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Original Article

Quantification of Blonanserin in Human Plasma Using Liquid Chromatography- Electrospray Ionization-Tandem Mass Spectrophotometry-Application to Pharmacokinetic Study

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ABSTRACT

Background: A novel LC-MS/MS method was developed for the estimation of blonanserin (BLN) in spiked human plasma. **Methods:** Liquid-liquid extraction (LLE) technique was adopted for the extraction of BLN from human plasma and chromatographic separation was performed on a waters symmetry shield, C18 (4.6mm id x 50 mm) analytical column using 7 Mm ammonium formate and acetonitrile (30:70) v/v as mobile phase. Positive ion mode was selected to obtain the product ion *m/z* 367.24 → 296.19 for BLN and *m/z* 326.8 → 269.07 for clozapine as internal standard (IS). **Results:** Calibration curve was linear over the range of 0.01 to 5 ng/ml. Developed method was satisfactory validated as per US-FDA guidelines for the bioanalytical study because it exhibits excellent intra and interday accuracy with % nominal 90 → 98.4 %, precision %CV ≤ 2% in all quality control levels, shows acceptable % extraction recovery (95.15 % → 97.04 %), demonstrated excellent matrix and analyte selectivity (% interference=0), matrix effect (matrix factor 0.981 at LQC and 1.02 at HQC level) and satisfactory stability study results in all types (% nominal 93.91 % → 99.58 %). Along with pharmacokinetic study rabbit plasma samples also analysed for one batch of accuracy, precision, matrix effect and rationalized the suitability of the developed method in other preclinical sample species **Conclusion**: Present method was successfully optimised, validated and applied favorable for the pharmacokinetic study of marketed formulation in rabbit blood samples in single oral human equivalent dose. The applicability of the developed method undoubtedly can further extend during preclinical and clinical trials.

Key words: Blonanserin, LCMS/MS, Validation, Plasma, Pharmacokinetic.

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INTRODUCTION

Schizophrenia is devastating psychiatric disorders characterized by positive, negative, affective and cognitive syndrome[1] which also leads to development of other co-morbid diseases.[2] It disrupts mental and social functioning, destroys the lives of a patients as well as their family, friends. A number of antipsychotics have been explored over last forty years but the outcomes for patients still unsatisfactory[3] Blonanserin (BLN) a newer atypical antipsychotic belongs to a series of 4 phenyl-2-(1-piperazenyl) pyridines, chemical structure shown in Figure 1, which acts as an antagonist at dopamine D_2, D_3 and $5HT_{2A}$ receptors.[4] With many second generation antipsychotics BLN is significantly more efficacious in the treatment of schizophrenia.[5] BLN is still not official in any pharmacopoeia and based on its wide acceptability throughout world, the detail analytical profile and method of quantification of it in human plasma must be reliable, economic and highly validated. Easy, reliable, economic and accurate method of drug analysis is always well accepted, because this finally minimized the cost of dosage from[6]. Keeping the focus on worldwide huge acceptability of BLN and availability of its very limited analytical methods in plasma, present work has been planned. A details review of literature reveals that there is an availability of very few methods for the determination of BLN in human plasma and urine. Four important factors must have to consider for plasma analysis of drug during the development of new method are reasonable elution time, an appropriate LLOQ, low cost, simple and efficient sample processing. In a single HPLC method[7] where the run time of each analytes were 6 minutes which is too long. The reported UPLC/MS method[8] utilized solid phase cartridges for extraction, which is generally costly and consider complex because of further recovery of analyte from the cartridges is require, where the chance of sample loss is high. Authors also followed complex gradient elution system for the elution of analyte. One reported GS-MS method[9] uses also solid phase extraction which is considered costly because of its cartridges, and complex because of analyte recovery and selection of proper washing solution, compared to simple LLE (liquid-liquid extraction) and reported method exhibits longer analysis time. Another reported method for the determination of BLN in urine[10] found less sensitive and shows retention time 4.2 minutes and 5 minutes run time, which generally consider too long in case of LC-MS analysis. One reported method[11] used protein precipitation for the extraction of sample form the matrix shows poor peak shape and peak intensity, also exhibits long run time and retention time for the analytes. Therefore these assay methods have several significant disadvantages such as low sensitivity, complexity in the method, larger volume of sample requirement, time consuming and expensive which limits there omnipresent acceptability in clinical trial and other studies. Herein a rapid, sensitive fast and easy method was developed with the use of 300 µl of plasma samples. Run time was 2.5 min for each sample with the use of simple Liquid-liquid extraction technique which yields better assay results. For LC–MS/MS analysis, there should be a proper extraction procedure is mandatory which can yields good recovery with minimal or no matrix effect[12], therefore authors established this method as a novel, and reliable then other reported methods for the determination of BLN in human plasma based on its empirical evidences shown in Figure 2 in the form of comparison chart and validated as per USFDA bioanalytical method development.[13] So that this method can better applicable for the further bioequivalence and toxicological study during clinical and preclinical trials.

