CHAPTER 4

PHARMACOKINETICS AND BRAIN PENETRATION STUDY OF CHLOROGENIC ACID IN RATS

Highlights of the Chapter

- *Earlier, finding using in vitro BBB model suggest that CGA exhibits a very low rate of BBB permeation and cannot considered for a direct effect on the central nervous system*
- *Present comparative study is designed to investigate the brain distribution and plasma pharmacokinetics profiles of CGA after IN and IV administration in rats*
- *Results revealed that CGA is rapidly available into brain (T_{max} = 30 minutes) IN administration.*
- *The exposure of CGA in the brain after IN administration ($AUC_{brain,\ IN}$) was significantly greater (4 times) as compared to the exposure of CGA in the brain ($AUC_{brain,\ IV}$) after IV administration*

Abstract

The present study is designed to investigate the brain distribution and plasma pharmacokinetics profiles of chlorogenic acid after intranasal administration in Charles-Foster rats to evaluate whether the CGA molecules are transported directly via the nose-to-brain path. The chlorogenic acid is administered intravenously (IV) and intranasally (IN) at the dose of 10 mg/kg. Further, its concentration in the plasma, cerebrospinal fluid (CSF) and the whole brain is analyzed by HPLC-UV method. The study observes that chlorogenic acid is rapidly absorbed in plasma with t_{max} of 1 min similar to IV route after IN administration. The peak plasma concentration and $AUC_{0\text{-}24}$ are higher by 3.5 and 4.0 times respectively in IV administration, compared to IN delivery that represents the significant less systemic exposure of chlorogenic acid in IN route. However, the concentration of chlorogenic acid in the brain is 4, 6.5, 5.3, 5.2 and 4.5 times higher at 30, 60, 120, 240 and 360 minutes respectively in IN administration

compared to IV administration. The exposure of CGA in the brain after IN administration ($AUC_{brain, IN}$) was significantly greater (4 times) as compared to the exposure of CGA in the brain ($AUC_{brain, IV}$) after IV administration reflecting significant brain uptake of chlorogenic acid through nasal route. Therefore, IN delivery of CGA can be a promising approach for the treatment of stroke and neurodegenerative disorders.

4.1. INTRODUCTION

Chlorogenic acid (CGA, IUPAC: (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid Fig. 2.6) is a major polyphenolic component of *Coffea arabica L*., *Coffea robusta* [214][215] and Maté (*Ilex paraguariensis* A. St-Hil.) [216]. It is also present to some extent in other plants including fruits, tea, vegetables [21], *Withania somnifera* (ashwagandha*)* [22]. Notably, recent experimental pieces of evidence shows that CGA has multiple neuroprotective [217] and neurotrophic activity [29]. For example, in vivo data demonstrated that CGA treatment improves survival of dopaminergic neurons and inhibits lipopolysaccharide (LPS)-induced microglial activation [32]; enhance transduction efficiency of PEP-1-rpS3 into CA1 region of the hippocampus and astrocytes [26]; reduce brain damage, brain edema and BBB damage by inhibiting matrix metalloproteinase (MMP)-2 and 9 expressions and antioxidant activities [218] as well as reduces neurological deficit score and cerebral infarction area in ischemia reperfusion injury rat models [128].

Previously, pharmacokinetic studies of CGA following oral [219], intravenous [220], intraperitoneal [221] administrations have been reported but any known study has not been performed to investigate the brain uptake and pharmacokinetics of CGA after intranasal (IN)

administration. The efficacy of CGA as a potent neuroprotectant has been demonstrated in *in-vivo* [222]*, in-vitro* [223] and *in-silico* [139] studies. Earlier, finding using *in vitro* BBB model suggest that CGA exhibits a very low rate of BBB permeation and cannot considered for a direct effect on the central nervous system [35]. Additionally, in the recent years, intranasal (IN) administration of the drug having biological effects on the CNS is gaining significant interest due to the importance of efficient drug delivery to the brain [224]. Nasal route is often considered as a safe and acceptable route of drug delivery for brain targeting and has some advantages including avoidance of hepatic first-pass metabolism and rapid absorption [225]. Intranasal drugs gain access to the brain via bypassing the blood-brain barrier (BBB) and direct brain delivery and utilizing the olfactory nerve tract via the olfactory epithelium [226]. Therefore, the present comparative study is designed to investigate the brain distribution and plasma pharmacokinetics profiles of CGA after IN and IV administration in Charles-Foster rats to evaluate whether the CGA molecules are transported directly via the nose-to-brain path through olfactory epithelium.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All experiments and surgical procedures were conducted as per the protocol for animal use and approved by the Central Animal Ethical Committee at Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration No. 542/02/ab/CPCSEA). Inbred Charles–Foster (CF) albino rats (250±30 g) were acclimatized for two weeks under conditions of 12-h light/dark cycle, constant humidity and controlled temperature (25±2 °C). Water and standard

pelleted diet were provided *ad libitum*. Animals have been starved of food for 8-10 hrs with free access to water before the experiment.

