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Simple and rapid method for the measurement of nitrite and nitrate in human plasma and cerebrospinal fluid by capillary electrophoresis

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Abstract

Nitrite and nitrate levels in physiological fluids are commonly used as an index of nitric oxide production. We developed simple and rapid method for the determination of these anions by capillary zone electrophoresis employing borate buffer (pH 10, 100 mmol/l) as running electrolyte. The anions were analyzed in plasma and cerebrospinal fluid (CSF) without deproteinization of the samples. Electrophoresis was carried out in a capillary (36.5 cm×75 μm) at a potential of 15 kV, with on-column UV detection at 214 nm. Mean retention times for nitrite and nitrates were 4.631 and 5.152 min, respectively. The method was linear (r=0.999) within a 1–500 μmol/l concentration range. Physiological levels of nitrate in plasma (40.2 μmol/l) and CSF (15.3 μmol/l) could be determined with good precision (coefficients of variation <6%) and accuracy (recoveries of added nitrate to plasma and CSF were 97.4 and 104.5%, respectively). Measurements of the physiological levels of nitrite in plasma (6.1 μmol/l) and CSF (0.9 μmol/l) were less precise and accurate. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Nitrite and nitrate determinations in biological fluids have increasingly being used as markers for the activity of the nitric oxide synthases [1]. These anions are final oxidized products of nitric oxide (NO) metabolism in vertebrates. NO is a ubiquitous intercellular messenger enzymatically synthesized from L-arginine [2], by different isoforms of NO synthases [3,4]. Two physiological roles for NO have been defined: cell–cell communications and cytotoxicity [5]. In low, physiological concentrations, NO is important physiological messenger that modulates blood flow and neural activity [6]. It could also have negative effects [6–8]. The process of NO production is a potential target of novel therapeutic approaches in different pathophysiological conditions [9–12].

Considering the physiological importance of NO, proper determination of its formation is a challenge for the clinical chemistry laboratory [13]. Direct measurement of in vivo NO formation is difficult due to its short halflife in physiological fluids, limited to a few seconds [2]. Measurement of nitrite and nitrate

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concentrations in blood or plasma could be used as an index of NO production [13].

Various methods for measurement of nitrite and nitrate in physiological fluids have been developed. The most widely used method applied Griess reagent which forms a purple azo dye with nitrite. Nitrate can be measured with the same reagent after its reduction by a cadmium column [14], cadmium particles [15], or enzymatically by nitrate reductase [1]. Whereas previous methods need different samples for nitrite and nitrate determinations, high-performance liquid chromatography permits measurement of these anions in a single run [16].

During recent years capillary electrophoresis (CE) has been introduced as an efficient technique for fast separation of small inorganic anions [17]. Various methods are currently used for separation and determinations of nitrate and nitrite by CE [17–22] with direct or indirect detection. Only a few are especially developed for nitrite and nitrate determinations in plasma, using deproteinized samples and different electrolytes (sodium chloride, sodium sulfate) with additives that reverse the electroosmotic flow during CE [21,22].

In the present paper we report conditions for rapid measurements of both nitrite and nitrate in human blood plasma and cerebrospinal fluid by CE. The present method differs from the others in that a strong electroosmotic flow is generated with no additives to the running electrolyte and sample deproteinization is not used.

2. Experimental

2.1. Capillary electrophoretic system

A P/ACE 5010 system (Beckman, Palo Alto, CA, USA) with P/ACE system software controlled by an IBM computer was used. Data analysis were performed on P/ACE system. The P/ACE 5010 system for CE contains built-in 200-, 214-, 254- and 280-nm narrow-band filters for on-line detection and quantification. In the present method 214-nm on-line detection was used. Electrophoresis was performed in uncoated fused-silica capillary tubing (Beckman PN 338454) total length 36.5 cm (30 cm from the window to inlet)$\times$75 μm I.D. The capillary was assembled in the cartridge with 100$\times$200 μm aperture and detection window located at 6.5 cm from the capillary outlet.

