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Original Article

Determination of Histamine H_3-Receptor Antagonist Conessine in Wistar Rat Plasma by a Rapid and Sensitive RP-UFLC Method: Application to a Pharmacokinetic Study

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ABSTRACT

Objectives: Epidemiological data suggests the highest density of histamine H3-receptors (H3R) in basal ganglia of central nervous system (CNS) wherein they function as inhibitory auto-receptors and control the release of many neurotransmitters. Inhibition of H3R increases the turnover of neurotransmitters, especially dopamine in basal ganglia and could be beneficial to treat Parkinson's disease. **Methods:** In this study, we formulated the brain targeting liposomes of conessine, a selective antagonist for H3R to increase bioavailability and developed and validated a rapid and sensitive reverse phase ultra-force liquid chromatographic (RP-UFLC) method and to quantitate conessine in Wistar rat plasma and tissue. Plasma and tissue samples were extracted by protein precipitation technique using acetonitrile (ACN) and aripiprazole as the internal standard. Chromatographic separation was performed on the Hibar C_{18} column with a mobile phase of Hexane Sulphonic acid (10 mM, pH 10.0 adjusted with ammonia) and methanol at a flow rate of 0.9 ml/min. **Results:** The lower limit of quantification of the developed method was 4.0 ng/ml and 6.0 ng/g in plasma and tissue samples, respectively. Liposomes of conessine (equivalent to 20 mg/kg) administered orally to animals, demonstrated remarkable absorption into the systemic circulation with maximum concentration (~8700 ng/ml) within 2.0 h. The order of area under curve was found to be kidney> brain> liver> lungs> spleen> heart. **Conclusion:** The liposomes of conessine were rapidly taken up into the brain and showed a good brain concentration after 2.0 h; sustenance up to 4.0 h was achieved which is better than conessine solution.

Key words: Conessine, RP-UFLC method, Pharmacokinetic, Tissue distribution, Validation.

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INTRODUCTION

Parkinson's disease (PD) is one of the commonly occurring neurodegenerative disorder which leads to muscular rigidity, bradykinesia, tremor of resting limbs and loss of postural balance.[1,2] The main characteristic of PD involves degeneration of pigmented neurons in substantia nigra resulting in depletion of dopamine (DA) and its metabolites level.[3,4] PD ranks among the most common late life neurodegenerative diseases, affecting approximately 1.5-2.0 % of the population which is older than 60 years.[5,6] Irrespective of enormous scientific research, exact physiological causes of PD, the second most common neurodegenerative disorder, are still largely unknown.[7] Age is the single greatest risk factor for PD with the number of world-wide cases increasing from an estimated 4.1 million (340,000 US cases) in 2005 to nearly 8.7 million (610,000 US cases) by 2030.[8]

A subclinical phase exists prior to the appearance of Parkinsonism; during this period striatal compensatory phenomena,[9,10] such as increased neuronal activity or sensitization of dopaminergic receptors are activated.[9,11] Therefore, PD is not clinically obvious before at least 50-70 % of the dopaminergic neurons get degenerated. The first clinical signs are only observed when the degeneration is already strongly advanced.[12] It follows that the onset time point of the degeneration cannot be determined. Thus, the etiology of PD remains difficult to establish leading to challenges in development of clinical tools.[13]

Histamine H_3-receptor (H3R) were first discovered in 1983 as a presynaptic autoreceptors for the neurotransmitter histamine.[14] A growing body of literature also supports a role for H_3R in influencing the release of a number of additional neurotransmitters like acetylcholine (Ach), dopamine (DA), glutamate, noradrenaline (NA) and serotonin (5-HT).[14-16] H3R are mainly located in cerebral cortex, hippocampus, amygdala, nucleus accumbens, globus pallidus, striatum and hypothalamus; however, the highest density of H3R are found in basal ganglia,[17,18] an important seat in the brain involved in coordination of information from sensorimotor, motivational and cognitive brain areas to control behaviors such as movement and reward learning. The selective antagonist/inverse antagonist of H3R such as thioperamide (THP) and ciproxifan (CPX),[19] potentiate neurochemical and behavioral effects of haloperidol (HAL) and enhance the turnover of dopamine in midbrain of rats. Literature supports that it also modulates the neurochemical and behavioral effects of meth-amphetamine.[20] This background suggests that H_3R are presynaptic auto-receptors which play an important role in modulating various neurotransmitters in the mid brain. A specific antagonist or inverse agonist of H3R may thus increase the level of dopamine in mid

brain and prove to be effective to treat Parkinsonism and other neurodegenerative diseases.

Conessine is a steroidal alkaloid found in a number of plant species from the Apocynaceae family, including *Holarrhena floribunda,*[21] *Halorrhena antidysenterica*[22] and *Funtumia elastica.*[23] It is one of the potent and selective H3R subtype antagonist.[24] It is well proven that conessine, an H3R antagonist exacerbated ethanol-induced psychostimulation by altering dopaminergic neurotransmission in the nigrostriatal pathway.[25] To the best of our knowledge, there are no detailed reports on the pharmacokinetic and tissue distribution properties of conessine using revers phase ultra-fast liquid chromatographic (RP-UFLC). Therefore, this study aimed to develop and validate a highly selective and sensitive method based on RP-UFLC for routine analysis of conessine in rat plasma. Also, we aimed to investigate the pharmacokinetics and tissue distribution profile of conessine in Wistar rats after oral administration at 20 mg/kg; the said dose having been established in ischemic renal injury in Wistar rats.[26]

