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Adaptation of the Griess Reaction for Detection of Nitrite in Human Plasma

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The determination of nitrite in human plasma or serum has been most frequently used as a marker of nitric oxide (NO) production. In addition, it has recently been suggested that nitrite could act as a vasodilating agent at physiological concentrations by NO delivery. Therefore, nitrite determination in biological fluids is becoming increasingly important. The most frequently used method to measure nitrite is based on the spectrophotometric analysis of the azo dye obtained after reaction with the Griess reagent. This method has some limitations regarding detection limit and sensitivity, thus resulting unsuitable for nitrite detection in plasma. We have identified some drawbacks and modified the original procedure to overcome these problems. By the use of the newly developed method, we measured 221 ± 72 nM nitrite in human plasma from healthy donors.

Keywords: Nitrite; Nitric oxide; Griess reaction; Human plasma

INTRODUCTION

Circulating nitrite (NO_2^-) is known to be a vasodilator and carrier of nitric oxide (NO), but its physiological role in NO-derived vascular regulation is still unclear. In particular, there is a general agreement that nitrite anions are direct or indirect (through NO release) vasodilators at higher physiological concentrations.[1,2] Plasma nitrite is present at submicromolar concentrations with a half-life of 100 s, being both removed by renal filtration and oxidized to nitrate by oxyhemoglobin.[3] The metabolic pathways that may regulate circulating nitrite concentrations have not been elucidated, but the possibility that nitrite could be a storage molecule for active NO (present at concentrations of one to two orders of magnitude over S-nitrosothiols and/or other supposed NO-carrier molecules) has focused the attention of many researchers. Mechanisms for the *in vivo* conversion of nitrite into NO have been examined: acidic disproportionation and reduction by xanthine oxidase have been proposed as main possible mechanisms.[4–6] However, some particular conditions (e.g. acidosis) and a nitrite concentration much higher than that found in the human plasma seem to be necessary to deliver enough NO from nitrite to produce vasodilation.[7,8] More recently, it has been shown that nitrite infusion into the human forearm brachial artery also causes a dose-dependent regional blood flow increase when near physiological nitrite concentrations are used (i.e. 2 μM).[9,10] The authors reported that this phenomenon is due to the interaction of nitrite with deoxyhemoglobin: the nitrite reductase activity of deoxyHb produces NO, which is not subjected to rapid reaction with ferrous Hb but can escape red cells and evoke vasodilation. These findings have focused on the importance of nitrite in the circulation and, though most of the regulative pathways are still obscure, it will be a future target for researchers in the field to determine whether and at what extent a modulation of blood nitrite levels can be correlated with the occurrence of pathologies at cardiovascular level. For this purpose, an accurate and precise nitrite detection in biological

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fluids is fundamental. A lot of methods have been proposed for NO_2^- measurement, e.g. flow injection combined with the Griess reaction,[11] chemiluminescence,[9] fluorescence,[12] chromatographic methods.[13–15] Most of them are time-consuming and/or require instruments that are unlikely in routine (clinical)-analysis laboratories. Moreover, many of these procedures require sample ultrafiltration, a step that has been reported to artificially increase measured nitrite levels because of delivery of nitrite from ultrafiltration devices.[16,17] The most frequently used method is based on the spectrophotometric analysis by the Griess reaction.[18] It consists in the reaction of nitrite under acidic conditions with aromatic amines in two steps, thus generating a purple azo dye. It is wide-spread, since it can be easily applied in every laboratory without a large and expensive analytical set up. However, this method has some limitations mainly owing to scarce sensitivity and high detection limit, thus producing unreliable results, and its use for nitrite detection in human plasma has been now almost completely abandoned. The main problem is probably represented by the presence of large quantities of proteins and/or other molecules that could compete with sulfanilamide for NO^+ binding.[15] This, coupled with the low concentration of nitrite in blood, render nitrite detection in blood/plasma by the Griess method very hard. In this work, we have tried to address these probable drawbacks of the Griess reaction by modifying the procedure to make it suitable for nitrite detection in human plasma.

MATERIAL AND METHODS

Materials

Sulfanilamide, N-ethylmaleimide (NEM), N-(1-naftil)-ethylendiamine, trichloroacetic acid (TCA), and all other reagents of highest purity available were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The saturated alcoholic sulfanilamide solution was obtained by slowly adding sulfanilamide to a stirring solution of 99% (v/v) ethanol until visible undissolved grains of sulfanilamide were present in the solution also after vigorous shacking. Dialysis tubing cellulose membrane (MW 12,400 cut off) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim). Before using, the membrane has been extensively washed (about 4 h) with an abundant volume of MilliQ water.

