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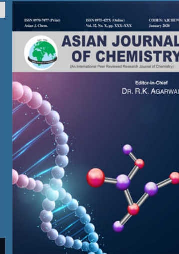

Development and Validation of UPLC-MS/MS Method for the Determination of Aripiprazole in Rat Plasma Using Liquid-Liquid Extraction: Pharmacokinetic and Bioequivalence Application

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A selective, simple, sensitive and rapid ultra-performance liquid chromatographic tandem mass spectrometric (UPLC-MS/MS) method for the detection of aripiprazole in rat plasma has been developed and validated using aripiprazole-D8 as internal standard (IS). A simple single step sample preparation process was accomplished by liquid-liquid extraction (LLE). The post-treatment samples were chromatographed and analyzed on a UPLC bridged ethyl hybrid (BEH) C-18 column using mobile phase composition of acetonitrile: 0.1% formic acid in water::70:30 (v/v). Aripiprazole was analyzed by MS detector in positive electrospray ionization mode (ESI). Multiple reactions monitoring (MRM) was employed to observed the transition for aripiprazole (*m/z* 448.35→285.09) and aripiprazole-D8 (*m/z* 456.2→293.2). The developed method was validated and found linear in the working range of 2-1025 ng/mL with correlation coefficient, r^2 = 0.99951 and quantification limit of 2.02 ng/mL. All validation parameters were in accordance with the ICH guidelines and met the acceptance criteria. The method was found to be accurate (recovery, 97.07 to 103.64%, precise (% CV, 2.68 to 7.70%), rapid (run time 4 min) and specific. The validated method was successfully used for the determination of plasma concentration of aripiprazole after single oral administration in rats and hence could be useful for *in vivo* pharmacokinetic study and bioequivalence testing of aripiprazole formulations.

Keywords: Aripiprazole, Mass spectrometric, Rat plasma, Ultra performance liquid chromatographic.

INTRODUCTION

Aripiprazole was given the US Food and Drug Administration (FDA) approval on November 15, 2002 [1] and associated with atypical or third generation antipsychotic (Fig. 1). It is orally indicated for treatment of major depressive disorder, bipolar I, schizophrenia, irritability associated with autism and Tourette's. It is also indicated as an injection for agitation associated with schizophrenia or bipolar mania [2]. Aripiprazole showed its effects through antagonism of 5-HT_{2A} α-adrenergic receptors and agonism of 5-HT_{1A} and dopaminic receptors [3]. It is partial agonist at 5-HT_{1A}/5-HT_{2C}/D_2 and antagonist at 5-HT_{2A}/5-HT_7/$D_{3/4}$ receptors, with fair H_1 antihistaminic, inhibitory serotonin transporter and α_1 adrenolytic activities [4]. Aripiprazole has molecular weight 448.385 g/mol and practically insoluble in water. Studies in healthy subjects demonstrated that aripiprazole exhibits favourable safety and tolerability prole with rapid absorption, high bioavailability (~ 87%) and high plasma protein binding (~ 99%) with albumin being the main binding component [5-7].

In the literature, few methods like UV-spectrophotometry [8], visible spectrophotometry [9,10], high performance liquid chromatography [11-19], column switching HPLC [20], ultra-high performance liquid chromatography coupled with mass spectrophotometer (UPLC-MS/MS) [21-28], comparative HPLC and UPLC method [29], capillary electrophoresis [14,30], gas chromatography coupled with mass spectrophotometer [31] and using electrochemistry technique [32] has been reported for the quantitation of aripiprazole in biological fluids. The major problem with many available methods is long run time for quantitation of aripiprazole in biological fluids [14,21, 27,29].

