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Short communication

Analysis of nitrate in biological fluids by capillary electrophoresis

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Abstract

Nitrite and nitrate represent the products of the final pathway of nitric oxide metabolism. These two ions were analyzed by capillary electrophoresis (CE) in serum, cerebrospinal fluid, urine and tissue homogenates by mixing the sample with acetonitrile containing NaBr as an internal standard, followed by centrifugation. The supernatant was injected hydrodynamically on a capillary 50 cm×75 μm (I.D.) and electrophoresed at 6 kV (reversed polarity) in 1.4% sodium chloride in phosphate buffer for 13 min with detection at 214 nm. In addition to removal of the proteins, acetonitrile caused sample stacking. Urinary nitrate analysis by CE was compared to that by the enzymatic *Aspergillus* nitrate reductase method, with a correlation coefficient of 0.96. © 1997 Elsevier Science B.V.

Keywords: Nitrate

1. Introduction

Nitric oxide has been identified as a mediator in many cellular functions such as vascular tone [1], signal transmission [2] and phagocytosis [3]. It is synthesized from arginine by three distinct forms of the enzyme, nitric oxide synthetase [4]. Nitric oxide is rapidly oxidized to nitrite and nitrate in the blood [5]. Clinically, serum nitrate increases after sepsis [6] and after infectious gastroenteritis [7]. Cerebrospinal fluid (nitrite+nitrate) levels increase after meningitis [8] and decrease in Parkinson's disease [9]. In urine, nitrate increases after minimal change nephrotic syndrome in children [10], and decreases after preeclampsia [11]. Urinary nitrate/creatinine ratios are elevated 3-fold in patients with rheumatoid arthritis and 10-fold in patients with infectious gastroenteritis compared with controls [7].

Colorimetric [12], HPLC [13] and chemiluminescence [14] methods have been described for the analysis of these two ions. These methods are time consuming. More recently, capillary electrophoresis has been shown to offer better specificity and speed for the analysis of these ions in serum [15,16]. Serum contains high concentrations of ions and proteins, which affect the separation of nitrate and nitrite by CE. Previously, we have shown that acetonitrile can be used to remove serum proteins for analysis by CE. In addition to removal of serum proteins, acetonitrile, owing to its low conductivity, produces concentration on the capillary, known as "stacking" [17–19]. Here we show that acetonitrile treatment is a convenient and suitable method for analysis of ions in general, and specifically for nitrite/nitrate. We also included an internal standard

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for better accuracy. In addition to that we compared the analysis of urinary nitrate by CE to an enzymatic assay [21].

2. Experimental

2.1. Chemicals

All chemicals including *Aspergillus* nitrate reductase were obtained from Sigma (St. Louis, MO, USA).

2.2. Instrumentation

Model 4000 Quanta (Waters Associates, Milford, MA, USA) was set at 214 nm and 6 kV (negative power supply). The current was about 180 μA. The capillary, 50 cm×75 μm, was washed for 2 min with NaOH, 0.2 *M*, and filled for 2 min with the running buffer: 1.4 g NaCl, 110 mg Na_2HPO_4, 50 mg, NaH_2PO_4 and 100 mg poly(ethylene glycol) 8000, in 100 ml water. The buffer was stored refrigerated.

Data was collected with the Data Shuttle (Omega Engineering, Stamford, CT, USA). Peak heights were used directly for calculation of the analyte concentrations.

2.3. Procedure

Serum, urine (diluted times five with water), tissue homogenates or standards (100 μl), were vortex mixed for 30 s with 200 μl acetonitrile containing 40 mg/l of sodium bromide, as an internal standard. The mixture was centrifuged for 1 min at 14 000*g* and the supernatant was injected hydrodynamically for 70 s onto the capillary.

2.4. Enzymatic assay

The enzymatic assay was based on the kinetic reduction of nitrate to nitrite by NADPH to NADP by the *Aspergillus* nitrate reductase and the oxidation of NADPH to NADP measured at 340 nm as described by Gilliam et al. [22]. The reaction rate was monitored for 40 min.

