Bioanalytical method development and validation of alimemazine in human plasma by LC-MS/MS and its application in bioequivalence studies

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ABSTRACT

Background: The use of anti-histaminic agents has been increased significantly from last decades and till now no method is available for quantitation of ALZ in human plasma which can be applied in a bioequivalence study using LC-MS/MS. **Objective:** The present study is concerned with the development and validation of ALZ in human plasma by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). **Materials and Methods:** Sample preparation involved the extraction with liquid-liquid extraction method by using ethyl acetate as an organic solvent. Chromatographic separation was performed on Atlantis® T3 5 μm 4.6 mm $\times$ 150 mm column with the mobile phase consisting of acetonitrile: (10 mm ammonium formate buffer: Formic acid: 99.9:00.1 v/v) 50:50 v/v. The interface used with the application programming interface 4000 LC-MS/MS was a turbo ion spray in which positive ions were measured in multiple reaction monitoring mode. The precursor to product ions transition of m/z 299.30 $\rightarrow$ 100.20 amu and 305.30 $\rightarrow$ 106.30 amu were used for ALZ and ALZ D6 respectively. **Results:** The method was validated over the concentration range of 20.013-10006.551 pg/mL. The mean percent recovery of ALZ was found 77.771% with a precision of 7.71% and the lower limit of quantification was 20.013 pg/mL. The intra- and inter-day precision of the method at three concentrations was 0.98-4.50% and 1.57-5.72% while the intra- and inter-day % accuracy was 99.02-93.82% and 101.78-106.96%. Stability of compounds was established in a series of stability studies. The application of this method was demonstrated in the bioequivalence study and was found suitable in a study of sample size as big as 30 enrolled volunteers. **Conclusion:** For the very first time, a sensitive, selective and robust Liquid Chromatography- Mass Spectrometry method for the determination of alimemazine (ALZ) in human plasma has been developed and validated using ALZ D6 as an internal standard.

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Alimemazine (ALZ), chemically, (3R,4R)-3,4-dihydroxyhexane-2,5-dione or N, N, 2-trimethyl-3-phenothiazin-10-ylpropan-1-amine is commonly provided as a tartrate salt.[1] ALZ is also known as methylpromazine or trimeperazine is a phenothiazine derivative that is generally used as an antipruritic agent.[2] Pharmacologically, it prevents itching from causes such as eczema or poison ivy, by acting as an antihistaminic agent.[3] ALZ binds at hemagglutinin (HA)-receptor sites, which competitively antagonizes the effects of histamine on HA-receptors, leading to a reduction of the negative symptoms brought on by histamine HA-receptor binding.[2] Moreover, ALZ also acts as a sedative, hypnotic and antiemetic for prevention of motion sickness.[3] Various studies have been performed for the quantitative analysis of ALZ in a different medium.[4-8] Kumazawa *et al*. performed the quantitative determination

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of phenothiazine derivatives including ALZ in human plasma using monolithic silica solid-phase extraction tips and gas chromatography-mass spectrometry in which the recoveries of the five phenothiazines spiked into plasma were 91-95% and the limits of quantification (LOQ) for each drug were between 0.25 and 2.0 ng/0.1 mL.[6] Similarly, various phenothiazine derivatives including ALZ were simultaneously determined in human whole blood and plasma by combining headspace solid-phase micro-extraction and gas chromatography with nitrogen-phosphorus detection. The study revealed that the extraction efficiency for the phenothiazine derivatives was 0.013-0.117% for both sample types.[7] In another study, drop-to-drop solvent microextraction coupled with gas chromatography/mass spectrometry for rapid determination of trimeprazine in urine and blood of rats was performed. Moreover, its application to pharmacokinetic (PK) studies was also performed. The results showed that the limits of detection (LODs) of trimeprazine were 0.05, 0.06, and 0.1 μg/mL in deionized water, urine and blood samples.[8] The present study is concerned with the development and validation of ALZ in human plasma by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) and its application in bioequivalence studies after single oral dose of in-house developed ALZ tartrate (5 mg) film coated tablets of test formulation versus ALZ tartrate (5 mg) film coated tablets of reference formulation in 30 healthy adult, human subjects, under fasting conditions.

Materials and Methods

Chemicals and materials

ALZ tartrate (mol.wt. 746.98; 99.3% w/w) and ALZ D6 maleate (mol.wt. 420.55, 99.52%) were obtained from Clearsynth Labs (P) Ltd. The structure of ALZ and ALZ D6 has been shown in Figure 1. Control buffered potassium salt of ethylene di-amine tetra acetic acid (K_2EDTA) human plasma was procured from Laxmi Sai Clinical Lab., India. All other reagents/chemicals were of analytical reagent grade.

