CHAPTER 5

RAPID DETERMINATION OF NITRATE IN BRAIN REGIONS AND CEREBROSPINAL FLUID OF RATS BY HPLC-UV

Highlights of the chapter

- *The study is reporting a HPLC-UV method for the determination of nitrate concentration in the cortex, cerebellum, hippocampi and CSF of normal and ischemic rats.*
- *The retention time for nitrate and nitrite were observed at 1.9 and 5.4 minutes respectively*
- *The concentration of nitrate in the cortex, hippocampi, cerebellum, and CSF of ischemic rats was 5.4, 7.6, 4, and 3 times higher respectively compared to normal rats.*
- *The present method is simple, rapid, and reproducible as well as suitable to analyze the nitrate level in the brain tissues and CSF in biochemical and pharmaceutical research.*

Abstract

Excessive production of nitric oxide during cerebral ischemic condition plays a detrimental role, resulting in neuronal cell death. Due to the very short half-life of nitric oxide, determination of nitrate and nitrite in the brain samples is a reliable strategy for indirect measurement of total NO production. Present simple, rapid and cost-effective study is designed to determine the nitrate in the cortex, cerebellum, hippocampi, and CSF of normal and ischemic rats. The global cerebral ischemia is performed by occluding both common carotid arteries for one hour followed by reperfusion of one hour. The level of nitrate is determined after one hour of reperfusion in the brain samples and CSF by HPLC coupled with UV detector at 212 nm. The mobile phase constitutes of 0.3M potassium phosphate monobasic, 20% acetonitrile, and 20 % methanol (pH 3.0) and 1.5 ml/min flow rate were kept for the analysis. The retention

time for nitrate and nitrite were observed at 1.9 and 5.4 minutes respectively. The concentration of nitrate in the cortex, hippocampi, cerebellum, and CSF of ischemic rats was 5.4, 7.6, 4, and 3 times higher respectively compared to normal rats. The method is suitable to analyze the nitrate concentration in the brain tissues and CSF in biochemical and pharmaceutical research.

5.1 INTRODUCTION

Nitric oxide (NO) is an essential neurotransmitter, plays a vital role in the control of cerebral blood flow [164][233], modulation of neuronal activity [234], inflammatory pathways of the brain [235]. It is synthesized from L-arginine by three nitric oxide synthases (NOS), i.e., nNOS, eNOS and iNOS [236][237] and is produced in neurons, endothelial cells, macrophages and glial cells. Under the ischemic condition, NO is produced in high concentration by the activation of neuronal NOS (nNOS) and inducible NOS (iNOS) that lead to neuronal cell death [238][45]. Since NO has a very short half-life (estimated to be a few seconds), its direct measurement is quite a challenge [46]. It is a highly reactive molecule and in the presence of oxygen, rapidly metabolized into nitrate (NO^{3-}) and nitrite (NO^{2-}). Nitrate is a stable, long-lasting end product of NO is used as a reliable biomarker of NO production [47]. Whereas, nitrite is usually a short-lived ion with a half-life of 110 seconds in whole blood [48]. Estimation of NO^{2-} and NO^{3-} is a reliable strategy for indirect measurement of total NO production. Various methods are implemented for measurement of NO^{2-} and NO^{3-} in biological samples. One of the most popular strategies is estimation by Greiss colorimetric assay [239]. In this reaction, nitrate needs to be reduced either enzymatically or chemically to nitrite to determine the NO that limits the sensitivity of the assay. Other indirect methods like fluorometry [240], chemiluminescence [241], electrochemical [242], and capillary electrophoresis [243] detection of nitrate and nitrite have also been reported. Most of these methods require a large volume of sample, are expensive

or constitutes of complex chemical reactions. Detection of NO_2^- and NO_3^- has also been reported by using high-performance liquid chromatography (HPLC) including ion-exchange HPLC coupled with UV detector [47], HPLC and Cloud-Point Extraction [244], HPLC with electrochemical detection [245]. Although, till date, no known simple method has been reported to determine the nitrate and nitrite level in the various brain samples and CSF of rats using HPLC UV method. Therefore, present study is designed to determine the nitrate and nitrite level in the various brain regions and CSF of normal and ischemic rat using HPLC coupled with UV detector.