2.2. Capillary electrophoretic procedures

During the run the cathode and capillary were immersed in the running electrolyte at the injection site, while anode and capillary outlet were immersed in the electrolyte at the receiving site. The running electrolyte was 100 mmol/l borate at pH 10. Constant voltage of 15 kV and temperature of 23°C were applied. Under these conditions a current of 122$\pm$4 μA was encountered. Samples were placed on the inlet tray of the P/ACE 5010 instrument and introduced into the capillary by pressure (0.5 p.s.i., 1 p.s.i.=6894.76 Pa) injection. Before each run the capillary was reconditioned with running buffer for 2 min. After a 5-s injection of the sample (sample volume, 56 nl), electrophoretic separation was performed for 6 min. Between runs the capillary was washed with sodium hydroxide solution (0.1 mol/l) for 2 min, followed by water for 2 min. All procedures were automatically controlled by the P/ACE system.

2.3. Chemicals and sample preparation

All chemicals were of analytical grade. Sodium nitrite, sodium nitrate, sodium hydroxide were purchased from Merck, and boric acid from Zorka, (Šabac, Yugoslavia). Solutions were prepared with Milli-Q water obtained by using Millipore Milli-Q water purification system (Waters, Millipore, Milford, MA, USA). The running electrolyte was 100 mmol/l boric acid titrated with sodium hydroxide solution to pH 10$\pm$0.1. Nitrite and nitrate stock solutions (1 mmol/l) were diluted in Milli-Q water to obtain the required concentrations.

Nitrate and nitrite were analyzed in blood plasma and cerebrospinal fluid (CSF). Blood samples were obtained from seven healthy subjects with food and drink allowed ad libitum. Heparinized blood plasma was obtained after centrifugation at 1575 *g* for 5 min. Lumbar CSF samples were obtained from five patients without subarachnoidal block subjected to diagnostic radiculography. CSF samples were frozen until analysis.

Intra- and inter-assay precisions and linearity were assessed from the analysis of standard nitrate–nitrite mixtures (1 to 500 μmol/l). Precision and recovery studies in plasma and CSF samples were carried out on pooled samples with or without addition of known concentrations of nitrate and nitrite. Prior to analysis plasma samples were diluted with Milli-Q water (50 μl sample plus 200 μl water). CSF samples were analyzed directly. Each sample was analyzed at least in duplicate. Nitrate and nitrite concentrations in plasma and CSF were calculated using peak area of daily analyzed standards considering dilution factors (the dilution factor for plasma was 5 and for CSF was 1).

Values are expressed as means±standard deviation (S.D.) with coefficients of variation (C.V.)

3. Results

Retention times of nitrate and nitrite were determined by separate injections. Under the described conditions examined anions migrated in sharp and symmetric peaks, completely resolved from each other (Fig 1. The limits of detection (LODs) in standard mixtures of each anion, based on signal-to-noise ratio of 3 were 0.57 and 0.43 μmol/l for nitrite and nitrate, respectively. The limits of quantification (LOQs) in standard mixtures of each anion, based on signal-to-noise ratio of 10 were 1.9 and 1.4 μmol/l for nitrite and nitrate, respectively.

A calibration curve was obtained using standard solutions with increasing concentrations of the anions (1 to 500 μmol/l). Concentration and peak area

