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Article

Development and Validation of an LC–ESI-MS Method for Quantitative Determination of Aripiprazole in Human Plasma and an Application to Pharmacokinetic Study

Sreedasyam Ravinder[1,2*], Akula Tukaram Bapuji[1], Khagga Mukkanti[2], Datla Rama Raju[1], Hamsa Laxmi Venkata Ravikiran[1] and Dendhi Chandrapal Reddy[1]

[1]Department of Clinical Pharmacology, APL-Research Centre, S.No.313, Bachupally, Quthubullapur mandal, Hyderabad-500090, AP, India, and [2]Institute of Science and Technology, JNT University, Kukatpally, Hyderabad 500085, AP, India

*Author to whom correspondence should be addressed. Email: r_sreedasyam@yahoo.com

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A selective, sensitive, high pressure liquid chromatography–positive electrospray ionization tandem mass spectrometry method was developed and validated for the quantification of aripiprazole in human K_2EDTA plasma using zolpidem tartrate as an internal standard. The analyte and internal standard were extracted from human plasma by solid-phase extraction using methanol. The eluted samples were chromatographed on a Grace Smart RP 18 4.6 × 100 mm, 3 μ column by using a 95:5 v/v mixture of methanol and ammonium acetate buffer (30 mM, pH 5.0 ± 0.05) as a gradient mobile phase at a flow rate of 0.6 mL/min, and analyzed by mass spectrometry in the multiple reaction monitoring mode using the $[M + H]^+$ ions m/z 448.03 → 285.14 for aripiprazole and m/z 308.13 → 235.25 for the internal standard (zolpidem tartrate), respectively. Calibration plots were linear over the concentration range of 0.20 to 60.01 ng/mL. Intra-day and inter-day precision (percent coefficient of variation) and accuracy (percent nominal) for quality control samples (0.60, 30.60 and 45.59 ng/mL) ranged between 2.28 and 8.93% and between 92.50 and 107.07%, respectively. Extraction recovery of aripiprazole from plasma was in the range 75.56–79.57%; mean recovery was 77.35%. The main pharmacokinetic parameters were T_{max} = (4.00 ± 2.336) C_{max} = (55.16 ± 13.490) and AUC = (1846.28 ± 484.686).

Introduction

Aripiprazole, 7-(4-[4-(2, 3-dichlorophenyl)-1-piperazinyl](butoxy)-3,4-dihydro-2(1H)-quinolinone (Figure 1), belongs to the chemical class of benzisoxazole derivatives, and is a novel, atypical antipsychotic drug for treatment of schizophrenia and schizoaffective disorders (1). It has potent partial agonist activity at dopamine2 (D2) receptors, partial agonist activity at serotonin1A (5-HT1A) receptors, and antagonist activity at 5-HT2A receptors. As a result, aripiprazole can improve both negative and positive symptoms of schizophrenia with lower propensity for extra pyramidal symptoms (EPS). In addition to these therapeutic advances, aripiprazole seems to have a good side-effect profile research that indicates that weight gain and sedation are minimal and there is no QTc interval prolongation or hyperprolactinemia compared with placebo (2–4).

High-performance liquid chromatography (HPLC) is the technique that is most commonly used for the determination of aripiprazole in human plasma (5–8). Other methods have been described on different instruments, such as gas chromatography–mass spectrometry (GC–MS) (9), capillary electrophoresis with HPLC (10) and LC–MS–MS (11–14). These methods are usually not sufficiently sensitive or specific to enable the determination of aripiprazole in schizophrenics' plasma, except for one sensitive analytical method along with its main metabolite, OPC-14857 (12). The previously mentioned methods have long run times (7.5 min), and the effects of other drugs used in combination with aripiprazole necessitates the treatment of two different methods. One method has relatively high price and a 7.5 min run time (12). In this paper, a rapid, specific, sensitive and inexpensive high-performance liquid-chromatographic–electrospray mass spectrometric (HPLC–ESI-MS) method is developed for the analysis of aripiprazole in plasma samples from patients suffering from schizophrenia. A low sample volume, simple solid-phase extraction (SPE) technique is used for extraction, which has 0.20 ng/mL as the limit of quantification (LOQ) and a short run time for the quantification of aripiprazole in human plasma. This assay has been successfully applied to a pharmacokinetic study involving the oral administration of 10 mg aripiprazole to 15 healthy human volunteers.

