & (721.30-----408.30 a.m.u) HITR and (383.10-----337.20 m/z) L to get a stable and reproducible response. The chromatography and S/N (Signal/Noise) ratio was optimised at LLOQ concentration.

Selectivity

All screened seven batches were free from significant interference from endogenous substances. Fig: 1, 2 & 3, illustrates the representative chromatogram for Aqueous Mix, blank and LLOQ.

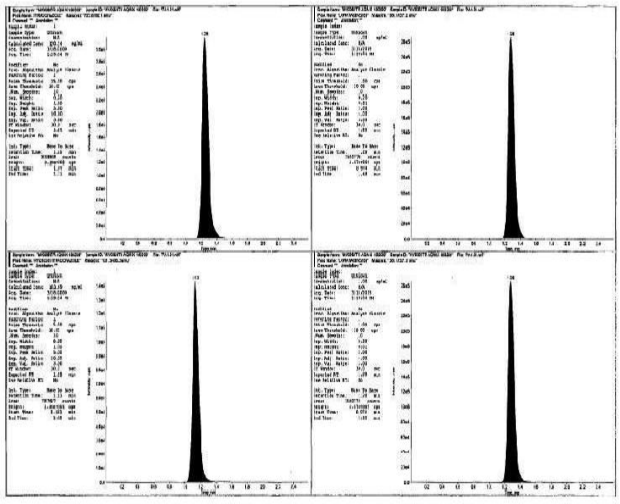

Fig 1: Representative chromatogram of Aqueous Mix

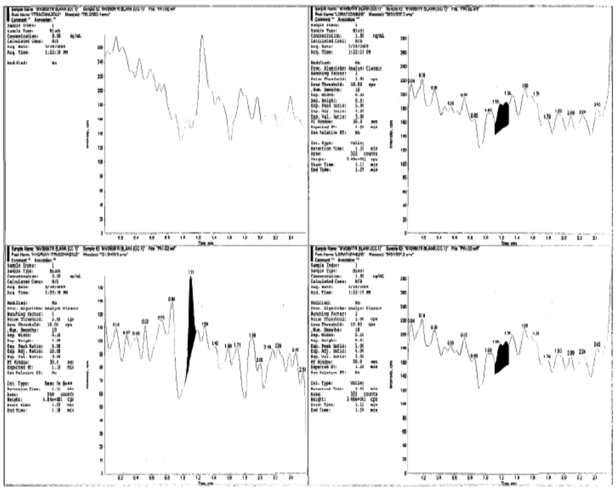

Fig 2: Representative chromatogram of blank sample

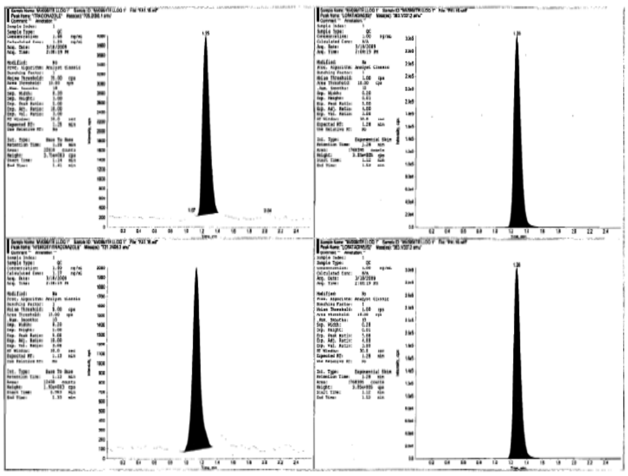

Fig 3: Representative Chromatogram of LLOQ

Matrix factor (MF)

Matrix factor experiment was performed by 'post processed spiking Method' at LQC & HQC level. The % CV of MF for the drugs was <15%. The IS Normalised MF was calculated and reported. The results are summarised in Table 1.

Table 1: IS-Normalised Matrix Factor

Analyte	Conc (ng/mL)	Mean MF ± S.D	% CV for MF
ITR	3.20	0.826±0.038	4.60
	316.42	0.830±0.024	2.90
HITR	3.24	0.967±0.041	4.29
	320.08	0.982±0.016	1.65

Linearity and sensitivity

The results of three precision and accuracy batch comply with the rule mentioned under section material and method and subsection linearity. The calibration curve data was subjected to goodness of fit analysis, resulting $1/x^2$ to be the best fit for regression. The coefficient of correlation (r) was >0.99 for calibration range ITR (1.08-403.28 ng/ml) and HITR (1.09-406.77 ng/ml). The observed S/N ratio was >5 throughout validation exercise. The precision and accuracy for sensitivity samples [LLOQ=1.08 ng/ml (ITR) & 1.09 ng/ml (HITR)] were within ± 20% limit. The results are summarised in table 2.

