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Capillary electrophoresis analysis of nitrite and nitrate in sub-microliter quantities of airway surface liquid

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Abstract

We developed a simple capillary electrophoresis (CE) method to measure nitrite and nitrate concentrations in sub-microliter samples of rat airway surface liquid (ASL), a thin (10–30 μm) layer of liquid covering the epithelial cells lining the airways of the lung. The composition of ASL has been poorly defined, in large part because of the small sample volume (~1–3 μl per cm^2 of epithelium) and difficulty of harvesting ASL. We have used capillary tubes for ASL sample collection, with microanalysis by CE using a 50 m*M* phosphate buffer (pH 3), with 0.5 m*M* spermine as a dynamic flow modifier, and direct UV detection at 214 nm. The limit of detections (LODs), under conditions used, for ASL analysis were 10 μ*M* for nitrate and 30 μ*M* for nitrite ($S/N=3$). Nitrate and nitrite were also measured in rat plasma. The concentration of nitrate was 102±12 μ*M* in rat ASL and 70±1.0 μ*M* in rat plasma, whereas nitrite was 83±28 μ*M* in rat ASL and below the LOD in rat plasma. After instilling lipopolysaccharide intratracheally to induce increased NO production, the nitrate concentration in ASL increased to 387±16 μ*M*, and to 377±88 μ*M* in plasma. The concentration of nitrite increased to 103±7.0 μ*M* for ASL and 138±17 μ*M* for plasma.

Keywords: Nitrite; Nitrate

1. Introduction

Nitric oxide (NO), an endogenous free radical mediator synthesized from L-arginine by three distinct forms of the enzyme nitric oxide synthase (NOS) plays a critical role in the regulation of various aspects of cellular physiology [1]. In varying degrees NO contributes to host defense [2], regulation of vascular tone [3], signal transmission [4] and inflammation [5]. Abnormalities in NO release have been implicated in various diseases ranging from hypertension and arteriosclerosis to septic shock and rheumatoid arthritis [6,7]. Furthermore, NO may also contribute to the regulation of mammalian lung airway smooth muscle tone [8]. Under inflammatory conditions, NO may also contribute to oxidative stress via the action of reactive derivatives. For example, NO can combine with superoxide (O_2^-) to generate peroxynitrite ($ONOO^-$), a cytotoxic inter-

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mediate [9] that can promote damage of lipids and proteins [10,11].

The lung is known to be a major source of NO, the production of which is increased in some diseases such as asthma [12,13]. Measurement of local nitrate and nitrite levels is essential to improve understanding of the metabolism of NO in the airway tree, especially in the airway surface liquid (ASL) that covers the apical surface of airway epithelial cells. Epithelial cells are themselves an important source of NO [14], which can regulate ciliary beat frequency [15] and contribute to the defense against some foreign pathogens [16]. The regulation of NO production in the airways is complex particularly in disease. For example, while epithelial expression of the inducible form of nitric oxide synthase (iNOS), the enzyme primarily responsible for NO production in inflammatory states, is increased in asthma [12,13,17], it is impaired in cystic fibrosis [18]. In addition, inflammatory cells, particularly macrophages [19], also contribute substantially to the generation of NO in the airway compartment. Despite its importance in a variety of disease states, the regulation of NO production in the airway remains incompletely understood.

Commonly used approaches to measuring NO output in the airways include detection of NO in exhaled air, although the relationship between exhaled NO and local concentrations of NO at the level of the airways themselves is unclear [20]. The nitrate and nitrite content of bronchoalveolar lavage fluid has also been measured [21], but this reflects NO production in both the airways and the lung tissue. Ideally, it would be useful to have an index of NO production at the level of the airway epithelium itself, by determining the concentration of stable metabolites of NO, nitrate and nitrite, in ASL. However, relatively little is known about the composition of ASL due to its limited quantity (ASL is present as a coating only 10–30 μm thick overlying the airway epithelium) and difficulties in collecting samples from such an inaccessible area without disturbing the underlying epithelium. We have previously developed a technique for harvesting ASL that involves the introduction of a small polyethylene capillary into the airways. ASL is then collected by capillary action. Although this results in the harvesting of only 100–300 nl of ASL, we have successfully used capillary electrophoresis (CE) to analyze salt and protein concentrations in ASL [22–25]. In the present study we describe the development of an improved CE protocol for nitrate and nitrite analysis suitable for use with sub-microliter samples of ASL. CE has previously been used for the analysis of nitrate and nitrite in biological samples such as plasma, urine, cerebrospinal fluid (CSF), synovial fluid and brain extracts [26–30]. Here we report the use of CE for microanalysis of nitrate and nitrite content of ASL under normal and inflammatory conditions.

