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Stable-isotope dilution GC–MS approach for nitrite quantification in human whole blood, erythrocytes, and plasma using pentafluorobenzyl bromide derivatization: Nitrite distribution in human blood☆

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ABSTRACT

Previously, we reported on the usefulness of pentafluorobenzyl bromide (PFB-Br) for the simultaneous derivatization and quantitative determination of nitrite and nitrate in various biological fluids by GC–MS using their ^{15}N-labelled analogues as internal standards. As nitrite may be distributed unevenly in plasma and blood cells, its quantification in whole blood rather than in plasma or serum may be the most appropriate approach to determine nitrite concentration in the circulation. So far, GC–MS methods based on PFB-Br derivatization failed to measure nitrite in whole blood and erythrocytes because of rapid nitrite loss by oxidation and other unknown reactions during derivatization. The present article reports optimized and validated procedures for sample preparation and nitrite derivatization which allow for reliable quantification of nitrite in human whole blood and erythrocytes. Essential measures for stabilizing nitrite in these samples include sample cooling (0–4 °C), hemoglobin (Hb) removal by precipitation with acetone and short derivatization of the Hb-free supernatant (5 min, 50 °C). Potassium ferricyanide ($K_3Fe(CN)_6$) is useful in preventing Hb-caused nitrite loss, however, this chemical is not absolutely required in the present method. Our results show that accurate GC–MS quantification of nitrite as PFB derivative is feasible virtually in every biological matrix with similar accuracy and precision. In EDTA-anticoagulated venous blood of 10 healthy young volunteers, endogenous nitrite concentration was measured to be 486 ± 280 nM in whole blood, 672 ± 496 nM in plasma (C_P), and 620 ± 350 nM in erythrocytes (C_E). The C_E-to-C_P ratio was 0.993 ± 0.188 indicating almost even distribution of endogenous nitrite between plasma and erythrocytes. By contrast, the major fraction of nitrite added to whole blood remained in plasma. The present GC–MS method is useful to investigate distribution and metabolism of endogenous and exogenous nitrite in blood compartments under basal conditions and during hyperemia.

1. Introduction

The major fraction of endogenously produced nitric oxide (NO) is oxidized to nitrate (NO_3^-) within the erythrocytes by oxyhemoglobin (HbO_2) [1]. NO partly autoxidizes to nitrite (NO_2^-) which is also oxidized to nitrate by HbO_2 in erythrocytes. However, in human blood the half-life of nitrite is about 6000 times longer than that of NO, i.e., about 12 min versus <0.1 s (reviewed in Ref. [1]).

Nitrite and nitrate circulate in blood, both in plasma and in red blood cells, and they are excreted in the urine. Under certain conditions nitrate may be an indicator of systemic NO production [2]. Recently, circulating nitrite has been suggested to reflect endothelial NO synthesis (reviewed in Ref. [3]). Pioneer work showed that nitrate and nitrite are evenly distributed between plasma and blood cells [4]. However, recent studies reported that nitrite is present in red blood cells at higher concentrations than in plasma, i.e., that nitrite accumulates within the erythrocytes, as measured by chemiluminescence [5]. Others reported that nitrite distribution in blood compartments is dependent upon the bicarbonate/CO_2 concentration in the blood [6], but this was not confirmed [7]. Distribution of nitrite in blood may be of particular importance, as erythrocytes may not only oxidize nitrite to nitrate, but they may also reduce nitrite to NO in certain conditions such as hypoxia [8,9].

