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Original Article

BIO ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF FLUPENTIXOL AND NORTRIPTYLINE HCL IN RAT PLASMA AND ITS PHARMACOKINETIC STUDIES BY LCMS

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ABSTRACT

Objective: An easy, quick, precise, active and reproducible Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) technique was developed for the bio-analytical method of flupentixol and nortriptyline HCl using zuclopenthixol as Internal Standard (IS).

Methods: This article summarizes the recent progress on bio-analytical LC-MS/MS methods using Luna Phenyl Hexyl column (250x4.6 mm, 5µ) and organic mobile phase of Ammonium acetate pH-3.0/Formic acid and Acetonitrile in 70:30 v/v. 5 min of run time was used in the analysis.

Results: The calibration curve was linear in the range of 5.0ng/ml to 200.0ng/ml (r^2= 0.99988±0.006) for nortriptyline HCl and 0.25ng/ml to 10.0ng/ml (r^2=0.99972±0.007) for flupentixol. Matrix effect, recovery and stability results were within the acceptable limit. An electrospray ionization source was used to study of nortriptyline HCl, flupentixol at m/z 300.8471 →73.2501, m/z 435.5225→103.4247 and m/z 400.9657→142.0087 for zuclopenthixol were ion pairs of mass analysis.

Conclusion: The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with United States Food and Drug Administration (USFDA) guidelines and applied effectively for the investigation of pharmacokinetic studies in rat.

Keywords: Flupentixol, Nortriptyline HCl, LC-MS/MS, USFDA guidelines, Rat plasma

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INTRODUCTION

Flupentixol, also known as flupenthixol (former BAN), marketed under brand names such as Depixol and Fluanxol is a typical antipsychotic drug [1, 2] of the thioxanthene class. In addition to single drug preparations, it is also available as flupentixol/melitracen—a combination product containing both melitracen (a tricyclic antidepressant) and flupentixol (marketed as Deanxit). Flupentixol's main use is as a long-acting injection given once in every two or three weeks to individuals with schizophrenia [3, 4] who have poor compliance with medication and have frequent relapses of illness, though it is also commonly given as a tablet. There is little formal evidence to support its use for this indication but it has been in use for over fifty years. Flupentixol is also used in low doses as an antidepressant [5, 6]. There is tentative evidence that it reduces the rate of deliberate self-harm [7, 8], among those who self-harm repeatedly. Extrapyramidal side effects such as (which usually become apparent soon after therapy is begun or soon after an increase in dose is made) Muscle rigidity [9], Hypokinesia, Parkinsonism [10, 11], Tremor [12, 13], Akathisia [14, 15], Dry mouth [16], Constipation [17, 18], Hypersalivation – excessive salivation, Blurred vision, Diaphoresis – excessive sweating [19], Somnolence [20], Restlessness, Insomnia [21, 22].

Nortriptyline, sold under the brand name Aventyl, among others, is a tricyclic antidepressant. This medicine is also sometimes used for neuropathic pain [23], Attention Deficit Hyperactivity Disorder (ADHD) [24], smoking cessation and anxiety. As with many antidepressants, its use for young people with depression and other psychiatric disorders may be limited due to increased suicidality in the 18–24 population initiating treatment. Nortriptyline is a less preferred treatment for ADHD and stopping smoking. It is taken by mouth. Common side effects include dry mouth, constipation, blurry vision, sleepiness, low blood pressure with standing, and weakness. Serious side effects may include seizures [25], an increased risk of suicide in those less than 25 y of age, urinary retention [26], glaucoma [27, 28], mania, and a number of heart issues. Nortriptyline may cause problems if taken during pregnancy. Use during breastfeeding appears to be relatively safe. It is a tricyclic antidepressant (TCA) and is believed to work by altering levels of serotonin and norepinephrine.

Till date, no method is available for bio-analysis of flupentixol and nortriptyline HCl in any type of biological matrix. The aim of the study was to develop a new rapid and sensitive LC-MS/MS method for the simultaneous estimation of flupentixol and nortriptyline HCl in rat plasma using zuclopenthixol as internal standard.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and Ammonium acetate, Formic acid, water (HPLC grade) were purchased from Merck (India) Ltd, Worli, Mumbai, India. All APIs of flupentixol and nortriptyline HCl as reference standards were procured from Zydus Cadila Health Care Ltd, Ahmedabad.

Equipment

An HPLC (High-Performance Liquid Chromatography) system (Waters Alliance e2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument was used. By the ABSCIEX software operation was performed [29, 30].

Pharmacokinetic study

Selection of animals

For *in vivo* pharmacokinetic studies, 6 healthy white New Zealand rats (app. 250 g) were obtained from Biological E Limited, Hyderabad, India. The protocol of animal study was approved by institute of animal ethics committee (Reg. No: 1074/PO/Re/S/22/CPCSEA). The animals were housed in identical laboratory circumstances, with a temperature of 20-26 °C and a humidity of 50-60%.