2. Experimental

2.1. Chemicals

Phosphoric acid (*ortho*, 85%) was purchased from Anachemia (Montreal, Canada), sodium hydroxide was from BDH (Toronto, Canada), sodium nitrite and sodium nitrate were from Fisher Scientific (Fair Lawn, NJ, USA), spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine, 97% pure) and lipopolysaccharide (LPS) (*Escherchia coli*, 0111:B4) were from Sigma (St. Louis, MO, USA). Buffer solutions were prepared from distilled and doubly deionized water (Milli-Q50 unit, Millipore, Montreal, Canada). All chemicals used were of analytical grade.

2.2. Animals

Sprague–Dawley rats (6–8 weeks, 200–250 g) were purchased from a commercial source (Charles River, St. Constant, Canada) and housed in a conventional animal care facility at the Meakins-Christie Laboratories. Protocols were approved by the local animal ethics committee.

2.3. Instrumentation

Experiments were performed on a CE unit from Applied Biosystems (Foster City, CA, USA), Model 270 A. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 150 or 365 μm O.D., 50 or 75 μm I.D., and 72 cm total length were used. The polymer coating was burned off 22 cm

from the cathode end of the capillary to form a detection window. Before use, new capillaries were washed with 0.5 *M* sodium hydroxide for 30 min, deionized water for 10 min, and background electrolyte (BGE) for 25 min. Samples were loaded into the capillary by automatic injection under a vacuum of 17 kPa for 1.5 s (injection volume ~3 nl) from sample vial (for plasma analyses) and from capillary tube (for ASL analyses). Separation was carried out at an applied electric field of −333 (plasma analyses) or $-347\ V\ cm^{-1}$ (all other analyses) (current ~65–75 μA). The capillary and buffer electrolyte temperature was 30°C and the UV detector was set at 214 nm during the analysis. Data were collected with an integrator (Model SP4600, Spectra-Physics, San Jose, CA, USA) and Spectra-Physics Winner software was used for data storage and manipulation. Time normalized peak areas were used for all quantitation.

2.4. Analysis of aqueous nitrate and nitrite solutions

Phosphate buffers with a range of pH from 2.5 to 4.0 were prepared by adjusting 50 m*M* phosphoric acid containing 0.5 m*M* spermine with 1 *M* NaOH. The best separation was found to be at pH 3.0, and this buffer was used for rat ASL and rat plasma analyses.

In-between runs, the capillary was washed with 0.5 *M* NaOH (2 min) followed by water (2 min) and then with running buffer (6 min). Calibration curves were made with standard nitrate and nitrite solutions, typically in the range of 5.0–1000 μ*M*. The limited sample volume precluded spiking the ASL with nitrate or nitrite standards.

2.5. Limit of detection, calibration range and reproducibility

Aqueous standard solutions of nitrate and nitrite were prepared at a concentration of 5–10 m*M*. These were diluted in water or plasma to prepare calibration and dilute standard solutions. The parameters related to quantitation such as limit of detection (LOD), calibration range and reproducibility were examined for both nitrite and nitrate in aqueous solutions. Because of the small volumes of ASL involved, spiked solutions for validation could not be prepared. The intra-day and inter-day reproducibilities for aqueous standards were determined using a solution containing 50 μ*M* of nitrate and nitrite. For intra-day reproducibilities, 10 successive runs were carried out on the same day and for inter-day reproducibilities, three successive runs were carried out on alternate days for a week.