LC-MS/MS instrumentation and settings

A HPLC system (Shimadzu Co., Kyoto, Japan) with Atlantis® T3 5 μm 4.6 mm × 150 mm column was used in this study. The column oven and auto-sampler temperature were maintained at 40 ± 2°C and 5 ± 1°C respectively while

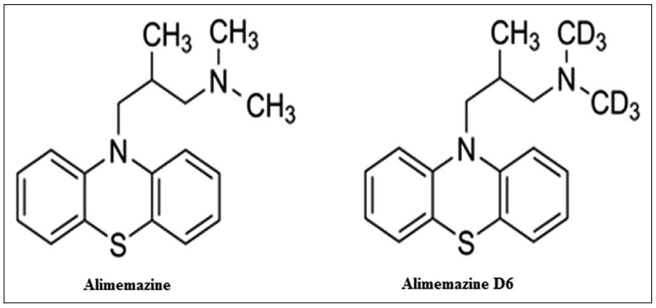

Figure 1: The structure of alimemazine and ALZ D6

and the flow rate was set at 1.000 mL/min. Ionization and detection of analyte and ion spray (IS) was performed on a triple quadrupole mass spectrometer, application programming interface 4000 LC-MS/MS equipped with turbo IS®, from MDS SCIEX (Toronto, Canada) operated in the positive ion mode. Quantitation was performed using the multiple reaction monitoring (MRM) mode to monitor protonated precursor to product ion transition of m/z 299.30 → 100.20 amu and 305.30 → 106.30 amu for ALZ and ALZ D6 respectively. All the parameters of HPLC and MS were controlled by analyst software version 1.4.2. The source dependent parameters maintained for analyte and IS were GS1: 50.00 psi, GS2: 50.00 psi, IS voltage: 2000.00 V, turbo heater temperature (TEM): 600.00°C, collision activation dissociation (CAD): 6.00 psi, curtain gas (CUR): 25.00 psi. The compound dependent parameters like declustering potential (DP) were optimized at 55.00 V, collision energy (CE) was 27.00 V, cell exit potential (CXP) was kept at 6.00 V and entrance potential (EP) was 10.00 V.

Preparation of standard stock and plasma samples

The standard stock solution of ALZ (1 mg/10 mL) and ALZ D6 (1 mg/10 mL) were prepared by dissolving in the requisite amount of methanol. Further dilutions from the stock solutions were prepared using the diluent solution (methanol: Milli-Q water: 50:50, v/v) for spiking in plasma to obtain calibration curve (CC) standards and quality control (QC) samples. CC standards consisted of a set of eight non-zero concentrations of 20.013 pg/mL, 40.026 pg/mL, 1250.819 pg/mL, 2501.638 pg/mL, 5046.039 pg/mL, 6585.508 pg/mL, 8552.608 pg/mL and 10006.551 pg/mL were prepared.

The QC samples consisted of ALZ concentrations of the lower limit of quantification quality control (LLOQQC) 20.017 pg/mL, low-quality control (LQC) 55.573 pg/mL, middle-quality control (MQC) 5006.576 pg/mL and high-quality control (HQC) 8259.148 pg/mL were prepared. After bulk spiking, 400 μL of spiked plasma samples were pipetted out in pre-labeled polypropylene tubes. The CC standards and QC samples were logged in ultra-low temperature deep freezer (temp range: −55°C to − 75°C) except 30 samples each of LQC and HQC, which were transferred for storage in cell frost deep freezer (temp range: −17°C to − 27°C) for the generation of long-term stability at −22°C ± 5°C. These samples were used for performing the method validation.

Preparation of mobile phase and liquid-liquid extraction method

A buffer solution was prepared by dissolving approximately 630.60 mg of ammonium formate in 1000 mL of milli-Q water and 1 mL of formic acid was added in the buffer solution. Mobile phase was prepared as the mixture of acetonitrile: Buffer solution in the ratio 50:50, v/v. For bio-analysis, a set of CC standards and/or QC samples were withdrawn from the deep freezer and allowed to thaw at room temperature. 50 μL of ALZ D6 as an internal standard (approximately 2000.000 pg/mL) was added into ria vials and 300 μL of plasma was aliquoted from

the pre-labeled polypropylene tubes into ria vials followed by vortexing the samples. LLE was performed using ethyl acetate as extraction solvent. Briefly, 2.0 mL of extraction solvent was added and vortexed for 10 min. Samples were centrifuged at 4000 rpm for 5 min at 4°C and flash freezed for approx. 0.2-2 min. The supernatant was decanted off and evaporated to dryness at 40°C (at constant pressure) in nitrogen evaporator. Residue was reconstituted in 500 µL of mobile phase and analyzed.

