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Simultaneous determination of nitrite and nitrate anions in plasma, urine and cell culture supernatants by high-performance liquid chromatography with post-column reactions

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Abstract

A high-performance liquid chromatographic method for the determination of nitrite and nitrate anions derived from nitric oxide in biological fluids is presented. After separation on a strong anion-exchange column (Spherisorb SAX, 250×4.6 mm I.D., 5 μm), two on-line post-column reactions occur. The first involves nitrate reduction to nitrite on a copper-plated cadmium-filled column. In the second, the diazotization-coupling reaction between nitrite and the Griess reagent (0.05% naphtylethylendiamine dihydrochloride plus 0.5% sulphanilamide in 5% phosphoric acid) takes place, and the absorbance of the chromophore is read at 540 nm. This methodology was applied to biological fluids. Before injection into the chromatographic system, the samples were diluted and submitted to suitable clean-up procedures (urine and cell culture supernatant samples are passed through C_{18} cartridges, and serum samples were deproteinized by ultrafiltration through membranes with a molecular mass cut-off of 3000). The method has a sensitivity of 30 pmol for both anions, as little as 0.05–0.1 ml sample volume is required and linearity is observed up to 60 nmol for each anion.

Keywords: Nitrite; Nitrate; Nitric oxide

1. Introduction

Nitric oxide (NO) is one of the endothelium derived relaxing factors (EDRF [1,2]). It is enzymically produced from the amino acid L-arginine [3] by a group of enzymes known as NO-synthases [4] present in a heterogeneous group of cells and tissues including endothelium [2,5], circulating monocytes and neutrophils [6] and neurons [7]. NO plays an important role as a modulator of several physiological (maintenance of vascular tonus [8], inhibition of platelet [9] and leukocyte [10] adhesion) and pathological (hypertension [11], septic shock [12]) events. NO is a free radical which is rapidly oxidized to nitrite and nitrate anions in oxygen-containing aqueous media [14] or in the presence of superoxide anions [13]. Nitrite and/or nitrate concentrations have been successfully correlated with NO release in some in vitro experiments [7]. However, such a correlation has not yet been definitely established in vivo. NO is released in the picomolar to nanomolar range depending on the biological system involved. For this reason, the quantitation of NO or its

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breakdown products requires highly specific and sensitive analytical procedures.

The most commonly employed method for the quantitation of nitrite (and nitrate after reduction) is the Griess reaction [15]. This spectrophotometric assay is based on the electrophilic attack by NO (released from nitrite in acid medium) of the NH_2 group of an aromatic amine with the posterior coupling of this intermediate to a nucleophilic aromatic compound, thereby giving rise to a resonant structure with a high molar absorption coefficient at 530–550 nm. However, this reaction is easily affected by interferences commonly found in biological matrices.

Extensive literature on chromatographic methods for nitrite and/or nitrate quantification is available. Most methods have been applied to the analysis of natural waters and foodstuffs.

Nitrite determination by pre-column derivatisation and posterior separation by either gas chromatography with electron capture detection [16,17] or reversed-phase high-performance liquid chromatography (HPLC) [18] have been reported for the analysis of natural waters. Other methods such as reversed-phase ion pair HPLC [19–22] and ion chromatography [23,24] have also been successfully applied to these matrices. In these HPLC methods, the detection of the species is made by conductivity, UV absorbance and even fluorescence [25]. Nitrite and/or nitrate anions have also been determined in foodstuffs by gas chromatography [26,27] and ion-exchange HPLC [28–35]. Nitrite and/or nitrate are generally present at high concentrations in natural waters and foodstuffs: thus, these methods do not offer the required sensitivity to study the L-arginine/NO pathway.

All published gas chromatographic methods for the analysis of mammalian body fluids (such as milk, urine and saliva), although sensitive, are only applicable to the quantitation of the nitrite anion [36–40] (or both nitrite and nitrate after proper reduction). Many HPLC methods applied to these biological matrices do not permit simultaneous quantification of both anions [41–44]. This makes these methods unsuitable for studying EDRF/NO metabolism in vivo.

