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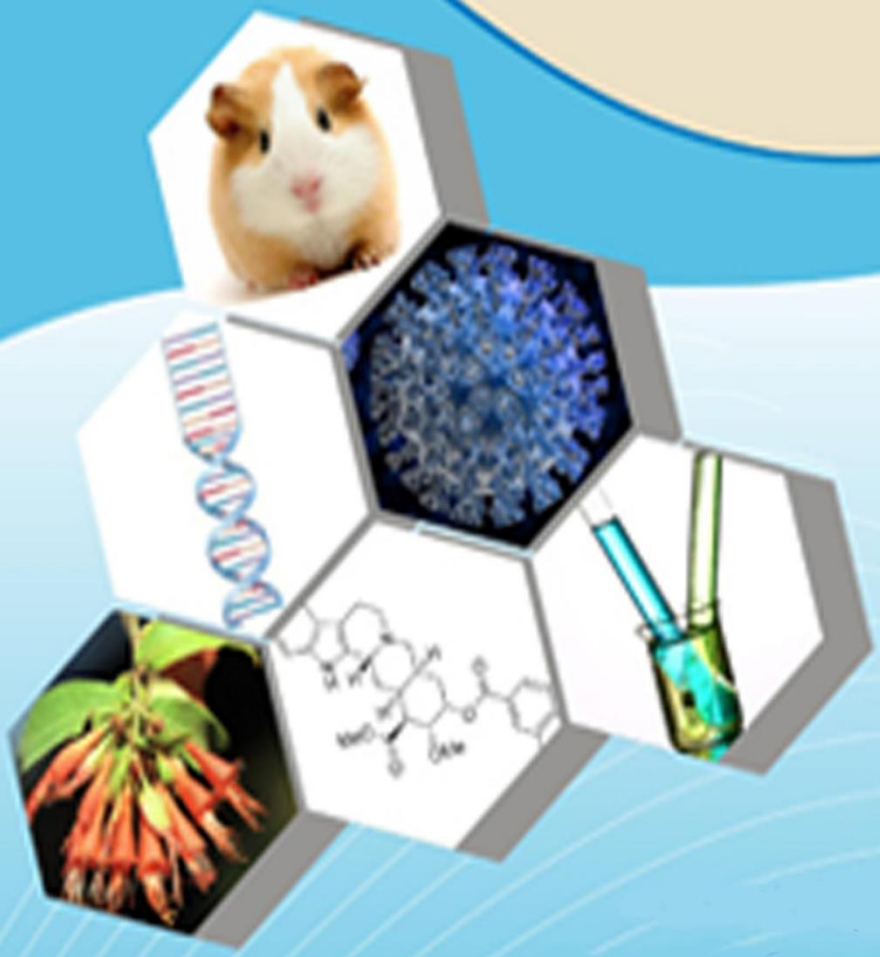

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DEPELOPMENT AND VALIDATION OF RP- HPLC METHOD FOR DETERMINATION OF ARIPRAZOLE IN HUMAN PLASMA

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ABSTRACT

The aripiprazole concentration in human plasma was determined utilizing a simple, precise, and accurate RP-HPLC (Reverse phase -high performance liquid chromatographic) method using voriconazole as the internal standard. Chromatographic settings included a phenomenex C18 (150 x 4.6 x 5 m) stationary phase, 0.01N potassium dehydrogenate phosphate (pH 4.8) mobile phase, acetonitrile and water at a 60:40 (v/v) ratio, a 1.0 ml/min flow rate, a 218 nm detection wavelength, and a 300 C column temperature. Results showed that Aripiprazole had a retention duration of 3,113 minutes and a coefficient of variation (CV) of less than 2%. The linearity of the suggested approach was studied throughout a concentration range of 10-400 ng/mL (r2 =0.999), and a recovery of 98.12% was determined. Detection levels as low as 10ng/mL were achieved, which is close to the concentrations of the substance that could be present in human plasma. Furthermore, the disclosed approach was shown to work in human plasma and verified in accordance with ICH principles. The disclosed approach was also verified in accordance with ICH recommendations and found to be within acceptable limits.

Key words: Aripiprazole; Voriconazole: RP-HPLC; Methods development; ICH Guidelines; validation.