3. Results and discussion

Acetonitrile is a simple and effective method for removal of serum proteins [18]. Fig. 1 illustrates the separation and the peak heights of nitrite and nitrate from standards prepared in water and saline solutions obtained in the absence and presence of acetonitrile. Salts tend to increase band diffusion, Fig. 1B; while water induces band stacking because of the low conductivity, Fig. 1A. Acetonitrile, Fig. 1C, causes sample stacking similar to that of water. This effect is also obtained when serum samples, which normally contain about 150 m*M* of sodium chloride, are deproteinized with acetonitrile, Fig. 1F. This stacking effect is also related to the low conductivity of acetonitrile as well as other factors [17–19] leading to higher field strength in the sample. Interestingly, the stacking is better in serum, Fig. 1F, than in pure saline samples, Fig. 1D. This is probably due to other contributing factors such as pH and other ions in the sample [17–19]. For example, we noticed that the addition of phosphate ions to the sample increases the peak height of nitrite and nitrate but not that of Br, Fig. 2. In an accompanying manuscript [20] we show the importance of ionic strength and pH in the sample on the stacking mechanism for small molecules in general.

Comparing this method to that based on removal of proteins by filtration [15], we see that the nitrite/nitrate peaks are sharper, i.e. have higher plate number by the present method compared to filtration (N=83 000 vs. 46 000 for nitrate). The addition of two volumes of acetonitrile should dilute the sample and thus reduce the peak height compared to filtration by a factor of three. However, because of the stacking, the peak heights for nitrite and nitrate are lower only by 14 and 32%, respectively, Fig. 3. Protein filters are relatively expensive compared to acetonitrile and require long periods of centrifugation. The capillary can be used for a greater number of injections when sample deproteinization with acetonitrile is employed.

In this method we have included bromide as an internal standard and eliminated the addition of compounds that reverse the electroosmotic flow, yet the analysis is rapid, within 13 min. The addition of 6 mg/l each of nitrite and nitrate to the serum vs. the deproteinized supernatant gave a recovery of 90 and

