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High sensitivity analysis of nitrite and nitrate in biological samples by capillary zone electrophoresis with transient isotachophoretic sample stacking

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Abstract

Tissue level of nitrate and nitrite are established indicators of altered nitric oxide metabolism under various pathological conditions. Determination of these anions in biological samples, in the presence of high chloride concentration, using capillary zone electrophoresis suffers from poor detection sensitivity. Separation conditions providing excellent resolution and submicromolar detection sensitivity of nitrate and nitrite have been developed and validated. Simple sample preparation was applied that maintains nitrite stability in tissue extracts and at the same time allows transient isotachophoresis stacking of the analytes. Nitrate and nitrite concentrations in rat brain and liver tissue samples were determined in control and lipopolysaccharide treated animals.

Keywords: Sample stacking; Isotachophoresis; Nitrite; Nitrate; Inorganic anions; Nitric oxide

1. Introduction

Nitric oxide (NO) is established as an important cell-signalling molecule and anti-infective agent in the mammalian tissues. Under physiological conditions its primary decomposition product is nitrite [1]. In the presence of oxidizing agents (e.g. oxyhaemoglobin) it is further oxidized to nitrate. Under various pathological conditions, characterized by oxidative stress of cells, NO combines with reactive oxygen species, like superoxide anion, and can give rise further series of compounds, called reactive nitrogen species (RNS). These derivatives can modify biopolymers and cause cell damage. Peroxynitrite is regarded as the most important nitrating agent in biological systems [2], which can be spontaneously decomposed to nitrate at physiological pH [3]. The nitrite and nitrate level, and their ratio in tissues are indicators of the disturbed NO metabolism, and can be used for the estimation of NO and RNS production. The direct quantitative assessement of these latter compounds otherwise is rather difficult, because of their very short half-lives. Analysis of plasma and urine levels of these anions has limited relevance in this regard, because of the oxyhaemoglobin catalyzed conversion of nitrite to nitrate, and the bias caused by the diet on the concentration of nitrate.

Determination of nitrite and nitrate in biological samples by capillary electrophoresis is difficult because of the high salt (chloride) content of the sample matrix and the relatively low analyte concentrations. In order to prevent peak distortion, high ionic strength separation buffers and low sample volumes should be used. Various capillary zone electrophoresis (CZE) separation conditions for quantitative determination of nitrite and nitrate in plasma and tissue samples, using direct UV detection at 214 nm, have been reported in the last several years [4–14]. Majority of these separations is based on migration of the anions in a high pH separation buffer

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containing modifiers to suppress or reverse the electroosmotic flow [4–9]. The applicability of low pH background electrolytes was also demonstrated [10–13]; higher selectivity of the nitrite and nitrate separation [10–12], and their ultra-rapid analysis by the use of an electroosmotic flow (EOF) modifier [11] could be achieved. Although these methods allow rapid separation of the analytes, some of them lack the needed detection sensitivity. Various on-capillary sample preconcentration techniques or sample clean-up procedures have been suggested to reach the required detection limit without the loss of separation efficiency. Field-amplified stacking of the analytes using diluted samples [5,7,13], creation of a transient pseudo-isotachophoresis (ITP) migration by inclusion of an organic solvent (acetonitrile) into the sample [14], or removal of chloride from the sample matrix (either by SPE sample clean-up, or reversed pre-electrophoresis) [13] provided limits of detection (LODs) between 2 and 10 μM of nitrite and nitrate in biological samples. Better quantitation limits could be achieved so far, when conductivity detection was applied and the chloride was removed from the sample [15].

High protein content of the biological samples, limited stability of nitrite under acidic conditions or in the presence of ferroproteins [16,17] and the prevalence of these anions in laboratory ware and reagents [4] also require simple and carefully designed sample preparation.