4.2.2 Chemicals and Reagents

CGA (99.4% pure) was purchased from Molekula (Molekula Limited, United Kingdom). Heparin sodium was obtained from Gland Pharma Ltd. Ultrapure water was obtained by a Milli-Q® water purification system (Merck Life Science Private Limited, India) and used for all preparations. Methanol and chromatographic reagents were purchased from TCI Chemicals (India) Pvt. Ltd. and Merck Life Science Pvt. Ltd. (India).

4.2.3 Preparation of CGA test formulations

For intravenous (IV) administration, CGA of strength 10 mg/ml, dose 10 mg/kg [218] and dose volume 1 ml/kg prepared by dispersing 50 mg CGA in 5 ml of 0.1 M phosphate buffer (PB; pH 7.4). For intranasal (IN) administration CGA of strength 125 mg/ml, dose 10 mg/kg and dose volume 80 µl/kg by dispersing 50 mg CGA in 400µl of 0.1 M phosphate buffer (PB; pH 7.4). The prepared dispersions were protected from light, vortexed for 30 seconds and immediately used for dosing.

4.2.4 Chromatographic system

The High-performance liquid chromatography (HPLC) system (Shimadzu Scientific Instruments, US) consisted of a chromatographic pump (LC-20AD), System Controller (CBM-20A), Column oven (CTO-20AC), Rheodyne injector and UV–Vis detector (SPD-20A); all operated at room temperature (24±1^{o}C). Data collection, calibration, and integration were done

using LC Solutions chromatography data analysis system. Kinetex Reversed Phase C_{18} column (250 x 4.6 mm, particle size 5 µm) was utilized for the separation. The mobile phase [227] consisted of acetonitrile and 0.2% aqueous phosphoric acid (10:90 v/ v); pH 3.0 adjusted with 0.2 g/ml sodium hydroxide at a flow rate of 2.5 ml/min. A Millipore filter system equipped with a 0.22-µm filter was used to filter the mobile phase prior using for the experiment. Further degassing was performed for 30 minutes immediately after filtration. CGA concentration was monitored at a wavelength of 327 nm throughout the experiments.

4.2.5 Standard and quality control (QC) samples

CGA stock solution (1 mg/ml) were prepared in methanol. The stock solution was further diluted using methanol to obtain working solutions of concentration (0.1, 10 and 500 µg/ml). The calibration standard solutions of concentrations (0.05-500 µg/ml) were prepared by diluting the working solution using blank plasma, brain and CSF samples. All these solutions were prepared daily and stored at 4ºC away from light. Further, quality control (QC) samples of high (500.00 µg/ml), medium (5.00 µg/ml), and low (0.05 µg/ml) concentrations were prepared to determine the accuracy (relative error, RE) and precision (relative standard deviation, RSD) of the HPLC method. The Limit of detection (LOD) and limit of quantification (LOQ) was calculated using linear regression of the calibration curve from the equation: $LoD = \frac{3SD}{b}$ and $LoQ = \frac{10SD}{b}$, where "SD" denotes the standard deviation of the response and "b" corresponds to the slope of the calibration curve [228]. After successful validation, the method was further implemented to determine the CGA levels in blood plasma, CSF, and brain of rats (both IV and IN groups).

4.2.6 Pharmacokinetic study

Eight (08) male Charles Foster rats were divided into two groups, i.e., intravenous (n=4) and intranasal (n=4). All animals were anesthetized using isoflurane during the experiment. For IV group (n=4) CGA was administered intravenously through tail vein at 10 mg/kg dose. For IN group (n=4) CGA was administered intranasally through nostril at 10 mg/kg dose (80 µl/kg of the CGA preparation). Each nostril of rat received 5 mg/kg dose of CGA dispersion via a hollow tubing (inserted upto10 mm) attached to a 25µl Hamilton syringe. Blood samples (0.2 ml) were successively collected from the retro-orbital plexus by using the heparinized capillary tube and kept in labeled tubes containing sodium heparin.

The following time intervals were used: 0 (pre-dosing), 1, 2, 5, 15, 30, 60,120, 240, 360, 480, and 1440 min after IV and IN administration. All animals were euthanized by a high dose of chloroform after completion of the study. The plasma was separated by centrifuging the obtained blood samples at 1400 RCF for 10 min at 4°C and transferred to labeled Eppendorf tubes for storage at −80°C till further analysis by HPLC.