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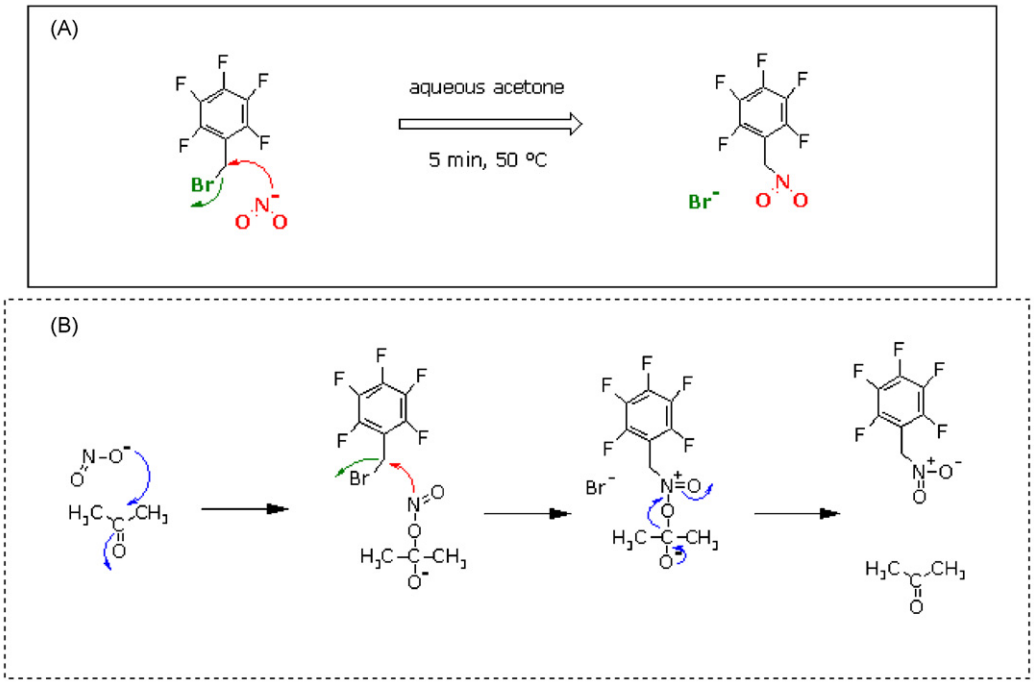

Scheme 1. (A) Alkylic nucleophilic substitution reaction between nitrite and pentafluorobenzyl bromide (α-bromo-pentafluorotoluene, PFB-Br) in aqueous acetone to form the pentafluorobenzyl derivative of nitrite (α-nitro-pentafluorotoluene) and bromide. (B) Proposed mechanism for acetone-catalyzed nucleophilic substitution of bromide in PFB-Br by nitrite in aqueous solution.

Thus, erythrocytic nitrite could be regarded as a storage form of NO-bioactivity within the vasculature. However, NO-related bioactivity of nitrite could be due to alternative Hb-independent mechanisms, such as the involvement of carbonic anhydrase activity [10,11]. The observations regarding nitrite's potential to mediate NO-related actions renewed the scientific interest in this inorganic anion, beyond its methemoglobinemic potential.

From the analytical point of view, blood is a problematic matrix for numerous analytes that are unstable in blood. For the measurement of endogenous and exogenous substances plasma or serum is preferentially used instead of whole blood. If nitrite is distributed unevenly between plasma and erythrocytes depending upon experimental conditions including kind of anticoagulation agent and blood concentration of bicarbonate/CO_2, measurement of nitrite in whole blood instead of plasma or serum could represent a more reliable method to assess NO synthesis in vivo. Nitrite has been determined by chemiluminescence in whole blood of healthy humans at a basal concentration of about 180 nM [5,12]. It has been reported that the use of potassium ferricyanide (i.e., $K_3Fe(CN)_6$) at very high concentrations (e.g., 800 mM), i.e., at a very high molar excess over erythrocytic Hb (about 10 mM), is necessary and sufficient to "stabilize" nitrite in blood until final detection.

We previously showed that pentafluorobenzyl (PFB) bromide (PFB-Br) is a useful agent for simultaneous derivatization and accurate quantification of nitrite and nitrate in several biological matrices by GC–MS in the electron-capture negative-ion chemical ionization (ECNICI) mode [13] (Scheme 1). Analyzed biological samples included plasma, serum, urine, saliva, and cell culture supernatants. However, the original procedures were not applicable to whole blood and erythrocytes because of considerable oxidation of endogenous nitrite and of the externally added internal standard ^{15}N-labelled nitrite. GC–MS methods for nitrite and nitrate have recently been reviewed by Helmke and Duncan [14]. By using PFB-Br as the derivatization agent, the aim of the present study was to develop a GC–MS method that allows accurate quantitative determination of nitrite in human whole blood and erythrocytes. By means of this method the distribution of endogenous and exogenous nitrite in blood plasma and erythrocytes of healthy subjects was investigated.

2. Experimental

2.1. Materials and chemicals

2,3,4,5,6-Pentafluorobenzyl bromide, sodium nitrite (purity 99.99 + %), sodium [^{15}N]nitrite (declared as 99 at.% at ^{15}N), and potassium hexacyanoferrate(III) (potassium ferricyanide, $K_3Fe(CN)_6$, purity > 99%) were obtained from Sigma–Aldrich (Steinheim, Germany). Toluene was purchased from Baker (Deventer, The Netherlands) and acetone was from Merck (Darmstadt, Germany). EDTA vacutainer tubes used for blood sampling were obtained from Sarstedt (Nümbrecht, Germany).

