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Short communication

Determination of nitrite in human blood by combination of a specific sample preparation with high-performance anion-exchange chromatography and electrochemical detection

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Abstract

All photometric or HPLC methods described to date have been unable to detect nitrite, a reliable marker of NO synthase activity, in human blood because of its rapid metabolism within the erythrocytes. We now elaborate on method to prevent nitrite degradation during sample preparation which in combination with high-performance anion-exchange chromatography and electrochemical detection allows a sensitive measurement of nitrite. A linear current response in the concentration range of 10–1000 nmol/l nitrite was observed yielding a correlation coefficient of 0.99. In addition, the combination of the electrochemical with a UV detector allowed us to simultaneously quantify nitrate within one analytical run, which is the end product of NO/nitrite metabolism. Basal levels for nitrate and nitrite in human blood were determined with 25 ± 4 μmol/l and 578 ± 116 nmol/l ($n = 8$), respectively and thus were in the same concentration range as expected from NO measurement in saline perfused isolated organs or cultured endothelial cells. Therefore, the presented method may be used to assess activity of endothelial constitutive NO synthase in humans under physiological and pathophysiological conditions.

Keywords: Sample preparation; Nitrite

1. Introduction

Nitric oxide (NO) is involved in the regulation of numerous biological functions such as vascular tone, immune response, and neurotransmission (for review see [1]). At baseline conditions NO is continuously formed by endothelial constitutive NO synthase (ecNOS) and released into the circulation at the low nanomolar concentration range. In contrast, the inducible iNOS, which produces NO at a more than hundred-fold higher rate, is not expressed at baseline [1]. Disturbances of ecNOS are suggested to play a key role for atherosclerotic lesion of the vascular system observed in arterial hypertension, hypercholesterolemia and diabetes mellitus. Thus, the measurement of NO or its metabolites in humans appears highly desirable. In the presence of oxygen, NO is oxidized via dinitrogen trioxide predominantly to nitrite following a pseudo-first order kinetic with a strict 1:1 stoichiometry [2]. As the direct measurement of NO in biological systems is hampered by its radical nature and, consequently, high reactivity, determination of its degradation product, nitrite, has frequently been used as a marker of NO production

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in aqueous solutions. Whereas in aqueous solutions and in human plasma nitrite is stable for up to several hours, it is rapidly converted to its end product, nitrate, within the erythrocytes in human blood [2,3]. However, the serum level of nitrate is an unreliable indicator of NO formation by ecNOS, as this parameter is influenced by a variety of exogenous factors such as dietary nitrate intake [4], bacterial synthesis of nitrate in the gastrointestinal tract [5], and inhalation of atmospheric gaseous nitrogen compounds [4]. Furthermore, the natural abundance of nitrate with a background concentration in the micromolar range in human blood would hamper exact evaluation of changes in the ecNOS activity via nitrate, as the amount of NO formed would yield only small changes in nitrate concentration within the nanomolar range.

Therefore, our aim was to develop an analytical method for the quantification of nitrite in human blood, which could represent a sensitive and specific measure of endogenous formation of NO by ecNOS in humans [2,6]. In order to prevent the rapid metabolism of nitrite to nitrate within the erythrocytes, a particular method for blood sample preparation had to be found.

2. Experimental

2.1. Chemicals and reagents

All solutions and the mobile phase were prepared with ultrapure LiChrosolv HPLC water from Merck (Darmstadt, Germany). Chromasolv acetonitrile and methanol were purchased from Riedel de Haën (Seelze, Germany), analytical grade sodium chloride, sodium hydroxide, sodium nitrite, sodium nitrate and phosphoric acid solution were obtained from Merck.