Simultaneous nitrite and nitrate determinations by HPLC have been applied to cell extract [45], serum [46–48], plasma [49], ocular fluid [46,47], gastric juice and urine [50] samples. The separation of the ions is achieved by either reversed-phase ion pair [48,49] or by ion-exchange chromatography [45–47,50], and the detection is made by either direct UV absorption [45,48,49] or conductimetry [46,47,50]. Despite the good sensitivity shown by some of these methods, the samples require several purification steps before injection into the chromatograph, in order to obtain good separation, and to eliminate interfering ions commonly found in these biological matrices at very high concentrations (such as chloride, bicarbonate and phosphate).

In this paper, we describe the development of an HPLC method which allows the separation of nitrate and nitrite anions and their specific quantification. The post-column reactions involve: (1) the reduction of nitrate to nitrite on a copper-plated cadmium powder-filled column, and (2) the formation of the diazo-compound after reaction with the Griess reagent. All these steps are carried out on-line and peak absorbances are read at 540 nm. The method is illustrated with data obtained from urine, serum and cultured macrophage supernatant samples.

2. Experimental

2.1. Solutions used for the diazotization-coupling reaction

All reagents employed were of analytical grade or higher purity, and the aqueous solutions were prepared with HPLC grade water (resistivity 18.2 MΩ/cm, Milli Q Plus, Millipore-Waters, Bedford, MA, USA). The Griess reagent consisted of a mixture of equal volumes of a 0.1% naphtylethylendiamine dihydrochloride in 5% phosphoric acid solution and 1% sulphanilamide (Sigma, St. Louis, MO, USA) in 5% phosphoric acid solution. This mixture was prepared immediately before the reaction. The solution used for conditioning the copper-plated cadmium column (referred to as "carrier solution") contained 100 g ammonium chloride (Reagen, Rio de Janeiro, RJ, Brazil), 20 g sodium tetraborate decahydrate (Merck, Rio de Janeiro, RJ, Brazil), 1 g Na_2EDTA (Sigma) and 0.2 g cupric sulphate pentahydrated (BDH, Poole, UK) added to 1000 ml water.

Cupric sulphate was excluded from this solution when batchwise NO_3^- reduction was performed (spectrophotometric method).

2.2. Preparation of the nitrate-reducing column

A 15-g weight of cadmium powder (300–340 mesh; Aldrich, Milwaukee, WI, USA) were suspended in 100 ml water and 600 ml 0.5% pentahydrated cupric sulphate was added under continuous stirring. The mixture was passed through filter paper (type Whatman No. 3) and the copper-plated cadmium precipitate was washed three times with water, once with 1 *M* hydrochloric acid and finally with water. After each wash the supernatant was decanted and discarded. The copper-plated cadmium precipitate was then resuspended in 50 ml carrier solution (Cu^{2+}-free). This suspension was employed in those experiments where nitrate reduction was performed batchwise.

The HPLC column (100×4.6 mm I.D.) was manually filled with the copper-plated cadmium (Cd/Cu) suspension and then connected to an HPLC pump. The carrier solution was pumped at 0.7 ml/min until the pressure remained stable (<1 bar).

