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Short communication

# Nitrite/nitrate balance during photoinduced cerebral ischemia in the rat determined by high-performance liquid chromatography with UV and electrochemical detection

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**Abstract**

A specific and simple method for the direct simultaneous detection of extracellular nitrite ($NO_2^-$) and nitrate ($NO_3^-$) has been developed, using high-performance liquid chromatography separation with UV and electrochemical detection in series. These stable endproducts of nitric oxide ($NO^\bullet$) were determined in dialysis perfusate obtained through in vivo brain microdialysis during and after experimental photoinduced cerebral ischemia in rats. The chromatographic conditions were optimized with a reversed-phase column (250×46 mm) using 10 m*M* *n*-octylamine pH 6.0 as a mobile phase. Absorbance was measured at 220 nm for $NO_3^-$ detection; electrochemical detection was performed at +0.7 V for $NO_2^-$ evaluation. This assay system holds the advantages of in vivo consecutive measurements, high precision, good reproducibility, technical simplicity, fast response (about 7 min), and wide availability. 

*Keywords:* Nitrite; Nitrate; Inorganic anions; Nitric oxide; Neurotransmitters

## 1. Introduction

Increases in extracellular neurotransmitter concentrations are related to the pathogenesis of ischemic cell damage [1,2]. Extracellular chemical events in several areas of the brain such as the hippocampus, cortex and striatum of laboratory animals, have received much attention, because these structures are particularly vulnerable to ischemia [3,4]. The importance of neuronal nitric oxide radical ($NO^\bullet$), as a neurotransmitter [5], in the pathophysiology of focal cerebral ischemia has been investigated, in the past [6]. Upon induction of ischemia, the $NO^\bullet$ level increases dramatically inside the cortex [7]. Several experiments with cultures of cortical and striatal neurons suggest NO may facilitate or modulate neurotoxicity by activating N-methyl-D-aspartate receptors via the hemodynamic mechanism [8]. $NO^\bullet$ is a highly reactive molecule and its short halflife makes it difficult to detect in the brain [9].

Many different methods for real-time detection of $NO^\bullet$ production (such as the NO-selective electrochemical microsensor or electrospin resonance) [10,9] have been reported. $NO^\bullet$ can also be detected by chemiluminescence [11] or by spectrophotometric assay in which $NO^\bullet$ reacts with ferrous oxyhemoglobin to yield nitrate ($NO_3^-$) and methaemoglobin [12]. These methods are technically complex and

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their in vivo application is difficult. $NO^{\bullet}$ production can be detected by determining of its stable end-products, namely nitrite ($NO_2^-$) and nitrate ($NO_3^-$). In body fluids most of the $NO_2^-$ is converted into $NO_3^-$; therefore, to determine $NO_2^-$ alone is meaningless. The most commonly used $NO_2^-$ assay is based on the Griess diazotation reaction [13], which is specific for $NO_2^-$. Hence $NO_3^-$ in samples must first be reduced to $NO_2^-$ either by reductase [14], or by hydrazine [15]. Determination of $NO_2^-$ and $NO_3^-$ in body fluids has been performed by means of high-performance liquid chromatography (HPLC) [16], capillary electrophoresis [17] or anion-exchange chromatography [18].

Recently, microdialysis was introduced as a very powerful in vivo sampling technique [19,20]. This technique (whose application is still to be fully explored) requires analyses to be performed on very small samples containing analytes in concentrations lower than for endogenous analytes.

To characterize extracellular changes in the NO metabolite within the context of an experimental model of focal cerebral ischemia (based on the photochemical induction of thrombotic stroke via Rose Bengal) [21], we developed a simple HPLC separation and designed a detection system for $NO_2^-$ and $NO_3^-$ anions which makes the analysis of these substances possible in microdialysates from the rat cortex. This was achieved by combining the direct injection of a small quantity (10 μl) of dialysis perfusate with high-performance ion-pair chromatography. In addition, two in-line connected detectors (UV and electrochemical detector) are needed to identify $NO_2^-$, $NO_3^-$ and other anions in perfusate. These may be useful tools when $NO_2^-/NO_3^-$ balance and excretion studies are required [22].