2.2. Equipment

Analysis of nitrite and nitrate was performed on a HPLC system consisting of a Sykam S1000 pump (Gilching, Germany), a Jasco 851-AS autosampler (Groß-Zimmern, Germany) with a Rheodyne injection valve 9010 (Cotati, CA, USA) and a 20-μl fixed filling loop. The analytical column was an anion-exchange column LC A08 125×4.6 mm I.D. (Sykam) filled with 5 μm silicon coated polysterol and dipropyldibutylamine as functional groups. A Spherisorb SAX 20×4.6 mm I.D. (Grom, Herrenberg, Germany) was used as a guard column. The columns were kept at a constant temperature of 20°C by a column thermal controller S4010 (Sykam). The analytical column was connected in line to a Linear UV detector UVIS 204 (Linear Instruments, Reno, NE, USA) for detection of nitrate in the micromolar range and an electrochemical detector ESA Coulochem 5200A (ESA, Chelmsford, MA, USA) for detection of nitrite in the low nanomolar range. UV absorption was constantly monitored at λ=220 nm at a range of 0.01 absorption unit full scale (AUFS) and a rise time of 0.1 s. The potential of the amperometric analytical cell 5040 with a glassy carbon target (ESA, Chelmsford, MA, USA) was maintained at +0.7 V with a range of 1 or 5 nA depending on sensitivity needed and a filter constant of 10 s. Data acquisition and integration was performed using the Pyramid Chromatography System (version 1.923, Axxiom, Moorepark, CA, USA).

2.3. Mobile phase

The mobile phase was helium-conditioned and consisted of acetonitrile–methanol–HPLC-water (70:10:20, v/v) and 40 mmol/l sodium chloride in the final solution. It was degassed and filtered through a 0.5-μm membrane filter (FHLP, Millipore, Eschborn, Germany) and pumped at room temperature at a flow-rate of 1.0 ml/min in a recirculating manner.

2.4. Sample preparation

Human blood (1.5 ml) was taken from the antecubital vein of healthy volunteers into a 3-ml syringe (Amefa, Kriftel, BRD) filled with 1.5 ml of a 0.1 mol/l sodium hydroxide solution (stop solution). After that the pH of the blood/stop solution mixture was set to 7.0 with a standard volume of 1 mol/l phosphoric acid. Next, the mixture was centrifuged at 14 000 *g* at room temperature for 5 min. The resulting supernatant was ultrafiltered with Centrisart I cut-off 10 000 (Sartorius, Göttingen, Germany) for 15 min at 2000 *g* and 20°C. The ultrafiltrate was either injected into the HPLC system or further

diluted up to tenfold (with HPLC water) depending on coeluting oxidizable contaminations. Samples were analyzed immediately or within 4 weeks after sampling (storage at −80°C). Data for basal levels of nitrite and nitrate in human blood were not corrected for loss of recovery during sample preparation.

3. Results

Chromatograms of aqueous standard solutions containing nitrite and nitrate applied to the HPLC setup used in the present study are given in Fig. 1. The potential of the electrochemical detector was set at 0.7 V, which gave maximal current response. A linear relation between the peak area and the concentration of standard sodium nitrite and nitrate solutions was observed at 100–1500 nmol/l and 5–50 μmol/l, respectively. The parameters of the calibration curves for the calculated area of the peak gave the following values for nitrite: slope 0.001 nA s/nmol/l, intercept −0.02 nA s and for nitrate: slope 5.3×10^{-5} AU s/μmol/l, intercept 4.8×10^{-5} AU s and a correlation coefficient of 0.99 for both anions.

In some samples of human blood taken from different individuals, unidentified blood constituents, running at 3 to 8 min, hampered exact chromatographic analysis of nitrite. Therefore, the ultrafiltrate of the prepared sample was diluted with HPLC-water up to tenfold if necessary. To compensate for the dilution factor the sensitivity of the assay had to be further increased. To achieve this goal the range of the ECD detector was set to 1 nA. Using this setup the parameters of the calibration curve were: slope 0.001 nA s/nmol/l, intercept 1.5×10^{-4} nA s, correlation 0.99. The final sensitivity of this combined assay for nitrite and nitrate under this experimental conditions was amounted to 3 nmol/l and 0.5 μmol/l for nitrite and nitrate at a signal-to-noise ratio of 3:1, respectively. The intra-assay coefficient of variance for retention times of nitrite (1000 nmol/l) and nitrate (10 μmol/l) were 0.3%, 0.2%, and for the measured concentrations 5.5%, and 4.9% ($n=10$), respectively. The day-to-day coefficient of variance for retention times of nitrite (1000 nmol/l) and nitrate (10 μmol/l) were 3.4%, 3.0%, and for the concentrations 8.0% and 7.5% ($n=8$), respectively.

