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A fast and sensitive method for the determination of nitrite in human plasma by capillary electrophoresis with fluorescence detection

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ABSTRACT

Analysis of nitrite, the indicator of nitric oxide (NO) generation *in vivo*, provides a useful tool to study NO synthesis *in vivo*. A fast and sensitive fluorometric CE method was developed for determination of nitrite in human plasma through its derivatization with 2,3-diaminonaphthalene (DAN). Nitrite in human plasma was easily reacted with DAN under acid conditions to yield the highly fluorescent 2,3-naphthotriazole (NAT). Fluorescence detection was optimized to achieve subnanomolar detection which allows a direct analysis of plasma samples unlike most CE–UV methods using sample stacking. Acetonitrile was used to remove the protein. Short-end injection and a high voltage (−30 kV) were used to shorten the analysis time. The good separation was achieved with 20 mM borate buffer at pH 9.23. The separation of NAT was obtained within 1.4 min. The deproteinized plasma sample was injected hydrodynamically for 5 s at −50 mbar into a 60 cm × 75 μm internal diameter uncoated fused-silica capillary. Excitation wavelength was selected with a broad-band filter (240–400 nm), and the emitted light was measured at 418 nm by the use of a cutoff filter. A good linearity (R^2=0.9975) was obtained in the range from 2 to 500 nM. The detection limit of nitrite was 0.6 nM in original plasma samples, which is 750 times lower than our previous CE–UV method. The developed fluorometric CE method offers the advantages of more simple system and lower cost compared with the current fluorometric HPLC methods without losing sensitivity. The detected mean nitrite concentration in human plasma by this method was consistent with the most frequently reported values.

1. Introduction

Recent studies suggest that nitrite has the potential to become a marker of NO synthesis under physiological and pathological conditions and could therefore be related as a biochemical parameter in experimental and clinical studies [1–3].

But up to now there is no consensus concerning the real nitrite concentration in human plasma. The reported levels of basal nitrite in human plasma have ranged from "non-detectable" to 26 μM. The most plausible nitrite concentration in human plasma ranges from 100 nM to 1 μM, the most frequently reported results ranging from 100 nM to 200 nM measured by most investigator groups [4]. Therefore, the determination of nitrite concentration in human plasma has become a challenge for analytical sciences. Both high sensitivity and precautions taken during the sample preparation procedure can improve the precision and accuracy of the measurements [5]. Fluorometric methods have been widely employed for sensitive analysis of nitrite. These methods involve the derivatization reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NAT). Several methods using HPLC with fluorescence detection were developed for the measurement of nitrite in water, urine, plasma and cell culture medium [6–9]. However, most fluorometric HPLC methods developed for biological samples require a complicated sample preparation procedure to remove matrix components and a guard column was also required. These additional preparation steps may introduce contamination by environmental nitrite. In addition, the fact that NAT is stable and has higher fluorescence intensity at alkaline conditions of pH 8.5–11.5 is not compatible with the stability of normal reversed phase HPLC columns. Because normal silica-based columns begin to dissolve and have short life time when the mobile phase pH is above 8 [10]. Therefore, a special column such as a polymer-based column is needed for analysis of nitrite in alkaline conditions. Even then, a rigorous cleaning procedure was needed after each HPLC run [9]. Capillary electrophoresis offers unique advantages in bio-analysis due to its simple sample preparation and cleaning procedure between runs. Although various CZE–UV methods combined with on-capillary preconcentration have been developed to achieve

Abbreviations: NO, nitric oxide; DAN, 2,3-diaminonaphthalene; NAT, 2,3-naphthotriazole

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the satisfactory sensitivity [11–13], there is no fluorometric CE method for analysis of nitrite until now.

The aim of the study was to develop a fast, simple and sensitive fluorometric CE method for the subnanomolar determination of nitrite in human plasma.

2. Material and methods

2.1. Chemicals

All chemicals used were of analytical grade. Sodium nitrite and disodium tetraborate decahydrate were from Merck (Darmstadt, Germany). Sodium hydroxide and acetonitrile were from Fisher Scientific (Loughborough, UK). DAN and fluorescein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid was from VWR International (Leuven, Belgium).

All solutions were prepared in distilled water purified by a Milli-Q Gradient system (Millipore, Molsheim, France). The buffer solution was filtered through 0.2 μm regenerated cellulose filter (Whatman, Dassel, Germany).