## 2. Experimental

### 2.1. Chemicals and reagents

$NaNO_2$, $NaNO_3$, *n*-octylamine were purchased from the Sigma (St. Louis, MO, USA). Other chemicals were at the highest analytical grade. The *n*-octylamine buffer was prepared by adding 1.6 ml of *n*-octylamine to 1 l of HPLC-grade water, twice filtered. After mixing for 30 min at least, the pH was corrected to 6.0±0.2 with 1 m*M* $H_2SO_4$.

Artificial cerebrospinal fluid was obtained by mixing 143.7 m*M* NaCl, 3 m*M* KCl, 10 m*M* $MgCl_2$, 14 m*M* $Na_2HPO_4$ and 1.26 m*M* $CaCl_2 \cdot H_2O$ (pH 7.4).

### 2.2. Chromatography

The HPLC system consisted of a Waters 510 A pump (Waters, MA, USA), equipped with a Rheodyne Model 7125 sample injector with a 10 μl sampling loop. Chromatographic separation was performed on a reversed-phase analytical column (250×46 mm), packed with LC-18-T, 5 μm particle size (Supelco, Bellefonte, PA, USA). Mobile phase was 10 m*M* *n*-octylamine containing 1 m*M* $H_2SO_4$ (pH 6.0) and the flow-rate was 1 ml/min. Detection was performed by means of a UV detector (Waters 484) and an electrochemical detector (Waters 460), in series. The absorbance and electrochemical response were measured at a wavelength of 220 nm and at a potential of +0.7 V, respectively. The peak height was used as a measure of the detector's response.

$NO_2^-$ and $NO_3^-$ quantitative determination was carried out using an external 50 μ*M* standard solution of either $NO_2^-$ and $NO_3^-$, obtained from a stock solution of 1 m*M* $NaNO_2$ and 1 m*M* $NaNO_3$ prepared in artificial cerebrospinal fluid (pH 7.4) and stored at −20°C for two weeks.

### 2.3. Animals

Male Wistar rats weighing 250–300 g were used. They were housed in groups in a room controlled at 22±2°C and maintained in an alternating 12-h light–dark cycle. Food and water were given ad libitum.

### 2.4. Brain microdialysis

Brain microdialysis was performed as described previously [23,24]. In brief, experimental ischemic group rats ($n$=8) were anesthetized intraperitoneally with Equitensine (pentobarbital, 29 mg/kg; chloral hydrate, 127.5 mg/kg; $MgSO_4$, 71.4 mg/kg; dihydroxypropane, 40%; ethanol, 10%) and placed on a Kopf–Carnegie's stereotaxic apparatus. A

Hospal–Cuprophan Bellco dialysis probe (4 mm long, 0.22 mm outside diameter, molecular mass cut-off at $M_r$ 10) was inserted horizontally: +0.2 mm from bregma and 2.0 mm under the skull surface to cross the frontoparietal cortical region. After injecting the vital dye Rose Bengal (1.7 mg/100 g mass) into the tail vein, the ischemia was induced for 20 min by irradiating the selected area with a halogen lamp (Endophare-Martin; 15 V, 150 W).

The control group ($n=6$) went through the same procedure described above, obviously without irradiation. This group was also made up of the rats on which the dye and light tests had no effect.

The probe was perfused at a rate of 2 μl/min (Carnegie Medicin CMA-110) with artificial cerebrospinal fluid and the perfusate was collected in 60-μl fractions and stored at −80°C until HPLC analysis was carried out.

### 2.5. Sample preparation

Under experimental photoinduced ischemia, samples were continuously collected, at room temperature, in test tubes according to the following schedule: 30 min time before anesthesia; 30 min time after induction of anesthesia (preischemia) and 20 min time during irradiation (ischemia). Within the subsequent 4 h (postischemia) samples were successively collected as above in 30 min intervals.

A small quantity of rat perfusate (10 μl) was directly injected into HPLC without pretreatment. Deproteinization is not necessary because brain microdialysate does not contain large molecules such as proteins.