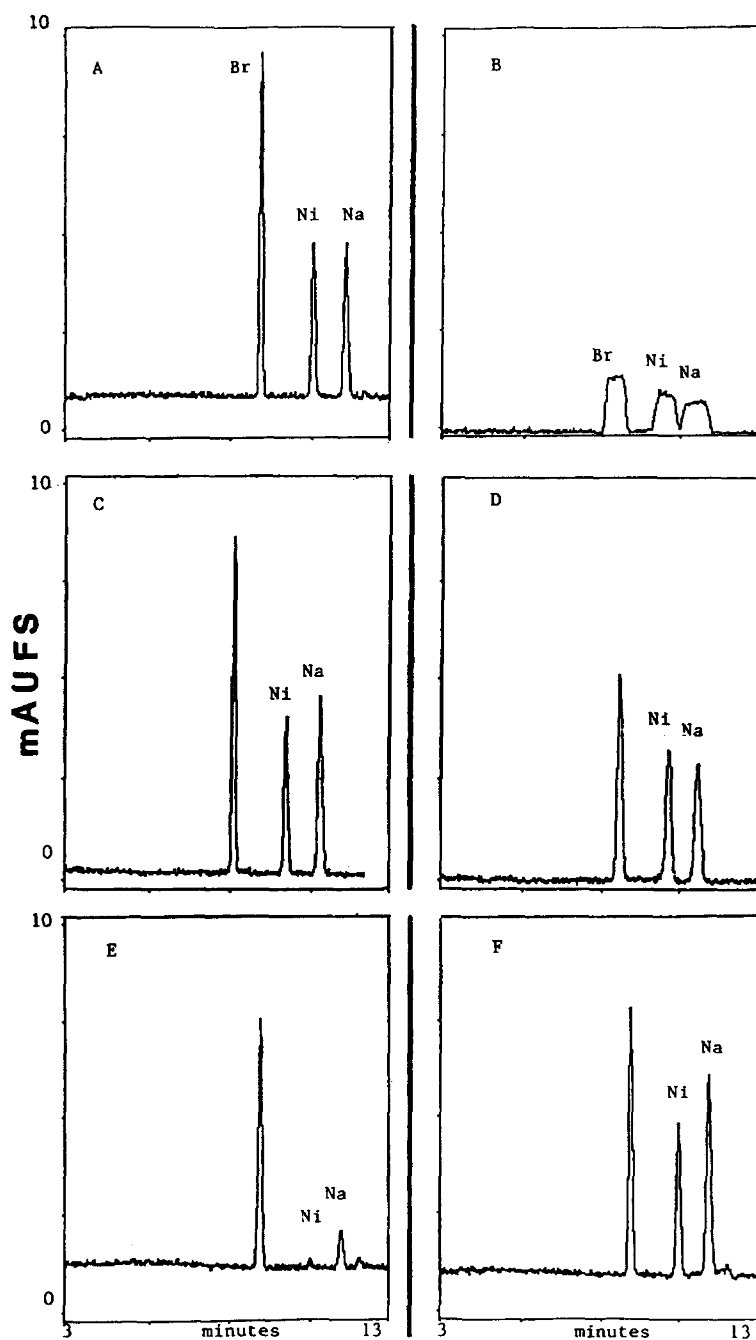

Fig. 1. Nitrite and nitrate (6 mg/l) added to: (A) water without acetonitrile, (B) 0.9% NaCl without acetonitrile, (C) 66% acetonitrile in water and (D) 66% acetonitrile in 0.9% saline. (E) Serum from an individual with low levels of nitrite and nitrate (for spiking), deproteinized with acetonitrile (66% acetonitrile final concentration) and (F) the same serum spiked with nitrite and nitrate (6 mg/l, 66% acetonitrile final concentration).

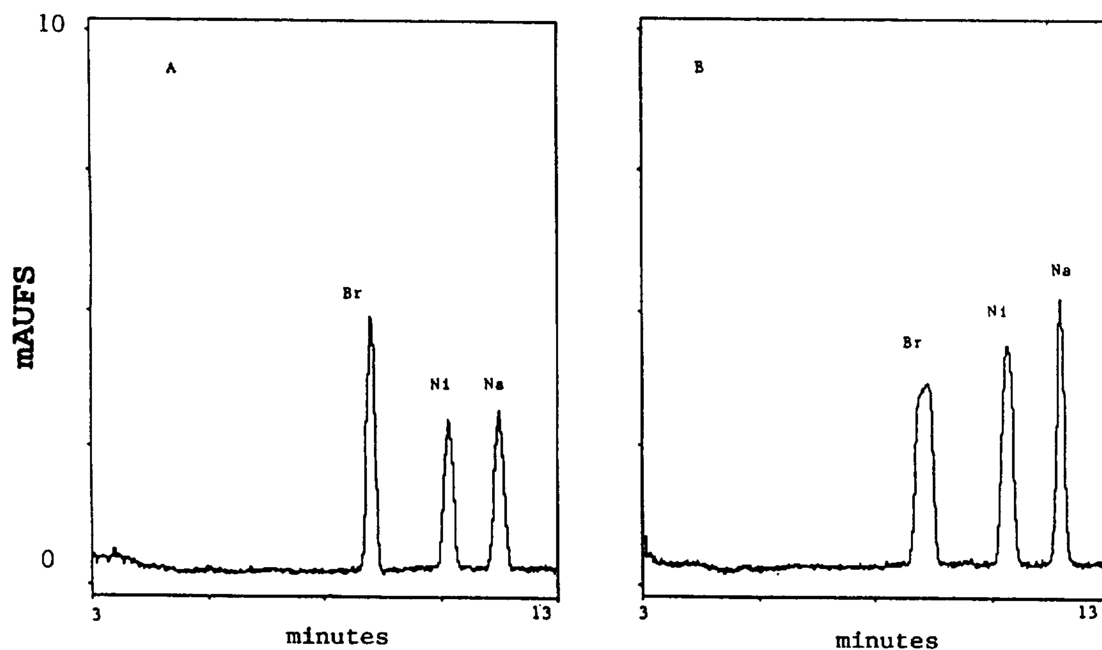

Fig. 2. Peak height of nitrite and nitrate in presence of (A) 0 and (B) of 15 g/l phosphate (pH 7.4). Samples were treated with acetonitrile.

