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Short communication

Determination of nitrate and nitrite by high-performance liquid chromatography in human plasma☆

Vera Jedličková*, Zoltán Paluch, Štefan Alušík

Department of Internal Medicine, Institute for Postgraduate Medical Education and Thomayer's Hospital, Vídeňská 800, Prague, Czech Republic

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Abstract

A new, accurate, fast and simple method has been implemented by which nitrite and nitrate ions, as stable forms of nitric oxide production were studied. A study of these two ions was carried out by a sensitive and accurate HPLC method with two detectors. The most important advantages of the reported method are: short time of analysis, minimal sample pre-treatment, long life of the analytical column and stable eluent solution. The photodiode array UV–Vis detector detected nitrite and nitrate ions at an absorbance of 212 nm. Much more sensitive electrochemical detection with a WE (glassy carbon) electrode was used for the detection of nitrite ions. An analytical chromatographic column was formed by a sorbent, containing strong base anion-exchange groups bound in Cl^- form in the hydrophilic hydroxyethyl methacrylate matrix. The anions were analysed in human plasma without deproteinization using 0.02 *M* sodium perchlorate monohydrate as eluent solution at pH 3.9. At this pH organic substances do not affect the analysis. The retention times for nitrite and nitrate were 3.62 and 3.72 min (by electrochemical detection) and 4.44 min, respectively. The method was linear (r=0.9992, 0.9998, 0.996) within a 1–100 (nitrate), 1–20 μmol/l (nitrite) concentration range.

Keywords: Nitrate; Nitrite

1. Introduction

Nitric oxide (NO) is a vital messenger in many cellular communication and control systems. Nitric oxide has a short lifetime in vivo (<5 s), then NO is converted quantitatively to nitrite and nitrite is rapidly oxidized to nitrate.

Classically, these ions have been determined by the Griess method, in which nitrite is diazotised with sulfanilamide and then reacted with *N*-1-naphthyl-ethylenediamine to form a coloured product [1]. It is necessary to reduce the nitrate to nitrite either chemically by a problematic reduction of cadmium (Cd) [2–5] or enzymatic reduction step [6]. These methods have a few disadvantages. They require a long and demanding pre-treatment of the sample, which makes the pre-treatment quite expensive.

Other methods have been reported for determining nitrate/nitrite in serum and plasma, including the ultraviolet spectrophotometric methods [7], ion chromatography (IC) [8–10], high-performance liquid

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*Corresponding author. Fax: +420-2-4172-1770.

E-mail address: vera.jedlickova@seznam.cz (V. Jedličková).

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chromatography (HPLC) [11], gas chromatography–mass spectrometry (GC–MS) [2,3,12] and capillary electrophoresis (CE) [13].

This work describes a method for the rapid, accurate, simple and cheap measurement of nitrite and nitrate in plasma. A study of these two ions was carried out by a sensitive and accurate HPLC method with two detectors analogous to the method of the Preik-Steinhoff and Kelm [14]. The present method used a different technique for determination of nitrite/nitrate ions. The differences between the present method and Kelm's method are: the pre-treatment of sample, the composition of eluent solution, the time of analysis, the composition of anion-exchange column and the type of HPLC system. The photodiode array UV–Vis detector detected nitrite and nitrate ions at an absorbance of 212 nm. Much more sensitive electrochemical detection with a WE (glassy carbon) electrode was used for the detection of nitrite ions. The anions were analysed in human plasma without deproteinization using 0.02 *M* sodium perchlorate monohydrate as eluent solution at pH 3.9. At this pH organic substances do not affect the analysis.

2. Experimental

2.1. Reagents

All chemicals were of analytical grade.

Sodium nitrite, sodium nitrate, *o*-phosphoric acid 85% (purchased from Lachema, Neratovice, Czech Republic and Merck, Prague, Czech Republic), sodium perchlorate monohydrate—$NaClO_4 \cdot H_2O$, (purchased from Merck). Solutions were prepared in Milli-Q water that had a resistance 18 MΩ (apparatus Watek, Ledec n/S, Czech Republic). Nitrite and nitrate stock solutions were diluted in Milli-Q water to obtain the required concentrations. The location of nitrite and nitrate peaks was determined from known standard solutions in water and was confirmed by spiking serum samples.

2.2. Samples

Venous blood samples from healthy male and female volunteers aged 20 to 40 years and from a group of old patients with cardiovascular diseases aged 70 to 95 years were drawn after fasting for 12 h and collected in heparinised tubes, immediately centrifuged at 2500 *g* for 10 min for separation of plasma.

Plasma was analysed after dilution with water 1:4.

2.3. HPLC system

The chromatographic system consisted of a Shimadzu Sil-10Advp autosampler, a Shimadzu LC-10Advp pump, a degasser DGU-14A, a UV–Vis photodiode array detector SPD-M10Avp, a Schimadzu LC-workstation (all instruments Shimadzu, Kyoto, Japan), and an electrochemical detector PROCEDOR that was connected in parallel (Antec Leyden, Zeoterwoude, The Netherlands).