Introduction

Medicines and their metabolites may be measured quantitatively in bodily fluids using bio-analytical techniques, which offer a novel strategy for the simultaneous detection and quantification of an analyzable unit. There are a many of ways to evaluate the ferocity of a coalesce. Considerations for choosing an analytical method include concentration levels, chemical features of the analyte, specimen matrix, cost, experimental speed, quantitative or qualitative measurement, required precision, and necessary equipment.

Several characteristics of a bio analytical method, such as its selectivity, accuracy, precision, recovery, sensitivity, and stability, are evaluated during validation.

Sample preparation:

Blood, plasma, serum, urine, etc. are all examples of biological media that may be used to conduct analyses. Puncturing a vein with a hypodermic syringe may draw up to five to ten ml of blood from a human body.7ml. Blood is extracted from a vein and placed in a tube containing an anticoagulant such heparin or potassium EDTA. Spin the plasma in a centrifuge at 4000 rpm for around 15 minutes, and you'll get plasma. It is expected that between 30 and 50 percent of the total amount would be retrieved...

Storage conditions for the biological samples:

Collecting, testing, and analyzing biological samples may be a lengthy procedure. Freezing the samples is necessary to prevent the samples from decomposing. The development of microbes in protein samples may be stifled by adding additives like 2mercaptoethanol and sodium aside, which obstruct cellular respiration...

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Preliminary treatment of biological sample:

Structure, partition coefficient, pH, functional groups, dissociation constant, polarity, and solubility are just some of the physicochemical properties that can be used to determine and optimize a sample preparation technique like liquid-liquid extraction, solid-phase extraction, or protein precipitation.

Liquid-liquid extraction (LLE):

The principle of differential solubility and partitioning equilibrium of sub-atoms between aqueous (the original specimen) and the organic component underlies this procedure. The extraction of a material from one liquid phase into another liquid phase is a common example of a liquid-liquid extraction process.

By binding the analytic to a solid support in a process known as solid phase extraction (SPE), the procedure is very selective and may be used to prepare specimens for analysis. The analysis is selectively eluted and the existing interference is rinsed away. SPE performance is influenced by a number of factors, including the kind and quantity of sorbent used, the volume of the loaded sample, and the makeup and amount of the washing and elution solutions.

Protein precipitation:

Proteins are often extracted using this method in standard biological analysis. The addition of an inorganic modifier might trigger the precipitation process. Perhaps a salt is meant by this modification. Changing the pH may cause precipitation as well. The protein solubility is altered as a result of this. The specimens are centrifuged and the supernatant portion may be introduced in to the LC system. Following that, it is dissolved in an appropriate solvent. The specimen eventually begins to converge. However, SPE is typically used in tandem with protein precipitation to get pure extract.

AIM AND OBJECTIVE

Aim:

The primary objective of the current research was to provide a method for the quick, accurate, sensitive, selective, repeatable, and exact quantification of Aripiprazole in human plasma.

The purpose of this study was to design and verify a novel reversed-phase high-performance liquid chromatography (RPHPLC) technique for the determination of aripiprazole concentrations in human plasma in accordance with current ICH recommendations. The goal is to create an RP-HPLC technique that is quick, accurate, selective, and sensitive, with little solvent and biological fluid use, and minimal extraction time.

DRUG PROFILE

Aripiprazole:

The atypical antipsychotic aripiprazole is taken orally and is effective against schizophrenia, bipolar I, major depressive disorder, autism-related irritability, and Tourette's syndrome. Both children and adults (ages 10-17) with schizophrenia or manic episodes from bipolar disorder may benefit from this injection, and it may be used as an auxiliary therapy for depression. Aripiprazole works by antagonizing alpha adrenergic receptors and agonistically activating dopamine and 5-HT1A receptors, while also agonizing 5-HT2A receptors. On November 15, 2002, the Food and Drug Administration officially approved aripiprazole for use.

Structure:

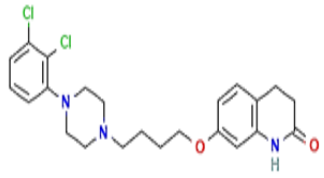

Table 1: Drug profile of Aripiprazole

IUPAC NAME:	7-(4-[4-(2,3-dichlorophenyl)-1-piperazinyl]butoxy)-3,4-dihydro quinolin-2(1*H*)-one.
CAS Number:	129722-12-9
Molecular weight:	448.39 g/mol
Molecular Formula:	$C_{23}H_{27}Cl_2N_3O_2$
Physical state:	Solid
pKa:	7.6
Solubility:	freely soluble in N,N-dimethylformamide, dichloromethane and insoluble in water.