Blood Collection and Plasma Separation

Human blood was obtained from healthy volunteers after informed consensus by venipuncture, using K_3EDTA as an anticoagulant. Blood was collected and rapidly centrifuged at 15,000*g* for 10 s to separate plasma. This rapid plasma separation was shown not to provoke any visible red cell hemolysis.

Nitrite Detection

After separation, 600 μl of plasma was immediately treated with 10 μl of 310 mM NEM, 300 μl of the saturated alcoholic sulfanilamide solution, and finally 150 μl of a solution containing 6% (w/v) sulfanilamide, 0.2% (w/v) N-(1-naftil)-ethylendiamine (NED), and 6% (v/v) phosphoric acid. After 5 min, 80 μl of 60% (w/v) TCA was added. A clear supernatant was then obtained by centrifugation at 15,000*g* for 3 min. The supernatant was transferred into optical glass cuvettes, and the 450–700 nm spectrum was recorded. A blank spectrum was obtained by treating plasma samples as previously described except that NED was omitted from the final solution. This procedure was compared with the conventional Griess assay.[18] Briefly, a solution containing 1% and 0.1% (w/v) sulfanilamide and NED, respectively, acidified with 2% (v/v) phosphoric acid was prepared, plasma samples were treated 1:1 with this solution, and the dye was allowed to develop for 15 min. All the solutions were freshly prepared before every experiment. Before starting each series of experiments spectra were recorded under the same experimental conditions, but substituting plasma with 600 μl MilliQ water to evaluate the possible interference of nitrite present as a contaminant. All experiments have been carried out at room temperature, unless differently indicated. All the reagent preparations gave negligible spectra under these conditions. A Jasco model V530 spectrophotometer and 2 cm path length cuvettes were used.

RESULTS

The first modification we set on is the use of 2-cm path length optical cuvettes, with the aim of decreasing the detection limit of the method, given the low nitrite concentration in human plasma.[17]

The first problem we encountered was that the addition of the conventional Griess reagent to a plasma sample[18] produces high turbidity, probably owing to denaturation of proteins by phosphoric acid contained in the solution. Only a large plasma dilution with the reagent minimized this phenomenon, but such a dilution was not compatible with the low concentration of nitrite, hampering its titration (not shown). Therefore, for a correct spectrum recording, plasma proteins needed to be removed by further acidification with TCA followed by centrifugation. In a first set of experiments, we treated plasma samples 1:1 with the conventional

Griess solution, and then we added 80 µl of 60% (w/v) TCA. The clear supernatant was tested for nitrite content; the resulting spectra revealed only a small peak at 540 nm (not shown), not corresponding to a nitrite concentration of 200–500 nM as was expected to be found in human plasma.[17] Some plasma samples were then extensively dialyzed against phosphate buffered saline solution and loaded with 500 nM nitrite to yield samples with known NO_2^- concentration. Spectra were then recorded on the clear supernatant obtained as previously described. Also in this case the absorbance was largely inferior than expected (Fig. 1, trace a). One of the main reasons for this inability to measure nitrite effectively could be represented by a coprecipitation of the azo dye together with plasma proteins, probably due to an aspecific binding to albumin. This can be easily verified: when higher concentrations of nitrite were added to plasma samples and treated with the Griess reagent, the successive TCA addition and centrifugation to remove proteins yielded a visible pink precipitate, suggesting that most of the dye was co-precipitated with proteins themselves. A successive crucial step was therefore to avoid this aspecific azo dye binding to proteins during protein precipitation.

We tested several different conditions and concluded that treatment of plasma with an alcoholic saturated solution of sulfanilamide and then with a concentrated aqueous solution of sulfanilamide, NED, and phosphoric acid represented the best condition (Fig. 1, trace b). The spectrum obtained for plasma samples dialyzed and loaded with 500 nM nitrite shows that this modified procedure largely increased the absorbance (Fig. 1, trace b). By the use of the same procedure, we assessed that the addition of 70 to 1400 nM nitrite to human, dialyzed plasma resulted in a dose-dependent absorbance increase in the 500–600 nm region (Fig. 2A). Plotting data obtained from this experiment, a linear ($r^2 = 0.997$) correlation between nitrite concentration and absorbance at 540 nm was observed (Fig. 2B).