MATERIALS AND METHODS

Chemicals and reagents

Conessine ≥97 % (HPLC) and Aripiprazole were obtained from Sigma-Aldrich (St. Louis USA). The chemical structure of conessine and aripiprazole are shown in Figure 1. The HPLC grade methanol and acetonitrile were purchased from SD Fine chemicals (Mumbai, India); Hexane Sulphonic acid, Trisaminomethane (TRIS), sodium chloride, Potassium chloride, ammonium solution and potassium dihydrogen phosphate of AR grade were procured from SD Fine chemicals (Mumbai, India). *Ortho*-phosphoric acid and di-methyl sulphoxide (DMSO) of AR grade were obtained from Qualigens Fine chemicals (Mumbai, India). The ultra-pure milli-pore water was obtained using a Milli-Q RO system (Millipore India, Bangalore, India).

Chromatographic conditions

The bioanalysis of conessine was carried out using a Shimadzu prominence UFLC system (Shimadzu Corporation, Kyoto, Japan). The UFLC instrument was equipped with a model series LC-20AD pump, SPD-M20A PDA detector and a Rheodyne 7752*i* injector with a 20 µl loop. The Hibar C_{18} column (250 mm × 4.6 mm; I.D., 5 µm) was used for separation and Lab solution chromatography software was used for data acquisition. The mobile phase consisted Hexane Sulphonic acid (10 mM, pH 10.0 adjusted with ammonia) and Methanol (10: 90 v/v) with a flow rate of 0.9 ml/min and Aripiprazole (100 µg/ml) was used as an internal standard (IS). The injected sample volume was 20 µl and detection wavelength 210 nm kept constant throughout the experiment. Before use, mobile phase was filtered through a 0.22 µm hydrophilic membrane filter and sonicated for 10 min. The whole experiment was performed at room temperature.

Preparation of calibration standards and quality control samples

The calibration standards were prepared by spiking the working standard solution of conessine into a pool of drug free rat plasma and through processed tissue homogenate to obtain the following concentrations 2.0, 12.0, 200.0, 1000.0, 2000.0, 8000.0, 20000.0, 32000.0 and 40000.0 ng/ml in plasma and 4.0, 10.0, 50.0, 100.0, 500.0, 1000.0, 2000.0 and 5000.0 ng/g in tissues. These solutions were labeled and stored at -70 ± 2°C until analysis. A minimum of three concentrations of conessine were prepared for quality control testing by spiking the working standard solution into a pool of drug free Wistar rat plasma and tissue to produce a concentration of 8.0, 16,000.0 and 32,000.0 ng/ml and 12.0, 2000.0 and 3200.0 ng/g respectively using Aripiprazole (100 µg/ml) as IS.

Tissue preparation

After treatment regimen, animals were sacrificed and blood was collected by cardiac puncture method into 4 ml tubes pretreated with sodium heparin (68 USP units) anticoagulant. The important vital organs (brain, liver, lungs, kidney, spleen and heart) were quickly removed and stored at -70 ± 2°C until analysis. For estimation, tissue samples were weighed accurately and homogenized using a glass tissue homogenizer after addition of Tris KCl buffer (10 mM pH 7.4); homogenised organs and blood were centrifuged at 10,500 X g for 20 min to obtain a clear supernatant and plasma.[27,28]

Extraction of plasma and tissue samples

A 500 µl of plasma, 500 µl of IS (100 µg/ml) and 500 µl of ACN were aliquoted into 2 ml eppendorf tube, vortexed for 30s then centrifuged (Remi Instruments, Mumbai) at 10,000 rpm for 10 min. The clear supernatant solution was transferred into a vial and 20 µl was injected to UFLC analysis. Similarly, tissue homogenates were processed and analyzed by UFLC.

Method validation

The UFLC method to determine biodistribution of conessine in Wistar rat plasma and tissue was validated as per the USFDA guidelines.[29,30] The method of determination was validated for specificity, extraction recovery, linearity, sensitivity, accuracy, precision and stability.

Specificity: is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The specificity was ascertained by the lack of interference peaks at the retention time of conessine and the aripiprazole (internal standard). The blank plasma and tissue samples were tested for interference and specificity was ensured at the lower limit of quantification (LLOQ).

Extraction recovery: of an analyte in an assay was determined by comparing the mean peak area obtained from either the extracted plasma or tissue samples with the peak area obtained by the direct injection of the corresponding spiked standard solutions. Different concentrations of conessine (8.0, 16000.0 and 32000.0 ng/ml in plasma and 12.0, 2000.0 and 3200.0 ng/g in tissue samples) were measured.

Linearity and sensitivity: calibration curves were generated for conessine in plasma and tissue samples. The linearity was tested over the concentration range of 2.0-40000.0 ng/ml in plasma and 4.0-5000.0 ng/g in tissues. The calibration curves were established by plotting the peak area ratio of conessine to IS versus the conessine concentration. The regression parameters of the slope, intercept and correlation coefficient were calculated by linear regression equation. The lowest limit of quantification (LLOQ) was set as the lowest amount of analyte in a sample that could be quantitatively determined with acceptable precision and accuracy (i.e. not to exceed 20 % coefficient of variation (CV) and ± 20 % nominal concentration in these assays, respectively).