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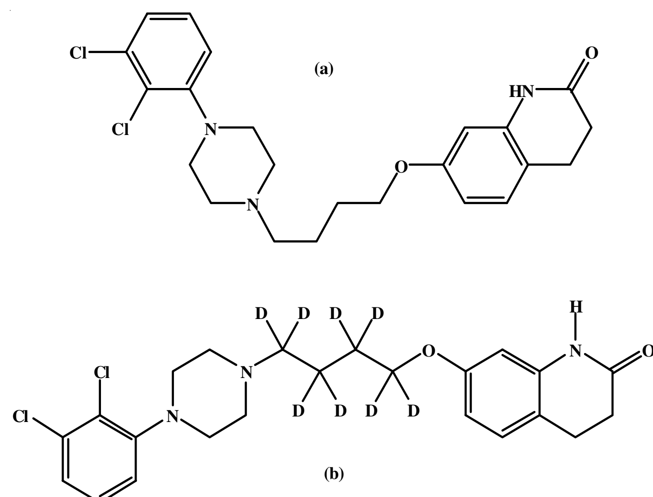

Fig. 1. Chemical structure of (a) aripiprazole and (b) internal standard aripiprazole-D8

Although UPLC-MS-MS is a good technique with wide operating power range up to 1200 bar pressure and selectivity but the method utilizes pre-cleanup using solid phase extraction procedures on samples for avoiding undesired interference from other matrix components. In this study, pre-cleanup using liquid extraction method followed with UPLC-MS/MS analysis for the determination of aripiprazole using aripiprazole-D8 (APD) as internal standard in rat plasma was developed and validated according to the ICH guidelines [33].

EXPERIMENTAL

Aripiprazole (drug) was received as gift sample from Sun Pharmaceutical Industries Ltd. (Gurugram, India) and aripiprazole-D8 as internal standard (IS) were purchased from Merck (Mumbai, India). Acetonitrile, ethyl acetate, methanol of HPLC grade and formic acid SQ grade were obtained from Merck (Mumbai, India). Milli Q HPLC water (Millipore, USA) was used for analysis. All other reagents were of analytical grade.

Liquid chromatographic and mass spectrometric conditions: Chromatography and analyses of samples were performed on Waters ACQUITY UPLCTM (Waters Corporation, MA, USA) with MS detector (Synapt; Waters, Manchester, UK). C-18 column (Waters ACQUITY UPLCTM BEH) with particle size of 1.7 µm and having dimension 2.1 mm × 100 mm was used in chromatographic conditions. LC-MS grade Degassed acetonitrile and 0.1% formic acid in water (70:30 v/v) was used as mobile phase with flow rate of 0.4 mL/min (gradient flow) and injection volume of 5 µL/min. The total run time for each sample was kept at 4 min. The quantization was done by using Waters XEVO-TQD (MA, USA) (triple quadrupole) mass spectrometer (Micromass MS Technologies, Manchester, UK and QCA896) under electrospray ionization (ESI) chamber in positive ion mode with quantification parameters as capillary (3.50 kV) and collision energy (58.0 eV). The operating conditions were set as; collision gas (Argon) with a pressure of 7.0×10^{-3} Pa having desolvation temperature and desolvation gas flow of 400 °C and 800 L/h, respectively. The transitions at *m/z* 448.35→285.09 and *m/z* 456.2→293.2 were adopted for quantification of aripiprazole and aripiprazole-D8, respectively under the MRM (multiple reaction monitoring modes). The Mass-Lynx software (V4.1, SCN918) was used for the analysis of data obtained.

Preparation of stock solutions, quality control and calibration samples: Aripiprazole and aripiprazole-D8 (IS) stock solutions (1 mg/mL) were prepared in acetonitrile. The final concentration for aripiprazole and internal standard (IS) was accounted for its potency and corrected by the actual amount weighed. The calibration curve was prepared by dilution of stock solution at spiking of 2% with drug free human plasma in the range of 2-1095 ng/mL producing calibration standards of concentration 2.03, 4.30, 35.80, 204.50, 415.10, 585.90, 780.70, 1091.28 ng/mL. The internal standard (IS) working solution of 200 ng/mL concentration was prepared *via* dilution of stock solution with acetonitrile-water (50:50, v/v) and from this stock fresh working IS dilutions were prepared as and when required. All solutions were stored in refrigerator at 2-8 ºC and brought to room temperature before analysis.

In plasma matrix three quality control dilutions were separately prepared in the similar way at the concentrations of 2.05, 5.76, 448.95 and 905.37 ng/mL as quality control at the limit of quantification quality control (LOQQC), low quality control (LQC), middle quality control (MQC) and high quality control (HQC), respectively. The spiked and unknown plasma samples were then extracted following the liquid-liquid sample preparation procedure.