The filling of the column was an ion exchanger based on styrene–divinylbenzene with quarternary amine in the Cl^- form of the HEMA-BIO 1000Q type, 10 μm (150×3 mm), with a guard column (30×3 mm) of the same type (Tessek, Prague, Czech Republic).

The eluent was 0.020 *M* $NaClO_4$, pH value 3.9. The flow-rate was 0.6 ml/min.

Detection of nitrate/nitrite anions was carried out by absorbance at 212 nm using UV–Vis detection and the detection of nitrite ions was carried out by the electrochemical type Procede detector, using amperometric detection with a WE (glassy carbon) electrode at +0.8 V. Nitrate ions are not electrochemically active.

3. Results and discussion

Fig. 1 shows a chromatogram obtained by using the strong base anion-exchange column in the Cl^- form of the human plasma (a, b) and the standards nitrite/nitrate in the water (c, d) with both absorbance at 212 nm (a, c) and using electrochemical detection (b,d) connected in parallel across a UV–Vis photodiode detector.

The chromatograms (b, d) show that the determination of nitrite with electrochemical detection is remarkably more sensitive than the determination with UV–Vis detection (approximately 100× more sensitive).

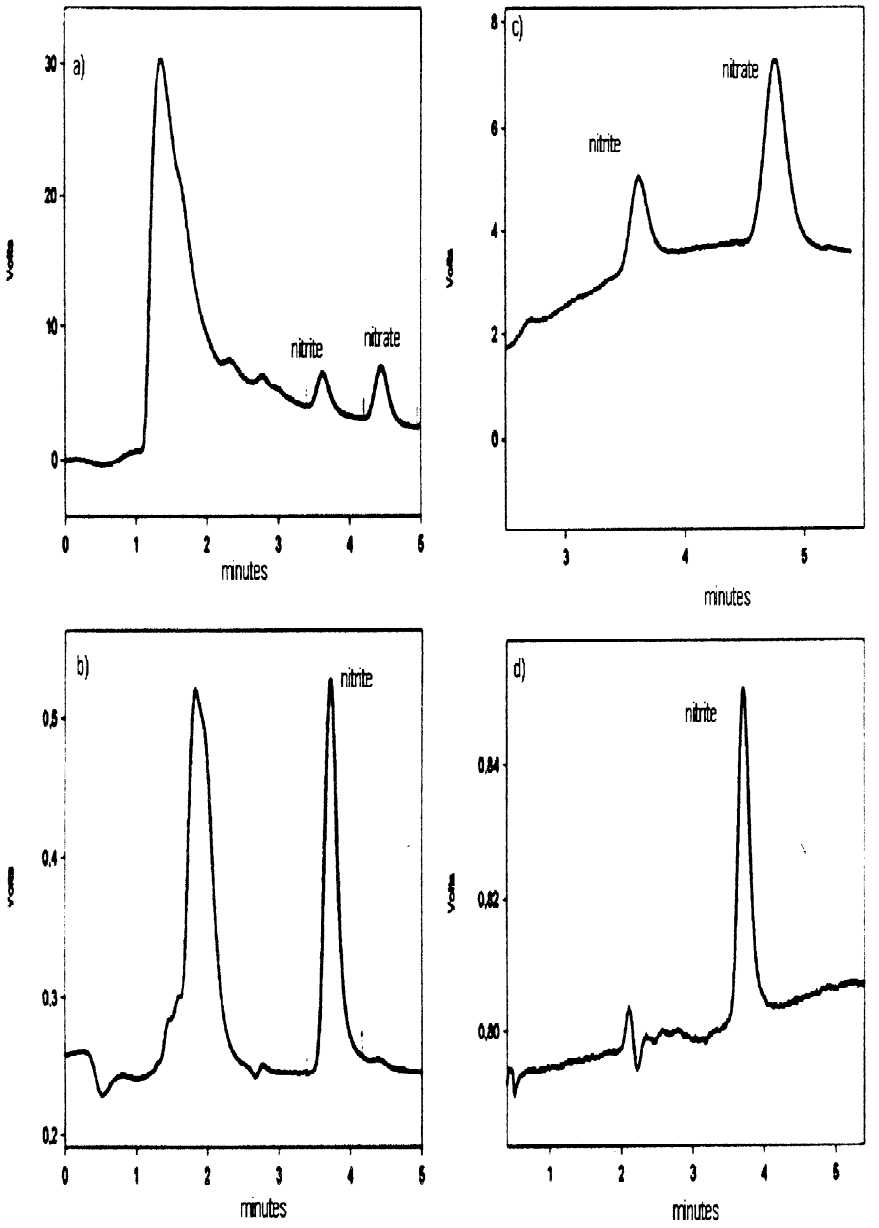

Fig. 1. (a) Chromatogram of nitrite and nitrate in human plasma. Plasma was analysed after dilution in water. Separation conditions: ion-exchange column eluent—sodium perchlorate, absorbance detection at 212 nm. (b) Chromatogram of nitrite in human plasma analysed by electrochemical detection with a WE electrode at +0.8 V. (c) Chromatogram of a standard containing 10 μmol/l nitrite and 50 μmol/l nitrate in water using the same separation conditions as in (a). (d) Chromatogram of the same standard of nitrite as in (c) analysed by electrochemical detection with a WE electrode at +0.8 V.