Accuracy and precision: The intra- and inter-day precision and accuracy in plasma and tissue samples were carried out at three different QC levels in six replicates on the same day and on three different days, respectively. Acceptable deviation was set within 15 % of the nominal concentration for accuracy and within 15 % of the CV for precision.

Stability: Testing of conessine in plasma and brain tissue were done by the analysis of QCs (8.0, 16000.0 and 32000.0 ng/ml in plasma and 12.0, 2000.0 and 3200.0 ng/g in tissue samples, *n*=6) subjected to different storage conditions like freeze thaw (3 cycles at -70 ± 2°C), short-term (at 25°C for 6 hr), long-term (at -70 ± 2°C for 30 days) and stock solution (at 25°C for 6 hr) stability. For freeze-thaw (3 cycles) stability, the spiked plasma and tissue samples were frozen at -70°C for 24 hr and thawed at room temperature. After completion the samples were refrozen for 12-

24 hr under the same conditions, the freeze-thaw cycle was repeated two more times, at the end of third cycle samples were analysed and compared with the freshly prepared QCs (n=6) in plasma and tissue. For the short-term and stock solution stability study, plasma and tissue QCs were kept at 25°C for 6 hr and samples were processed, analysed and compared with the freshly prepared QCs. The long-term stability was evaluated by analyzing stored plasma and tissue samples. The samples were considered to be stable, when the deviation from the nominal values was within ± 15%.

Pharmacokinetics and tissue distribution study

The male Wistar rats weighing 180-220 g were used for pharmacokinetic and tissue distribution studies of conessine. The animal house was well ventilated and the animals were maintained on a 12:12 hr light and dark cycle in large specious cages throughout the experiment. The animals were provided with food and water *ad libitum* and fasted for 12 hr before starting the experiment. The experimental protocol was approved by Institutional Animal Ethical Committee. Fifty-four Wistar rats were randomly assigned into nine groups of six animals in each group. The liposomal formulation of conessine[31-33] and pure conessine were dissolved in Tris KCl buffer (pH 7.4) and then orally administered to rats at a dose equivalent of 20 mg/kg. Aliquots of approximately 0.5 ml of blood samples were collected via cardiac puncture at time intervals of 0 min (predose), 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 and 12 h post-dose. The vital organs of interest were collected immediately after cervical dislocation at above prescribed time periods and weighed accurately. The tissue was washed with Tris KCl buffer (pH 7.4) to remove blood or content and blotted dry with tissue paper and stored at -70 ± 2°C until analysis.

The pharmacokinetic parameters were calculated by non-compartmental analysis after calculating extravascular input of individual concentration–time data using pK solver software (AGAH Working group PK/PD modelling). The pharmacokinetic parameters such as maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained directly from the plasma concentration time curve. The elimination rate constant (K_{el}) was calculated from parameters of the multiexponential fit of the plasma concentration–time profile, elimination half-life ($T_{1/2}$) was calculated as 0.693/ K_{el}, area under the plasma concentration time curve from 0 to 12 hr ($AUC_{0-12\,h}$) was calculated by the linear trapezoidal rule and area under the curve from 0 h extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{(0-12h)} + C_z/K_{el}$ where C_z represents the observed or calculated plasma concentration at the last measurable sampling time. The apparent total plasma clearance (CL/F) was calculated as the drug dose divided by $AUC_{(0-12h)}$ and the apparent volume of distribution (V_z/F) was calculated as total dose given by orally divided by the last concentration-time point. All the values are expressed as means ± standard deviation except for the T_{max}, which is expressed as the median.[27]

RESULTS

Ultra-force liquid chromatographic analysis

The conessine with a molecular weight of 356.6 g/mol partly polar in nature, was found to be slightly water soluble, sparingly soluble in methanol, ACN and ethanol. Reverse phase mode was used for analysis with a Hibar C_{18} column.[34] The λ_{max} for conessine was found to be 210 nm by using UV-Visible spectroscopy. The retention time for conessine and IS with 90 % methanol and 10 % hexane sulphonic acid (10 mM, pH 10.0 adjusted with ammonia) at the flow rate 0.9 ml/min with a run time of 12 min was found to be 3.18 min and 8.6 min, respectively.

Validation of Ultra-force liquid chromatographic method

Specificity: The chromatographic peaks of conessine and IS showed no interference from endogenous components in the retention time of observed drug. Conessine and IS showed very good resolution with employed chromatographic conditions. The standard UFLC chromatograms for blank plasma, tissue and spiked plasma are shown in Figure 2.

Extraction recovery: The percentage mean recovery of conessine ranged from 92.82 to 95.63 % and 91.83 to 94.69 % in plasma and brain respectively. The recovery for IS at 150 µg/ml was found to be 94.84 and 92.22 % in plasma and tissue respectively. The extraction recoveries of conessine in plasma and various tissue samples are shown in Table 1.

Linearity and sensitivity: The conessine was spiked into plasma and tissue sample to determine the calibration curve. The linearity of each calibration curve was ascertained by plotting the response factor *versus* concentration of standard solution. Linear calibration curves for conessine in plasma and various tissue samples were observed at 4.0-40000.0 ng/ml and 6.0-5000.0 ng/g respectively. The result for linear regression analysis is listed in Table 2 and showed that the correlation coefficient of the calibration curves for all the tissue samples were greater than 0.99.