Indication:

Treatment of irritability in autistic spectrum disorders, therapy of schizophrenia, treatment of Tourette's disease, and adjunctive treatment of major depressive disorder are all possible uses for aripiprazole...

Mechanism of action:

Although the precise mechanism of aripiprazole's antipsychotic activity has not been established, it is thought to include agonism of D2 and 5-HT1A receptors. Effects on other receptors might account for some of the unwanted side effects. Orthostatic hypotension, for instance, may be due to antagonism of adrenergic alpha1 receptors.

Absorption:

Peak plasma concentration of aripiprazole occurs between three and five hours after dosing. It has a maximum concentration in the blood after three hours when taken without food and up to twelve hours when taken with a high-fat meal. The oral bioavailability is 87%.

Protein binding:

Serum albumin and alpha-1-acid glycoprotein bind more than 99% of the plasma proteins..

Metabolism:

Dehydro-aripiprazole, the primary metabolite of aripiprazole, has affinity for D2 receptors comparable to that of the parent molecule. This pharmacologically relevant property is a result of the enzymes CYP3A4 and CYP2D6.

Half-life:

The elimination half-life of aripiprazole is 75 hours, whereas that of its primary metabolite, dehydro-aripiprazole, is 94 hours.
Clearance:0.8mL/min/kg[2]. Other studies have reported a clearance rate of 3297±1042mL/hr[3]

Dosage form:

Table No.2: Dosage form

Drug name	Label claim	Manufacturer
ABILIFY	2mg	Otsuka pharmaceuticals

INTERNAL STANDARD:

Voriconazole:

structure:

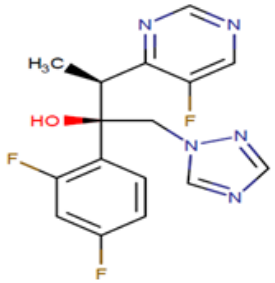

Table No.3 Internal standard profile

IUPAC NAME:	(2R,3S)-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1- (1H-1,2,4-triazol-1-yl)butan-2-ol
CAS Number:	137234-62-9
Molecular weight:	349.3105g/mol
Molecular Formula:	$C_{16}H_{14}F_3N_5O$
Physical state:	Solid
pKa:	12.71
Solubility:	organicsolventssuchasmethanol,ethanolandDMSO.
Mechanism of action	Voriconazolebindsandinhibitsergosterolsynthesisbyinhibiting CYP450-dependent14-alphasteroldemethylase.Theinhibitionof14-alphasteroldemethylaseresultsinadepletionof ergosterol infungal cell membrane.
Pharmacokinetics	
Absorption	96%
Protein binding	58%
Metabolism	Hepatic and intracellular
Excretion	Urine

MATERIALS AND METHODS

Materials:

API:

Hetero Labs in Hyderabad generously provided a sample of their API for both aripiprazole and voriconazole.
Blood plasma from a human donor:

TableNo.4: Human plasma

K_2EDTA Control Plasma	Deccan Pathological labs, Hyderabad

Chemical

TableNo.5: Chemicals and Solvents

S.No.	Chemicalname	Grade	Manufacturingcompany
1	Milli-Q water	HPLC	Millipore
2	Acetonitrile	Gradient Grade	Thermo Fisher scientific India Pvt.Ltd
3	Phosphatebuffer	Laboratory Reagent	Standard reagents
4	Methanol	Gradient Grade	Thermo Fisher scientific India Pvt.Ltd
5	Sodiumdihydrogenphosphate	AnalyticalReagent	Standard reagents
6	Ortho-phosphoricacid	AnalyticalReagent	Rankem

TableNo.6: Instruments and Equipment

S.No.	Instrument	Make
1	Electronicbalance	Shimadzu
2	pHmeter	Polmon
3	Sonicator	Labman
4	Centrifuge	Thermo Fisher
5	Vertex	RemiCM101
6	HPLC	Shimadzu LC-2030C plus

METHODOLOGY

Diluents: The medicines' solubility informed the choice of diluent: 0.1% orthophosphoric acid and 50% acetonitrile.

Preparation of Aripiprazole Stock solution (100µg/ml):

Put 10 milligrammes of aripiprazole into a 100 milliliter volumetric flask and dilute it to 100 micrograms per milliliter.