### 2.6. Statistical analysis

Values are expressed as mean±S.D.. The temporal profile of each variable was evaluated by Friedman's two-way analysis of variance test for repeated measures. The Wilcoxon signed rank test was used to compare control and ischemic groups. Statistical differences were considered significant at $P<0.5$

## 3. Results

### 3.1. Chromatographic separation

The method described in this paper ensures an excellent separation of $NO_2^-$ and $NO_3^-$ with retention times standing about 4.8 and 6.3 min, respectively as shown in Fig. 1.

These compounds were identified by comparing

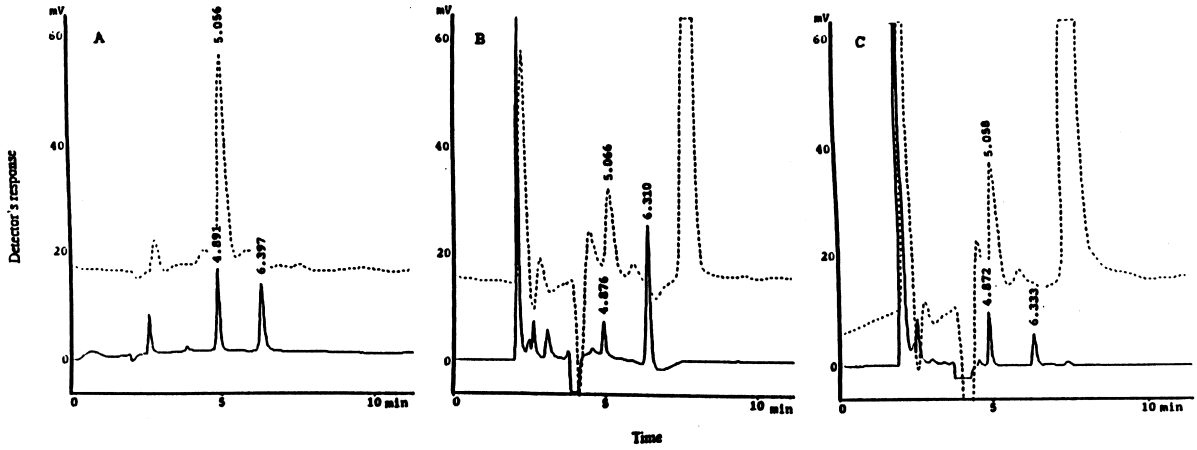


Fig. 1. (A) Typical chromatograms of 50 μ*M* standard compounds of $NO_2^-$ ($t_R=4.8$) and $NO_3^-$ ($t_R=6.30$); (B) a basal condition of microdialysis sample from the rat cortex; (C) a microdialysis sample collected 60 min after photoinduced ischemia. Solid line=UV detection; dashed line=electrochemical detection. Separation was obtained on a LC-18-T, 5 μm column, using a mobile phase at pH 6.0±0.02. Detector's response was monitored at a wavelength of 220 nm for UV detection and at a potential of +0.7 V for electrochemical detection.

their retention times with those of the pure compounds and by adding the latter to perfusate samples. Other identified compounds, such as tyrosine, 6-OH-dopamine, serotonin, dopamine, 5-OH-tryptophan and 3-nitrotyrosine were proved not to interfere.

Retention of ionic compounds in a reversed-phase system could be achieved by applying a ion-pairing mechanism. The use of *n*-octylamine [25] as an ion-pair reagent improves the retention and resolution of $NO_2^-$ and $NO_3^-$ on a $C_{18}$ HPLC column. The mobile phase composition was chosen following a thorough assessment of the influence of each constituent on chromatographic separation. In particular, the eluent pH (6.0), the absence of organic modifier concentrations, and *n*-octylamine concentration (10 m*M*, adjusted with sulphuric acid) all significantly improved separation reproducibility. Under these conditions, analysis time was sufficiently short to allow several samples to be assayed per day.

An analytical 250×46 mm column, at a flow-rate of 1.0 ml/min, was preferred to a shorter column despite being associated with slower analysis,in order to rule out interference from other perfusate compounds. The absorbance spectrum of $NO_2^-$ and $NO_3^-$ performed in the range 205–300 nm, shows a maximum at 205 nm. However, the 205 wavelength presents a large solvent front, so that a 220 nm wavelength was preferred, despite being associated with lower sensitivity.

