Chapter 2

Evaluation of pharmacokinetic and pharmacodynamic properties of I3C in MCAO rats

2 Introduction

Indole-3-Carbinol (I3C) is a primary glucobrassicin breakdown product found in cruciferous vegetables (Aggarwal & Ichikawa, 2005). Conventionally, I3C was administered through the oral route and reported immunomodulation (Exon, South, Magnuson, & Hendrix, 2001), nephroprotection (El-Naga & Mahran, 2016), cardioprotection (Ramakrishna & Krishnamurthy, 2022), hepatoprotection (Garikapaty et al., 2005), and neuroprotection (El-Naga et al., 2014). I3C intravenous (i.v.) and intraperitoneal (i.p.) injections exhibited anticancer activity (Garikapaty et al., 2005), inhibited liver fibrosis (Ping, Gao, Xu, Li, & Wang, 2011), and prostate tumor growth (Souli, Machluf, Morgenstern, Sabo, & Yannai, 2008). Recently, oral treatment of I3C has been reported to exhibit neuroprotection against middle cerebral artery occluded (MCAO) rats (P. Paliwal et al., 2018). To ischemic stroke patients, instantly, rtPA is administered intravenously. Injectable or oral anticoagulants are utilized as first-line therapy in ischemic stroke patients resistant to rtPA (Bansal et al., 2013). In agreement with the stroke treatment academic industry roundtable (STAIR) (Fisher et al., 2009), I3C possesses antiplatelet and antithrombotic (Park, 2008; Ramakrishna & Krishnamurthy, 2022) activity to exhibit neuroprotection in ischemic stroke. However, there is no data on the pharmacokinetics of I3C to rationalize the route and dose for the treatment of ischemic stroke.

I3C and its acid condensation products, notably 3, 3-diindolylmethane (DIM), are found in the plasma and other tissues within 15 minutes of oral consumption (Anderton et al., 2003; Anderton et al., 2004; De Kruif et al., 1991; Moussata, Wang, & Wang, 2014; Shertzer & Senft, 2000). It was believed that I3C protective effects are may due to high levels of DIM in plasma (Anderton et al., 2004) and the nucleus (Staub et al., 2002). Therefore, I3C and DIM

pharmacokinetics determination may provide clear discrimination of therapeutic effects of I3C and DIM. Further, the lack of pharmacokinetic data of test compounds in preclinical studies is one of the main reasons for the failure of clinical studies for stroke treatment (Y. Li et al., 2016). The unavailability of I3C pharmacokinetic and pharmacodynamic data after intravenous injection is limiting its use in treating cancer and other diseases. Given the importance of pharmacokinetics and pharmacodynamics in stroke treatment, we evaluated these pharmacokinetics and pharmacodynamics of I3C simultaneously to discover the most effective delivery route for protecting the brain from ischemia injury. Consequently, the objectives of this study were to determine the pharmacokinetics and brain penetration of I3C in sham and MCAO group rats with oral and intravenous administration and to compare the pharmacological activities of I3C (50 mg/kg) with oral and intravenous administration in MCAO rats.

2.1 Materials and methods

2.1.1 Chemicals

Indole-3-carbinol (Sigma Aldrich, USA), diindolylmethane (TCI chemicals, Japan), high-performance liquid chromatography (HPLC) grade water (Merck), acetonitrile (Merck India), dimethyl sulfoxide (DMSO) (Merck, India), methanol (Merck, India), thiopentone sodium (Neon laboratories, India), 0.45µm filters (Rankem laboratories, India), dichlorvos (Krushikendra, India), Evans blue (EB) (Sigma Aldrich, USA), and triphenyl tetrazolium chloride (TTC) (Sigma Aldrich, USA) were obtained.

2.1.2 Experimental animals and drug treatment

Adult healthy male Wistar rats (240–260 gm, 12-13 weeks old) were obtained from the IMS, Banaras Hindu University, Varanasi, India. Rats were kept in laboratory conditions, with free access to food and water and a 12-hour light-dark cycle. Before the study began, the Institutional

Animal Ethics Committee approved the experimental procedures (protocol no. Dean/2016/CAEC/31). A two weeks acclimatization period was given before the experiments. The sample size was calculated using a preliminary G* power analysis. The experiment was divided into two parts: evaluation of the I3C pharmacokinetics (total number of animals 150) and determination of pharmacological activity on stroke animals (total number of animals 54) (figure 2.1). Animals involved in the pharmacokinetic studies are randomly divided (standard = RAND () function in Microsoft Excel) into the following groups; sham (oral I3C), sham (intravenous (i.v.) I3C), MCAO + oral I3C, MCAO + I3C i.v. (n=36 for each group). All the animals were kept overnight fasting before surgical procedures. I3C was dissolved in DMSO and administered orally to the experimental groups. The total DMSO concentration was not exceeded 0.5 % (v/v). In the pharmacological investigation, I3C was administered just before the filament removal, and a single dose of I3C per day was administered for each 24 hours. An equal voulme of DMSO was adminsitred to the MCAO rats. Six animals have died during or after MCAO surgery. The remaining animals (144) were used to determine the pharmacokinetics of I3C at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hr after I3C administration (n=4 for each time point). The dose of I3C of 50 mg/kg was chosen from the previous study (P. Paliwal et al., 2018). Animals were anesthetized with thiopentone sodium (40 mg/kg, intraperitoneal) (Ramakrishna, Srinivasan, & Sharma, 2021). Blood was collected from the retro-orbital plexus in heparinized centrifuge tubes. The animals were then euthanized, and tissues (heart, liver, brain, and lungs) were collected at above mentioned time points. The plasma and tissue homogenates were used for I3C and DIM quantification. The rats that died during or after MCAO surgery were excluded from the study and data analysis. The complete experimental protocol was illustrated in figure 2.2.

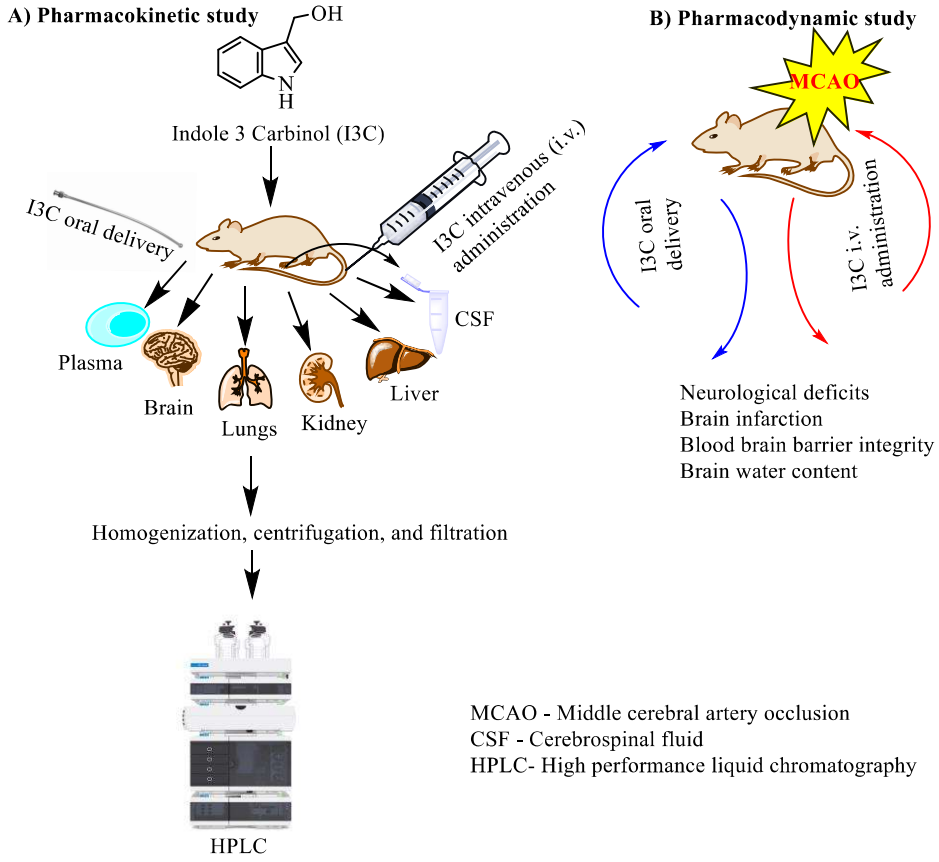

Figure 2.1. Objectives of the study.

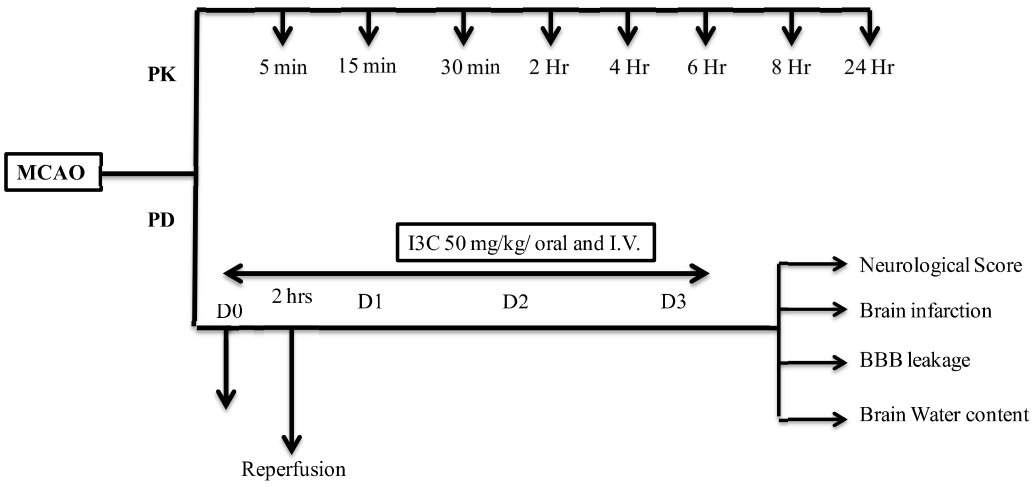

Figure 2.2. Study design. D-day, MCAO- middle cerebral artery occlusion, I3C-indole-carbinol, PK-pharmacokinetics, PD-pharmacodynamics, BBB-blood brain barrier, I.V.-intravenous.

