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New method for analyzing the nitrite level in PC12 cells using capillary electrophoresis

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Abstract

Nitric oxide (NO) is a gaseous molecule shown to have signal transmitter properties in organisms. Direct measurement of NO in physiological conditions has been difficult due to its short lifetime and low concentration. Nitrite has been used as a marker for NO formation in biological systems. Capillary electrophoresis (CE) has been recently used to measure nitrite in biological fluids. The purpose of this study is to analyze nitrite in PC12 cells (pheochromocytoma cell line) using CE. Optimal CE performance was employed with 150 m*M* Tris–phosphate, 6 μ*M* hexadecyltrimethyammonium chloride buffer at pH 7.0 and a fused-silica column of 57 cm×75 μm I.D. The signal was measured with a UV detector at 214-nm wavelength and negative potential of 10 kV was applied for nitrite analysis. Under the optimum conditions, we monitored the changes in the concentration of the nitrite levels through synergistic stimulation of tumor necrosis factor alpha plus γ-interferon in PC12 cells.
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1. Introduction

No other single molecule has as many biological effects as nitric oxide (NO). This simple molecule, which acts in a fairly specific manner, is now recognized as playing an integral part in various homeostatic mechanisms—including those of the central and peripheral nervous systems and the cardiovascular system, as well as host–defense interactions—where it can directly alter vital functions; it also affects gene expression [1]. NO is formed directly from the guanidine nitrogen of L-arginine by the enzyme nitric oxide synthase (NOS) through a process that consumes five electrons and results in the formation of L-citrulline [2,3].

The direct measurement of NO in physiological systems has been difficult. The half-life of NO in biological fluids is very short (<5 s) as it is rapidly oxidized to nitrite and nitrate. Consequently, nitrite, the end products of NO metabolism, is usually measured as a marker for NO formation in vivo and in vitro due to its greater stability.

Formerly, this ion has been determined by the Griess method, in which nitrite is diazotized with sulfanilamide and then reacted with *N*-1-naphthylethylenediamine to form a colored product [4,5]. This method, however, has a few disadvantages. It requires a long and demanding pre-treatment of the

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sample, which makes the pre-treatment quite expensive. Other methods such as ion chromatography (IC) [6,7], high-performance liquid chromatography (HPLC) [8], and gas chromatography–mass spectrometry (GC–MS) [9,10] have also been reported for determining nitrite in serum and plasma.

Although plenty of other techniques are available for the detection and quantification of ions, only capillary electrophoresis (CE) offers the potential for examination [11]. Therefore, in the modern period the capillary electrophoresis (CE) method has been developed. CE is one of the most powerful tools for separating ionic species, particularly for charged aqueous and biological samples [12,13]. Major advantages of this technique are its ability to handle extremely small samples without loss of sensitivity and its rapid and efficient separation ability, making it one of the most attractive techniques for biological sample research work.

Cytokines are proteins secreted into the extracellular fluid. Most cytokine proteins have, on a molar basis, very high biological activities, one of which is inducing nitric oxide in various kinds of cells. NO, a radical gas, is known as an important regulator of hundreds of biochemical pathways and a component of many neuropathologies. Therefore, the monitoring of NO is very useful in investigating neuronal diseases.

Rat pheochromocytoma (PC12) cells are a useful model for studying the mechanisms of neuronal cells [14,15]. When PC12 cells are exposed to cytokine, in contrast to the immune system, they produce small amounts of NO. Even a small dose of NO plays a critical role in the neuronal system. For instance, NO induces neurodegenerative diseases such as Alzheimer's and Parkinson's. Therefore, quantification of nitrite in PC12 cells is very important in research on neuronal disease.

In the present study, we describe a CE technique for the quantification of small amounts of nitrite in neuronal PC12 cells. The goal of this work was to establish optimal conditions such as the running buffer pH, temperature and additive concentration using sodium nitrite for nitrite monitoring by CE coupled to UV detection, and to apply the conditions to the analysis of nitrite production in cytokine-treated PC12 cells.