Method validation

The method has been validated for selectivity, sensitivity, linearity, matrix effect, CC standards and QC samples, precision and accuracy batches.[9,10] The results of various stabilities i.e. (stock dilution stability at refrigerator temperature and room temperature, standard stock solution stability in refrigerator temperature and room temperature and photo degradation test in light, auto-sampler stability, re-injection reproducibility, freeze-thaw stability, long-term stability at 65°C ± 10°C and at −22°C ± 5°C, reagent stability, bench top stability, dry ice stability, dry extract stability, extended bench top stability, wet extract stability in refrigerator, lipemic and hemolyzed plasma stability), blood stability, effect of potentially interfering drugs (PIDs), dilution integrity, recovery, ion suppression through infusion, ruggedness, robustness and extended batch verification meeting the acceptance criteria as per the US Food and Drug Administration guidelines (Food and Drug Administration, 2001).[9,10] Selectivity was performed in 8 lots of normal, 4 lots of lipemic and 4 lots of hemolyzed plasma containing K_2EDTA as an anticoagulant. Sensitivity of the method was determined in six LLOQ samples. For matrix effect, 12 blank samples were processed from 6 normal plasma lots (two aliquots prepared from each plasma lot) and 6 blank samples were processed from 3 lipemic plasma lots and six blank samples were processed from 3 hemolyzed plasma lots respectively. After drying these processed blank samples from each plasma lot were reconstituted with aqueous LQC and aqueous HQC dilution respectively. For comparison of matrix effect same prepared aqueous LQC and HQC dilution were used and six replicates were injected from each prepared aqueous LQC and HQC. Matrix effect was calculated as per the following formula:

Matrix factor = (peak response in the presence of matrix ions)/(peak response in the absence of matrix ions)

% matrix effect = (1 − mean of matrix factor) ×100

The precision of the assay was calculated as percent coefficient of variation (CV) over the concentration range of LLOQQC, LQC, MQC and HQC samples respectively. The accuracy of the assay was calculated as the ratio of the calculated mean values of the LLOQQC, LQC, MQC and HQC samples to their respective nominal values. The data of three precision and accuracy batches were subjected for goodness of fit analysis. The back-calculated concentrations of CC standards using $1/x$ and $1/x^2$ weighing were considered for finding the best fit for regression. Linearity was calculated using a regression equation with a weighting factor of $1/x^2$ for the drug to IS concentration to produce the best fit for the concentration-detector response relationship for ALZ. Stock solution and stock dilution stability in the refrigerator for ALZ and ALZ D6 IS was carried out for 11 days while stock solution and stock dilution stability at room temperature was carried out for 72 h. Photo degradation test of analyte and IS was performed for 72 h in light. For all the aqueous related stability studies, two aqueous mixtures were prepared, one from the stability standard stock solution and the other from fresh standard stock solution (comparison stock). Six replicates of aqueous mixture from each, stability stock and comparison stock were injected. The response of stability sample was corrected using a correction factor.

Correction factor = (conc. of fresh standard sol.)/(conc. of stability standard sol.)

Corrected response = stability stock response × correction factor

% Change = (mean response of comparison samples − mean corrected response of stability samples)/(mean response of comparison samples) ×100

Aqueous recovery comparison samples (LQC, MQC, and HQC) were prepared by adding 6 µL each of aqueous dilution of ALZ from respective QC samples, 50 µL of internal standard dilution (~2000.000 pg/mL) and 444 µL of mobile phase (representing 100% extraction). The aqueous samples (LQC, MQC, and HQC) of ALZ were compared against 6 sets of processed LQC, MQC, and HQC samples. Recovery of internal standard was compared at LQC, MQC, and HQC level.

% Recovery = (mean peak area response of extracted sample)/(corrected mean peak area response of unextracted sample) ×100

The effect of PIDs i.e. ibuprofen, caffeine, acetaminophen and acetyl salicylic acid on ALZ analysis was performed by spiking PID's at their approximately C_{max} concentration in the LLOQ sample in triplicate.

Bench top stability was determined for 12 h using six sets each of LQC and HQC samples while extended bench top stability was determined in spiked samples to assess the stability of ALZ at each step of extraction. The freeze-thaw stability was determined for five freeze-thaw cycles. Six sets of LQC and HOC samples were analyzed after five freeze-thaw cycles. Long-term stability (at −65°C ± 10°C and −22°C ± 5°C) was carried out in plasma for 32 days by using six sets of LQC and HQC. Dry extract stability was carried out by processing six sets of LQC and HQC, stored at −22°C ± 5°C without reconstitution while wet extract stability was carried out by processing the six sets of LQC and HQC, stored at 2-8°C after reconstitution. The samples of wet extract and dry extract stabilities were analyzed after 75 h storage. All stability QC's were analyzed against the freshly spiked CC standards and six sets of freshly spiked LQC and HQC (prepared from the freshly weighed stock solution) to calculate the % change between the stability QC's and Comparison QC's.

For robustness six sets of LQC and HQC were analyzed against a CC standards at different chromatographic conditions, i.e., robustness experiment was performed at different column temperatures (38°C and 42°C), at different flow rates (0.950 mL/min and 1.050 mL/min) and at different mobile phase compositions acetonitrile: (10 mm ammonium formate buffer: Formic acid: 99.9:00.1 v/v) 48:52 v/v and 52:48 v/v. To evaluate ruggedness, precision and accuracy batch was processed against CC standards and analyzed by a different analyst using the different column and different sets of solutions.

