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Development of a novel running buffer for the simultaneous determination of nitrate and nitrite in human serum by capillary zone electrophoresis

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Abstract

In order to improve NO_2^- peak height and obtain a convenient buffer system for the assay of nitrogen monooxide metabolites, we developed a novel running buffer for the simultaneous determination of nitrite and nitrate in human serum by capillary electrophoresis. The addition of cetyltrimethylammonium chloride to the running buffer resulted in high-speed separation using reverse electroosmotic flow. Highly sensitive determination was also achieved using stacking with 10-fold diluted sample solutions. The samples were injected hydrodynamically for 100 s into a 50 cm×75 μm I.D. capillary. The separation voltage was 10 kV (negative polarity). UV detection was performed at 214 nm. We obtained complete separation of nitrite and nitrate in deproteinized human serum within 6 min with optimum analytical conditions. Linear calibration curves for nitrite and nitrate for both peak height and peak area were obtained with standard addition method. The limits of detection obtained at a signal-to-noise ratio of 3 for nitrite and nitrate were 4.1 and 2.0 μ*M*, while the values of relative standard deviation of peak height were 2.4 and 2.6%, respectively.

Keywords: Buffer composition; Stacking; Nitrate; Nitrite; Inorganic anions; Nitric oxide

1. Introduction

Nitrogen monooxide (NO) is synthesized from L-arginine in mammalian cells by a family of three NO synthases (NOSs) [1]. NOSs are classified into three isoforms as neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Constitutive NOSs, nNOS and eNOS, synthesize relatively small quantities of NO, while iNOS synthesizes relatively large quantities of NO. NO has been identified as a mediator in many physiological functions, including regulation of vascular tone [2], signal transmission [3], phagocytosis [4], etc. In order to correlate changes in volume of NO with disease, it is important for human health to monitor NO synthesis. NO levels were changed in serum by sepsis [5] and infectious gastroenteritis [6], in cerebrospinal fluid by meningitis [7], and in urine by

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preeclampsia [8]. It is important to determine NO, however, NO is known to be rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) in the blood [9]. Due to its short half-life, NO assay in biological fluids mostly measures NO_2^- and NO_3^- as markers of NO release. Moreover, NO is one of the typical oxidative stress related compounds. Peroxynitrite ($ONOO^-$) is an NO metabolite [10] and is synthesized from NO and superoxide anion (O_2^-) usually by superoxide dismutase (SOD), a disproportionating enzyme which changes a low level of O_2^- formed in the body to H_2O_2 and O_2. This reaction is very rapid and is called diffusion controlled reaction. However the formation reaction of $ONOO^-$ is equally as fast. $ONOO^-$ is quickly changed by protonation to ONOOH, which has a large capacity for oxidation. This capacity for oxidation is equal to that of hydroxy radical. NO_2^- and NO_3^- are also $ONOO^-$ metabolites. . Since NO_2^- and NO_3^- are NO and $ONOO^-$ metabolites, we can measure NO total formation volume by determining NO_2^- and NO_3^-.

The Griess reaction is the most widely used method for the determination of NO_2^- and/or NO_3^- in human blood [11,12]. Determination by chemiluminescence [13], high-performance liquid chromatography [14], ion chromatography [15] and gas chromatography–mass spectrometry [16] has also been developed, but these methods are time-consuming because of complicated chemical reactions of samples.

Recently, capillary electrophoresis (CE) has attracted much attention because of its high speed and ease of use. Since the 1990s anions in fresh environmental waters have been analyzed by capillary zone electrophoresis (CZE) using a commercial buffer system (NICE-Pak OFM Anion-BT; Waters, Milford, MA, USA). However, it is not as easy to analyze anions in biological fluids because anions in a highly ionic strength sample matrix are easily diffused over the injected sample zone. The determination of NO_2^- and NO_3^- using CZE was investigated by several authors [17–20].