The linearity of this method was assessed using aqueous standards. Calibration curves for both nitrite (0–20 μmol/l) and nitrate (0–100 μmol/l) were found linear over the range. The linearity was expressed by the following equation: (a) $\text{nitrite}_{\text{UV–Vis detection}}$ $y=6698.5+13\,228.7x$ ($r=$

Table 1
Concentrations of nitrite and nitrate in the plasma of healthy volunteers and old patients

Volunteers	Age range (years)	Nitrite (mean±SD) (μmol/l)	Nitrate (mean±SD) (μmol/l)	*n*
Healthy volunteers (males and females)	From 20 to 40	1.317±0.840	19.16±6.35	32
Males	From 20 to 40	1.19±0.644	20.07±7.62	15
Females	From 20 to 40	1.428±0.987	18.34±5.06	17
Old patients (males and females)	From 70 to 95	1.372±0.914	45.40±10.88	26
Males	From 70 to 95	1.359±0.968	44.16±9.14	13
Females	From 70 to 95	1.384±0.897	46.63±12.64	13

0.996), (b) $\text{nitrite}_{\text{ELCHEM.detection}}$ $y=74\,054+3\,374\,306.5x$ ($r=0.9998$), (c) nitrate $y=21\,191.5+17\,123.7x$ ($r=0.9992$) (y=peak area, x=concentration of corresponding added anion). The mean values of retention time±SD for nitrite and nitrate determined in standard mixtures were: (a) $\text{nitrite}_{\text{UV–Vis detection}}$ 3.623±0.012 min (RSD=3.3%), $\text{nitrite}_{\text{ELCHEM.detection}}$ 3.725±0.047 min (RSD=1.26%), (c) nitrate 4.445±0.0062 min (RSD=1.39%).

The recovery of $\text{nitrite}_{\text{UV–Vis detection}}$ was 98.37±1.95%, $\text{nitrite}_{\text{ELCHEM.detection}}$ 96.53±4.58%, and nitrate 98.23±2.12%. The detection limit for $\text{nitrite}_{\text{UV–Vis detection}}$ was 0.1 μmol/l, $\text{nitrite}_{\text{ELCHEM.detection}}$ was 0.001 μmol/l and nitrate 0.2 μmol/l.

The concentration values of nitrite (determined by electrochemical detection) and nitrate in the plasma of group 1 (healthy volunteers, both males and females aged 20 to 40 years) and group 2 (old patients, both males and females aged from 70 to 95 years) are presented in the Table 1. The concentrations of nitrate in plasma of female volunteers in group 1 were slightly lower than those of males in the same group of age. In group 2 the values of nitrate concentrations were twice as high as those in group 1. In group 2 the concentrations of nitrate in plasma of female volunteers were slightly higher than those of men in the same age group.

Table 2 presents values of concentration of nitrite and nitrate as published by various authors using different analytical methods. It is difficult to say which method is more accurate, however it is known [1,17] that plasma pre-treatment can considerably influence the results. Our attempt was to reduce the manipulation of plasma to a minimum, in order to avoid negative influence in the plasma pre-treatment. Therefore the analysis was done without deproteinization [13] and without plasma filtration.

4. Conclusions

The aim of this work was to introduce a sensitive, accurate, simple and cheap HPLC method for the analysis of nitrite and nitrate ions in plasma.

Table 2
Normal range values of nitrite and nitrate in plasma as published by other authors (compare with Table 1)

Author	Method	Nitrite (mean±SD) (μmol/l)	Nitrate (mean±SD) (μmol/l)	*n*
Tsikas et al. [2]	GC–MS	1.8±0.4	38.0±11.0	10
Monaghan et al. [9]	IC	4.2±3.9	39.9±22.0	200
Stratford et al. [10]	IC	0.71±0.46	47.8±6.1	5
Žunič et al. [13]	CE	6.1±2.3	40.2±10.3	7
Preik-Steinhoff and Kelm [14]	HPLC	0.58±0.12	25.0±4.0	8
Tsikas et al. [15]	GC–MS	0.55±0.16	27.4±3.3	8
Radisavljevic et al. [16]	HPLC	3.1±0.4	10.3±0.3	22

It has been found that nitrate concentration in the group of old patients aged 70–95 years was twice as high as that found in the group of healthy volunteers aged 20–40 years.

The advantages of the reported method can be summarized as follows:

(a) High sensitivity of electrochemical detection for the determination of nitrite ions: electrochemical detection is significantly more sensitive than UV–Vis detection (about 100×).
(b) Short time of analysis (4.5 min).
(c) Minimal sample pre-treatment.
(d) Long life of the analytical column: the operating life of the analytical column can be extended by its regeneration/cleaning with a solution of 2% NaCl+0.1% NaOH followed by 2% NaCl+5% methanol wash. The first regeneration of the column was carried out after 850 analyses, where retention time shifted by approximately 10%. The next regeneration was carried out after 200 analyses.

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