2.3. Instrumentation

The chromatographic system consisted of a pump (ABI 400, Applied Biosystems, Foster City, CA, USA; P1 in Fig. 1) pumping the mobile phase (0.06 *M* NH_4Cl, pH 2.8, flow-rate 0.7 ml/min) to separate the anions on a strong anion-exchange column (functional group quaternary ammonium, particle size 5 μm; Spherisorb SAX, 250×4.6 mm I.D., Sigma). The exit of this column was connected to a zero dead-volume tee (Supelco, Bellefonte, PA, USA) where the effluent was mixed with the carrier solution to be delivered at a flow-rate of 0.7 ml/min by a second pump (LKB Bromma, Model 2150, Pharmacia-LKB, Uppsala, Sweden, P2 in Fig. 1). This mixture passed through the Cd/Cu column, and the effluent of this last column was combined with the Griess reagent in a second tee connector.The Griess reagent was in turn delivered by a third pump (ABI 400, Applied Biosystems) at a flow-rate of 0.7 ml/min. The effluent of this tee passed through a reactor (Teflon tubing, 30 cm×0.5 mm I.D., filled with acid washed 250 mm beads, Supelco) and a delay tube (300 cm×0.5 mm I.D., Supelco). The delay tube allows the contact between the Cd/Cu column effluent and the Griess reagent for an additional period of 17 s before entering the detector. The absorbance of the final mixture was monitored at 540 nm by a UV–Vis photodiode array detector (1000 S, Applied Biosystems) with the slit width set at 25 nm. The signal output was recorded either by a serial printer (Panasonic Model KX P1180, Osaka, Japan) or by a computerized integrator (CI 4000, Milton-Roy, Riviera Beach, FL, USA). The samples were manually injected through an injector valve (Rheodyne 7125, Rheodyne, Cotati, CA, USA) equipped with a 2000-μl loop. Fig. 1 shows the connections of the complete system.

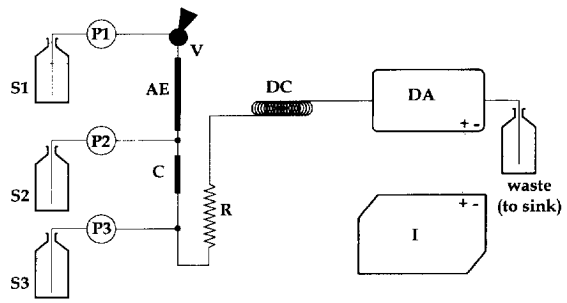

Fig. 1. Schematic representation of the HPLC system used for the simultaneous analysis of nitrite and nitrate. P1 = pump for the ion-exchange column mobile phase; P2 = pump for the Cd/Cu column carrier solution; P3 = pump for the Griess reagent; S1 = ion-exchange column mobile phase; S2 = Cd/Cu column carrier solution; S3 = Griess reagent; V = injection valve; AE = ion-exchange column; C = Cd/Cu reducing column; R = reactor; DC = delay tube coil; DA = photodiode array detector; I = integrator (printer).

Aqueous $KNO_3 + KNO_2$ standards were directly injected into the system (volume 400 μl). Biological samples were subjected to purification before injection. Urine and cell culture supernatant samples were diluted six-fold with water, passed through an octadecyl silica column [50] (Bond Elut C_{18}, Analytichem International, Harbor City, CA, USA) and 400 μl of the effluent were injected into the chromatograph. Plasma samples were diluted six-fold with water and deproteinized by ultrafiltration across a 3 kDa cut-off membrane using an Amicon Micro-ultrafiltration System (Amicon, Beverly, MA,

USA). Four hundred microliters of the ultrafiltrate were injected into the chromatographic system.

2.4. Spectrophotometric determination of nitrite and nitrate in urine samples

For nitrite quantification, aqueous KNO_2 standards (Sigma, concentration range 0.9–56.5 μM) or urine samples were diluted 1:2 with water and passed through octadecyl silica columns. To a 1-ml volume of the eluates, 500 μl of the Griess reagent were added. The absorbances were read at 540 nm in a Uvikon 810 spectrophotometer (Kontron, Schlieren, Switzerland) and corrected for any non-specific absorbance caused by the reagents and samples. Urine nitrite concentrations were calculated from the calibration curve obtained by linear regression between the nitrite concentration and the absorbance of the aqueous standards. For nitrate quantification, aqueous KNO_3 standards (Sigma, concentration range 1.3–81.9 μM) or urine samples were diluted 1:2 with the Cu^{2+}-free carrier solution and passed through octadecyl silica columns. A 100-μl volume of Cd/Cu suspension in Cu^{2+}-free carrier solution was added to a 1-ml volume of the eluates and incubated at room temperature for 15 min. The tubes were then centrifuged (2000 *g*, 10 min), the supernatants separated and mixed with 500 μl of the Griess reagent, and the absorbances then were read at 540 nm as above. The total nitrate plus nitrite concentrations were calculated from the calibration curve obtained by linear regression. Urine nitrate concentrations were calculated by subtraction of the nitrite values.

