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# Determination of nitrate by the IE-HPLC-UV method in the brain tissues of Wistar rats poisoned with paraquat

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*Abstract:* This work was a part of an initial study regarding the involvement of reactive nitrogen species (RNS) in paraquat (PQ) neurotoxicity. The nitrate concentration in the vulnerable regions of the brain (*cortex, striatum* and *hippocampus*) of Wistar rats was used as a measure of nitric oxide (NO) production or catabolism of the formed RNS. The tissue homogenates were deproteinized with acetonitrile and then centrifuged. Nitrate was measured in filtrated supernatants by simple and rapid isocratic ion-exchange high performance liquid chromatography with UV detection (IE-HPLC-UV) at 214 nm. The mobile phase (pH 8.5) consisted of borate buffer/gluconate concentrate, methanol, acetonitrile and deionized water (2:12:12:74, v/v/v/v), and the flow rate was 1.3 mL/min. Physiological nitrate levels (18.8 ± 6.1 nmol/mg of proteins), as well as a diverse range of nitrate concentrations could be determined with good precision ($CV$ = 2.2 %) and accuracy (recovery of spiked samples was 99 ± 4 %) in the brain tissue homogenates. Linearity was achieved in the range of nitrate from 0–80 μM. The retention time of nitrate anion was 5.3 ± 0.3 min.

*Keywords*: nitrate, IE-HPLC-UV, brain, paraquat.

## INTRODUCTION

The role of nitric oxide (NO) as an important physiological messenger that modulates blood flow and neural activity is well known.[1–3] However, NO radical ($NO^{\bullet}$), peroxynitrite anion ($ONOO^-$) and some other reactive nitrogen species (RNS) could have negative effects on biomolecules, and in particular, in oxidative stress induced by poisons.[1–3] The developed methods for $NO^{\bullet}$ determination are technically complex and their *in vivo* application is difficult.[4]

Nitrate, as a stabile, long-lasting endproduct of NO oxidative metabolism is used as a reliable biomarker of NO production by NO-synthase (NOS) and/or ca-

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tabolism of RNS in tissues, in addition to biological fluids.[4–10] From an analytical point of view, the stability of nitrate is an important property, which assures data interpretation.[2,8–10]

In order to reveal the involvement of NO in the neurotoxicity developed after intrastriatal *(i.s.)* administration of paraquat (PQ) to Wistar rats, nitrate levels were measured in homogenates of the vulnerable regions of the brain (*cortex, striatum* and *hippocampus*). As a redox cycling compound, PQ undergoes oxidative metabolism, depletes molecular oxygen ($O_2$) and forms the superoxide anion radical ($O_2^{\bullet-}$).[11–14]

NO, as a highly reactive molecule with a short half-life, reacts readily with $O_2^{\bullet-}$ ($6.7\times10^9$ $M^{-1}s^{-1}$) and produces the peroxynitrite anion ($ONOO^-$), a potent oxidant and nitrating agent.[1,4] Possibly, $ONOO^-$ formation is one of the crucial nitrosative pathways that outcompetes dismutation of $O_2^{\bullet-}$ by the enzyme superoxide dismutase (SOD).[15] $ONOO^-$ hydroxylates aromatic amino acids, can nitrate tyrosine (3-nitrotyrosine is a biomarker of NO-dependent oxidative stress) and oxidizes thiols and lipids.[1,4,11] Peroxynitrite is not the only RNS formed in the chain radical reaction between NO and ROS but it is probably the most important one. Peroxynitrite decomposition to nitrite and molecular oxygen leads to nitrate production. In body fluids most of the nitrite is converted into nitrate, therefore, nitrate, as a final metabolic product of NO metabolism, is more reliable to determine.[1,4,11,12,15]

Several HPLC methods for nitrate measurements in biological fluids have been published.[4,6,8–10,16–20] Rizzo *et al.* have reported results in relation to extracellular nitrate contents in the brain *cortex, hippocampus* and *striatum* dialysates, although, to date, there has been no evidence regarding nitrate concentrations in brain tissue homogenates.[4]

In this study, for the first time, a sensitive, cost-effective, and simple IE-HPLC-UV method for nitrate measurement in the homogenates of the vulnerable regions of the brain of Wistar rats *i.s.* poisoned with PQ was developed.