Accuracy and precision: The intra and inter-day accuracy and precision of conessine in plasma and tissue samples carried out at three different QC levels in six replicates were found to be within the acceptable limit. These results indicate that the assay method was accurate and precise for replicate analysis of conessine in plasma and tissue samples (Table 1).

Stability: The chemical stability of conessine in plasma and brain under different conditions for given time intervals were carried out by measuring the concentration changes in QCs (n=3). The results indicate that conessine was stable under all the stability conditions. Table 3 depicts the percentage changes in the mean concentration of conessine under all tested conditions.

Pharmacokinetic study

The pharmacokinetic parameters of liposomal formulation of conessine and pure conessine solution in rat plasma and brain are shown in Table 4. The quantitative estimation of conessine in plasma and tissue samples were carried out by validated chromatographic conditions following oral administration at the dose of 20 mg/kg. The mean plasma concentration-time profile for liposomal formulation of conessine and pure conessine solution is shown in Figure 3a and 3b. After oral administration of conessine solution it got absorbed into systemic circulation and showed the maximum concentration 10124.75 ng/ml at 2.0 hr. The liposomal formulation of conessine after oral administration achieved maximum concentration 8672.42 ng/ml after 2.0 hr. The $AUCs_{(0-12h)}$ in plasma for conessine solution and conessine liposomes was found to be 51920.10 ± 478.27, 44613.53 ± 410.72 ng/h/ml and plasma $T_{1/2}$ 1.58 ± 0.18, 3.818 ± 0.16 hr respectively which suggests that liposomal formulation shows good systemic absorption with prolonged action. The pharmacokinetic parameters for liposomes in plasma of C_{max}, $AUC_{(0-12h)}$, $AUC_{(0-inf)}$, CL/F, $T_{1/2}$, T_{max}, K_{el}, Vz/F and MRT was found to be 8672.42 ± 251.66 ng/ml, 44613.53 ± 410.72 ng/h/ml, 51246.67 ± 458.91 ng/h/ml, 39.03 ± 9.04 ml/h/kg, 3.818 ± 0.16 h, 2 h, 0.181 ± 0.01 1/h, 214.99 ± 21.78 ml/kg and 6.08 ± 1.28 h, respectively.

Tissue distribution study

The tissue distribution study of liposomal formulation of conessine were determined in various tissues of Wistar rats like brain, liver, lung, kidney, heart and spleen are shown in Table 5. Figure 4 shows the concentration-time curve of conessine in various tissues following oral administration at 20 mg/kg. The AUCs of conessine in kidney (16567.12 ± 524.90, 16640.43 ± 576.81 ng/h/g), liver (10500.27 ± 476.32, 10657.41 ± 514.26 ng/h/g) and brain (14732.45 ± 308.42, 15121.04 ± 326.74 ng/h/g) were larger than in other organs. The least AUCs were found in heart (1381.90 ± 129.3, 1426.22 ± 117.19 ng/h/g) and spleen (1624.025 ± 108.59, 1776.63 ± 125.71 ng/h/g). The possible reason for these results is that the

Table 1: Recovery, accuracy and precision for determination of conessine in plasma and tissues (*n* = 6).

				Intra-day		Inter-day	
Biological matrix	QCs (ng/ml or g)	Mean concentration found (ng/ml or g)	Recovery (%)	Accuracy (% nominal)	Precision (% CV)	Accuracy (% nominal)	Precision (% CV)
Plasma	8	7.54 ± 0.28	93.16	94.24	4.6	90.84	6.4
	16000	15325.4 ± 764.25	92.82	95.78	4.2	87.91	5.9
	32000	30571.9 ± 1392.91	95.63	95.53	4.9	91.50	6.7
Brain	12	11.02 ± 0.73	91.83	94.43	6.7	86.64	5.1
	2000	1904.21 ± 138.27	95.21	95.84	5.8	91.97	7.2
	3200	3027.6 ± 227.65	94.59	95.32	6.2	90.89	4.2
Liver	12	11.39 ± 1.08	94.73	94.14	6.1	91.33	7.3
	2000	1865.80 ± 168.5	93.29	93.64	5.7	93.51	5.6
	3200	2973.44 ± 291.22	92.62	96.47	6.8	88.58	7.1
Lung	12	11.16 ± 0.96	93.06	93.24	4.6	91.74	5.9
	2000	1915.21 ± 168.6	95.76	94.28	5.4	87.78	6.8
	3200	3062.72 ± 211.7	95.71	96.22	5.9	90.44	7.9
Kidney	12	10.92 ± 0.72	91.00	95.71	5.2	92.83	6.7
	2000	1928.0 ± 155.05	96.40	93.45	4.6	87.64	7.4
	3200	3016.32 ± 179.78	94.26	94.97	6.7	86.55	6.2
Spleen	12	10.80 ± 0.91	90.06	95.36	5.2	89.12	6.8
	2000	1904.8 ± 118.98	95.24	94.25	5.8	91.31	7.6
	3200	2992.96 ± 221.21	93.53	95.30	6.2	89.86	6.9
Heart	12	11.02 ± 0.87	91.86	94.19	4.8	90.34	5.7
	2000	1859.4 ± 118.32	92.97	95.68	4.6	91.32	6.3
	3200	2961.92 ± 170.32	92.56	93.28	4.2	93.02	6.5