The aim of this study was to find separation conditions providing improved detection sensitivity and better reproducibility of nitrite and nitrate measurements in tissue samples. In this paper separation conditions providing excellent resolution and increased detection sensitivity by sample-induced transient ITP stacking of the analytes are described. Special care was taken to maintain nitrite stability during sample preparation and analysis. The method yields good reproducibility and accuracy, the analysis time is short. The composition of the separation medium, and the sample clean-up is simple, there are no need of using buffer modifier or expensive and time-consuming sample preparation. Nitrate and nitrite concentrations in rat brain and liver tissue extracts were determined in control and lipopolysaccharide (LPS) injected animals to demonstrate the increased tissue level of these analytes following induction of NO synthesis.

2. Experimental

2.1. Instrumentation and separation conditions

All experiments were performed with a Prince (Prince Technology, Emmen, The Netherlands) capillary electrophoresis system equipped with a UV detector, set at 214 nm. Separations were carried out in uncoated fused silica capillaries 75 μm i.d., 365 μm o.d., 70 cm total length, 55 cm to the detector (Polymicro Technology, Phoenix, AZ, USA). The capillary was washed successively with 1 M sodium hydroxide, water and the separation buffer (200 kPa, 1 min each) between runs. Axxiom 727 (Axxiom Chromatography, Moorpark, CA, USA) software was used for data collection.

Thirty mM Sulfate-β-alanine pH 3.8 buffer was used for the separations (when other pH is not indicated). Hundred mM formate–NaOH buffer pH 7 containig 0.09% NaCl or 50 mM acetate–NaOH buffer pH 7 containig 0.09% NaCl was used for the preparation of model samples of nitrite, nitrate and bromate (IS) to induce their transient ITP migration. The separations were performed at constant current of −75 μA (if otherwise is not indicated).

2.2. Reagents

Solutions were prepared using ultrapure water from a Milli-Q water system (Millipore, Bedford, MA, USA). Ultrapure analytical grade potassium nitrate, sodium nitrite and potassium bromate were purchased from Fluka (Buchs, Switzerland). Sodium hydroxide, sodium chloride, sulfuric acid, formic acid and acetic acid of highest purity were from Merck (Darmstadt, Germany). 0.1 M stock solution of standards were prepared and kept at −20 °C. Lipopolysaccharide from *E. coli* and β-alanine were obtained from Sigma (St. Louis, MO, USA).

2.3. Animals

Male Wistar rats (6-weeks old, 180–200 g) were supplied by the Animal Center of the Semmelweis University. All animal procedures were performed according to the *Guidelines of care and use of laboratory animals* of the Semmelweis University. The animals were housed in a climate controlled (22 ± 2 °C) animal room with 12-h light and 12-h dark cycle. Standard laboratory rodent chow and tap water were ad libitum. The rats were treated intraperitoneally with a single 10 mg/kg dose of LPS or saline (control animals). Tissue samples were obtained 24 h after treatment.

3. Results and discussion

3.1. Optimization of the separation method

3.1.1. Buffer composition

Majority of the previously published CZE methods used high pH separation buffer, where both nitrite and nitrate possess high, but similar electrophoretic mobilities. The use of EOF modifier to suppress and reverse the bulk flow was needed to have rapid separations applying negative polarity [4–9], but the analytes migrated rather close to each other. At lower pH, the resolution of the analytes can be increased, because of the selective retardation of nitrite (p*K* of nitrite is 3.15), while the mobility of nitrate does not change [11]. In the previously described methods, polyacrylamide coated capillary [10] or EOF reversal [11] were used to get rapid anionic separations in acidic background electrolytes. Using

low separation pH allows the adjustment of the resolution of nitrate and nitrite, furthermore reasonably rapid analysis time can be achieved even without using EOF modifier, as is demonstrated in our present experiments. Thirty mM sulfate-β-alanine buffer was chosen to perform the separations, because the mobility of sulfate is similar to those of the analytes, and is between those of the intended leading and terminating ions, thus allows the sample-induced transient ITP migration of the analytes [18]. We have studied the resolution of nitrite, nitrate and bromate (IS) in the sulfate-β-alanine buffer of various pH (between pH 2 and 4) using uncoated fused silica capillary; electropherograms are shown in Fig. 1. The separation was too slow at pH 3 and below, since high portion of nitrite was protonated, and had low electrophoretic mobility. At pH 3.5 nitrite and bromate co-migrated, thus further increase of pH was needed to get the desired resolution and shorter migration time. Eventually, pH 3.8 was chosen to perform rapid separations with excellent resolution of the analytes. The EOF velocity was rather constant in the studied pH range, as is shown by the unchanged migration times of nitrate and bromate between pH 3 and 4.