Sample preparation: Aripiprazole-D8 (100 µL, 200 ng/mL) and 2 mL of ethyl acetate was added to 100 µL thawed plasma sample, which was then vortexed at 2000 rpm for 20 min. The vortexed sample was centrifuged at 4 ºC at 4000 rpm for 5 min. The supernatant was collected and dried at 50 °C and 20 psi pressure in nitrogen evaporator. Dried sample was reconstituted with acetonitrile: 0.1% formic acid in water (70:30, v/v) as mobile phase. Then samples were injected into LC-MS/MS system *via* labeled auto sampler vials.

Method development and validation procedure: 10 lots of plasma samples including haemolyzed plasma with EDTA and one lipaemic were screened and analyzed to determine if the endogenous components would interfere with determinations of aripiprazole and aripiprazole-D8 (IS). The plasma batch with least interference was used to spike and prepare 6 samples at limit of quantification (LOQ) level. Processed blank plasma samples were analyzed against selectivity LOQ samples processed with IS and area response of each extracted blank plasma sample was evaluated against mean area response of selectivity LOQ samples at retention time of aripiprazole and aripiprazole-D8.

For assessment of reproducibility, three precision and accuracy batches were analyzed. The accuracy of the assay was defined as the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentage (% nominal).

Extraction recoveries of aripiprazole were measured at three QC levels and determined by measuring the mean peak area response of spiked (extracted) QC samples (LQC, MQC and HQC) against the mean peak area response of aqueous

(non-extracted) QC samples (LQC, MQC and HQC) of approximately same concentrations. The percentage recovery of aripiprazole-D8 was determined by measuring the mean peak area response of spiked (extracted) QC sample (MQC) against the mean peak area response of aqueous (non-extracted) IS dilution of approximately same concentration.

Dilution integrity was determined by assaying six replicate QCs spiked approximately two times the concentration of LOQ, previously frozen and thawed over multiple cycles. The first Freeze Thaw cycle for dilution integrity consisted of freezing for at least 24 h at -20 ± 10 ºC storage temperature followed by unassisted thawing at room temperature. For the second and third freeze thaw cycles, the samples were frozen for a minimum of 12 h at a storage temperature of -20 ± 10 ºC and thawed. The freeze thaw stability were processed by diluting an appropriate factor (2 and 4) prior to extraction using pooled plasma after completion of third freeze thaw cycle and analyzed against freshly spiked calibration curve standard samples processed simultaneously.

Matrix effect was calculated by spiking analyte (aripiprazole) and aripiprazole-D8 at LQC and HQC samples into each of blank plasma extracts from six different batches of matrix, respectively and analyzed in duplicate along with six replicate injections of aqueous samples (representing 100% concentration of analyte and aripiprazole-D8) at the level of LQC and HQC.

The freeze-thaw stability (3 freeze-thaw cycles), bench-top stability (for a duration of 6.5 h), in-injector stability (samples were processed and kept in auto-injector and analyzed after 73.47 h) and long term stability in rat plasma (samples stored for 21 days in cold room at -20 ± 10 ºC) were determined for aripiprazole. The stability samples were processed along with four sets of freshly spiked and prepared QC samples (comparison samples) at concentration level of LQC and HQC and analyzed against freshly spiked and prepared calibration curve standards.

The stock solution stability of aripiprazole and aripiprazole-D8 was evaluated at room temperature and refrigerated temperature (2-8 ºC). All stability samples were stored at intended duration at applicable storage conditions. Stock solution stability was performed to check the stability of the analyte and aripiprazole-D8 by storing aripiprazole and aripiprazole-D8 stock solutions at room temperature for a minimum of 6 h at room temperature and approximately for one week at refrigerated temperature. The ruggedness of the extraction procedure and chromatographic method was evaluated by analysis of a batch of six sets of QC samples and a set of calibration standard (one precision and accuracy batch) using a different column (same type) by another analyst. ICH guidelines were followed for validation of all parameters [33].