MATERIALS AND METHODS

Blonanserin standard (99.84% pure) and Clozapine (99.87% pure) was kindly provided by Zydus Cadila healthcare Ltd, Ahmadabad, India, as a gift sample. Ammonium formate buffer was obtained from Sigma-Aldrich, Hyderabad, India.

High Performance Liquid chromatographic operating conditions

An isocratic elution technique was adopted with the mobile phase (7 mM ammonium formate: acetonitrile, 30:70) derived at a flow rate of 600 µl/minute using waters Symmetry shield RP C-18 column with 4.6 mm internal diameter, 5 µm particle size, 100A^0 pore size. Under these condition retention time of BLN was 1.00 ± 0.3 min and 1.10 ± 0.3 min for internal standard clozapine Column effluent was introduced in to mass chamber and temperature of the auto sampler was maintained 4^{0}C. Run time was up to 2.5 minutes.

Mass spectrometry operating conditions

Mass spectrometry was performed using an API-3000 triple quadrupole mass spectrometer (AB SCIEX, Foster city, CA/concord, Ontario, Canada) was equipped with an electrospray ionisation source (ESI), operating in the positive ion mode at 800^{0}C desolvation temperature. The ion source parameters for examples capillary voltages 3.30 kV, cone voltages 35 kV, source temperature 400^{0}C, desolvation gas flow 800 L/h and other parameters were also set for the analysis BLN and internal standard were summarised in Table 1. Detection of the ions were carried out in multiple reaction monitoring by monitoring the transition pairs of m/z 367.24–296.19 and m/z 326.8-269.07 for internal standard was shown in Figure 3 and 4.

Preparation of calibration curve and quality control samples

The calibration curve and quality control samples of BLN were prepared from the aqueous dilutions separately. Stock solution (10000 ng/ml) was prepared by dissolving 1mg of BLN in 1 ml of acetonitrile and make 100 ml with diluent (mobile phase). From the stock solution aqueous calibration curve dilutions were made to obtain the concentrations 100, 50, 15, 10, 5, 2, 1, 0.2 ng/ml. Finally plasma samples were prepared by diluting 0.25 ml aliquot form different aqueous dilutions. 0.75 ml of plasma matrix were added to each tubes and volumes were made up to 5 ml with diluent to obtain the final concentration 5 to 0.01 ng/ml. Quality control samples were also prepared in same way from the different aqueous QC dilutions to obtain the final concentrations at LLOQ (0.01 ng/ml), LQC (0.25 ng/ml), MQC (2.5 ng/ml), HQC (5 ng/ml), DIQC (25 ng/ml) levels.

Sample preparation

A simple liquid-liquid extraction technique was adopted for the sample preparation. The plasma samples of the analytes and internal standard for calibration curve and quality control samples were thawed at room temperature and vortexed the samples to ensure the complete mixing. 250µl of plasma sample was pipette out and placed in to 15 ml of Stoppard tube. 25 µl of internal standard from 0.25 µg/ml clozapine dilution was added to each Stoppard tube and vortexed except the blank plasma samples where 25 µl of diluent (mobile phase) was added. 5 ml of tetra butyl methyl ether as extracting solvent was added to each tube and shaken for 20 minutes on reciprocating shaker at 200 rpm. Samples were then centrifuged at 4000 rpm for 10 minutes at 25^{0}C. The supernatant organic layer was transferred into pre labelled tubes and evaporated to dryness under nitrogen gas at 40^{0}C. All the samples were reconstituted with 300 µl of mobile phase and transferred in to auto sampler loading vials and 10 µl of sample was injected in to the LC-MS/MS system.

Method validation

The optimised method was validated as per sated guidelines of bioanalytical method development and validation of by USFDA. The developed method was validated for matrix effect, carry over effect, accuracy, precision, recovery, sensitivity and different stability parameters.

Carry over effect

This was performed to investigate the effect of carrying analytes and internal standard in different sections of the system. Six replicates of the extracted blank matrix and six replicates of the extracted high concentration of analytes at the calibration curve range (ULOQ), six extracted LLOQ concentrations of the analytes and internal standard were prepared and injected as per sequence The sequence is, first six injections was extracted LLOQ, followed by extracted ULOQ and extracted blank plasma. ULOQ and blank plasma samples were injected alternatively. Response of interfering peaks in blank samples at the retention time of the analytes must be ≤ 20 % of average drug response and for internal standard must be ≤ 5% of average internal standard response in LLOQ samples.

Matrix effect

It was determined at two concentration level (LQC and HQC) in eight replicates for analyte and internal standard. One haemolytic and one lipemic plasma lots has been selected. One set of each of samples were spiked with blank matrices (haemolytic), another set of each samples (LQC and HQC) with internal standard was spiked with lipemic plasma was prepared. Six replicates of aqueous samples equivalent to final LQC and HQC concentrations were prepared by spiking the analyte with internal standard to reconstituted solution and injected individually. IS normalized matrix factor was calculated by dividing peak response area in presence of matrix ion and mean peak area response ratio in absences of matrix ion. The variability in IS-normalized factor as measured by the coefficient of variation which should be less than 15%.