2.6. Sampling and analysis of rat ASL

Rat ASL samples were collected as previously described [22–24]. Briefly, the rats were sedated with xylazine (0.08 ml/100 g body mass, intraperitoneal) and then anaesthetized using pentobarbital (0.053 ml/100 g body mass, intraperitoneal). An intubation tube (6 cm×1.67 mm I.D.×2.42 mm O.D.; Becton-Dickinson, Sparks, MD, USA) was inserted into the trachea and the animal placed supine. The polyethylene sampling capillary (10 cm×280 μm I.D.×610 μm O.D.; Becton-Dickinson) was passed through the intubation tubing, which reduces the risk of picking up liquid in the upper airways. The sampling catheter was then left in contact with the epithelium approximately 0.5 cm below the level of the main carina for 3–5 min before removal. The volume of ASL samples obtained in this way was typically around 100–300 nl per collection. Samples were analyzed immediately after harvesting.

To inject the ASL sample onto the CE, the sampling capillary (280 μm I.D.) was fitted over the end of the separation capillary (150 μm O.D.×50 μm I.D.). The separation capillary was inserted until it just touched the liquid within the sampling capillary and a vacuum of 17 kPa was applied to the outlet end of the capillary for 1.5 s to inject ~3 nl of sample. The sample capillary was then removed, and the separation was carried out as described above.

2.7. Sampling and analysis of rat plasma

After ASL sampling the rats were decapitated and blood was collected into a 10-ml sterile, heparinised container (Vacutainer tube, Becton-Dickinson). The samples were spun at 2000 *g* for 6 min at 4°C. The plasma was then carefully removed and centrifuged

at 12 000 *g* for an additional 10 min at 4°C. The plasma was then passed through a 0.2 μm sterile membrane filter and analyzed as described earlier. Sampling and analyses were carried out in less than 1 h for each sample. Capillaries used for plasma analysis were 365 μm O.D.×75 μm I.D.

2.8. Intratracheal instillation of lipopolysaccharide

To demonstrate that this approach could detect increases in NO production, we treated additional rats with LPS as follows. Rats were anaesthetized as described above and then a volume of 0.15 ml of phosphate-buffered saline (PBS) containing 150 μg of LPS (0.5 mg kg^{-1} body mass) was instilled intratracheally into the lungs to induce inflammation. After 30 min incubation, ASL and plasma samples were collected and analyzed as before.

3. Results and discussion

3.1. Selection of electrolyte, electroosmotic flow (EOF) modifier and pH

A strategy generally used in CE to rapidly analyze anionic compounds is to decrease or reverse EOF by using an EOF modifier, with injection of the sample at the negative terminal and detection near the positive terminal. Polyacrylamide or other neutral coatings may be used to permanently coat the capillary and thereby eliminate the EOF [27]. Alternatively, cationic surfactants such as cetyltrimethylammonium or tetradecyltrimethylammonium salts have been used as dynamic flow modifiers to reduce or reverse the EOF [30,31]. Though these compounds are effective EOF modifiers, problems may arise when analyses of proteinaceous biological samples are performed because proteins can precipitate on contact with cationic surfactants [32]. To avoid these problems, we used a low-molecular-mass amine compound, spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine), as a dynamic flow modifier to control EOF. Unlike some surfactants, spermine does not precipitate proteins. Furthermore, spermine has negligible absorption at 214 nm and provides efficient dynamic coating to reduce EOF [33]. This UV transparency is highly desirable in the present analysis of nitrite and nitrate, which is based on detection at 214 nm. The use of phosphate buffers also minimizes the background UV absorption at 214 nm. Optimal resolution between nitrite and nitrate was observed at pH 3.0 with a total analysis time of less than 10 min compared to pH 2.5 and 4.0 (Fig. 1). At pH 3.0, nitrite migrates towards the anode much more slowly than the nitrate because nitrous acid is a weaker acid than nitric acid and is only partially dissociated [27]. Although these separations are slow compared to ones optimized for speed [34], the limitation in ASL analysis is sampling rather than separation, so the run time is acceptable for the intended purpose.