4.2.7 Brain distribution study

Forty (40) male Charles Foster rats were divided into two groups, i.e., intravenous (20 rats/group) and intranasal (20 rats/group). Each group was further subdivided into five groups, i.e., 30 minutes (n=4), 60 minutes (n=4), 120 minutes (n=4), 240 minutes (n=4) and 360 minutes (n=4). Anesthesia and dosing were performed as discussed for pharmacokinetic study. CSF (≈50 µl) was collected in labeled tubes from the cisterna magna without making an incision in this region [229] and stored at −80°C. Serial samples of systemic blood (0.5 ml) were collected by cardiac puncture and placed in labeled tubes containing sodium heparin. The

brains were cautiously removed from the skull after the sacrifice by cervical dislocation and immediately rinsed with normal saline and used for the sample preparation for the HPLC analysis. The plasma was prepared and stored for further analysis by HPLC as discussed above for pharmacokinetic study.

4.2.8 Plasma, CSF and brain sample preparation Plasma and HPLC grade methanol was mixed in the ratio of 1:2 and vortexed thoroughly for 30 seconds. The mixture was then centrifuged at 800 RCF for 10 min at 4°C to obtain a clear supernatant. 10 µl of the collected supernatant was injected into the HPLC system for the determination of CGA concentration. CSF was collected from the cisterna magna and 10 µl volume was injected for HPLC analysis. The whole rat brain was homogenized in methanol (1:3 w/v) using a Dounce tissue grinder in an ice-cold bath. Subsequently centrifugation of the whole brain homogenate–methanol mixture was performed at 2100 RCF for 20 min at 4°C in a refrigerated centrifuge (Eppendorf Centrifuge 5418 R). The clear supernatant was aliquoted, and 10 µl was used for HPLC analysis.

4.2.9 Data analysis

The time to reach the maximum plasma concentration (t_{max}) and peak plasma concentrations (C_{max}) values were measured from the concentration–time data of plasma. Other pharmacokinetic parameters such as elimination half-life ($t_{1/2}$), the area under the curve from time zero to twenty-four hours ($AUC_{0\text{-}24hr}$), the volume of distribution (V_{ss}), mean residence time (MRT), and Total body clearance (Cl) were calculated by non-compartmental methods using software PK Solver.

The linear/logarithmic trapezoidal rule was used for calculating the area under the plasma concentration–time curve. Pharmacokinetic parameters and brain penetration data were expressed as the mean ± standard deviation (SD). The following equation was used to calculate the absolute bioavailability of CGA after IN administration:

$$Bioavailability\ F(\%) = \frac{AUC_{IN}}{AUC_{IV}}\ X\ \frac{Dose_{IV}}{Dose_{IN}}\ X\ 100$$

Statistical comparisons among intranasal and intravenous groups were performed with GraphPad Prism version 5 (San Diego, CA) using two-way analysis of variance (ANOVA) followed by Bonferroni posttests and unpaired t-test. A P-value below 0.05 was considered to indicate a significant difference.

4.3 RESULTS

4.3.1 Chromatography

The retention time of CGA was noted to be 6.75 minutes at 2.5 ml/min flow rate and 19.6 MPa pressure, and no co-eluting peaks were observed in the vicinity of the CGA peak on the chromatogram of spiked blank plasma (Fig. 4.1). A linear calibration curve (peak area vs. concentration) was obtained for the concentration range 0.05–500.000 µg/ml of CGA in rat plasma. The mean correlation coefficient (R) of the standard curve for plasma, brain and CSF were 0.9999, 0.9999 and 0.9997 respectively. The HPLC method parameters including LOQ; LOD; average percentage recovery of QC samples of CGA at three concentrations; relative error (%) and relative standard deviation (%) of lowest concentration (0.05 µg/ml) of plasma, brain and CSF are shown in Table 4.1.

Table 4.1 PK parameters of CGA after IN and IV administration to rats

PK parameter	IN	IV
Dose (Dose volume)	10mg/Kg (0.08 ml/kg)	10mg/Kg (1 ml/kg)
C_{max} (µg/ml)	128.140±31.332	442.498±29.217*
T_{max} (min)	1	1
AUC $_{0-24}$ (µg min/ml)	1803.355±297.463	7194.085±320.493*
$t_{1/2}$ (min)	579.649±118.296	448.897±113.834
MRT (min)	-	216.741±31.249
Cl (L/Kg/min)	-	0.001±0.000
Vss (L/Kg)	-	0.289±0.035
Bioavailability F[a] (%)	24.969±3.043	-

Data are presented as mean±S.D. (n= 4).
[a] Bioavailability F(%) calculated based on AUC from 0 to 24 hours.
* Significant difference (P<0.05)