2.5. Biological samples

2.5.1. Rat urine

Twenty-four hour rat urine samples were collected from animals with diabetes, with 5/6 of renal ablation, or which had been sham-operated. After centrifugation (2000 *g*, 10 min) to remove solid matter, the samples were stored at −20°C until analysed.

2.5.2. Rat serum

Venous blood samples were obtained from non-treated or chronically L-NAME-treated rats (60 mg/kg/day, p.o., for 7 days; L-NAME is the competitive NO-synthase inhibitor N^{ω}-nitro-L-arginine methyl ester, Sigma). After allowing the blood to clot at room temperature, the sera were separated by centrifugation (2000 *g*, 10 min) and stored at −20°C until analysed.

2.5.3. Murine macrophage culture supernatants

Balb/c mice received either saline or a suspension of *Mycobacterium bovis* (BCG; i.p.). Seven days after treatment, the animals were sacrificed and peritoneal exudate cells were obtained according to the method described by Takema et al. [51]. Adherent macrophage monolayers devoid of non-adherent cells were prepared by incubating peritoneal exudate cells in 96-well microculture plates at a density of 1×10^6/well for 2 h at 37°C. The macrophages were cultured in fresh RPMI medium for 24 h in a humidified CO_2/air atmosphere at 37°C. After this period, the supernatants were separated and kept at −20°C until analysed. In these samples, only nitrite concentrations were determined since the cell culture medium contains 1 mM $Ca(NO_3)_2$.

3. Results and discussion

A schematic representation of the HPLC system is shown in Fig. 1. Fig. 2 shows that using the mobile phase described above the nitrite and nitrate peaks were successfully resolved from pure salt aqueous solutions (panel a) and from urine (panel b), cell supernatant (panels c and d) and plasma (panels e and f) samples. Under these conditions, the retention times for NO_2^- and NO_3^- were 9.8±0.5 min and 13.2±0.5 min, respectively.

The choice of the anion in the mobile phase (Cl^-) was based on its small size, its monovalent charge and its inability to be polarized. This results in a weaker interaction with the highly cationic groups of the stationary phase than would otherwise occur with the highly polarized and hydrated NO_2^- and NO_3^-. NH_4Cl concentrations higher than 0.06 M resulted in poor resolution of these peaks.

Low pH values are mandatory for a differential interaction between the anions and the here employed stationary phase. The weak nitrous acid (HNO_2, pK_a 5.2) is always in equilibrium with NO_2^-,