Bioequivalence study

To compare the bioavailability and characterize the PK profile for assessment of bioequivalence of the test formulation (ALZ tartrate 5 mg film coated tablet) with reference formulation (ALZ tartrate 5 mg film coated tablet) in healthy adult, human subjects, under fasting conditions. The protocol was approved by the relevant institutional ethics committee. Plasma samples of all evaluable subjects completing all the periods of the clinical phase of the study were to be analyzed. All participants gave written consent and were informed of the aims and risks of the study. Inclusion criteria comprise age (18-45 years), body mass index (18.5-30.0 kg/height^2) and absence of abnormalities on physical examination along with normal electrocardiogram and laboratory tests. Exclusion criteria comprise allergy to ALZ, alcoholism, psychosis, smoking, diabetes or any disease, which could compromise the hemopoietic, gastrointestinal, renal, hepatic, cardiovascular and respiratory or central nervous systems. Moreover, all procedures were based on the international conference on harmonization, E6 good clinical practice (International Conference on Harmonization, E6GCP) guidelines. As per the study protocol clinical blood samples were to be collected from 30 subjects during two period of the study. The clinical blood samples were collected in K_2EDTA vacutainer from the subjects and intermediately stored in the deep freezer at −22°C on the days of plasma sample collection. Plasma was obtained by centrifugation at 4500 rpm at 4°C for 10 min and was stored at −65°C until assayed.

Results and Discussion

Method development and optimization

The scanning and acquisition of the parent and the product ions for ALZ and ALZ D6 was performed by continuous infusion (5 μL/min) of the approximately 200 pg/mL concentration of ALZ and ALZ D6 one by one through a Harvard syringe pump and sorting out the appropriate polarity of ions $[M - H]^+$ in positive ion mode. The parent ion and product ion mass spectra of the $[M - H]^+$ ions of ALZ and ALZ D6 are shown in Figure 2. The optimization of compound dependent parameters i.e., DP, EP, CE and CXP was carried out by continuous infusion (5 μL/min) of ALZ and ALZ D6 aqueous mixture sample. All parameters such as DP, EP, CE and CXP were ramped to get the better intensity of parent ion as well as productions.

After that in MRM mode, all source dependent parameters

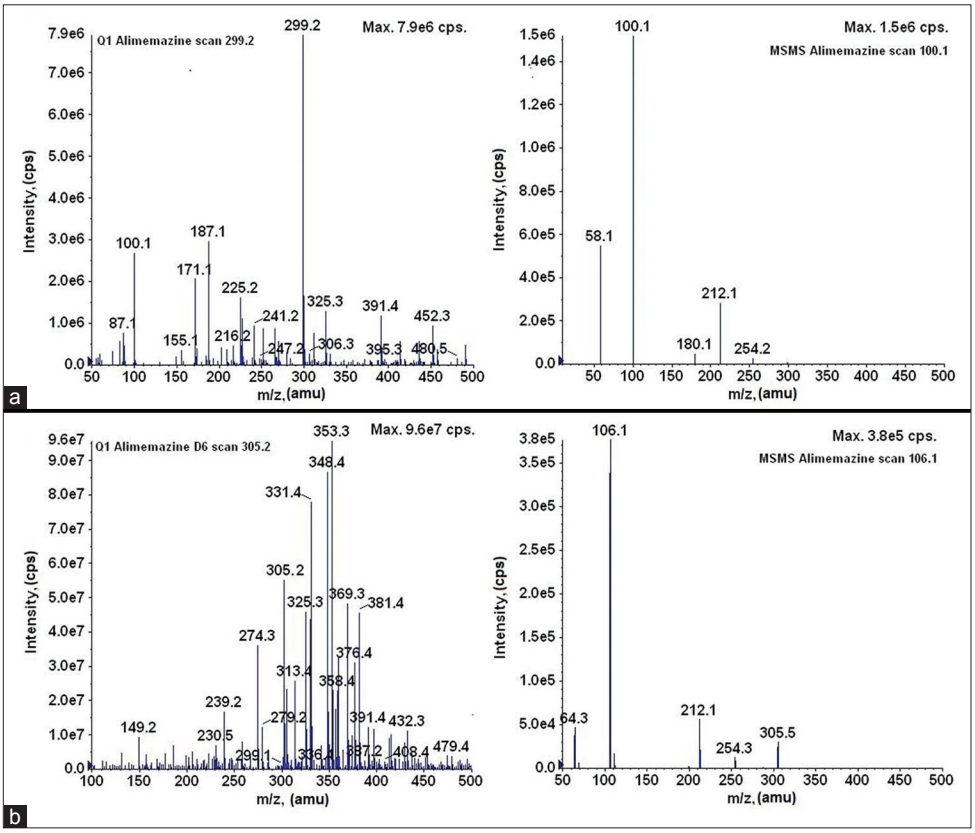

Figure 2: The parent and product ion mass spectra of the $[M - H]^+$ ions of (a) alimemazine and (b) ALZ D6

were optimized one-by-one by flow injection analysis by using a union instead of column, i.e. CUR, CAD, IS voltage, source temperature TEM, GS1 (heater gas) and GS2 (auxiliary gas) to achieve the better intensity of parent ions as well as product ions. Electro spray ionization provided the high ionization efficiencies for both analyte and IS in positive ion mode, which resulted in admirable sensitivity of the method.