## EXPERIMENTAL

*IE-HPLC system*

The IE-HPLC system configuration included: HPLC pump (LKB 2150, Bromma, Sweden); sample loop, 50 μL; UV-diode array detector (UV-DAD) (LKB 2152, Bromma, Sweden); Anion column IC-PAK$^{TM}$ (based on quaternary amines) 50 mm × 4.6 mm, 10 μm particle size (Waters, Millipore, Milford, MA, USA); and Anion Guard-PAK$^{TM}$ (Waters, Millipore, Milford, MA, USA). Wavesan EG/Nelson commander (LKB, Bromma, Sweden) was used for data processing.

*Chromatographic conditions*

The chromatography was performed at room temperature with a mobile phase (pH 8.5) composed of borate buffer/gluconate concentrate, methanol, acetonitrile and deionized water (2:12:12:74, v/v/v/v), with a flow rate of 1.3 mL/min. The borate buffer/gluconate concentrate consisted of 0.07 mol/L sodium gluconate, 0.3 mol/L $H_3BO_3$, 0.1 mol/L $Na_2B_4O_7$ and 3.8 mol/L glycerol in deionized water

(1.6 g sodium gluconate, 1.8 g $H_3BO_3$, 2.5 g $Na_2B_4O_7$ and 25 mL glycerol were dissolved in deionized water to 100 mL). The borate buffer of the mobile phase served to adjust the pH of the mobile phase to 8.5. Calibration of the pH meter (HI 9321, Hanna Instruments, Vila do Conde, Portugal) at pH 7.01 and 10.01 was performed with standard buffers HI 7710P (Hanna Instruments, Vila do Conde, Portugal). The mobile phase was filtrated prior to use. Diode-array detection was performed at 214 nm. The injected volume of the samples was 50 μL.

A standard mixture of nitrite and nitrate was used to determine the retention times and separation of the peaks. Nitrite and nitrate concentrations were equal in the mixture solution and were in the range of 0–80 μmol/L.

*Reagents*

All chemicals were of analytical grade. Paraquat – Galokson® (200 g/L), was purchased from Galenika (Zemun, Serbia); Pentobarbiton-Na – Vetanarcol® (0.162 g/mL) was purchased from Werfft-Chemie (Wien, Austria); NaCl *solutio infundibile* (0.9 %) was purchased by the Hospital Pharmacy (Military Medical Academy, Belgrade, Serbia). $NaNO_2$ was purchased from Arachem (Kuala Lumpur, Malaysia) and $NaNO_3$ from Mallinckrodt Chemical Works (St. Louis, USA). Sodium gluconate, EDTA, $Na_2HPO_4$, $KH_2PO_4$, glycerol, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany), sodium tetraborate and boric acid were purchased from Zorka (Šabac, Serbia). Deionised water was prepared by Millipore milli-Q water purification system (Waters, Millipore, Milford, MA, USA).

*Animals*

The experiments were conducted on 11 week old Wistar rats of both sexes with body weights ~250 g. The animals were accommodated in separate cages with free access to food and water. For adaptation, the animals were kept seven days prior to the experiment at room temperature and a circadian regimen of light/dark ratio of 13/11.

Before treatment, the Wistar rats were intraperitoneally *(i.p.)* anesthetized with pentobarbital-Na in doses of 40.5 mg/kg body weight. 10 μL of an aqueous PQ solution (0.25 g/L), which corresponds to an applied dose of 50 mg/kg, and 10 μL of 0.9 % NaCl (for the sham operated animals) were directly administrated in the *striatum* of Wistar rats. A liquid overload (amounts greater than 10 μL) could provoke a brain tissue oedema.[21] The administration was performed using a Hamilton syringe, with a stereotaxic instrument for small laboratory animals (coordinates: 8.4 A, 2.6 L and 4.8 V).[22]

The experimental animals were treated according to the Guidelines for Animal Study No. 282/12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia).

