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Simultaneous determination of nitrate and nitrite in biological fluids by capillary electrophoresis and preliminary study on their determination by microchip capillary electrophoresis

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Abstract

In order to develop a highly sensitive and high-throughput screening method for nitrogen monoxide metabolites in biological fluids, we have investigated the simultaneous determination of nitrite and nitrate, using capillary electrophoresis and microchip capillary electrophoresis. In capillary zone electrophoresis, a running buffer based on human serum components with high ionic strength has been developed for the determination of nitrite and nitrate in human serum and human saliva. We obtained successful separation of nitrite and nitrate in the serum and the saliva within 7 min under optimum analytical conditions. Linear calibration curves for nitrite and nitrate for both peak height and area were obtained by a standard addition method. The limits of detection obtained at a signal-to-noise ratio (*S*/*N*) of 3 for nitrite and nitrate in the serum were 2.6 and 1.5 μM, respectively. The values of the relative standard deviation of peak height for the serum with 9.2 μM nitrite and 20.9 μM nitrate were 5.7 and 4.1%, respectively. For on-site analysis with high-throughput screening, a microchip capillary electrophoresis method using a microchip made of quartz with a UV detector was developed. In this high-throughput format, using a running buffer with an electroosmotic flow modifier, the peaks of nitrite and nitrate in an artificial serum sample were obtained within 8 s. In high-resolution mode, using the buffer without electroosmotic flow modifier, the separation of nitrite and nitrate was obtained within 15 s. In high-resolution mode, using an artificial serum sample with 50 μM NO_2^- and 50 μM NO_3^-, the limits of detection (*S*/*N* = 3) of 41 μM for NO_2^- and 26 μM for NO_3^- were obtained. The method was applied to human serum and saliva. We obtained peaks due to nitrite and nitrate in 10-fold diluted saliva.

Keywords: Nitric oxide; High throughput; High resolution; Cross-type chip; Quartz chip

1. Introduction

Nitrogen monoxide (NO) is synthesized from L-arginine in mammalian cells by a family of three NO synthases [1]. NO has been identified as a mediator in many physiological functions, including the regulation of vascular tone [2], cell-to-cell communication [3], and phagocytosis [4]. In order to correlate changes in the amount of NO with disease, it is important for human health to monitor the amount of NO. NO levels in serum are changed by sepsis [5] and infectious gastroenteritis [6]; in cerebrospinal fluid, by Parkinson's disease [7]; and, in urine, by nephritic syndrome in children [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, most NO assays in biological fluids measure NO_2^- and NO_3^- as markers of NO release.

There are many reports in the literature for the determination of NO_2^- and NO_3^- [10]. For the determination of

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NO_2^- and/or NO_3^- in human blood, the Griess reaction is widely used [11,12]. Moreover, the separation methods for determination of NO_2^- and/or NO_3^- in saliva, plasma and urine by high performance liquid chromatography with an electrochemical detector [13], in saliva by ion chromatography (IC) with conductivity detection [14] and in serum using a sequential injection analysis flow system with a spectrophotometer [15] have been developed. Capillary electrophoresis (CE) has also been widely used. However, it is not easy to analyze anions in liquids, such as biological fluids and seawater, because anions in a sample matrix with high ionic strength easily diffuse over the injected sample zone. The determination of NO_2^- and NO_3^-, using CE, has been investigated by several authors [16–18]. The separation methods, using CE for simultaneous determination of NO_2^- and NO_3^- in human extracellular fluids, in hair and skin samples obtained from a victim of gunshot and in neuronal tissues have been reported. In order to obtain highly sensitive determination, some devices are presented. In order to eliminate a baseline dip caused by bromide ions, tetradecyltrimethylammonium bromide (TTAB) has been used as an electroosmotic flow (EOF) modifier: TTAB solution was passed through an anion exchange cartridge to replace bromide ions with hydroxide ions [16]. Chloride in the sample was removed by passing the sample through a solid-phase extraction cartridge [18].