Then chromatographic conditions were optimized to look for better sensitivity, peak shape and chromatographic run time. The selection of mobile phase was preformed taking into account the symmetric peak shape with shorter run time that further leads to low consumption of organic solvent altogether making the method cost-effective. Results obtained from the several combinations showed that acetonitrile: (10 mM ammonium formate buffer: Formic acid: 99.9:00.1 v/v) 50:50 v/v serves the desired purpose with utmost effectiveness. By increasing the composition of buffer solution in the mobile phase, decrease in matrix effect has been observed in the extracted plasma samples. An increase in organic content in the mobile phase aids in creating better spraying condition within the ionization source. But, later on during the extraction of drug from plasma matrix effect has been observed with solid-phase extraction method as well as in LLE method. Hence, it has been decided to optimize the composition of mobile phase to remove the matrix effect. Various compositions of mobile phase have been tried and out of that acetonitrile: (10 mm ammonium formate buffer: Formic acid: 99.9:00.1 v/v) 50:50 v/v was observed best and no matrix effect has been observed in the extracted samples at this composition. Than source temperature has been increased to 600°C for getting the better spray within the ionization source. Addition of buffer as the aqueous component of the mobile phase helps in adjusting the pH of the mobile phase within the range of the pKa of the analytes. An Atlantis® T3 5 μm 4.6 mm × 150 mm column was found to be most suitable for analysis from an array of different varieties available as its bonded phase offers high percentage of hydrophobic retention, which helps in getting the good chromatography at 50% aqueous composition of mobile phase. Retention time (RT) of ALZ and ALZ D6 was found to be 3.28 ± 0.3 min and 3.27 ± 0.3 min respectively. The use of proper internal standard was done to eliminate the quantitative bias caused by matrix effect and instrumental variation. ALZ D6 was selected, which has similar ionization condition, appropriate RT and recovery compared with ALZ leading to better tracking of analyte during the course of experiments.

Extraction procedure optimization

LLE method was used for sample preparation because of relatively low cost, good extraction efficiency as well as a simple procedure. Various extraction solvents were tried, but 100% ethyl acetate was found to be most effective for extraction of both ALZ and ALZ D6 with minimal interference and good recovery. Due to the good response of ALZ in processed LLOQ sample 300 μL of processing volume has been used and final dried samples were reconstituted in 500 μL of mobile phase.

Method validation

Selectivity and matrix effect

Figure 3 shows typical MRM chromatograms of a blank plasma sample, a plasma sample spiked with ALZ at the LLOQ (20.013 pg/mL) and a plasma sample from a healthy volunteer 2.0 h after the oral administration of the ALZ tablet. No significant interference was observed from endogenous substances at the RTs of the analyte and internal standard in normal, hemolyzed and lipemic plasma. The variability of matrix factor (reported as %CV of matrix factor) was 3.94% QC) and 5.50% LQC for ALZ and 4.81% LQC and 5.10% HQC for ALZ D6 and the variability of IS-normalized matrix factor on normal plasma (reported as %CV of matrix factor) was 1.28% (HQC and 3.60% LQC. The % matrix effect ranged from −0.27% HQC to 0.67% LQC. The results were within the acceptance criteria and indicate that ion suppression or enhancement due to the plasma matrix was consistent and would not interfere with the quantitation of analytes.

Linearity and sensitivity

The LOD is defined as the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise while LLOQ is defined as the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The lowest standard on the CC is accepted as the LOQ if the analyte response at the LLOQ is at least 5 times the response compared with the processed blank response and the analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80-120%. The correlation coefficients (r^2) were greater than 0.99 over the concentration range of 20.013 pg/mL to 10006.551 pg/mL. Typical equations of CC are as follows:

$y = 0.002450x + (-0.002079)$, $r^2 = 0.9965$

Where y represents the analyte\I.S. peak area ratio and x represents the plasma concentration of the analyte. The precision and accuracy for ALZ at LLOQ was 6.84% and 102.49%, respectively revealing a prodigious sensitivity of the method.