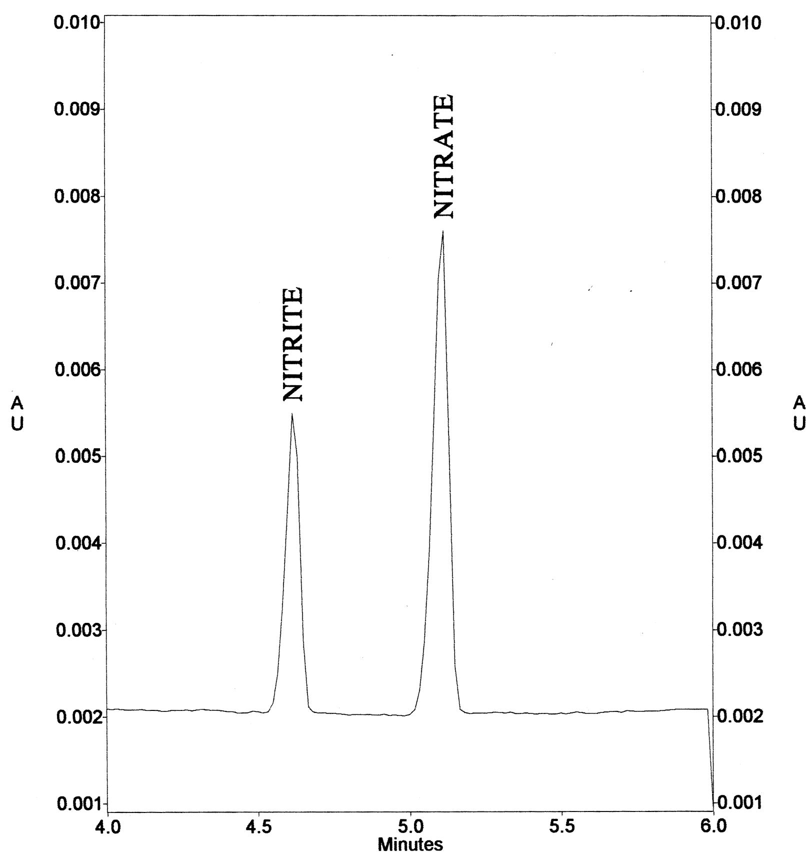
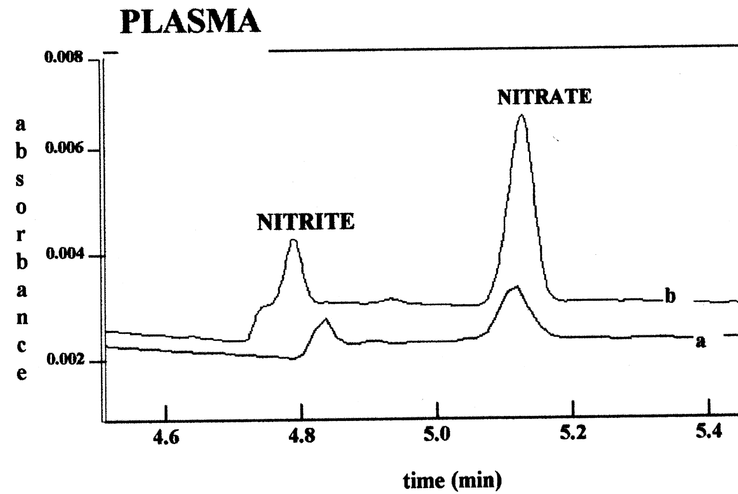


Fig. 1. Electropherogram of standard solution mixture of nitrate and nitrite. Pattern of standard solution of nitrite and nitrate at individual concentration of 50 μmol/l. Separation conditions: 28 cm×75 μm I.D. fused-silica capillary, 100 mmol/l borate running electrolyte at pH 10, detection at 214 nm. AU (ordinate)=absorbance.



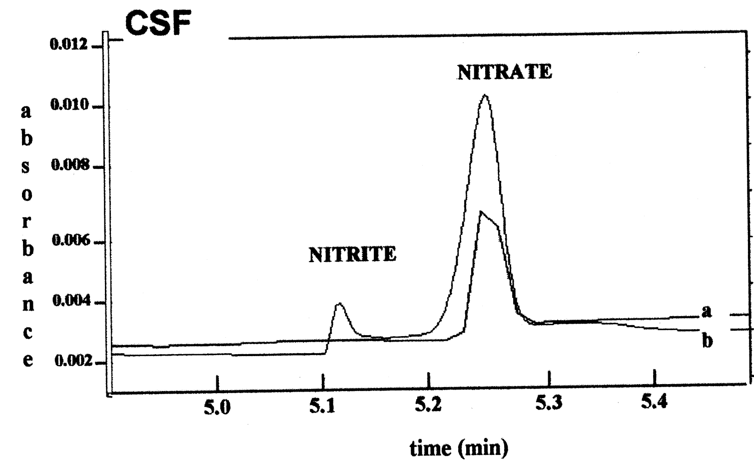


Fig. 2. Capillary zone electrophoresis of nitrite and nitrate in human plasma and cerebrospinal fluid (CSF) before (a) and after addition (b) of the examined anions. Plasma was analyzed after dilution with water (one volume plasma plus four volumes of water) and CSF directly. For details of separation conditions see Fig 1.

correlated linearly in the examined range. The linearity, obtained with the mean values from five determinations, was expressed by the following equations: nitrite $y=2.096+0.0039x$ ($r=0.9999$, Sa=2.49, $P>0.05$); nitrate $1.117+0.0023x$ ($r=0.9999$, Sa=2.46, $P>0.05$) (y=peak area, x=concentration of corresponding added anion).