2.1.3 Preparation of standard solutions

The stock solution was prepared by dissolving 1 mg of I3C and DIM in acetonitrile. Then the stock solution was diluted to the working solution range of 0.05 to 125 µg/ml. A stock solution of dichlorvos (8 mg/ml (in methanol)), was added to plasma and tissue samples to prevent I3C oxidation during the storage (Moussata et al., 2014).

2.1.4 Preparation of calibration and quality control samples

The blank plasma and tissue homogenates were used to make the calibration and quality control (QC) samples. The plasma was obtained by centrifuging the blood at 4000rpm/10min/4°C from untreated animals. The tissue homogenates were prepared by homogenizing fresh tissue obtained from untreated animals. Calibration samples at 0.05, 1, 5, 25, and 125 µg/ml for I3C and DIM were prepared by spiking a specific volume of corresponding working solutions to 300µl of blank plasma/tissue homogenates containing 10µl of dichlorvos (80µg/ml). QC samples were prepared at 0.05, 5, and 125 µg/ml concentrations for I3C and DIM.

2.1.5 Extraction of I3C and DIM

The I3C and DIM from the plasma and other tissue homogenate samples were extracted according to previous reports with modifications (Moussata et al., 2014). Briefly, plasma (100µl) samples were mixed with dichlorvos (10µl) and vortexed for 15 sec. The tissues were thawed and weighed into plastic tubes and ice-cold phosphate buffer saline (PBS, pH 7.4) was added to homogenise the samples. Dichlorvos (10µl) was added to 100µl of homogenate and vortexed for 15 sec. Then, to plasma and tissue homogenates, 15 µl of 70% acetonitrile was added and vortexed for 1 min. Finally, these samples were centrifuged at 13,000rpm/10min/4°C and obtained supernatant was passed through 0.45µm filters. A 5µl of filtered solution was injected into the HPLC system.

2.1.6 HPLC system

The HPLC method for the quantification of I3C and DIM was developed and validated using Agilent Technologies HPLC, Infinity 1260 II, consisting of a degasser, a quaternary pump, and an autosampler. The system includes a photodiode array (PDA) detector, a computer running Agilent openLAB CDS software for data acquisition and processing. The chromatographic separation was achieved using a C8 column (4.6 X 250 mm, 5µm). The analytes I3C and DIM were quantified by a PDA at 280 nm. The mobile phase consisted of water (A) and acetonitrile (B) (30:70). The isocratic elution was maintained at a 1 ml/min flow rate for 6 min.

2.1.7 Pharmacokinetics

Maximum plasma concentrations (Cmax) and time to reach the maximum plasma concentration (Tmax) values were obtained directly from the plasma concentration-time curves of I3C. PK Solver software (Brand, Orr, Perevoshchikova, & Quinlan, 2013) was used to measure the pharmacokinetic parameters, including area under the curve (AUC), mean residence time (MRT), half-life (t1/2), volume of distribution (Vz), clearance (CL). Bioavailability was calculated as follows (equation 1):

$$\text{Bioavailibility}(\%) = \text{AUC (oral)} \times \text{Dose (i.v.)} \div \text{AUC (i.v.)} \times \text{Dose (oral)} \times 100$$

2.1.8 Middle cerebral artery occlusion and reperfusion injury

The experimental animals (n=54) were randomly divided into sham, MCAO, MCAO + I3C 50 mg/kg (oral), and MCAO + I3C 50 mg/kg (intravenous) using standard = RAND() function in Microsoft Excel. The right MCAO method of focal ischemia was induced in rats. General anesthesia in rats was induced by injecting thiopentone sodium (40 mg/kg/intraperitoneal) (Ramakrishna et al., 2021). A middle neck incision was made and the right common carotid artery, external (ECA) and internal carotid arteries (ICA) were separated. The external carotid

artery was ligated at both ends and cut between the ligations. A nylon monofilament (3-0, 18-23 mm) was inserted into the ECA and forwarded through ICA to block the MCA. After the occlusion, the monofilament was ligatured, and the incision was sutured. The monofilament was removed after 2 hours and blood flow was resumed to the MCA region, and the incision was closed. In sham animals, monofilament was inserted and removed immediately (Belayev, Alonso, Busto, Zhao, & Ginsberg, 1996a; Longa et al., 1989). The body temperature was maintained at 37 °C by using a rectal probe and a heating pad (RWD Life Sciences, China). The proper post-operative care was given to all groups of animals. I3C was administered before the reperfusion and treatment continued for up to 72 hours. Six animals died during or after MCAO surgery and the remaining animals (n=48) were used for the determination of neurological deficits (Longa et al., 1989), brain infarction (Shah et al., 2019), blood-brain barrier integrity (W. Zhang et al., 2016), and brain water content (Maleki, Aboutaleb, & Souri, 2018) as per earlier reports.

2.1.9 Neurological deficits

Neurological deficits were counted on five points after 22 h of reperfusion, as reported earlier. 0 = No neurological deficit, 1= failure to extend right paw fully, 2 = circling if pulled by the tail, 3=spontaneous circling, 4 = did not walk spontaneously and had a depressed level of consciousness (Longa et al., 1989).

2.1.10 Brain infarction

The rats were euthanized, and brain tissues were collected frozen at − 20 °C for 20-30 min and 2 mm-thick coronal sections were made using the brain matrix. After that, slices were immersed in triphenyl tetrazolium chloride (TTC, 2%) solution in normal saline for 15-20 min at 37 °C. Uniform staining was achieved by flipping the slices after 7 min anteriorly and posteriorly. After

that, brains were fixed in 10% formalin solution, and images were captured within 48 hrs of incubation. ImageJ software was used to measure the infarction area. The total infarct area was calculated by the sum of the infarct area of all slices. Brain edema was known to alter the actual infarct area and obtained infarct area was corrected. The corrected infarct area was calculated as follows: Corrected infarct area =contralateral hemisphere area – (ipsilateral hemisphere area – infarct area) (Shah et al., 2019).

2.1.11 Evaluation of blood brain barrier integrity

Blood-brain barrier (BBB) integrity was determined using the Evans blue extravasation method. Evans blue (2%, 4 mL/kg) dye solution was administered intravenously. After four hours of infusion, animals were perfused with normal saline, and brains were isolated. Brains slices were made and soaked in formamide (10 ml/kg) solution for 48 hrs. After that, brain slice images were captured, and then brains were homogenized and centrifuged at 14,000 rpm for 30 min. The collected supernatant was subjected to measure the absorption at 620 nm. The EB content was calculated as μg/g of brain tissue using a standardized curve (W. Zhang et al., 2016).

2.1.12 Brain water content

A wet/dry method was used for the determination of the brain water content. Briefly, rats overdosed with thiopentone sodium and brains were collected. Carefully, the brainstem, cerebellum, and olfactory bulb were removed. After that, brains were divided into ischemic (ipsilateral hemisphere) and non-ischemic (contralateral hemisphere) hemispheres. The ipsilateral hemisphere weighed (wet weight) and placed in a hot air oven for 24 hrs at 110 °C and again weighed (Dry weight). The brain water content was calculated using the following formula (wet weight - dry weight)/wet weight × 100 (Maleki et al., 2018).

2.1.13 Data Analysis

Pharmacokinetic parameters and brain penetration data are expressed as mean ± SD. Graph-Pad Prism version 5 (San Diego, CA, USA) was used for data analysis. Plasma, brain, and CSF concentrations of I3C and DIM were analysed by two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. PK Solver software (Brand et al., 2013) was used to measure the pharmacokinetic parameters, such as the area under the curve (AUC), mean residence time (MRT), half-life (t 1/2), volume of distribution (Vz), clearance (Cl). Neurological deficits, brain infarction, BBB permeability, and brain water content were analysed by one-way ANOVA followed by Tukey's test post hoc test. A Student's t-test was used to analyse the pharmacokinetic parameters. A level of $p < 0.05$ was considered statistically significant.

2.2 Results

2.2.1 Linearity and sensitivity

Plasma and tissue homogenate standard calibration curves were found to be linear with the correlation coefficients (r2) >0.998. LOD and LOQ were found to be 0.029 µg/ml, and 0.031 µg/ml for I3C and DIM. LLOQ of I3C and DIM was identified as 0.04 µg/ml.

2.2.2 Precision and accuracy

Precision and accuracy are illustrated in table 2.1. We have observed the intra-day accuracy values were 98.3-101.2% and 97.61-100% for I3C and DIM, respectively, and inter-day accuracy values ranged from 95.7–100%, 96.3–100% for I3C and DIM, respectively. Percentage of relative standard deviation (RSD%) was calculated to represent the precision. The overall RSD% of I3C and DIM was ranged from 4.4-12.3% (I3C) and 4.7-11.8% (DIM) for intra-day, 5.3-9.8 % (I3C) and 3.8–10.4% (DIM) for inter-day precision.