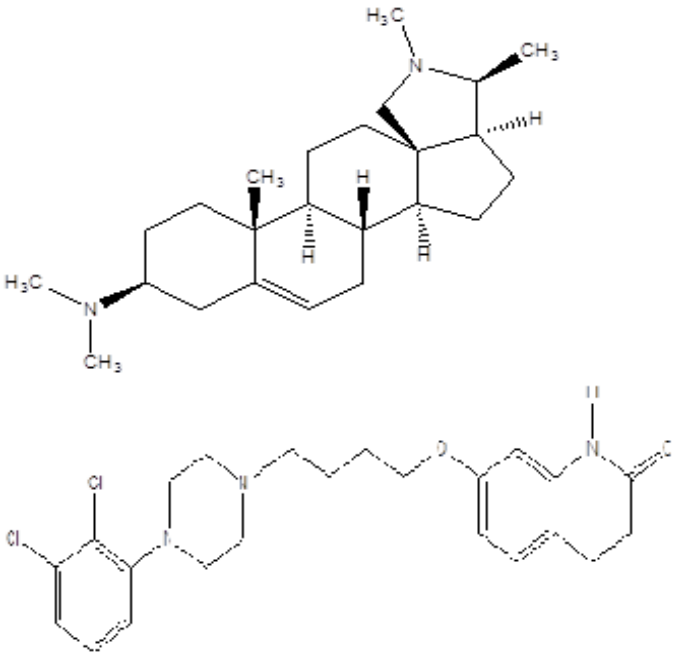

Figure 1: Chemical structure of a) Conessine and b) Aripiprazole.

Table 2: Equations of linear regression analysis of Conessine for plasma and various tissues.

Biological Sample	Concentration range (ng/ml or g)	Equation	R^2
Plasma	4.0-40,000.0	y = 0.00006x + 0.00129	0.999
Brain	6.0-5000.0	y = 0.0004x + 0.0025	0.999
Liver	6.0-5000.0	y = 0.0002x + 0.0048	0.997
Lung	6.0-5000.0	y = 0.0001x + 0.0040	0.998
Kidney	6.0-5000.0	y = 0.0003x + 0.0054	0.999
Spleen	6.0-5000.0	y = 0.0001x + 0.0043	0.997
Heart	6.0-5000.0	y = 0.00004x + 0.0059	0.995

Table 3: Summary of stability testing of conessine in Wistar rat plasma under various storage condition (*n = 6*).

	Biological matrix							
	Plasma				Brain			
Stability test	QCs (ng/ml)	Mean ± SD (ng/ml)	Accuracy (% nominal)	Precision (% CV)	QCs (ng/ml)	Mean ± SD (ng/ml)	Accuracy (% nominal)	Precision (% CV)
Freeze-thaw (3 cycles at -70 ± 2°C)	8	7.09 ± 0.51	88.62	6.7	12	10.72 ± 0.65	89.33	6.3
	16000	14695 ± 1043.6	91.84	5.4	2000	1825.8 ± 108.9	91.29	5.9
	32000	29450.8 ± 1465.7	92.03	6.1	3200	2963.2 ± 223.7	92.6	6.1
Short-term (at 25°C for 6 h)	8	7.55± 0.43	94.37	5.8	12	11.21 ± 0.7	93.41	5.8
	16000	15017.8 ± 1063.2	93.86	5.6	2000	1868.4 ± 152.4	93.42	5.9
	32000	29890.4 ± 1902.6	93.40	6.2	3200	2991.2 ± 190.5	93.47	5.6
Long-term (at-70 ± 2°C for 30 days)	8	7.17± 0.7	89.62	6.8	12	10.32 ± 0.69	86.0	6.9
	16000	14218.5 ± 989.6	88.86	6.7	2000	1830.8 ± 136.5	91.54	6.3
	32000	28565.49 ± 1739.2	89.26	6.9	3200	2917.3 ± 196.9	91.16	6.6
Stock solution (at 25°C for 6 h)	8	7.69 ± 0.31	96.12	4.2	12	11.71 ± 0.34	97.58	3.9
	16000	15498.9 ± 803.4	96.86	3.8	2000	1935.6 ± 103.8	96.78	4.1
	32000	30994.48 ± 1218.4	96.85	4.1	3200	3096.8 ± 182.4	96.77	4.2

Table 4: The mean pharmacokinetic parameters for Conessine solution and nanoformulation in plasma and brain following oral administration at 20 mg/kg.

Parameter	After Conessine Solution		After Formulations	
	Brain	Plasma	Brain	Plasma
C_{max} (ng/g or ml)	1095.4 ± 61.25	10124.75 ± 218.92	3243.8 ± 141.2	8672.42 ± 251.66
$AUC_{(0\text{-}12h)}$ (h*ng/g or ml)	3365.82 ± 76.23	51920.10 ± 478.27	14732.45 ± 308.42	44613.53 ± 410.72
$AUC_{(0\text{-}inf)}$ (h*ng/g or ml)	3469.45 ± 84.9	52526.02 ± 518.60	15121.04 ± 326.74	51246.67 ± 458.91
CL/F (ml or g/kg/h)	576.45 ± 28.2	38.07 ± 7.38	132.26 ± 14.52	39.03 ± 9.04
$T_{1/2}$ (h)	1.219 ± 0.11	1.58 ± 0.18	1.95 ± 0.14	3.818 ± 0.16
T_{max} (h)	2	2	2	2
K_{el} (1/h)	0.568 ± 0.01	0.438 ± 0.02	0.354 ± 0.01	0.181 ± 0.01
Vz/F (ml or g/kg)	1014.23 ± 105.6	86.83 ± 24.08	373.52 ± 32.56	214.99 ± 21.78
MRT (h)	3.35 ± 0.21	3.83 ± 0.12	4.41 ± 0.18	6.08 ± 1.28

systemic circulation of conessine is limited as a significant amount is distributed to kidney and liver, where it is metabolised and eliminated from body. Increased conessine exposure results in maximal concentrations in the kidney suggesting renal excretion is the main elimination route.

