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Modified method for the determination of capillary electrophoresis nitric oxide-correlated nitrate in tissue homogenates

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Abstract

A modified capillary electrophoretic method for the determination of nitric oxide correlated nitrate in several tissue homogenates is described in this study. The method was developed using a running buffer consisting of 200 m*M* lithium chloride and 10 m*M* borate buffer at pH 8.5, in a fused-silica column total 82 cm, effective 43 cm length and 75 μm I.D. The signal was measured at 214 nm and controlled current of 200 μA (equivalent to 12.7 kV) was applied in the reversed polarity direction. The sample was injected by vacuum pressure 50 ms (25 nl). In these conditions, bromide as internal standard and nitrate appeared at 7.2 and 8.9 min, respectively. Whole validation procedures were applied and satisfactory results were obtained. The nitrate levels of the tissue homogenates of control and L-NAME applied (heart, brain, kidney, stomach, lung, testis and liver) were monitored by the present method and it was decided that the method is precise and accurate.

Keywords: Capillary electrophoresis; Nitric oxide-correlated nitrate; Tissue homogenates

1. Introduction

Nitric oxide (NO) has been accepted as one of the important cellular mediators for almost 10 years. Since the endothelium-derived relaxing factor (EDRF) has been determined to be identical to NO, a lot of research is being performed to elucidate certain physiological or patho-physiological phenomena. To date, it has been demonstrated that NO is responsible for a number of physiological mechanisms [1–4].

NO is enzymatically synthesized from L-arginine by nitric oxide synthase (NOS) and this enzyme can be inhibited by nitro-L-arginine methyl ester (L-NAME). Three isoforms of this enzyme have been isolated such as inducible (iNOS), neuronal (nNOS) and endothelial (eNOS). The production of small quantity of NO by NOSs provides the tonus of the vessels, communications between cells and responses from very meaningful physiological mechanisms [5,6].

The chemistry of nitrogen atom is very interesting and complicated. The oxidation states of nitrogen can be at several values between +5 and −3, in the compounds. The valence of N atom in NO is +2 and it tends to increase its oxidation states [7,8]. Therefore, it is very unstable and has very short half-life to be oxidized to nitrite, especially in aqueous media. Nitrite is also an unstable compound and it is easily converted to the nitrate by super oxide radical that is

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present in water and blood, therefore, nitrate is the last product in the pathway of NO oxidation and it is highly stable in the aqueous solutions. Starting from this point of view, most of the researchers prefer to measure nitrate as a product of NO.

Certain studies have been attempted to monitor the real-time levels of NO in vivo systems. To achieve this, electroanalytical methods have been employed using microelectrodes [9–13]. Other methods have been reviewed [1] and the research carried out that was based on the measurement of the products of NO. Capillary electrophoresis and certain techniques are also employed to determine the level of nitrite and nitrate in urine, synovial fluid, plasma and several tissue homogenates [14–20].

The aim of the present study was to improve a new and modified CE method for the analysis of nitrate in the body fluids or homogenates and to compare it to the present methods.

The results were statistically evaluated and the application of the method was examined by measuring nitrate level in tissue homogenates. Validation of the method was achieved following the suggestions of SFSTP Commission [21] and an article review on the strategies and validation rules for capillary electrophoresis [22].

2. Experimental

2.1. Chemicals

All of the chemicals (Merck, Darmstadt, Germany) and acetonitrile were of analytical and HPLC grade (Carlo Erba, Rodano, Italy). Double distilled water was prepared in our laboratory using all glassware.

2.2. Apparatus

CE experiments were conducted using a SpectraPhoresis 100 system capillary electrophoresis equipped with a high-voltage power supply, a modular injector and a model of SpectraFOCUS scanning detector (Thermo Separation Products, CA, USA) cabled to a model Etacomp 486 DX4-100 computer processing the data using PC 1000 (version 2.6) working under OS/2 Warp Program (version 3.0). The analyses were realized in a 75 μm I.D. fused-silica column 82 cm total and 43 cm effective length (Phenomenex, CA, USA). The pH of the solutions were measured by employing a model Multiline P4 pH meter with a model SenTix glass electrode (WTW, Weilheim, Germany). All of the solutions including sample were degassed employing a B-220 model ultrasonic bath (Branson, CT, USA) and used after centrifugation.