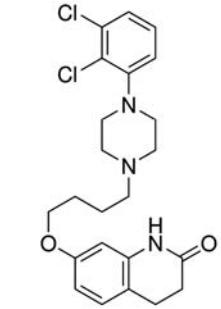
Materials and Methods

Chemicals and reagents

Aripiprazole reference standard (potency w/w, 99.8%) was procured from Aurobindo Pharma (India). Zolpidem tartrate (Figure 1) reference standard (internal standard; IS) (potency w/w, 97.1%) was procured from Aurobindo Pharma (India). HPLC-grade methanol, formic acid and n-hexane, and GR-grade ammonium acetate, were purchased from Merck Specialties (Mumbai, India). High-purity water was prepared with a Milli-Q water purification system obtained from Millipore (Bangalore, India). HLB 3 cc/60 mg cartridges (Waters-Oasis) were procured from Waters Corporation (Ireland). Blank (drug-free) human plasma was obtained from Cauvery Diagnostics and Blood Bank (Secunderabad, India) and stored at −20°C before use.

HPLC operating conditions

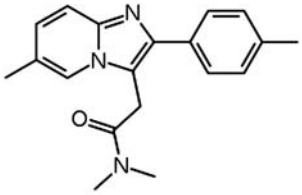
An HPLC system, Alliance 2695 (Waters) interfaced with Waters Quattro Micro MS-MS, was used as the chromatographic separation module. The mobile phase was 95 parts methanol and 5 parts 30 mM ammonium acetate (pH 5.0 ± 0.05), which was degassed ultrasonically for 10 min and delivered at a flow



Aripiprazole Formula $C_{23}H_{27}Cl_2N_3O_2$

7-{4-[4-(2, 3-dichlorophenyl)

piperazin-1-yl] butoxy}-3, 4-dihydroquinolin-2(1*H*)-one



Zolpidem formula $C_{19}H_{21}N_3O$ (IS)

Figure 1. Chemical structures of aripiprazole and IS.

rate of 0.600 mL/min (gradient flow) into the ESI-MS chamber. The retention times of the analyte and IS were 2.05 ± 0.61 and 1.50 ± 0.45 min, respectively.

Mass spectrometry operating conditions

Quantitation was achieved by using Waters Quattro Micro (Micro Mass) LC–MS-MS detection in positive ion mode for the analyte and IS using a mass spectrometer, equipped with an ESI interface at 400°C desolvation temperature. The ion source parameters, capillary 3.50 kV, cone 35 v, extractor 5 v, RF lens 0.0 v, source temperature 100°C, desolvation temperature 400°C, desolvation gas flow 800 L/h and cone gas flow 70 L/h, for the analyte and IS are shown in Table I. Detection of the ions was carried out in the multiple reaction monitoring modes (MRM), by monitoring the transition pairs of m/z 448.03 → 285.14 for aripiprazole and m/z 308.13 → 235.25 for the IS. The obtained analysis data were processed using Mass Lynx software (version 4.2).

Preparation of stock solutions of analyte and IS

Primary stock solutions of aripiprazole for preparation of standard and quality control (QC) samples were prepared from separate weighing. Primary and stock solutions of aripiprazole (1 mg/mL) were dissolves in approximately 5.000 mL of 0.5% formic acid in methanol and made up to 10.000 mL with methanol to get 1.000 mg/mL (Figure 2). The final concentration of aripiprazole was corrected to account for its potency and the actual amount was weighed. The primary stock solution of IS (1 mg/mL) was prepared in methanol. The stock solutions of aripiprazole and IS were stored at 2–8°C, and were found to be stable for 8.90 days.

Table I
Optimized Mass Spectrometry Parameters for Analyte and IS

Channel	Parent (Da)	Daughter (Da)	Dwell (s)	Cone (V)	Collision (eV)
Aripiprazole	448.03	285.14	0.500	35.00	25.00
Zolpidem	308.13	235.25	0.500	50.00	35.00
MS parameters					
ES^+ source					
Capillary (kv)					3.50
Cone (v)					35
Extractor (v)					5
RF lens (v)					0.0
Source temperature (°C)					100
Desolvation temperature (°C)					400
Desolvation gas flow (L/h)					800
Cone gas flow (L/h)					70
Analyzer					
LM resolution 1					13.5
HM resolution 1					13.5
Ion energy 1					0.5
Entrance					1
Collision					25
Exit					2
LM resolution 2					13.5
LM resolution 2					13.5
Ion energy 2					1.0
Multiplier					650

One set of working stock solutions of aripiprazole made with 5.000 mL of 0.5% formic acid in 5 mL of methanol [from primary calibration curve (CC) stock] was successively diluted with methanol to prepare appropriate working solutions to prepare CC standards. Another set of working stock solutions of aripiprazole made in methanol (from primary QC stock) was successively diluted with formic acid in 5 mL of methanol to prepare appropriate dilutions for preparation of QC samples. A working IS solution was prepared in methanol (w/v).

Preparation of CC standards and QC samples

Calibration samples and QC samples were prepared by spiking 20 μL of an appropriate working solution of aripiprazole into 980 μL of control human plasma. The CC consisted of a set of eight non-zero standard concentrations ranging from 0.20 to 60.00 ng/mL. The QC samples were prepared at concentrations of 0.20 (lower limit of quantification, LLOQ), 0.60 (low quality control, LQC), 30.00 (medium quality control, MQC) and 45.59 ng/mL (high quality control, HQC). Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations and 500-μL plasma aliquots were distributed into different tubes. All spiked samples were stored at −20 ± 5°C.