DISCUSSION

In previously described method Garg S and Bhutani KK[35] developed HPLC method to quantify conessine for quality control of marketed Kutajarista samples, whereas Brás Heleno de Oliveira[36] described a importance of HPLC analytical methods to estimates important classes of alkaloids including conessine. But, until now no method has been developed to estimate conessine in bioanalytical samples which could provide important insights for bioavailability and pharmacokinetic studies. In the present study we developed simple, sensitive and robust analytical method to determine conessine in biological samples using UFLC. While developing this method we utilized different combinations of solvent as mobile phase. Initial combination of mobile phase ACN: water (70:30) showed good peak area but very low retention time. However, mobile phase Methanol: Ammonium formate (75:25) at pH 6.5 showed good resolution between conessine and IS, but IS showed tailing effect. The best resolution factor was observed at a ratio of 90 % methanol and 10 % hexane sulphonic acid (10 mM, pH 10.0 adjusted with ammonia) with a flow rate 0.9 ml/min. The IS is highly sensitive to a pH and wavelength; a slight change in either leads to major changes in resolution of IS. For extracting the drug from biological samples various extraction procedures have been tried such as solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PPT) to determine the percentage recovery for conessine. The limit of detection of 4 ng/ml was obtained with SPE as well as PPT when ACN was used as a protein precipitating agent, but LLE did not show credible limit of detection for conessine. As PPT technique is easier and less time consuming in comparison to SPE which involves multiple purification steps rendering it time intensive, further extraction of drug were carried out with PPT technique.

The present method is accurate and precise for both the analytes in plasma and as seen from the inter- and intraday precision and accuracy, it is reproducible. Day-to-day reproducibility is demonstrated by the intra- and inter-batch analysis of the QCs. The lowest limit of quantification (LLOQ) in plasma and tissue samples were determined by analyzing different levels of concentration ranging from 4.0-100.0 ng/ml or g and

Table 5: The mean pharmacokinetic parameters for Conessine nanoformulation in main visceral organ after oral administration at 20 mg/kg.

Parameter	Heart	Spleen	Lungs	Liver	Kidney
C_{max} (ng/g)	408.75 ± 69.21	465.1 ± 39.94	936.7 ± 57.29	2806.9 ± 131.43	3672.7 ± 140.01
$AUC_{(0\text{-}12h)}$ (h*ng/g)	1381.90 ± 129.3	1624.025 ± 108.59	3266.37 ± 207.46	10500.27 ± 476.32	16567.12 ± 524.90
$AUC_{(0\text{-}inf)}$ (h*ng/g)	1426.22 ± 117.19	1776.63 ± 125.71	3298.33 ± 216.11	10657.41 ± 514.26	16640.43 ± 576.81
CL/F (g/kg/h)	1402.3 ± 85.32	1125.72 ± 99.04	606.36 ± 49.05	187.66 ± 23.51	120.18 ± 18.43
$T_{1/2}$ (h)	1.30 ± 0.11	1.90 ± 0.15	1.571 ± 0.12	1.70 ± 0.14	1.19 ± 0.10
T_{max} (h)	2	2	2	2	2
K_{el} (1/h)	0.532 ± 0.08	0.364 ± 0.02	0.441 ± 0.03	0.405 ± 0.06	0.582 ± 0.12
Vz/F (g/kg)	2633.38 ± 152.57	3089.84 ± 169.4	1374.48 ± 93.72	462.22 ± 37.02	206.36 ± 21.67
MRT (h)	3.19 ± 0.84	3.90 ± 1.34	3.53 ± 1.63	3.78 ± 1.72	3.78 ± 1.06