Preparation of Aripiprazole Spiking Solutions (10 ng/ml to 400 ng/ml):

In order to manufacture 10ng/ml, 20ng/ml, 30ng/ml, 160ng/ml, 20ng/ml, 240ng/ml, 320ng/ml, and 400ng/ml, aliquots of the aforesaid Aripiprazole stock solution were pipetted into 8 separate 10 ml volumetric flasks, and the remaining volume was filled with diluent. In order to generate 10 ng/ml, 20 ng/ml, 30 ng/ml, 160 ng/ml, 200 ng/ml, 240 ng/ml, 320 ng/ml, and 400 ng/ml calibration standards and quality control (QC) samples, working stock dilutions of analyses were added to blank plasma.

Preparation of internal standard solution (Voriconazole):

Stock-1: Put 5 milligrammes of voriconazole in a 100 millilitre volumetric flask and add enough solvents to get to 50 milligrammes per millilitre.

Stock-2: To prepare 5 g/ml solutions, transfer 1 ml of the aforementioned solution to a 10 ml volumetric flask and adjust the volume with the diluent.

Concentration: take 0.5 ml of the aforementioned solution and spike 1 g/ml ISD concentration into blank plasma using working stock dilutions of analyse...

Extraction procedure

Put 750 ml of plasma, 500 ml of internal standard, and 250 ml of aripiprazole from their respective spiking solutions into a centrifuge tube, add 1 ml of acetonitrile, and run the mixture through a cyclomixer for 15 seconds. Then, centrifuge for 5 minutes at 3200 rpm while in the vertex position. As soon as the centrifugation process is complete, take the filtered sample and inject it into a 10 Lento HPLC.

Method Development

HPLC Operating conditions:

The Phenomenex C18 (150 mm x 4.6 mm, 5) column was utilized in a Shimadzu LC-2030 high-performance liquid chromatography (HPLC) system at 30 degrees Celsius. With an injection volume of 20 L, a flow rate of 1.0 ml/min, UV detection at 218 nm, and a run duration of 6 minutes, the mobile phase is a 60:40 combination of 0.01N potassium dihydrogen ortho phosphate (pH 4.8) and acetonitrile.

Method Validation:

System suitability: Six duplicates of analyte and

internal standard were injected into the system, and its appropriateness was determined by analyzing the retention time and area ratio. A number of characteristics, including the tailing factor, the relative standard deviation of retention times and peak areas, the resolutions, and the theoretical plates, were calculated to assess the system's viability.
Acceptance standards require that the acquired percent CV of retention duration be no more than 5%, and that the obtained percent CV of area ratio be no more than 2%.

The auto-sampler was tested by introducing a series of both raw and processed samples into it. For the Auto-sampler carry over test, we injected and then measured the area under the curve of two blank solutions, two reference solutions, one extracted ULOQ, LLOQ standard, and two extracted blank matrices in a specific sequence.
Standardized measures of acceptability: When compared to the high standard sample, the percentage of carry over that was acquired does not exceed 0.5% of the blank sample regions.
A Look at the Matrix Factors: The matrix effect is a critical component of pharmacokinetic study evaluation. There was no evidence of ionization suppression or enhancement in the plasma samples, as measured by the internal standard normalized matrix factor, which ranged from 0.90 to 0.99.
Samples for quality assurance: Acceptable and typical chromatograms were seen over the course of Aripiprazole testing for standard zero (standard blank with internal standard), QC-LLOQ, QC-LQC, QC-MQC, and QC-HQC samples.

Selectivity: During validation, we looked for potential interference at the retention time of Aripiprazole and Internal standard owing to endogenous plasma components to determine the selectivity of the technique. Six lots of K2EDTA blank plasma were tested for selectivity, with the detection of extracted blank plasma demonstrating excellent selectivity for both the drug and the internal standard.
Pure solution calibration curves were tested for linearity across a concentration range of 10-400 ng/mL to ensure linearity. Twenty microliters (l) of each sample were injected into the chromatographic apparatus, and the resulting chromatograms were captured. By comparing the peak area to the drug concentration, a calibration curve was generated. Peak area was found to be linearly related to drug concentration across the studied range (concentration was plotted in ng/mL on the X-axis, and the peak area response was shown on the Y-axis).