Ueda et al. [17] reported determination of NO_2^- and NO_3^- in human blood plasma using 750 m*M* sodium chloride containing 5% commercial electroosmotic flow (EOF) modifier (NICE-Pak OFM Anion-BT) as buffer and a 60 cm×75 μm I.D. capillary with detection wavelength at 214 nm and applied voltage at 20 kV. Friedberg et al. [18] reported determination of NO_2^- and NO_3^- in biological fluids using phosphate-buffered saline and polyethyleneglycol (for suppression of EOF) as buffer and a 50 cm×75 μm I.D. capillary with detection wavelength at 214 nm and applied voltage at 6 kV. With these methods, they obtained simultaneous determination of NO_2^- and NO_3^-, however, run time was more than 13 min. Leone et al. [19] reported the determination of NO_2^- and NO_3^- in plasma using 25 m*M* sodium sulfate containing 5% commercial EOF modifier (NICE-Pak OFM Anion-BT) as buffer and a 72 cm×75 μm I.D. capillary with a detection wavelength of 214 nm and an applied voltage of 6 kV. Bories et al. [20] reported determination of NO_2^- and NO_3^- in biological fluids using 15 m*M* sodium sulfate containing 2.5% EOF modifier ($OFM\text{-}OH^-$),(adjusted to pH 8.0 with 1.5 *M* boric acid, as buffer and a 60 cm×100 μm I.D. capillary with detection wavelength at 214 nm and applied voltage at 15 kV. In these methods, they obtained simultaneous determination of NO_2^- and NO_3^- within 6 min, however, detection of NO_2^- was difficult because of insufficient limit of detection for NO_2^-.

We have investigated anion analysis in seawaters using a novel buffer solution similar in composition to artificial seawater [21] in order to reduce the very high chloride peak and also to concentrate the sample zone by stacking and/or transient isotachophoresis [22–26], and obtained good results. We also tried analyzing anions in human serum, because human serum is a sample matrix with high ionic strength and is fairly similar to seawater in composition. Using artificial seawater as running buffer, we investigated high speed separation by adding EOF modifier, and highly sensitive determination by using the stacking concentration technique. We obtained simultaneous determination of NO_2^- and NO_3^- in human serum by CE with artificial seawater as running buffer [27], but the NO_2^- peak was small and preparation of the buffer was complex. In this paper, we described a novel running buffer for simultaneous determination of NO_2^- and NO_3^- in human serum using CZE, developed in order to improve NO_2^- peak height and obtain a convenient buffer system.

2. Experimental

2.1. Chemicals and solutions

A prepared electrolyte similar to human serum was adjusted with 0.1 *M* hydrochloric acid to pH 7.4, the pH of human serum. A running buffer was prepared consisting of the electrolyte plus 1 m*M* cetyltrimethylammonium chloride (CTAC) as EOF modifier for high speed assay by CE. CTAC was obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical grade and used without further purification. All solutions were prepared in distilled water purified by Milli-Q Jr. (Millipore, Bedford, MA, USA).

Nitrate and nitrite standard solutions were prepared with the corresponding sodium and potassium salts, because sodium and potassium ions are similar electrolytes of human serum.

2.2. Instrumentation

A capillary ion analyzer (Waters) was used. Separation voltage was 10 kV (negative polarity at inlet side) and detection wavelength was set at 214 nm. A 375 μm O.D.×75 μm I.D. fused-silica capillary was used for separation. Its total length was 50 cm and effective length was 42.5 cm. The data were collected and analyzed by an 805 data station (Waters). An HM-60V pH meter (Toa, Tokyo, Japan) and a Model-320 UV spectrophotometer (Hitachi, Tokyo, Japan) were used.

2.3. Sample preparation of human serum

Pooled human serum (NESCOL-X; Kaketsuken, Kumamoto) was deproteinized with a sequential centrifugal ultrafiltration at 2000 *g* using Biomax-100K, Biomax-30K and Biomax-5K (Millipore). The deproteinized pool human serum was then diluted by a factor of ten with distilled water for use in the stacking concentration technique, because the effect of stacking is effective when there is a greater than 10-fold difference in electric field, corresponding to ionic strength, between the sample and buffer zones [28]. The 10-fold diluted sample was injected for 100 s compared to the non-diluted sample which was injected for only 10 s. The diluted sample gave higher peak and lower noise level than the non-diluted sample. As a result, the diluted sample was injected with gravity injection for 10 cm×100 s (~174 nl) onto the capillary.

3. Results and discussion

3.1. Development of running buffer

We have carried out CZE with bromide-free artificial seawater as running buffer and found it effective in analysing anions in seawater [22–26]. Therefore, we expected that using an electrolyte similar to human serum as running buffer would be effective in determining NO_2^- and NO_3^- in human serum by CZE. Moreover, we found that addition of CTAC to running buffer was effective in high speed assay, and that the stacking concentration technique using a 10-fold diluted sample was effective for highly sensitive determination. Therefore, in this study, we used CTAC and stacking.