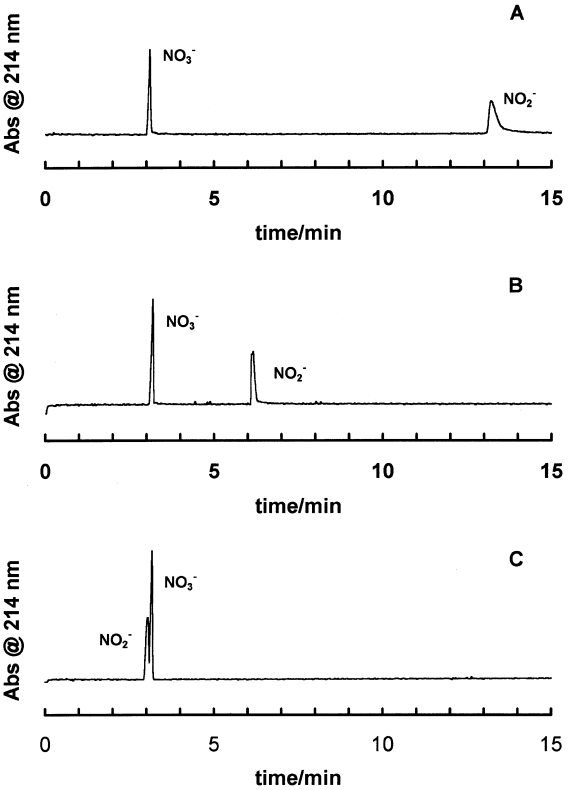

Fig. 1. Electropherograms for the separation of 1 m*M* nitrate and 2 m*M* nitrite at (A) pH 2.5 (B) 3.0 and (C) 4.0. BGE: 50 m*M* phosphate with 0.5 m*M* spermine. Applied voltage: -347 V cm^{-1} (I=~75 μA).

3.2. Limit of detection, calibration range and reproducibility

A limitation of the present study was our inability to study the LOD in the matrix of interest because of the impossibility of harvesting a sufficient quantity of ASL. Nevertheless, we were able to carry out spiking experiments in plasma that confirmed identification of peaks in the electropherogram (Fig. 2). The LOD ($S/N=3$) for aqueous solution of nitrate and nitrite were found to be 6.0 μ*M* and 10.0 μ*M*, respectively on using the 365/75 μm capillary whereas the LOD were found to be 10.0 μ*M* for nitrate and 30.0 μ*M* for nitrite on using the 150/50 μm capillary.

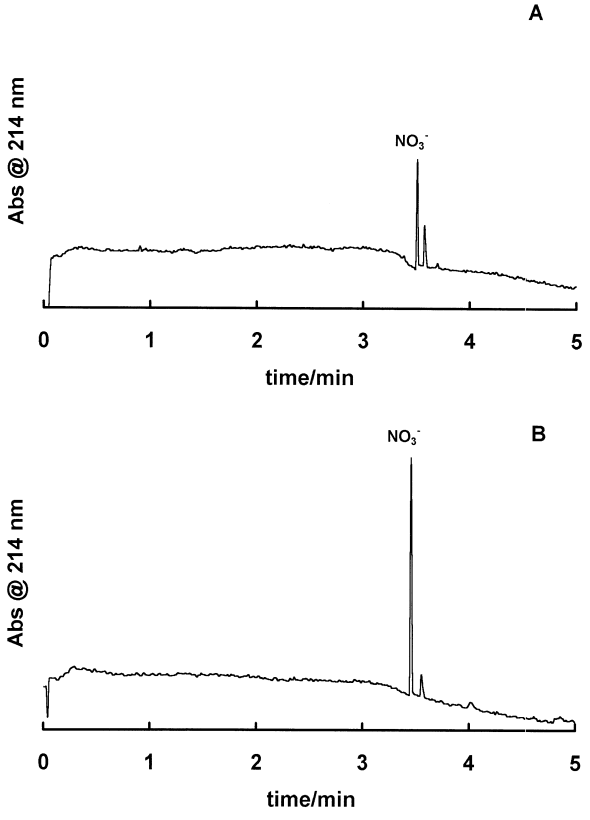

Fig. 2. Electropherograms for the analysis of nitrate in (A) rat plasma and (B) rat plasma spiked with standard nitrate (90 μ*M*). BGE: 50 m*M* phosphate with 0.5 m*M* spermine, pH 3.0. Applied voltage: -333 V cm^{-1} (I=~65 μA).