*The brain tissues preparation*

Since NOS is concentrated in the vulnerable brain structures (*cortex, hippocampus* and *striatum*) of Wistar rats, these regions were selected for nitrate measurements. The animals were decapitated 30 min, 24 h and 7 days after treatment and the heads were frozen immediately (–70 °C). The brain structures – *cortex, hippocampus* and *striatum* were dissected on ice, and each tissue slice (approximately 0.1 g) was transferred into a tube of 1 ml of cold, buffered sucrose medium (0.25 mol/L sucrose with 0.1 mmol/L EDTA in 50 mM K–Na phosphate buffer, pH 7.2). Homogenization of the tissue in the sucrose medium was performed by a homogenizer (Tehtnica, Železniki, Slovenia) at 800 rotation/min, on ice. The homogenates were centrifuged at 1000 × g, for 15 min at 4 °C. The precipitates were redispersed in sucrose medium and centrifuged again. The supernatants were centrifuged at 2500 × g for 30 min at 4 °C and the obtained precipitates were redispersed in 1.5 mL of deionized water. After the one hour of incubation, the samples were centrifuged at 2000 x g for 15 min at 4 °C and the supernatants (crude mitochondrial fractions) were stored at –70 °C.[23] Proteins were determined by the Lowry method using bovine serum albumin as the standard.

After protein precipitation with acetonitrile (sample: acetonitrile, 2:1, v/v) and filtration, nitrate was chromatographically determined.

*Application of the method*

Nitrate was measured in the vulnerable regions of the brain of Wistar rats, *i.s.* poisoned with one single dose of PQ (50 mg/kg, *i.e.,* 2.5 μg/10 μL), 30 min, 24 h and 7 days after treatment. Control nitrate values were obtained from sham operated Wistar rats, to determine the effect of PQ on NO production.

*Statistical analysis*

The program STATISTICA 5.0 was used to perform one-way ANOVAs and post hoc Tukey tests (0.05 confidence value).

## RESULTS AND DISCUSSION

### *Method validation*

Under the given chromatographic conditions, analysis of the standard mixture solution of nitrite and nitrate (40.3 μmol/L, middle of the employed concentration range) displayed good separation of the peaks ($Rt$ = 3.6 and 5.15 min, respectively, separation coefficient, 1.49) (Fig. 1). The obtained retention times for nitrite and nitrate were 3.8 ± 0.3 min and 5.3 ± 0.3 min, respectively. The specificity of the method was tested by comparing chromatograms (retention times) and UV spectra of the parent compound. The retention time of $NO_3^-$ obtained from control samples (intact brain tissues) was identical to that of the standard solution (5.3 ± 0.3 min). Confirmation of endogenous $NO_3^-$ ($Rt$ = 5.2 min) was performed by comparing the UV spectra (max. absorbance at 214 nm) with spiked control samples (Fig. 2). A peak was observed at a retention time of about 2 min in the control and spiked samples, probably originating from some extracted endogenous compound, which, however, does not interfere with the nitrate determination. No interference with any peaks was observed. The linearity of the method was checked with nitrate concentrations ranging from 0–80 μmol/L, because nitrate concentrations in this range were expected. The obtained nitrate values of the filtered supernatants were within the employed concentration range. Since the protein content in the tissue homogenates varied, the nitrate concentration measured in the filtered deproteinized homogenates were calculated per mg of protein.

Five calibrants (each of them was repeated three times) were used to construct a calibration curve. The regression analysis was expressed by the equation: $y = 4886.8\,x + 52089.2$ ($Sa$ = 2811.9, $P$ < 0.00034) and a coefficient of correlation of 0.9981.

The within-assay precision was checked by repeated measurements (twenty) of samples spiked with 4.0 μmol/L $NO_3^-$, and the coefficient of variation, $CV$, was found to be 2.2 %.