Sample preparation

A simple SPE method was followed for the extraction of aripiprazole from human plasma. To an aliquot of 0.500 mL plasma, IS solution (50 μL of 200 ng/mL) and 2% of formic acid buffer solutions were added and mixed for 15 s on a cyclomixer (Spinx Instruments; Mumbai, India). Before sample extraction, HLB SPE cartridges (3 cc, 60 mg, Waters Oasis) were conditioned with 1 mL of methanol followed by 1 mL of water. Plasma (prepared samples) was loaded onto the SPE cartridges and samples were eluted completely under slow vacuum. SPE

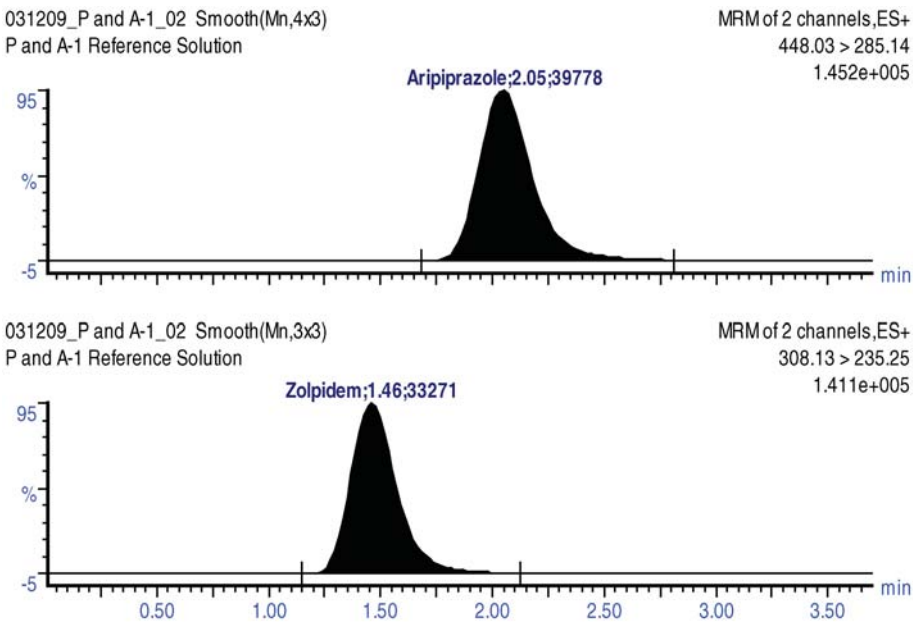


Figure 2. Reference solution.

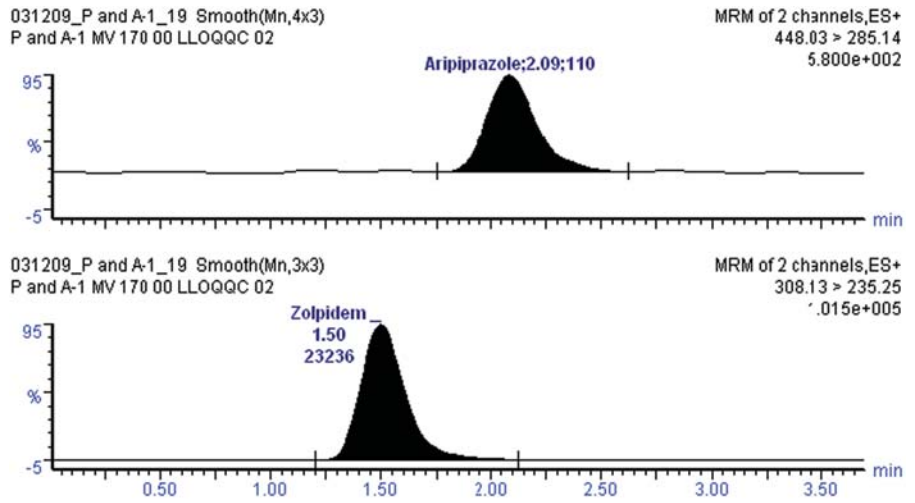


Figure 3. LLOQ.

cartridges were then washed with 1 mL of Milli-Q-water followed by 1 mL of n-hexane and dried for 2 min. The analyte and IS were eluted from SPE cartridge with 0.500 mL of methanol, and the elution sample was collected into a prelabeled ria-vial tube, transferred into autosampler vials and 20 μL was injected onto the LC–MS-MS system.

Validation Parameters

Validation of analytical method for the assay in human plasma was carried out according to the United States Food and Drug Administration guidelines.