2.2. Sample preparation

The venous blood samples collected from seven healthy male volunteers (no dietary restriction) were drawn into lithium-heparin tubes and plasma was separated by centrifugation (3478*g* for 10 min at 4 °C) within 3 min after sample collection. Plasma was then isolated and immediately frozen at −80 °C until later analysis. After thawing at room temperature, 100 μL plasma sample was deproteinized by adding 100 μL acetonitrile [14], and the mixture was centrifuged at 14,100*g* for 5 min in a Minispin Plus microcentrifuge (Eppendorf, Hamburg, Germany). Then, 100 μL supernatant was adjusted to pH 6 by adding 5 μL 0.62 M HCl and then taken for the derivatization reaction. In total 100 μL sample consisting of 50 μL of reaction mixture and 50 μL of 0.1 μM fluorescein solution used as internal standard, was directly used for injection. The study was approved by the local Ethics Committee (KU Leuven) and the informed consent was obtained from all subjects.

2.3. Reaction of nitrite with DAN to form NAT

The reaction of nitrite with DAN was performed according to the method of Misko et al. [15]. DAN reacts rapidly with nitrite under acidic conditions to form the highly fluorescent product NAT [16], which is stable in alkaline solution [17]. For the DAN stock solution, DAN was dissolved in 0.62 M HCl at a concentration of 2 mg/ml and stored at −20 °C in the dark. DAN working solution (0.05 mg/ml) was prepared by a 40-fold dilution of the stock solution in 0.62 M HCl, which was stored at 4 °C in the dark. 100 μL nitrite standard or plasma sample was incubated at 25 °C with 10 μL of DAN working solution in a Thermomixer Comfort (Eppendorf, Hamburg, Germany) for 15 min, followed by addition of 10 μL of 1.4 M NaOH.

2.4. Instrument and CE conditions

All experiments were performed on a HP ^{3D}CE system (Agilent Technologies, Waldbronn, Germany). Separations were carried out using an uncoated fused-silica capillary (60 cm × 75 μm i.d.; effective length 43.5 cm, 16.5 cm from the outlet to fluorescence detection window) at a voltage of −30 kV. The capillary was thermostated at 25 °C and coupled to an Argos 250 B fluorescence detector (Flux Instruments, Basel, Switzerland) as external detector. The Argos 250 B light source is fed by a xenon-mercury lamp. Excitation is done using perpendicular irradiation of the glass capillaries of a CE system. The emitted light is guided along the capillary by total reflection and focussed using a patented light core, which is connected to a photo-multiplier using a light guide [18]. The fluorescence intensity was examined at 418 nm emission wavelength using the broad band (200–400 nm) filter for excitation. Short-end injection was performed hydrodynamically for 5 s at −50 mbar. Prior to the injection of plasma samples, the capillary was flushed with 0.1 M NaOH (2 min) and running buffer (2 min).

The pH value of borate buffers was measured with a Metrohm 691 pH-meter (Metrohm, Herisau, Switzerland).

3. Results and discussion

3.1. Development of fluorometric CE method

In view of previous reports of an increase in fluorescence intensity in alkaline conditions and a loss of fluorescence signal in acidic conditions, borate buffer was tested in the range from pH 9 to 10 keeping the fluorescence gain value at 1100. There was no significant increase in fluorescence intensity with increasing pH at a gain value of 1100. An increased pH also leads to an increased current. The concentration of borate buffer was tested at 10, 20 and 30 mM. Finally, 20 mM borate buffer without pH adjustment was selected as a compromise of current and ionic strength. The gain value of the fluorescence detector was tested from 800 to 1100, and the highest signal was obtained at a gain setting of 1100. The fluorescence intensity was examined at 418 and 450 nm emission wavelength and the maximum signal was at 418 nm using the broad band (200–400 nm) filter for excitation. The derivatization time was also tested at 1, 5, 10, 15, 20 and 30 min by using 250 nM standard nitrite solutions and 250 nM nitrite spiked plasma samples. The reaction time was kept at 15 min due to the fact that there is no obvious increase of product intensity beyond 15 min in both cases. The separation time could be made almost three times shorter by short-end injection compared with the normal end injection. Typical electropherograms of plasma sample with short end injection and normal end injection have been shown in Fig. 1.