The achieved limit of detection ($LOD$), 0.4 μmol/L, and the limit of quantification ($LOQ$), 1.2 μmol/L, were calculated mathematically by multiplying the standard deviation ($Sd$) by three and ten, respectively. However, the statistical calculation for the validation of the method was expressed as μmol/L for $NO_3^-$, unlike the obtained results regarding the nitrate values in the tissue homogenates, where

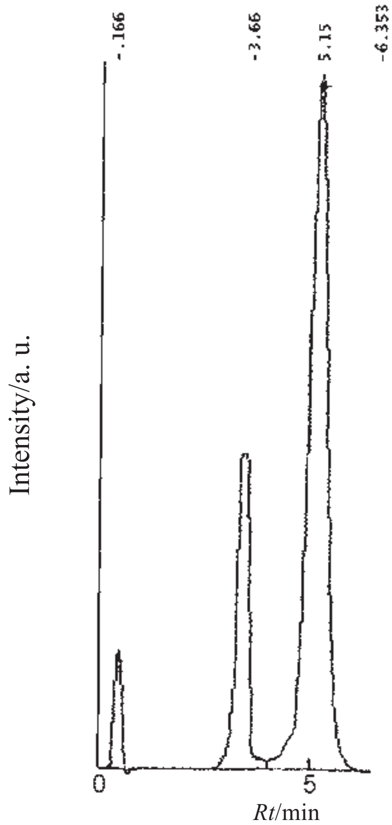


Fig. 1. Chromatogram of a mixed nitrite and nitrate standard solution, concentrations of nitrite and nitrate were both 40.3 μmol/L. Chromatographic conditions: anionic column IC-PAK$^{TM}$, 50 mm × 4.6 mm, 10 μm particle size (4.6×10 μm) and guard column; mobile phase: borate buffer/gluconate concentrate : methanol : acetonitrile : deionized water (2:12:12:74, v/v/v/v), pH 8.5; flow rate 1.3 mL/min; diode-array detection at 214 nm; room temperature.

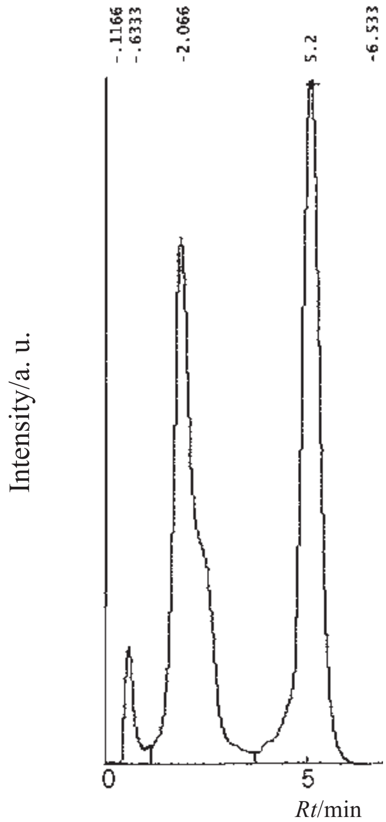


Fig. 2. Chromatogram of nitrates in spiked samples of brain tissue. Homogenate of the intact *cortex* of Wistar rats supplemented with nitrate, 40.3 μmol/L, was analyzed after protein precipitation with acetonitrile and centrifugation at 3000 × g, for 30 min. After protein precipitation with acetonitrile and filtration, nitrate was chromatographically determined. 50 μL of the filtrate was injected. Retention time of nitrate was 5.2 min.

the concentration was expressed as nmol/mg proteins for $NO_3^-$. Some of the cited authors obtained lower *LOQ* values, which may be due to the different biological material, sample preparation and chromatographic conditions.[4,5,19]

The *LOQ* for $NO_3^-$ determination (1.2 μmol/L) achieved using the present method was acceptable because the physiological nitrate levels measured in the brain tissue homogenates were 2.8 ± 0.9 μmol/L (*i.e.,* recalculated per mg of proteins: 18.8 ± 6.1 nmol/mg proteins for $NO_3^-$).