Chromatographic conditions

To achieve Chromatographic separation, using Luna Phenyl Hexyl (250 x 4.6 mm, 5 micron) columns, was administered in isocratic mode at room temperature. A mobile phase mixture of Ammonium acetate pH-3.0/Formic acid and acetonitrile at 70:30 v/v with a flow of 1.0 ml/min was used. 10µl was the injection rate and the run time was 5 min.

Preparation of standard and internal standard samples

Preparation of nortriptyline HCl Parent stock solution

8 mg of the nortriptyline HCl working standard was taken into a 100 ml volumetric flask and 70 ml of diluents was added and sonicated for 10 min to dissolve the contents completely and made up to the mark with diluent. Further dilution was done by taking 0.5 ml into 10 ml volumetric flask.

Preparation of flupentixol parent stock solution

5 mg of the flupentixol working standard was transferred to into a 100 ml volumetric flask and 70 ml of diluents was added and sonicated for 10 min to dissolve the contents completely and made up to the mark with diluent. Further dilutions were made by taking 0.4 ml into 10 ml volumetric flask.

Preparation of standard stock solution

1.0 ml of nortriptyline HCl parent stock solution and 0.1 ml of flupentixol parent stock solution were transferred into 10 ml volumetric flask and made up to the mark with diluents.

Preparation of zuclopenthixol stock solution

8 mg of the zuclopenthixol working standard was taken into a 100 ml volumetric flask and added 70 ml of diluents and sonicated for 10 min to dissolve the contents completely and made up to the mark with diluent. Further dilution was made by taking 0.5 ml into 10 ml volumetric flask. Further dilution was made by taking 1.0 ml into 10 ml volumetric flask.

Preparation of standard solution

For standard preparation 200µl of plasma was taken and 300µl of acetonitrile (ACN) into a 2 ml centrifuge tube and 500µl of standard stock solutions and 500µl of IS and 500 µl of diluents were added and vortexed for 10 min. These samples further subjected for centrifuge at 4000rpm for 20 min. Collect the solution and filter through 0.45µ nylon syringe filter and the clear solution was transferred into vial and injected into a system.

Bio-analytical method validation

The method was validated [31-39] in selective, sensitive, linearity, accuracy and precise, matrix condition, recovery study, re-injection reproducibility and stability.

Selectivity

By analyzing the six different rat's plasma samples and to check interference at the retention time, selectivity was conducted.

Matrix effect

By comparing the height area ratio from the six various drug free plasma samples for nortriptyline HCl and flupentixol to get matrix effect [40, 41]. Experiments were performed at MQC levels in triplicate with six different plasma lots with the suitable precision of ≤ 15 %.

Precision and accuracy

Precision and accuracy [42, 43] was determined by replicate analysis of internal control samples at a Lower Limit of Quality Control (LLOQC), Low-Quality Control (LQC), Medium Quality Control (MQC), High-Quality Control (HQC) levels. The % CV (Coefficient Variance) should be less than 15 % and accuracy should be within 15% except LLOQ where 20%.

Recovery

The analysis of six samples reproduce at each internal control concentration is by extracting the nortriptyline HCl and flupentixol. By comparing the height areas of extracted standards to the height areas of unextracted standards, recovery is evaluated [44].

Carryover

Carryover [45, 46] deals with the analyte retained by the chromatographic system during the matrix with an analyte concentration Upper Limit of Quality Control (ULOQC) and above the diluting this sample with the blank matrix.

Dilution integrity

By spiking the matrix with an analyte concentration above the ULOQC and diluting this sample with blank matrix, the dilution integrity [47] should be explained.

Stability

By comparing the act of stock solution stability [48] under the stability sample with the sample from the fresh stock sample preparation. Sample Stability studies in plasma were performed at the LQC and HQC concentration levels using six replicates at each level. Analyte was considered stable if the change is smaller amount than 15 % as per US FDA guidelines [49]. The perfectness of spiked rat plasma stored at room temperature was evaluated for twenty-four hrs. The stability of spiked rat plasma stored at RT in an autosampler was evaluated for twenty-four hrs. The autosampler stability (LQC, MQC and HQC) was evaluated by comparing the extract plasma samples that were injected immediately with the samples that were re-injected after storing with wet extract stability at room temperature after 12 h and 18 h at 2-8 °C the reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately, with the samples that were re-injected after storing in the dry extract stability at room temperature after 12 h and 18 h at-20±3 °C the freeze-thaw stability was conducted by comparing the steadiness samples that had been frozen at-31 °C and thawed 3 times, with freshly spiked internal control samples. The short-term stability was conducted 7 d at 7 °C. For long-term stability evaluation, the concentrations obtained after 24 h were compared with initial concentration.