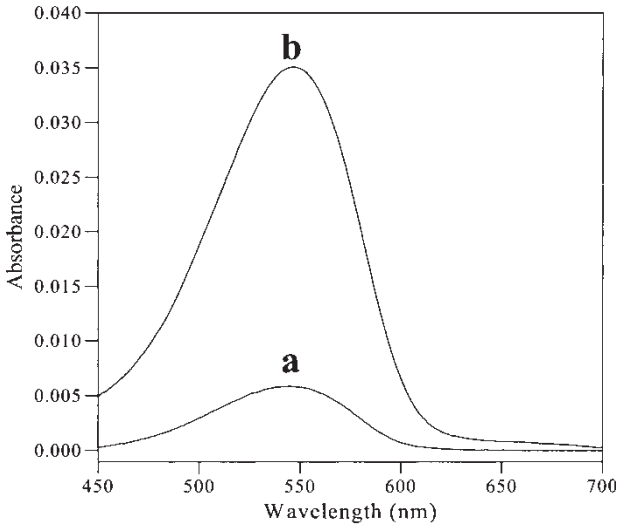

FIGURE 1 Nitrite determination after standard nitrite solution (500 nM) addition to human plasma. Human plasma was dialyzed for 4 h against a large volume of Na^+/K^+ phosphate buffered saline (145 mM NaCl, 10 mM Na^+/K^+ phosphate, pH 7.4). Successively, 500 nM nitrite (50 µM in H_2O) was added to 600 µl plasma pretreated with 5 mM NEM (300 mM in H_2O) and detected by the conventional Griess reaction (A) or by our modified procedure (B). Both traces are representative of five different experiments.

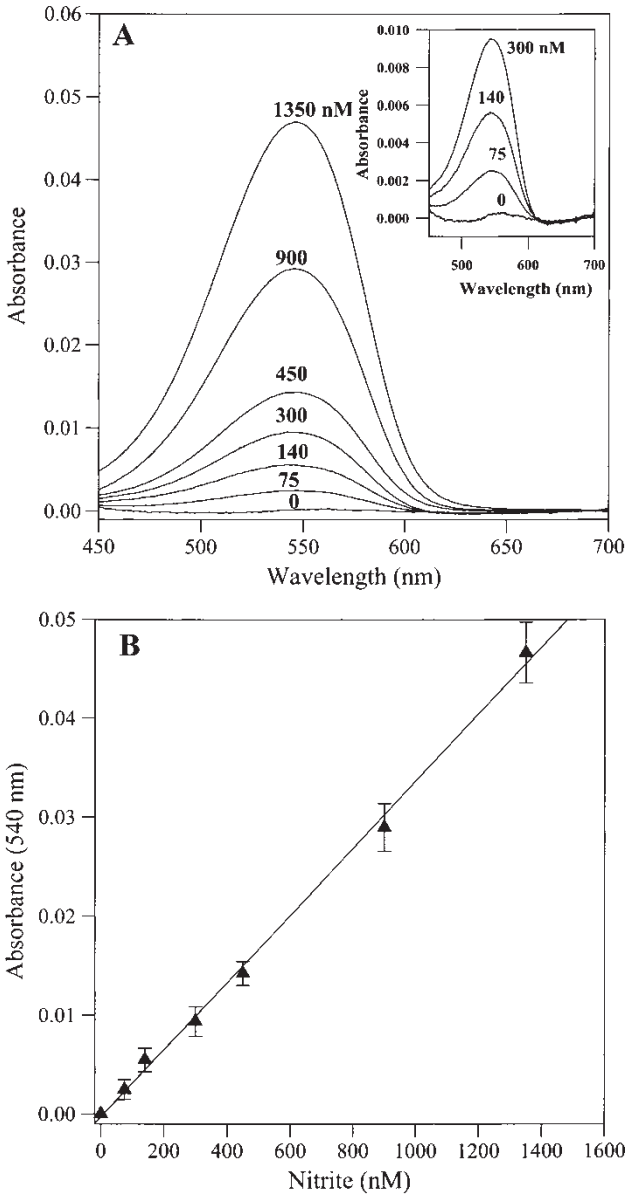

FIGURE 2 Standard curves obtained by addition of nitrite solutions (0 to 1400 nM) to human plasma. Human plasma was dialyzed for 4 h against a large volume of Na^+/K^+ phosphate buffered saline (145 mM NaCl, 10 mM Na^+/K^+ phosphate, pH 7.4). Successively, known amounts of nitrite were added to 600 µl of human plasma samples pretreated with 5 mM NEM. Nitrite was then determined by our method. A: spectra obtained with samples loaded with different nitrite concentrations (0–1400 nM); inset shows the lowest nitrite additions. B: standard curve obtained. Values are means ± SD of 10 measurements.