Previously, we have investigated anion analysis in seawater by capillary zone electrophoresis (CZE), using a running buffer similar to artificial seawater composition [19] in order to reduce the very high chloride peak and also to concentrate the sample zone by stacking and/or transient isotachophoresis (tITP) [20–24]. Therefore, based on this methodology, we have developed a novel running buffer based on human serum components after deproteinization, because human serum is, like seawater, a sample matrix of high ionic strength. In addition, using artificial seawater as running buffer, we investigated high-speed separation by addition of another EOF modifier, cetyltrimethylammonium chloride (CTAC), and highly sensitive determination by using the stacking concentration technique. In the study presented here, we obtained the simultaneous determination of NO_2^- and NO_3^- in human serum, using the CE method with the novel running buffer with added CTAC and sample stacking [25]. However, an improvement in limit of determination of NO_2^- is required.

Moreover, microchip CE (MCE) has attracted much attention, because of its usefulness in high-throughput screening and requiring only μL level sample volumes [26,27]. Kikura-Hanajiri et al. [28] has developed a microchip made of poly (dimethylsiloxane) (PDMS) with electrochemical detection (ECD) for the measurement of NO. Nitrite was determined within 45 s by MCE–ECD, however, nitrate must be reduced to nitrite by a chemical reaction using Cu-coated Cd granules. Goto et al. [29] has developed a bioassay system for the determination of NO from cells, using a glass microchip based on the Griess reaction. The assay time was reduced by optimization of some conditions, however it was still 2 h. In many literature reports on the determination of the components in biological fluids, using MCE, laser-induced fluorescence detection, ECD and conductivity detection were used. The use of UV detection has not been reported as far as we know.

In this paper, we describe that improvement in the limit of determination by suppression of the system peak resulted from the running buffer [25], and the preliminary study on determination of NO_2^- and NO_3^- in biological fluids using MCE with the improved running buffer. In particular, in MCE, the selection of high-throughput and high-resolution modes was investigated by active control of EOF, using running buffer with/without CTAC. Moreover, the method was applied to human serum and saliva.

2. Experimental

2.1. Chemicals and solutions

A running buffer B as given in Table 1B was prepared and adjusted to pH 7.4 with 0.1 M hydrochloric acid. As a running buffer for CZE and the high-throughput mode in MCE, the running buffer B with 1 mM CTAC (running buffer A, Table 1, column A) was used. For the high-resolution mode in MCE, the running buffer B was used. An artificial serum as given in Table 2 was prepared and adjusted to pH 7.4 [25]. As an artificial serum sample in MCE, the electrolyte with added NO_2^- and NO_3^- was used. CTAC was obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical grade and used without further purification. All

Table 1
Composition of running buffers

Component	A: Running buffer A concentration (mM)	B: Running buffer B concentration (mM)
Na_2HPO_4	2.00	2.00
KCl	12.0	12.0
NaCl	412	412
Urea	5.43	5.43
Glucose	4.72	4.72
CTAC	1.00	–

Running buffer A was used for CZE and the high-throughput mode in MCE. Running buffer B was used for the high-resolution mode in MCE. Running buffers were adjusted to pH 7.4 with 0.1 M hydrochloric acid.

Table 2
Composition of artificial serum

Component	Concentration (mM)
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

The artificial serum sample for use in MCE was adjusted to pH 7.4 with 0.1 M hydrochloric acid and known additions of NO_2^- and NO_3^- were used.

solutions were prepared in distilled water purified by Milli-Q Jr. (Millipore, MA, USA).

2.2. Instrumentation

A capillary ion analyzer (Waters, MA, USA) was used. The 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. Gravity injection at 10 cm height was employed for 100 s (ca. 156 nL) to the serum and for 30 s (ca. 46 nL) to the saliva. The data was collected and analysed, using a 805 data station (Waters).

A MCE-2010 (Shimadzu, Kyoto, Japan) and microchip Type-U (Shimadzu) made of quartz were used for high-throughput analysis. The microchip was cross-type with a sample injection channel, a separation channel and four reservoirs at the ends of each channel as shown in Fig. 1. Electrophoretic sample injection and separation was performed by applying a voltage to the sample and buffer reservoirs of the chip, using computer-programmed sequencing of the MCE-2010. The separation behavior was observed using direct UV detection at 214 nm for the whole separation channel, using a linear photodiode array detector.