Table 2: Sensitivity at LLOQ level

Analyte	LLOQ	Replicate	Observed Conc.(µg/ml)	%Nominal	Mean	Mean%nominal	%CV
ITR	1.08ng/ml	1	1.26	116.67			
		2	1.25	115.74			
		3	1.06	98.15	1.162	107.59	7.61
		4	1.12	103.70			
		5	1.12	103.70			
HITR	1.09ng/ml	1	1.08	99.08			
		2	1.01	92.66			
		3	0.93	85.32	0.986	90.46	6.36
		4	0.98	89.91			
		5	0.93	85.32			

Table 3: Intra-day and Inter-day precision and accuracy

			Inter Day(n=6)			Inter Day(n=18)		
Analyte	Level	Nominal (µg/ml)	Mean Concentration(µg/ml)	Mean % nominal	%CV	Mean Concentration(µg/ml)	Mean % nominal	%CV
ITR	LLOQQC	1.08	1.230	113.89	1.63	1.107	102.47	10.73
	LQC	3.20	3.133	97.92	3.48	3.142	98.19	4.68
	MQC	202.51	209.687	103.54	3.24	198.966	98.25	4.86
	HQC	316.42	308.040	97.35	0.84	295.151	93.28	3.48
HITR	LLOQQC	1.09	1.210	111.01	2.02	1.138	104.38	10.62
	LQC	3.24	3.270	100.93	5.20	3.174	97.98	6.86
	MQC	204.85	228.278	111.44	2.22	203.828	99.50	9.37
	HQC	320.08	314.602	98.29	1.17	294.653	92.06	5.23

Precision and accuracy

The Intra and Inter batch precision were within ≤15% at all the concentration levels, except LLOQQC (±20%). The % nominal calculated for a batch and between batches were within +15% at all the concentrations, except LLOQQC (±20%). The assay performance is summarized in Table 3. The % interference at the RT of drugs was <20% throughout the validation. The % interference at the RT of IS was always <5%.

Stability

In a study where the results are directly influenced by analyte stock solutions, it is recommended that the acceptance difference between the absolute responses of fresh stock and aged stock be tighter (within 5-7 %), rather (15-20%) normally applied to bioanalytical results [37]. The % Change for Stock solution stability at refrigerated temperature range (2-8^{0}C) and stock dilution stability at room temperature were within ±7%. Stability of drugs in plasma, which includes BT, FT, LT and AI stability were within the acceptance limit. Results are demonstrated in table 4 & 5.

Recovery

The mean % recovery was 70.92% and 68.93% for ITR and HITR respectively. The % recovery for IS was 75.66%. Precision among % recoveries at Low, medium and higher QC level were 6.73% and 5.41% for OXC and MHD, respectively.

Extended Batch Verification

The method validation precision and accuracy run should contain enough test samples to mimic the actual run time of a production batch; this is a current industry standard and FDA expectation [38]. Six batches, totalling 288 samples were run against a calibration curve. The batch size was defined at SPE unit. A batch contained 2 QC set (LQC, MQC and HQC) and 42 plasma samples, processed together. In a batch, 2/3 of the total QC samples and 1/2 at each concentration level were within ±15% for accuracy. None of the previously reported method established this parameter.

Table4: Stability data of OXC and MHD under different storage and processing conditions (n=6)

Stability	Analyte	Nominal concentration (μg/ml)	Mean concentation (μg/ml)	%Nomanal	%CV
Freeze thaw(3cycles)	ITR	3.20	3.310	103.44	2.42
		316.42	300.215	94.88	1.35
	HITR	3.24	3.257	100.51	4.12
		320.08	294.712	92.07	0.70
Bench Top(8Hours)	ITR	3.20	3.355	104.84	1.75
		316.42	302.733	95.67	2.04
	HITR	3.24	3.287	101.44	4.39
		320.08	295.502	92.32	1.02
Long term-70Degree(15days)	ITR	3.20	3.187	99.58	1.98
		316.42	290.718	91.88	1.61
	HITR	3.24	3.225	99.54	4.07
		320.08	290.222	90.67	4.97
Long term-25degree(15days)	ITR	3.20	3.177	99.27	3.04
		316.42	287.318	90.80	1.66
		3.24	3.092	95.42	6.10
		320.08	286.786	89.58	2.78
Autosampler (24Hours)	ITR	3.20	3.272	102.24	3.61
		316.42	301.027	95.14	1.10
	HITR	3.24	3.305	102.01	3.65
		320.08	301.798	94.29	2.77

Table5: Summary of stability exercises carried out in aqueous phase (n=6)

Stability experiment	Analyte	Mean peak area (stability sample)	Mean peak area (comparison sample)	%CV(stability sample)	%CV(comparison sample)	%Change
STSRF(10days)	ITR	2645802.16	2689164.00	1.12	1.32	1.61
	HITR	1179858.95	1232644.33	1.18	0.58	4.28
	L	1296325.65	1280152.83	0.95	1.17	-1.26
STSRF(24 Hour)	ITR	2657343.80	2689164.00	1.24	1.32	1.18
	HITR	1176837.83	1232644.33	2.19	0.58	4.53
	L	1201639.81	1280152.83	0.51	1.17	6.13
STDSRT(24Hour)	ITR	2749691.21	2689164.00	0.94	1.32	-2.25
	HITR	1240147.20	1232644.30	0.73	0.58	-0.61
	L	1226593.56	1280152.80	0.56	1.17	4.18

STSRF: Stock solution stability in refrigerator; STSRT: Stock solution stability at room temperature; STDSRT: Stock dilution stability at room temperature

Other parameters

The samples were reinjectable after storage of 24 hours in autosampler at 5^{0}C, ruggedness under the change of solution/reagents and column was established. Dilution integrity (DI-10) was reproducible and within the acceptable limits.

CONCLUSION

The objective of the study was to interpret the guidelines correctly, understand the expectation of the regulatory bodies in relevance, and to perform the experiments based on the current industry best practices. The full validated method was compliant to, not only FDA/EMEA, but also with AAPS whitepapers and other relevant journals. The method is applicable to BA/BE and pharmacokinetics studies.

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