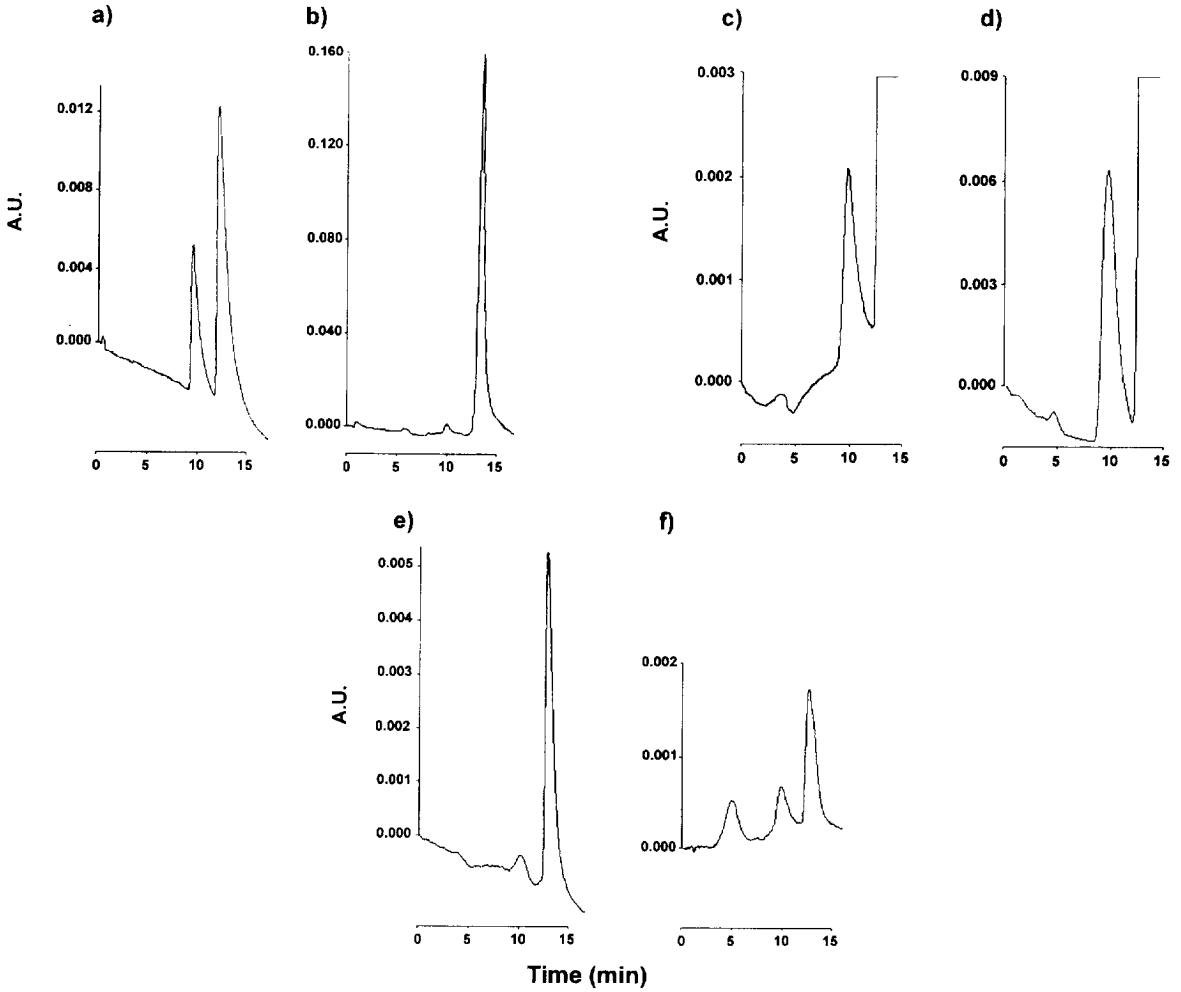

Fig. 2. Typical chromatograms obtained after the injection of 400 μl aqueous potassium nitrite (4.08 μ*M*) and nitrate (6.86 μ*M*) solutions (panel a), six-fold diluted rat urine (2.9 μ*M* nitrite, 147.8 μ*M* nitrate; (panel b), six-fold diluted supernatants from 24-h murine peritoneal macrophage culture obtained seven days after an intraperitoneal injection of 100 μl of saline solution (panel c: 9.0 μ*M* nitrite) or BCG suspension (panel d: 38.8 μ*M* nitrite), and six-fold diluted rat sera obtained after seven days of treatment with water (panel e: 0.8 μ*M* nitrite, 14.4 μ*M* nitrate) or 60 mg/kg/day of L-NAME (panel f: 0.5 μ*M* nitrite, 5.8 μ*M* nitrate, 19.1 μ*M* L-NA).

but as the pH decreases it becomes the predominant species. As a result, the interaction with the column is weaker and the NO_2^- retention time is shortened with respect to NO_3^-. This behaviour was observed at pH values below 3.0. However, given the good separation observed and in order to avoid exposing the analytical column to extremely acid conditions, we routinely employed a pH of 2.8.