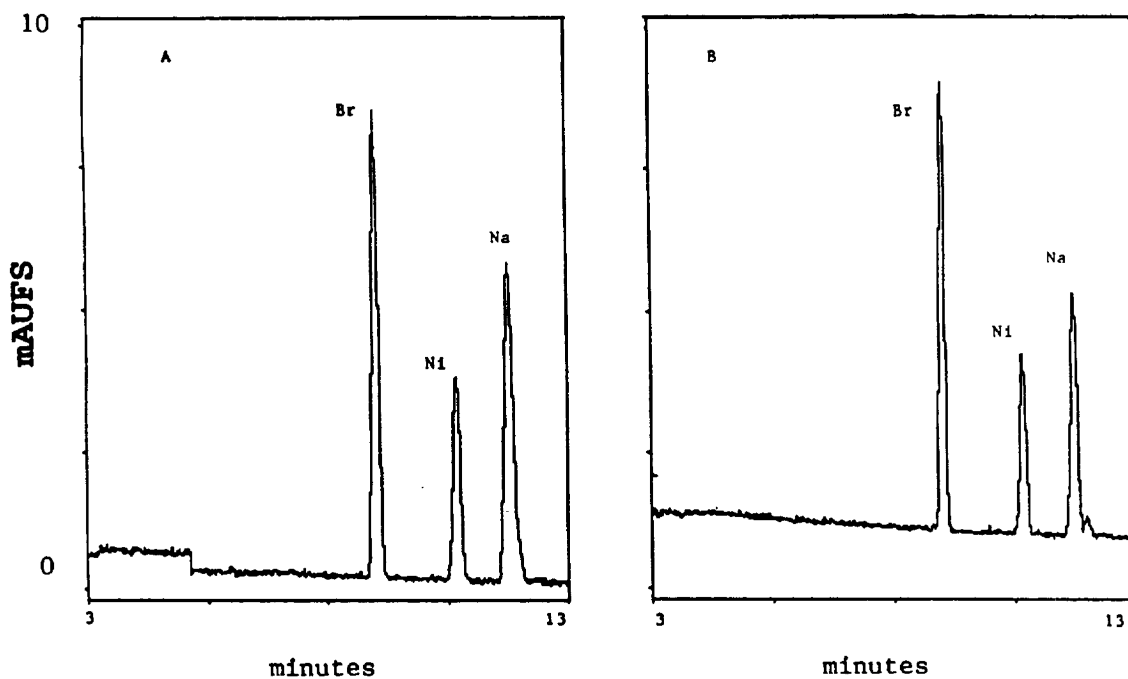

Fig. 3. Serum spiked with 6 mg/l of nitrite and nitrate by (a) filtration, (b) acetonitrile deproteinization.

89%, respectively, indicating that these ions do not co-precipitate with the proteins. The test was linear by peak height between 0.3 to 12 mg/l, with the lowest detection limit (3×baseline noise) being 0.3 mg/l. The R.S.D. for peak heights for nitrite and nitrate were 3.7 and 5.7 ($n=10$), respectively. Because of the difference in peak height between aqueous and serum samples we prepared the standard directly in serum samples (Fig. 1E), or the fluid being analyzed, provided the sample is low in these two ions.

Fig. 4A,B illustrates the separation of these two ions in serum and urine. The method is applicable to analysis of nitrite and nitrate in other fluids including animal and plant tissues, Table 1. Urine has much higher levels of nitrate compared to serum or cerebrospinal fluid. Patients with sepsis have higher mean levels of serum nitrate compared to healthy individuals, Table 1. Serum, urine and cerebrospinal fluid values are close to those in the literature [7,15,16,22], [7,11,22] and [8], respectively. Unfortunately the data concerning these ions in human or animal tissues is scarce. As listed in Table 1, some tissues such as the lung have high levels while others such as the liver have low levels. We also applied this method to analysis of plant tissues such as leaf lettuce and Soya bean.

Previous studies [15,16,23] did not compare the nitrite/nitrate by CE to other methods. Since the

Table 1
Nitrite and nitrate level in different biological fluids and tissues (μ*M*)

Fluid/tissue	*n*	Nitrite±S.D.	Nitrate±S.D.
Serum normal	21	6.6±11	34±18
Serum sepsis	10	–	43±25
Urine/l	22	–	493±338
Urine/g creatinine	20	–	611±389
CSF	10	3.4±3.1	7.6±4
Synovial fluids	10	–	58±31
Human tissues (μM/kg wet mass)			
Pancreas	4	21±23	128±45
Brain	3	111±63	200±75
Lung	3	152±126	480±182
Thyroid	3	13±23	280±92
Kidney	3	69±23	230±75
Liver	3	111±128	120±52
Spleen	3	13±23	160±92