We analysed human serum after deproteinization. Table 1 shows the expected average components of human serum for men aged 20–29 years as described in Ref. [29]. When we prepared an electrolyte based on Table 1, uric acid precipitated in the electrolyte, because it is hard to dissolve in water. Therefore uric acid was omitted from the buffer composition. Furthermore, magnesium and calcium, which tend to precipitate in highly saline solution, were also omitted. Running buffer A as described in Table 2 was

Table 1
Average components of human serum for men aged 20–29 years

Component	Concentration (g/l)
Glucose	0.85
Lactic acid	0.13
Urea nitrogen	0.152
Uric acid	0.054
Na^+	3.311
K^+	0.164
Ca^{2+}	0.094
Mg^{2+}	0.021
Cl^-	3.723
HPO_4^{2-}	0.192
HCO_3^-	1.464

Table 2
Composition of running buffer A

Component	Concentration (m*M*)
Na_2HPO_4	2.00
$NaHCO_3$	24.0
KCl	4.20
NaCl	94.4
Sodium lactate	1.44
Urea	5.43
Glucose	4.72
Sodium acetate	20.2

Table 3
Composition of running buffer B

Component	Concentration (m*M*)
Na_2HPO_4	2.00
$NaHCO_3$	24.0
KCl	4.20
NaCl	420
Urea	5.43
Glucose	4.72

prepared. Sodium acetate was added to control the concentration of sodium ion. When we determined NO_2^- and NO_3^- in human serum by CE with running buffer A, we obtained the electropherogram shown in Fig. 1; the NO_3^- peak was clearly detected, but no NO_2^- peak was detected.

This result suggested that high UV absorption of the buffer was interfering with detection of NO_2^-. When we measured UV absorption of the buffer, we obtained high UV absorption at 214 nm, and when we measured the UV absorption of each component, we found that sodium acetate had high UV absorption. Therefore, we changed sodium acetate to sodium sulfate. However, we still could not detect the NO_2^- peak. Sodium lactate was then omitted from the buffer and broad and incomplete peak separation of NO_2^- and NO_3^- was obtained. This result suggested that concentration by stacking was not sufficient. Better concentration can be obtained with higher salinity, so the volume of sodium chloride in the buffer was increased to that in artificial seawater. Furthermore, sodium sulfate was omitted from the buffer because the concentration of sodium ion no longer needed to be controlled due to addition sodium chloride and decrease in UV absorption. As a result of these experiments, running buffer B as shown in Table 3 was prepared. Analysis of

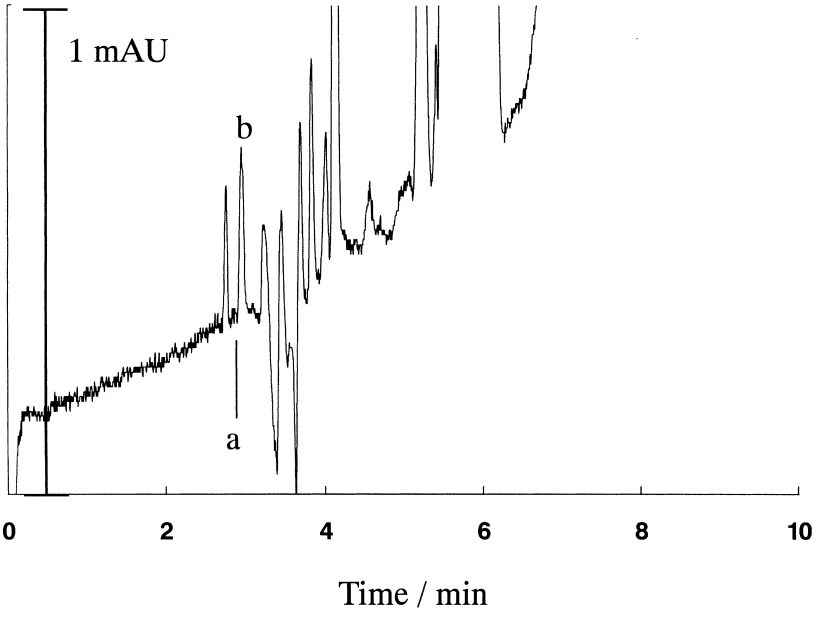

Fig. 1. Electropherogram of a sample of 10-fold diluted human serum with running buffer A including CTAC. Analytical conditions: capillary, 50 cm×75 μm I.D. fused-silica capillary; detection at 214 nm; applied voltage, 10 kV (negative polarity at inlet side); injection, gravity for 10 cm×100 s (~174 nl). Peaks: (a) NO_2^-; (b) NO_3^-.