Direct injection of perfusate may cause the occurrence of many interfering peaks in 2 and 8 min time spans, endangering the accuracy of $NO_2^-$ determination. Specificity of the separation was evaluated through UV and electrochemical detection in series. The electrochemical-to-absorbance signal ratio can be used as a tool for $NO_2^-$ purity peak evaluation.

Moreover, the low recovery of both anions through the dialysis membrane requires sensitivity to be increased. The use of an electrochemical detector, set at +0.65–0.7 V, solved this problem by increasing sensitivity to $NO_2^-$ by three times compared to the UV signal.

To avoid possible contamination or sample carryover between injections, great care was taken to clean the injection loop with methanol–deionized water (30:70, v/v). By following the above procedures, an adequate resolution was obtained for the peaks of interest and no problems arose with late eluting peaks.

### *3.2. Assay performance*

The linearity of this HPLC assay was tested for both anions and the calibration curves of peak height versus injected amount were linear within the range of 1.0–1000 μ*M* for both anions.

The in vitro recovery of $NO_2^-$ and $NO_3^-$ through the dialysis membrane was 27.9% for $NO_2^-$ and 41.2% for $NO_3^-$ (five determinations), tested by immersing the dialysis probes into 37°C artificial cerebrospinal fluid (pH 7.4) containing 1 m*M* $NaNO_2$ and $NaNO_3$ and perfusing for 60 min at a rate of 2 μl/min. The concentration of $NO_2^-$ and $NO_3^-$ in perfusate was not corrected for loss of recovery during probe perfusion

The precision and repeatability of the system were examined by $NaNO_2$ and $NaNO_3$ injections ($n=10$) of a 50 μ*M* standard solution. The mean value was 0.498±0.017 μ*M*, giving a R.S.D. of 3.4%. The injection of artificial cerebrospinal fluid, used as a blank, did not yield any detectable signal.

The absolute detection limit (defined as three times the baseline noise) of this combined assay for $NO_2^-$ and $NO_3^-$ was 0.9 n*M* and 4.4 n*M*, respectively.

The stability of $NO_2^-$ and $NO_3^-$ in a pool spiked perfusate (at concentrations of about 50 and 100 μ*M*) stored for 1 month at −80°C was studied. In addition, the stability of aliquots of the same pool perfusate stored for 12 h at room temperature and at 4°C was also evaluated. The compounds proved to be stable under all conditions examined, allowing a nonrefrigerated autosampler to be used.

### *3.3. Extracellular $NO_2$ and $NO_3$ concentration*

In the rat cortex dialysis perfusate ($n=14$), the $NO_2^-$ basal levels were in the range 15–27 μ*M* (mean 22.15±3.21). $NO_3^-$ basal levels were found in the range 16–61 μ*M* (mean 42.78±16.6).

As shown in Table 1, extracellular concentrations of $NO_2^-$ and $NO_3^-$ before, during and after ischemia show no significant difference between ischemic and control groups. The temporal profile of the $NO_2^-$/

Table 1
Extracellular levels of $NO_2^-$ and $NO_3^-$ in microdialyzed samples collected before, during and after experimental photoinduced ischemia

| | $NO_2^-$ | | $NO_3^-$ | | $NO_2^-$:$NO_3^-$ | |
|---|---|---|---|---|---|---|
| | Controls Mean±S.D. | Ischemics Mean±S.D. | Controls Mean±S.D. | Ischemics Mean±S.D. | Controls Mean±S.D. | Ischemics[a] Mean±S.D. |
| Preischemia | | | | | | |
| 30 min before anesthesia | 21.9±2.24 | 22.28±4.1 | 39.4±16.7 | 45.14±17.5 | 0.66±0.33 | 0.64±0.44 |
| anesthesia | 17.5±6.58 | 20.0±12.5 | 31.6±17.6 | 31.3±13.5 | 0.70±0.45 | 0.66±0.46 |
| Ischemia | | | | | | |
| 20 min | 15.9±7.24 | 26.1±16.1 | 23.8±10.3 | 46.7±20.3 | 0.73±0.29 | 0.57±0.33 |
| Postischemia | | | | | | |
| 1 h | 12.20±6.06 | 39.3±28.6 | 16.4±7.99 | 37.3±18.5 | 0.77±0.18 | 1.13±0.65 |
| 2 h | 12.6±5.81 | 31.2±21.6 | 18.7±9.12 | 27.5±13.6 | 0.72±0.25 | 1.27±0.81 |
| 3 h | 14.3±6.65 | 30.0±14.9 | 17.8±4.43 | 27.6±8.1 | 0.80±0.28 | 1.07±0.57 |
| 4 h | 16.7±8.58 | 29.7±21.7 | 18.7±7.0 | 36.1±12.02 | 0.96±0.55 | 0.91±0.80 |