Matrix selectivity and specificity

Matrix selectivity was evaluated by analysing the plasma of six different lots, including one haemolytic and one lipemic plasma to investigate the interference the retention time of the analytes and internal standard. The interference at the retention times of the drugs by comparing the response in the blank plasma, against the response of LLOQ was evaluated. And the interference at the retention time of internal standard also evaluated against the response of the extracted internal standard in LLOQ sample. The response of the interfering substance will be acceptable if it is less than 20% of the mean drug response in LLOQ sample and less than 5% in case of internal standard.

Analyte selectivity

Analytes selectivity was also analyzed to investigate the internal standards interference at analytes retention time. The investigate this six replicates of matrix blank with internal standard was injected, if any area for analytes was found was compared with mean area of the analytes obtained with LLOQ concentration injected. Similarly internal standards selectivity was also investigated for this six replicates of matrix blank with drugs was in injected at ULOQ level and area of internal standard if obtained, compared with mean area of internal standard at LLOQ level.

Precision and accuracy

The intra and interday precision and accuracy were estimated using several QC samples at the level of LLOQ, LQC, MQC and HQC in six replicates and the concentrations in these level was calculated followed

by standard deviation, % CV for precision and % nominal for accuracy for each replicates. The acceptance criteria for accuracy (% nominal) is ±15% and ±20% only for LLOQ level and for precision (%CV) should be within ±15% and only for LLOQ sample it is ±20%.

Linearity

The developed method was assessed for linearity in the concentration range of 0.01-5 ng/ml. Eleven CC (calibration curve) samples has been prepared by spiking human plasma and processed. The CC has been constructed by using a regression equation with a weighing factor $1/(\text{concentration ratio})^2$ of the drug to internal standard concentration to produce best fit for the concentration/response relationship. The acceptance criteria for the linearity is the r^2 (coefficient of correlation) should be ≥0.98.

Dilution integrity

To investigate dilution integrity of the developed method 12 sets of QC stock solution were prepared by spiking 1.5 times of the highest standard concentration. Six sets of dilution integrity samples were prepared by diluting 2 times and another six samples by 4 times dilution. These samples were analyzed and concentrations were calculated by multiplying suitable dilution factors, 2 (for two times dilution) and 4 (for four times dilution). For each dilution level at least 67% (4 out of 6) of the QC samples should be 15% of their respective nominal.

Recovery study

This study was conducted to express the extraction efficiency of an analytical process, by comparing the peak response from extracted and non extracted samples. Six LQC, MQC and HQC samples has been prepared freshly and these samples were processed by adding internal standard and injected. Foe non extracted samples 18 blank matrix samples were spiked with six sets of each LQC, MQC and HQC with internal standard and injected. Six non extracted samples of each 3 levels were prepared by spiking 10 µl of analytes and 10 µl of internal standard in extracted blank plasma. Mean overall % recovery was calculated, and % of difference should not be more than 25% between highest and lowest % recovery.

Ruggedness

To investigate the ruggedness of the developed method, one precision and accuracy batch of samples were processed and analyzed with different columns of the same make and with different reagent lots.

Stability studies

In the stability study (Bench top, wet extract, freeze thaw, autosampler, short term and long term stability) of BLN was performed using freshly prepared calibration curve samples and quality control samples were prepared at low middle, high level and analysed. Concentration response linearity data was collected and used to calculate the concentration of stability samples.

Room temperature stability study

It was performed using the stock solution prepared for at least a period of 6 hours. Fresh stock solution of analyte and internal standard were prepared. The final dilution of stock solution (stability samples) and fresh stock solution (comparison sample) was done, which is equivalent to final middle quality control analytes and internal standard. Six replicates of fresh and comparison samples were injected immediately and % of stability was calculated. It must be between 95%-105% and %CV should be less than 10%.

Refrigerator stock solution stability

To accessed this type of stability six replicates of stock solution was prepared and stored at refrigerator at 2-8^{0}C for 4 days. On the day of evaluation fresh standard stock solution was prepared (comparison sample) equivalent to final MQC concentration of the analytes with final concentration of internal standard in reconstituted solution. All comparison and stability samples were injected immediately. Percentage of stability was also calculated for the analytes and internal standard and it must be between 95%-105% and %CV must be less than 10%.

Bench top stability

Six sets of LQC and HQC samples were collected from the deep freezer and placed unprocessed for a period of 12 hours. After that period six sets of fresh quality control samples (low, middle, high) and calibration samples were prepared. Bench top stability samples were processed analyzed along with fresh samples. Concentration was calculated from the linearity data.

Autosampler stability

To access this type stability six sets of quality control samples were prepared in LQC and HQC level and kept in autosampler for 3 days, all the stability samples were quantified against the freshly prepared spiked calibration curve and quality control samples at low middle and high level.

Freeze thaw stability

It was determined by four freeze thaw cycles. Six replicates of LQC and HQC samples were prepared and stored in deep freezer at -70^{0}C. After 24 hours first six samples were withdrawn and thawed at room temperature and refreeze again. In the similar way remaining samples also withdrawn after next 12 hours followed by another 12 hours and refreeze again. All samples were processed after suitable 4 cycles. Stability samples were analyzed by quantifying along with freshly spiked calibration samples and quality control samples at low mid and high level.