Specificity and selectivity

The specificity of the method was evaluated by analyzing human plasma samples from six different lots to investigate the potential interferences at retention times of the analytes (aripiprazole) and IS. The responses of the interfering substances or background noises at the retention time of aripiprazole were acceptable if they were less than 20% of the response of the lowest standard curve point or LLOQ (Figure 3). The responses of the interfering substances or background noise at the retention time of the IS are acceptable if they are less than 5% of the mean response of the IS in LLOQ samples.

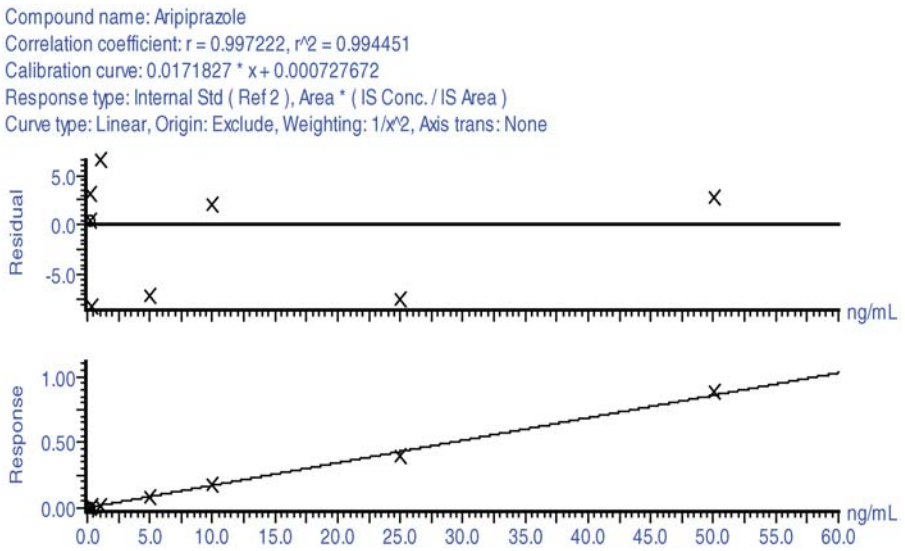


Figure 4. Calibration curve.

Calibration curve

Linearity was assessed in the concentration range of 0.20 to 60.01 ng/mL by weighted linear regression ($1/x^2$) of analyte-IS peak area ratios based on four independent CCs prepared on two different days using an eight-point CC. The CC needed a correlation coefficient (r) of 0.99 or better (Figure 4). The acceptance limit of accuracy for each of the back-calculated concentrations was $\pm$15%, except for LLOQ, at which it was $\pm$20%. The calibrators used for the analyte were 0.20, 0.40, 1.00, 5.00, 10.00, 25.01, 50.01 and 60.01 ng/mL. The samples were run in the order from low to high concentration. In addition, blank plasma samples were analyzed to confirm the absence of direct interferences, but these data were not used to construct the CC. For a calibration run to be accepted, at least 75% of the standards, including the LLOQ and upper limit of quantification (ULOQ), were required to meet the acceptance criterion, otherwise the CC was rejected.

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing aripiprazole at four different QC levels, LLOQ, LQC, MQC and HQC, in human plasma. The inter-assay precision and accuracy were determined by analyzing six replicates at four different QC levels on four different runs. The acceptance criteria included accuracy within $\pm$15% deviation from the nominal values, except LLOQ QC, at which it should be $\pm$20% and a precision of $\leq$15% relative standard deviation (RSD), except for LLOQ QC, at which it should be $\leq$20%.

Recovery

The efficiency of the aripiprazole and IS extraction from human plasma was determined by comparing the responses of the analyte extracted from replicate QC samples ($n = 6$) with the response of analyte from post-extracted plasma QC samples at equivalent concentrations. Recoveries of analyte was determined at LQC, MQC and HQC concentrations (aripiprazole: 0.60, 30.00 and 45.59 ng/mL), whereas the recovery of the IS was determined at a single concentration of 200.00 ng/mL. The mean overall recovery of the analytes and IS was determined by comparing the peak areas of extracted plasma QC samples to the peak areas of post-extraction plasma samples spiked at corresponding concentrations.

Matrix effect

The matrix effect was investigated to ensure that precision, selectivity and sensitivity were not compromised by the screened matrix. The matrix effect was investigated by analyzing six replicates of post-extracted QC samples at low and high concentrations (prepared by spiking aqueous solutions into extracted, processed blank plasma samples from six different volunteer matrix lots containing EDTA as anticoagulant) along with equivalent, similarly prepared aqueous samples (neat samples) at low and high concentrations. The percent matrix effect was determined by comparing the peak areas of post-extracted plasma QC samples to the peak areas of neat samples spiked at corresponding concentrations. The matrix effect was expressed as the percentage ratio of mean peak area response of post-extracted and aqueous (neat) samples. Matrix effect was not considered significant if percent responses were within 85%–115% and precision $\leq$15% RSD or percent coefficient of variation (CV%)].