As with any trace analysis, contamination of equipment or reagents with nitrite is a serious pitfall in the analysis of nitrite in biological fluids [14]. For the derivatization of nitrite and the subsequent extraction and GC–MS analysis we tested two commercially available glass vials (2 ml) and polypropylene Eppendorf tubes (1.8 ml) for potential nitrite contamination. For safety reasons we decided to use closely lockable autosampler glass vials from Macherey-Nagel (Düren, Germany) and Agilent Technologies (Waldbronn, Germany). Crimp vials (N11-1 HP clear) and crimp caps (N11 TB/0A-0.9) were from Macherey-Nagel. Snap top vials (5182-0544) and snap caps (5182-0550) were from Agilent Technologies. Contaminating nitrite was quantified by analyzing unspiked and spiked quality control pooled human plasma by GC–MS [13]. Fig. 1 shows that the use of crimp vials and caps was associated with considerably less nitrite contamination than snap top vials and caps. Similar results were obtained for nitrite in unspiked and spiked quality control pooled human urine (data not shown). Because of these findings crimp vials and crimp caps were used exclusively in the present study.

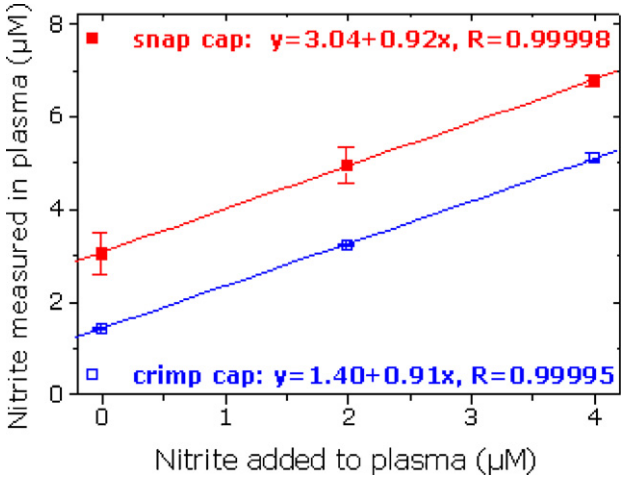

Fig. 1. Contribution of contaminating nitrite from glassware and septa to plasma nitrite. Relationship between measured and added nitrite in quality control pooled human plasma using (i) crimp vials and crimp caps or (ii) snap top vials and snap caps for derivatization and GC–MS analysis. Insets show the regression equations from linear regression analysis between measured (y) and added (x) nitrite. Plasma samples (100 μl) were spiked with nitrite at the indicated concentrations and with the internal standard [^{15}N]nitrite at a fixed final added concentration of 4 μM. Derivatization (5 min, 50 °C) and GC–MS analysis of nitrite were performed as described [13].

1) Prepare mixtures under vortexing and store in ice bath	400 μL acetone 10 μL 8 M $K_3Fe(CN)_6$ 10 μl 50 μM $Na^{15}NO_2$
2) Add the samples	100 μL of sample (stored on ice)
3) Vortex and store in ice bath	5 s
4) Centrifuge	800×g, 4 °C, 5 min
5) Decant the supernatants	300 μL aliquots
6) Add PFB-Br and vortex	10 μL, 5 s
7) Derivatize nitrite	5 min at 50 °C
8) Evaporate acetone	N_2 stream, ca. 5 min
9) Extract reaction products	1-min vortexing with 1000 μL toluene
10) Centrifuge	800×g, 4 °C, 5 min
11) Decant the supernatants	700 μL aliquots
12) Analyse by GC-MS (1-μL aliquots)	splitless injection ECNICI SIM (50 ms each) of *m/z* 46 and *m/z* 47

Scheme 2. Schematic of the procedures used in the GC–MS quantitative determination of nitrite in human whole blood, erythrocytes and plasma samples. For nitrite analysis in plasma, steps 4 and 5 are omitted. In the blood samples, the final concentration of the internal standard [^{15}N]nitrite is 5 μM and that of $K_3Fe(CN)_6$ 800 mM with respect to the sample volume (for more details, see the text).

2.2. Subjects, sample and derivatization procedures

Routinely, blood was drawn from antecubital veins of healthy volunteers using 9-ml syringes that contained EDTA as the anticoagulation agent. The monovette was gently shaken and put immediately in an ice bath. Erythrocytes and plasma were obtained by centrifugation (800 × g, 4 °C, 5 min). Erythrocytes and plasma fractions were stored separately in an ice bath until further treatment. Analyses were performed immediately. Approval from the local Ethics Committee of the Medical School of Hannover was obtained.