Using the described sample preparation, nitrite and nitrate in human blood were clearly separated by anion-exchange chromatography (Fig. 2). Addition of nitrite and nitrate standards confirmed their identity and the clear separation from any kind of disturb-

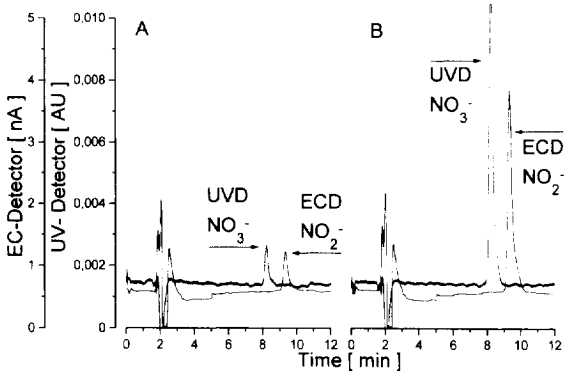

Fig. 1. Representative chromatograms of simultaneous measurement of a standard solution containing sodium nitrite and nitrate analysed by means of EC- and UV detection in a single analytical run. Concentrations are: A= nitrite 100 nmol/l and nitrate 5 μmol/l; B= nitrite 1000 nmol/l and nitrate 50 μmol/l, respectively. The EC-detector sensitivity was maintained at 5 nA range.

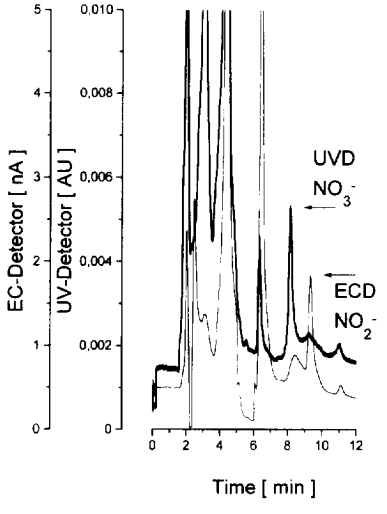

Fig. 2. Representative chromatogram of a human blood sample with simultaneous measurement of nitrite and nitrate. In consideration of dilution factor 2 (1 ml human blood plus 1 ml stop solution) the basal concentration of nitrite and nitrate in this human blood sample was found to be 487 nmol/l and 31 μmol/l, respectively. The EC-detector sensitivity was maintained at 5 nA range.

ing peak. In the sample depicted, nitrite and nitrate were detected at concentrations of 487 nmol/l and 31 μmol/l, respectively. The recovery of added nitrite and nitrate in the physiological relevant concentration range (nitrite: 100–500 nmol/l; nitrate: 10–50 μmol/l) was found to be 75.9±12.1% and 91.7±8.5%, respectively, each $n=6$.

The reproducibility of the described method including sample preparation and analysis was determined as 91.9±6.0%. Measurement of nitrite and nitrate in venous blood samples of eight healthy volunteers showed concentrations of 578±116 nmol/l and 25±4 μmol/l, respectively.