Pharmacokinetic study and statistical analysis: The protocol for *in vivo* studies was approved by Institutional Animal Ethics Committee of Institutional Animal Ethics Committee of the Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO. Pharmacokinetic study (PK) was performed in six Sprague-Dawley rats (weigh approx. 250 mg) of either sex. The purpose of this study was to accurately estimate the pharmacokinetic parameters of aripiprazole in biological samples with this developed and validated analytical method. A dose of 50 mg/Kg of aripiprazole was given orally in form of suspension to rats (n = 6). After anesthetization of rats using diethyl ether, the blood samples were collected into sodium-heparin tubes from the retro-orbital plexus at pre-determined time intervals *i.e.* 0.24, 0.48, 0.72, 1, 2, 4, 6, 12 h, 1, 1.5, 2, 3, 4, 6 and 7 days. Plasma was separated out using REMI centrifuge at 5000 rpm for 10 min and stored at -20 °C till analysis. The Phoenix WinNonlin software (version 8.3) was used for the analysis of data obtained.

RESULTS AND DISCUSSION

Sample preparation and method development: For *in vivo* pharmacokinetic and bioequivalence studies, determination of analyte with sensitivity and selectivity is perquisite. Sample preparation proves to be a vital factor in determining analyte in blood plasma or serum. In this research, sample preparation by LLE method using acetonitrile was found suitable for the determination of aripiprazole in rat plasma by UPLC-MS/MS. All possible interfering substances were eliminated using a simple sample preparation method based on liquid extraction and well defined chromatograms of aripiprazole and aripiprazole-D8 were obtained as depicted in Fig. 2. Nowadays in biological analysis, UPLC-MS/MS is emerging as powerful and widely used technique with increased sensitivity due to equipped with mass detector. The C-18 column (Waters Acquity UPLCTM BEH) with small particle size was used for chromatography as it displayed good peak shape and response for aripiprazole.

Several trials were carried out using different composition of mobile phase and other parameters for getting good symmetric peaks and intensity of response. Ionization agents like formic acid, ammonium acetate and acetic acid were tested and among these formic acid was found to give best intensity of response. The mobile phase composition of acetonitrile: 0.1% formic acid (70:30, (v/v)) with flow rate of 0.4 mL/min

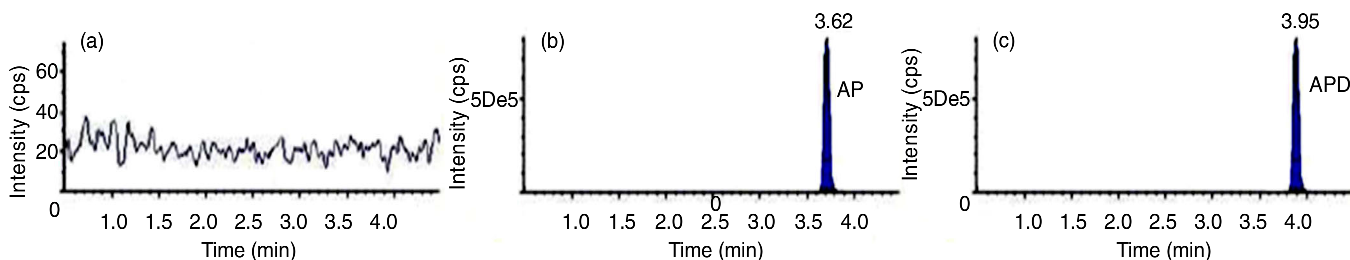

Fig. 2. UPLC Chromatograms of (a) blank plasma sample; (b) aripiprazole, MQC sample and (c) aripiprazole-D8, MQC sample

TABLE-1
PERCENTAGE RECOVERY FOR ARIPIPRAZOLE AND ARIPIPRAZOLE-D8 IN PLASMA (n = 6)

QC	Analyte	A (% CV)	B (% CV)	Extraction Recovery (%)
LQC	Aripiprazole	1561 (2.39)	1738 (2.75)	89.81
	Aripiprazole-D8	58316 (4.61)	66439 (5.61)	87.77
MQC	Aripiprazole	86725 (4.97)	95862 (4.35)	90.46
	Aripiprazole-D8	60498 (5.19)	71691 (5.95)	84.38
HQC	Aripiprazole	174952 (6.05)	168137 (5.73)	104.05
	Aripiprazole-D8	61369 (4.43)	63728 (4.49)	96.30