The accuracy of the method is shown in Table I. The samples were prepared according to the procedure given in the Experimental section.

TABLE I. Recovery values of nitrate in spiked homogenates of the *cortex* tissue, taken from untreated Wistar rats

| Added nitrate/μmol $L^{-1}$ $NO_3^-$ | Obtained nitrate/μmol $L^{-1}$ $NO_3^-$ | Coefficient of variation % | Recovery of added nitrate/% |
|---|---|---|---|
| 8.1 | 7.9 ± 0.3 | 3.3 | 99 ± 3 |
| 16.1 | 15.1 ± 0.4 | 2.7 | 93 ± 2 |
| 20.2 | 19.9 ± 1.1 | 5.7 | 99 ± 6 |
| 40.3 | 41.9 ± 0.7 | 1.7 | 104 ± 2 |

The baseline of $NO_3^-$ (endogenous $NO_3^-$, physiological level), in the *cortex*, before supplementation was 2.8 ± 0.9 μmol/L (18.8 ± 6.1 nmol $NO_3^-$/mg proteins). Number of repetitions for each amount of nitrate added to the homogenates $n = 6$. Recovery of added nitrate to the samples was 99 ± 4 % ($r = 0.9892$, $y = 0.9875\ x + 0.79$, $y$ = obtained concentration, $x$ = added nitrate concentration).

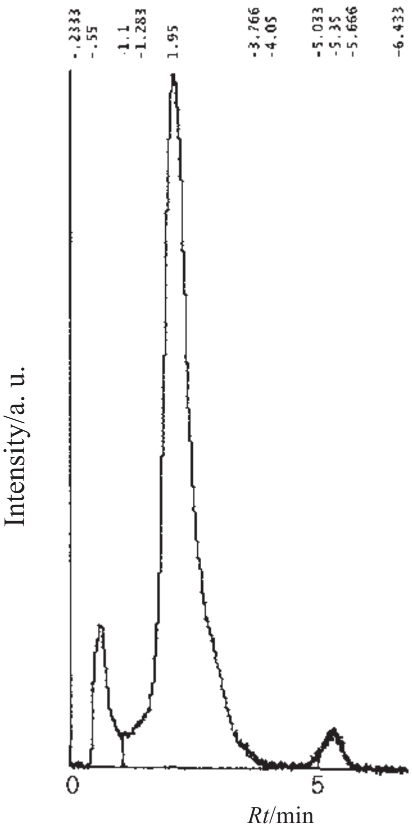


Fig. 3. Chromatogram of the intact brain tissue of Wistar rats (*cortex*, control sample). Retention time of nitrates was 5.35 min. For details of chromatographic conditions, see Fig. 1. The homogenates were prepared according to the protocol described in the Experimental.

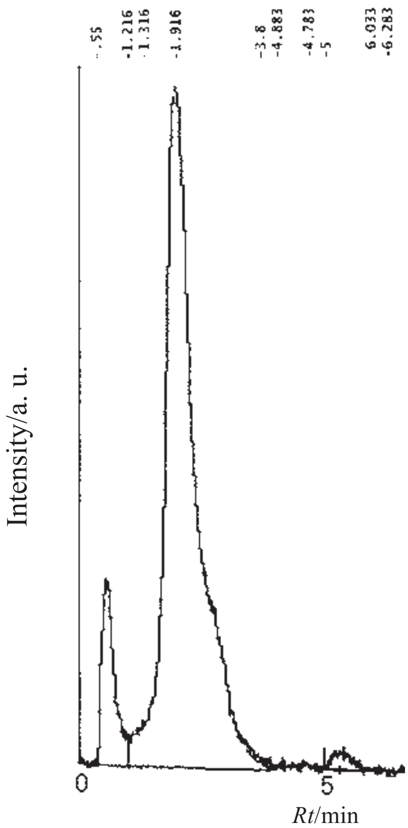


Fig. 4. Chromatogram of the brain tissue (*cortex*, treated group of Wistar rats, 30 min after the treatment). The homogenates of cortex taken from Wistar rats *i.s.* poisoned with PQ (50 mg/kg) were prepared according to the protocol described in the Experimental. Obtained retention time of the nitrate was 5.01 min. For details of chromatographic conditions, see Fig. 1.

