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Short Communication

Rapid and simple method for the determination of nitrite in synaptosomal superfusates by liquid chromatography with electrochemical detection

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ABSTRACT

A rapid and simple technique using reversed-phase high-performance liquid chromatography with electrochemical detection (HPLC–ED) was developed for the determination of nitrite. Standard solutions of sodium nitrite produced a linear current response over the concentration range 1.5–30 μM. The chromatographic peaks of sodium nitrite were superimposable with peaks obtained after injection into the chromatograph of supernatants from rat cerebellar synaptosomes. The concentrations of nitrite in synaptosomes measured with HPLC–ED were similar to those found with a widely used colorimetric procedure. Therefore, this new method may represent a useful means of measuring nitrite in biological samples.

INTRODUCTION

There is increasing evidence that nitric oxide (NO) is a chemical messenger that plays a role in the control of a series of physiological processes such as smooth muscle relaxation, platelet inhibition, immune regulation, neurotransmission and penile erection [1]. The synthesis of NO occurs in cells through the action of the nitric oxide synthase (NOS), a calcium-dependent enzyme that utilizes L-arginine as a substrate and requires tetrahydrobiopterin and nicotinamide-adenine dinucleotide phosphate (NADPH) [2]. The lifespan of NO is very short, in that it spontaneously and rapidly breaks down to nitrite (NO_2^-) and nitrate (NO_3^-) [3]. One of the approaches in studying the metabolism of NO in tissues is the evaluation of NO_2^-/NO_3^- in biological fluids. Griess's colorimetric method has been extensively used for the measurement of nitrite in cell cultures [4], laboratory animals and humans [5,6]. Moreover, several groups have reported the use of high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection for the determination of nitrite and nitrate in standard solutions [7] and in cured meats [8–10].

Since the transition $NO \rightarrow NO_2^- \rightarrow NO_3^-$ is a reversible electrochemical reaction, in this investigation we decided to use an electrochemical

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detector to measure NO_2^- concentrations, both in standard solutions and in supernatants from rat cerebellar synaptosomes. The separation of nitrite was obtained by using a co-poly(styrene–divinylbenzene) reversed-phase column. The NO_2^- levels measured by HPLC in the analysed samples were compared with data obtained by means of a colorimetric procedure using Griess reagent as described by Green *et al.* [11].

EXPERIMENTAL

Chemicals and reagents

Sulphanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, L-arginine, N-methyl-D-aspartic acid (NMDA), sucrose and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA); sodium nitrite ($NaNO_2$), HPLC-grade acetonitrile, 1-heptanesulphonic acid sodium salt and phosphoric acid (H_3PO_4) were from Carlo Erba (Milan, Italy); dihydronicotinamide-adenine dinucleotide phosphate tetrasodium salt (NADPH-Na_4) and glucose were from Merck (Darmstadt, Germany). All other reagents were of analytical reagent grade.

Equipment

The chromatographic analyses were carried out on a Kontron (Milan, Italy) HPLC computer-assisted system. The acquisition and the analysis of the data were performed by a Kontron Data System 450 multitasking computer program. The system was equipped with a Rheodyne 7125 injection valve with a fixed 20-μl sample loop (Cotati, CA, USA). The analytical column was a reversed-phase co-poly(styrene–divinylbenzene) (PLRP-S) (5 μm particle size, 150 mm × 4.6 mm I.D.) column from Polymer Labs. (Church Stretton, UK) equipped with a LiChrosorb RP-8 guard column (7 μm particle size, 30 mm × 4 mm I.D.) from Merck. The analytical column was connected to a Model 5100A Coulochem detector with a Model 5010 analytical cell from ESA (Bedford, MA, USA). The potential of the porous graphite electrode was maintained at +400 mV, relative to a H_2/H^+ ion couple reference electrode.

The colorimetric measurements were carried out by means of an LKB UV–VIS 4050 spectrophotometer (Biochrom, Cambridge, UK).

Mobile phase

Sodium dihydrogenphosphate solution (5 m*M*, pH 3.3 ± 0.03) containing 0.15% 1-heptanesulphonic acid and 0.1% acetonitrile was prepared from analytical-grade reagents. The flow-rate was 1.0 ml/min. The solution was pumped at room temperature during chromatography.