For rat plasma, calibration curves were made both with plasma spiked (with standard nitrate) and with aqueous solution of standard nitrate and nitrite, over the range 5–1000 μ*M* and analyses were done with the 365/75 μm capillary. Both plasma spiked and aqueous standards gave very similar calibration data and there was no significant matrix effect on the calibration. We have also previously shown that errors due to viscosity differences between samples and standards are not significant with measurement of ASL inorganic ions [22]. With nitrate and nitrite concentration expressed in μ*M*, the equations of typical standard curves were as follows. Area(nitrate)=(25.6±0.8)[nitrate]−(0.8±0.4), r^2=0.996 (n=6); area(nitrite)=(25.3±0.9)[nitrite]+(191±53.5), r^2=0.996 (n=5). For rat ASL, calibration curves were made with aqueous solution of nitrate and nitrite, over the range 15–500 μ*M* and analyses were done with the 150/50 μm capillary. With nitrate and nitrite concentration expressed in μ*M*, the equations of typical standard curves were as follows. Area(nitrate)=(4.82±0.21)[nitrate]+(9.9±49.6), r^2=0.992 (n=6); area(nitrite)=(7.83±0.07)[nitrite]+(84±20), r^2=0.999 (n=5). The differences in slope for nitrate and nitrite in two different capillaries is due to the different detection path lengths and alignment due to different sizes of the capillaries.

Intra-day and inter-day reproducibility measurements for aqueous samples (50 μ*M*) of nitrate and nitrite were carried out with the 365/75 μm capillary. The intra-day relative standard deviations (RSDs) (n=10) for peak area were 2.9% (nitrate) and 5.4% (nitrite), and inter-day (1 week) were 3.8% (nitrate) and 9.9% (nitrite), respectively. The intra-day RSDs for migration time were 0.8% (nitrate) and 5.0% (nitrite), and for inter-day runs were 0.9% (nitrate) and 8.0% (nitrite), respectively. Intra-day RSDs for peak area of triplicate runs carried out with the 150/50 μm capillary were 0.9% (nitrate) and 5.9% (nitrite) for 50 μ*M* aqueous samples. The corresponding RSDs for migration were 0.5% (nitrate) and 1.4% (nitrite). Inter-day reproducibilities for nitrate and nitrite with 150/50 μm capillaries were not determined out due to the possibility of variations in the alignment of capillary window in the detector, which is optimized for use of the 365 μm O.D. capillary.

3.3. Rat ASL and plasma nitrate and nitrite analyses

Nitrate and nitrite were found to be the major metabolites of NO in rat ASL whereas in rat plasma, nitrate was the major metabolite of NO. The concentration of nitrite was found to be very low (<10 μ*M*) in plasma, presumably due to oxidation of nitrite to nitrate [35]. Nitrate and nitrite in rat ASL migrated approximately 3.2 min and 6.0 min, respectively, under an applied electric field of −347 V cm^{-1} (Fig. 3) whereas nitrate in rat plasma migrated at a time of approximately 3.5 min under an applied electric field of −333 V cm^{-1}, which was confirmed by spiking rat plasma with standard nitrate (Fig. 2). A slightly lower electric field (−333 V cm^{-1}) was used for plasma analyses using 365/75 μm capillaries to keep the current below 75 μA. We also consistently detected a minor peak migrating close to nitrate in both ASL and plasma. We considered the possibility that this peak could be accounted for by bromide, which has a similar mobility to nitrate and also absorbs around 214 nm [31]; human plasma is known to contain ~40 μ*M* bromide [36]. However, spiking rat plasma with bromide failed to confirm the peak's identity and therefore it remains unidentified. One of the advantages of using direct detection at 214 nm is that we avoid interference from chloride, which is present in high concentration (~50–100 m*M*) in both ASL [22,23,25] as well as in plasma [37] and migrates close to nitrate [22,33].