UV diode-array detection is preferable because of the possibility to scan the spectra of compounds, including nitrate (max. of absorption at 214 nm), within the range of wavelengths 190–370 nm. Neither sulfate nor chloride anions, normally present at high concentration in tissue homogenates, absorbed at this wavelength.[9] Under the given chromatographic conditions, a nitrite peak was not detected in the analzyed samples (Figs. 3 and 4).[4,6,16,17,19] The obtained *LOQ* for nitrite in the analyzed standard nitrite and nitrate mixture was 4.2 μmol/L, which means that the nitrite concentrations in the samples were below the *LOQ* for nitrite. Also, some authors could not detect nitrite in cerebrospinal fluid.[9] A good separation of nitrite and nitrate peaks was achieved (separation coefficient, 1.49) (Fig. 1). A run time of 7.02 min was acceptable, related to the retention time of nitrate, around 5 min.

*Applicability of the method*

The method was applied for nitrate determination in brain tissue homogenates of Wistar rats poisoned with PQ. A wide range of nitrate concentration was used for regression analysis, covering the highest and lowest measured nitrate values in the samples (74.1 μmol/L, *i.e.,* 29.0 nmol/mg protein, and 3.4 μmol/L, *i.e.,* 0.6 nmol/mg proteins, in the *cortex* 30 min and 24 h after PQ poisoning, respectively).

The nitrate concentrations were measured at three time points after the treatment and the obtained results are shown in Table II. The large standard deviations obtained for the samples of PQ *i.s.* poisoned rats were expected, based on the wide nitrate variations obtained from the controls. Other authors also found similar results for nitrate (wide variation) measured in various biological samples. Smith measured nitrate in human plasma and found them in the range of 46.8 ± 18.6 nmol/L[5] and Rizzo measured nitrate in rat brain perfusates in the range of 1–1000 μmol/L. Moreover, for basal rat *cortex* nitrate levels, Rizzo obtained 42.78 ± 16.6 μmol/L.[4] Preik-Steinhoff measured nitrate in human blood in the range of 5–50 μmol/L.[16] Everett measured nitrate in plasma and in rat tumour perfusates and obtained values of 7.9 ± 2.6 μmol/L and 19.0 ± 11.0 μmol/L, respectively.[19] Ellis measured nitrate in human plasma and serum in the range of 30–200 μmol/L and 20–80 μmol/L, respectively.[17] Nitrate (and nitrite) are rapidly distributed throughout the body, therefore, it is likely than the largest portion of tissue nitrate comes from NO which had previously been oxidized in red blood cells.[15,16] Considering the wide range of nitrate concentration in blood, it could presumably be one of the key reasons for the large standard deviations obtained in the examined samples.

Nitrate levels in all three brain structures measured 24 h after PQ poisoning were significantly lower than the values observed 30 min after the poisoning. Also the nitrate measured 24 h after PQ poisoning in the *hippocampus* and *striatum* were significantly lower than in the controls (see Table II).