Wet extract stability

To investigate wet extract stability six replicates of LQC and HQC samples were prepared, processed and kept for one day at room temperature (20 ± 5^{0}C). After the suitable stability period the samples were injected with freshly spiked calibration curve samples and quality control samples at low middle and high level. The amount of analytes in stability samples were calculated in compared with freshly prepared samples.

Short term stability

Six sets of quality control samples at low and high level were prepared and stored at deep freezer at -20^{0}C after spiking. Three days later on the day of evaluation samples were processed along with freshly prepared quality control samples in all levels and calibration curve samples. The concentration of the stability samples were calculated in comparison to freshly prepared samples.

Long term stability

This was investigated with LQC and HQC samples kept for 30 days at -70^{0}C. On the day evaluation six sets of long term quality control samples (LQC and HQC) were withdrawn and processed them with freshly prepared calibration curve and quality control samples. All the stability samples were quantified from the calibration curve data. For all stability samples mean % nominal concentration at each quality control level must be between 85% and 115% and the precision must be ≤15% of the CV %. At least 67% of the stability QC samples should be within 15 % of their respective nominal values.

Pharmacokinetic study

In vivo Pharmacokinetic study was performed using NZ white rabbit (n=3, 2kg each, 2 male, male: female 2:1) model. It was performed as per local animal ethical committee guidelines. To assess the applicability of the developed method, it was carried at 0.18 mg/kg dose for BLN. The orally administered dose was human equivalent dose to particular this

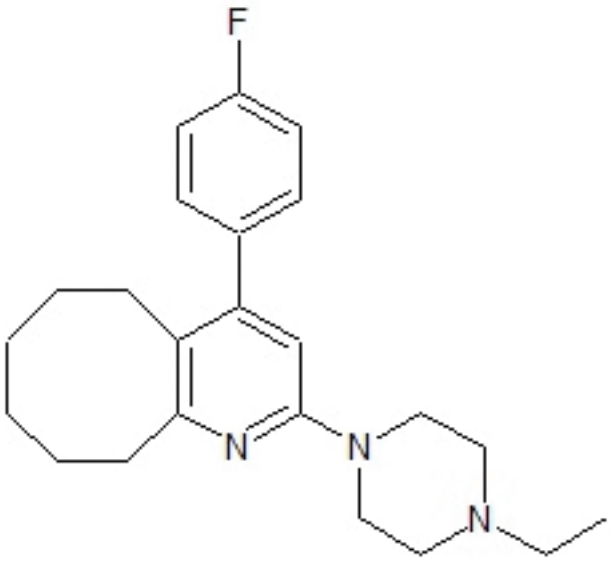

Figure 1: Chemical structure of Blonanserin.

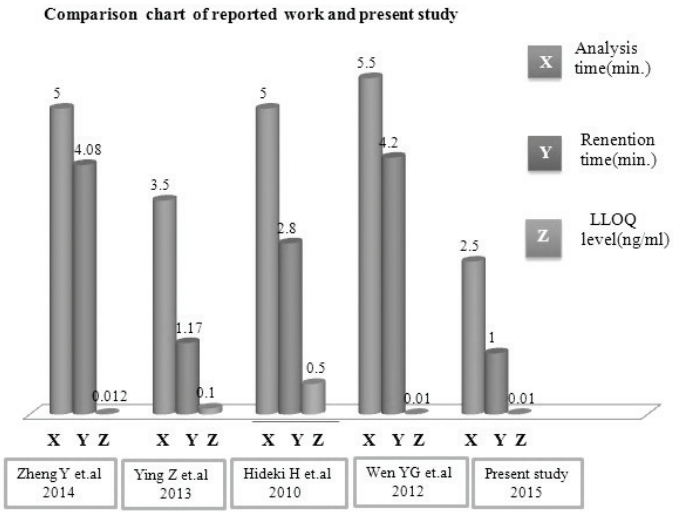

Figure 2: Comparison chart between reported and present work.

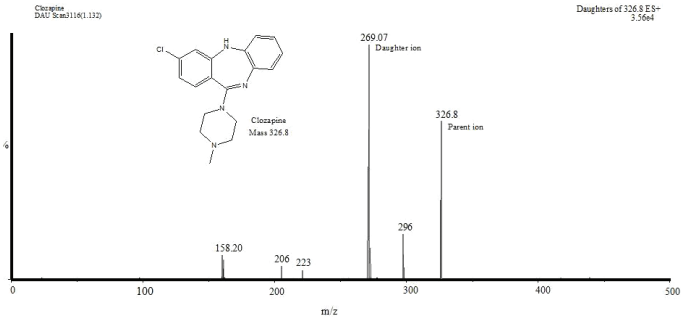

Figure 3: Product ion mass spectra of $[M+H]^+$ of Blonanserin.