Analysis of nitrite in blood was performed with modifications of procedures previously developed, validated and applied to other matrices [13]. Scheme 2 shows the major steps of the procedure used in the present study for the quantitative determination of nitrite in human whole blood. An essential modification is that all materials including whole blood and the solvent acetone were stored in an ice bath during the various steps except for centrifugation (4 °C) and derivatization (50 °C). All steps until the first centrifugation for Hb removal (step 4 in Scheme 2) were carried out using 1.8-ml polypropylene tubes. For all subsequent procedures 2-ml autosampler glass vials were used. Deviations from these procedures are reported explicitly in the text.

2.3. Method optimization and validation

2.3.1. Effect of ferricyanide

The effect of ferricyanide on the concentration of nitrite in human whole blood was investigated by adding in duplicate various volumes (0–20 μl) of an 8 M stock solution of aqueous $K_3Fe(CN)_6$ to mixtures of acetone and [^{15}N]nitrite to achieve final added $K_3Fe(CN)_6$ blood concentrations in the range between 0 and 1600 mM. Subsequently, whole blood was added to the samples which were further processed as shown in Scheme 2.

2.3.2. Effect of erythrocytic hemoglobin

In previous work we found that plasmatic proteins do not affect the quantitative determination of nitrite in human plasma when measured by GC–MS as PFB derivative [13]. Potential effects of erythrocytic Hb on nitrite analysis in whole blood were investigated by storing acetone-treated blood samples for up to 30 min in an ice bath and by derivatizing the samples at 50 °C for 5 min in the presence (i.e., no centrifugation) and in the absence (i.e., after centrifugation and decantation of the supernatant) of plasmatic and erythrocytic proteins. In non-centrifuged samples, the entire acetone–blood mixture including the protein precipitate was subjected to derivatization. For centrifuged samples, 300-μl aliquots of the supernatants were decanted (step 5 in Scheme 2) and processed further.

2.3.3. Intra-assay validation of the method for nitrite in whole blood, erythrocytes and plasma

As inter-assay validation experiments of the method for nitrite in whole blood and erythrocytes samples are difficult to perform, we limited method validation to intra-assay experiments.

Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for nitrite in whole blood, erythrocytes and plasma were investigated by using a procedure considered to be optimum (see Scheme 2 and Section 3). Optimum experimental conditions include storage of samples in an ice bath, where applicable, use of potassium ferricyanide at a final concentration of 800 mM, and removal of proteins by centrifugation prior to derivatization. Intra-assay validation in whole blood was performed using a pooled whole blood (about 18 ml) donated by a healthy volunteer and a constant added final blood concentration of 5 μM for [^{15}N]nitrite. Unlabelled nitrite was added as 10-μl aliquots of solutions of sodium nitrite in distilled water at concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 80, 100 μM, resulting in

added relevant concentrations in the blood of 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 μM. Seven replicates were performed for unspiked and spiked blood samples for each nitrite concentration investigated.

Intra-assay validation in erythrocytes and plasma was performed using a pooled whole blood (about 16 ml) donated by a healthy volunteer. Blood was centrifuged immediately. Plasma was decanted, pooled and frozen at −80 °C until further analysis on next day. Erythrocytes were analyzed immediately. A constant added final concentration of 5 μM for [^{15}N]nitrite was used in both matrices. Unlabelled nitrite was added as described above for whole blood to achieve added nitrite concentrations of 0, 1, 2, 3, 4, 6, 8, 10 μM both in erythrocytes and in plasma samples. Each four replicates were performed for unspiked and spiked samples.

All samples in the validation experiments were treated by a single person within about 2 h in each experiment. All derivatized samples were analyzed by GC–MS on the same day overnight within a single run each (about 6 analyses per hour). Recovery (%) was calculated as: [(measured nitrite concentration − basal nitrite concentration): added nitrite concentration] × 100.