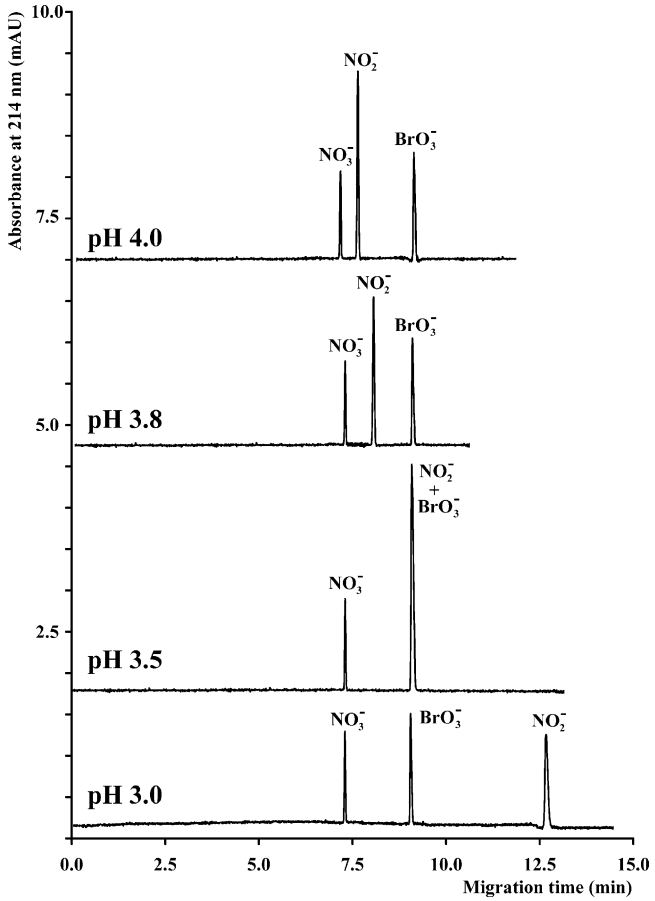

Fig. 1. Separation of nitrate, nitrite and bromate (10, 20, and 100 μM in distilled water, respectively) in 30 mM sulfate-β-alanine buffers of various pH. Voltage: −14 kV. Injection: 2.5 kPa, 30 s.

3.1.2. Injection conditions

Separation performance is usually degraded by the presence of salts in biological samples, causing alterations in peak heights and peak distortion associated with an enhanced zone dispersion [14,19]. High salt content of the sample allows only small injection volume without the deterioration of separation efficiency, however, this results in unsatisfactory detection limits when direct UV detection of analytes at 214 nm is used. Two approaches of the on-capillary sample concentration was examined in our further experiments.