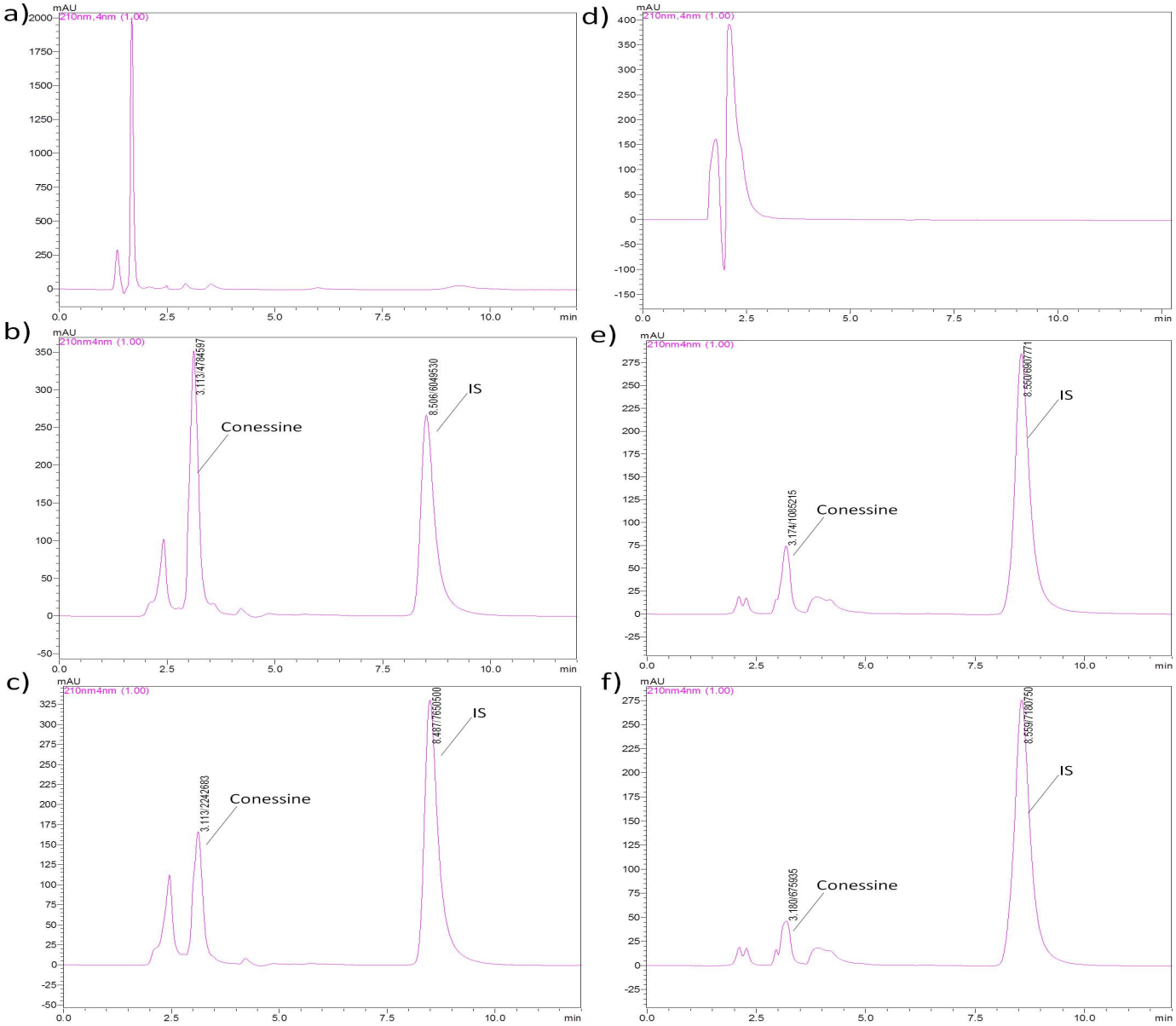

Figure 2: Representative chromatograms of a) Blank Plasma, b) Plasma spiked with liposomes and IS, c) Plasma Sample after oral administration of liposomes, d) Blank brain tissue, e) Brain tissue spiked with liposomes and IS, f) Brain tissue sample after oral administration of liposomes.

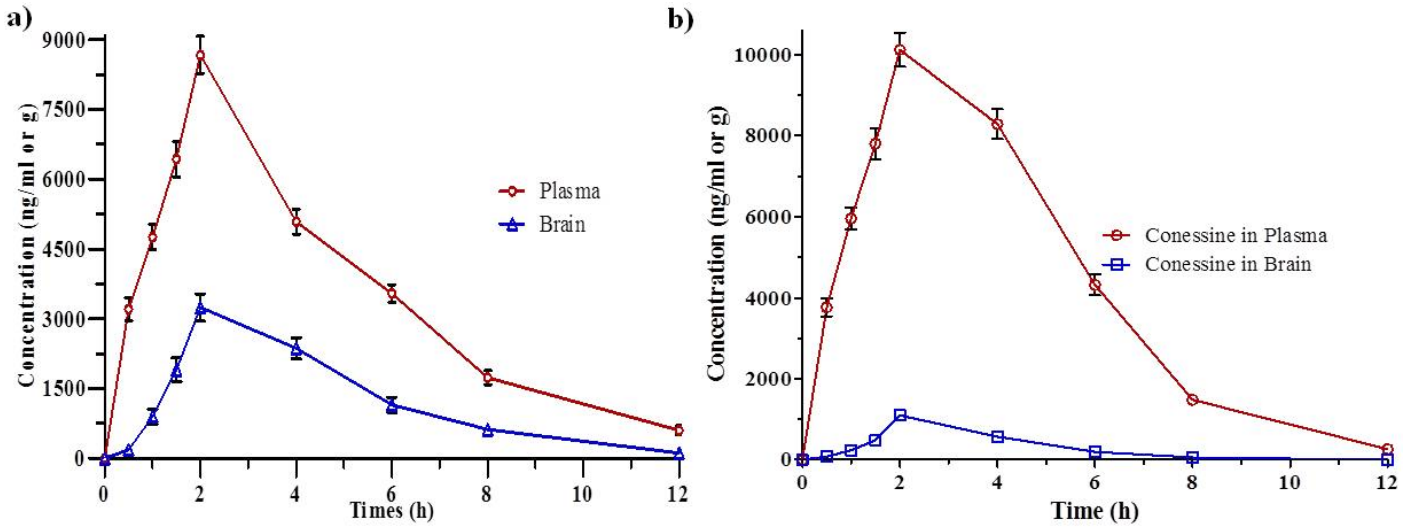

Figure 3: Concentration-time curves for plasma and brain after a) oral administration of single dosage of conessine liposome (equivalent of 20 mg/kg) and; b) conessine solution (20 mg/kg). Data are represented as means ± SD (*n* = 6).

were found to be 4.0 ng/ml and 6.0 ng/g, respectively, with accuracy of 95.02 % with 4.6 % precision in plasma and 91.10 % of accuracy with 6.0 % precision in tissue. The result shows that LLOQ of conessine was found to be within the acceptable accuracy and precision range. The limit of detection (LOD) for conessine in plasma and tissue samples was found to be 1.2 ng/ml and 2.0 ng/ml, respectively which is adequate for determining the pharmacokinetics of conessine.