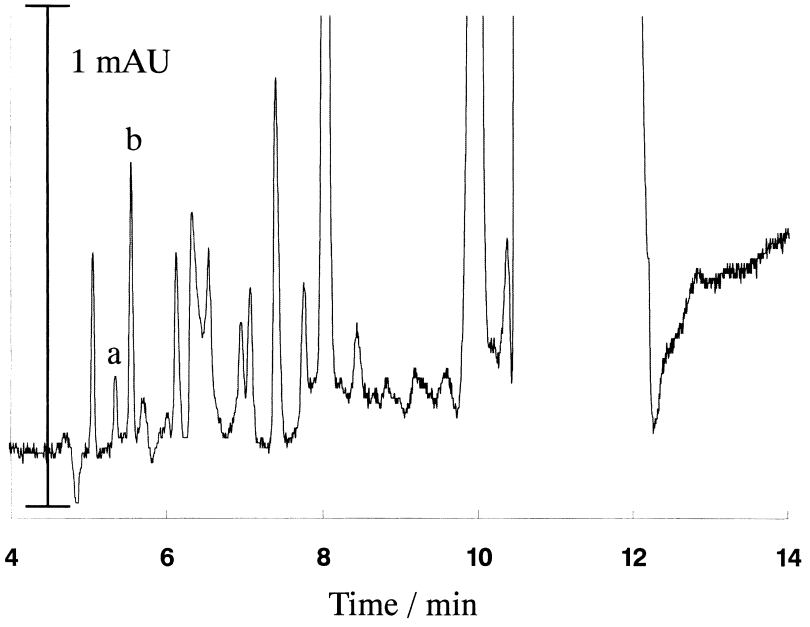

Fig. 2. Electropherogram of a sample of 10-fold diluted human serum with running buffer B including CTAC. Analytical conditions as in Fig. 1. Peaks: (a) NO_2^-; (b) NO_3^-.

human serum by CE with running buffer B produced sharp and complete peak separation of NO_2^- and NO_3^- as shown in Fig. 2.

3.2. Calibration curves of NO_2^- and NO_3^- using standard addition method

In order to obtain calibration curves for NO_2^- and NO_3^- using both peak height and peak area, we applied standard addition method for determining NO_2^- and NO_3^- in 10-fold diluted pooled serum. Calibration curves for 0.5–2.0 μM NO_2^- were fairly linear and correlation coefficients for peak height and peak area were 0.999 and 0.998, respectively. In the case of 1.0–4.0 μM NO_3^-, correlation coefficients were 0.998 and 0.998 for peak height and peak area, respectively.

3.3. Reproducibility and limits of detection for CE determination of NO_2^- and NO_3^- in human serum

Reproducibility and the limits of detection (LODs) for peaks of NO_2^- and NO_3^- were investigated in 10-fold diluted pooled serum. Although the NO_2^- peak was small, we obtained a fairly good relative standard deviation value (RSD; $n=10$) of 2.4% for peak height and an LOD value ($S/N=3$) of 4.1 μM for NO_2^-, and an RSD of 2.6% and an LOD of 2.0 μM for NO_3^-. Friedberg et al. reported concentrations of NO_2^- and NO_3^- in normal serum of 6.6 ± 11 and 34 ± 18 μM, respectively [18]. The LOD for NO_2^- needs further improvement. To obtain higher peak height for NO_2^-, the UV absorption of the buffer should be reduced to decrease baseline noise. This improvement is now under investigation.

4. Conclusions

We have established a method to simultaneously determine the NO metabolites, NO_2^- and NO_3^-, in human serum by CZE using a novel running buffer including the EOF modifier CTAC, and also using the stacking concentration technique with 10-fold diluted deproteinized human pooled serum. We obtained complete separation of NO_2^- and NO_3^- within 6 min. We also developed a convenient buffer system. However, sensitivity was not much higher than that obtained with artificial seawater. The LOD of NO_3^- was sufficient for determination in human

serum with less than 3% RSD. However, because the NO_2^- peak is still small, we are investigating improvement of the novel running buffer to obtain higher NO_2^- peak height.

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