Under control conditions, the nitrate concentration in ASL was found to be 102±12 μ*M* (*n*=9), for plasma it was 70±1.0 μ*M* (*n*=7). Under the same conditions, the concentration of nitrite in ASL was 83±28 μ*M* (*n*=6) but undetectable in plasma (Table 1). The stability of nitrite in plasma and serum depends on the time between sampling and processing as nitrite is oxidized to nitrate by oxyhemoglobin [30,38,39]. Since the sampling and analyses were carried out in less than 1 h for each plasma sample, with the sample stored at 4°C, stability of nitrite is not a problem in our analysis [30,39] and could not account for the low level (below LOD) of nitrite. The higher concentrations of nitrate and nitrite we detected in ASL compared to plasma are expected given that NO is synthesized locally in airway epithelial cells [14,40]. The present values for ASL nitrate and nitrite concentrations are consistent with our earlier measurements by CE with conductivity detection [22].

Although we are aware of no other attempts to measure nitrate and nitrite directly in ASL, other approaches have been used to estimate their concentrations in airway lining fluids. For example, bronchoalveolar lavage (BAL) fluid of lung airways of human [41] or animal [21] and sputum samples of human [10,42] have been analyzed either by Griess reaction or by ozone induced chemiluminescence. The measured values of NO metabolites (nitrite/nitrate) were in the range of ~100–550 μ*M* and are quite similar to those reported here. Likewise, our measurements of plasma nitrate concentration are comparable to those reported for human plasma (~41 and 90 μ*M*) by similar CE methods [26,28].

To further validate our method, we attempted to

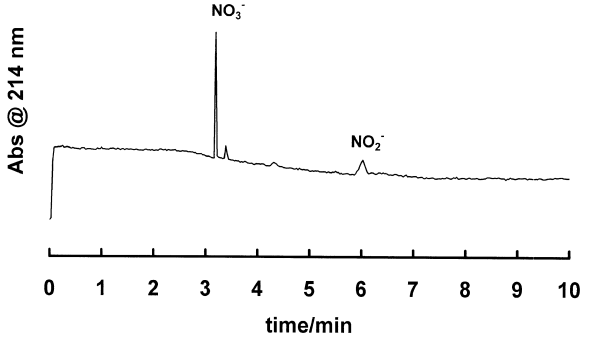

Fig. 3. Electropherogram for the analysis of nitrate and nitrite in rat ASL. BGE: 50 m*M* phosphate with 0.5 m*M* spermine, pH 3.0. Applied voltage: −347 V cm^{-1} (*I*=~60 μA).

Table 1
The composition of nitrite and nitrate determined in ASL and plasma from Sprague–Dawley rats

Analyte	Rat ASL (μ*M*)		Rat plasma (μ*M*)	
	Normal	LPS induced	Normal	LPS induced
Nitrate	102±12[a]	387±16[c]	70±1.0[e]	377±88[c]
Nitrite	83±28[b]	103±7.0[d]	–	138±17[c]

Error values are standard deviations on the mean of the measurements.
[a] *n*=9.
[b] *n*=6.
[c] *n*=4.
[d] *n*=3.
[e] *n*=7.

detect the increase in nitrite and nitrate that is expected after inflammatory induction of NOS expression by intratracheal instillation of LPS. LPS induces inflammatory processes by the recruitment of various cytokines and in turn this process induces iNOS to produce more NO [21]. Intratracheal instillation of LPS (0.5 mg kg^{-1} body mass) led to a marked increase in nitrate level to 387±16 μ*M* (*n*=4) from 102±12 μ*M* (*n*=9) in ASL and to 377±88 μ*M* (*n*=4) from 70±1.0 μ*M* (*n*=7) in plasma. The concentration of nitrite was changed from 83±28 μ*M* (*n*=6) to 103±7.0 μ*M* (*n*=3) for rat ASL whereas the value was increased to 138±17 μ*M* (*n*=4) from an undetectable amount in rat plasma (Table 1). The relative increases we found are comparable to those found in other studies [21,43] using LPS treatments. This is also consistent with the observation of higher levels of NO synthase activity from patients with inflammatory lung diseases compared to healthy ones [44]. It remains controversial whether the increased NO production under inflammatory condition is beneficial or toxic. However, the ability of our methodology to measure the changes in NO level at micromolar concentrations in ASL and plasma under different physiological conditions with nano- or microliter samples is significant and provides an impetus for the study of the various roles of NO in lung airways and systemic diseases.

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