2.3. CE experiments

The column was washed and conditioned by rinsing, in turn, 5 min each with 0.1 *M* NaOH, double distilled water and background electrolyte. Besides, 2-min washing with background electrolyte was made between each of the experiments.

In the present study, fused-silica capillary was filled with the background electrolyte consisting of 200 m*M* lithium chloride, 10 m*M* borate buffer at pH 8.5. All of the solutions were prepared in a nitrate-free double distilled water. The dilutions of nitrate were made from the stock solutions of 1.06×10^{-3} *M* KNO_3 and 4.39×10^{-4} *M* KBr as internal standard (I.S.). The final concentration of I.S. was always 2.92×10^{-4} *M* in the calibration and sample solutions.

The detection was made at 214 nm where monochromatic light is absorbed maximum by the related anions. The injection time was 50 ms (corresponds to almost 25 nl) using vacuum injection mode and reversed polarity controlled current of 200 μA corresponds to 12.7 kV was applied.

2.4. Physiological experiments

Twelve Spraque–Dawley male rats that have 200–250 g were employed to examine the performance of the method. The rats were anesthetized by uretan injecting 1.5 g kg^{-1} intraperitoneally and they were left spontaneous breathing because endotracheal entubation was not applied. Left femoral veins of the rats were canuleted by a PE-50 catheter. After the process 20 min was waited for the stabilization. To the member of the groups, serum physiologic without or with L-NAME (50 mg kg^{-1}) was applied to each rat and then they were killed by cervical dislocation and the tissues of heart, brain, kidney,

stomach, lung, testis and liver were obtained and they were kept in a deep freeze at −80°C. The tissues were homogenized in a 3-ml isotonic buffer solution at pH 7.4 and rest of the process was followed as explained under the topic of CE experiment.

3. Results and discussion

Quantitative measurements of released NO during the in vivo studies are very difficult due to the short half-life of mentioned compound. Since it is rapidly oxidized to nitrite and nitrate in body fluids or tissue homogenates, most studies have been achieved to measure nitrite and/or nitrate as markers of NO generation.

Nitrite and nitrate in biological material as indices of NO generation and the measurement of their levels are very complicated by several factors. Most significantly human and animals get these by dietary intake. Therefore, the researchers have to consider there would be an offset for the measurement of NO products in the organism.

3.1. Optimization of the method

Highly concentrated background electrolytes consisting of inorganic salts and buffer components have been tried to provide good resolution and short analysis time [14,15,17,19,20]. Since the ions move very fast, some researchers employed certain viscous materials to diminish the electrophoretic mobility of the system as well [15]. Actually, the ions are resolved very well from each other and no interference is observed, but in the use of NaCl, crystallization can be seen because of its low solubility in organic solvents during the experiments, especially in the sample solution containing acetonitrile. Therefore, the methods have been performed, previously modified by the combinations of their advantage parameters. The crystallization was eliminated by using LiCl because of more soluble in organic solvents.

Current-controlled system was employed to provide the electrical field in the reverse direction. This kind of application avoided waving of the signals, shifting of the peaks and variations at integration values. The applied current to the system as an electrical field was 2.44 μA cm^{-1} and it was corresponding to −12.7 kV. Thus, the Joule Heating was also diminished by applying low electrical field.

CE is very similar to chromatographic methods considering the analytical procedures. Therefore, I.S. should be used to provide the actual and reproducible results. Usually, bromide was used as an I.S. for the determination of nitrite and nitrate [15].

However, only one study has been monitored at 200 nm [17], all the other publications report that they have detected the ions at 214 nm [14–16,18–20]. Well-resolved and sharp peaks were obtained in the use of 200 m*M* LiCl and 10 m*M* borate buffer at pH 8.5, detecting at 214 nm in our study. High efficient separation was observed with their 572 000 plates m^{-1}. The peaks were rather symmetrical with 0.97 and 1.08 CFR asymmetry factor and they appeared at the migration times of ($t_M \pm$RSD%) 7.2±1.1 min and 8.9±1.01 min for bromide and nitrate, respectively. The reproducibility of the t_M of nitrate and bromide seemed very high. It can be attributed to the success of optimization and conditioning of the capillary.

The electropherogram of standard bromide and nitrate is given in Fig. 1.