A HM-60V pH meter (DKK-Toa, Tokyo, Japan) was also used.

2.3. Sample preparation

Pooled human serum (NESCOL-X; The chemo-sero-therapeutic research institute, Kumamoto, Japan) was deproteinized by sequential centrifugal ultrafiltration at 2000 × *g*, using Biomax-100K, Biomax-30K and Biomax-5K filtration units (Millipore, MA, USA) in that order. Human saliva was collected with a Salivette (Sarstedt, Germany). In CZE, these samples were diluted 10-fold with distilled water to achieve the sample stacking effects. In MCE, the saliva with already known concentrations of NO_2^- and NO_3^- from CE, of 637 and 5437 μM, respectively [30], was used.

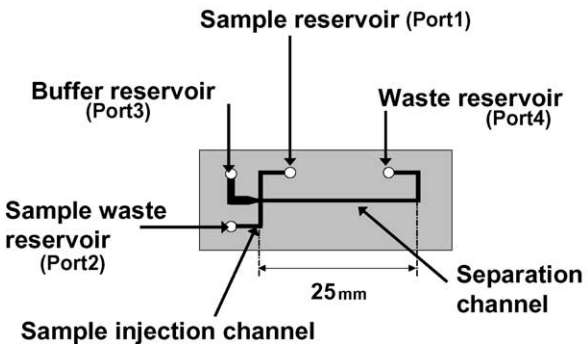

Fig. 1. Structure of the cross-type microchip: a sample injection channel, a separation channel and four reservoirs at the ends of each channel.

3. Results and discussion

3.1. Capillary electrophoresis

In CZE, for the determination of NO_2^- and NO_3^- in human serum, running buffer A (Table 1A) was used. The characteristic of the running buffer is that the proportion of sodium to potassium ions in the artificial serum was equal to the proportion of these ions in human serum, in order to obtain a stable baseline. When we determined NO_2^- and NO_3^- in the serum using CE with the running buffer, we obtained successful separation of NO_2^- and NO_3^- peaks within 6 min as shown in Fig. 2.

In order to obtain calibration curves for NO_2^- and NO_3^-, a standard addition method for NO_2^- and NO_3^- in the serum