Dilution integrity

The dilution integrity exercise was performed with the aim of validating the dilution test to be carried out on higher analyte concentrations above the ULOQ during real-time analysis of subject samples. Dilution integrity experiment was carried out at 2.0 times the ULOQ concentration for the analyte. Six replicates, each of half and quarter concentrations, were prepared by 2-times and 4-times dilution with blank plasma and their concentrations were calculated by applying the dilution factors 2 and 4.

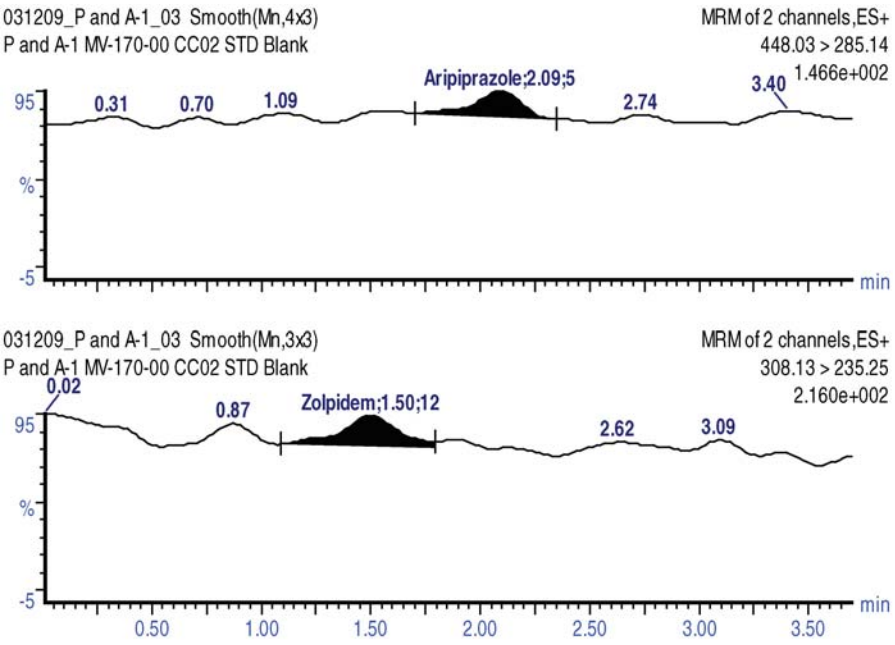


Figure 5. Extracted standard blank plasma.

Stability experiments

The stability of aripiprazole and IS in the injection solvent was determined periodically by injecting replicate preparations of processed plasma samples for up to 76.83 h (in the autosampler at 10°C) after the sample loading. Wet extract stability was successfully assessed by analyzing six replicates of wet extract stability samples stored at a temperature below 10°C for 72.97 h at low and high concentrations. The stability of the analyte (aripiprazole) in plasma during 9.93 h (bench-top) was determined at ambient temperature (~25°C) at two concentrations (LQC and HQC) in six replicates. The stability of aripiprazole in human plasma following five freeze-thaw cycles was assessed, during which the samples were stored at -20 ± 5°C and -70 ± 5°C between freeze/thaw cycles and the samples were thawed by allowing them to stand (unassisted) at room temperature for ~1.5 h. The samples were then returned to the freezer. Freezer stability (long-term) of the analyte in human plasma was assessed by analyzing the LQC and HQC samples stored at -20 ± 5°C and -70 ± 5°C.The samples were processed using the same procedure as described previously. Samples were considered stable if the assay values were within the acceptable limits of accuracy ($\pm 15\%$) and precision ($\leq 15\%$ RSD or CV%).

Pharmacokinetic study

A pharmacokinetic study was conducted on 15 healthy, adult, male, human subjects under fasting conditions ($n = 15$) following oral administration of a 10-mg tablet. The venous blood samples were withdrawn at pre-dose (0.00) and at 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 7.00, 8.00, 10.00, 12.00, 18.00, 24.00, 36.00, 48.00 and 72.00 h post dose using K_2EDTA vacutainer collection tubes (BD; Franklin, NJ). The tubes were centrifuged at 2,500 RCF, 4°C for 10 min and the plasma was collected. The collected plasma samples were stored at −20°C until use. Plasma samples were spiked with IS and processed using the extraction procedure described previously. Along with clinical samples, QC samples at low, middle and high concentrations were assayed in duplicate and distributed among the unknown samples in the analytical run. The analytical runs were accepted if not more than 33% of the QC samples were greater than $\pm 15\%$ of the nominal concentration. Plasma concentration-time profile of aripiprazole was analyzed by the non-compartmental method using WinNonlin Version 5.2 (Pharsight Corporation; Mountain View, CA). The linear mean plot of aripiprazole plasma concentration versus time under fasting conditions is shown in Figure 7.