Sample preparation

Synaptosomes were prepared from the cerebella of male Sprague–Dawley CD-COBS rats (weighing 300–350 g) by means of ultracentrifugation of crude synaptosomal fractions of tissue homogenates in a sucrose gradient density, as described by Erin *et al.* [12]. This work was performed according to the "Guidelines for use of animals in biomedical research" [13]. The concentration of membrane protein was determined by means of Bio-Rad reagent using the BSA solution as a standard. To activate the L-arginine NO production in synaptosomes, the membranes (1 mg of protein per ml) were incubated in Krebs–Ringer solution (pH 7.4) for 1 h in the presence of 0.5 m*M* NADPH, 100 μM L-arginine and 100 μM NMDA [14]. The reaction was stopped by adding a concentrated sodium hydroxide solution up to a final pH of 9.0, which is optimal for hydrolysing S-nitrosothiols [15]. The proteins were precipitated and separated by centrifugation at 12 000 *g* for 30 min at 4°C in an ALC 4224 centrifuge (Milan, Italy). Aliquots of the supernatant (20 μl) were injected into the chromatograph for analysis. To assay NO_2^- by the Griess reaction, 500-μl aliquots of the supernatant were incubated with 500 μl of 1% sulphanilamide and 500 μl of 1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4 at room temperature for 5 min. Absorbance at 543 nm was measured. The standard solutions containing 1.5–30 μM sodium nitrite were prepared in modified Krebs–Ringer buffer (138 m*M* NaCl, 11 m*M* $NaHCO_3$, 5 m*M* KCl, 1 m*M* CaCl, 1 m*M* $MgCl_2$, 11 m*M* glucose, pH 9.0).

The standard solutions were injected into the HPLC system at a constant volume of 20 μl and at increasing concentrations. The detector response (nA), calculated by automatically measuring the height of the chromatographic peaks, was used to construct the calibration regression line. The calculation of nitrite concentration in the supernatant of the synaptosomes was made by using a linear regression program running on a Macintosh computer.

RESULTS AND DISCUSSION

The chromatograms of standard solutions containing increasing concentrations of sodium nitrite are shown in Fig. 1. The potential of the electro-

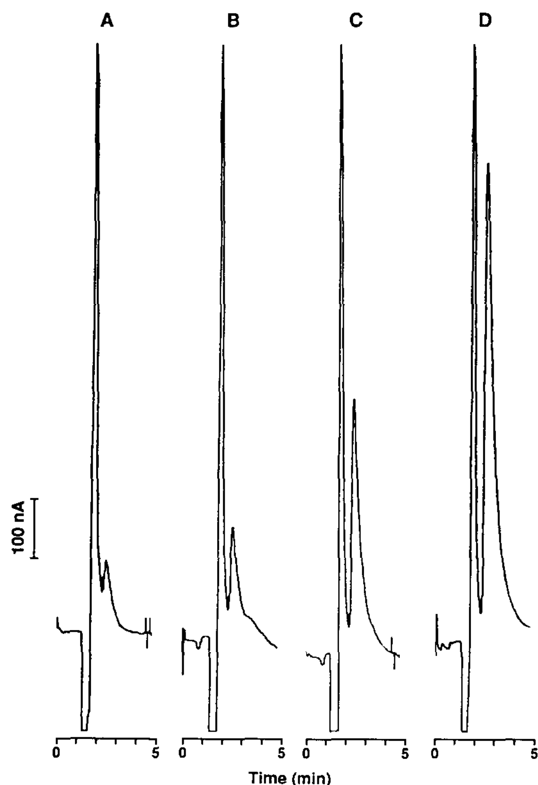

Fig. 1. Chromatograms of sodium nitrite standard solutions obtained by HPLC–ED. Sodium nitrite concentrations: A, 3 μ*M*; B, 5 μ*M*; C, 15 μ*M*; D, 30 μ*M*. Chromatographic conditions: reversed-phase PLRP-S column, 5 μm particle size, 150 mm × 4.6 mm I.D. Mobile phase: sodium dihydrogenphosphate (5 m*M*)–1-heptanesulphonic acid (0.15%)–acetonitrile (0.1%), pH 3.33. Flow-rate: 1.0 ml/min. Electrochemical detection: +400 mV. Injection volume: 20 μl.