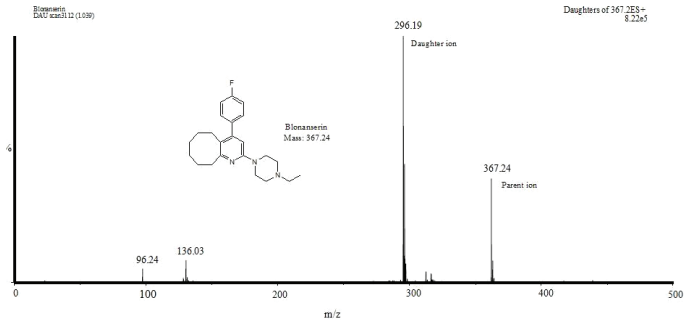

Figure 4: Product ion mass spectra of $[M+H]^+$ of Clozapine.

marketed formulation, has been calculated as per US-FDA guidelines for equivalent dose calculation.[14] The oral dose was administered via silicon rubber gastric intubation tube to their respective groups. Blood samples (1.5 ml) was collected from the marginal ear vein into polypropylene tube (K2 EDTA, J.K diagnostic- Rajkot) at 0, 1, 3, 5, 8, 12, 16, 20, 24 hour post dose. Plasma was separated immediately by centrifugation (2000 rpm, 5 min) and stored at -80^{0}C until analysis. The plasma samples were spiked with internal standard and processed as per sample preparation technique described earlier. The time and plasma concentration data was analysed with a non compartment model, using PK solver.2 a menu driven adding programme for MS excel.[15] One batch of accuracy, precision and matrix effect has also performed taking rabbit plasma samples to justify that the developed method is good enough in other preclinical species plasma.

RESULT AND DISCUSSION

Mass spectrometry

Proper tuning of all mass parameters has been done in both positive and negative ion mode for the analyte and internal standard; finally positive ion mode with multiple reaction monitoring mode was selected for specificity. Optimized mass parameters have been cited in Table 1. Protonated analyte and internal standard $[M+H]^+$ ion was the parent ion in the Q1 segment and use as precursor ion to obtained Q3 product ion spectra.

Method development

To develop the method several chromatographic trials were conducted using different mobile phases with different volume ratio and columns. Various combinations of acetonitrile and buffers has been used during initial trials, different types of columns like C-8, C-18 of hypersil, Zorbax, kromasil has been used. Finally waters symmetry shield, C_{18} (4.6 mm id x 50 mm) analytical column (waters milford, MA, USA) with the mobile phase composed of acetonitrile and 1% formic acid in the volume ratio of (80:20) v/v has been selected. In this optimized condition peak shape was found satisfactory for analyte and internal standard even at very low quality control sample. Initially several compounds were investigated to select a suitable internal standard, finally clozapine was selected as the retention time and other values were very selective and lack of interference with analyte. Under this optimized condition the retention time of BLN was found 1.00 ± 0.3 min, and for internal standard 1.10 ± 0.3 min was found, shown in Figure 5.

Validation

The results of carryover test shows there was no interference at the retention time of the analyte and internal standard at ULOQ and LLOQ level. The obtained response for blank samples is 0 and calculated % carry over is also 0% which satisfied the acceptance criteria. In the result of matrix