2. Materials and methods

2.1. Reagents

Trizma-base, potassium phosphate, sodium nitrite and hexadecyltrimethyammonium chloride (CTAC) were obtained from Sigma (St. Louis, MO, USA). All chemicals were of analytical reagent grade. RPMI 1640 medium, type IV collagen, fetal bovine serum (FBS), horse serum (HS), penicillin G sodium and streptomycin sulfate were purchased from Gibco BRL (Gaithersburg, MD, USA), while recombinant rat tumor necrosis factor α (TNFα) and γ-interferon (IFNγ) were from R&D systems (Minneapolis, MN, USA).

2.2. Sample preparation

The rat pheochromocytoma PC12 cell line, obtained from the American Type Cell Collection (ATCC, Rockville, MD, USA) was grown as a monolayer in RPMI 1640 medium, supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, penicillin G sodium (100 U/ml) and streptomycin sulfate (0.1 mg/ml). Cells were cultured at 37 °C in 95% humidified air with 5% CO_2. For experiments, 12-well plates were coated with type IV collagen (1 μg/ml) and washed twice with serum free medium. Then $2 \cdot 10^5$ cells were seeded per dish and the attached cells were washed with serum free medium after 24-h incubation. For serum deprivation, the medium was replaced with RPMI 1640 medium which does not contain serum. After 12-h incubation, the RPMI 1640 medium was replaced with fresh serum-free medium which removed phenol-red to avoid light interference. The sample was then treated with 50 ng/ml TNFα and 50 ng/ml IFNγ for 1 day. For nitrite analysis using CE, the sample supernatant was taken and subjected to CE coupled to UV detection. For quantification of nitrite, a standard curve prepared with $NaNO_2$ was used.

2.3. CE apparatus

All experiments were performed on an automated P/ACE 5000 capillary electrophoresis system (Beck-

man Instrument, Fullerton, CA, USA). The instrument was equipped with a 214-nm wavelength UV detector. Separation was performed using an untreated fused-silica 57 cm×75 μm I.D. capillary column. The running buffer consisted of 150 m*M* Tris–phosphate buffer and 6 μ*M* CTAC buffer at pH 7.0. Sample injections were performed under gravity for 20 s and an applied negative potential of 10 kV. To obtain reproducibility of the migration time and the peak height, the capillary tube was washed before each run for 5 min with 0.5 *M* NaOH solution and flushed with the running buffer solution for 20 min. All buffer and sample solutions were sonicated, passed through a 0.2-μm filter and degassed before use. A standard curve for nitrite was constructed by injecting a RPMI 1640 medium mixture containing known amounts of $NaNO_2$. A component of the RPMI 1640 medium always migrated after nitrite and was taken as indicating the nitrite peak.

3. Results and discussion

3.1. Optimization of conditions for nitrite detection

To achieve optimum conditions using CE coupled with UV detector, we studied the influence of running buffer pH, surfactant concentration and temperature on nitrite detection. Nitrite was detected in RPMI 1640 medium containing 20 μ*M* sodium nitrite up to set optimum conditions. In this study, the specifications of the diameter and length of capillary, and running buffer concentration were fixed in all experiments as a 57 cm×75 μm I.D. fused-silica column and 150 m*M* Tris–phosphate buffer were used throughout. The signal was measured with a UV detector at 214-nm wavelength and a negative potential of 10 kV was applied for nitrite analysis. For quantification, sodium nitrite was used as standard nitrite. A peak appeared at the same time in every electropherogram, and therefore, we decided it indicated the nitrite peak.