3.1.2.1. Field amplified sample stacking. On-capillary sample concentration with field amplified stacking can be achieved by dilution of the sample with deionized water. Longer plug of the diluted, low conductivity sample can be injected hydrodynamically into the capillary, filled with the separation buffer of higher conductivity. After the high voltage is applied, the ions in the low-conductivity sample plug experience a higher field strength than those in the high-conductivity separation buffer, thus the sample ions move quickly through the sample matrix and slow down when enter the separation electrolyte, resulting in a narrow zone of analytes at the boundary of the two solutions [20]. Using field amplified sample stacking, up to 10% of the separation capillary can be filled with sample without a decrease in the separation efficiency. When higher volume is injected, zone broadening and increase in the migration times occur because only a small portion of the field strength drops across the separation electrolyte and there is a mismatch in the electroosmotic velocity in the different parts of the capillary [20]. Larger injection volumes can be used, when the removal of the low-conductivity sample matrix from the capillary is possible after stacking is performed. Burgi and Chien used polarity-switch to reverse the EOF and eliminate the sample water into the inlet vial, while keeping anionic analytes in the capillary [21]. In our present work, the separation is performed in reversed polarity mode; the migration of the anionic analytes is opposite to the direction of the weak EOF. Under these conditions, large sample volume can be filled into the capillary, because the low conductivity sample matrix is pumped out by the EOF during the field amplified sample stacking. This can be achieved because of the uneven distribution of field strength, which provides higher EOF velocity in the sample plug compared to the separation electrolyte. Separation of a model sample containing nitrite, nitrate and bromate dissolved in physiological saline is shown in Fig. 2A. Good separation efficiency was only achievable by loading small volume of this high salt content sample into the capillary (about 1% of the capillary volume), which resulted in unacceptably high detection limit. However, almost the total capillary length could be filled with the sample when it was highly diluted with deionized water. Excellent separation efficiency was gained when ninty percent of the capillary was loaded with the sample after its 100 times dilution, since the low-conductivity sample matrix was removed by the EOF through the capillary inlet (Fig. 2B). This procedure provides considerable sample

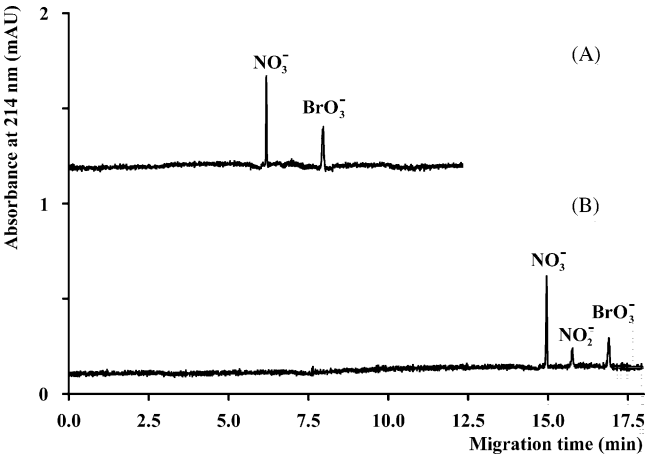

Fig. 2. Separation of nitrate, nitrite and bromate (20, 4 and 200 μM in 0.9% NaCl, respectively). Injection: (A) 1 kPa, 15 s and (B) 10 kPa, 135 s of the 100× diluted (with deionized water) sample. Separation buffer: 30 mM sulfate-β-alanine pH 3.8, voltage: −14 kV.

concentration; 0.04 μM sample nitrite on capillary could be well detected (Fig. 2B). The estimated LOD achievable in the original (undiluted) biological specimens is about 2 μM nitrite. Although this procedure provides acceptable detection limits, the rather long analysis time, because of the lengthy procedure of the sample stacking, is a disadvantage of this method. Thus, the possibility of sample concentration by a transient ITP migration of the analytes has also been studied.

3.1.2.2. Sample induced transient ITP migration. On-capillary transient ITP migration usually permits the injection of large volume (about 30–50% of capillary volume) of sample into the capillary, without the restriction of having low sample conductivity, characteristic of the field amplified sample stacking [22]. When high mobility ion (in this case chloride) is present in the sample at a concentration of several orders of magnitude higher than the analyzed species (nitrite, nitrate), sample induced transient ITP stacking can be expected [18,22]. The zones of the minor sample components become stacked (concentrated) and migrate in ITP mode for some time behind the zone of the macrocomponent prior to their migration in zone electrophoresis. Usually the co-ion of the background electrolyte is chosen to be the terminator ion [23]. Artificial samples containing 0.09% NaCl (10 times diluted physiological saline) were created to model separation efficiency of the biological samples. Low mobility terminator ion as co-ion of the background electrolyte (50–250 mM formate-β-alanine or 50–250 mM acetate-β-alanine pH 3.8 as separation buffer) was first chosen, but under these conditions, the zones of the sample components reached the detector earlier than they were unstacked, thus the individual components could not be detected (data not shown). Simultaneous stacking of sample components can also be achieved when the co-ion of the background electrolyte possesses an intermediate mobility and both the leading and terminating ions are present in the injected sample [18]. In order to create