Precision and accuracy

The precision of the analytical method describes the closeness of repeated individual measures of analyte and is defined as the ratio of standard deviation/mean (%). Precision is expressed as the CV. Precision should be demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs, i.e. using the same runs and data as for the demonstration of accuracy. The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the analyte, the QC samples. Table 1 summarizes

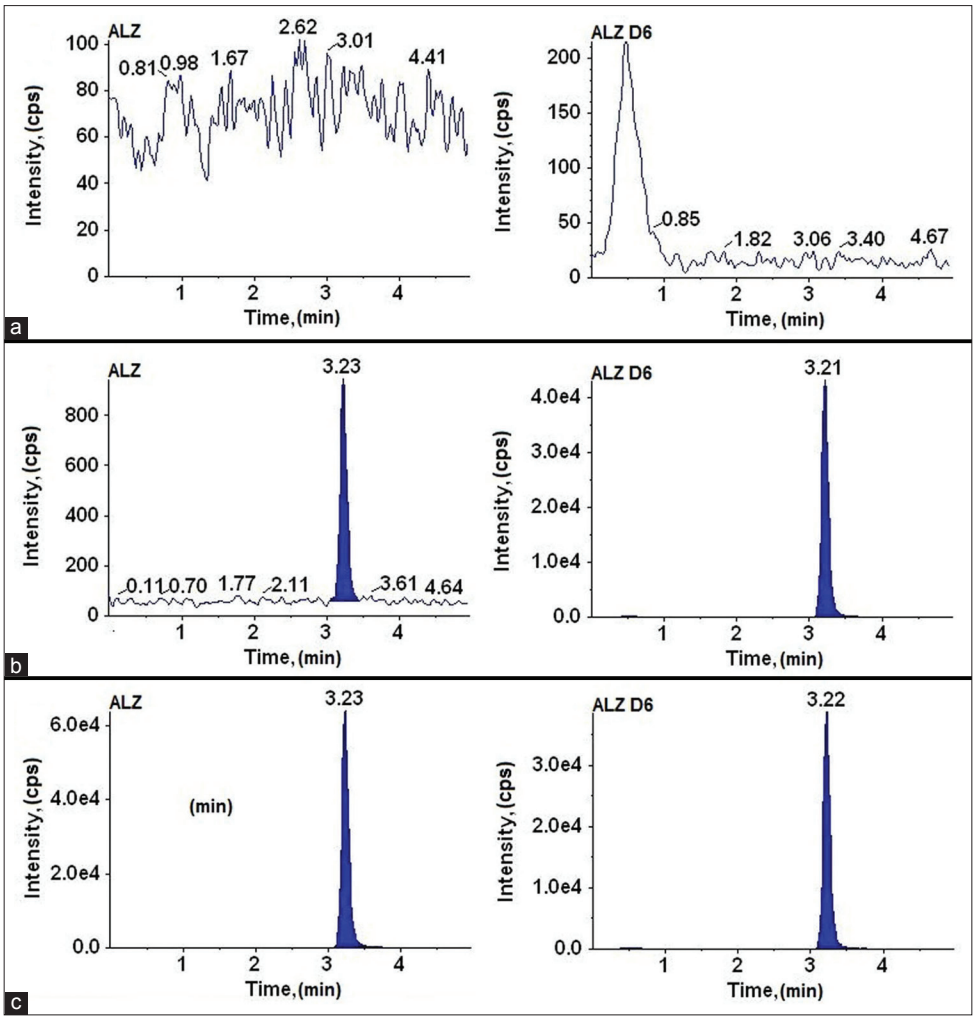

Figure 3: Representative multiple reaction monitoring chromatograms of (a) Blank plasma sample, (b) A plasma sample spiked with alimemazine (ALZ) at the lower limit of quantification (20.013 pg/mL) and (c) A plasma sample from a healthy volunteer at 2.0 h (left panel of the figure showing the ALZ and right panel of the figure showing the ALZ D6)

Table 1: Back calculated concentration of the calibration curve standards for ALZ ($n=3$)

Standards	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	Slope	Intercept	r^2
ALZ (ng/mL)	20.013	40.026	1250.819	2501.638	5046.039	6585.508	8552.608	10006.151	-	-	-
Mean	19.066	43.767	1293.814	2601.095	5120.172	6432.295	8128.117	9385.738	0.002450	−0.002079	0.9965
SD	0.172	0.691	5.456	16.271	42.495	62.818	18.249	77.147	-	-	-
% CV	0.90	1.58	0.42	0.63	0.83	0.98	0.22	0.82	-	-	-
% Nominal	95.27	109.35	103.44	103.98	101.47	97.67	95.04	93.80	-	-	-

ALZ: Alimemazine, CV: Coefficient of variation, SD: Standard deviation, STD: Standard