Through analysis of six replicates at four distinct QC levels (LLOQ, LQC, MQC, and HQC), we were able to determine the accuracy and precision both within and between days. Six duplicate analyses were conducted at each of four concentrations (10ng/ml (LLOQ), 30ng/ml (LQC), 200ng/ml (MQC), and 320ng/ml) of Aripiprazole to assess the accuracy and precision of the procedure (HQC).
Aripiprazole recovery was calculated by comparing the peak areas of extracted blank plasma spiked with standards having the same area with a known dose of the drug to the peak areas obtained from prepared plasma samples. The average percentage of drug recovery for Aripiprazole and Voriconazole was determined.

Aripiprazole's Stability on the Laboratory Bench Analysis of quality control samples at both low and high concentrations allowed for accurate assessment of the Zero-day stability of Aripiprazole in human K2EDTA plasma. Calibration curve standards were only generated and used to calculate the concentrations of QC samples. For the purpose of determining the matrix's long-term stability at -20°C, these recalculated values will serve as the nominal concentrations. Analyses of six duplicates of LQC and HQC samples (30 and 320ng/ml) were performed at room temperature on the laboratory bench for 9 hours to determine stability.
The 37-day freezer-proofness of matrix samples: The stability of an Aripiprazole stock solution at the LQC-HQC concentration was evaluated during 37 days of refrigeration at -20°C.

RESULTS

METHOD DEVELOPMENT:

Additional testing was done since neither peak shape was acceptable and the system suitability parameters were outside of the acceptable range.

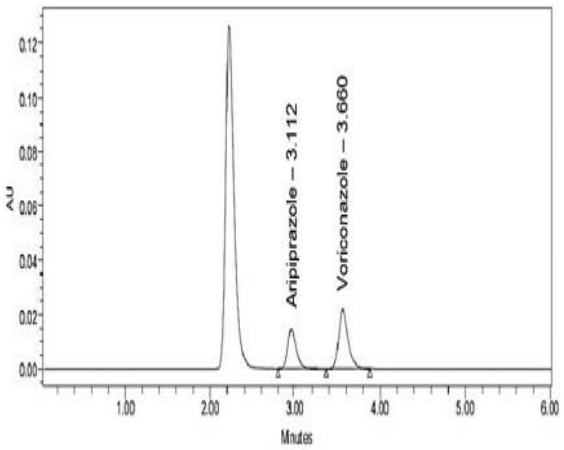

Fig 1: Chromatogram of Aripiprazole

Table.7: Observation of chromatogram

Replicate No.		Matrix factor	LQC	Matrix factor
1	32553	0.98166	3268	0.99
2	32510	0.97207	3285	0.987
3	32568	0.98942	3293	0.997
4	32533	0.96896	3266	0.987
5	32625	0.98881	3277	0.985
6	32462	0.98128	3299	0.994
N	6	6	6	6
Mean	32541.8	0.98037	3281.33	0.99
SD	55.1195	0.00842	13.3666	0.0045
% CV	0.16938	0.85926	0.40735	0.457

S.NO.	Peak Name	RT	Area	USPPlate count	USP tailing	USP resolution
1	Aripiprazole	3.112	21234	4741.4	1.1	3.2
2	Voriconazole	3.66	551182	5377.0	1.3	

Final conc (ng/ml)	Aripiprazole (peak area)
0	0
10	1088
20	2023
30	3251
160	16812
200	21292
240	25103
320	32351
400	41038

All criteria of system appropriateness fell within acceptable ranges, as defined by ICH recommendations. Both the Retention time (RT) and the area ratio (analyte area/IS area) had a coefficient of variation (CV) between 0.14 and 0.30% during the system suitability test.

Matrix factor evaluation:

Table 8: Matrix factor evaluation (absence of matrix factor)

Table 9: Matrix factor evaluation (presence of matrix factor)

Linearity: Calibration was found to be linear over the

Aripiprazole concentrations might be anything from 10 ng/mL to 400 ng/mL. In every instance examined, the value of the correlation coefficient (r2) was shown to be larger than 0.999. This demonstrates that the findings are linear and that there is a strong association between the peak area and the concentration of the analyte. Figure 2 displays a typical example of a calibration curve.