The statistical evaluation of integrated area of 7.1×10^{-5} *M* nitrate and 2.92×10^{-4} *M* bromide was made by three procedures to investigate the repeatability of peak areas using: (a) only the ratio values of nitrate and bromide (I.S. no P.N.); (b) only peak normalization values of nitrate (P.N. no I.S.); (c) the ratio of peak normalization values nitrate to I.S. (I.S. and P.N.) as evaluated elsewhere [21,22]. The brief statistical results and relevant information are given in Table 1.

It is concluded that the employment of the ratio of peak area normalization values (I.S. and P.N.) provide better results comparing to the others. Therefore, same procedure was used for all quantification studies.

The selectivity of the method was calculated as $\alpha=1.17$ and the resolution values were 9.7 and 3.2 for the adjacent peaks of bromide and nitrate. These show that the method could be employed for the analysis of nitrate, confidently.

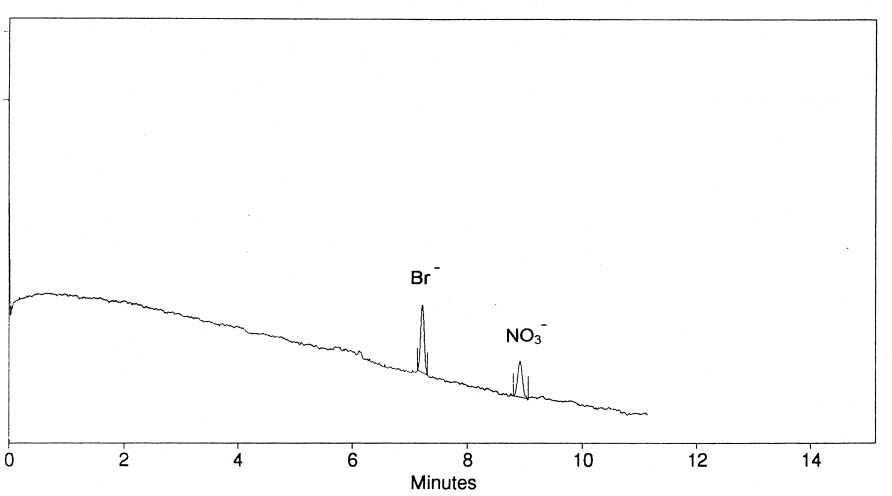

Fig. 1. Electropherograms of bromide and nitrate. Conditions: 200 m*M* LiCl and 10 m*M* borate (pH 8.5); injection, hydrodinamically 0.5 s; applied voltage. −12.7 kV; capillary, uncoated fused-silica, 75 μm I.D., 82-cm total and 43-cm effective length; detection wavelength, 214 nm. Sample concentration 2.92×10^{-4} *M* bromide and 2.82×10^{-5} *M* nitrate in acetonitrile–water (1:2, v/v).

3.2. Validation of the method

In these conditions, the linearization studies were performed in the range of 1.41×10^{-5}–7.05×10^{-5} *M* nitrate and fix concentration of 2.92×10^{-4} *M* bromide. The calibration equations were computed considering the data of inter-day and intra-day studies using the ratio values of normalized nitrate and bromide areas. Statistical evaluation of the calibration studies was tried to validate the method. The related results covering accuracy of the method in the range mentioned and mean recoveries are at the level of 95% confidence interval are in Table 2.

The limit of the detection (LOD) as concentration was calculated to be 5.70×10^{-7} *M* according to the criteria of the division of 3.3 times of standard deviation of intercept to the slope of the calibration curve. The limit of quantification (LOQ) was computed as 1.73×10^{-6} *M* via the division of 10 times of standard deviation of intercept to slope of the calibration curve [23]. The results obtained above indicate that it has a good precision with the analytical points of view and they are almost identical as those of some studies [19,20].

The precision of the method was computed by measuring repeatability and time different intermediate precision. These were achieved by at the three concentration levels doing the experiments 3 days. The RSD values were estimated from repeatability and time-different intermediate precision variances, respectively. Higher RSD values were observed at higher concentration. The precision of the method at various concentrations is demonstrated in Table 3.