CV: Co-efficient of variation; A: Mean area response of six replicate samples prepared by spiking before extraction; B: Mean area response of six replicate samples prepared by spiking in extracted blank plasma.

and total run time 4 min was optimized to achieve symmetrical peak shapes, diminished background noise, reduced chromatographic run time and reduced the matrix effect. The retention time of aripiprazole and aripiprazole-D8 were found to be 3.62 and 3.95, respectively. The aripiprazole and aripiprazole-D8 analysis was done under positive ionization mode using ESI as the ionization source and enhanced selectivity was achieved using tandem MS analysis *via* MRM functions. Full MS spectra reveals the abundance of positive ionization product of aripiprazole with *m/z* 448.35 and aripiprazole-D8 with *m/z* 456.2. Collision energy of 58 eV, resulted in a major fragment of aripiprazole at *m/z* 285.09 and aripiprazole-D8 at 293.2.

Selectivity and specificity: Chromatogram for blank plasma and plasma spiked with aripiprazole and aripiprazole-D8 (IS) are shown in Fig. 2. The method is selective and specific since there are no significant interferences from endogenous substances at the retention time of aripiprazole and aripiprazole-D8.

Linearity: The linearity of aripiprazole was tested in plasma using eight-point standard curve by least square regression analysis. At each concentration level, peak area ratio was measured and calibration curve with least-squares linear regression and correlation coefficient was obtained. The coefficient of correlation (r^2) was 0.99951, average slope was 0.00406428 and intercept was -0.000621497 in the concentration range of 2-1095 ng/mL for aripiprazole.

Limit of quantification: The present UPLC-MS method provided quantitation limit of 2.02 ng/mL for aripiprazole. At quantitation limit, the accuracy (% recovery) and precision (% CV) were found to be 99.68% and 4.81%, respectively. These results showed that aripiprazole in plasma can be estimated accurately at the concentrations obtained during *in vivo* pharmacokinetic and bioequivalence studies.

Recovery: The percent recovery at low quality control (LQC), middle quality control (MQC) and high quality control (HQC) samples were 89.81%, 90.46%, 104.05% for aripiprazole and 87.77%, 84.38%, 96.30% for aripiprazole-D8, respectively as shown in Table-1. The mean recovery percentage of 94.77% for aripiprazole and 89.48% for aripiprazole-D8 indicates that the extraction procedure did not require further improvement.

Accuracy and precision: The intra-day accuracy for aripiprazole ranged from 97.30% to 107.81% and inter-day accuracy from 98.85 to 104.69% (Table-2). The intra-day and inter-day precision, calculated as percent coefficient of variation (% CV) over the concentration range of LQC, MQC and HQC was found out in range from 3.87 to 6.28% and 2.39 to 4.26%, respectively (Table-2).

TABLE-2
INTRA-DAY AND INTER-DAY PRECISION AND ACCURACY OF ARIPIPRAZOLE IN PLASMA (n = 6)

Nominal amount (ng/mL)	Amount found (ng/mL)	Precision		Accuracy (%)
		SD	% CV	
Intra-day				
2.05	2.14	0.11	5.14	104.39
5.76	6.21	0.39	6.28	107.81
448.95	436.83	18.57	4.25	97.30
905.37	964.51	37.29	3.87	104.49
Inter-day				
2.05	2.11	0.09	4.26	102.93
5.76	6.03	0.24	3.98	104.69
448.95	443.79	10.62	2.39	98.85
905.37	898.64	26.41	2.94	99.26

$$\text{Precision as \% CV} = \frac{\text{Standard deviation}}{\text{Mean amount found}} \times 100$$

$$\text{Accuracy} = \frac{\text{Amount found}}{\text{Nominal amount}} \times 100$$

Extended precision and accuracy batch: Extended precision and accuracy batch was run to establish the performance of bioanalytical method throughout the anticipated batch run time having sample size of 118 samples including calibration curve standards and QC samples. The results are shown in Table-3. The batch accuracy ranged from 97.07 to 103.64% and batch precision ranged from 2.68% to 7.70% for aripiprazole.