2.4. Kinetics of exogenous nitrite in whole blood

Venous EDTA blood from a healthy volunteer was used to study the kinetics of nitrite externally added to whole blood. Immediately after blood draw, an aliquot of 8 ml pooled whole blood was transferred into a 12-ml polypropylene tube and put in an ice bath for about 20 min; from time to time the blood sample was shaken gently. A 10-μl aliquot of an aqueous 10-mM nitrite solution was added to the blood under gentle vortexing to achieve an added whole blood nitrite concentration of about 10 μM. Prior to transferring the spiked blood sample to a water bath, constantly held at 37 °C under gentle shake, two 100-μl aliquots and one 700-μl aliquot of blood were taken to determine nitrite concentration at time "zero" in whole blood, plasma and erythrocytes, respectively. After 5, 10, 20, 30, 45 and 60 min of incubation in the water bath each two 100-μl aliquots and one 700-μl aliquot of blood were taken to determine nitrite concentration in the respective matrices. For measurement of nitrite in plasma and erythrocytes, the 700-μl aliquots of blood were immediately centrifuged (800 × *g*, 5 min, 4 °C). Whole blood, erythrocytes and plasma samples were analyzed in duplicate following the same procedure shown in Scheme 2. [^{15}N]Nitrite was used at a constant added final concentration of 5 μM in all matrices.

2.5. Kinetics of endogenous nitrite in whole blood and plasma during reactive hyperemia

In order to test the utility of the proposed method for assessing changes in concentration of endogenous nitrite in blood upon intervention, we quantitated nitrite in whole blood and plasma during reactive hyperemia, after stimulation of endothelial NO synthase by shear stress, in a healthy volunteer. Local circulatory stasis was induced by inflation (240 mm Hg, 5 min) of a blood pressure cuff placed around the upper arm. Whole blood and plasma nitrite concentrations in the draining antecubital vein were measured (in triplicate) at rest, immediately after ischemia, and every 10 s during a 1-min period. Blood was drawn from a catheter placed in the antecubital vein and collected in 5-ml heparinized monovettes. Immediately, at the bedside, blood samples were processed for analysis of whole blood and plasma nitrite (Scheme 2). Nitrite concentration in erythrocytes (C_E) was calculated from measured whole blood (C_B) and plasma (C_P) concentrations (see Section 3). During the experiment, forearm blood flow was measured by impedance plethysmography (NICCOMO MEDIS GmbH, Ilmenau, Germany).

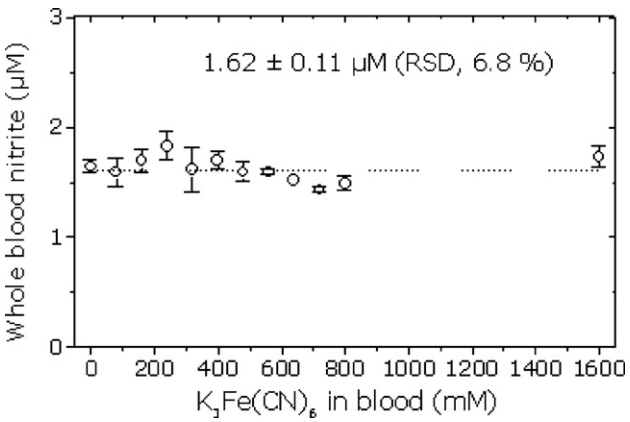

Fig. 2. Effect of various concentrations of $K_3Fe(CN)_6$ added to blood (calculated final concentrations are indicated) on the concentration of endogenous nitrite in whole blood samples of a volunteer. A single whole blood sample (about 9 ml) stored in an ice bath was used for the entire experiment. Sample treatment was performed according to the procedure shown in Scheme 2 and as described in Section 2. The internal standard [^{15}N]nitrite was used at a calculated final added concentration of 5 μM in the blood. Derivatization was performed after removal of acetone-precipitated plasmatic and erythrocytic proteins by centrifugation. The horizontal line indicates the mean nitrite concentration if all samples analyzed are considered.

2.6. Gas chromatography–mass spectrometry

GC–MS analyses were performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). A fused-silica capillary column Optima-17 (15 m × 0.25 mm i.d., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots (1 μl) of the toluene phases were injected in the splitless mode and quantification was performed by selected-ion monitoring (SIM) of *m*/*z* 46 for [^{14}N]nitrite and *m*/*z* 47 for [^{15}N]nitrite with a dwell time of 50 ms for each ion (Scheme 2). The following oven temperature program was used with helium as the carrier gas at a constant flow rate of 1 ml/min: 1 min at 70 °C, then increased to 135 °C at a rate of 30 °C/min, and to 280 °C at a rate of 70 °C/min; the oven temperature of 280 °C was held for 1 min. Interface, injector and ion source were kept at 260, 200 and 250 °C, respectively. Electron energy and electron current were set to 70 eV and 100 μA, respectively, for ECNICI with methane as the reagent gas at a flow rate of 2.4 ml/min.