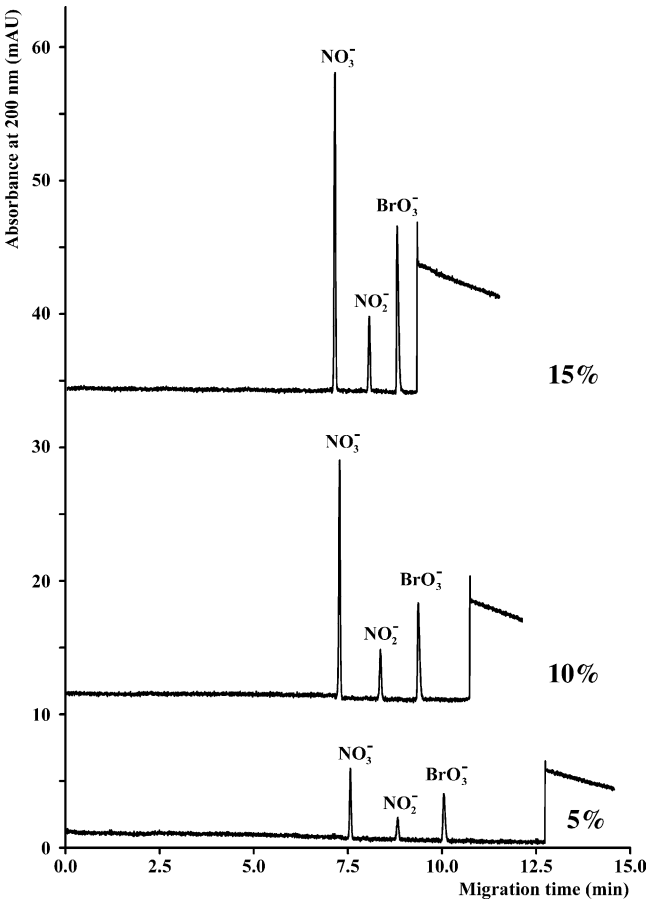

Fig. 3. Separation of nitrate, nitrite and bromate 5, 5 and 50 μM, respectively, in 100 mM formate–NaOH buffer pH 7 containig 0.09% NaCl. Separation buffer: 30 mM sulfate-β-alanine pH 3.8, constant current: −75 μA. Injection: 2.5 kPa, for 30, 60 and 90 s, corresponding to 5, 10 and 15% of capillary volume, respectively.

this condition, 30 mM sulfate-β-alanine pH 3.8 background electrolyte was used, and the sample was supplemented with the intended terminator ion; the analyte standards were dissolved either in 50–100 mM formate–NaOH or 50–100 mM acetate–NaOH buffer pH 7 containing 0.09% NaCl, respectively. The macrocomponent chloride, like in biological samples, serve as the leading ion. Under these conditions, when both leading and terminator ions were present in the sample, transient ITP migration and concentration of the analytes were observed. Both formate and acetate were proved appropriate terminator ions, however, the application of the slower acetate allowed higher sample injection volume. The mobility of formate was close to that of the sample components of interest, thus the zone of formate unstacked just before the zone of bromate, allowing injection volume not more than 15% of the capillary volume (Fig. 3). Having the acetate in the sample, 40% of the capillary volume could be filled with the sample and still good resolution of the analytes could be achieved (Fig. 4). However, when 40% injection volume was used, the zone of nitrate destacked just before reaching the detector, which limited the further increase of sample injec-