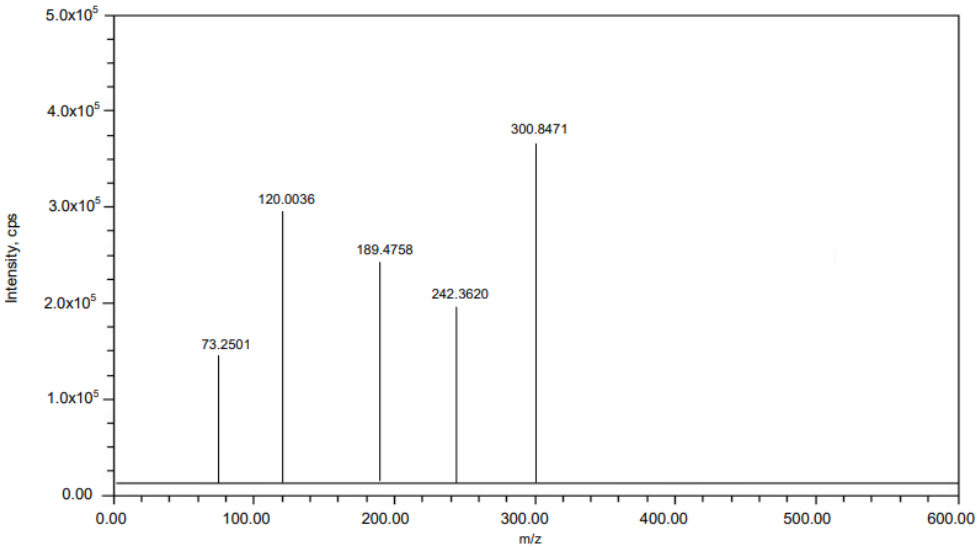
Pharmacokinetic study

Before experimentation, all animals are starved overnight and had water ad-libitum. Topical anesthetic procedure was used. Pharmacokinetic evaluation was performed for nortriptyline HCl and flupentixol formulations. The samples were administered to each rat under fasting conditions. After oral administration of nortriptyline HCl and flupentixol, blood samples were collected from rat marginal ear vein using a 25-guage, 5/8 inch needle by clipping the marginal ear vein with a paper clip with volume of 0.3 ml at 1, 2, 4, 8, 16, 24, 32 and 40 h. The blood was collected in Eppendorf containing 10% EDTA (Ethylene Diamine Tetra Acetic acid) solution. Blood was centrifuged at 4000 rpm for 20 min at 2-8 °C temperature. The clear supernatant plasma were collected and stored at-30 °C till its analysis. The plasma samples were treated for liquid-liquid phase extraction and analyzed for drug content with developed analytical method. After the study, the animals were returned to animal house for rehabilitation.

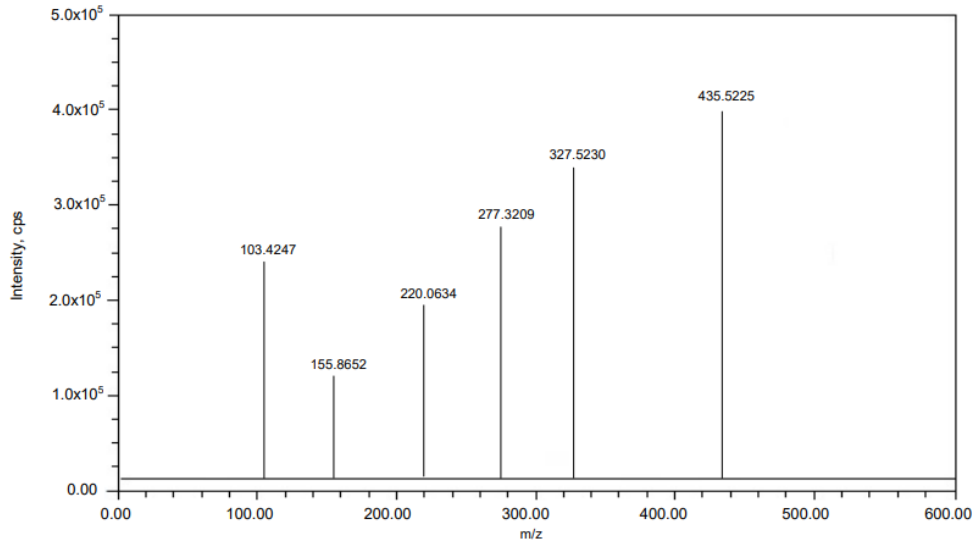
The pharmacokinetic parameters for nortriptyline HCl and flupentixol oral administration were determined from plasma concentration data. Pharmacokinetic parameters like AUC (Area Under the Curve), C_{max} (Maximum Concentration), T_{max} (Time to reach peak concentration) the time at which C_{max} occurred, Data was measured by the trapezoidal rule method from time zero to infinity of the concentration-time curve. C_{max} and T_{max} were obtained from the graph. All values are expressed in mean±SD. (SD – Standard Deviation).