Results

Mass spectrometry

To obtain optimum sensitivity and selectivity, the ESI technique operated in the positive ion mode was used for the LC–MS-MS MRM analysis. The protonated form of the analyte and IS $[M + H]^+$ ions were the parent ions in the Q1 spectrum and used as the precursor ion to obtain Q3 product ion spectra. The optimized compound parameters, i.e., capillary (KV), cone (v), extractor (v) and desolvation temperature are presented in Table I. The product ion mass spectrum of the positively charged ion of aripiprazole was monitored at m/z 448.03 → 285.14 and 308.13 → 235.25 for IS (Figure 8).

Selectivity and Chromatography

The degree of interference by endogenous plasma constituents with analyte and IS was assessed by inspection of the chromatograms derived from the processed blank plasma sample. As shown in Figures 5 and 6, no significant interferences in the blank human plasma traces were found from

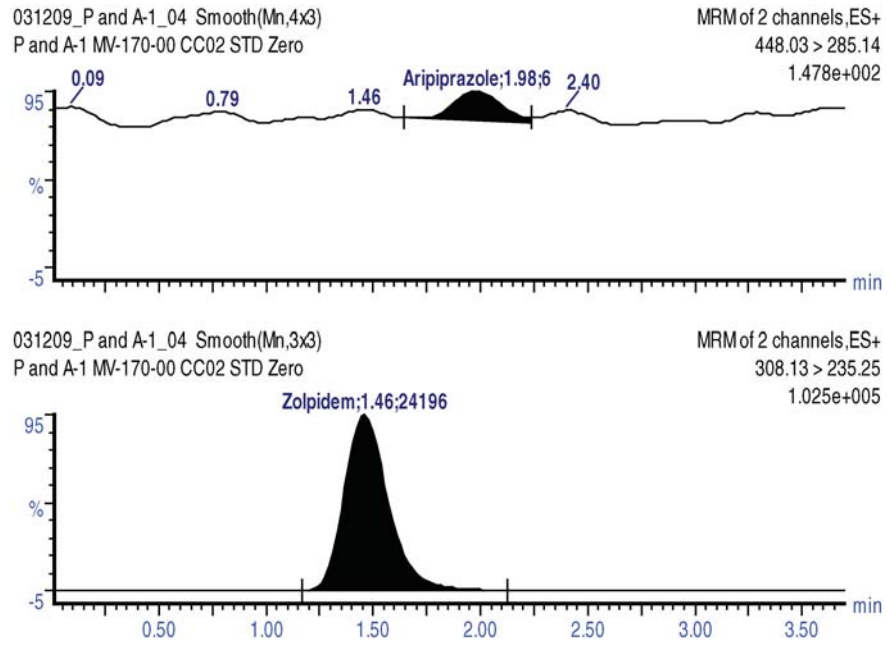


Figure 6. Extracted blank plasma with IS.

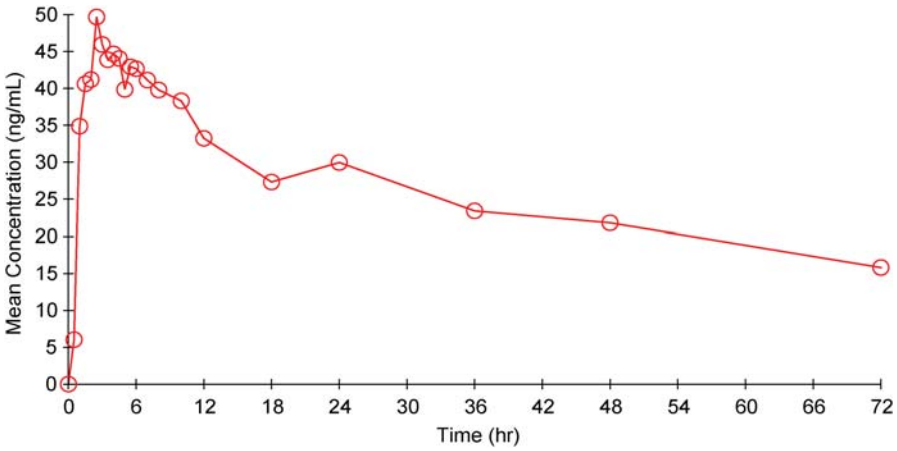


Figure 7. Linear mean plot of aripiprazole plasma concentration versus time under fasting conditions.

endogenous components in drug-free human plasma at the retention times of the analyte and IS.

Sensitivity

The lowest limit of reliable quantification for analyte was set as the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 9.08% and 98.33%.

Extraction efficiency

A simple SPE with 95 parts of methanol and 5 parts of 30 mM ammonium acetate (pH 5.0 ± 0.05) proved to be robust and provided the cleanest samples. The recoveries of the analytes and IS were good and reproducible. The mean overall recoveries (with the precision range) of aripiprazole were $77.35\% \pm 2.040$ (0.99–11.96%). The recovery of IS was 75.55%.