3. Results

3.1. Optimization and validation of the method for whole blood, erythrocytes and plasma nitrite

3.1.1. Effect of ferricyanide on endogenous whole blood nitrite

Ferricyanide has been suggested and used as a stabilizing agent in a chemiluminescence-based analysis of nitrite in whole blood [5,12]. In the present study we investigated the effect of ferricyanide (0–1600 mM) on the concentration of endogenous nitrite in a pooled whole blood sample of a healthy volunteer using the procedure described in Scheme 2. Fig. 2 shows that ferricyanide had no significant effect on the nitrite concentration measured in the whole blood. Considering all samples of this experiment, a mean nitrite concentration of 1.62 μM (RSD, 6.8%) is calculated in the pooled blood. The area of the peak of the internal standard did not change significantly upon addition of ferricyanide (data

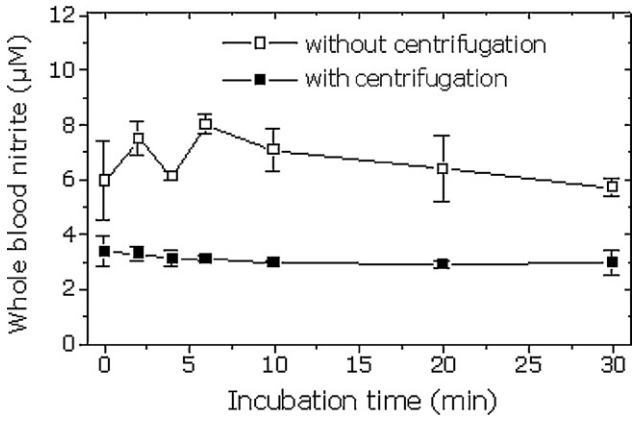

Fig. 3. Concentration of endogenous whole blood nitrite measured by PFB-Br derivatization in the presence (without centrifugation) and in the absence (with centrifugation) of plasmatic and erythrocytic proteins. Incubation time refers to the time of storage of acetone-treated blood samples in the ice bath until further treatment. A pooled whole blood sample of a healthy volunteer was used for all samples which were analyzed in duplicate. The calculated final concentration in the blood of [^{15}N]nitrite and $K_3Fe(CN)_6$ was 5 μM and 800 mM, respectively.

not shown), suggesting that $K_3Fe(CN)_6$ did not interfere with the derivatization of nitrite by PFB-Br under the conditions of the procedure used, i.e., in the absence of Hb which had been removed by centrifugation prior to derivatization.

In addition, 100-μl aliquots of physiological saline in phosphate buffer (100 mM, pH 7.4), which was measured to contain about 450 nM of nitrite by GC–MS, were incubated with PFB-Br and heated at 50 °C for up to 60 min in the absence and in the presence of 800 mM of $K_3Fe(CN)_6$. In both cases, all samples remained colorless and very similar, statistically insignificantly different nitrite concentrations were measured, even after a 60-min incubation time (i.e., 460 ± 35 nM after 60 min versus 458 ± 25 nM at time zero, each $n = 2$), strongly suggesting that $K_3Fe(CN)_6$ itself does not affect at all determination of nitrite as PFB derivative by this GC–MS method in Hb-free aqueous buffer. For these reasons and in order to be able to compare our results with those reported by other groups having used ferricyanide in their analyses, we decided to use $K_3Fe(CN)_6$ in quantitative nitrite analyses (step 1 in Scheme 2).

3.1.2. *Effect of erythrocytic hemoglobin*

Fig. 3 shows that PFB-Br derivatization of nitrite in whole blood in the presence of precipitated plasmatic and erythrocytic proteins yields considerably and constantly higher nitrite concentrations as compared to deproteinized samples, i.e., in the absence of acetone-precipitated proteins. Also, this figure shows considerable variation in nitrite concentration in non-centrifuged blood samples, i.e., notably in the presence of precipitated Hb. Fig. 3 suggests that the differences in nitrite concentrations measured in centrifuged and non-centrifuged blood samples are likely to be due to interferences by proteins, even though being precipitated. These interferences seem to be largely independent of the incubation time of acetone-treated blood samples in the ice bath. It is more likely that proteins contribute to nitrite during the derivatization step, i.e., during sample heating for 5 min and 50 °C. It is worth mentioning that in all GC–MS analyses from blood samples derivatized in the presence of precipitated proteins the peak areas of the internal standard [^{15}N]nitrite (m/z 47) were smaller and the peak areas of [^{15}N]nitrite-derived [^{15}N]nitrate (m/z 63) were considerably higher as compared to the samples being derivatized in the absence of precipitated proteins (data not shown). These findings indicate partial oxidation of [^{15}N]nitrite to [^{15}N]nitrate.