5.2 EXPERIMENTAL SETUP

5.2.1 Chemicals and reagents

Sodium nitrate, Sodium nitrite, phosphoric acid and potassium phosphate monobasic were obtained from BR Biochem Life Sciences Pvt. Ltd. (New Delhi, India). Acetonitrile, methanol, and HPLC-grade water were procured from Merck Life Science Pvt. Ltd. (India). Ethyl ether and chloroform were obtained from Sisco Research Laboratories Pvt. Ltd (Maharashtra, India).

5.2.2 Animals

Experiments and surgical procedures were performed according to the guideline of the Institute of Medical Sciences, Banaras Hindu University Committee on Animal Welfare (Registration No. 542/02/ab/CPCSEA). Ten in-breed Charles Foster albino male rats (240 ± 30 g) were obtained from the animal house of the institute and randomly divided into two normal (n=5) and ischemic (n=5) groups.

5.2.3 Surgical procedure to induce global cerebral ischemia

The cerebral ischemia was induced by BCCAO or 2VO with some modification [246]. Anesthesia to male albino rats were provided by a combination of ketamine (50 mg/kg b.w.)

and xylazine (10 mg/kg b.w.). The anesthetized rats were placed and fixed on the surgical table in the supine position. The body temperature (37±0.5°C) was maintained using a heating pad. The ventral neck's fur was clean-shaven and Povidone-iodine was applied on the skin. A vertical midline-incision (~ 1.5 cm length) in neck skin was performed to expose the common carotid arteries (CCAs). The salivary glands were carefully detached to identify the left and right CCA's. Vagal nerves associated with both CCAs were cautiously separated from both CCAs. By using a loose cotton suture (5-0), a circle was made around each CCA and a piece of saline-soaked cotton was kept in between the CCA and suture. The suture was tightened strongly, skin incision stitched and blood vessels were kept occluded for one hour. After one hour of occlusion, reperfusion for one hour was carried out by cutting the suture loop carefully using a sharp surgical blade, both cotton and suture was removed and skin was stitched again after application of povidone-iodine ointment on wound area. After completion of the surgical process, animals were transferred to their cages to recover from anesthesia and regain the righting reflex. The sham-operated (normal) animals received all surgical procedures except the ligation of CCAs.

5.2.4 Brain Samples and CSF preparation

After one hour of reperfusion, brain and cerebrospinal fluid (CSF) were collected. The CSF (≈50 µl) was collected from the cisterna magna [229]. All animals were decapitated after a high dose of chloroform and brains were collected on a plate containing the chilled saline solution (0.9%). The hippocampi (0.2 gm), cerebellum (0.5 gm), and cortex (0.5 gm) were extracted and homogenized in 0.5, 1 and 1 ml of HPLC-grade water respectively. The centrifugation of brain homogenate was performed at 2100 RCF for 20 min at 4°C. After centrifugation, clear supernatant was cautiously sampled and filtered with 0.22 µm Millipore

filters and transferred in labeled tubes for storage at -20°C. The samples were diluted with water (1:1), and 10 µl was used for the HPLC-UV analysis.

5.2.5 Standard and quality control (QC) samples

Stock solutions of sodium nitrate and sodium nitrite (1000 µg/ml) were prepared in HPLC grade water and incubated at 4°C. The working solutions of concentrations (10, 50, and 500 µg/ml) were prepared from the stock solutions with HPLC water. Further, the standard calibration solutions of concentrations (5–250 µg/ml) were prepared daily by diluting the working solution (1:1) using blank cerebellum, cortex, hippocampus and CSF samples of normal rats and stored at 4°C till analysis. The quality control (QC) samples of high (250.00 µg/ml), medium (25.00 µg/ml), and low (5 µg/ml) concentrations were also prepared for the determination of the accuracy (relative error, RE) and precision (relative standard deviation, RSD) of the HPLC method. The limit of quantification (LOQ) and limit of detection (LOD) was calculated according to Kumar et al. 2018 [247].

5.2.6 Chromatographic conditions

The HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan) with System Controller (CBM-20A), UV–Vis detector (SPD-20A), chromatographic pump (LC-20AD), Column oven (CTO-20AC) and Rheodyne injector operated at room temperature (24 ± 1°C). The LC solutions chromatography data analysis system was used for data collection, integration, and calibration. The analytes were separated on Kinetex Reversed Phase C18 column (250x4.6 mm, particle size 5 mm). The mobile phase consists a mixture of 20% acetonitrile, 20 % methanol and potassium phosphate monobasic (0.3M). The pH of mobile phase was adjusted 3.0 using phosphoric acid). The mobile phase was vacuum filtered through a 0.22 µm nylon

filter. The column temperature and flow rate was set to 30°C and 1.5 mL/min respectively. The nitrate and nitrite concentration was monitored at a wavelength of 212 nm throughout the experiments.