Most authors who use Cd/Cu to reduce NO_3^- to NO_2^- in flow injection analysis (FIA) employ Cd filings with a larger particle size (20–40 mesh) than those of Cd powder [52–54]. Such filings can be successfully Cu-plated using $CuSO_4 \cdot 5H_2O$ concentrations in the range of 2–5%. Under our conditions, the use of these concentrations led to the quantitative reduction of Cu^{2+} to metallic Cu, as evidenced by the colour change and the loss of reducing activity towards NO_3-. These difficulties were overcome when $CuSO_4.5H_2O$ was used at a concentration of 0.5%. The advantage of Cd powder over Cd filings is the finer adjustment of the granulometry and the higher purity (≥99%).

The Cu^{2+}-free carrier solution for the Cd/Cu reagent was based on that suggested by Stainton [55]. Although the Cd/Cu reagent showed 100% efficiency when tested batchwise, this efficiency fell dramatically when employed in the whole system on line. The addition of 0.02% $CuSO_4 \cdot 5H_2O$ to the Cd/Cu carrier solution reversed this decline (Fig. 3). The calibration curve slope values were 344.8±2.7 and 343.1±1.9 (r^2=0.9996 and 0.99992 for NO_2^- and NO_3^- respectively). Both lines had intercepts not significantly different from zero. As the slope values were not different, we conclude that the reducing efficiency of the Cd/Cu column was 100%. For a 400-μl injection volume, the method had a sensitivity of 75 n*M* for both NO_2^- and NO_3^- (taken as five-fold the baseline) and a linearity up to 150 μ*M*.

Fig. 4 shows the relationship between the HPLC and the colorimetric Griess reaction method when applied to the quantification of urinary NO_2^- (panel a) and NO_3^- (panel b). Regression analysis by the least squares method showed slope values of 1.35±0.21 (r^2=0.865, n=8) for NO_2^- and 0.265±0.026 (r^2=0.941, n=8) for NO_3^-. In the case of NO_2^-, the slope value was not significantly different from unity. From the slope value for NO_3^-, it can be calculated that the batch reduction of NO_3^- to NO_2^- is approximately 26% of that obtained on-line. This deficiency, which reflects a loss in the reducing capacity of Cd/Cu, can be explained by a possible inactivation of the reagent by some urinary component(s). These observations disqualify the colorimetric Griess reaction as suitable for NO_3^- quantification in urine samples when using Cd/Cu as the reducing reagent.

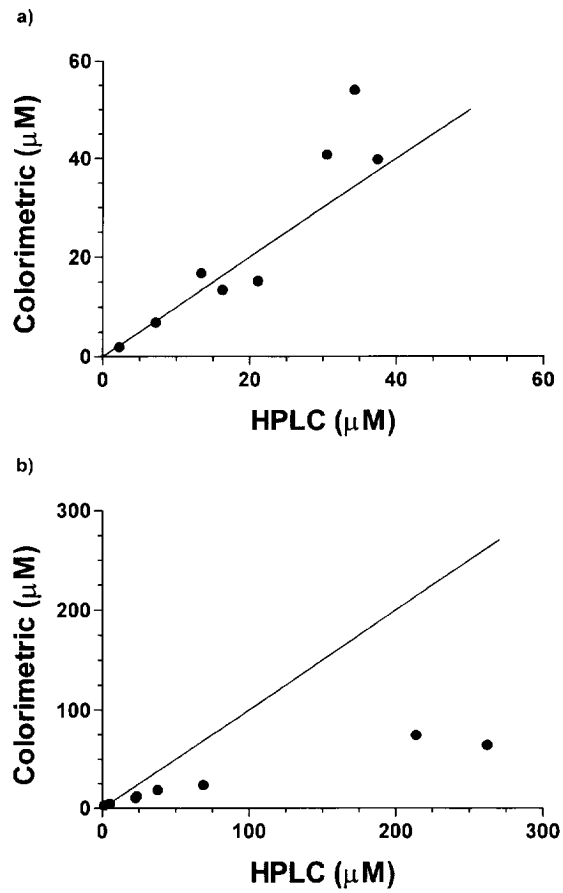

Fig. 4. Correlation between the HPLC and the spectrophotometric Griess reaction concentrations of nitrite (panel a) and nitrate (panel b) in rat urine samples. The straight lines represent identity (slope = 1.00).