Table 2: Inter-day and intra-day precision and accuracy of the method for ALZ

Levels	Concentration added (ng/mL)	Inter-day ($n=6$)			Intra-days ($n=18$)		
		Mean concentration found (ng/mL)	% Nominal	% CV	Mean concentration found (ng/mL)	% Nominal	% CV
LLOQQC	20.017	18.779	93.82	4.50	19.768	98.76	5.72
LQC	55.573	55.881	100.55	0.98	57.021	102.61	2.00
MQC	5006.576	5239.301	104.65	1.81	5260.990	105.08	1.70
HQC	8259.148	8178.043	99.02	0.98	8276.525	100.21	1.57

ALZ: Alimemazine, CV: Coefficient of variation, LQC: Low-quality control, HQC: High-quality control, MQC: Middle-quality control, LLOQQC: Lower limit of quantification quality control

back calculated concentrations of CC standards for ALZ whereas Table 2 represents the intraday and inter days precision and accuracy data. The intra- and inter-day precision of the method at three concentrations was 0.98-4.50% and 1.57-5.72% while the intra- and inter-day accuracy was 99.02-93.82% and 101.78-106.96%. The results showed that method is fairly precise and accurate within the acceptable limits.

Recovery

The mean % recovery of ALZ was 77.771% with a precision of 7.71% while ALZ D6 showed mean % recovery of 82.228% with a precision of 1.13%. The data shows that the simple LLE procedure efficiently extracts analyte as well as IS from human plasma.

Stability and other parameters

Table 3 represents stability data and shows there were no stability-related issues that might cause problems in the application of the assay to PK studies. The outcomes of other parameters such as ruggedness, reinjection reproducibility, effect of PID, dilution integrity, extended batch verification

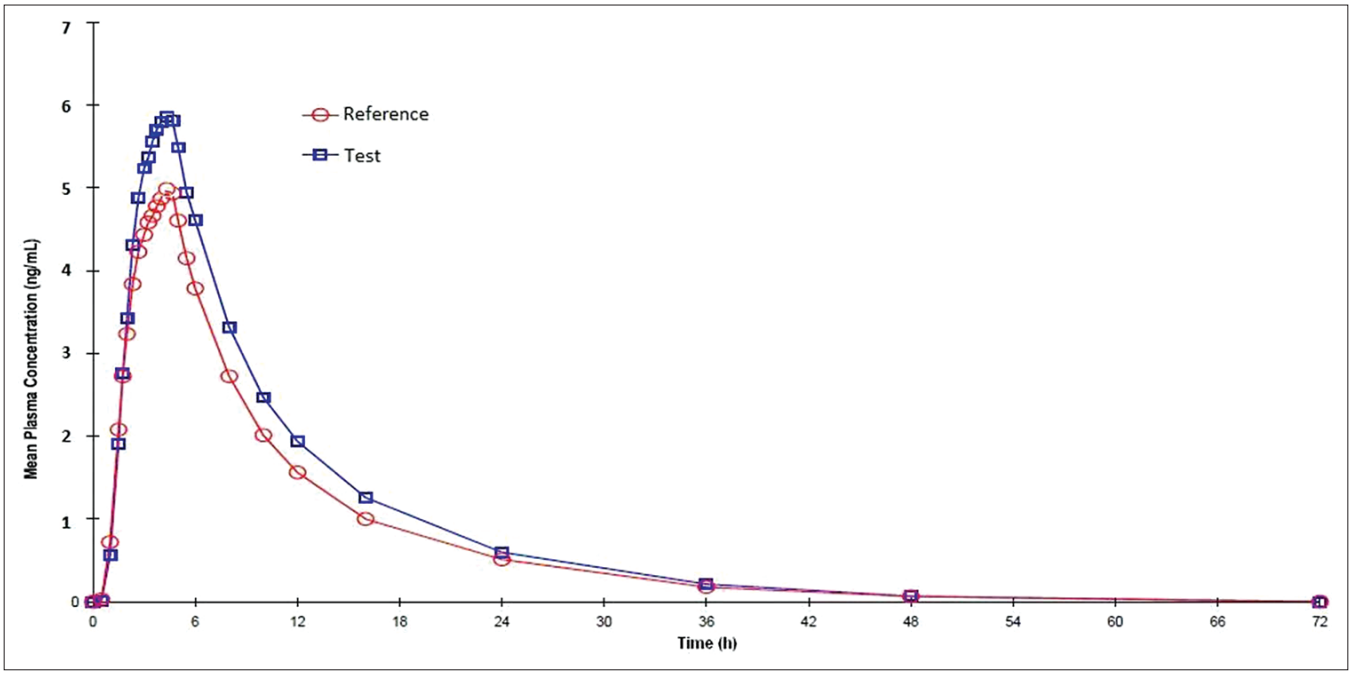

Figure 4: Mean plasma concentration versus time profile for alimemazine (ALZ) when compared with standard ALZ film coated tablet

Table 3: Stability data of ALZ in processed QC samples for different stability activities at different conditions ($n=6$)