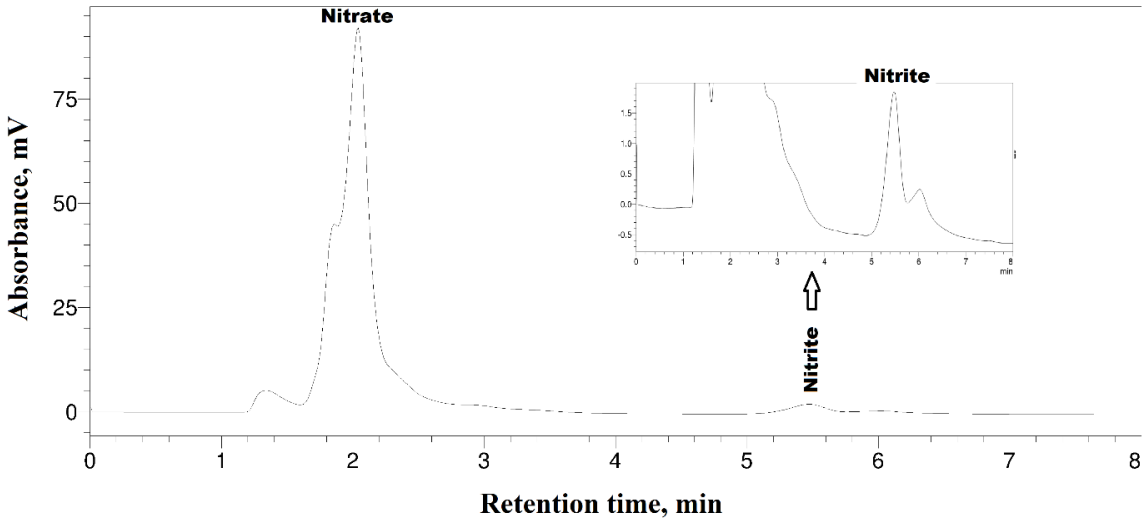

Fig. 5.1: Blank Hippocampi of normal rat spiked with Nitrate and Nitrite (5 µg/ml)

5.3 RESULTS

5.3.1 Chromatogram

The retention time for nitrate and nitrite were observed at 1.9 and 5.4 minutes respectively according to above mentioned chromatographic conditions. There are no co-eluting peaks observed in the vicinity of the nitrate and nitrite peak on the chromatogram of spiked blank brain samples and CSF (Fig. 5.1). Both nitrate and nitrite were separated entirely within 10 min, and peaks for both analytes are well resolved from each other.

5.3.2 Linearity, precision, recovery and detection limit

Before plotting calibration curve, the area of standard subtracted with area obtained from respective blank samples of normal rats. A linear calibration curve (peak area vs. concentration) was plotted for the concentration range 5-250 µg/ml of nitrate and nitrite in rat brain samples and CSF. Precision (relative standard deviation) and accuracy (relative error) were assessed by repeating five consecutive runs inter-day and intra-day using standard nitrate and nitrite neurotransmitters. Percentage average recovery was calculated by spiking known amounts of a mixture of nitrate and nitrite standards into the brain homogenates and CSF. The regression equations of the calibration curves, relative errors of concentrations, relative standard deviations of the peak areas, average percentage recoveries, LOQ and LOD were calculated and are listed in Table 5.1. The calibration curves for nitrate and nitrite yielded good linearity with correlation coefficients of >0.999. For nitrate, the relative standard deviations were from 3.16 to 4.35 % for inter-day determination and from 1.87 to 2.92 % for intra-day determination. For nitrite, the relative standard deviations were from 2.69 to 3.02 % for inter-day determination and from 1.31 to 1.98 % for intra-day determination. The average percentage recovery (%) of QC samples were from 97.08 to 102.17% for nitrate and from 100.77 to 102.22 % for nitrite. The LOD ranged from 1.80 to 1.99 µg/mL for nitrate and from 1.55 to 1.95 µg/mL for nitrite. The LOQ ranged from 6.00 to 6.63 µg/mL for nitrate and from 5.16 to 6.49 µg/mL for nitrite.