		0.05 µg/ml		5 µg/ml		125 µg/ml	
Plasma	**Intra-day variation**	**I3C**	**DIM**	**I3C**	**DIM**	**I3C**	**DIM**
	Cal. Conc.	0.047	0.046	4.83	4.79	124.09	123.667
	SD	0.001	0.002	0.23	0.19	1.168	1.98127
	Precision (%)	3.620	4.561	4.76	4.037	0.94142	1.60211
	Accuracy (%)	94.4	93	96.69	95.8	99.272	98.9333
	Inter-day variation						
	Cal. Conc.	0.04767	0.0485	4.81467	4.81467	122.22	122.997
	SD	0.00153	0.00071	0.1978	0.13632	0.6449	2.5305
	Precision (%)	3.2046	1.45795	4.10825	2.83144	0.52766	2.05737
	Accuracy (%)	95.3333	97	96.2933	96.2933	97.776	98.3973
Brain	**Intra-day variation**						
	Cal. Conc.	0.049	0.04667	4.958	4.80533	123.14	122.36
	SD	0.001	0.00208	0.0518	0.19869	1.63022	3.21977
	Precision (%)	2.04082	4.46071	1.04473	4.13471	1.32388	2.63139
	Accuracy (%)	98	93.3333	99.16	96.1067	98.512	97.888
	Interday variation						
	Cal. Conc.	0.04633	0.04633	4.94133	4.77933	122.69	122.823
	SD	0.00153	0.00115	0.05132	0.14674	0.55462	2.08246
	Precision (%)	3.29682	2.49216	1.03851	3.07028	0.45205	1.69549
	Accuracy (%)	92.6667	92.6667	98.8267	95.5867	98.152	98.2587
Lungs	**Intra-day variation**						
	Cal. Conc.	0.04733	0.047	4.91067	4.807	124.453	121.303
	SD	0.00153	0.002	0.10542	0.20829	0.67241	3.04484
	Precision (%)	3.22717	4.25532	2.14685	4.33297	0.54029	2.5101
	Accuracy (%)	94.6667	94	98.2133	96.14	99.5627	97.0427
	Inter-day variation						
	Cal.Conc.	0.04867	0.04533	4.559	4.899	122.033	122.447
	SD	0.00058	0.00208	0.27185	0.15589	2.57016	1.53184
	Precision (%)	1.18634	4.59191	5.96283	3.18203	2.10611	1.25103

	Accuracy (%)	97.3333	90.6667	91.18	97.98	97.6267	97.9573
Liver	**Intra day variation**						
	Cal.Conc.	0.048	0.04567	4.879	4.74433	123.063	120.813
	SD	0.00265	0.00351	0.16523	0.06615	2.74835	0.71598
	Precision (%)	5.51198	7.69026	3.3865	1.39438	2.23328	0.59264
	Accuracy (%)	96	91.3333	97.58	94.8867	98.4507	96.6507
	Inter day variation						
	Cal. Conc.	0.04867	0.04767	4.88467	4.726	123.39	122.773
	SD	0.00058	0.00153	0.09224	0.26309	2.78216	2.76386
	Precision (%)	1.18634	3.2046	1.88837	5.56689	2.25477	2.25119
	Accuracy (%)	97.3333	95.3333	97.6933	94.52	98.712	98.2187
Kidney	**Intra-day variation**						
	Cal.Conc.	0.04933	0.04533	4.80333	4.761	121.94	122.233
	SD	0.00058	0.00153	0.17793	0.20103	2.62429	2.35069
	Precision (%)	1.1703	3.36954	3.7042	4.22243	2.15212	1.92312
	Acuracy (%)	98.6667	90.6667	96.0667	95.22	97.552	97.7867
	Inter-day variation						
	Cal. Conc.	0.04633	0.04633	4.78167	4.709	124.687	122.407
	SD	0.00306	0.00115	0.12716	0.1815	0.45884	1.96187
	Precision (%)	6.59363	2.49216	2.6593	3.85437	0.36799	1.60275
	Accuracy (%)	92.6667	92.6667	95.6333	94.18	99.7493	97.9253

Table 2.1: Precision and accuracy of I3C and DIM. Calculated concentration (Cal.conc.), standard deviation (SD). All precision and accuracy values run in triplicates (n=3)

2.2.3 Stability studies

The stability of QC samples of I3C and DIM are depicted in table 2.2. QC samples of I3C and DIM were stable in plasma and tissue homogenates and fall within ±15%. The overall stability of the I3C and DIM was ranged from 90 to 99 % (table 2).

		Plasma			Brain			Lungs			Liver			Kidney		
Benchtop	**Known conc.**	**Cal. Conc.**	**Pre (%)**	**Acc (%)**	**Cal. Conc.**	**Pre (%)**	**Acc (%)**	**Cal. Conc.**	**Pre (%)**	**Acc (%)**	**Cal. Conc.**	**Pre (%)**	**Acc (%)**	**Cal. Conc.**	**Pre (%)**	**Acc (%)**
I3C	0.05	0.05	1.27	90.67	0.05	2.04	98.00	0.05	2.34	98.67	0.05	2.08	96.00	0.05	1.19	97.33
	5.00	4.88	1.16	97.77	4.81	3.71	96.13	4.91	0.74	98.18	4.80	2.78	96.01	4.78	4.81	95.53
	125.00	124.17	0.57	99.34	123.44	1.64	98.75	124.19	1.06	99.35	123.39	2.26	98.71	123.36	2.27	98.69
DIM	0.05	0.05	2.04	98.00	0.05	4.63	90.00	0.05	4.17	96.00	0.05	1.19	96.67	0.05	2.08	96.00
	5.00	4.96	0.10	99.27	4.59	3.97	91.81	4.77	3.42	95.35	4.91	2.11	98.15	4.75	6.14	95.09
	125.00	124.85	0.14	99.88	124.10	0.82	99.28	124.50	0.38	99.60	123.48	1.99	98.78	123.63	1.12	98.90
Autosampler																
I3C	0.05	0.05	2.17	92.00	0.05	2.46	94.00	0.05	4.34	94.53	0.05	4.49	92.67	0.05	1.19	96.67
	5.00	4.86	0.98	97.22	4.60	1.65	91.96	4.84	1.96	96.79	4.63	3.06	92.69	4.76	1.89	95.20
	125.00	124.30	0.73	99.44	122.18	1.15	97.75	123.29	2.07	98.63	124.81	0.15	99.85	122.88	2.03	98.31
DIM	0.05	0.05	4.49	92.67	0.05	1.28	90.00	0.05	2.34	98.67	0.05	3.20	95.33	0.05	6.74	95.33
	5.00	4.77	5.41	95.31	4.68	1.54	93.68	4.92	1.39	98.31	4.78	2.43	95.61	4.67	2.15	93.38
	125.00	123.14	1.98	98.51	123.77	1.24	99.01	124.81	0.13	99.85	122.73	1.72	98.19	122.69	2.17	98.15
Freeze thaw																
I3C	0.05	0.05	4.35	92.00	0.05	6.49	92.67	0.05	2.42	95.33	0.05	5.43	92.67	0.05	5.17	97.33
	5.00	4.65	1.49	92.98	4.67	5.75	93.31	4.72	4.80	94.38	4.70	2.32	94.04	4.66	2.80	93.17
	125.00	123.02	1.65	98.41	122.26	1.48	97.80	122.59	2.13	98.08	124.09	0.56	99.28	123.22	2.04	98.58
DIM	0.05	0.05	4.17	96.00	0.05	2.41	96.00	0.05	2.10	95.87	0.05	1.22	94.67	0.05	3.61	96.00
	5.00	4.81	2.79	96.21	4.68	4.47	93.55	4.86	3.17	97.22	4.81	4.67	96.29	4.73	2.29	94.64

	125.00	124.66	0.32	99.72	124.07	0.07	99.25	123.15	1.75	98.52	124.13	0.50	99.30	121.46	1.97	97.17
Long term																
I3C	0.05	0.05	3.37	90.67	0.05	3.32	92.00	0.05	2.39	96.67	0.05	3.69	94.00	0.05	3.23	94.67
	5.00	4.63	2.47	92.65	4.71	1.05	94.19	4.83	1.66	96.57	4.69	5.16	93.81	4.66	3.93	93.23
	125.00	122.49	1.32	97.99	123.27	1.76	98.62	122.29	1.58	97.83	121.48	1.67	97.19	121.80	2.11	97.44
DIM	0.05	0.05	3.61	96.00	0.05	0.42	91.33	0.05	6.45	94.67	0.05	2.42	95.33	0.05	2.08	96.00
	5.00	4.71	4.45	94.26	4.55	1.97	90.93	4.71	3.39	94.12	4.67	3.86	93.45	4.80	3.07	96.09
	125.00	123.11	1.90	98.49	124.01	0.70	99.21	122.34	1.82	97.87	123.42	1.96	98.74	123.31	1.98	98.65

Table: 2.2 Stability of I3C and DIM at different conditions. Known concentration (Known conc.), Accurcay (Acc), Precision (Pre). All values represented as mean (n=3).

2.2.4 Recovery

The recovery of I3C and DIM from the quality control samples is depicted in Table 2.3. The recoveries of I3C and DIM from plasma and tissue samples ranged from 91.09 to 99.66 %.