RESULTS AND DISCUSSION

The maximum response on air pressure chemical ionization mode selected in this method is by having the electrospray ionization. The mobile phase flow of 10 µl/min nortriptyline HCl and flupentixol are highly responsive in the positive ion mode to offer sensitivity and signal stability with continuous flow to electro spray ion. Fig. 1 gives the mass spectras of nortriptyline HCl, flupentixol and zuclopenthixol.



A



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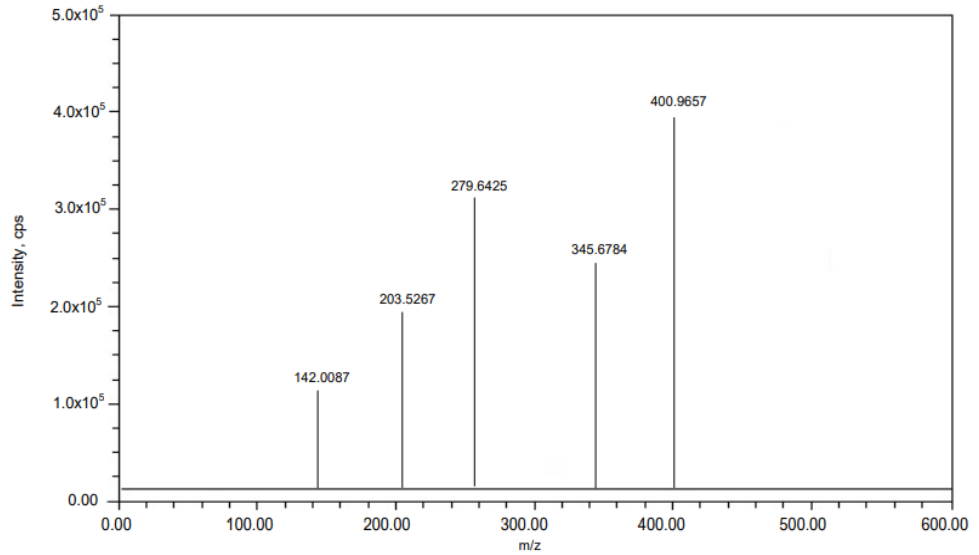


C

Fig. 1: Mass spectras of (A) Nortriptyline HCl (B) Flupentixol and (C) Zuclopenthixol

Specificity

The specificity [50] of the method to research nortriptyline HCl and flupentixol simultaneously is proved. The chromatograms of blank and standard as shown in fig. 2, 3. The chromatograms of blank rat plasma and standard having no interference peaks were observed.

Matrix effect

Percent RSD (Relative Standard Deviation) for within the signal, ion suppression/enhancement was observed as 1.0 percent for nortriptyline HCl and flupentixol in LC-MS/MS, suggesting that under these circumstances, the matrix effect [51] on analyte ionization is within an acceptable range of ionization. In matrix effect, LQC and HQC of nortriptyline HCl were 95.38 and 98.06 and flupentixol were 95.24, 98.25%. %CV of the both drugs at LQC level were 1.92, 4.74 and HQC level is 0.24, 1.93 respectively. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

Linearity

The peak area ratio of calibration standards was proportional to the concentration. The concentration range of nortriptyline HCl is 5-200 ng/ml and flupentixol is 0.25-10 ng/ml. Linearity results of nortriptyline HCl and flupentixol were shown in following table 1 and their calibration plots were shown in fig. 4 [52]. The calibration curves were appeared linear and coefficient of correlation was found to be 0.999 for nortriptyline HCl and flupentixol.