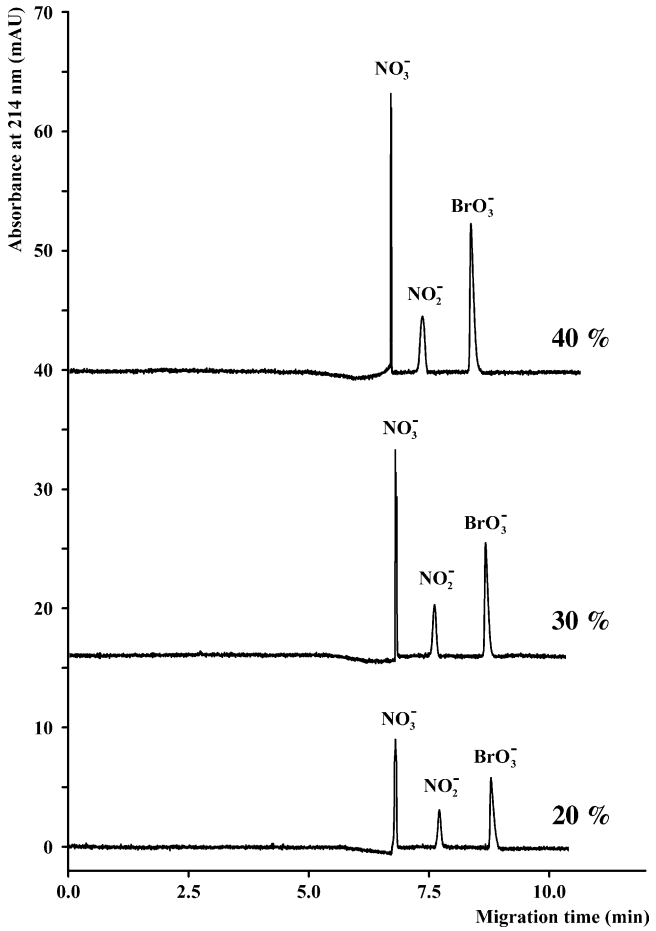

Fig. 4. Separation of nitrate, nitrite and bromate 5, 5 and 50 μM, respectively, in 50 mM acetate–NaOH buffer pH 7 containig 0.09% NaCl. Separation buffer: 30 mM sulfate-β-alanine pH 3.8, constant current: −75 μA. Injection: 10 kPa for 30, 45 and 60 s corresponding to 20, 30 and 40% of capillary volume, respectively.

tion. 30% of the capillary volume was chosen as injection size in the analysis of biological extracts containing similar amount of acetate. When transient ITP preconcentration was intended to perform, the commonly used internal standard of anion analysis, bromide was not appropriate, because it is faster than chloride, thus another UV absorbing anion, bromate was chosen as IS. Sulfate, the co-ion of the background electrolyte has similar mobility to nitrite, thus destacking of analyte zones does not follow the familiar pattern of the transient ITP migrations (where destacking of zones starts either from the high-mobility or from the low-mobility analyte zones and follows according to the order of the mobilities). Under our separation conditions, the first and last zones (nitrate and bromate) are sharper, and the zone of nitrite is a little bit more dispersed. First destacking and more dispersion of the zone of analyte having similar mobility to the co-ion of the background electrolyte were predicted based on theory, and have proved by Krivankova et al. [24]. Otherwise, under our separation conditions, the pH difference between the sample and the background electrolyte zones may contribute to the stacking of nitrite, because of its pH dependent mobility. Nitrite is faster in the higher pH sample zone, and slows down reaching the saparation buffer of lower pH, thus zone sharpening is induced. Using the pH 3.8 sulfate-β-alanine separation buffer and hydrodynamic injection of artifical sample of standards dissolved in 50 mM acetate–NaOH pH 7 buffer containing 0.09% sodium chloride, limit of detection for nitrite in the submicromolar range (about 0.2 μM) can be expected.