TABLE-3
EXTENDED PRECISION AND ACCURACY ANALYSIS OF ARIPIPRAZOLE

Nominal Amount (ng/mL)	Amount found (ng/mL)	% CV	Accuracy (%)
2.05	1.99 ± 0.10	5.02	97.07
5.76	5.97 ± 0.46	7.70	103.64
448.95	441.25 ± 11.83	2.68	98.28
905.37	885.49 ± 33.74	3.81	97.80

Stability: Stability was evaluated at 5 different parameters of stability in freeze-thaw, in-injection stability, short-term, long term and standard stock solution stability. They were analyzed by comparing LQC and HQC (stability samples) with comparison quality control samples which analyzed against freshly spiked calibration curve standards and freshly spiked QC samples. The comparative In-Injector stability, bench-top

TABLE-4
STABILITY DATA FOR ARIPIPRAZOLE UNDER DIFFERENT CONDITIONS (n = 6)

QC sample	Comparison sample			Stability sample			Stability (%)
	Mean ± SD	% CV	% Nominal	Mean ± SD	% CV	% Nominal	
Freeze thaw cycle, after 3 cycles at -20 ± 10 °C							
LQC	5.93 ± 0.23	3.87	102.95	5.87 ± 0.21	3.58	101.91	98.99
HQC	901.72 ± 16.39	1.82	99.60	914.59 ± 19.10	2.08	101.02	101.43
Bench top stability; 6.5 h at 25 ± 5 °C							
LQC	6.04 ± 0.18	2.98	104.86	6.11 ± 0.27	4.41	106.07	101.16
HQC	894.13 ± 19.86	2.23	98.76	897.61 ± 22.15	2.46	99.14	100.39
Injector stability; 73.47 h							
LQC	5.69 ± 0.22	3.86	98.78	5.66 ± 0.20	3.53	98.26	99.47
HQC	876.14 ± 14.93	1.70	96.77	869.68 ± 16.47	1.89	96.06	99.26
Long term stability in plasma; after 21 days							
LQC	5.63 ± 0.19	3.37	97.74	5.52 ± 0.17	3.08	95.83	98.05
HQC	909.85 ± 24.38	2.68	100.49	902.29 ± 30.12	3.34	99.66	99.17

TABLE-5
STOCK SOLUTION STABILITY DATA FOR ARIPIPRAZOLE AND ARIPIPRAZOLE-D8 UNDER DIFFERENT CONDITIONS (n = 6)

Storage condition	Comparative samples		Stability samples		Stability (%)
	Mean ± SD	% CV	Mean ± SD	% CV	
Aripiprazole					
Room temperature	90296 ± 2591.49	2.87	88319 ± 2684.90	3.04	97.81
2-8 °C	92731 ± 1780.43	1.92	92704 ± 2419.57	2.61	99.97
Aripiprazole-D8					
Room temperature	61459 ± 1327.51	2.16	61693 ± 1536.16	2.49	100.38
2-8 °C	60831 ± 1052.37	1.73	61501 ± 1703.58	2.77	101.10

stability, freeze-thaw stability and long-term stability of aripiprazole in rat plasma ranged from 99.47 to 99.26%, 101.16 to 100.39%, 98.99 to 101.43% and 98.05 to 99.17%, respectively (Table-4).

Further as shown in Table-5, the percent stability at room temperature for stock solution of aripiprazole and aripiprazole-D8 was 97.81 and 100.38%, respectively. Similarly, the percent stability of the stock solution analyzed after keeping at 2-8 °C for 7 days for aripiprazole and aripiprazole-D8 was 99.97% and 101.10%, respectively (Table-5).

Ruggedness: The ruggedness of the extraction procedure and chromatographic method was evaluated by analyzing a set of calibration standard and a lot of six sets of QC samples by another analyst but using a different column (same type). The results are shown in Table-6. The intra batch accuracy ranged from 97.92 to 102.93% for aripiprazole and within batch precision ranged from 2.39 to 6.16%. Thus, the results are within limits indicating that the batch met the acceptance criteria of accuracy, precision and linearity.