3.2. Influence running buffer pH

In CE analysis, the pH of the running buffer has a key effect on the precise detection of the analytes, so the selection of the pH of the running buffer is very important in obtaining accurate data. In the first phase of this work, the effect of the running buffer pH on the nitrite peak intensities was studied using 150 m*M* Tris–phosphate buffer of various pH values ranging from 4.0. to 10.0. Fig. 1 shows the influence of buffer pH, clearly indicating that nitrite absorbances were pH-sensitive. It was not possible to detect nitrite under low-pH conditions, in this case pH 4. Silanol groups were in a slightly protonated state and there was possible capillary wall–analyte interaction. When the buffer pH was increased from 4 to 10, it became possible to detect nitrite. With buffer pH increase, peak intensities showed an increase up to pH 7, after which they started to decrease. Although shorter migration times were achieved at pH 8 and 10 due to high electroosmotic flow (EOF), we selected running buffer pH as 7.0, because of its higher intensity and better efficiency.

3.3. Influence of CTAC concentration

Generally at low pH range, it was not possible to detect nitrite ions in uncoated silica capillary, since negatively charged nitrite ions became absorbed into the positively charged capillaries (protonated silanol groups). In order to overcome this absorption problem we selected a cationic surfactant, CTAC. CTAC diminishes analyte–capillary wall interactions by

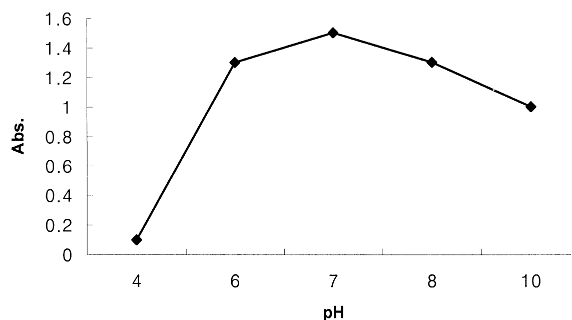

Fig. 1. Influence of pH of 150 m*M* Tris–phosphate. CE conditions: untreated 57 cm×75 μm I.D. fused-silica capillary, hydrodynamic injection for 20 s, UV detector at 214 nm, −10 kV applied voltage. Sodium nitrite concentration is 20 μ*M*.

forming a cationic layer on the surface of the capillary, allowing efficient separations and symmetrical peak shapes [16]. Under reserved-polarity conditions (negative potential at the source vial), CTAC induces electroosmotic flow towards the anode, presumably due to the creation of a cationic layer on the inner surface of the fused-silica capillaries. It may be used over a wide range of pH values without loss of electroosmotic flow. In order to achieve good resolution, we studied the influence of CTAC concentration by testing various concentration ranging from 0 to 10 μ*M*. Fig. 2 depicts the effect of CTAC concentration on resolution. Nitrite was not detected in this CE system in the absense of CTAC, but in the presence of CTAC nitrite migrated at ~8 min. As the critical micelle concentration of CTAC (1 m*M*) increased, interactions with the pseudo-stationary micellar phase also increased Therefore, as CTAC concentration increased, resolution became more effectual. However, by increasing the concentration of CTAC to 10 μ*M*, a high current was observed due to maximum Joule heating with poor resolution. At 6 μ*M* CTAC, the nitrite peak was well resolved with high efficiency. Hence based on the results of our experiments, we decided that 6 μ*M* was the optimal concentration of CTAC surfactant.

3.4. Influence of temperature

Another important separation parameter in CE analysis is temperature. In the present study, in order to obtain high efficiency, we investigated the effect of temperature. The temperatures studied were 15, 20 and 25 °C. The effects of different temperatures on migration time and resolution are shown in Fig. 3. Migration time decreased with increasing temperature. This phenomenon is common in capillary electrophoresis because temperature induced viscosity changes result in enhanced electroosmotic flow. Nitrite and indicator peaks co-migrated at 25 °C, but they separated at 15 and 20 °C; a temperature difference of 5 °C affects the mobility of analyte resulting in co-migration. The best resolution with high intensity peaks was obtained at 20 °C.