Concentration (µg/ml)	**Plasma**		**Brain**		**Lungs**		**Liver**		**Kidney**	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
I3C										
0.05	91.43	2.6	92.93	2.29	92.74	2.11	93.17	1.38	91.09	3.59
5	98.54	6.65	99.13	5.23	94.01	4.5	97.10	5.8	95.43	8.7
125	98.18	4.18	98.95	2.15	97.84	3.87	97.84	1.84	95.67	2.27
DIM										
0.05	92.54	6.52	95.21	8.19	91.99	6.7	92.96	4.6	96.34	1.8
5	97.34	2.2	98.01	3.4	92.37	2.9	96.19	1.5	94.50	4.1
125	97.14	2.6	96.87	2.29	98.66	2.11	97.42	5.2	99.66	1.59

Table 2.3: Recovery of I3C and DIM in plasma and other tissues. Relative standard deviation (RSD). All values are expressed as mean (n=3).

2.2.5 Plasma concentration of I3C and DIM

The plasma concentrations of I3C and DIM are illustrated in figures 2.3 and 2.3.1. Two-way ANOVA studies revealed that I3C and DIM concentrations in plasma significantly differed among the groups. Post hoc analyses revealed that plasma concentrations of I3C did not alter between the sham and MCAO groups after oral delivery (0.5 to 15.30 µg/ml and 0.35 to 16.20 µg/ml, $p > 0.05$) and between sham and MCAO rats after intravenous delivery (1.5 to 40.31 µg/ml and 1.5 to 38.31 µg/ml, $p > 0.05$). However, I3C plasma concentrations were significantly

higher in I3C intravenously administrated sham and MCAO rats than in orally administered sham ($p < 0.0001$) and MCAO rats ($p < 0.0001$). There were no significant changes in DIM plasma concentrations between sham and MCAO rats (0.45 to 25.90 µg/ml and 0.40 to 27.10 µg/ml, $p > 0.05$) after oral administration of I3C and between sham and MCAO animals after intravenous delivery of I3C (0.10 to 10.29 µg/ml and 0.15 to 11.82 µg/ml, $p > 0.05$). However, oral administration of I3C had higher plasma DIM levels in sham rats than intravenously administered sham rats ($p < 0.0001$). Similarly, MCAO animals given I3C orally had increased plasma DIM levels than MCAO rats administered I3C intravenously ($p < 0.0001$).

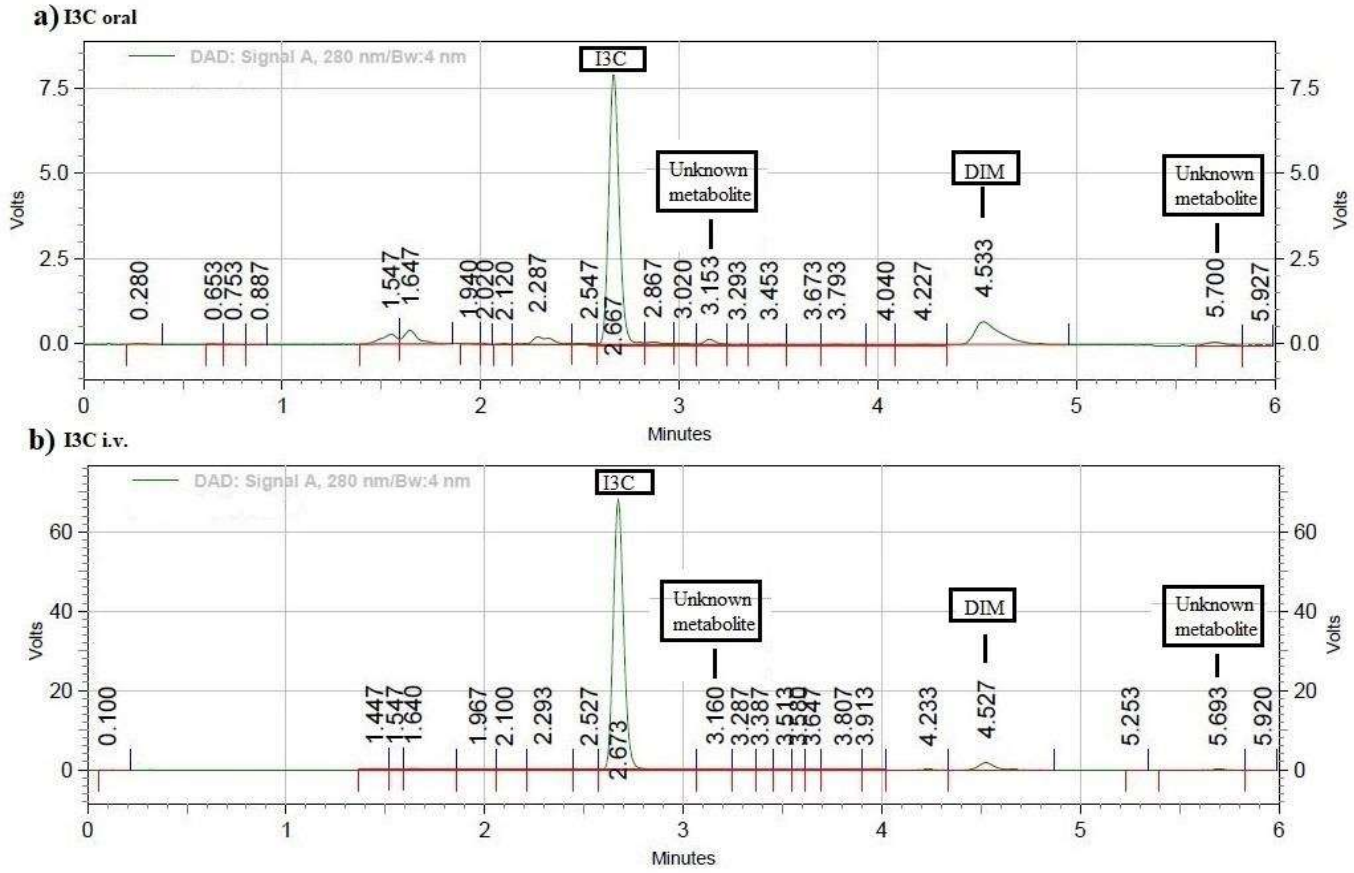

Figure 2.3. *Chromatograms of rat plasma samples. Chromatograms of the peak plasma*

concentration of I3C and its metabolites after oral administration of I3C (a) and after its intravenous administration (b). I3C-indole-3-carbinol, DIM-diindolylmethane.

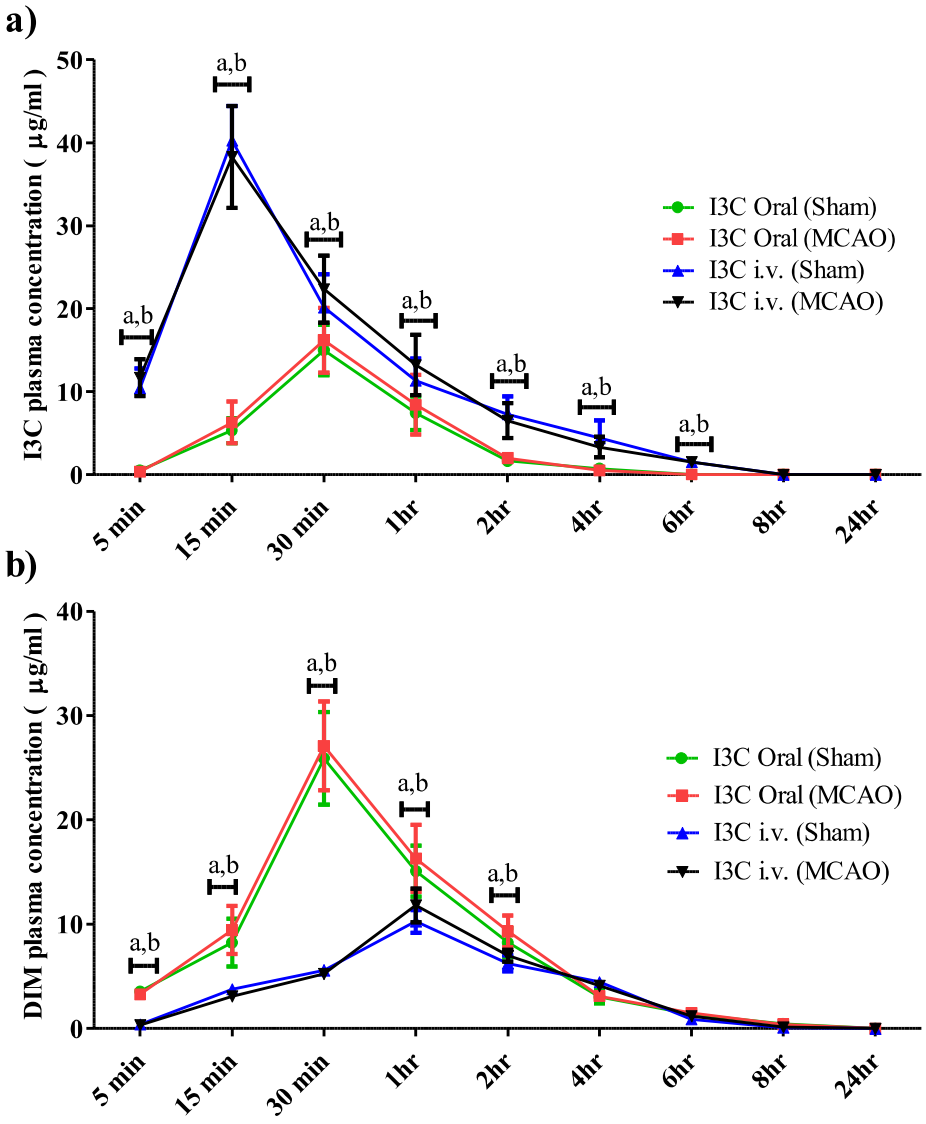

Figure 2.3.1. Plasma concentration of I3C and DIM in sham and MCAO rats. Figure 2.3.1a represents the plasma concentration of I3C in sham and MCAO rats. Figure 2.3.1b indicates the plasma concentration of DIM in sham and MCAO rats after I3C administration. [a]p<0.001 vs.