Table 1
Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS method for nitrite in whole blood of a healthy volunteer.

Added nitrite (μM)	Measured nitrite (μM) (mean ± SD, $n = 7$)	Recovery (%)	Imprecision (%)
0	1.47 ± 0.16	N.A.	10.9
0.5	1.93 ± 0.31	92.0	16.1
1	2.42 ± 0.32	95.0	13.2
2	3.42 ± 0.28	97.5	8.2
3	4.31 ± 0.22	94.7	5.1
4	5.28 ± 0.18	95.3	3.4
5	6.42 ± 0.49	99.0	7.6
6	7.22 ± 0.26	95.8	3.6
8	9.24 ± 0.31	97.1	3.4
10	11.2 ± 0.38	97.3	3.4

N.A., not applicable.

3.1.3. *Intra-assay accuracy and imprecision in whole blood, erythrocytes and plasma of healthy humans*

The results from the intra-assay validation experiments are summarized in Table 1 for human whole blood and in Table 2 for human erythrocytes and plasma. In the investigated nitrite concentration range, nitrite was quantified in human whole blood by the present method with recovery and imprecision rates ranging between 92–99% and 3.4–16.1%, respectively (Table 1). Linear regression analysis between measured (y) and added (x) nitrite concentration (μM) yielded a straight line with the regression equation $y = 1.45\ (0.03) + 0.97\ (0.01)\ x$, with $R = 0.99987$ (SD, 0.05) and $P < 0.0001$. The lowest added nitrite concentration of 0.5 μM was determined with a recovery of 92% and an imprecision of 16.1%, suggesting that the lower limit of quantitation (LLOQ) of the method is below 0.5 μM for a whole blood sample with a basal nitrite concentration of about 1.5 μM. The relative lower limit of quantification (rLLOQ) of the method [15] is calculated to be 33.3%. The rLLOQ expresses the percentage fraction of the analyte which, upon addition to the biological samples that contains this analyte, can be measured therein with acceptable accuracy and precision [15]. GC–MS chromatograms from the quantitative determination of nitrite in an unspiked blood sample and in the same blood sample spiked with 0.5 μM of nitrite are shown in Fig. 4. Virtually, these chromatograms are almost identical with those obtained by the original method in erythrocytes and plasma (present study, not shown), and in plasma, urine, saliva and other matrices in

Table 2
Intra-assay accuracy (recovery) and imprecision (RSD) for nitrite in erythrocytes and plasma of a healthy volunteer.

Added nitrite (μM)	Measured nitrite (μM) (mean ± SD, $n = 4$)	Recovery (%)	Imprecision (%)
(A) *Erythrocytes*			
0	2.43 ± 0.26	N.A.	10.9
1	3.38 ± 0.55	95.0	16.2
2	4.41 ± 0.22	99.0	5.0
3	5.73 ± 0.26	110.0	4.5
4	6.87 ± 0.16	111.0	2.3
6	9.19 ± 0.46	104.0	5.0
8	10.6 ± 0.13	102.0	1.2
10	12.2 ± 0.29	97.7	2.4
(B) *Plasma*			
0	1.48 ± 0.003	N.A.	0.2
1	2.59 ± 0.08	110.3	3.3
2	3.42 ± 0.11	97.0	3.2
3	4.42 ± 0.10	98.0	2.3
4	6.00 ± 0.28	113.0	4.7
6	7.71 ± 0.15	103.8	1.9
8	9.64 ± 0.28	102.0	2.9
10	11.6 ± 0.22	101.2	1.9

N.A., not applicable.