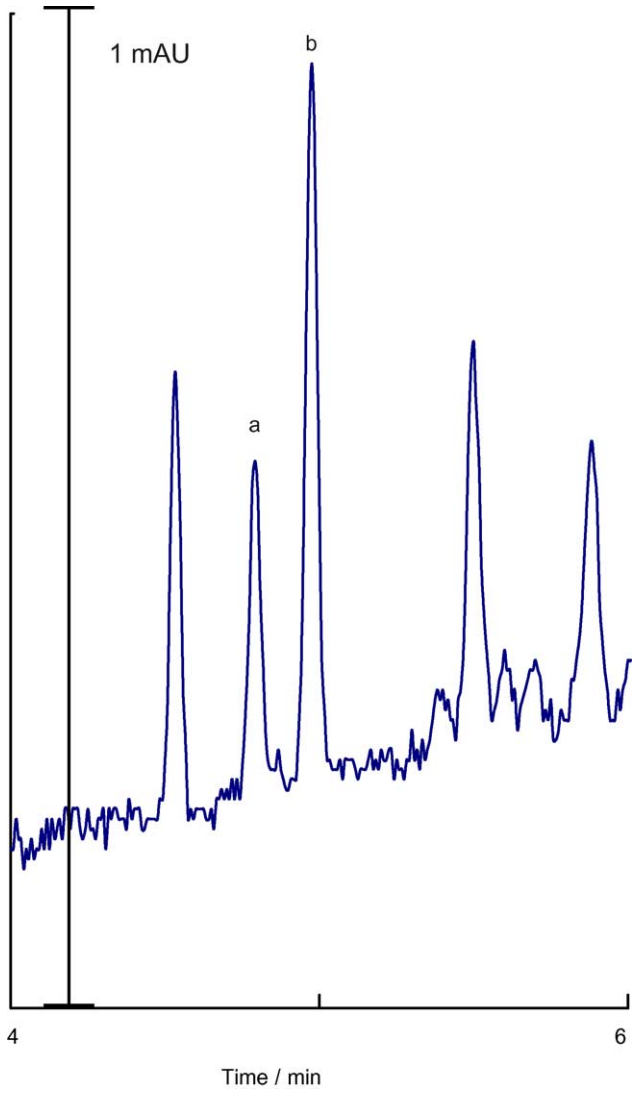

Fig. 2. Electropherogram of 10-fold diluted human serum as a sample with running buffer A. Analytical conditions: capillary, 75 μm i.d. × 50 cm fused-silica capillary; detection, at 214 nm; applied voltage, 10 kV (negative polarity at inlet side); injection, gravity for 10 cm × 100 s (ca. 156 nL). Peaks: a, NO_2^-; b, NO_3^-.

Table 3
R.S.D. of within-run and between-run, LOD in CE

	R.S.D. (%)				LOD (μM)
	Within-run (n = 10)		Between-run (n = 4)		
	Peak height	Peak area	Peak height	Peak area	
NO_2^-	5.7	6.6	5.2	2.8	2.6
NO_3^-	4.1	4.2	6.0	6.9	1.5

was applied. A standard solution was added to the serum to yield concentrations of 5, 10, 15 and 20 μM NO_2^-. The serum with a standard solution was diluted 10-fold and used. Calibration curves for NO_2^- were fairly linear and correlation coefficients were 0.996 and 0.997 for peak height and peak area, respectively. Moreover, in the case of NO_3^-, a standard solution to yield concentrations of 10, 20, 30 and 40 μM NO_3^- was added into the serum, correlation coefficients were 0.994 and 0.995 for peak height and peak area, respectively. Using calibration curves, the concentrations of NO_2^- and NO_3^- in the serum were 9.2 and 20.9 μM, respectively.

Using the serum, the reproducibility and the limits of detection (LODs) of NO_2^- and NO_3^- were obtained as shown in Table 3. Relative standard deviation (R.S.D.; n = 10) of within-run (repeatability) was calculated from the peak height and area for NO_2^-, and NO_3^-. R.S.D. (n = 4) of between-run (between-day) was calculated from the peak height and area. LODs (S/N = 3) were 2.6 μM for NO_2^- and 1.5 μM for NO_3^-.

Recoveries for NO_2^- and NO_3^- using the serum are shown in Table 4. The serum, containing a standard solution was diluted 10-fold and used. Average results based on three data points at each condition were used.

In addition, we tried to apply the CE method to human saliva. Saliva sampling has the advantage of non-invasiveness in comparison to blood sampling. This method might be capable of evaluating a stress-induced exercise tolerance test by monitoring the concentrations of some components in human saliva. Takahashi et al. [31] reported that the concentration of NO_3^- in human blood fluctuated in the exercise tolerance test. The concentrations of NO_2^- and NO_3^- in human saliva might be changed corresponding to the concentrations of these in human blood. The running buffer was applied to the determination of NO_2^- and NO_3^- in human saliva. When the saliva was introduced by gravity injection for 30 s (ca. 46 nL) onto the capillary, the best result was obtained. The unstable current on the capillary was caused by the larger injection volume. Because the conductivity and the viscosity of the saliva differ from those of the serum, the saliva was not deproteinized. It is thought that the causes of instability were the low conductivity and the high viscosity. We achieved successful separation of NO_2^- and NO_3^- in the saliva within 7 min as shown in Fig. 3. Using calibration curves, the concentrations of NO_2^- and NO_3^- in the saliva as shown in Fig. 3 were 118.5 and 532.0 μM, respectively. The concentrations of NO_2^- and NO_3^- in human saliva, 80–148 and 169–290 μM, respectively, have been reported previously [14]. In other literature, in human saliva, 125–270 μM NO_2^- and 579–884 μM NO_3^- was reported [32]. In comparison, the concentrations of NO_2^- and NO_3^-