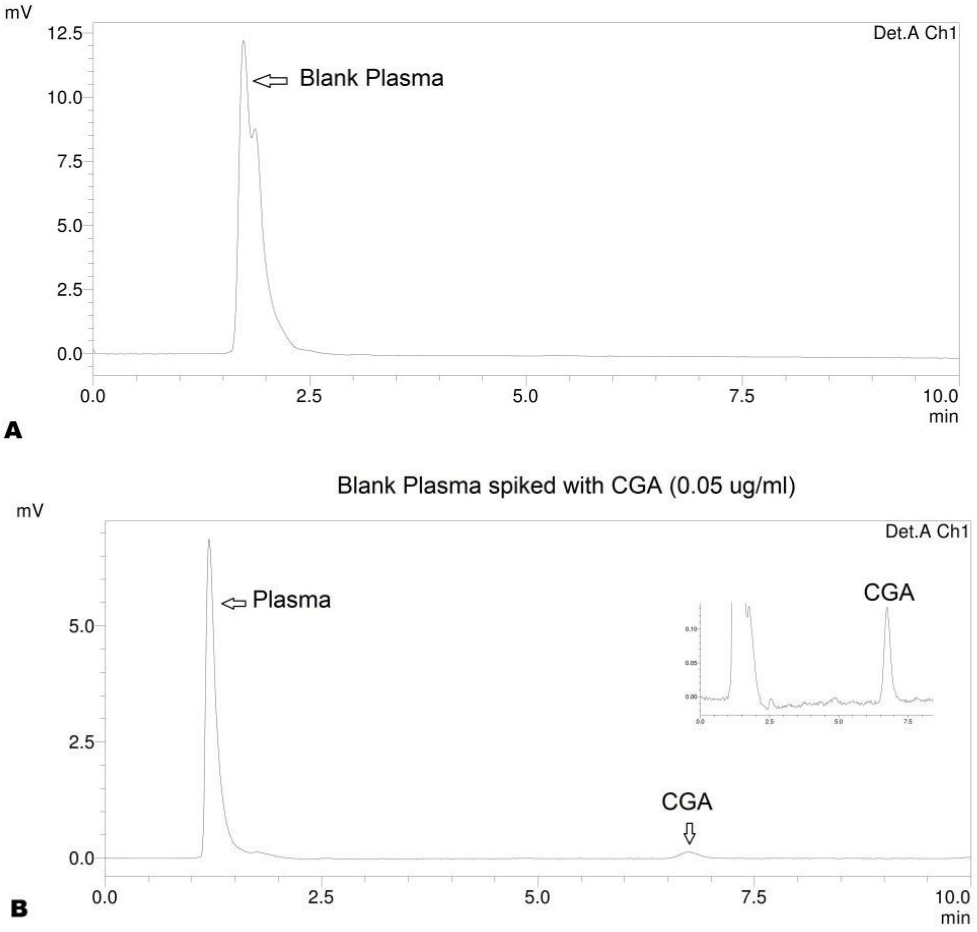

Fig. 4.1: Typical chromatogram of (A) Blank plasma and (B) Chlorogenic acid (Lowest QC concentration, 0.05 µg/ml) spiked in blank plasma at 327nm and 2.5 ml/min flow rate.

4.3.2 Pharmacokinetics of CGA after IN and IV administration

The mean plasma concentration vs. time curve of CGA following intranasal and intravenous administration in rats at single doses 10mg/kg are presented in Fig. 4.2. Two-way ANOVA shows that there are significant differences in the concentration-time profile at 1, 2, 5 and 15 minutes post dose of CGA obtained from intranasal and intravenous administration [$F(10, 66) = 117.4$, $p < 0.05$]. PK Solver software determined the resultant non-compartmental pharmacokinetic parameters which are summarized in Table 4.2. Further unpaired t-test analysis shows that there are significant differences in the pharmacokinetic parameters (C_{max} and AUC_{0-24}) obtained from intranasal and intravenous administration.

Table 4.2 HPLC method parameters for calibration, accuracy and precision of CGA

Sample	R	P Value	RSD (%) of Lowest QC sample Concentration (0.05 µg/ml) (N=6)		RE (%) of Lowest QC sample Concentration	Average Percentage recovery (%) of three QC samples	LOQ (µg/ml)	LOD (µg/ml)
			Intra-day	Inter-day				
Plasma	0.9999	<0.0001	5.3	6.7	6.4	96.26	0.055	0.016
CSF	0.9999	<0.0001	5.8	7.2	4.2	87.58	0.051	0.015
Brain Homogenate	0.9997	<0.0001	6.4	7.1	5.8	91.73	0.059	0.018

R: Correlation Coefficient; RSD: Relative Standard Deviation; RE: Relative Error; LOD: Limit of Detection