I3C (oral) sham, [b]p<0.001 vs. I3C (i.v.) sham. [c]p<0.001 vs. I3C (oral) stroke. All the results are expressed as Mean ± SD (n=4). Two-way ANOVA followed the Bonferroni post hoc test. I3C-indole-3-carbinol, DIM-diindolylmethane, MCAO-middle cerebral artery occlusion, i.v.-intravenous.

2.2.6 Pharmacokinetic parameters

Pharmacokinetic parameters of I3C are depicted in table 2.4. One-way analysis revealed that there were significant differences observed after I3C oral and intravenously administration between sham groups: Cmax (16.06 ± 3.33 and 40.17 ± 3.74, p< 0.001) Tmax (0.5 and 0.25, p< 0.001), t half (0.74 ± 0.11 and 1.78 ± 0.50, p< 0.001), AUC (15.85 ± 4.21and 45.65 ± 7.38, p< 0.001), MRT (1.16 ± 0.1 and 2.36 ± 0.38, p< 0.001) , CL (3.05 ± 0.45and 1.02 ± 0.22, p < 0.001), and Vz (3.02 ± 0.5and 2.36 ± 0.38, p< 0.05). There were significant differences between MCAO groups after oral and intravenous administration of I3C: Cmax (16.84 ± 3.74 and 39.23 ± 6.21, p < 0.001) Tmax (0.5 and 0.25, p< 0.001), t half (0.76 ± 0.15 and 1.82 ± 0.40, p< 0.001), AUC (16.35 ± 3.45 and 44.6 ± 6.56, p< 0.001), MRT (1.2 ± 0.1 and 2.25 ± 0.52, p< 0.001) , CL (2.88 ± 0.46 and 0.98± 0.11, p< 0.001), and Vz (2.96 ± 0.27 and 2.40 ± 0.4, p< 0.05). I3C pharmacokinetics did not differ between sham and MCAO rats following I3C oral administration (p> 0.05) or between sham and MCAO rats following I3C intravenous administration (p> 0.05). Further, we did not find any significant difference of I3C bioavailability between sham (35.31 ± 5.44%) and MCAO (36.39 ± 5.67%).

Parameter	I3C Oral (Sham)	I3C Oral (MCAO)	I3C i.v. (Sham)	I3C i.v. (MCAO)
Cmax (µg/ml)	16.06±3.33	16.84±3.74	40.17±7.20[a,b]	39.23±6.21[a,b]
Tmax (hr)	0.5	0.5	0.25	0.25
t ½ (hr)	0.74±0.11	0.76±0.15	1.78±0.50[a,b]	1.82±0.4[a,b]
$AUC_{0\text{-total}}$ (µg/ml × hr)	15.85±4.21	16.35±3.45	45.66±7.38[a,b]	44.6±6.56[a,b]
MRT (hr)	1.16±0.1	1.2±0.1	2.32±0.2[a,b]	2.25±0.52 [a,b]
Vz (mg/kg)/ (µg/ml)	3.02±0.5	2.96±0.27	2.36±0.38[a,b]	2.48±0.4[a,b]
CL (mg/kg)/ (µg/ml)/h)	3.05±0.45	2.88±0.46	1.02±0.22[a,b]	0.98±0.11[a,b]
$Bioavailability_{abs}$(%)	35.31±5.44		36.39±5.67	

Table 2.4. Pharmacokinetic parameters of I3C in MCAO rats. [a]p<0.001 vs.I3C oral (sham) and [b]p<0.001 vs.I3C oral (MCAO). All the results are expressed as Mean ± SD (n=4). Students t-test. I3C-indole-3-carbinol, MCAO- middle cerebral artery occlusion, Cmax- maximum concentration, Tmax- time to reach, *t ½ - half-life,* MRT- *mean residential time, Vz-volume of distribution, CL-clearance, AUC- area under the curve, i.v.- intravenous, abs-absolute.*

2.2.7 Tissue distribution

The tissue distribution of I3C and DIM are depicted in table 2.5. The concentrations of I3C and DIM were higher in the liver, followed by the kidney and lungs. I3C oral administration was found to have high amounts of DIM liver, kidney, and lungs than intravenous administration of I3C.

Organ		I3C (Oral)	I3C (i.v.)
	I3C	17.10±1.32	26.21±1.10
Liver	**DIM**	14.80±1.10	10.4±1.12
	I3C	13.00±1.16	22.32±1.14
Kidney	**DIM**	11.32±1.09	8.60±1.12
	I3C	8.29±1.21	16.20±1.32
Lungs	**DIM**	5.40±1.12	3.50±1.29

Table 2.5: Tissue distribution of I3C and DIM. Peak concentrations of I3C and DIM (µg/ml) in Liver, Kidney, and Lungs. All the results are expressed as Mean ± SD (n=4).

2.2.8 Brain concentrations of I3C and DIM

The brain concentrations of I3C and DIM are illustrated in figure 2.4. Two-way ANOVA analyses found significant differences in brain levels of I3C and DIM levels among groups. The brain concentrations of I3C of sham rats (0.25 to 6.5 µg/g/ml) were significantly lower than MCAO rats (0.6 to 8.6 µg/g/ml, $p< 0.001$) after oral administration. The brain concentrations of I3C were significantly higher in MCAO rats (2.34 to 18.4 µg/g/ml) than sham rats (1.31 to 13.31 µg/g/ml, $p< 0.001$) after its intravenous administration. The DIM levels were significantly higher in MCAO rat brains (1.80 to 13.20 µg/ml) than sham rats (0.5 to 11.30 µg/g/ml, $p<0.001$) after

I3C oral delivery. Similarly, I3C intravenously administered MCAO rats had higher amounts of DIM (0.15 to 7.10 μg/g/ml, $p < 0.001$) than sham-operated rats (0.09 to 5.0 μg/g/ml). DIM levels in the brains of I3C orally administered sham and MCAO rats were significantly higher in the brains of I3C intravenously administered sham and MCAO rats ($p < 0.001$).