Table 4
Recoveries for NO_2^- and NO_3^- in CE

Component	Concentration (mM)		Recovery (%)
	Spiked	Found	
NO_2^-	5.0	5.42	108.4
	10.0	9.76	97.6
	15.0	13.88	92.5
	20.0	20.85	104.3
NO_3^-	10.0	8.01	80.1
	20.0	22.57	112.9
	30.0	29.31	97.7
	40.0	39.73	99.3

The serum spiked with NO_2^- and NO_3^- was diluted 10-fold.

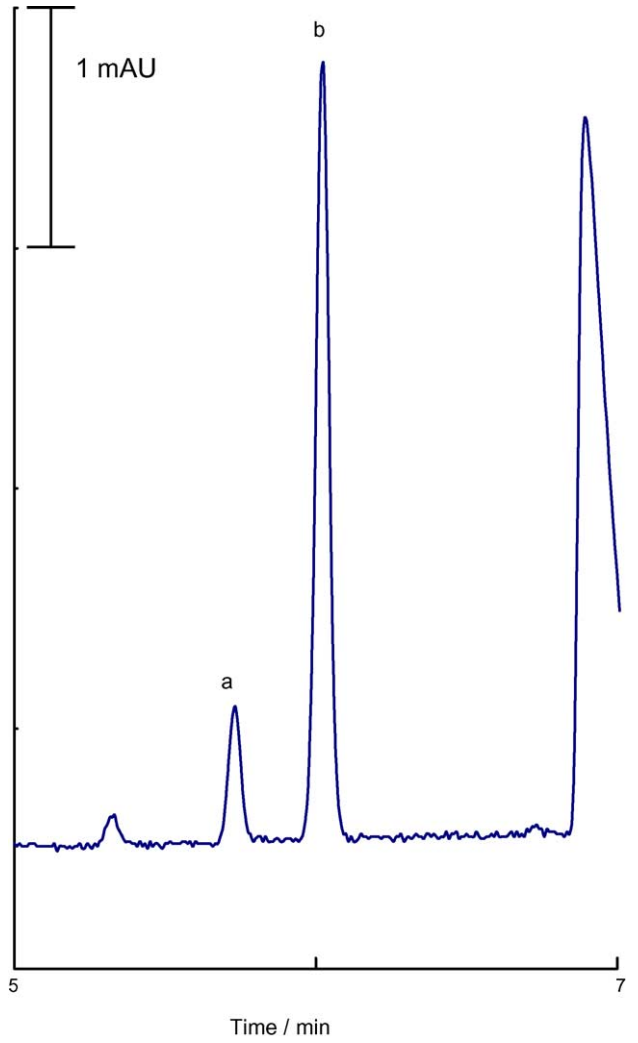

Fig. 3. Electropherogram of 10-fold diluted human saliva as a sample with gravity injection for 10 cm × 30 s (ca. 46 nL). Other analytical conditions are the same as Fig. 2. Peaks: a, NO_2^-; b, NO_3^-.

in human serum have been reported as 5–20 and 14–52 μM respectively [15]. It is clear that the amounts of NO_2^- and NO_3^- in the saliva are larger than those in the serum.

3.2. Microchip capillary electrophoresis

MCE system is useful for on-site analyses with high-throughput screening. We made preliminary studies of high-throughput separation of NO_2^- and NO_3^- using a microchip made of quartz with direct UV detection at 214 nm for the whole separation channel, using a linear photodiode array detector.

For the high throughput, running buffer A as well as CE was used. When using cross-type chip, pinched injection is generally used for sample injection. However, as injection volume is low, sensitivity is low. Therefore, an injection method similar to floating injection was used. The applied voltage conditions for each ports were as follows; in case of sample injection mode, 0.00, 0.30, 0.15 and 0.15 kV were applied to ports 1 to 4, and in case of sample separation mode, 0.15, 0.15, 0.00 and 1.00 kV were applied to ports 1–4, respectively. Under these conditions, the peaks of NO_2^- and NO_3^- in the artificial serum sample with 200 μM NO_2^- and 200 μM NO_3^- were obtained within 8 s as shown in Fig. 4A. The electropherogram was obtained for the distance of the separation channel from cross-channel in the system. Therefore, the ion with short migration time (NO_2^-) is detected behind the ion with long migration time (NO_3^-).