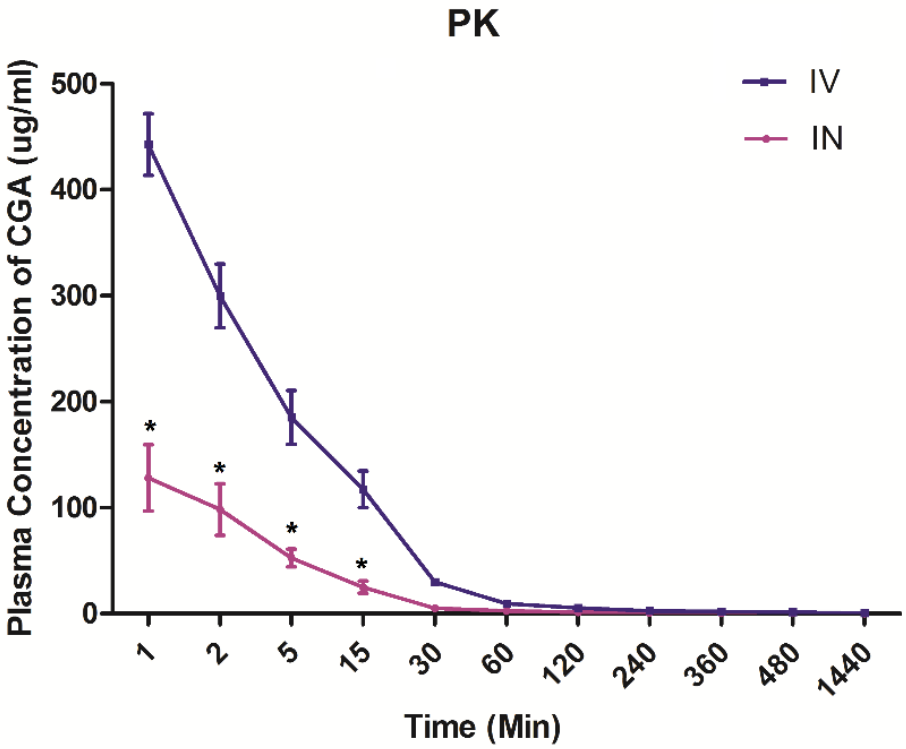

Fig. 4.2 Mean plasma concentration–time plot of CGA after IN and IV administration of 10 mg/kg doses in rats. Data present in the mean ± S.D. (n= 4). *Significant difference ($p < 0.05$)

4.3.3 Brain penetration of CGA

The distribution of CGA in brain tissue was assessed from the ratio of CGA concentration in brain homogenate over the CGA plasma concentration (C_b/C_p). CGA concentration in brain [$F(4, 30) = 64.39$, $p < 0.05$] at time points 30, 60, 120 and 240 minutes as well as in CSF [$F(4, 30) = 3.860$, $p < 0.05$] and Cb/Cp [$F(4, 30) = 16.88$, $p < 0.05$] at time points 30, 60, 120, 240 and 360 minutes, increased significantly in intranasal group compared to intravenous group after two-way ANOVA followed by Bonferroni posttests (Fig. 4.3). CGA concentration in systemic plasma at time points 30, 60 and 120 minutes, decreased significantly in IN group compared to IV group. The exposure of CGA ($AUC_{0\text{-}6\ hr}$) in the brain and CSF were significantly higher in IN route compared to IV route (Table 4.3). The drug targeting efficiency

(DTE) of CGA in the brain was assessed from the ratio of exposure in whole brain homogenate over the systemic blood plasma, i.e., $AUC_{brain}/AUC_{systemic\ plasma}$. $AUC_{brain}/AUC_{systemic\ plasma}$ values was 27 times higher in IN group compared to IV group. The exposure of CGA ($AUC_{0\text{-}6\ hr}$) in systemic plasma were significantly higher in IV administration compared to IN administration (Table 4.3).

Table 4.3: Exposure of single CGA dose of 10mg/kg at 30, 60, 120, 240 and 360 minutes post administration

AUC	IN	IV
$AUC_{systemic\ plasma}$ (µg min/ml)	596.94±18.75*	4106.79±579.57
AUC_{CSF} (µg min/ml)	1963.27±108.35*	1019.46±159.11
AUC_{brain} (µg min/ml)	31944.85±3808.71*	7977.65±1288.06
$AUC_{brain}/AUC_{systemic\ plasma}$	53.58 ±6.76*	1.97 ±0.40

Data are presented as mean±S.D. (n= 4).
* Significant difference ($P<0.05$)