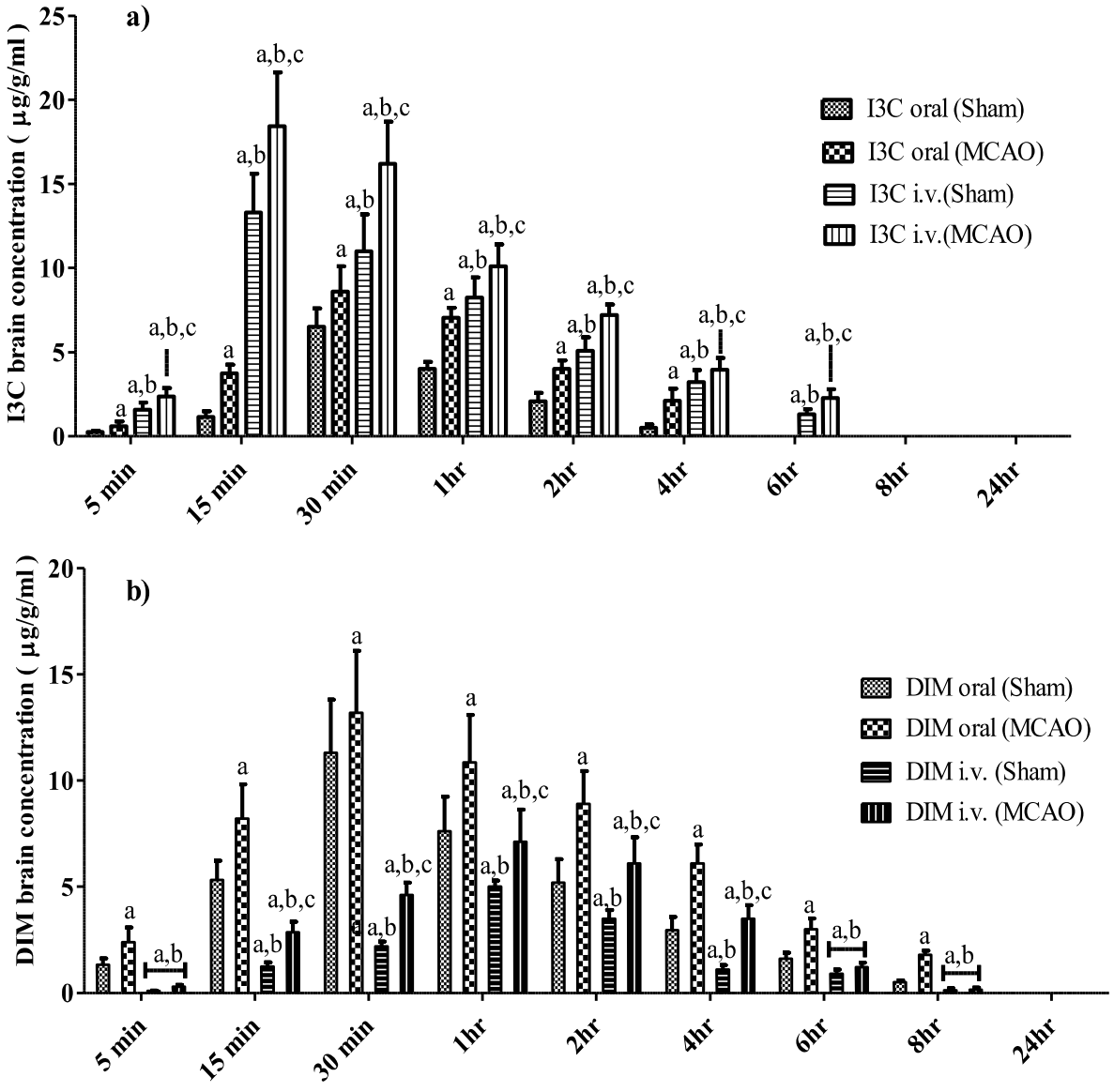

Figure 2.4. I3C and DIM concentration in the brains of sham and MCAO rats. Figure 2.4a represents the brain concentration of I3C in sham and MCAO rats. Figure 2.4b indicates the brain concentration of DIM in sham and MCAO rats after I3C administration. $^{a}p<0.001$ *vs. I3C (oral) sham,* $^{b}p<0.001$ *vs. I3C (i.v.) sham, and* $^{c}p<0.001$ *vs. I3C(oral) MCAO. All the results are expressed as Mean ± SD (n=4). Two-way ANOVA followed the Bonferroni post hoc test. I3C-*

indole-3-carbinol, DIM-diindolylmethane, MCAO-middle cerebral artery occlusion, i.v.-intravenous.

2.2.9 I3C and DIM concentrations in CSF

Changes in I3C and DIM concentrations in CSF are illustrated in figure 2.5. Two-way ANOVA revealed that I3C levels in CSF of sham animals (0.2 to 2.7 μg/ml) were significantly lower than I3C orally administered MCAO rats (0.5 to 4.1 μg/ml, $p< 0.05$). I3C intravenous administered sham rats had significantly lower levels of I3C in CSF (0.7 to 6.20 μg/ml) compared to MCAO rats (1.4 to 7.45 μg/ml, $p< 0.05$). I3C orally administrated sham and MCAO rats had significantly lower levels of I3C in CSF compared to I3C levels in sham and MCAO rats ($p < 0.001$) of I3C intravenous delivery. The I3C oral delivery had significantly high levels of DIM in the CSF of MCAO rats (0.5 to 10.32 μg/ml, $p< 0.001$) than in the sham rats (0.1 to 6.3 μg/ml). I3C intravenously administered sham animals had significantly lower levels of DIM in CSF (0.1 to 3.20 μg/ml) compared to MCAO rats (1.4 to 7.45 μg/ml, $p< 0.001$). The DIM levels were significantly higher in the CSF of sham and MCAO rats after oral administration than in the intravenously administered sham and MCAO rats ($p< 0.001$).

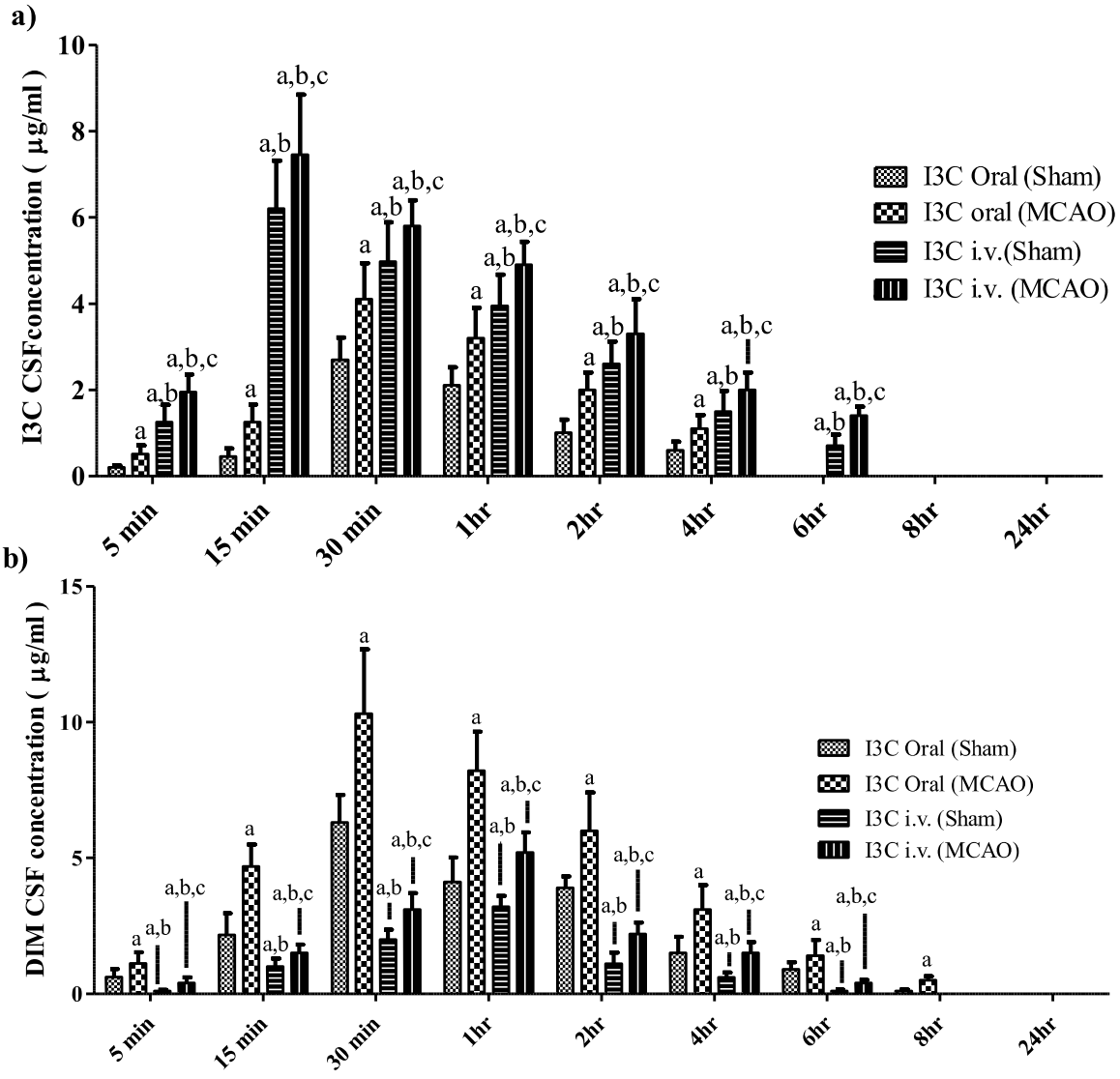

Figure 2.5. I3C and DIM concentrations in CSF of sham and MCAO rats. Figure 2.5a indicates the CSF levels of I3C in sham and MCAO rats. Figure 2.5b indicates the CSF levels of DIM in sham and MCAO rats after I3C administration . $^{a}p<0.001$ *vs. I3C (oral) sham,* $^{b}p<0.001$ *vs. I3C (i.v.) sham, and* $^{c}p<0.001$ *vs. I3C(oral) stroke. All the results are expressed as Mean ± SD (n=4). Two-way ANOVA followed the Bonferroni post hoc test. I3C-indole-3-carbinol, DIM-*

diindolylmethane, MCAO-middle cerebral artery occlusion, i.v.- intravenous, CSF-cerebrospinal fluid.

2.2.10 I3C alleviated the neurological deficits

I3C-induced changes in the neurological deficits are depicted in figure 2.6. One-way ANOVA showed that there were significant differences in neurological deficits among groups. The oral treatment of I3C significantly ameliorated the neurological deficits compared to intravenously treated MCAO rats ($p < 0.001$) and untreated MCAO rats ($p < 0.0001$). Similarly, I3C intravenous treatment significantly reduced neurological deficits in MCAO rats compared to untreated rats ($p < 0.0001$). The sham animals did not show any neurological deficits.