The mean value of retention time±S.D. for nitrite and nitrate determined in standard mixtures ($n=48$) with various concentrations ranging from 1 to 500 μmol/l were 4.631 min±0.077 (C.V.=1.66%) and 5.152 min±0.099 (C.V.=1.92%), respectively. Peak areas obtained in the replicate with the same standard solution had different coefficients of variation depending on the concentration range. Namely, for the concentrations ranging from 5 to 500 μmol/l coefficients of variation for peak areas were <10%. Peak areas of 1 μmol/l nitrite (169±40.7) and 1 μmol/l nitrate (197.4±39.5) are between LOD and LOQ, while measurements are less precise (C.V.>20%).

The methods were tested on human blood plasma and CSF samples. Under the described conditions nitrite and nitrate were completely separated in the examined physiological fluids (Fig. 2). We assesed the precision of the method by repeated analysis of plasma and CSF samples with added known concentrations of nitrite and nitrate (Table 1). Variation of nitrate and nitrite retention times were <2%. Precision of nitrate measurment in plasma were similar regardless the added nitrate with C.V.s <6%. However, precision of nitrite measurement in plasma increased with its concentration (Table 1). Recoveries of added nitrite and nitrate to plasma sample were 86.6% ($r=0.9989$, $y=4.61+0.866x$, Sa=1.2) and 97.4% ($r=0.9998$, $y=35.52+0.974x$, Sa=5.2), respectively (y=expected concentration; x=measured concentration μmol/l).

The precision of nitrate determination in CSF samples is similar to the plasma measurements (Table 1). However, nitrite measurements in the pooled CSF sample were not as precise as nitrate determinations (Table 1). This was probably due to the low nitrite level, not measurable in every run at physiological level. Higher nitrite levels improved precision of the measurements (Table 1). Recoveries

Table 1
Reproducibility of the retention times and peak areas of nitrite and nitrate in plasma and cerebrospinal fluids (CSF) without (a) and with (b) known concentrations of the anions[a]

Compound	Fluid	Added nitrite/nitrate (μmol/l)	Retention time (min)	Concentration (μmol/l)
Within run ($n=3$)				
Nitrite	Plasma a	0	4.828±0.020 (0.4)	6.8±0.6 (9.5)
	Plasma b	50	4.809±0.018 (0.4)	49.1±0.6 (1.3)
	CSF a	0	NQ	NQ
	CSF b	5	5.150±0.001	7.2± 0. 7 (9.4)
Nitrate	Plasma a	0	5.110±0.069 (1.4)	41.1±2.4 (5.9)
	Plasma b	100	5.173±0.006 (0.1)	136.1±0.7 (0.5)
	CSF a	0	5.189±0.074 (1.4)	12.0±0.4 (3.4)
	CSF b	20	5.301±0.009 (0.2)	39.8±0.9 (2.2)
Between run ($n=5$)				
Nitrite	Plasma a	0	4.829±0.023 (0.5)	6.7±0.6 (8.2)
	Plasma b	50	4.791±0.026 (0.6)	49.2±1.5 (2.9)
	CSF a	0	NQ	NQ
	CSF b	5	5.149±0.021 (0.4)	7.1±0.4 (6.2)
Nitrate	Plasma a	0	5.107±0.056 (1.1)	40.5±1.1 (2.8)
	Plasma b	100	5.158±0.010 (0.2)	132.1±3.7 (2.8)
	CSF a	0	5.256±0.040 (0.8)	12.4±0.7 (5.4)
	CSF b	20	5.298±0.028 (0.5)	39.8±0.7 (1.7)

[a] Plasma was analyzed after dilution with water (one volume plasma plus four volumes of water) and CSF directly. Values are mean±S.D.; values in parenthesis are coefficients of variation (%); NQ, not quantified.