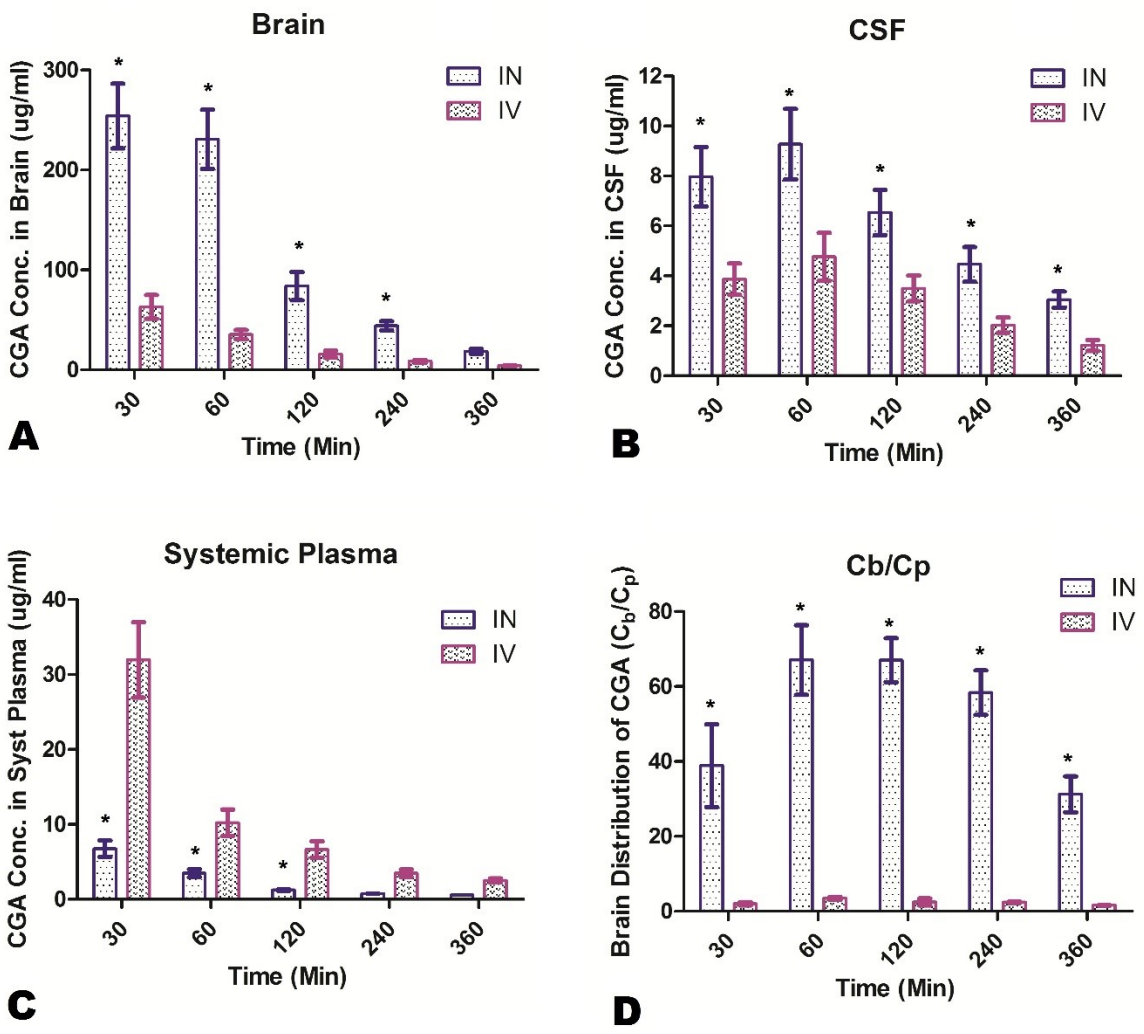

Fig. 4.3 Brain Penetration data of CGA following intravenous and intranasal administration at single doses (10mg/kg); (A) Brain, (B) CSF, (C) Systemic Plasma and (D) C_b/C_p. Data are presented as mean±S.D. (n= 4). * Significant difference ($P<0.05$)

4.4 DISCUSSION

The use of intranasal route for drug administration to deliver neurotherapeutics to the CNS is beneficial for the treatment of stroke and neurodegenerative disorders. Several neurotherapeutic drugs previously discovered has been rendered inefficient for ameliorating memory dysfunction and neurodegeneration due to the obstacle imposed by BBB, limiting the drug from entering the CNS [224]. There are several invasive methods such as intra-cerebral

implantation, interstitial delivery, BBB disruption (BBBD) strategies and biological tissue delivery which are used to deliver the drug into the brain by bypassing the BBB. Limitations of use of these methods in humans include safety, convenience, cost, and ethics related issues. To overcome such restrictions, drug delivery via nasal cavity proves to be an efficient alternative for delivering the neurotherapeutics directly into the CNS overcoming the obstacle posed by BBB [230]. Noninvasive administration through intranasal cavity targets neurotherapeutics to the brain, reducing systemic exposure as well as its side effects. For IN delivery, chemical modification of neurotherapeutic drugs is not required to target the CNS although delivery via this route to the brain is fast, occurring within few minutes [231]. Moreover, the drugs administered through intranasal cavity immediately reach to the brain via the olfactory and trigeminal neural path because nasal cavity is innervated by olfactory and trigeminal nerves that provide a direct connection with the CNS [232].

The PK results demonstrate that the pharmacokinetic parameters of IN group including C_{max} and AUC_{0-t} were significantly reduced indicating reduced peripheral exposure of CGA in the plasma. The plasma C_{max} following IV administration was 3.5 times higher compared to that after IN delivery. Further, the time to reach maximum plasma concentration for IN and IV administration of CGA was found to be 1 minute. The bioavailability of the CGA was found to be 24.97% by the AUC_{0-24h} values calculated after IN delivery of the CGA (10mg/kg dose). The plasma AUC_{0-24h} following IV administration was 4 times higher compared to IN delivery which represents a larger amount of CGA exposure in the body when administered through IV route. There was no significant difference ($P<0.05$) observed in T_{max} and $t_{1/2}$ followed by IV and IN administration.