3.5. Optimized conditions

Based upon the above findings the optimal con-

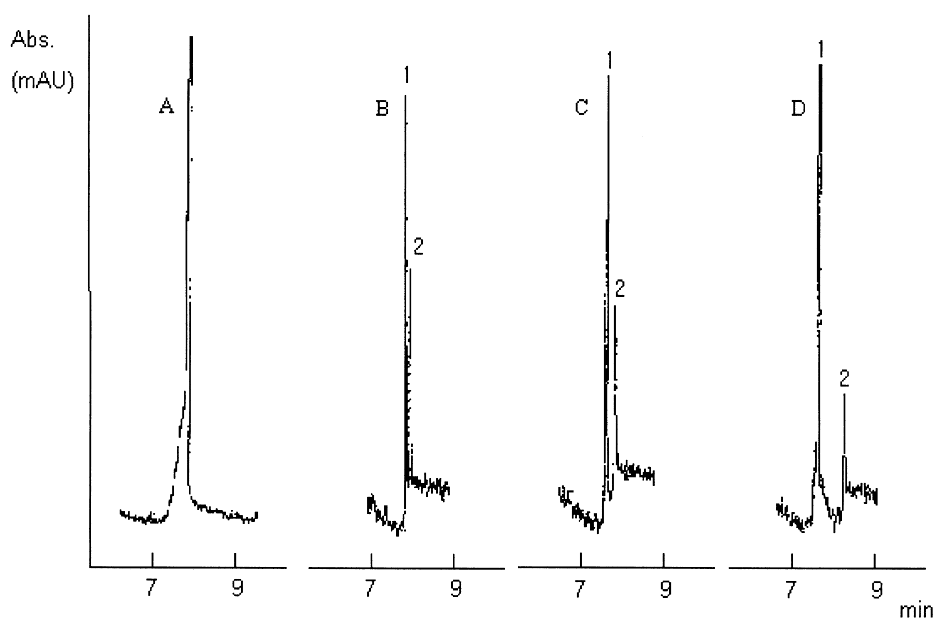

Fig. 2. Influence of CTAC in 150 m*M* Tris–phosphate. (a) CTAC 0 μ*M*, (b) CTAC 4 μ*M*, (c) CTAC 6 μ*M*, (d) CTAC 10 μ*M*. Peaks: 1=nitrite; 2=internal standard. CE conditions: untreated 57 cm×75 μm I.D. fused-silica capillary, hydrodynamic injection for 20 s, UV detector at 214 nm, −10 kV applied voltage. Sodium nitrite concentration is 20 μ*M*.

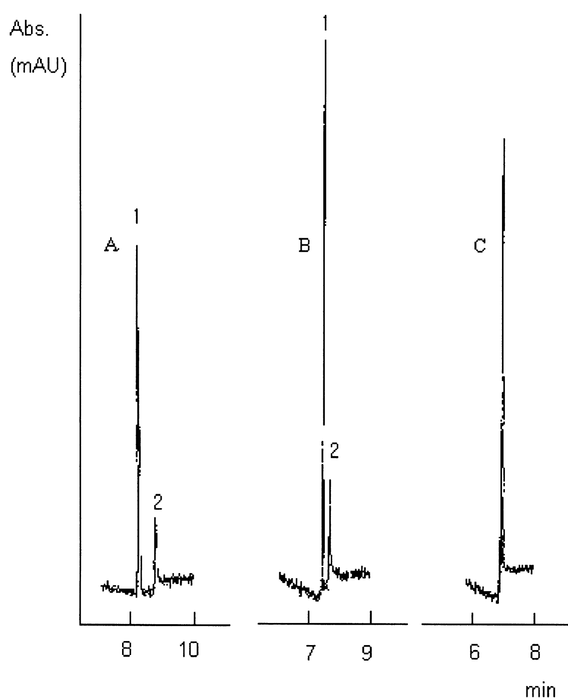

Fig. 3. Effect of incubation temperature on the analysis of nitrite: (a) 15 °C, (b) 20 °C, (c) 25 °C. Peaks: 1=nitrite; 2=internal standard. CE conditions: untreated 57 cm×75 μm I.D. fused-silica capillary, hydrodynamic injection for 20 s, UV detector at 214 nm, −10 kV applied voltage. Sodium nitrite concentration is 20 μ*M*.