Table 10: Linearity of Aripiprazole

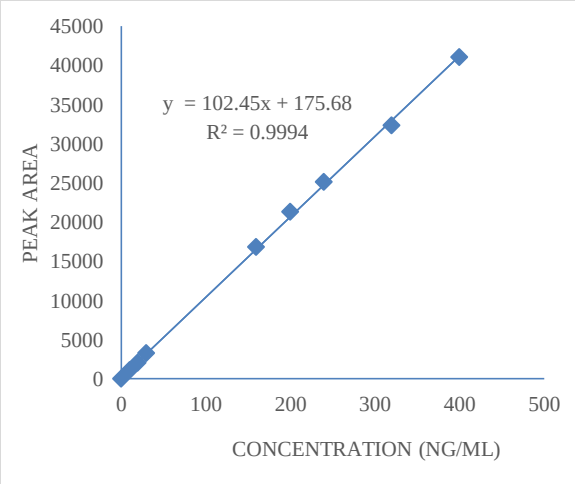

Fig 2: Calibration curve of Aripiprazole

Precision (intra-day and inter-day runs of Aripiprazole): When comparing plasma samples taken on different days, researchers found that the samples were 99.01% accurate on average, with a range of 99.51%-101.29%, and 100.07% accurate, with a range of 99.51%-102.23%. Analyte and plasma sample precision (%CV) values ranged from 0.45% to 2.7% intraday and from 0.44% to 1.97% interday, respectively.

Table 11: Precision data for intra-day and inter-

Replicate No.	HQC	LQC
1	33161	3301
2	33444	3327
3	32916	3301
4	33575	3306
5	32994	3324
6	33081	3318
N	6	6
Mean	33195	3313
SD	260.31	11.65
% CV	0.78	0.35

day runs of Aripiprazole

Sensitivity: The retention durations of the analytes and the internal standard showed no interferences. Typical chromatograms for a pooled plasma standard blank and a blank with internal standard.

Mean recovery after treatment with Aripiprazole was 98.12 percent. Overall, Voriconazole resulted in a mean recovery rate of 99.00 percent.

For LQC, the computed mean stability was 100.03%, whereas for HQC it was 99.99%. respectively. This result was shown in the Table 12.

Table 12: Stability of Aripiprazole (zero day)

	HQC		LQC	
	Comparison samples	**Stability samples**	**Comparison samples**	**Stability samples**
N	6	6	6	6
Mean	320.955	320.87	31.12833	31.13417
SD	0.716121	0.736261	1.004098	1.016948
% CV	0.223122	0.229852	3.225671	3.27898
%Mean Accuracy	100.2984	100.1	103.7611	103.3806
% Mean Stability	99.99		100.03	

Matrix samples stability at -20 ± 5°C for 37 days: The % mean stability of the Aripiprazole was found to be 99.81% & 99.98% for HQC & LQC at -20 ± 5°C.

Table 13: Matrix samples stability at -20 ± 5°C for 37 days

	HQC		LQC	
	Comparison samples	**Stability samples**	**Comparison samples**	**Stability samples**
N	6	6	6	6
Mean	320.9117	320.3033	30.5637	30.5577
SD	1.46489	0.55655	2.04532	0.52195
% CV	0.46	0.17	6.69	1.71
%Mean Accuracy	100.28	100.09	101.88	101.86
% Mean Stability	99.81		99.98	

CONCLUSION

The concentration of Aripiprazole in human plasma was determined utilizing a straightforward, accurate,

Acquisition Batch	**HQC**	**MQC**	**LQC**	**LLOQ**
N	6	6	6	6
Intra-day precision				
%CV	0.45	1.65	2.70	0.47
% Mean Accuracy	100.16	100.96	101.29	99.01
Inter-day precision				
%CV	0.44	1.52	1.9	1.97
% Mean Accuracy	100.07	101.48	102.23	100.27

and exact technique that relied on Voriconazole as an internal reference. Aripiprazole had a retention time of 3.111 seconds. The coefficient of variation (CV) for Aripiprazole was shown to be less than 2.0. Recovering 98.12% of what was lost was the result. Concentrations between 10 and 400 ng/mL are linear (r2 = 0.999). Quantitative minimum detectable levels for both medications in human plasma were set at 10ng/mL. The disclosed approach was also verified in accordance with ICH recommendations and found to be within acceptable limits. The suggested approach is convenient for clinical labs to use for therapeutic and pharmacokinetic drug monitoring since it is quick, easy, accurate, and exact.

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