For the high-resolution mode, running buffer B (Table 1, column B) was used. The above-mentioned conditions of applied voltage for each port were used. Under these conditions, the injection method was electrophoretic injection. NO_2^- and NO_3^- migrated against the EOF. The separation of NO_2^- and NO_3^- in an artificial serum sample was obtained within 15 s as shown in Fig. 4B.

As in the MCE results, it was found that the EOF control enabled the selection of the high-throughput mode or the high-resolution mode. In both, the high-throughput mode and the high-resolution mode, the validity of the electrophoretic mobilities of NO_2^- and NO_3^- in MCE were investigated in comparison with these in conventional CE. Assuming that the structure of channel was simple, the mobilities in MCE were 1.3–1.7 times larger than the ones in CE. It is thought that the difference in electric field strength between MCE and CE is caused by the geometrical structure of channel and the applied voltage conditions.

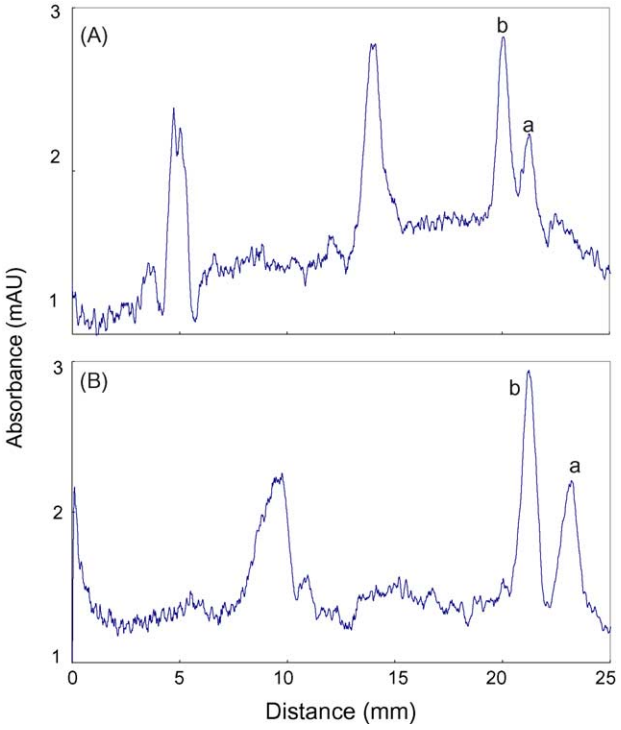

Fig. 4. The separation profile of NO_2^- and NO_3^- in the artificial serum sample with 200 μM NO_2^- and 200 μM NO_3^-, using direct UV detection at 214 nm for the whole straight part of the separation channel. A: high-throughput mode, running buffer A was used; separation time was 8 s. B: high-resolution mode, running buffer B was used; separation time was 15 s. Peaks: a, NO_2^-; b, NO_3^-.

Using the artificial serum sample, reproducibility and the limits of detection (LODs) of NO_2^- and NO_3^- were obtained as shown in Table 5. Using the artificial serum sample with 200 μM NO_2^- and 200 μM NO_3^-, in the high-throughput mode and the high-resolution mode, R.S.D. (n = 8) of within-run was calculated from the peak height and peak area for NO_2^- and NO_3^-. It is predicted that the cause of high R.S.D. is error in injection volume. In the high-throughput mode, using the artificial serum sample with 100 μM NO_2^- and 100 μM NO_3^-, LOD (S/N = 3) of 67 μM for NO_2^- and 35 μM for NO_3^- were obtained. In the high-resolution mode, using the artificial serum sample with 50 μM NO_2^- and 50 μM NO_3^-, LOD (S/N = 3) of 41 μM for NO_2^- and 26 μM for NO_3^- were obtained.