3.1.3. Sample clean-up

In aqueous solutions nitrite is stable for up to several hours, but it is rapidly converted to nitrate by oxyhaemoglobin or other ferrohemoproteins in tissues, thus rapid deproteinization of the samples is required. However, nitrite is also unstable under acidic conditions, where it is converted to NO, and subsequently lost to the gaseous phase, thus acidic deproteinization cannot be used [16]. Acetonitrile was applied to remove sample proteins by Friedberg et al. [14], and in addition to deproteinization, low conductivity acetonitrile in the sample induced stacking of the analytes after their injection. We have tried this favourable method to achieve both deproteinization and on-capillary sample concentration, but did not get the expected concentrating effect. The explanation of this observation remains to be clarified. Although it should be noted, that according to our knowledge, pseudo-ITP, induced by acetonitrile in the sample, have not been demonstrated, so far in separation electrolytes of acidic pH. Thus, a strong base was used for the rapid inactivation of the sample proteins [16]. Tissue samples were homogenized in 5 volumes of 0.1 M NaOH to immediately destroy proteins, then the pH of an aliquot was adjusted to about 7 by an equal volume of 0.09 M acetic acid (in accordance with our previously established injection conditions). The samples were heated in boiling water bath for 3 min and after cooling the precipitated proteins were removed by centrifugation. The supernatants were used for sample injection. The stability of nitrite and nitrate during this sample clean-up procedure was assessed at three concentration levels (2.5, 5 and 10 μM) of the analytes. The normalized peak areas of nitrite and nitrate were compared when the aqueous samples and the samples following the clean-up procedures were analyzed. Using the *t*-test, the normalized peak areas were not found significantly different. This sample clean-up procedure is appropriate to use for the extraction of nitrite from the tissue extracts without its loss or conversion. In the previously published papers, ultrafiltration was commonly used to remove sample proteins [4–6,9], although it is a rather expensive and time-consuming method.

3.2. Method validation

Calibration curves were constructed by addition of nitrate (0.3–6 μM) and nitrite (0.2–4 μM) standards to extracted tissue (rat liver) samples containing 50 μM IS. Equations of typical calibration curves were as follows: area (nitrate)/area (IS) = 3.534 (±0.104) [nitrate] + 0.317 (±0.111), $r = 0.9957$;

Table 1
Reproducibility data of nitrate and nitrite measurements in tissue samples

	Added nitrate/nitrite (μM)	Within run (n = 5)		Between run (n = 3)	
		Precision area R.S.D. (%)	Accuracy concentration (%)	Precision area R.S.D. (%)	Accuracy concentration (%)
Nitrate	1.5	3.36	112.7	9.56	114.6
	3	5.32	106.4	8.93	109.4
	6	2.47	105.9	10.39	113.9
Nitrite	0.5	9.04	109.0	9.79	101.2
	1	3.29	101.2	8.74	107.6
	2	2.12	113.6	10.25	108.2

area (nitrite)/area (IS) = 4.303 (±0.104) [nitrite] − 0.044 (±0.050), r = 0.9962. The sensitivity of the assay was also evaluated; the limit of detection, LOD (S/N = 3) in aqueous solution of nitrate and nitrite, prepared in acetate buffer containing sodium chloride, were found to be 0.1 and 0.15 μM, respectively. The lower limit of quantification (R.S.D. less than 20%) was 0.2 and 0.3 μM for nitrate and nitrite, respectively. The intra- and interday precision and accuracy were determined at three concentration levels of nitrate and nitrite (1.5, 3, 6 μM and 0.5, 1, 2 μM added to the tissue, respectively). The intra-assay relative standard deviations of peak area ranged from 2.5 to 5.3% for nitrate, and 2.1 to 9.0% for nitrite. The accuracy was between 105.9 and 112.7% for nitrate, and 101.2 and 113.6% for nitrite. The between-day precision ranged from 8.7 to 10.4% and accuracy between 101.2 and 114.6% for the two analytes. Data are summarized in Table 1.