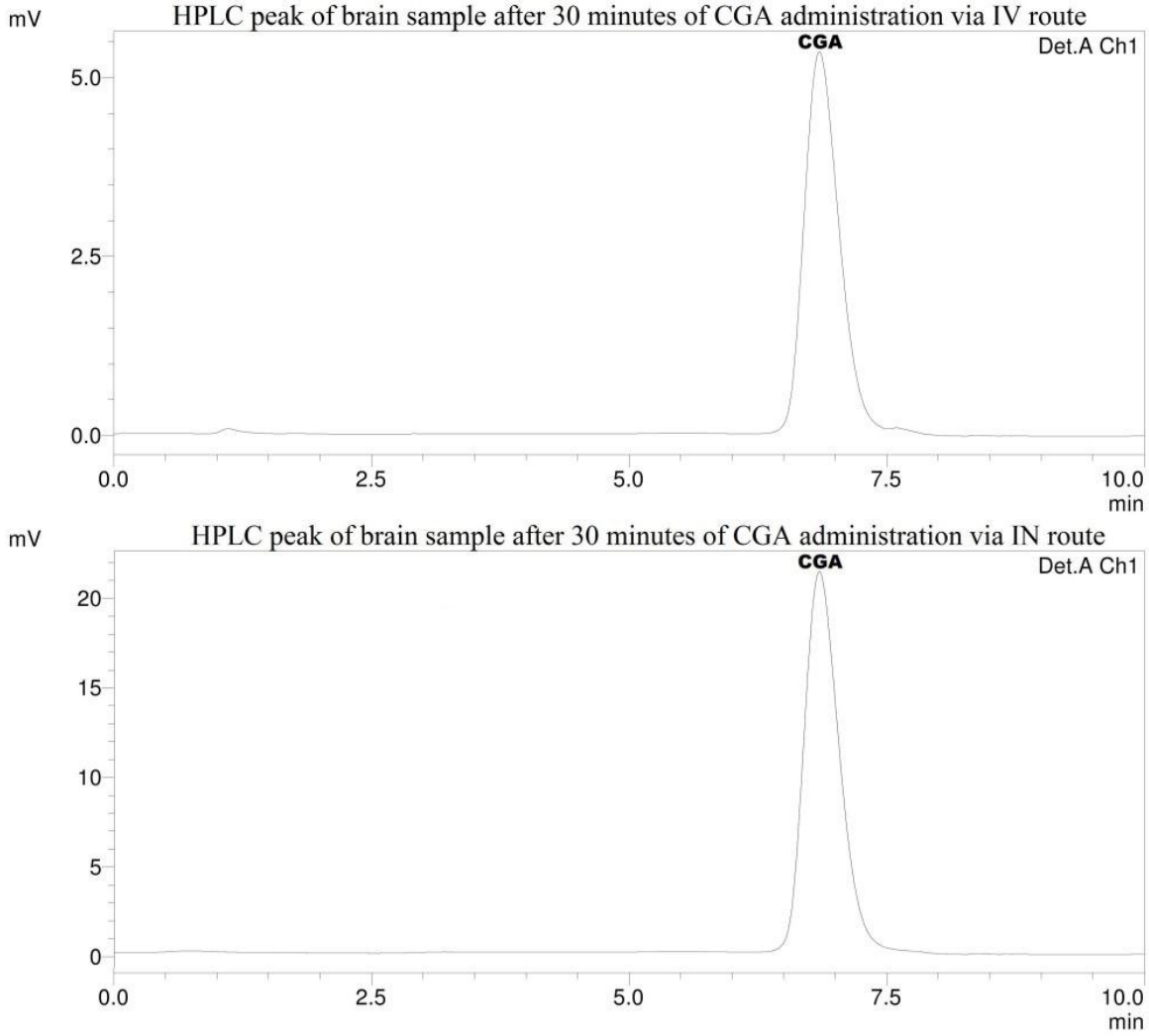

Fig. 4.4: HPLC peaks of brain sample after 30 minutes of CGA administration via IV and IN route.

The brain uptake study was performed to investigate the role of nasal CGA delivery into the brain and CSF after IN administration. For evaluating the effect of CGA on CSF and brain distribution and its nasal systemic absorption, we concurrently determined the CGA levels in systemic plasma, CSF and whole brain after IN and IV administration. Fig. 4.4 shows the HPLC peaks of brain sample for both IV and IN route after 30 minutes of CGA administration. Results of the brain penetration study revealed that the concentration of CGA in the brain, CSF and Cb/Cp shows a significant increase in IN group compared to IV group indicating significant CNS exposure of CGA in the brain when administered by the intranasal route. In

addition, the concentration of CGA in the brain at 30, 60, 120, 240, and 360 minutes was 4, 6.5, 5.3, 5.2, and 4.5 times higher respectively, compared to the values obtained after IV administration reflecting higher brain intake of CGA through nasal route.