5.3.3 Optimization of the chromatographic conditions

To develop a rapid, accurate and reproducible method for detecting the nitrate and nitrite in the different parts of the rat brain and CSF, the chromatographic conditions were optimized. We found that the change in pH of the mobile phase altered the shape of the nitrate/nitrite

peaks. As the percentage of the organic phase increased, the retention time of nitrate and nitrite changed. The lower the pH of the mobile phase, the better peak shape was observed. Finally, acceptable chromatographic conditions that exhibited relatively short analysis time, satisfactory resolution and good peak shapes, were 0.3M potassium phosphate monobasic, 20% acetonitrile, and 20 % methanol (pH 3.0) and 1.5 ml/min flow rate.

Table 5.1. The HPLC method parameters for calibration, accuracy, and precision

Analyte in the tissue sample		Regression Equation	R	RE %	RSD of lowest QC sample, n=6, %		Average recovery of QC samples, %	LOQ, µg/ml	LOD, µg/ml
					Intra-day	Inter-day			
Nitrate	Cortex	y = 58345x + 631042	0.99999	8.95	2.92	4.26	102.17	6.15	1.84
	Hippocampi	y = 49195x + 63573	0.99999	3.02	1.87	3.78	100.58	6.21	1.86
	Cerebellum	y = 52647x + 449136	0.99998	9.05	1.94	3.16	97.96	6.63	1.99
	CSF	y = 63120x + 39835	0.99999	9.03	2.25	4.35	97.08	6.00	1.80
Nitrite	Cortex	y = 11961x - 18351	0.99999	4.21	1.84	2.69	102.22	5.34	1.60
	Hippocampi	y = 9941.8x - 3539.6	0.99999	9.73	1.67	2.74	102.16	6.11	1.83
	Cerebellum	y = 10783x - 9564.8	0.99999	0.76	1.31	2.77	100.77	6.49	1.95
	CSF	y = 12056x + 6712.8	0.99999	2.94	1.98	3.02	101.81	5.16	1.55

RSD: relative standard deviation; R: Correlation coefficient, RE: relative error of lowest QC sample concentration; LOD: limit of detection; LOQ: limit of quantification

5.3.4 Sample determination

The rat brain samples and CSF were prepared and analyzed for the nitrate and nitrite as described in the chromatographic method (Fig. 5.2). The stability of the nitrate and nitrite in the samples were also studied by calculating peak areas of brain samples and CSF that had

been kept at -20ºC for 1, 2, 4 and 6 hours (*data not shown*). Nitrate was successfully determined in the rat brain tissues and CSF using the above-mentioned chromatographic procedure. The mean concentrations of nitrate in rat brain samples and CSF of normal and ischemic groups are listed in Table 5.2. The concentration of nitrate in the cortex, hippocampi, cerebellum, and CSF of ischemic rats was 5.4, 7.6, 4, and 3 times higher respectively compared to normal rats. There was no significant decrease in peaks of the nitrate at 1-4 hours. The nitrite concentration was also determined in the rat brain tissues and CSF when processed and analyzed same day (*data not shown*). Significant changes were observed in the nitrite concentration with respect of time and different sample storage conditions (-20ºC, -80ºC and 4ºC). The nitrite concentration decreases significantly within 2 hours making quantification difficult. Therefore, we have not reported nitrite concentration in the present study. This method is sensitive, rapid and reliable for the determination of nitrate concentration in the rat brain in normal and ischemic condition.

Table 5.2. The mean concentrations of the nitrate in rat brain samples and CSF (after 1h of ischemia followed by 1h of reperfusion)

Sample	**Nitrate Concentration, µg/ml**			
	Cortex	**Hippocampi**	**Cerebellum**	**CSF**
Normal	21±1.34	11±0.88	23±1.97	06±0.45
BCCAO	114.97±18.72	84.29±12.34	92.58±19.96	18.07±2.15