Table 1
Precision of peak areas (RSD%) of 7.05×10^{-5} *M* nitrate and 4.39×10^{-4} *M* bromide[a]

	Repeatability %	Intermediate precision %
I.S.	2.46	7.49
P.N.	7.87	7.87
I.S. and P.N.	0.08	6.18

[a] *k* (day)=3; *n* (no of experiments)=6.

Table 2
Results of inter-day and intra-day calibration studies for linearity and accuracy of the method

Regression parameters	
Linearity	
r^2	0.9986; 0.9974; 0.9978
Intercept (mean±SD, *n*=3)	0.048±0.006
Slope (mean±SD, *n*=3)	16405±584
Accuracy	mean recovery±CL (%)
50%	100.5±13.1
100%	101.3±8.2
150%	100.6±3.4

Table 3
Precision of the method at various concentrations

Concentration	Repeatability (k=3; n=6, RSD%)
(50%) 1.41×10^{-5} M	0.1495
(100%) 4.23×10^{-5} M	0.2336
(150%) 7.05×10^{-5} M	0.0833
ConcentrationIntermediate precision (k=3; n=6, RSD%)	
(50%) 1.41×10^{-5} M	0.1495
(100%) 4.23×10^{-5} M	0.2336
(150%) 7.05×10^{-5} M	6.1873

Nitrite also appeared in the electropherogram as in Fig. 2. As is known, nitrite is very unstable in the aqueous solutions and it changes to nitrate with a rate constant [16]. Thus, it is obviously that the measurement of nitrite level only gives a respective value, even if it is detected. It has not already appeared in our samples as demonstrated in Fig. 3, and this result confirms the selectivity, as well.

Since the background electrolyte is highly concentrated to provide the high electrophoretic mobility, the sample stacking may also be observed for such studies. It is reported that stacking provides 10–100 times higher sensitivity comparing to common CZE. Salt stacking normally provides to concentrate the solutes before electrophoresis but there may be some problems if organic solvent is used for the precipitation of proteins, e.g., in the use of acetonitrile, there may be crystallization in the capillary. To avoid from the crystallization, a salt of lithium chloride that is more soluble in acetonitrile was made composed to the background electrolyte. Thus, both stacking and non-crystallization was provided at the same running buffer system. In the system we used, the ions move to the detector side by electrophoretic effect. These show that the sample bias is not a problem when analyzing the ions in the background electrolyte employed.

3.3. Application of the method to the tissue homogenates

CE techniques are very appropriate for the biological analysis. During the processes, only precipitation of proteins is realized. A group of chemicals is used for the precipitation of proteins for the material of because proteins usually cover the peak of the compounds to be detected. Acids can not be available in the use of borate buffered background electrolyte system and sample containing acids can cause the crystallization of tetraborate in the capillary column. The best precipitation agents are acetonitrile or ethanol for the CE analysis. Acetonitrile was used for precipitating agent of proteins in this study. Besides, acetonitrile makes the peaks sharp and symmetrical and decrease the electroosmotic mobility as well.

The applicability of the method was tested using

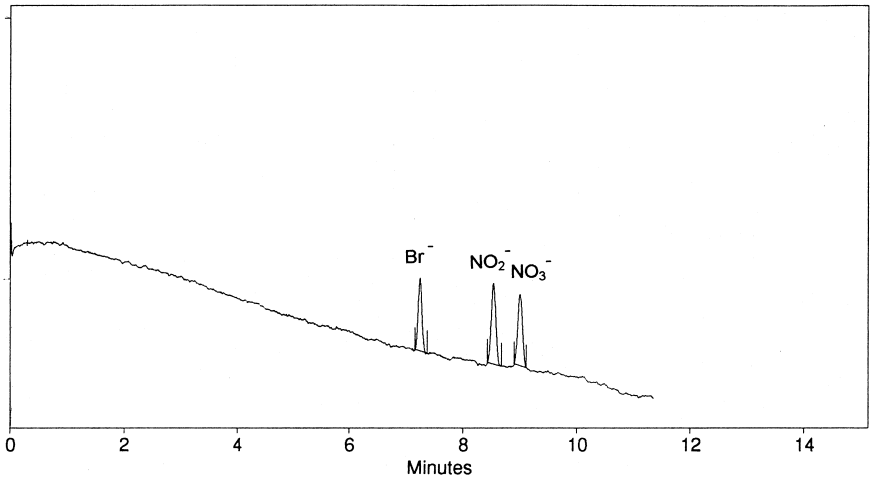

Fig. 2. Electropherograms of bromide (2.92×10^{-4} M), nitrite (8.88×10^{-5} M) and nitrate (5.64×10^{-5} M). Other conditions as described in Fig. 1.