As shown in Table 4.3, when single dose of CGA was administered through IN and IV route, the significant difference observed between $AUC_{sytemic\ plasma,IV}$ and $AUC_{sytemic\ plasma,IN}$ indicates higher peripheral exposure of CGA with IV administration. Further, the exposure of CGA in the brain after IN administration ($AUC_{brain,\ IN}$) was significantly greater (4 times) as compared to the exposure of CGA in the brain ($AUC_{brain,\ IV}$) after IV administration. Similarly, the exposure of CGA in the CSF after IN administration ($AUC_{CSF,\ IN)}$ was significantly greater (1.9 times) as compared to the exposure of CGA in the CSF ($AUC_{CSF,\ IV}$) after IV administration. These results indicate higher CNS exposure with limited peripheral exposure of CGA following intranasal administration. Fig. 4.5 briefly depicts the findings of pharmacokinetics and brain uptake of CGA after IN and IV administration.

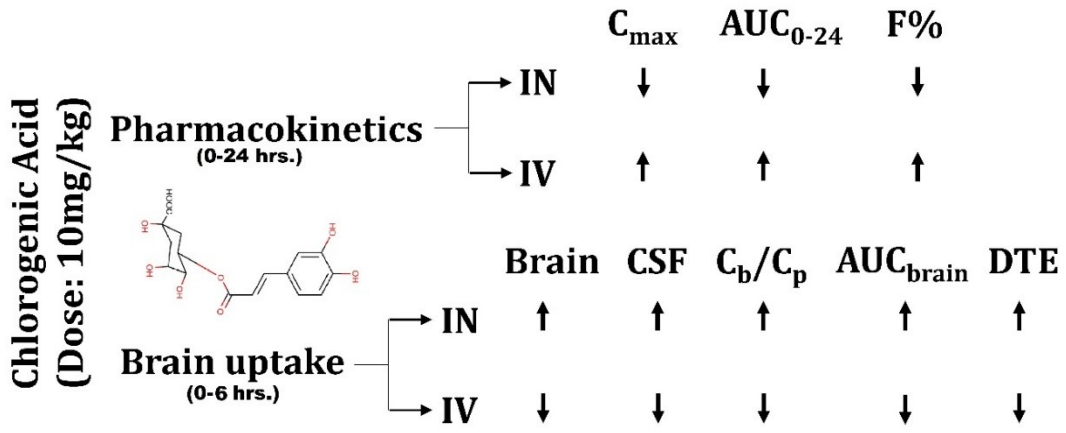

Fig. 4.5 Summary of PK and brain uptake analysis of CGA at single dose (10 mg/kg)

The results revealed that CGA is rapidly available into the systemic circulation (T_{max} = 1 min) and brain (T_{max} = 30 minutes) after IV and IN administration. Also after IN administration

CGA enter freely into the brain tissues, transporting via nasal cavity-to-brain pathway as the major means of transportation route. To determine whether CGA is transported to the brain via a direct nasal cavity-to-brain transport pathway, the drug targeting efficacy was calculated from the brain uptake data obtained after IN and IV delivery of CGA in Charles Foster rats. The CGA targeting efficiency is defined as the ratio of $AUC_{brain/}AUC_{sytemic\ plasma.}$ In rats, the $AUC_{brain/}AUC_{sytemic\ plasma}$ value (53.58 ±6.76) of CGA after IN administration was 27 times higher than the $AUC_{brain/}AUC_{sytemic\ plasma}$ value (1.97 ±0.40) obtained after IV administration. Higher exposure of CGA in brain tissue along with significantly greater drug targeting efficiency (DTE= $AUC_{brain/}AUC_{plasma}$) for the IN route of delivery as compared to IV administration of CGA demonstrates the nasal route to be a significant contributing factor in the direct provision of the drug from the nasal cavity to the CNS. Thus, CGA finds an increased opportunity of contact with the olfactory mucosa leading to higher brain penetration and $AUC_{brain/}AUC_{systemic\ plasma}$ ratio.

4.5 CONCLUSION

Rapid absorption of CGA along with higher exposure in Charles Foster rat brain following IN administration was revealed from brain distribution and PK studies. Also, the brain distribution studies suggest that nasal route mediates direct nose-to-brain transportation of CGA efficiently as compared to IV administration. Increased penetration and high exposure of the CGA in the brain tissue as well as significantly higher drug targeting efficiency (DTE= $AUC_{brain/}AUC_{plasma}$) were found in IN group as compared to the IV group. These studies prove that IN delivery system of CGA can be a promising approach for use in the treatment of stroke and neurodegenerative disorders.