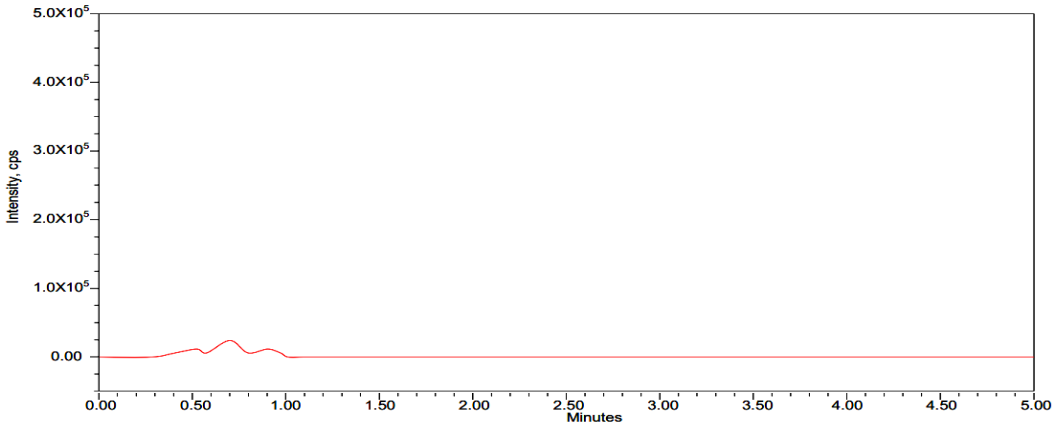


Fig. 2: Chromatogram of blank

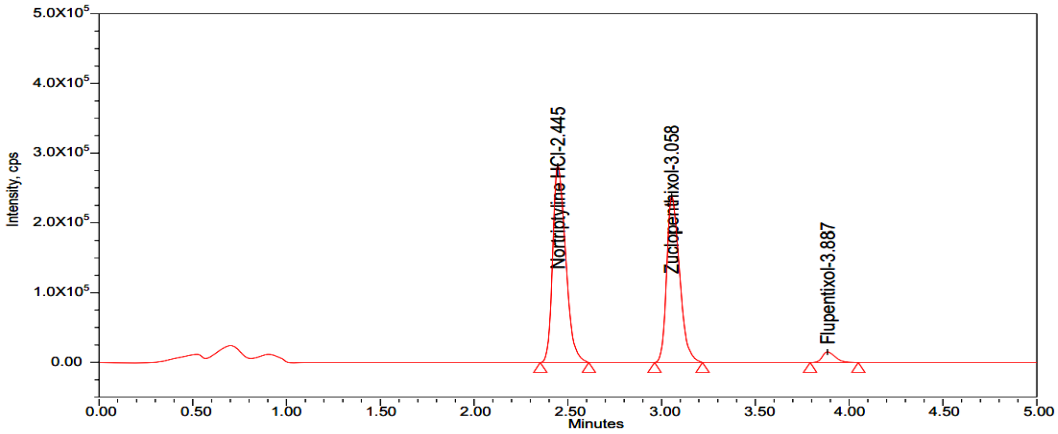
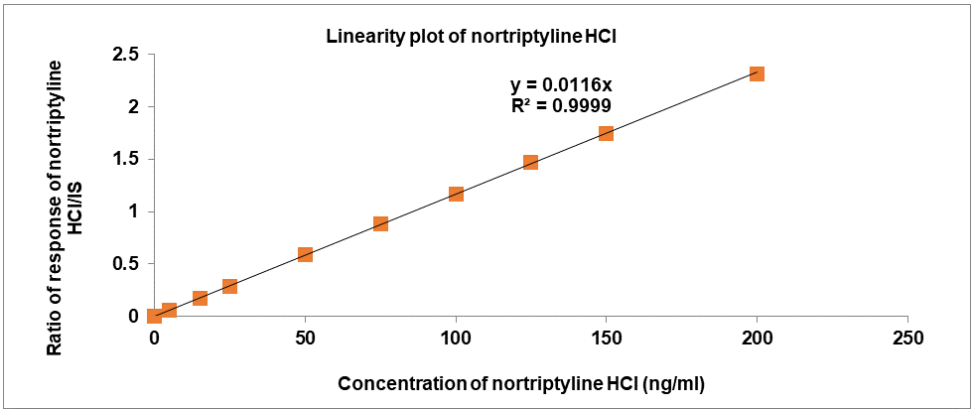