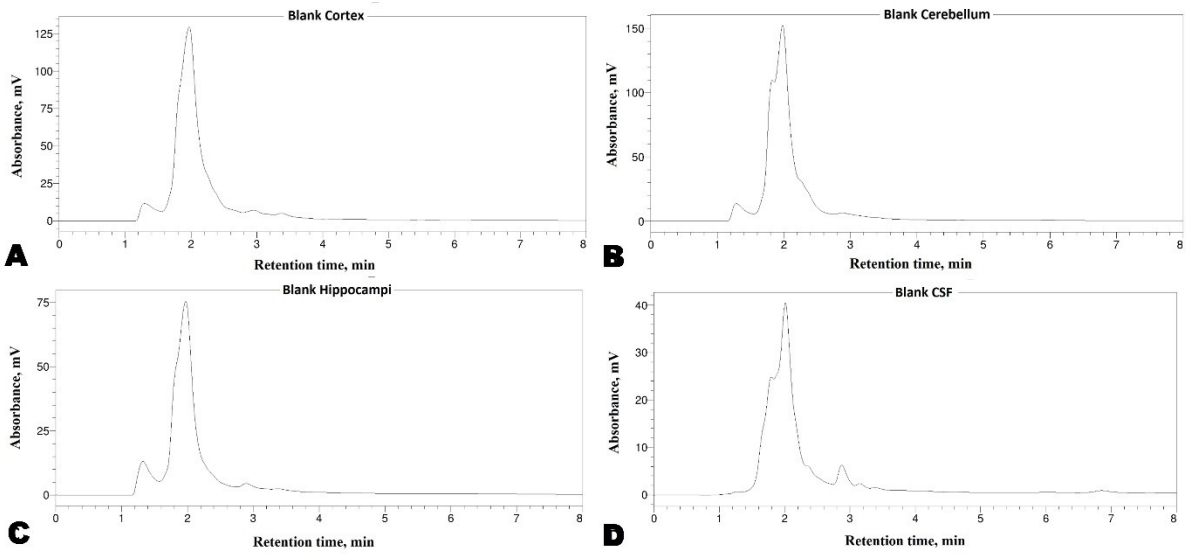

Fig. 5.2: HPLC chromatograms of nitrate in (A) Cortex; (B) Cerebellum; (C) Hippocampi and (D) CSF of normal rat.

5.4 DISCUSSION

The NO and nitrite have short half-lives as they are oxidized into nitrate by oxyhaemoproteins (oxygenated haemoglobin or myoglobin) [248] making it difficult for their analysis using simple procedures with HPLC UV. A wide range of analytical methods for the detection of nitrite and nitrate are available in biological samples but most of them are expensive, labor intensive or require a series of sample and chemical preparation. Therefore, a simple, rapid, and robust procedure is rendered essential for the measurement of nitrate.

Indirect measurement of NO by determination of its stable oxidized metabolite nitrate using HPLC UV in the rat brain samples has been successfully performed in the present work. Brain samples including cortex, hippocampi and cerebellum as well as CSF were processed carefully and level of nitrate was determined. For nitrate, the RSD was below 5% for inter-day determination and from 3% for intra-day determination and relative error was below 10%. For nitrite, the RSD was below 4% for inter-day determination and below 2% for intra-day

determination and relative error was below 10%. The LOD and LOQ for both nitrate and nitrite was observed below 2 μg/mL and 7 μg/mL respectively. The study performed by Ćurčić-Jovanović et al. [47] to determine nitrate in the brain samples using ion-exchange HPLC UV demonstrated that LOQ of nitrate is 1.2 mmol/L. However, in the present study LOQ is ≤ 0.1 mmol/L (≈ 6.63 μg/ml). This difference might be due to nitrate determination method, laboratory conditions, and sample processing. Charles Foster rats have been reportedly used as laboratory experimental models like diabetes [249], stroke [250], toxicity [251] and pharmacokinetics [247]. But no known scientific studies have reported the concentration of nitrate in brain regions of Charles Foster rats. In the present study, the concentration of nitrate in the cortex, hippocampi, cerebellum, and CSF of normal rats was 21, 11, 23, 6 μg/ml respectively**.** Rizzo et al. [252] reported extracellular nitrate basal levels in the rat cortex dialysis perfusate is in the range of 16–61 μM. Gao et al. [243] reported nitrate concentration in brain perfusate to be 15.51 μM. These values are lower than that obtained in the present study which might be attributed to species variation since this is the first study on the levels of nitrate in Charles Foster rats. However, after ischemia, the concentration of nitrate in the cortex, hippocampi, cerebellum, and CSF was 114, 84, 92 and 18 μg/ml respectively which is significantly higher as compared to normal rats.

5.5 CONCLUSION

In the present study, an optimized HPLC-UV method for determination of nitrate in rat cortex, cerebellum hippocampi, and CSF was developed. The advantages of this method are simple mobile phase composition, inexpensive equipment, and rapid analysis. This method is suitable to analyze nitrate in the brain tissues and CSF in biochemical and pharmaceutical research. Further study is needed to stabilize the nitrite level in the biological sample for its determination by HPLC UV.