Table 2
Nitrite and nitrate in blood plasma and cerebrospinal fluid samples[a]

Physiological fluid	Nitrite (μmol/l)	Nitrate (μmol/l)
Plasma ($n=7$)	6.1±2.3 (37.6)	40.2±10.3 (25.5)
CSF ($n=5$)	0.9±0.8 (85.2)	15.3±4.8 (31.7)

[a] Values are mean±S.D.; values in parenthesis are coefficients of variation (%).

of the added nitrite and nitrate to CSF sample were 92.6% ($r = 0.9977$, $y = 3.117 + 0.926x$, Sa = 1.01) and 104.5% ($r = 0.9956$, $y = 23.525 + 1.045\ x$, Sa = 1.58), respectively.

Mean nitrate levels in plasma and CSF in the examined population, were 40.2 and 15.3 μmol/l, while nitrite levels were more than ten times lower (Table 2).

4. Discussion

The presented method permits determination of nitrate and nitrite from the same sample in a single run, like the other methods using CE [17,19,21,22]. Under the described analytical conditions nitrate and nitrites are completely separated both in standard mixture and examined physiological fluids. The separation was due to the significant mass-to charge difference between these anions, achieved with borate electrolyte without addition of any electroosmotic flow modifier. In the other methods commercial alkylammonium compounds are usually added to the running electrolyte in order to reverse the electroosmotic flow [21,22]. The presented method is a few-fold faster than some methods [21] but slower than others [22] employing CE for nitrate and nitrite determinations in plasma. Sensitivity, repeatability and reproducibility of retention times and peak areas obtained by this method were similar to the other CE methods [21,22].

In addition, the presented method is suitable for nitrite and nitrate determination both in plasma and CSF without prior deproteinization of the samples. The other methods used ultrafiltration for plasma deproteinization [21,22]. It was reported that omitting the ultrafiltration step in analysis of plasma samples resulted in increase of the nitrite–nitrate migration times due to plasma protein effects on the capillary wall [22]. We noticed changes in the current at constant voltage, accompanied by alterations in nitrite and nitrate migration times, after about 20 analyses. However, nitrite and nitrate migrations were improved with fresh electrolyte and no serious contamination of the capillary occurred after more than 200 analyses. This may be due to the high ionic strength and pH 10 of the borate buffer which enables CE of plasma proteins, that are on-line detected at 200 or 206 nm [23]. We used on-line detection at 214 nm for nitrite and nitrate determinations, as did Refs. [21,22]. Neither chloride nor sulfate anions normally present at high concentrations in plasma, absorbed at this wavelength [22].

We examined the influence of some working parameters on the nitrate and nitrite migrations during CE. Voltage influenced retention times but not the order of migration of the examined anions. A decrease in pH at the same ionic strength of the electrolyte resulted in increased retention times.

The basal value of plasma nitrate in normal volunteers determined in the present investigations was 40.2 μmol/l, ranging from 29.2 to 59.2 μmol/l. It was within the limits reported by other authors [14,15,21,22,24,25]. However, the basal nitrite level of 6.05 μmol/l (ranging from 3.3 to 8.2 μmol/l) was within the limits reported by some authors [24], but higher than the values reported by others [21,22]. The differences might be caused by different sample pretreatment procedures, which might have particularly influenced nitrite levels [13]. Plasma nitrate could be measured with good precision (C.V. $<$ 10%) whereas nitrite determinations in plasma were less precise due to the low basal level of this anion in plasma. Higher nitrite concentrations improved precision of the measurements. Thus, good precision of nitrite measurement should be obtained in plasma of patients with increased nitrite levels only. High nitrate and nitrite levels were observed in different pathophysiological states [25].

The concentration of nitrate in CSF was 15.3 μmol/l (range from 7.7 to 19.8 μmol/l) while nitrite level was very low, often $<$1 μmol/l. Total nitrite and nitrate in CSF samples, calculated from the presented results, were within the range of values reported for healthy subjects by other workers [26]. However, the physiological level of nitrite was near

the LOQ and could not be determined with good precision. Samples with added nitrite at a concentration of 5 μmol/l were analyzed with satisfactory precision.

In conclusion, the presented method provides good sensitivity, precision and accuracy for nitrate measurement in plasma and CSF at physiological levels. Measurements of the nitrite at the physiological level in these fluids were less precise.

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