The high-resolution mode was applied to human serum and saliva. When using the non-diluted serum, the peaks of

Table 5
R.S.D. of within-run, LOD in MCE

	High-resolution mode			High-throughput mode		
	R.S.D. (%)		LOD (μM)	R.S.D. (%)		LOD (μM)
	Peak height (%)	Peak area (%)		Peak height (%)	Peak area (%)	
NO_2^-	16.2	20.9	41	21.0	25.9	67
NO_3^-	16.4	21.3	26	9.6	9.7	35

R.S.D.: n = 8.

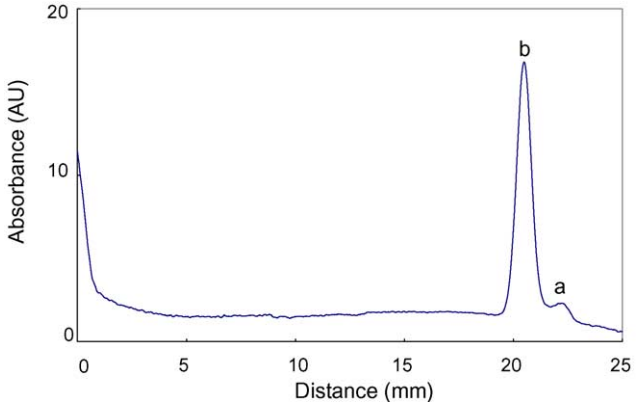

Fig. 5. The separation profile of NO_2^- and NO_3^- in the 10-fold diluted saliva, using the high-resolution mode. Peaks: a, NO_2^-; b, NO_3^-.

NO_2^- and NO_3^- were not obtained because of the low concentrations of NO_2^- and NO_3^- in the serum. However, when using the 10-fold diluted saliva, we obtained the peaks of NO_2^- and NO_3^- at ca. 20.5-mm and ca. 22-mm from the cross-channel of the separation channel as shown in Fig. 5.

In this study, saliva with high concentrations of NO_2^- and NO_3^- was used. Hence, it is necessary to obtain high sensitivity for the determination of NO_2^- and NO_3^- by MCE. A general method for obtaining high sensitivity is to increase the volume of injected sample. The large injection volume is obtained, using a different design of chip, such as a double T-type chip. However, using that method, it is predicted that incomplete separation of NO_2^- and NO_3^- is obtained with broad peaks. Complete separation is necessary for investigation of applying high voltage and on-line concentration. Moreover, it is essential for correction of injection volume, because the measurement error was caused by the error of injection volume.

However, the assay time of MCE is much shorter than that of CE, IC and the Griess reaction kit. The sample volume of MCE is much smaller than that of IC and the Griess reaction kit.

Hence, having established the determination of NO_2^- and NO_3^- in biological fluids using MCE, we will investigate improvement of the running buffer with large buffer capacity and the development of on-line concentration techniques, such as tITP, for highly sensitive determination. Moreover, in order to obtain good reproducibility, the selection of an internal standard for correction of injection volume will be investigated.

4. Conclusions

The simultaneous determination of NO metabolites, NO_2^- and NO_3^-, in human serum and saliva, using CE was established. In comparison with a previous study [25], the LOD of NO_2^- and NO_3^- was obtained with 1.6 and 1.3-fold improvements, respectively.

For on-site analysis with high-throughput screening, we made preliminary studies into high-throughput separation, using MCE. Using the running buffer with CTAC for the high throughput, the peaks of NO_2^- and NO_3^- in the artificial serum sample were obtained within 8 s. Moreover, using the running buffer without CTAC for the high-resolution, the separations of NO_2^- and NO_3^- were obtained within 15 s. We obtained the separation of NO_2^- and NO_3^- in the saliva within 15 s. In order to establish the determination of NO_2^- and NO_3^- in biological fluids using MCE, improvement of the running buffer to apply high voltages, development of on-line concentration and selection of an internal standard for correction of injection volume will be investigated.

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