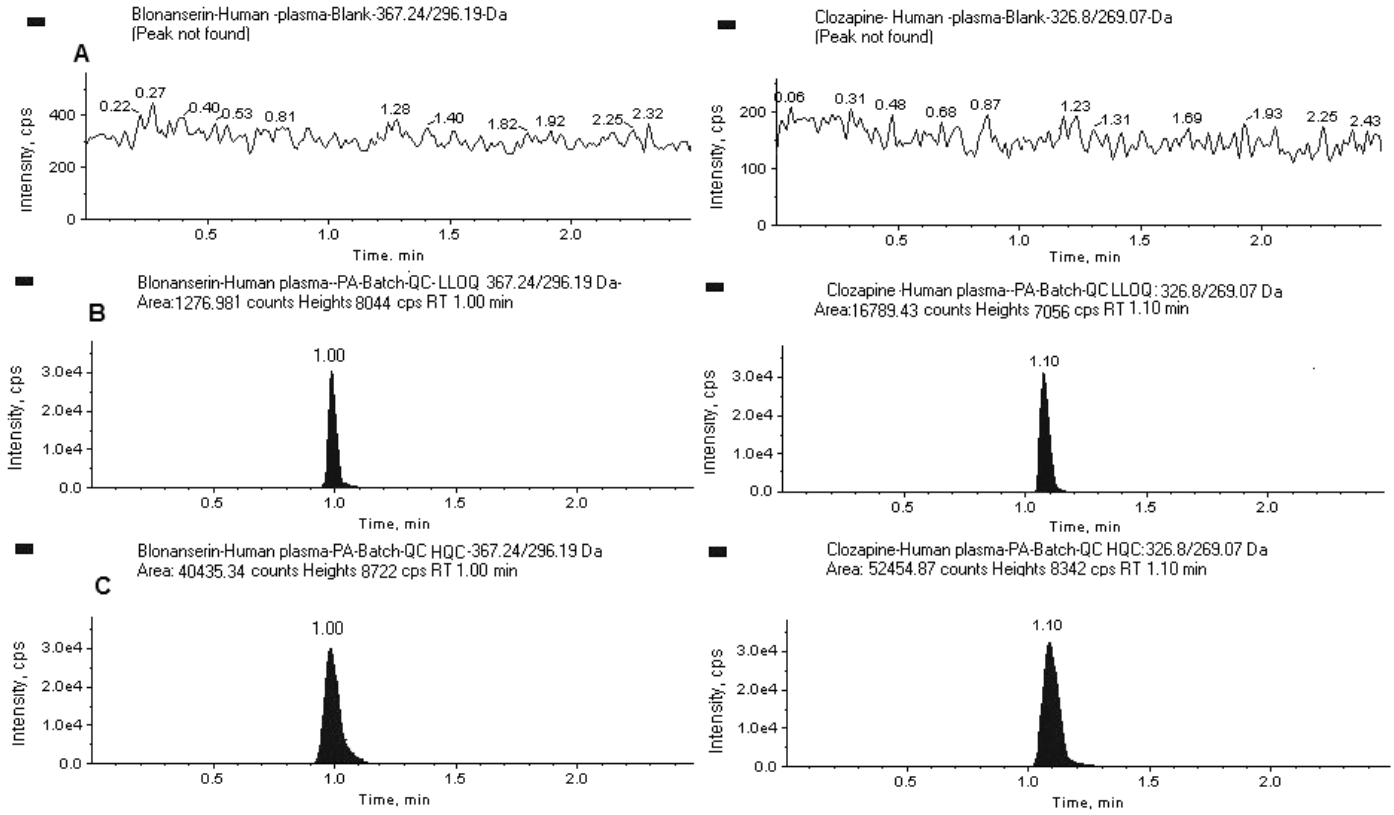

Figure 5: MRM chromatograms of Blonanserin, Blank plasma (A), LLOQ samples with internal standard [right panel] (B), HQC samples with internal Standard [right panel] (C).

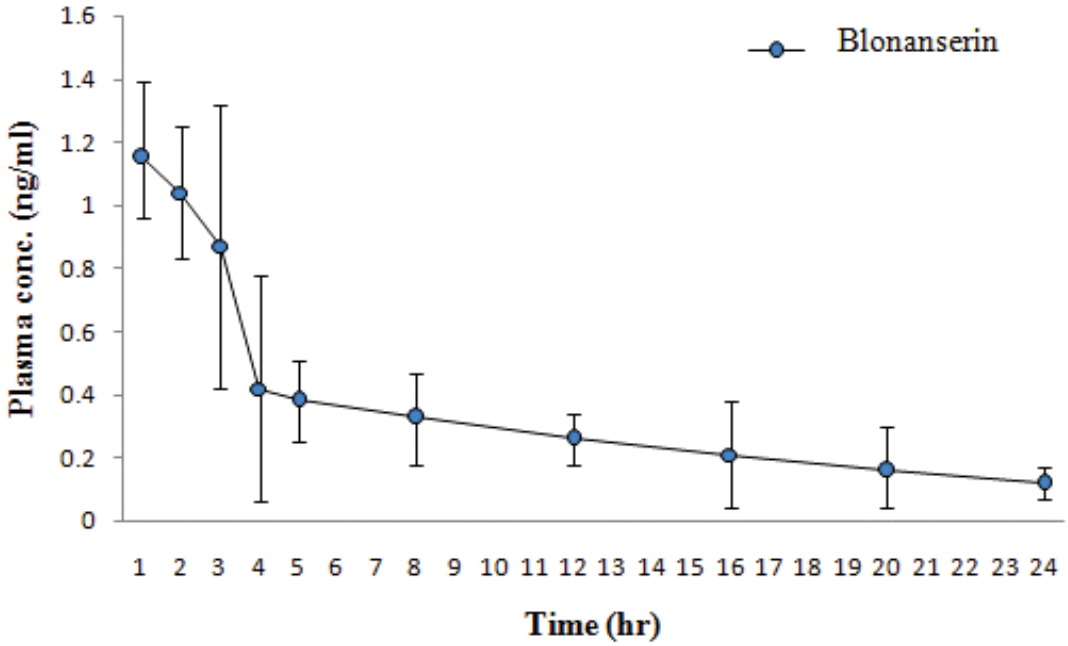

Figure 6: Mean plasma concentration –time profile of BLN after oral administration of BLN tablet human equivalent dose.

Table 1: LC-MS/MS operating conditions (Positive ion mod

Parameters	Blonanserin	Internal standard
MRM	Parent (Da) m/z 367.24	Parent (Da) m/z 326.8
	Daughter (Da) m/z 296.19	Daughter (Da) m/z 269.07
Ion spray voltage	4000 V	5500 V
Source temperature	400^{0}C	400^{0}C
Dewll time (msec)	200	200
Curtain gas1	20 psi	20 psi
Declustering potential (DP)	87V	87V
Capillary (kv)	3.50	3.50
Cone (v)	35	35
Collision energy (CE)	37	37
Collision exit potential(CXP)	20	12
Extractor(v)	5	5
RF lens(v)	0	0
Desolvation temperature (^{0}C)	800	800
Desolvation gas flow(L/h)	75	75

MRM= multiple reaction monitoring.