The calculated extinction coefficient was 34,500 $M^{-1} cm^{-1}$ which is a value very close to that we obtained for nitrite in aqueous solutions (41,000 $M^{-1} cm^{-1}$). We next tested the modified Griess method to detect nitrite in human plasma; 12 samples were thereby analysed and the mean nitrite concentration obtained was 221 ± 72 nM (Fig. 3). The intra-assay coefficient of variation obtained from repetitive measurements on the same sample was less than 6%. Finally, since the time elapsing between blood collection and sample treatment could be of fundamental importance, because nitrite is transformed into nitrate by reaction with hemoglobin,[3] we measured the time course of nitrite levels after blood collection. Blood samples were loaded with extra nitrite to a final concentration of 2.5 μM (we started with a supraphysiological nitrite concentration to better appreciate the kinetic of its disappearance), and plasma nitrite concentration was measured with time. We observed a rapid nitrite consumption with a $t_{1/2}$ close to 3 min; after 60 min, nitrite concentration was negligible (Fig. 4). To confirm these data, samples previously analyzed for nitrite concentration (Fig. 3) were re-assayed after 120 min from drawing, but none visible peak could be detected (not shown).

Finally, to confirm that the recorded spectra resulted from the presence of nitrite, 600 μl of freshly obtained plasma samples were pre-treated with 50 μl of NH_4-sulfamate (200 μM in water), acidified at pH 3.5 with a concentrated (85% v/v) solution of phosphoric acid, and then measured with our proposed method. In all the analysed samples ($n = 5$) no evident peak was obtained under the 450–700 nm wavelength range (data not shown).

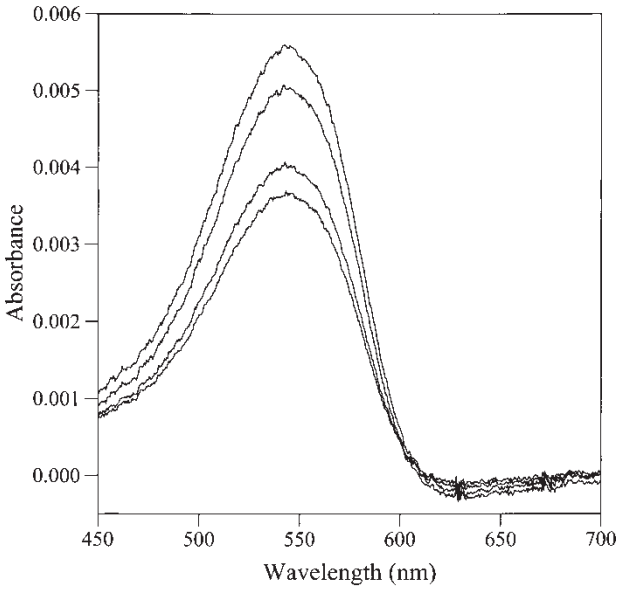

FIGURE 3 Nitrite in human plasma. Blood was obtained from healthy donors and plasma was immediately separated and pretreated with 5 mM NEM (300 mM in H_2O), then nitrite determination was carried out. Exemplificative spectra from four blood donors are shown.

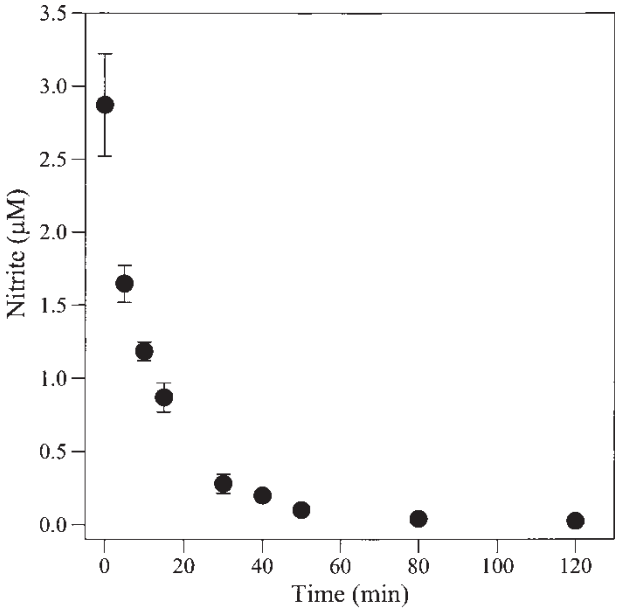

FIGURE 4 Time course of nitrite consumption in plasma after blood collection. Blood was obtained from healthy donors and kept at 37°C under gentle rotation. It was loaded with 2.5 μM nitrite (50 μM in saline physiological solution). At indicated times after blood treatment plasma was separated, pretreated with 5 mM NEM (300 mM in H_2O), and then 600-μl plasma aliquots were analyzed for nitrite content by our developed procedure ($n = 5$).