Matrix effect

No significant matrix effect was observed in any of the six batches of human plasma for the analytes at LQC and HQC concentrations. The precision and response for aripiprazole at LQC were found to be 2.10 and 99.62%; at HQC, the precision and response were found to be 7.28 and 103.51%.

Linearity

After comparing the two weighting models, i.e., $1/x$ and $1/x^2$, a regression equation with a weighting factor of $1/x^2$ of analyte

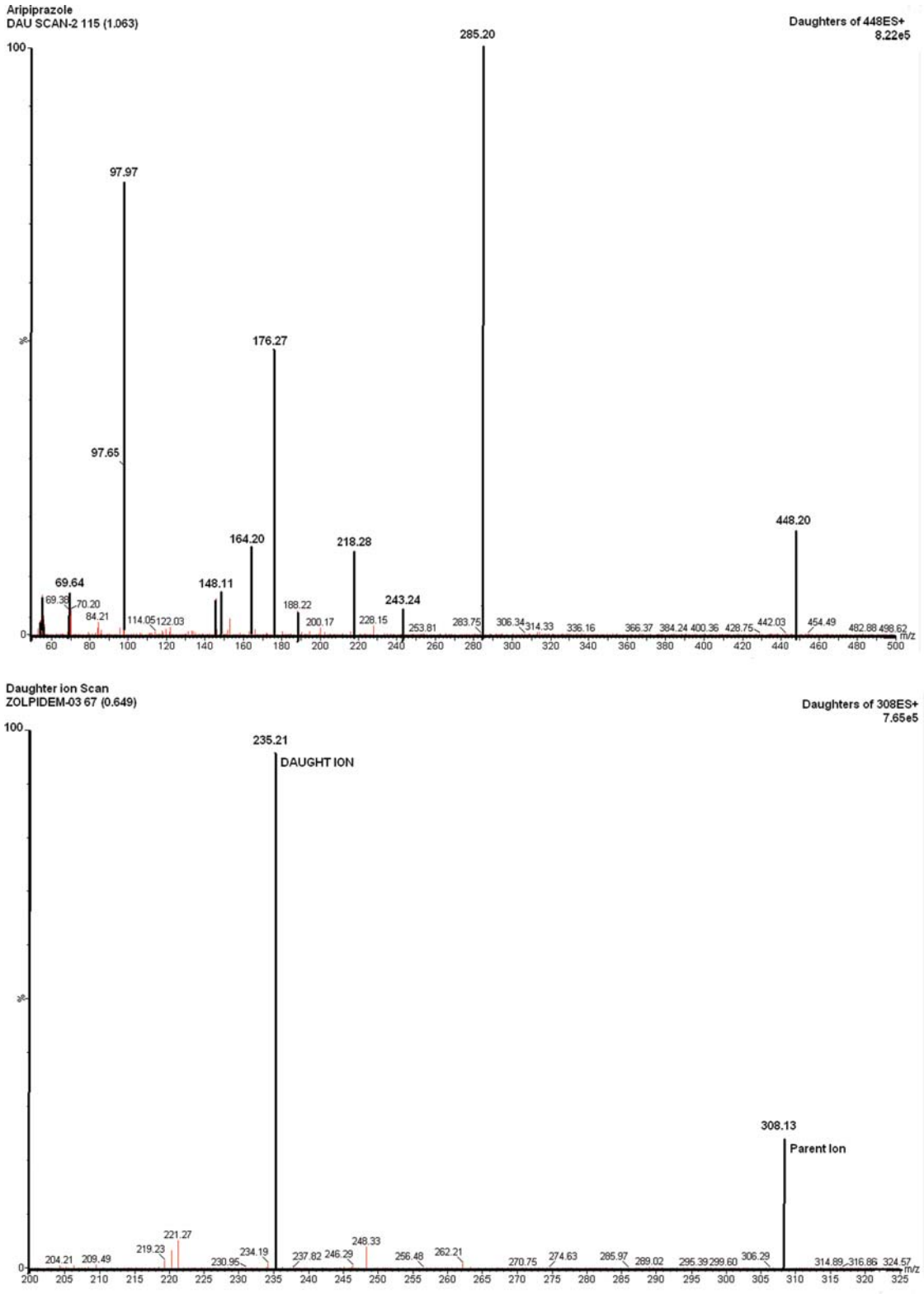


Figure 8. Product ion mass spectra of $[M+H]^+$ of aripiprazole and zolpidem (IS).

to IS concentration was found to produce the best fit for the concentration-detector response relationship for the analyte in human plasma. By using the recommended $1/x^2$ model, values for correlation coefficient (r^2) were found to be $\geq$0.99, which indicates linearity over the whole calibration range for the analyte. Additionally, the mean value of r was