Table 2: Accuracy and precision for determination of Blonanserin in human plasma

QC levels	Blonanserin measured concentration (ng/ml)				
	Run*	Mean	SD	%CV	% Nominal
		Between the Batch Intra day			
LLOQ	1	0.00933	0.001835	19.6677	93.30
	2	0.00935	0.001862	20.0215	93.00
	3	0.00931	0.001861	20.6801	90.00
	4	0.00922	0.001049	11.6552	90.00
LQC	1	0.24383	0.004875	1.96801	97.60
	2	0.23380	0.028176	12.0123	93.52
	3	0.24166	0.016108	6.66221	96.40
	4	0.23533	0.031392	13.3312	94.00
MQC	1	2.41016	0.144235	5.98011	96.40
	2	2.33001	0.119191	5.21722	93.20
	3	2.41084	0.214962	8.91603	96.40
	4	2.41083	0.214930	8.87924	96.42
HQC	1	4.70553	0.473671	10.0645	94.01
	2	4.81651	0.357633	7.42865	96.20
	3	4.92900	0.312611	6.32324	98.40
	4	4.52101	0.412404	9.12182	90.42
		Within batch Interday			
LLOQ		0.0098	0.00145	14.28	98.21
LQC		0.2461	0.00389	1.58	98.40
MQC		2.3552	0.13120	5.57	94.20
HQC		4.5830	0.43610	9.51	91.66

*each run includes six replicates.

Table 3: Calibration curve concentrations and obtained percentage accuracy of individual concentration

Calibration curve Concentrations (ng/ml)	Mean of back Calculated concentrations* (ng/ml)	Standard Deviation (SD)	% Accuracy
0.01	0.0106	0.0011	96.36
0.02	0.0205	0.0031	97.15
0.03	0.0310	0.0110	101.17
0.05	0.0516	0.0030	102.00
0.10	0.0866	0.0015	86.00
0.25	0.2730	0.0115	109.20
0.50	0.4560	0.0551	90.00
0.75	0.6561	0.0310	87.46
1.00	1.0530	0.0250	105.00
2.50	2.6760	0.0551	106.80
5.00	4.8710	0.0410	97.40

*Mean of three runs of each concentration.

Table 4: Stability data of Blonanserin

QC levels		Blonanserin			
	Type of stability	Mean*	SD	%CV	%Nominal
LQC	Bench Top	0.23633	0.01165	4.93	94.53
	Freeze thaw	0.24266	0.00943	3.88	97.06
	Autosampler	0.23701	0.01332	5.62	94.80
	Wet extract	0.24033	0.01320	5.49	96.13
	Short term	0.23834	0.00876	3.68	95.20
	Long term	0.23133	0.02324	10.04	92.52
HQC	Bench Top	4.92833	0.32455	6.58	98.56
	Freeze thaw	4.77833	0.46799	9.79	95.56
	Autosampler	4.69566	0.36635	7.80	93.91
	Wet extract	4.78433	0.24153	5.04	95.68
	Short term	4.85633	0.35484	7.30	97.12
	Long term	4.95833	0.22578	4.55	99.16

*mean of six replicates.

Table 5: Interday accuracy and precision data of rabbit blood sample

Analyte	Blonanserin				
Level	LLOQ 3 ng/ml	LQC 15 ng/ml	MQC-2 150 ng/ml	MQC-1 750 ng/ml	HQC 1500 ng/ml
Mean*	2.89	14.69	146.93	751.8	1518.01
SD	0.54	0.09	0.18	0.85	1.43
%CV	0.18	0.61	0.12	0.11	0.09
%Nominal	96.33	97.93	97.95	100.24	101.20

Table 6: Pharmacokinetic parameters of BLN in rabbit plasma

Pharmacokinetic parameters	Blonanserin
C_0(ng/ml)	0.81±1.65
C_{max} (ng/ml)	1.45±0.46
t_{max}	2.01±1.22
$AUC_{0-\infty}$ (h* ng/ml)	10.20±3.09
AUC_{0-t} (h* ng/ml)	8.62±6.12
$t_{1/2}$ (h)	9.11±6.62
V_d (L/kg)	9217.19±112
Cl (L/h/kg)	1178.65±43.51

AUC (Area under the curve), V_d (Volume of distribution), Cl (Clearance).