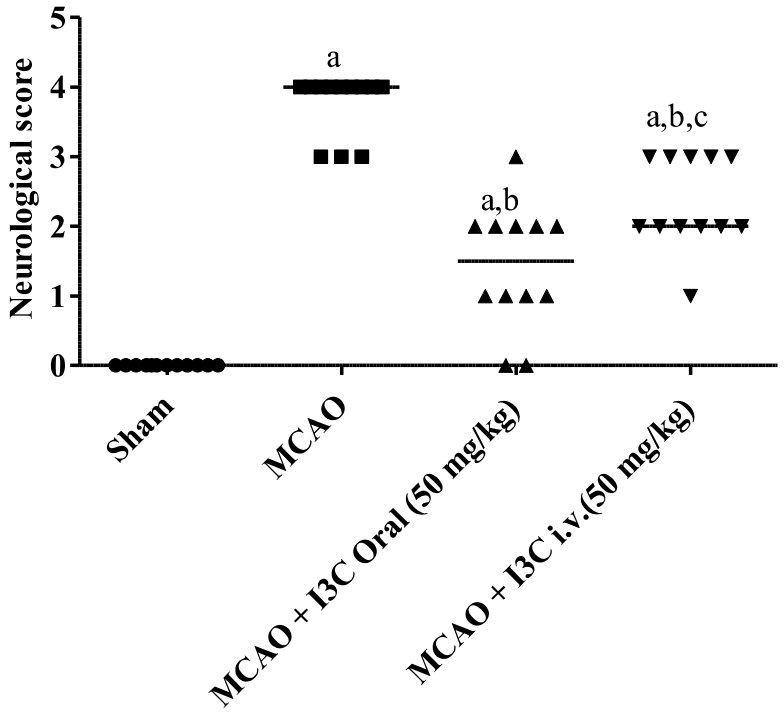

Figure 2.6. Effect of I3C treatment on neurological deficits. [a]p<0.001 vs. sham, [b]p<0.001 vs. MCAO, and [c]p<0.001 vs. MCAO+ I3C oral (50 mg/kg). All the results are expressed as Median (n=12 for each group). One-way ANOVA followed by Tukey's post hoc test. I3C-indole-3-carbinol, MCAO-middle cerebral artery occlusion, i.v.- intravenous.

2.2.11 I3C ameliorated the brain infarction

The changes in the brain infarct size after treatment with I3C are depicted in figure 2.7. One-way ANOVA analysis revealed that there were significant differences in brain infarction among groups. The infarct size was significantly ameliorated in I3C orally treated MCAO rats (17.15 ± 2.9%) compared to intravenously treated MCAO rats ((28.74 ± 3.84 %, p< 0.0001) and untreated MCAO rats (39.89 ± 2.59 %, p< 0.0001). The intravenous administration of I3C in MCAO rats

significantly decreased the infarct size compared to untreated MCAO rats ($p < 0.001$). Sham-operated animals had no infarction (0 ± 0).

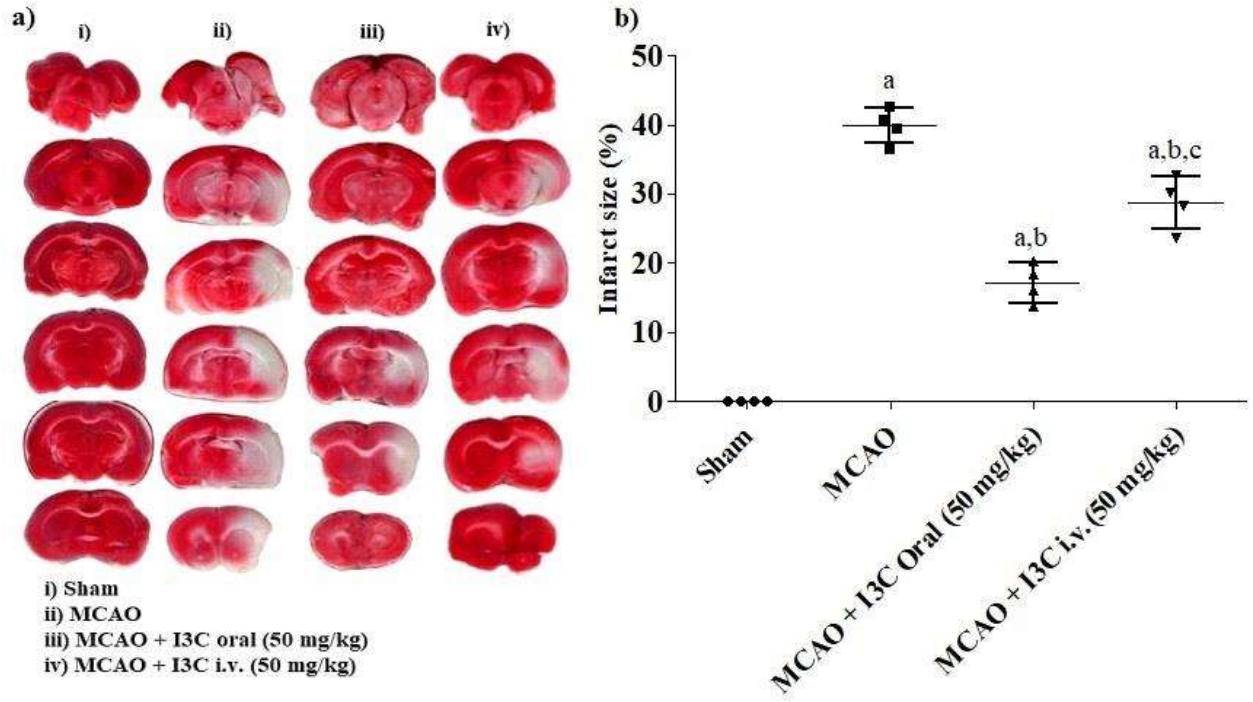

Figure 2.7. Effect of I3C treatment on infarction size. Figure 2.7a indicates the representative images of brain infarction with respective to their groups and figure 2.7b represents the brain infarction (%). [a]$p<0.001$ vs. sham, [b]$p<0.001$ vs. MCAO and [c]$p<0.001$ vs. MCAO+ I3C oral (50 mg/kg). All the results are expressed as Mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test. I3C-indole-3-carbinol, MCAO-middle cerebral artery occlusion, i.v.- intravenous.

2.2.12 I3C mitigated the BBB leakage

The alteration in I3C-induced BBB permeability is depicted in figure 2.8. One-way ANOVA indicated significant differences in EB levels among groups. MCAO rats had significantly higher brain EB levels (41.65 ± 5.11 µg/g) than sham animals (0.4 ± 0.1 µg/g, $p < 0.0001$). I3C oral

treatment significantly reduced the EB levels than intravenously treated MCAO rats (14.5 ± 3.39 µg/g, $p< 0.05$), untreated MCAO rats ($p< 0.0001$), and sham-operated rats ($p< 0.001$). Similarly, I3C intravenous treatment significantly reduced the EB levels compared to untreated MCAO rats ($p< 0.0001$) and sham-operated animals ($p< 0.001$).

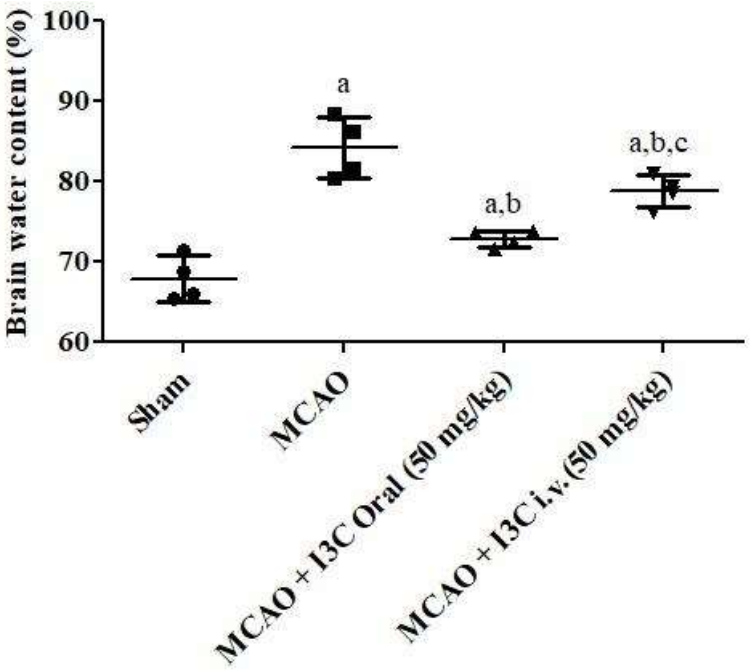

Figure 2.8. Effect of I3C treatment on blood-brain barrier permeability. Figure 2.8a indicates the representative images of BBB leakage respective to their groups and figure 2.8b represents the Evans blue concentration. [a]$p<0.001$ vs. sham, [b]$p<0.001$ vs. MCAO, and [c]$p<0.001$ vs. MCAO+ I3C oral (50 mg/kg). All the results are expressed as Mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test. I3C-indole-3-carbinol, MCAO-middle cerebral artery occlusion, i.v.- intravenous.

2.2.13 I3C alleviated the brain edema

Changes in brain water content after I3C treatment in MCAO rats is illustrated in figure 2.9. One-way ANOVA indicated the significant differences in brain water content among groups. MCAO rats significantly had higher brain water content (84.2 ± 3.78 %) than sham animals (67.9 ± 2.84 %, $p < 0.0001$). I3C oral treatment significantly decreased the brain water content (72.89 ± 1.35 %) compared to intravenously treated MCAO rats (78.61 ± 2.0 %, $p < 0.05$), untreated MCAO rats ($p < 0.001$), and sham-operated rats ($p < 0.05$). Similarly, I3C intravenous treatment significantly decreased the brain water content in MCAO rats than untreated MCAO rats ($p < 0.05$) and sham-operated rats ($p < 0.0001$).