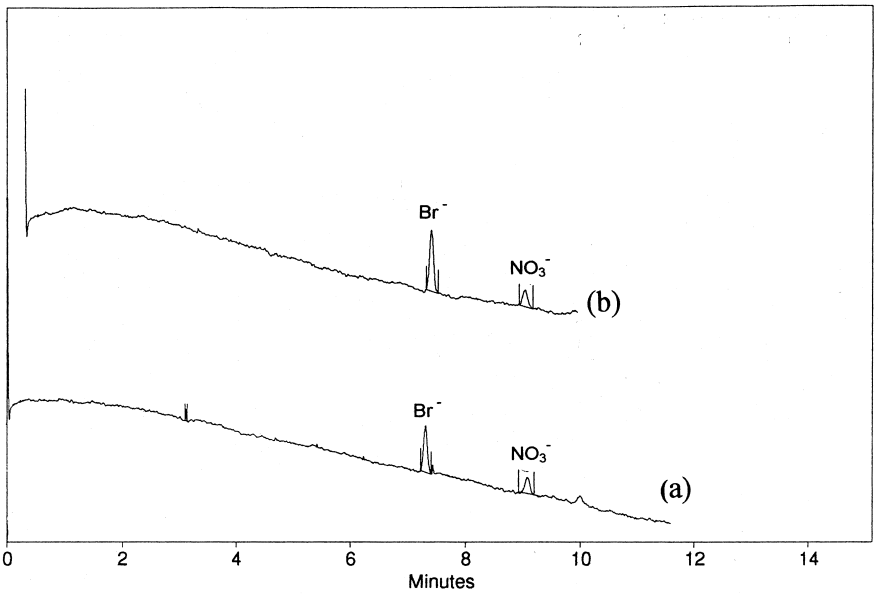

Fig. 3. Electropherograms of stomach tissue homogenates of rat (a) control group and (b) L-NAME applied group. Other conditions as described in Fig. 1.

certain tissue homogenates of rats such as kidney, brain, heart, stomach, lung, testis and liver. The groups were consisting of control and group to which is applied 50 mg kg^{-1} L-NAME. Any interference is not observed during the analysis of tissue homogenates at all. Although, the t_M values of the standard bromide and nitrate shift a little due to the conditioning of the capillary surface. Therefore, the integration values of the peaks needed to be corrected as presented in the validation processes. The original electropherogram of stomach tissue homogenate of rat were demonstrated in Fig. 3a control group and 3b L-NAME applied group. The level of nitrate was measured as the details given in the experimental sections. The brief results for the tissues are demonstrated in Table 4.

Thus, the applicability of the method is presented in this way. Some chemicals or agents may cause the increase in the level of NO when they were injected into the body such as sodium nitroferricyanide, certain vasodilators and sildenafil citrate that it is used for the treatment of impotence. On the contrary, L-NAME decreases NO production depending on the NOS inhibition [3]. This application was achieved to investigate whether the method was effected by any factor in biological studies. As is seen from the results, L-NAME significantly causes decrease of nitrate levels, which corresponds to NO production.

In conclusion, the method is worthwhile for the analysis of nitrate especially in the body fluids and tissue homogenates. It is precise, accurate, cheap and a single analysis takes about 15 min. All of the points mentioned above exhibit the advantageous of the study.

Table 4
Brief results of nitrate levels±SD in the relevant tissue homogenates of rats as nmol g^{-1} wet weight of tissue (n=3 for each)

Tissue	Nitrate level (mean±SD) (nmol g^{-1}, w/w)		Rate of decrease (%)
	Control	L-NAME	
Kidney	21.8± 6.0	16.0± 5.5	26.8
Brain	20.4±4.6	17.6±8.9	13.7
Heart	19.3±5.1	9.2±9.2	52.4
Stomach	43.1±15.1	12.8± 7.7	70.3
Lung	50.7±26.1	8.8±6.6	82.6
Testis	16.4±2.7	4.2±2.6	74.5
Liver	17.7±6.5	7.5±2.4	57.4

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