effect, internal standard normalized matrix factor were calculated for the analyte in presence of matrix ion. No significant effect of matrix was found in eight batch includes haemolytic and lipemic plasma. Internal standard normalized factor was calculated, the %CV of Is normalized factor was found 3.97% for LQC and 1.70% for HQC samples, satisfies acceptance criteria. Intraday within batch precision (%CV) for LLOQ, LQC, MQC and HQC samples of BLN were found within 1% to14%. Intraday accuracy for LLOQ, LQC, MQC and HQC was found in the range of 91% to 98%. Between the batch accuracy (% Nominal) results for the levels of LLOQ, LQC, MQC and HQC were between 90% to 98.4% and precision (%CV) values were between1.968% to 13.33%, details were cited in Table 2. Which indicates that the present method is highly accurate and precise. In the study of matrix selectivity no interference was found at the retention time of analyte and internal standard when compared with blank matrix against the mean response of extracted LLOQ samples and the response of interfering peaks at the retention time of analyte and internal standard was found 0% of the mean drug response. Analyte selectivity results also demonstrated that there was no interference at the retention time of analyte in presence of internal standard and also shows no interference at the retention time of internal standard in the presence of analyte. Overall matrix and analyte selectivity study demonstrated that developed method was found selective because of 0% interference. A regression equation with weighing factor, 1/(concentration ratio)^2 of drugs to internal standard concentration shows best fit relationship with a correlation coefficient r^2=0.998 in the concentration range of 0.01 to 5 ng/ml for BLN, with regression equation Y=0.623x) confirms the linearity of the developed method. The mean back calculated concentrations for linearity with mean accuracy values has been accumulated and cited in Table 3 .In the dilution integrity study of BLN at ULOQ level was quantified. %CV and % nominal was found 3.179% and 98.04% at two times dilution and 0.84 and 96.08 at four times dilution in comparison with the undiluted calibration curve samples which demonstrated about the integrity of analyte in dilution. In recovery study the mean overall recovery of BLN was found 96.01% with a precision (%CV) range of 1.19 to 3.8 and % difference between height and lowest % recovery was 1.89%. The mean recovery of internal standard was 94.13%. Therefore the mean overall recovery results indicates that BLN and IS in LQC, MQC and HQC level were foind well satisfactory recovery and %difference is also within acceptance level. The result of within batch precision (%CV) for ruggedness study was found 11.89%, 11.27%, 4.37%, and 7.12%. The accuracy values for ruggedness study were 95%, 103.22%, 95.29%, 95.68% at LLOQ, LQC, MQC and HQC levels respectively. Hence in the ruggedness study accuracy and precision values demonstrated that the developed method was found rugged on changing different column and set of reagents.

Stability studies

The results of room temperature (20 ± 5^{0}C) stock solution stability study shows % stability for BLN and internal standard were 98.13% and 100.79%. In refrigerator stock solution stability study at 2-8^{0}C for 4 days, the calculated % of stability was found 98.19 % for BLN and 96.29% for internal standard which indicates that the values were found satisfactory and within the limit and confirms the stability of the developed method. The % nominal for Bench top stability study results were within 94 to 98% at LQC and HQC level. The autosampler stability study (72 hours of suitable stability period in autosampler) and the results of four cycles freeze thaw stability sample exhibit an excellent range of acceptability in both LQC and HQC level of Blonanserin. In wet extract stability study results % nominal value for BLN was more than 95%. The short term stability study result satisfied the acceptance limit because the calculated % nominal for BLN were more than 95% at LQC and HQC level. The mean % nominal for long term stability study (30 days at -70^{0}C) were found 92.52 for LQC and 99.16% for HQC samples. The summary of all stability study results were demonstrated in Table 4. In bench top, wet extract, freeze thaw, autosampler, short and long term (-70^{0}C, 30 days) stability studies, stability samples were compared with freshly prepared samples and concentration was back calculated from the calibration curve samples. In all category of stability studies the mean % of nominal values were found within 94.53% to100.79% and %CV values were within 10%, which strongly demonstrated about the stability of all quality control samples and stability of the developed method.

In vivo pharmacokinetic study

The pharmacokinetic parameters obtained from the study using non compartmental model, were area under the curve (AUC_0-∞) for BLN=10.20 ± 3.09 hr.ng/ml. Elimination half life ($t^{1/2}$) = 9.11 ± 6.02 hr. BLN maximum concentration in plasma (C_{max}) = 1.45 ± 0.46 and t_{max} was achieved at 5 ± 1.53 hr. Along with pharmacokinetic study rabbit plasma samples also analysed for one batch of accuracy, precision, and matrix effect, where all values are within the acceptance limit and rationalized the suitability of the developed method in other preclinical sample species. Accuracy and precision data on rabbit plasma samples were demonstrated in Table 5 and pharmacokinetic data was summarized in Table 6. The newly developed validated method was well implemented during the analysis of pharmacokinetic samples. The plasma concentration and time is smooth enough to derive all parameters (Figure 6). The clearance and elimination half life were well fitted with one compartmental model. The selectivity, sensitivity and specificity of the present method was sufficient enough for the characterization of Pharmacokinetic profile of BLN in *NZ* white rabbit plasma.

CONCLUSION

The present method shows high throughput than the few reported LCMS/MS methods because the requirement of total analysis time is very less with less run time. A convenient liquid-liquid extraction procedure made this method more practically workable and cost effective. Empirical evidences of all validation results demonstrated that the method is highly validated and easy as all the parameters are within acceptance limit as per the US-FDA guidelines. So this present cheap attractive, easy, reliable novel method can undoubtedly highly applicable for the quantitative analysis of BLN during clinical trials, preclinical trials, forensic and toxicological study.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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