3.3. Determination of nitrite and nitrate concentration in biological samples

Nitrate and nitrite concentrations in liver and brain tissue samples of rats were determined in control and LPS treated animals. LPS is a well-known inducer of the inducible isoform of nitric oxide synthase (NOS), thus increases the NO production. Previously, considerable enhancement of nitrate and nitrite levels in airway surface liquid and plasma of rats after intrathecal instillation of LPS have been demonstrated [12]. In the present experiments, LPS was administered intraperitoneally (10 mg/kg), and the anions were measured 24 h after its administration. In the tissues of LPS treated animals the level of the anions increased, although high interindividual differences were observed (Table 2). Representative electropherograms of brain tissue samples of control and LPS treated rats are shown in Fig. 5. Previously reported concentrations of nitrite and nitrate in rat brain tissues were about one order of magnitude higher [5], than found in our present experiments. The standard error of the measurements was also much bigger than in our study when basal tissue levels of nitrate and nitrite were determined. Contamination of laboratory wares and reagents with these anions as a possible cause of this discrepancy cannot be ruled out.

Table 2
Nitrate and nitrite concentrations in rat liver and brain samples

	Rat liver (μmol/kg ± S.E.M.)		Rat brain (μmol/kg ± S.E.M.)	
	Control	LPS treated	Control	LPS treated
Nitrate	32.8±5.59	68.5 ± 47.9	16.5±1.07	29.1 ± 15.1
Nitrite	8.78±0.93	21.81 ± 4.81	4.53±0.21	15.65 ± 10.5

The effect of LPS treatment, n = 5 in each group.

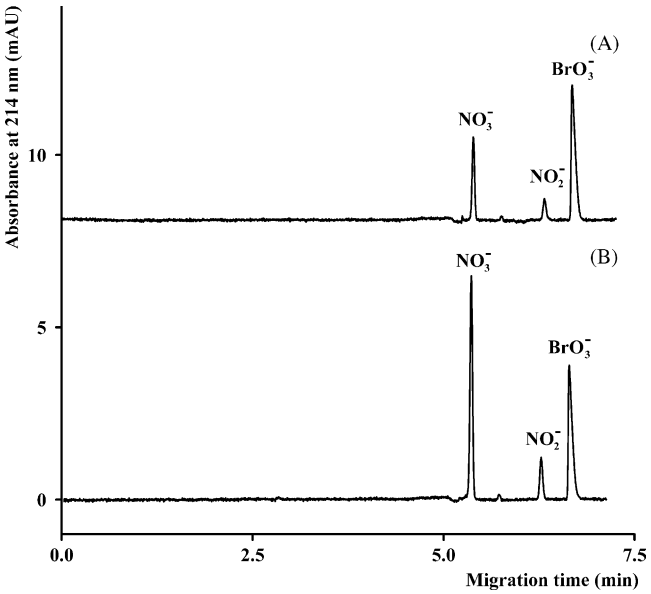

Fig. 5. Electropherograms of brain tissue samples of control and LPS treated rats. Tissues were homogenized in 5 volumes of 0.1 M NaOH containing 100 μM IS, pH of an aliquot was adjusted by addition of an equal volume of 0.09 M acetic acid. The sample was placed into boiling water bath for 3 min to precipitate proteins, after cooling and centrifugation supernatants were injected: 10 kPa for 45 s. Separation buffer: 30 mM sulfate-β-alanine pH 3.8, constant current: −75 μA.

4. Conclusions

CZE determinations of low amount of nitrite and nitrate in biological samples, in the presence of high salt content, requires the concentration of the analytes to reach the needed detection sensitivity. In our present experiments, separation conditions allowing field-amplified concentration of large volume of diluted sample, and sample preparation providing transient ITP stacking have been demonstrated. Both procedures allow determination of submicromolar concentrations of nitrate and nitrite. The method allowing the sample-induced ITP stacking is suitable for the analysis of these

anions in tissue extracts with excellent precision and accuracy.

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