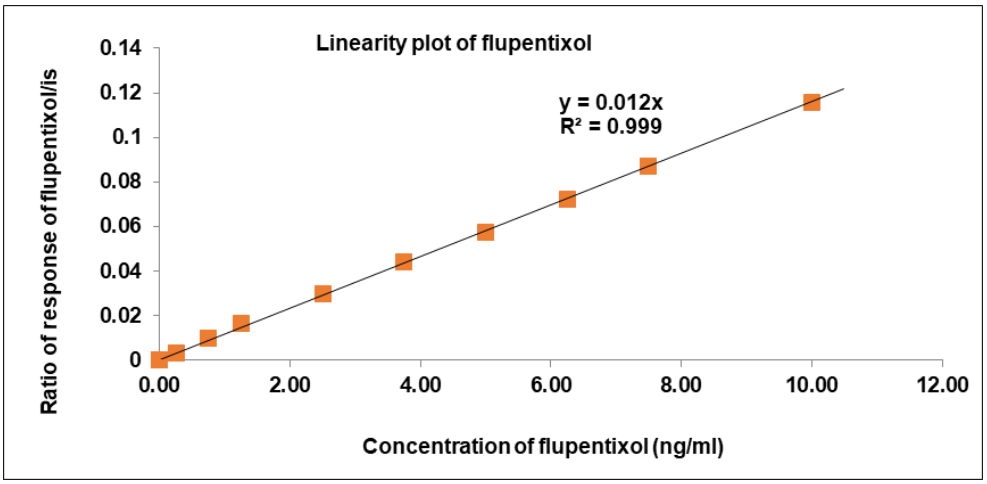
Fig. 3: Chromatogram of standard

Table 1: Results of linearity

Linearity	Nortriptyline HCl		Flupentixol	
	Conc. (ng/ml)	Area response ratio	Conc. (ng/ml)	Area response ratio
1	5.00	0.059	0.25	0.003
2	15.00	0.174	0.75	0.010
3	25.00	0.290	1.25	0.017
4	50.00	0.594	2.50	0.030
5	75.00	0.881	3.75	0.044
6	100.00	1.166	5.00	0.057
7	125.00	1.470	6.25	0.072
8	150.00	1.748	7.50	0.087
9	200.00	2.312	10.00	0.116
Slope		0.0117	Slope	0.0118
Intercept		0.00014	Intercept	0.00056
CC		0.99988	CC	0.99972



A



B

Fig. 4: Calibration plots of (A) Nortriptyline HCl and (B) Flupentixol

Precision and accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision [53, 54] were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. The precision results of nortriptyline HCl and flupentixol was shown in table 2, 3. Nortriptyline HCl accuracy results in quality control samples 94.41-98.00 and flupentixol accuracy results in quality control samples 90.23-98.50. Nortriptyline HCl and flupentixol CV is<5% of total internal control samples.

Table 2: Precision and accuracy of nortriptyline HCl

S. No.	HQC	MQC	LQC	LLQC
	Nominal concentration (ng/ml)			
	150	100	15	5
	Analyte peak area			
1	$4.044x10^5$	$2.699x10^5$	$0.401x10^5$	$0.128x10^5$
2	$4.048x10^5$	$2.712x10^5$	$0.398x10^5$	$0.127x10^5$
3	$4.033x10^5$	$2.689x10^5$	$0.411x10^5$	$0.133x10^5$
4	$4.051x10^5$	$2.695x10^5$	$0.389x10^5$	$0.132x10^5$
5	$4.042x10^5$	$2.701x10^5$	$0.405x10^5$	$0.129x10^5$
6	$4.037x10^5$	$2.697x10^5$	$0.403x10^5$	$0.131x10^5$
n	6	6	6	6
Mean	$4.043x10^5$	$2.699x10^5$	$0.401x10^5$	$0.130x10^5$
SD	0.00672	0. 00765	0.00739	0.00237
% CV	0.17	0.28	1.84	1.82
% Accuracy	97.87%	98.00%	97.07%	94.41%

Data are expressed as mean+SD, n=6.

Recovery

The recoveries for nortriptyline HCl and flupentixol at LQC, MQC and HQC levels the results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recoveries for nortriptyline HCl (96.10%-98.08%) and flupentixol (90.23%-98.75%) at LQC, MQC and HQC levels and % CV ranged from 0.06-1.36 for nortriptyline HCl and 1.44-4.14 for flupentixol. the results demonstrated that the bio analytical method had good extraction efficiency.

Table 3: Precision and accuracy of flupentixol

S. No.	HQC	MQC	LQC	LLQC
	Nominal concentration (ng/ml)			
	7.50	5.00	0.75	0.25
	Analyte peak area			
1	$0.192x10^5$	$0.134x10^5$	$0.019x10^5$	$0.007x10^5$
2	$0.197x10^5$	$0.131x10^5$	$0.020x10^5$	$0.006x10^5$
3	$0.199x10^5$	$0.139x10^5$	$0.017x10^5$	$0.006x10^5$
4	$0.188x10^5$	$0.128x10^5$	$0.018x10^5$	$0.006x10^5$
5	$0.194x10^5$	$0.129x10^5$	$0.021x10^5$	$0.006x10^5$
6	$0.195x10^5$	$0.124x10^5$	$0.019x10^5$	$0.006x10^5$
n	6	6	6	6
Mean	$0.194x10^5$	$0.131x10^5$	$0.019x10^5$	$0.006x10^5$
SD	0.00387	0.00519	0.00141	0.00041
% CV	1.99	3.97	7.44	6.62
% Accuracy	97.24%	98.50%	95.24%	90.23%

Data are expressed as mean+SD, n=6.

Ruggedness

The percent recoveries and percent CV of nortriptyline HCl and flupentixol determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC and MQC samples. The results proved method is ruggedness. The percent recoveries ranged from 96.83 – 98.09% for nortriptyline HCl and 95.24%-98.25% for flupentixol. The %CV values ranged from 0.09-0.95 for nortriptyline HCl and 1.28 – 3.93 for flupentixol. The results proved method is ruggedness [55, 56].