Earlier described HPLC methods [ref] for the determination of conessine in polyherbal formulation exhibited linearity in the concentration range of 0.1–1.0 mg/ml. This sensitivity could be adequate to for the estimation of conessine in polyherbal formulation but for the bioanalytical estimation high sensitivity is required. As per the US FDA guidelines, bioequivalence study protocols generally recommend plasma sample collection for a time period, corresponding to three to four times the drug plasma elimination half-life, which brings terminal concentration values of ~6% of the peak concentration value. The mean peak plasma concentration of conessine was 30571.9 ng/ml and its plasma concentration followed by four half-lives was 1894.75 ng/ml. The pharmacokinetic parameters for liposomes in plasma of C_{max}, $AUC_{(0\text{-}12h)}$, $AUC_{(0\text{-}inf)}$, CL/F, $T_{1/2}$, T_{max}, K_{el}, Vz/F and MRT were calculated and these values suggests conessine liposomes are rapidly taken up into the brain reaching a maximum concentration (3243.8 ± 141.2 ng/g) after 2.0 hr, which is much higher than conessine solution (1095.4 ± 61.25 ng/g) after 2.0 hr. This indicates that liposomal formulation can easily cross blood brain barrier than conessine solution and remains stable for up to 3-4 h. The pharmacokinetic and tissue distribution of conessine was analysed by non-compartment extra-vascular model. The order of AUC was found to be kidney> brain> liver> lungs> spleen> heart. The order of the maximum conessine concentration in tissue was kidney> brain> liver> lungs> spleen> heart.

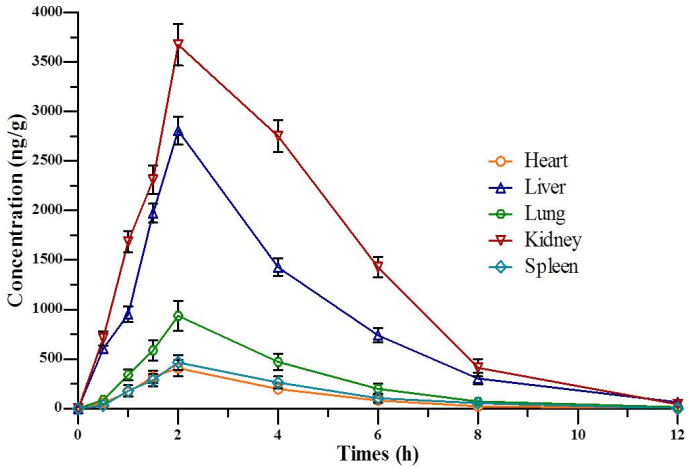

Figure 4: Concentration-time curve for important visceral organs after oral administration of single dosage of conessine solution (20 mg/kg). Data are represented as means ± SD (*n* = 6).

CONCLUSION

A selective, sensitive and simple UFLC method was developed and validated for the estimation of conessine in Wistar rat plasma. The easy and simple PPT technique was employed for analysis of plasma and tissue samples; also, by the employed UFLC method, good sensitivity of detection (4.0 ng/ml in plasma and 6.0 ng/ml in tissue) was achieved. The pharmacokinetic and biodistribution data shows that the liposomal formulation of conessine have better systemic absorption and possess better ability to cross blood brain barrier. Also, liposomal formulation of conessine showed prolonged half-life of conessine compared to conessine solution alone. This pharmacokinetic study offers significant promise and should be further explored to study effect of conessine a potent H3R antagonist over turnover of dopamine in the substantial nigra of brain region. The developed method was successfully utilized for analysis of conessine in rat plasma and tissues after oral administration at the dose of 20 mg/kg. This developed method could be very useful for quantitative and qualitative estimation of conessine in biological samples and polyherbal formulations. Furthermore, the sensitivity and simplicity of the method make it suitable for bioavailability and bioequivalence studies.

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CONFLICT OF INTEREST

There are no conflicts to declare.

ABBREVIATIONS

H3R: Histamine H3-receptors; **CNS:** Central nervous system; **RP-UFLC:** Reverse phase ultra-force liquid chromatographic; **ACN:** Acetonitrile; **PD:** Parkinson's disease; **DA:** Dopamine; **TRIS:** Trisaminomethane; **DMSO:** Di-methyl sulphoxide; **IS:** Internal standard; **LLOQ:** Lower limit of quantification; **LOD:** Limit of detection; **CV:** Coefficient of variation; **HPLC:** High-performance liquid chromatography; **C_{max}:** Maximum concentration recorded; **T_{max}:** Time take to reach C_{max}; **AUC:** Area under the curve; **CL/F:** Clearance; **T1/2:** Half-life; **Kel:** Elimination rate constant; **Vz/F:** Volume of distribution during terminal elimination; **MRT:** Mean Residence Time.

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