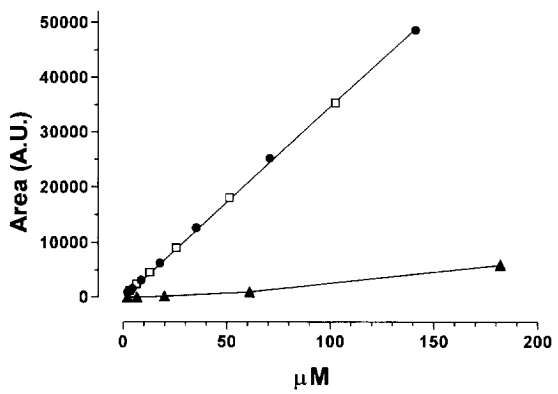

Fig. 3. Calibration curves for nitrite (●) and nitrate in the absence (▲) and presence (□) of 0.02% $CuSO_4 \cdot 5H_2O$ in the Cd/Cu carrier solution.

Many HPLC methods for the determination of NO_2^- and NO_3^- have been published. These methods have been successfully applied to the analysis of waters [18–24], vegetables [28,31,34,35,57] and for controlling the content of these anions added to meats [29,30,32,35] and cheeses [26–28,30]. Most of them make use of the UV absorption of these ions at 200–220 nm. However, the concentration of NO_2^- and NO_3^- in the above mentioned samples is in the micromolar to millimolar range, while in situations where nitric oxide participation is implicated the concentration is in the nanomolar to micromolar.

The method described by Gennaro and Bertolo [56] employs an ion-pair reversed-phase separation of inorganic anions. When this method was tested by adding different amines and quaternary ammonium compounds to the mobile phase, a good separation was achieved (data not shown), but the reduction of NO_3^- to NO_2^- on the Cd/Cu column did not occur. It is worth mentioning that such compounds were not studied by Davison and Woof [58] in their paper on the susceptibility of the Cd/Cu reagent to several interfering substances. Homogeneous reducing agents such as hydrazine [59] and titanium (III) chloride [60] were reported in the analysis of nitrite and nitrate anions by FIA. However, these reactions are too slow to be coupled on-line after separation by HPLC. The Berthelot reaction [61,62] was considered as an alternative to the Griess reaction, but this reaction was also too slow to be coupled (in our hands, the reaction was completed in 20 min).

As shown in the serum chromatograms obtained from rats chronically treated with the NO-synthase inhibitor L-NAME, but not for samples from non-treated rats (Fig. 2, panels f and e, respectively), a peak eluting at 5.0±0.5 min appears. This peak co-elutes with true N^{ω}-nitro-L-arginine (L-NA), the active L-NAME metabolite, and reflects the fact that L-NA (and L-NAME) has a nitro group attached to one of the nitrogen atoms of the guanidino group. This nitro group decomposes to NO_2^- upon passing through the Cd/Cu column. This is the first report of this reaction in the literature, and the underlying mechanism is currently being investigated. The finding is of great importance given the widespread use of these compounds in experiments where NO release is studied. Biological fluids in which NO_3^- and NO_2^- concentrations are measured in the presence of L-NA or L-NAME (and probably any other N-nitro guanidino compound) will give misleading results if the Cd/Cu reagent is used without a previous separation of the species. Despite the reaction of N^{ω}-nitro-L-arginine with the Cd/Cu reagent not being stoichiometric (i.e. 1 mol L-NA produces less than 1 mol nitrite anion), L-NA concentration is linearly related to the peak area and this fact allowed the calculation of serum L-NA concentrations in rats chronically treated with L-NAME (data not shown).

Based on the above results, we conclude that the method presented here is appropriate for the simultaneous measurement of nitrate and nitrite anions in biological samples where the participation of NO is being studied. The main advantages of this method over the colorimetric Griess reaction (even when performed as FIA [53]) include the small sample volume required, the sensitivity of the assay and, principally, the possibility of overcoming potential problems arising from the presence of interfering substances.

Acknowledgments

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