Autosampler carryover

Peak area response of nortriptyline HCl and flupentixol wasn't observed within the blank rat plasma samples after successive injections of LLQC and ULQC at the retention times of nortriptyline HCl and flupentixol. In autosampler carryover, this method doesn't exhibit autosampler carryover [57, 58].

Stability

Nortriptyline HCl and flupentixol solutions were prepared with diluents for solution stability [59, 60] analysis and placed in a refrigerator at 2-8 °C. Fresh stock solutions were associated with stock solutions that were prepared 24 h earlier. The plasma stability of the bench top and auto sampler was stable for twenty-four hours and 24 h at 20 °C in the auto sampler. It became apparent from future stability that nortriptyline HCl and flupentixol were stable at a storage temperature of-30 °C for up to 24 h. The overall stability results of nortriptyline HCl and flupentixol have been stated in the below table 4, 5.

Table 4: Stability results of nortriptyline HCl

Stability experiment spiked plasma		Mean area±SD	% CV	% Recovery
Benchtop stability	LQC	$0.400x10^5$±0.00331	0.96	96.83
	MQC	$2.671x10^5$±0.00418	0.16	96.99
	HQC	$4.045x10^5$±0.00360	0.08	97.92
Autosampler stability	LQC	$0.397x10^5$±0.00510	1.33	96.10
	MQC	$2.675x10^5$±0.01126	0.42	97.13
	HQC	$4.049x10^5$±0.01066	0.26	98.02
Long term (Day28) stability	LQC	$0.346x10^5$±0.00390	1.13	83.76
	MQC	$2.321x10^5$±0.00378	0.16	84.28
	HQC	$3.519x10^5$±0.00523	0.15	85.19
Wet extract 18 H stability	LQC	$0.398x10^5$±0.00335	0.84	96.34
	MQC	$2.675x10^5$±0.00258	0.10	97.13
	HQC	$4.049x10^5$±0.00288	0.07	98.02
Dry extract 18 H stability	LQC	$0.397x10^5$±0.00452	1.14	96.10
	MQC	$2.677x10^5$±0.00383	0.14	97.20
	HQC	$4.050x10^5$±0.00374	0.09	98.04
Freeze thaw stability	LQC	$0.397x10^5$±0.00423	1.07	96.10
	MQC	$2.671x10^5$±0.00258	0.10	96.99
	HQC	$4.048x10^5$±0.00394	0.10	97.99
Short term stability	LQC	$0.384x10^5$±0.00327	0.85	92.96
	MQC	$2.616x10^5$±0.00360	0.14	94.99
	HQC	$3.955x10^5$±0.00314	0.08	95.74

Data are expressed as mean+SD, n=6.

***In vivo* pharmacokinetic evaluation**

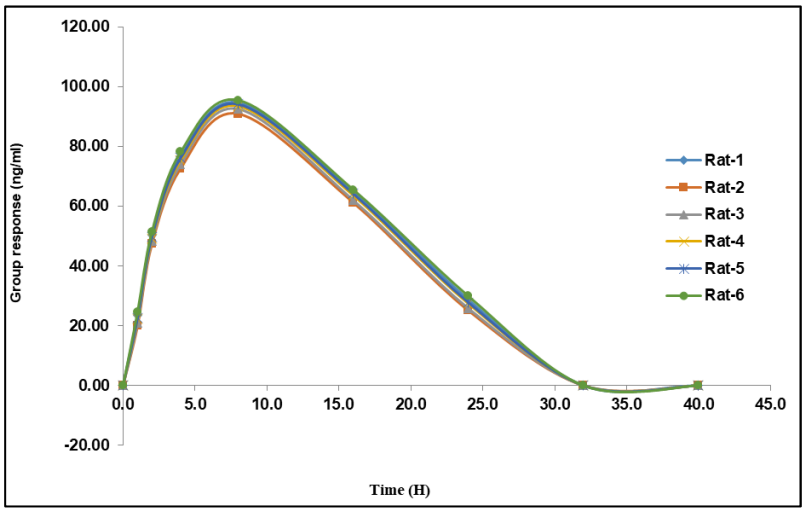
The plasma concentration-time profiles of nortriptyline HCl and flupentixol in rat are shown in fig. 5. The graph indicated bell shaped curve in both the cases of experimental formulation. nortriptyline HCl and flupentixol could be traced to be present in the blood for 24 h and 32 h after oral and intravenous administration, which indicates the effectiveness of drug release from the formulation.