3.2. Linearity, detection and quantification limit, precision and accuracy of the fluorometric CE method

A linearity relationship (r^2=0.9975) was obtained by standard addition of nitrite (0, 2, 20, 40, 200, 400, 500 nM) to deproteinized plasma samples injected in triplicate. Fluorescein 50 nM was used as internal standard (I.S.). The linearity regression equation was y=0.0084x+0.2031, $S_{y,x}$=0.01. Where x represents the concentration of nitrite added to the deproteinized plasma samples and y represents the relative peak area (the corrected peak area of NAT divided by the corrected peak area of I.S. peak), $S_{y,x}$ represents standard error of y estimate. The limit of detection and limit of quantification for nitrite in original plasma were 0.6 nM and 2 nM based on a signal to noise ratio of 3 and 10, respectively when using the Argos 250 B fluorescence detection with maximum setting of the gain at 1100. The sensitivity of the developed CE method for the analysis of nitrite was better than previous fluorometric HPLC methods [6,9], but inferior to the LODs of 0.01 and 0.27 nM reported in [7,8]. The precision of the method which was determined on 250 nM nitrite spiked plasma samples was expressed as relative standard deviation (% R.S.D.) of the relative peak areas. The intra-day and inter-day precision for duplicate injection on three days (n=6) were found to be 0.2% and 1.1%, respectively. The accuracy of the method for the analysis of nitrite was evaluated with known amounts of sodium nitrite. Average results based on three data points at each concentration were used. The recoveries for different

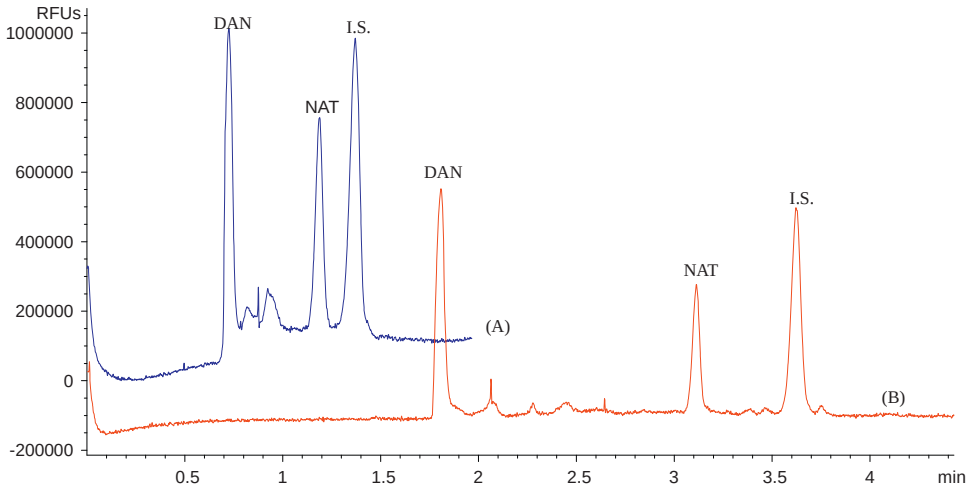

Fig. 1. Typical electropherograms of plasma sample containing 137 nM nitrite and 50 nM I.S. with (A) short-end injection and (B) normal end injection (DAN: 2,3-diaminonaphthalene; NAT: 2,3-naphthotriazole; I.S.: Internal standard).

Table 1
Recoveries of nitrite in human plasma samples ($n=3$).

Component	Concentration (nM)		Recovery (%)
	Added	Found	
Nitrite	20.8	20.7	99.6
	208	177.2	85.3
	416	467.1	112.3

concentrations of nitrite were within the range of 85.3–112.3%, indicating acceptable accuracy of the method (see Table 1).

3.3. Application of the developed method

The proposed method was applied to determine the concentrations of nitrite in human plasma samples. Plasma samples of seven healthy young male volunteers were analyzed for nitrite. The mean basal nitrite concentration measured by this method was 159.4 ± 58.5 nM, which is in agreement with the most frequently reported results ranging from 100 nM to 200 nM measured by most investigator groups [4].

4. Conclusion

A fast and sensitive method for the determination of nitrite in human plasma by CE with fluorescence detection has been successfully developed and validated. The new CE method offers the advantages of simple operation and low cost compared with the existing fluorometric HPLC methods using special columns combined with rigorous cleaning procedures. It could also prove useful in the detection and quantification of nitrite in other biological systems and serve as a useful tool for investigating the role of NO synthesis in both normal and pathological processes.

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