DISCUSSION

The interest in the quantitative measurement of nitrite in biological fluids has recently been increased exponentially by the identification of nitrite role in the regulation of vasal tone (see, for review Refs. [9,10]). Since nitrite levels under different pathological conditions and, moreover, how diet, lifestyle, age, circadian rhythm, and other physiological situations can influence nitrite blood concentration are far from being effectively established, inexpensive and not time-consuming methods are needed for its large-scale determination. Commonly used procedures to detect nitrite in human plasma are represented by HPLC, capillary electrophoresis, DAN fluorescence method, chemiluminescence, and flow injection methods.[9,11–15,19,20] In analogy to what frequently happens for other NO-related molecule detection (e.g. S-nitrosothiols), not all methods appear free from artefacts; moreover, values obtained by different procedures are frequently discordant.[15,21] The Griess reaction is surely a widely known method to detect nitrite in biological samples, however, some interfering reactions and its low detection limit render it unsuitable for being used with human plasma.[12,15] The first step in the Griess procedure requires the reaction of nitrite with aromatic amines

such as sulfanilamide. For this reaction to take place, the sample must be acidified to yield NO^+ from nitrite. This could represent a weak point in that some nucleophilic groups, in particular protein or non protein SH groups, but also tryptophan, tyrosine, and amino groups, can interfere by their binding with NO^+.[15] SH groups can be easily blocked before acidification by reaction with NEM, while the other reactions are not easily prevented, though they appear to take place more slowly.[15] In addition to the above-mentioned interfering reactions, we also observed that the azo dye resulting from the reaction of acidified nitrite with sulfanilamide and NED co-precipitates with proteins (Fig. 1). Some of these problems can be avoided by sample ultrafiltration,[12] but this step can be time-consuming and, moreover, some additional nitrites can be artifactually generated.[16,17]

We thereby developed a new method for nitrite detection in human plasma by modifying the Griess reaction. Our proposed method couples the simplicity and rapidity of the original Griess reaction with the possibility to eliminate interfering reactions and decrease the detection limit. First of all, 2-cm path length optical cuvettes were used, and free thiols were blocked by treating plasma with NEM. Then, to the samples we added an ethanol solution saturated with sulfanilamide and, finally, an aqueous acidified solution containing sulfanilamide and NED. These modifications, as a whole, appeared fundamental in that greatly increased the absorbance at 540 nm (Fig. 1) when a standard nitrite solution (500 nM) was added to plasma samples. The use of a high sulfanilamide concentration probably allows to overcome the problems resulting from the competing reaction of nitrite with other molecules and in particular with the unspecific binding to albumin. The use of an alcoholic solution decreases the unspecific protein binding to the dye once colour develops, thus preventing the azo dye co-precipitation with proteins. We tested the method in the physiological concentration range of nitrite (100–1000 nM)[17] and found a linear relationship between absorbance and nitrite concentration (Fig. 2). The concentration of nitrite present in human plasma from healthy donors measured with our proposed method was 221 ± 72 nM, in good agreement with recent findings.[20]

Finally, we used this proposed method to study the kinetics of nitrite consumption after blood drawing. Obtained data ($t_{1/2} = 180$ s) suggested that it is of fundamental importance to immediately separate plasma and measure nitrite after blood collection.

In conclusion, we have developed a spectrophotometric assay that allows quantification of nitrite in human plasma. We can infer that our suggested protocol could be also applied (with some modifications) for other NO-derived molecules, such as S-nitrosothiols (RSNOs). The unspecific bind of the Griess reagents to NOx allows the detection of different compounds depending on sample manipulation. However, even if the determination of total NOx can be easily performed, we can expect that the measurement of each single compound is much more difficult. It is known, for example, that plasma RSNO levels are very low, about one order lower than nitrite,[21] thus their determination could be hardly performed with our method. In any case, since nitrite can be eliminated by sulfamate incubation, our method could be helpful to measure S-nitrosothiols under particular conditions where their levels are increased (e.g. S-nitrosothiol infusion) and/or to verify that their basal concentrations are under the detection limit. Further analyses are necessary, anyway, to verify these possible additional applications of the method.

Thanks to its low detection limit (close to 50 nM), rapidity, and simplicity, we suggest this assay could be suitable for studies of (patho) physiological or pharmacological alterations of nitrite levels in human blood. In fact, even if routine analyses laboratories are not generally equipped with spectrophotometers with 2-cm pathlength cells, we think this upgrade should be arranged easily and at low cost thus allowing large scale determinations.

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