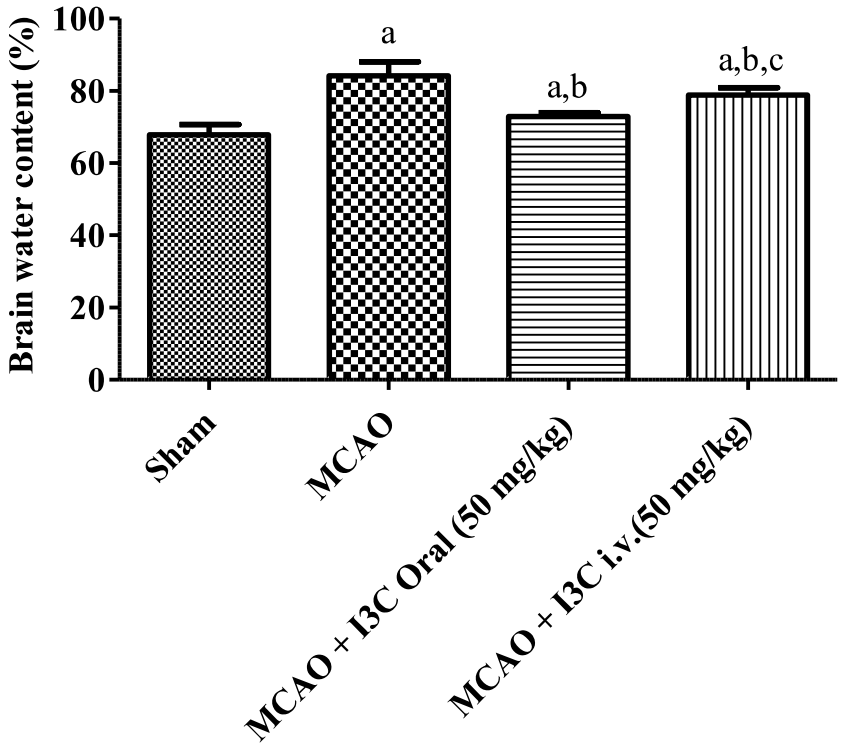

Figure 2.9. Effect of I3C treatment on brain water content. [a]p<0.05 vs. sham, [b]p<0.05 vs. MCAO, and [c]p<0.05 vs. MCAO+ I3C oral (50 mg/kg). All the results are expressed as Mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test. I3C-indole-3-carbinol, MCAO-middle cerebral artery occlusion, i.v.- intravenous.

2.3 Discussion

The present study evaluated the pharmacokinetic and pharmacodynamic properties of I3C in sham as well as MCAO animals with two different routes of administration (oral and intravenous). We found that the pharmacokinetic properties of I3C remain similar, but brain penetration of I3C is elevated in MCAO group animals. The oral treatment of I3C significantly exhibited the neuroprotection in MCAO rats than i.v. treatment.

The Food and Drug Administration (FDA) states that linearity, sensitivity, accuracy, precision, recovery, and stability should be counted to validate a novel method (FDA, 2001). Our study demonstrated that linearity, precision, accuracy, and recovery values are within the range per the international council for harmonization (ICH) and FDA guidelines. Further, stability studies indicate that the I3C and DIM QC samples stability lies within ±15%. Therefore, our results suggest that benchtop, autosampler, freeze-thaw, and long-term storage conditions did not affect I3C and DIM quantification. Therefore, our study recommends that rat plasma and tissue homogenates, consisting of I3C and DIM, can be stored at −80 °C for one month without significant loss.

Understanding pharmacokinetics to establish dose regimens is necessary to determine the therapeutic activity of a compound (Martinez, Papich, & Drusano, 2012). I3C pharmacokinetics such as Cmax, Tmax, half-life, MRT, AUC, clearance, and volume of distribution was identical in sham and MCAO rats, indicating that cerebral ischemic injury did not alter the pharmacokinetics of I3C. I3C, under acidic environments (the stomachs of rodents), forms acid condensation products leading to the generation of high levels of DIM and low levels of I3C in plasma (Anderton et al., 2003; Anderton et al., 2004; Grose & Bjeldanes, 1992). Similarly, we have observed a high amount of DIM and a low amount of I3C in plasma after the oral administration of I3C, whereas intravenously delivered I3C generated a low amount of DIM and a high amount of I3C remained in its native form. This indicates that I3C is rapidly metabolized after oral administration. It was reported that I3C undergo acid condensation at stomach due to acidic pH and liberates DIM. Therefore, this may be the cause to elevation in DIM levels after oral administration of I3C than the intravenous administration. Further, we found a significant

194 rise in plasma AUC_{0-24} of intravenously delivered I3C (3 folds higher) than I3C delivered via the
195 oral route, indicating no changes in the gastrointestinal (GI) absorption of I3C after ischemic
196 insult. Further, the Cmax has decreased in I3C orally administered rats compared to
197 intravenously administered rats (2.5 folds), indicating that I3C has lower absorption after its oral
198 administration. We found high Vz with oral administration of I3C, suggesting that I3C shows
199 high efficiency in escaping the bloodstream and circulating extravascularly. Moreover, I3C and
200 DIM are well distributed through all other highly perfused organs (liver > kidney > brain > lung)
201 (Supplementary Table 4). Further, we found that I3C was rapidly cleared from the body
202 following oral administration. Furthermore, in both sham and MCAO rats, I3C bioavailability
203 was identical. The low bioavailability (32%) could be attributed to a breakdown in an acidic
204 environment and rapid elimination from the body. Given the significance of I3C's
205 pharmacokinetic features, we determined that after oral administration of I3C to sham and
206 MCAO rats, I3C is rapidly degraded, absorbed, and excreted. I3C pharmacokinetics following
207 intravenous treatment in sham and MCAO rats are unaffected. These data suggest that I3C
208 pharmacokinetics following cerebral ischemic reperfusion (I/R injury) are identical and that the
209 pathological conditions of a stroke may not impact I3C availability. The ability of drugs to enter
210 the brain is the essential prerequisite for treating ischemic stroke (Y. Deng et al., 2016). Previous
211 results imply that I3C can cross the BBB (Anderton et al., 2004). Similarly, I3C and DIM levels
212 were found in the brain and CSF of oral and intravenous administration of I3C, indicating that
213 I3C can cross the BBB in normal and pathological stroke conditions. BBB disruption potentially
214 affects the therapeutic window and brain exposure of a compound (Mukherjee, Kumar, &
215 Patnaik, 2020). Therefore, these elevated levels of I3C and DIM brain and CSF could be due to

BBB compromise. Hence, pathological changes due to focal ischemia may not limit the entry of I3C into the brain.

I3C oral treatment alleviates neurological abnormalities and brain infarction in MCAO rats (P. Paliwal et al., 2018). Similarly, we found that I3C treatment, both oral and intravenous, reduced neurological impairments and brain infarction in MCAO rats. In fact, when compared to intravenous injection of I3C, oral delivery of I3C substantially reduced these abnormalities in MCAO rats. Stroke compromises the BBB's integrity and induces structural and functional abnormalities in the brain resulting in increased permeability of substances and, eventually, cerebral edema (Jin et al., 2008). We found that I3C administration (oral and i.v.) reduces BBB leakage and brain water content. However, the oral administration of I3C showed much more BBB protection than the i.v. I3C administration, implying that the oral I3C administration protects BBB integrity. Further, I3C decreased brain water content could be due to preserving the BBB integrity by reducing vascular permeability into the brain.

Conventionally, oral delivery of I3C has been reported to treat cancer and other diseases (Singh et al., 2021). Apart from oral delivery of I3C, intravenous and intraperitoneal administration also exhibited anticancer activities (Garikapaty et al., 2005; Souli et al., 2008). For the first time, our results also show that intravenous delivery of I3C also exhibits neuroprotection in ischemic stroke. However, i.v. I3C delivery protected the brain from ischemia injury but not as much as oral I3C treatment. Further, brain penetration studies uncovered that I3C and DIM availability in the brain differed for oral and intravenous administration. Even though I3C and its metabolite DIM are present in the brains of MCAO group rats, we found that I3C oral treatment significantly ameliorated the structural and functional outcomes in the MCAO

rats than intravenous treatment. Moreover, it is noteworthy to mention that I3C was not found in the tissues of I3C-treated rats, implying that DIM and other acid condensation products have physiological effects (Ahmad, Sakr, & Rahman, 2011; Anderton et al., 2004; Stresser et al., 2000). We have observed brain protection after oral administration of I3C as well as high amounts of DIM in plasma and brain than I3C i.v delivery, indicating that DIM mediates the pharmacological activities of I3C. However, the role of DIM in protecting the brain against cerebral ischemia has yet to be discovered. Furthermore, it is unknown if the production of acid condensation products is beneficial or detrimental to the efficiency of I3C, which necessitates further research. The ischemic stroke can be treated with thrombolytic agents (intravenous) and oral antiplatelets/anticoagulants (Bansal et al., 2013). I3C (50 mg/kg) was found to have no thrombolytic activity (Ramakrishna, Singh, & Krishnamurthy, 2022). Therefore, I3C cannot be used in place of thrombolytic therapy to treat stroke. On the other hand, thrombolytic-resistant ischemic strokes are commonly treated with oral antiplatelet/anticoagulants (Bansal et al., 2013). Furthermore, I3C given orally was more beneficial than I3C given intravenously in the treatment of stroke.

2.4 Summary

- I3C pharmacokinetics in sham and MCAO rats did not alter, but brain penetration of I3C was higher in MCAO rats, possibly due to a compromised BBB.
- DIM levels were high after oral administration of I3C than i.v. administration.
- Pharmacodynamic studies showed that oral administration of I3C exhibited potent pharmacological activity than intravenous administration.

- The better pharmacological activity of I3C in the oral route may be due to high levels of DIM.
- Hence, this study provides a scientific basis for the use of oral administration of I3C for treating cerebral ischemic stroke.