Apparently, PQ metabolism interferes with NO and contributes to PQ neurotoxicity, probably through $ONOO^-$ production, which triggers lipid peroxidation

TABLE II. Nitrate in the *cortex, hippocampus* and *striatum* of Wistar rats, 30 min, 24 h and 7 days after intrastriatal administration of PQ (50 mg/kg)

| | Control group | | 30 min | | 24 h | | 7 days | |
|---|---|---|---|---|---|---|---|---|
| | µmol $NO_3^-$/L | mg pr/mL hom nmol $NO_3^-$/mg pr | µmol $NO_3^-$/L | mg pr/mL hom nmol $NO_3^-$/mg pr | µmol $NO_3^-$/L | mg pr/mL hom nmol $NO_3^-$/mg pr | µmol $NO_3^-$/L | mg pr/mL hom nmol $NO_3^-$/mg pr |
| *Cortex* | 2.8±0.9 | 18.8±6.1 | 54.6±33.1 | 1.6±0.6 | 15.7±13.0 | 2.9±2.6 | 27.2±3.4 | 36.2±33.7 |
| | | 6.7±5.4 | | 31.9±22.6 | | 4.9±0.7 | | 1.0±0.6 |
| | (*n*=5) | | (*n*=5) | | (*n*=5) | | (*n*=6) | |
| *Hippocampus* | 3.4±0.8 | 18.0±5.0 | 50.5±38.5 | 32.7±12.2 | 13.3±6.0 | 3.3±2.9* | 21.7±11.1 | 14.5±6.9 |
| | | 5.3±3.9 | | 1.6±0.7 | | 4.5±1.5 | | 1.2±0.2 |
| | (*n*=5) | | (*n*=7) | | (*n*=7) | | (*n*=6) | |
| *Striatum* | 3.1±1.9 | 16.4±4.6 | 42.5±32.5 | 17.0±11.7 | 15.7±8.0 | 3.8±2.3* | 14.2±10.2 | 9.8±8.0 |
| | | 51.±2.3 | | 1.5±0.8 | | 3.7±0.8 | | 1.2±0.3 |
| | (*n*=5) | | (*n*=7) | | (*n*=6) | | (*n*=6) | |

The numbers in parenthesis indicate the number of the experimental animals. Control group represent sham operated animals. Nitrate values were calculated per mg of proteins, due to the different protein contents in the brain tissue homogenates. Sample preparation and chromatographic condition are described in the Experimental section.

mg pr = mg proteins

mg pr/mL hom = mg proteins/mL of homogenates;

* statistically significant lower values *vs.* the control group ($p < 0.05$)

in the neuronal membranes. Reaction between NO and $O_2^{\bullet-}$ probably contributes to endogenous NO depletion (significant at the $24^{th}$ hour, see Table II). Time and space propagation of OS was achieved almost identically in the *cortex*, *hippocampus* and *striatum,* which is in accordance with literature data regarding the acessibility of $ONOO^-$ to biological targets (permeability coefficient for $ONOO^-$ is $8 \times 10^{-4}$ cm/s, 400 times greater than that for $O_2^{\bullet-}$) which are restricted by their decomposition at the physiological pH ($t_{1/2}$ = 1s, 37 °C).[1] Therefore, nitrate, as one of the final decomposition products of $ONOO^-$ could be used as a measure of NO involvement in PQ neurotoxicity.[4,6]

## CONCLUSION

In conclusion, for the first time, a method for nitrate determination in brain tissue homogenates was validated in terms of sensitivity, specificity, linearity, precision, and accuracy. The lower quantification limit, *LOQ* of 1.2 mmol/L shows that this method is reliable and sensitive for the determination of nitrate below patho/physiological levels in brain tissues of Wistar rats.