Table II
Intra-Day and Inter-Day Precision of the Determination of Aripiprazole in Human Plasma

QC	Run	Measured concentration of aripiprazole (ng/mL)			
		Mean	SD	%CV	%Nominal
Intra-day variation (six replicates at each concentration)					
LLOQ (0.20 ng/mL)	1	0.21	0.028	13.47	105.00
	2	0.22	0.020	9.08	108.33
	3	0.20	0.033	16.92	98.33
	4	0.21	0.023	10.94	105.83
LQC (0.60 ng/mL)	1	0.63	0.030	4.77	104.72
	2	0.58	0.052	8.93	96.94
	3	0.56	0.043	7.79	92.50
	4	0.62	0.022	3.60	103.06
MQC (30.00 ng/mL)	1	32.12	0.732	2.28	107.07
	2	30.28	1.059	3.35	100.94
	3	29.07	1.070	3.68	96.90
	4	30.92	1.654	5.35	103.06
HQC (45.59 ng/mL)	1	47.43	2.457	5.18	104.04
	2	45.39	1.689	3.72	99.57
	3	43.57	2.515	5.77	95.56
	4	46.05	1.761	3.82	101.01
Inter-day variation (20 replicates at each concentration)					
LLOQ	0.21	0.026	12.42	104.38	
LQC	0.60	0.047	7.86	99.31	
MQC1	30.60	1.572	5.14	101.99	
HQC	45.61	2.450	5.37	100.04	

greater than 0.99 in the concentration range of 0.20 to 60.01 ng/mL.

Precision and accuracy

Accuracy and precision data for intra-day and inter-day plasma samples for aripiprazole are presented in Table II. The assay values on both the occasions (intra-day and inter-day) were found to be within the accepted variable limits.

Dilution integrity

The upper concentration limits can be extended to 120.01 ng/mL for aripiprazole by 1/2 or 1/4 dilution with screened human blank plasma. The mean backcalculated concentrations for 1/2 and 1/4 dilution samples were within 85–115% of their nominal concentrations. The %CV for 1/2 and 1/4 dilution samples were 3.79 and 4.14%, respectively, and the nominal percentages for 1/2 and 1/4 dilution samples were 91.77 and 95.78%, respectively.

Stability studies

The stability studies of the analyte aripiprazole in human plasma over five freeze-thaw cycles indicate that the analytes are stable in human plasma when stored at below $-70 \pm 5^{\circ}C$ and thawed at room temperature. Results of bench-top (9.93 h), autosampler (76.83 h), wet extract (72.97 h) and freeze-thaw (5 cycles) stability are presented in Table III. The long-term stability of the analyte in human plasma stored for a period of 94.72 days at $-20 \pm 5^{\circ}C$ compared with zero-day stability showed reliable stability behavior. The results of the tested samples were within the acceptance criteria.

Table III
Stability Data of Aripiprazole QCs in Human EDTA Plasma

QC	Type of Stability	Mean	SD	% CV	%Nominal
LQC	Bench top (9.93 h)	0.55	0.031	5.58	91.39
(0.60 ng/mL)	Injector (76.83 h)	0.60	0.31	5.22	99.72
	Wet extract (72.97 h)	0.67	0.042	6.29	110.82
	Freeze and thaw (5 cycles)	0.61	0.023	3.88	100.83
	LT stability in EDTA plasma (94.72 days)	0.64	0.037	5.76	106.11
HQC	Bench top (9.93 h)	40.79	2.552	6.26	89.48
(45.59 ng/mL)	Injector (76.83 h)	48.15	1.990	4.13	105.60
	Wet extract (72.97 h)	47.21	5.272	11.17	103.55
	Freeze and thaw (5 cycles)	43.55	1.763	4.05	95.52
	LT stability in EDTA plasma (94.72 days)	47.32	0.617	1.30	103.79

Discussion

Validated methods are essential for the quantitative estimation of aripiprazole concentrations in human plasma for clinical pharmacokinetic studies. The validated method is simple, rugged and rapid due to its short run time of 3.70 min for each sample analysis. Here, the method was developed and validated for the determination of aripiprazole in human plasma with good and reasonable sensitivity (LLOQ 0.20 ng/mL) for the quantification of aripiprazole in plasma samples. The method used very a simple sample preparation procedure using SPE followed by direct injection.

Conclusions

In summary, a selective, reproducible and high-throughput LC–MS-MS (Quattro Micro) method was developed and validated to quantify aripiprazole using zolpidem as IS. To the best of our knowledge, the cost-effectiveness, simplicity of the assay using SPE and sample turnover rate of 3.70 min per sample make it an attractive procedure in high-throughput bioanalysis of aripiprazole. From the results of the validation parameters, we can conclude that the developed method can be useful for BA/BE studies and routine therapeutic drug monitoring with desired precision and accuracy.

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