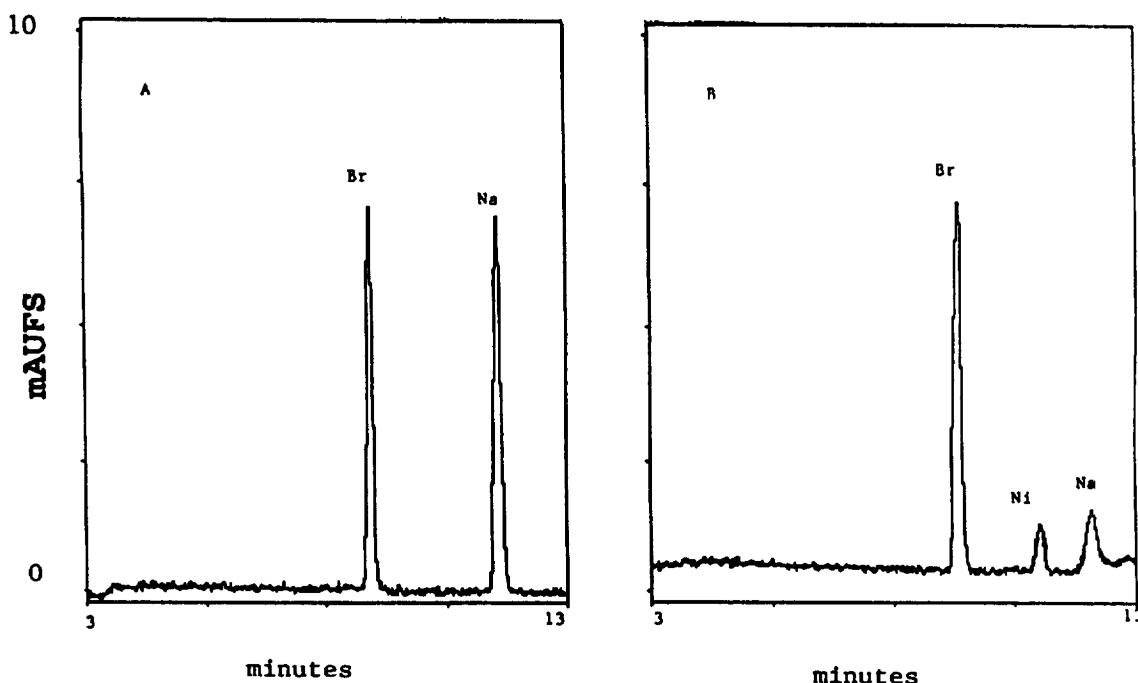

Fig. 4. Nitrite and nitrate in: (a) urine sample, (b) CSF sample.

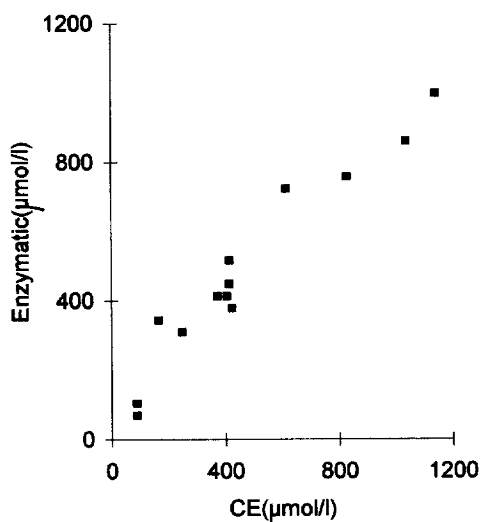

Fig. 5. Comparison of the CE nitrate to the enzymatic nitrate reductase method [20].

urine in general contains much more interference than other biological fluids we compared the urinary nitrate by CE to a kinetic enzymatic assay based on the *Aspergillus* nitrate reductase [20]. As illustrated in Fig. 5 the results of the two methods compared well, with a correlation coefficient of 0.96 ($n=13$). The advantage of CE over the enzymatic method is its low cost. In addition to that, the method can measure other ions at the same time, e.g., Br (after being excluded from the acetonitrile), oxalate (migration time 15 min) and methyl malonic acid (migration time 17 min).

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