Values (mean±S.D.) are expressed in μmol/l.
[a] $P<0.05$ at Friedman's test.
Experimental groups: controls $n=6$; ischemics $n=8$.

$NO_3^-$ ratio, instead, significantly increases ($P<0.5$) in the ischemic group compared to the controls.

## 4. Conclusions

The present study describes a specific HPLC method for the measurement of $NO_2^-$ and $NO_3^-$, as indices of endogenous $NO^\bullet$ production, in rat cortical perfusate, obtained through microdialysis. This is a very flexible technique which allows simultaneous measurement of a number of biological substances such as amino acids, monoamines, and superoxide derived from the brain tissues. In addition, the resulting dialysate sample does not contain large molecules such as proteins and no clean-up is necessary. However, the direct injection of perfusate entails many problems associated with $NO_2^-$ at low concentration ranges (the dialysate fraction produced is very small) and with laboratory contamination with $NO_2^-$. The use of UV and electrochemical detectors in series is useful in obtaining good accuracy and specificity. Moreover, high precision over repeated measurements and good reproducibility make this assay highly suitable for consecutive brain $NO^\bullet$ production monitoring with a relatively short resolution time.

The present study presents the indirect measurements of the $NO^\bullet$ in the rat brain, by evaluating the $NO_2^-/NO_3^-$ balance. This parameter can better explain the alteration of $NO^\bullet$ during and following the course of photoinduced cerebral ischemia, than single $NO_2^-$ or $NO_3^-$ determination. As a matter of fact, in our experiments, a statistically significant difference in ischemic rat cerebral perfusate, compared to controls, was observed only when the $NO_2^-/NO_3^-$ ratio was considered, while neither $NO_2^-$ nor $NO_3^-$ alone proved to be useful in highlighting such differences.

As shown in Table 1, an increase in the $NO_2^-/NO_3^-$ ratio over time, during and after focal cerebral ischemia in rats, followed by a slow decline to baseline levels, was observed in our model. This findings are consistent with other ischaemia models, described in Refs. [26–30].

Regarding the biological significance of this increase in NO production (which we demonstrated to be more reliably described by $NO_2^-/NO_3^-$ variations over time), it could be interpreted as a compensatory mechanism. Indeed, neuronal cells try to obviate the dangerous condition of ischemia by producing a large amount of $NO^\bullet$, a potent natural vasodilator [31]. This mechanism has been well recognized in a number of physiopathological conditions, such as

atherosclerosis and different models of thrombosis [32–34].

The fast $NO_2^-/NO_3^-$ increase we observed after ischemia, may be attributed to the production of free radicals in the ischemic area and to the reaction of $NO^\bullet$ with superoxide anions, which decompose to generate highly toxic hydroxyl- and nitrogen dioxide-radicals [35]. The slow decline in the $NO_2^-/NO_3^-$ ratio, during the last postischemic period, may be attributed to limited NO consumption by chemical reactions.

Our data demonstrate that the methodology is reliable in determining the metabolites of $NO^\bullet$ such as $NO_2^-$ and $NO_3^-$ in body fluids and tissue specimens. In particular, our findings suggest that most $NO^-$ in the microdialyzed fraction is of neuronal origin, thus raising the possibility that variations in $NO^\bullet$ metabolism could be used as an index of significant alterations in the homeostatic mechanism in the ischemic area.

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