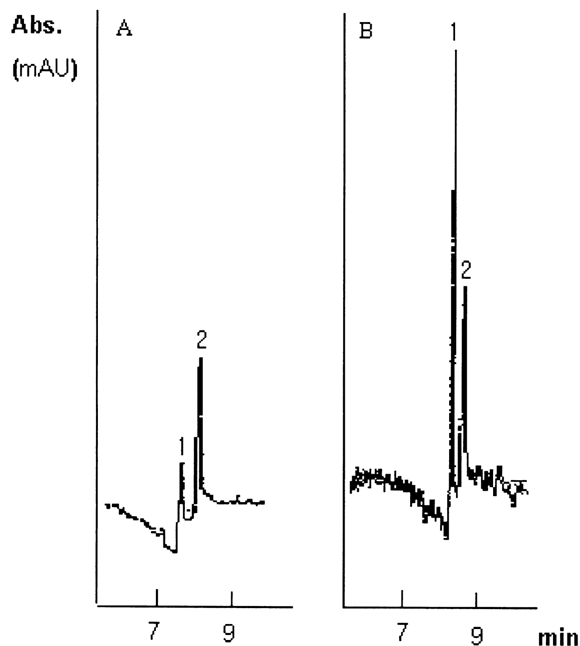

Fig. 4. Electropherogram of nitrite analysis in optimized conditions: (a) medium only (RPMI 1640), (b) standard 20 μ*M* $NaNO_2$ in medium. Peaks: 1=nitrite; 2=internal standard. CE conditions: untreated 57 cm×75 μm I.D. fused-silica capillary, hydrodynamic injection for 20 s, UV detector at 214 nm, −10 kV applied voltage.

ditions for the analysis of nitrite were 150 m*M* Tris–phosphate buffer, pH 7.0 containing 6 μ*M* CTAC, in an uncoated 57 cm×75 μm I.D. fused-silica column at a temperature of 20 °C. The signal was measured with a UV detector at 214-nm wavelength and negative potential of −10 kV was applied. Fig. 4 shows the electropherograms of a standard nitrite separated under optimal conditions. The optimized conditions allowed the detection of nitrite with a detection limit of ~25 p*M*.

3.6. Calibration curve and quantification of nitrite from cytokine-treated PC12 cells

In order to quantify nitrite, we first constructed a standard calibration curve using sodium nitrite. By employing optimal conditions, the calibration curve was generated for a concentration range of 0.025–20 μ*M*. The normalized peak heights of nitrite were plotted against nitrite concentration and the data points were fitted to a second order polynomial equation. Fig. 5 shows the calibration curve used for quantification. Each data point represents the average

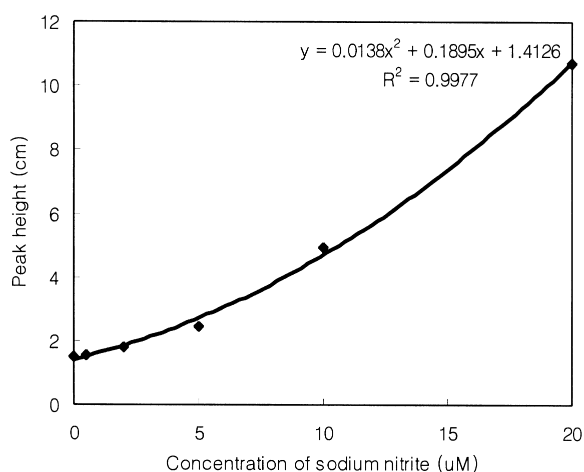

Fig. 5. Standard calibration curve, R^2=0.998. Each data point corresponds to an average of three electrophoretic runs.