The pharmacokinetic parameters C_{max}, T_{max}, $T_{1/2}$, AUC_{0-t}, $AUC_{0-\infty}$, were calculated and the data is shown in table 6. The C_{max} for nortriptyline HCl and flupentixol were found to be 93.6 ng/ml and 4.7 ng/ml, respectively. The T_{max} for nortriptyline HCl and flupentixol were found to be 8.0 h and 8.0 h, respectively. The t½ values were 24.0 h and 32.0 h, respectively for nortriptyline HCl and flupentixol. The AUC0-t for nortriptyline HCl and flupentixol were found to be 1503 and 87ng-h/ml, respectively. The pharmacokinetic parameters were shown in table 6.

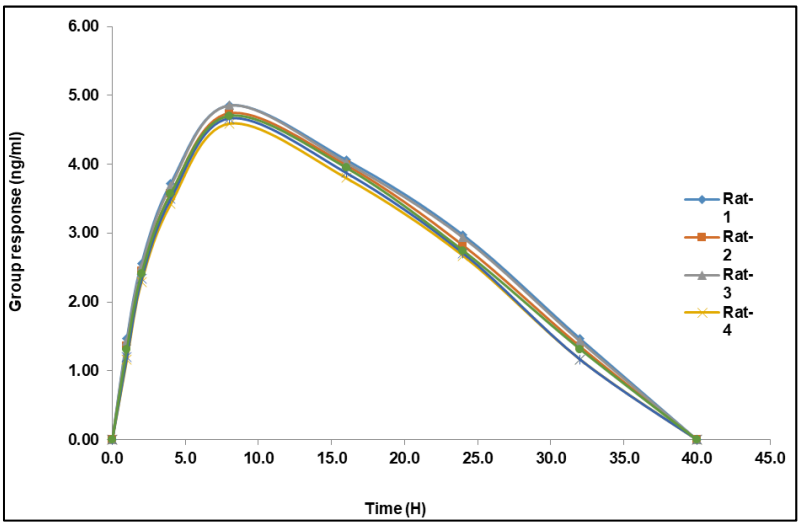
Table 5: Stability results of flupentixol

Stability experiment spiked plasma		Mean area±SD	% CV	% Recovery
Benchtop stability	LQC	0.019x10^5±0.00141	7.44	95.24
	MQC	0.130x10^5±0.00207	1.58	97.74
	HQC	0.197x10^5±0.00183	0.93	98.75
Auto sampler stability	LQC	0.019x10^5±0.00062	3.35	95.24
	MQC	0.131x10^5±0.00248	1.82	98.50
	HQC	0.195x10^5±0.00337	1.13	97.74
Long term	LQC	0.017x10^5±0.00052	3.10	85.21
(Day 28) stability	MQC	0.112x10^5±0.00456	1.65	84.21
	HQC	0.169x10^5±0.00331	1.96	84.71
Wet extract 18 H stability	LQC	0.019x10^5±0.00075	3.93	95.24
	MQC	0.131x10^5±0.00412	1.83	98.50
	HQC	0.196x10^5±0.00179	0.91	98.28
Dry extract 18 h stability	LQC	0.019x10^5±0.00092	4.02	95.24
	MQC	0.130x10^5±0.00408	1.39	97.74
	HQC	0.196x10^5±0.00250	1.28	98.25
Freeze thaw stability	LQC	0.019x10^5±0.00063	3.33	95.24
	MQC	0.130x10^5±0.00419	1.48	97.74
	HQC	0.194x10^5±0.00383	1.98	97.24
Short term stability	LQC	0.018x10^5±0.00075	4.14	90.23
	MQC	0.126x10^5±0.00380	1.03	94.74
	HQC	0.186x10^5±0.00186	1.00	93.23

Data are expressed as mean+SD, n=6.



A



B

Fig. 5: Recovery plot (A) Nortriptyline HCl and (B) Flupentixol

Table 6: Pharmacokinetic parameters of nortriptyline HCl and flupentixol

Pharmacokinetic parameters	Nortriptyline HCl	Flupentixol
AUC_{0-t}	1503ng-h/ml	87ng-h/ml
C_{max}	93.6ng/ml	4.7ng/ml
$AUC_{0-\infty}$	1769ng-h/ml	114ng-h/ml
t_{max}	8.0 h	8.0 h
$T_{1/2}$	24.0 h	32.0 h

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity, AUC_{0-t}: Area under the curve up to the last sampling time, C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration

CONCLUSION

For the primary time higher sensitive HPLC-ESI-LCMS/MS method was developed and validated for the determination of nortriptyline HCl and flupentixol in rat plasma. Here the described method is rugged, fast, reproducible bio-analytical method. This method was validated according to USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

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AUTHORS CONTRIBUTIONS

Bharath has collected the literature and information about the drug. Visalakshamma and Sri Ramudu has carried out the research samples and prepared the manuscript. Khasim Sharif supported solution preparation in analysis. Ramachandran check the data and reviewed the article.

CONFLICTS OF INTERESTS

Author declares that there have been no conflicts of interest.

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