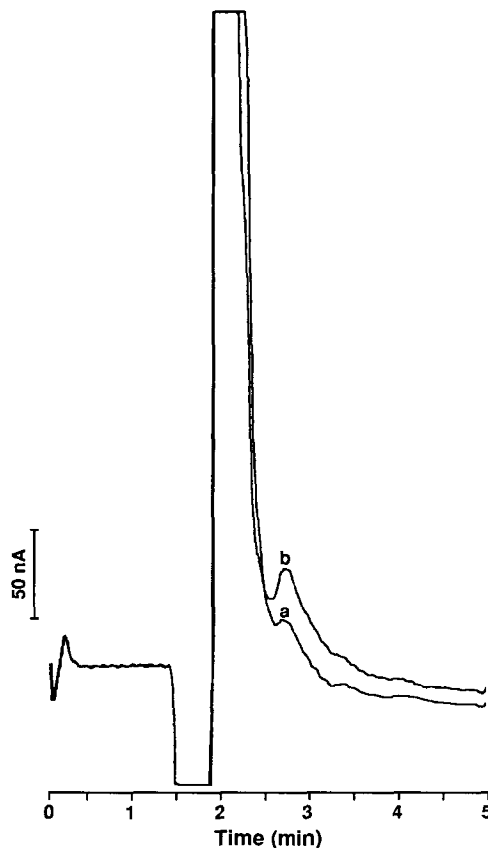

Fig. 2. Separation of nitrite in supernatants of cerebellar synaptosomes. Overlay plot of chromatograms obtained by HPLC–ED: (a) control, (b) NMDA-treated synaptosomes. All synaptosomal preparations were incubated with 0.5 m*M* NADPH and 0.1 m*M* L-arginine. The chromatographic conditions were as described in Fig. 1.

chemical detector was set at +400 mV, which gave the maximal current response (not shown). With the present chromatographic procedure sodium nitrite had a retention time of 2.53 ± 0.03 min (mean ± S.D.) ($n = 6$). The regression of the detector response (measured as current response) *versus* concentration of the standard was linear over the 1.5–30 μ*M* range, the correlation coefficient being 0.999 (not shown). The injection of Krebs–Ringer solution, used as a blank, did not produce any detectable signal (not shown). Fig. 2 shows the chromatograms obtained after injection into the HPLC system of the supernatants of cerebellar synaptosomes incubated in the absence

TABLE I

COMPARISON OF CHROMATOGRAPHIC AND COLORIMETRIC METHODS FOR DETERMINATION OF NITRITE IN THE SUPERNATANT OF CEREBELLAR SYNAPTOSOMAL MEMBRANES

Synaptosomes were incubated in Krebs–Ringer solution containing 0.5 m*M* NADPH and 0.1 m*M* L-arginine in the absence (saline) or in the presence of NMDA for 1 h. The number of replications varied from 3 to 10.

Treatment	Nitrite concentration (mean ± S.D.) (μmol/mg/ml)	
	HPLC	Colorimetry
Saline	1.41 ± 0.39	1.77 ± 0.16
NMDA	2.85 ± 0.47^{a}	2.44 ± 0.15^{a}

[a] $p < 0.01$ compared with saline, Student's *t*-test.

and in the presence of NMDA, which caused a significant increase in the concentration of nitrite (Table I). The retention time of nitrite in the analysed samples (2.6 ± 0.1; mean ± S.D.) was similar to that measured for nitrite in the standard solution. The molar concentration of nitrite in cerebellar synaptosomal preparations measured by HPLC was similar to the values calculated using the colorimetric procedure (Table I).

Ion-pair reversed-phase HPLC coupled with electrochemical detection was shown by Lookabaugh and Krull [16] to be a useful technique for the analysis of nitrite in food. These authors used a positively charged ion-pair agent, and the pH of mobile phase was 6.8. However, pH 6.8 is known to be optimal for nitrite binding to the thiol groups of proteins to form S-nitrosothiols, which leads to a decrease in nitrite quantity in the samples [15]. To prevent S-nitrosothiol formation, we used a mobile phase with pH less than 4.0 and found new conditions for nitrite separation using a PLRP-S reversed-phase column. This method of determination, which is simple, fast and reliable, can be used for the study of NO metabolism in various biological systems.

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