4. Discussion

The present study describes a new HPLC method for measurement of nitrite in human blood, a specific marker of endogenous NO production. To our knowledge, this is the first HPLC method which allows reliable quantification of nitrite in human blood. Considering the activity of the constitutive NO synthase, biologically relevant concentrations of NO and, consequently, nitrite can be estimated to be in the nanomolar range [2,6]. The combination of the specifically developed sample preparation with high-performance anion-exchange chromatography and electrochemical detection yields an extraordinary low detection limit of 3 nmol/l in aqueous solution and 60 nmol/l in human blood which is below the concentrations expected under normal or pathological conditions in vivo. However, determination of nitrite in this low concentration range is rendered difficult by nitrite contamination of laboratory ware and solutions which at least in part may account for the calculated intercept of the calibration curves. Therefore, calibration and determination of nitrite contamination of the used laboratory ware and solutions were calculated daily to make a reliable calculation of the nitrite concentration in the biological sample, e.g. human blood, possible.

There were two major problems inherent to sample preparation which prevented the reliable measurement of nitrite in human blood by means of HPLC to date: (i) the rapid metabolism of NO to nitrite and nitrate must be arrested immediately during sampling and (ii) sufficient, complete protein removal to ensure a sufficient lifespan of the columns without loss of nitrite and nitrate. To solve these problems, we alkalized the human blood with an equal volume of 0.1 mol/l sodium hydroxide solution, thus immediately disrupting all blood cells and irreversibly denaturing all proteins. Subsequent centrifugation and ultrafiltration of the alkaline-arrested samples prevented the loss of nitrite during the preparation process. This deproteinisation method was chosen because commonly used acidic denaturation would have rapidly converted nitrite to NO with its subsequent loss into the gaseous atmosphere.

Furthermore, it was important to consider that human blood contains chloride and bromide in the micromolar concentration range [7,8]. Both ions share some UV-absorption characteristics with nitrate. In addition, both ions can be oxidized if high potentials are used for electrochemical detection. Therefore, we selected an anion-exchange column and a buffer system especially adapted to the analysis of samples containing high amounts of chloride and bromide anions to prevent interference of both anions with the nitrite/nitrate peaks in the ECD or in the UV detector signal.

In addition to quantification of nitrite, the simultaneous determination of nitrate may serve as a useful adjunct when nitrate balance and excretion studies are desired [9]. In our methodological setup the use of two in-line connected detectors was an important prerequisite to demonstrate the separation of nitrite and nitrate and other anions in human blood. Moreover, the simultaneous determination of the two degradation products of NO was necessary to measure the interruption in the metabolic breakdown from nitrite into nitrate by the developed stop solution.

Several methods have been developed to estimate endothelial synthesis of NO or nitrite using photometric assays such as the Griess reaction or the hemoglobin assay [10–13], chemiluminescence [14], HPLC [15–17] and capillary electrophoresis [18,19]. However, these methodological approaches can only be applied to experimental approaches in which aqueous buffer solution are present. In contrast, the determination of NO or nitrite in human blood has been impossible until very recently. Vallance et al. [20] adapted a porphyrinic microsensor for measurement of NO in human veins. However, measurement

of NO by either Clark-type electrode or the porphyrinic sensor is highly susceptible to motion artifacts due to changes in flow velocity. Furthermore, the signal of the one-electron transfer reflects the NO production only in the immediate vicinity of the probe, but does not allow calculation of NO formation throughout the systemic or organ circulation.

Rhodes et al. [6] were the first to exactly measure nitrite in human blood derived from L-arginine using a sophisticated GC–MS assay, which, however, does not appear to be applicable to routine analysis in clinical settings. The concentrations of nitrite in human blood are in the same order of magnitude as measured in the present study. The HPLC technique presented herein can be reproduced with an affordable effort and may thus represent a suitable alternative for measuring nitrite and nitrate as metabolites of NO in human blood.

In conclusion, we have developed an HPLC assay that allows quantification of nitrite in human blood, a marker of endogenous NO production in human blood. Due to its high sensitivity, this assay offers the possibility to analyze the activity of the constitutive NO synthase, which produces NO in the low nanomolar range. Thus, this assay will be suitable for further studies of (patho-)physiological or pharmacological alterations of NO production in humans.

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