Pharmacokinetic application of validated method: The plasma concentrations of aripiprazole in rats were estimated after single oral administration of 50 mg/Kg of aripiprazole suspension. The plasma concentration *versus* time curve is shown in Fig. 3 whereas the mean *in vivo* kinetic parameters are summarized in Table-7. Aripiprazole concentrations increased quickly and reached a maximum value 263.59 ng/mL within 4 h followed by a rapid clearance. The $t_{1/2}$ value for aripiprazole was found to be 61.87 h. The plasma concentrations at all sampling points were measurable for aripiprazole with the help of developed analytical method. Hence, the method could be useful for *in vivo* pharmacokinetic and bioequivalence studies.

TABLE-6
RUGGEDNESS DATA ANALYZED USING A DIFFERENT COLUMN OF SAME TYPE BY ANOTHER ANALYST (n = 6)

Nominal amount (ng/mL)	Amount found (ng/mL)	% CV	% Accuracy
2.05	2.11 ± 0.13	6.16	102.93
5.76	5.62 ± 0.22	3.91	97.92
448.95	447.39 ± 16.51	3.69	99.65
905.37	899.41 ± 21.50	2.39	99.34

Conclusion

A novel UPLC-MS/MS method with liquid-liquid extraction (LLE) procedure having high sensitivity and repro-

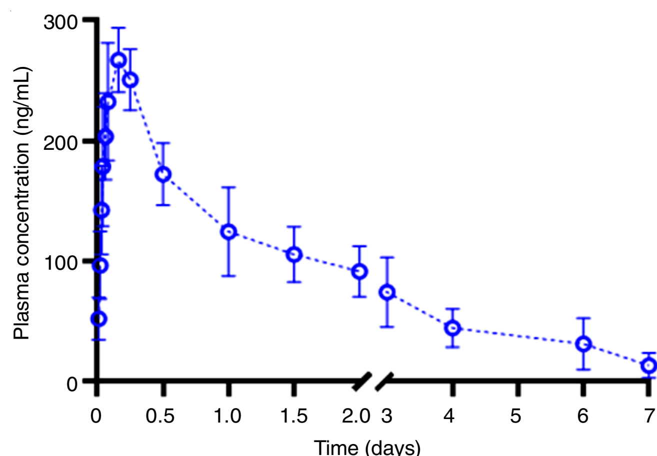

Fig. 3. Plasma concentration *vs*. time profile of aripiprazole in rats (n=6) after peroral administration

TABLE-7
in vivo PHARMACOKINETIC PARAMETERS OF ARIPIPRAZOLE AFTER PERORAL ADMINISTRATION (n = 6)

Pharmacokinetic parameters	Mean ± SD
C_{max} (ng/mL)	263.59 ± 35.1
T_{max} (h)	4.00 ± 0.82
AUC_{0-t} (ng h/mL)	12731.57 ± 473.9
$AUC_{0-\infty}$ (ng h/mL)	13937.81 ± 685.7
K_e (h^{-1})	0.0112 ± 0.03
$t_{1/2}$ (h)	61.87 ± 7.4
Cl (L/h/Kg)	8.96 ± 2.1
V_d (L)	800.74 ± 61.5

C_{max} = Maximum observed serum concentration; AUC_{0-t} = Area under the concentration-time curve from time 0 to time 't'; $AUC_{0-\infty}$ = Area under the concentration-time curve from time 0 to infinity; T_{max} = Time to maximum serum concentration; K_e = Elimination rate constant; $t_{1/2}$ = Elimination half-life; V_d = Volume of distribution; Cl = Clearance.

ducibility for the quantification of aripiprazole in rat plasma was developed. The method has been validated successfully in terms of linearity, specificity, stability, accuracy and precision for determination of aripiprazole concentration in rat plasma. The UPLC coupled with mass detector allows fast and sensitive estimation of compounds compared to HPLC. The advantages of our method are high sensitivity (QL: 2.02 ng/mL), the short analysis time (4 min) and a simple sample extraction procedure. The developed and validated method was successfully applied in estimation of aripiprazole plasma concentrations and pharmacokinetic parameters after peroral administration of aripiprazole suspension in rats. The selectivity, specificity, sensitivity and rapidness (short run time) of the method allows it to be used for bioequivalence studies of aripiprazole preparations.

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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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