of three consecutive runs. A correlation coefficient of 0.997 was obtained which is acceptable for quantification. To quantify nitrite in biological samples, we used the PC12 cell line treated with a cytokine mixture. PC12 cells cultured in RPMI 1640 medium and treated with a cytokine mixture were spiked with a known amount of standard nitrite and subjected to CE analysis under optimum conditions. However, nitrite could be detected without any cytokine treatment. The reason is that the RPMI 1640 medium originally contains arginine which produces nitric oxide. Therefore, the calibration curve does not pass through the coordinate origin. Quantitative analysis was accomplished by means of the calibration curve. Fig. 6 shows the electropherogram of nitrite from cytokine-treated PC12 cells.

In PC12 cells, a cell line that has the characteristics of a neuron, cytokine treatment causes NO production. We treated the cells with 50 ng/ml TNFα plus 50 ng/ml IFNγ and incubated them for 1 day. The cultured medium was then subjected to CE–UV monitoring. An increase of extracellular nitrite after treatment with cytokine mixture is observed in Fig. 6. These results show that treatment with the cytokine mixture induced an increase of NO production in PC12 cells. The precise nitrite concentration was 2.215 μ*M* if there was no cytokine treatment (Fig. 6a). However, nitrite concentration increased to 8.687 μ*M* in cytokine-treated PC12 cells (Fig. 6b). Accurate quantification analysis was performed by calibration curve (Fig. 5).

In the past, the Griess method has been mostly used for determination. Therefore, we compared the developed method using CE and the conventional method using Griess reagent. We obtained a good correlation between the Griess method and the CE method. Using Griess reagent, the nitrite concentration was from 2.064 to 2.302 in the case of untreated mixture, and from 8.593 to 8.790 in cytokine-treated PC12 cells. As shown by this result, the Griess method produces more variable results than the CE method.

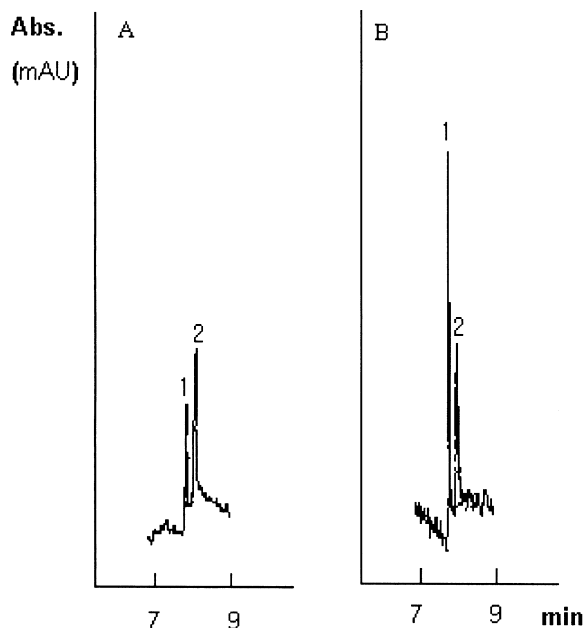

Fig. 6. Electropherograms of nitrite in PC12 cells: (a) untreated and (b) treated for 24 h with 50 ng/ml IFNγ plus 50 ng/ml TNFα. Peaks: 1=nitrite; 2=internal standard. CE conditions: untreated 57 cm×75 μm I.D. fused-silica capillary, hydrodynamic injection for 20 s, UV detector at 214 nm, −10 kV applied voltage.

4. Conclusions

In the present work, we established optimal and reproducible separation conditions for nitrite analysis in cytokine-treated PC12 cells using CE–UV. As shown by the results, our optimized method requires little effort in sample preparation and offers rapid analysis time and very precise quantification in measuring the production of nitrite in PC12 cells. Therefore, we believe that this method could be applied to quantify nitrite production in mammalian cells under various conditions.

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