Stability	Storage condition	Levels	DRN				
			Mean comparison concentration (ng/ml, $n=6$)	% CV	Mean stability concentration (ng/mL, $n=6$)	% CV	% Stability (% mean change)
Bench top	Room temperature for 13 h	LQC	59.400	3.07	58.948	4.01	0.75
		HQC	8084.889	0.52	8081.685	0.55	0.03
Auto sampler	Auto sampler at 5°C for 74 h	LQC	59.400	3.07	58.717	2.62	1.14
		HQC	8084.889	0.52	8043.295	0.96	0.51
Freeze and thaw	After 5th cycle at −70°C	LQC	55.053	1.27	55.701	1.77	−1.18
		HQC	8186.121	1.07	8000.415	0.68	2.26
Long-term	22 days at −70°C	LQC	53.746	11.73	56.050	0.64	−5.18
		HQC	8199.991	1.50	7907.636	4.10	3.70
	22 days at −22°C	LQC	53.746	11.73	56.055	2.37	5.19
		HQC	8199.991	1.50	7907.636	4.10	3.55
Dry extract	51 h at −22°C	LQC	59.400	3.07	58.551	2.68	1.42
		HQC	8084.889	0.52	8074.181	1.00	0.13
Wet extract	51 h at 2-8°C	LQC	59.400	3.07	57.217	2.56	3.67
		HQC	8084.889	0.52	8013.716	1.17	0.87
Haemolysed impact	2 days at −70°C	LQC	55.053	1.27	55.270	2.57	−0.40
		HQC	8186.121	1.07	7997.992	0.54	2.29
Lipemic impact	2 days at −70°C	LQC	55.053	1.27	55.256	3.01	−0.38
		HQC	8186.121	1.07	8019.556	0.45	2.03

ALZ: Alimemazine, CV: Coefficient of variation, QC: Quality control, LQC: Low-quality control, HQC: High-quality control

and robustness were found to be within the acceptance criteria as per USFDA guidelines.[9-10]

Carryover test

The MRM chromatograms of blank (ALZ and ALZ D6) were analyzed by following the upper LOQ samples had showed that there was no carryover.

Application of the method

The validated method was successfully applied to quantify the concentration of ALZ in human plasma samples obtained from the PK study of test formulation (ALZ Tartrate 5 mg film coated tablet) with reference formulation (ALZ Tartrate 5 mg film coated tablet) in healthy adult, human subjects, under fasting conditions. Blood samples were collected using K_2EDTA vacutainer at different time points as per protocol. PK parameters were calculated from the subjects who had successfully completed periods I and II of the study. The mean plasma concentration versus time profile is shown in Figure 4.

Conclusion

For the very first time a sensitive and selective HPLC-MS/MS method using a LLE sample preparation procedure has been developed and validated for the determination of ALZ in human plasma using HPLC–MS/MS with turbo-IS in positive ion mode. The extraction procedure and HPLC-MS/MS conditions were optimized in order to improve the sensitivity and robustness of the method. The procedure was fully validated as per USFDA guidelines and found to be well-suited for the PK study.

References

1. Available from: http://www.drugbank.ca/drugs/DB01246 [Last accessed on 2012 Dec 25].
2. Lutka A, Koziara J. Interaction of trimeprazine with cyclodextrins in aqueous solution. Acta Pol Pharm 2000;57:369-74.
3. Bello LL. Factors affecting efficacy of oral trimeprazine sedation for dental procedures in children: A retrospective study. Saudi Dent J 2011;23:87-90.
4. Hu OY, Gfeller E, Perrin JH, Curry SH. Relative bioavailability of trimeprazine tablets investigated in man using HPLC with electrochemical detection. J Pharm Pharmacol 1986;38:172-6.
5. Kintz P, Villain M, Cirimele V. Determination of trimeprazine-facilitated sedation in children by hair analysis. J Anal Toxicol 2006;30:400-2.
6. Kumazawa T, Hasegawa C, Uchigasaki S, Lee XP, Suzuki O, Sato K. Quantitative determination of phenothiazine derivatives in human plasma using monolithic silica solid-phase extraction tips and gas chromatography-mass spectrometry. J Chromatogr A 2011;1218:2521-7.
7. Shinmen N, Lee XP, Kumazawa T, Hasegawa C, Ishiwata Y, Sato K, *et al.* Simultaneous determination of some phenothiazine derivatives in human blood by headspace solid-phase microextraction and gas chromatography with nitrogen-phosphorus detection. J AOAC Int 2008;91:1354-62.
8. Agrawal K, Wu HF. Drop-to-drop solvent microextraction coupled with gas chromatography/mass spectrometry for rapid determination of trimeprazine in urine and blood of rats: Application to pharmacokinetic studies. Rapid Commun Mass Spectrom 2007;21:3352-6.
9. Guidance for Industry. Bionanalytical method validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM). May 2001.
10. US Department of Health and Human Services Food and Drug Administration. Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER). Guidance for Industry: ICHE6 Good Clinical Practice, 1996. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM073122.pdf. [Last accessed on 2012 Dec 28].

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