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## ИЗВОД

## ОДРЕЂИВАЊЕ НИТРАТА IE-HPLC-UV МЕТОДОМ У МОЖДАНИМ ТКИВИМА WISTAR ПАЦОВА ТРОВАНИХ ПАРАКВАТОМ

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Презентовани рад је део започете студије о укључености реактивних врста азота (RNS) у неуротоксичност параквата (PQ). Садржај нитрата у селективно осетљивим можданим регијама (*cortex, striatum* и *hippocampus)* Wistar пацова може се користити као мерило продукције азотмоноксида или катаболизма других RNS. Хомогенизати можданог ткива су најпре депротеинизовани, затим центрифугирани. Нитрати су одређивани у филтрираном супернатанту брзом и једноставном изократском методом високо ефикасне течне хроматографије са diode-array детекцијом (IE-HPLC-UV) на 214 nm. Коришћена је мобилна фаза састава: боратни пуфер/глуконат концентрат : метанол : ацетонитрил : дејонизована вода (2:12:12:74, v/v/v/v), pH 8,5, при протоку 1,3 mL/min. Широк опсег концентрација нитрата као и њихови физиолошки нивои (18,8 ± 6,1 nmol/mg протеина) могу се мерити са добром прецизношћу (*CV* = 2,2 %) и тачношћу (recovery оптерећених узорака 99 ± 4%) у хомогенизатима можданих ткива. Линеарност је добијена у опсегу 0–80 μmol/L нитрата док је ретенционо време било 5,3 ± 0,3 min.

(Примљено 17. фебруара, ревидирано 5. јула 2006)

## REFERENCES

1. J. P. Eiserich, R. P. Patel, V. B. O'Donnell, *Mol. Aspects Med.* **19** (1998) 221
2. K. B. Wallace, *Free radical toxicology,* Taylor & Francis Ltd., London, 1997, pp. 11, 26
3. B. Halliwell, J. M. C. Gutteridge, *Free radicals in biology and medicine,* Clarendon Press, Oxford, 1985, pp. 206, 226
4. V. Rizzo, L. Montalbetti, A. L. Rozza, W. Bolcani, C. Porta, G. Balduzzi, E. Scoglio, R. Moratti, *J. Chromatogr. A* **798** (1998) 103
5. C. C. T. Smith, L. Stayner, D. J. Betteridge, *J. Chromatogr. B* **779** (2002) 201
6. D. Tsikas, *Free Radic. Res.* **39** (2005) 797
7. J. A. Timbrell, *Toxicology* **129** (1998) 1
8. Z. Radisavljevic, M. George, D. J. Dries, R. L. Gameli, *J. Liq. Chromatogr.* **19** (1996) 1061
9. G. Žunić, S. Spasić, Z. Jelić-Ivanović, *J. Chromatogr. B* **727** (1999) 73
10. V. Jedlickova, Z. Paluch, S. Alusik, *J. Chromatogr. B* **780** (2002) 193
11. S. Stojanovic, D. Stanic, M. Nikolic, S. Raicevic, M. Spasic, V. Niketic, *J. Serb. Chem. Soc.* **70** (2005) 601
12. C. K. Hallstrom, A. M. Gardner, P. R. Gardner, *Free Radic. Biol. Med.* **37** (2004) 216
13. Z. E. Suntres, *Toxicology* **180** (2002) 65
14. J. A. Timbrell, *Principles of biochemical toxicology*, Taylor & Francis Ltd., London, 2000, p. 107
15. B. Mayer, S. Pfeifer, A. Schrammel, D. Koesling, K. Schmidt, F. Brunner, *J. Biol. Chem.* **273** (1998) 3264
16. H. Preik-Steinhoff, M. Kelm, *J. Chromatogr. B* **685** (1996) 348
17. G. Ellis, I. Adatia, M. Yazdanpanah, S. K. Makela, *Clin. Biochem.* **31** (1998) 195
18. D. Connolly, L. Barron, B. Paul, *J. Chromatogr. B* **767** (2002) 175
19. S. A. Everett, M. F. Dennis, G. M. Tozer, V. E. Prise, P. Wardman, M. R. Stratford, *J. Chromatogr. A* **706** (1995) 437
20. B. K. Yang, E. X. Vivas, C. D. Reiter, M. T. Gladwin, *Free Radic. Res.* **37** (2003) 1
21. T. Fukushima, K. Yamada, N. Hojo, A. Isobe, K. Shiwaku, Y. Yamane, *Exper. Toxicol. Pathol.* **456** (1994) 437
22. G. Paxinos, C. Watson, *The rat brain in stereotaxic coordinates*, Academic Press, New York, 1986, p. 10–56.
23. J. W. Gurd, L. R. Jones, H. R. Mahler, W. J. Moore, *J. Neurochem.* **22** (1974) 281.