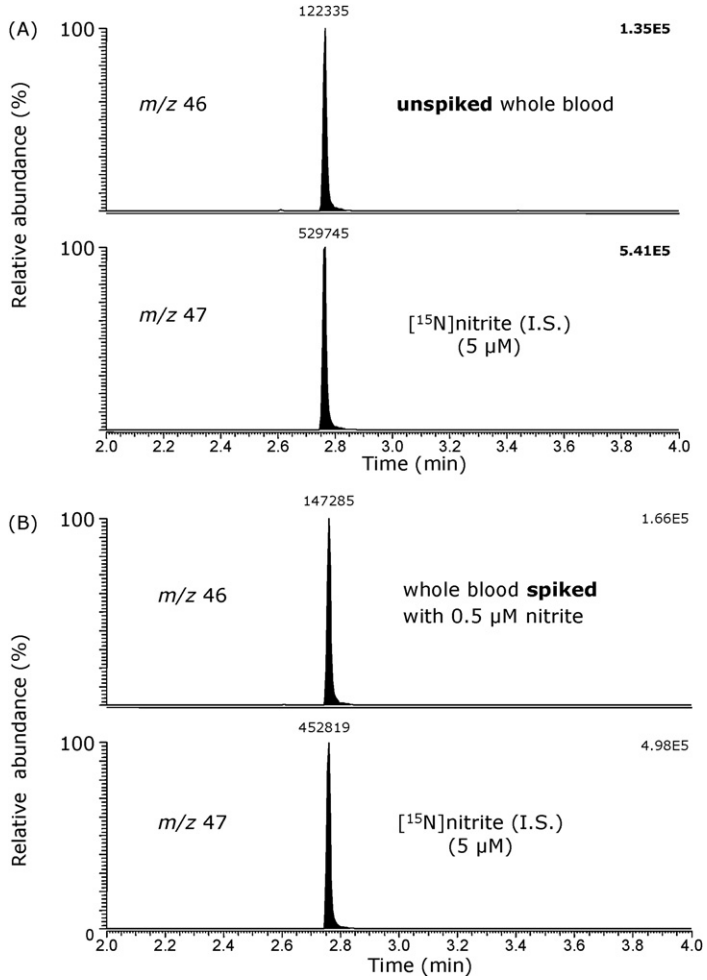

Fig. 4. Partial GC–MS chromatograms from the analysis of nitrite in whole blood of a healthy volunteer before (A) and after (B) addition of nitrite to the blood sample at a final added concentration of 0.5 μM. [^{15}N]Nitrite was present in both samples at a final concentration of 5 μM each. Selected-ion monitoring of *m*/*z* 46 ($^{14}NO_2^-$) and *m*/*z* 47 ($^{15}NO_2^-$) was performed. The PFB derivatives of unlabelled and labelled nitrite emerged from the GC column each at 2.77 min. The concentration of nitrite was determined to be 1.16 μM in the unspiked blood sample (A) and 1.62 μM in the blood sample spiked with 0.5 μM of nitrite (B).

a previous study [13]. SIM of *m*/*z* 46 for unlabelled nitrite and *m*/*z* 47 for the internal standard [^{15}N]nitrite provides two peaks with the same retention time of 2.76 min in the time-window of interest (i.e., 2–4 min) due to the PFB derivatives of [^{14}N]nitrite (upper traces in Fig. 4) and of [^{15}N]nitrite (lower traces in Fig. 4).

Nitrite was quantified in erythrocytes and plasma of a healthy volunteer with intra-assay recovery and imprecision rates ranging between 95–113% and 0.2–16.2%, respectively (Table 2). Linear regression analysis between measured (y) and added (x) nitrite concentration (μM) yielded straight lines with the regression equations: $y_1 = 2.59\ (0.21) + 1.00\ (0.04)\ x_1$, with $R = 0.99564$ (SD, 0.35) and $P < 0.0001$ for erythrocytes, and $y_2 = 1.53\ (0.11) + 1.02\ (0.02)\ x_2$, with $R = 0.99878$ (SD, 0.19) and $P < 0.0001$ for plasma. The data of Table 2 suggest that in the blood of the young (23 years of age), healthy female volunteer used in this validation experiment endogenous nitrite is slightly unevenly distributed between plasma and erythrocytes, with the erythrocytic nitrite concentration being about 1.6 times higher than the plasmatic nitrite concentration.

Table 3
Measured basal endogenous nitrite concentration (nM) in whole blood (C_B), plasma (C_P) and erythrocytes (C_E) of ten healthy fasted volunteers and calculated C_E-to-C_P ratio.

No.	Gender	Age (years)	Hct (%)	Whole blood	Plasma	Erythrocytes	C_E-to-C_P ratio
1	Female	43	40	312	481	578	1.20
2	Female	27	38	645	915	781	0.85
3	Female	24	39	331	420	417	0.99
4	Female	23	41	191	248	233	0.94
5	Female	23	40	439	353	473	1.34
6	Female	24	41	241	292	294	1.01
7	Male	28	47	979	1791	1171	0.65
8	Male	31	49	685	997	994	0.99
9	Male	24	49	829	975	1032	1.06
10	Male	31	46	207	247	222	0.90
Mean ± SD		27.8 ± 6.2		486 ± 280	672 ± 496	620 ± 352	0.993 ± 0.188

3.2. Basal concentrations of nitrite in whole blood, erythrocytes and plasma of healthy humans

The GC–MS method was applied to measure nitrite in whole blood, erythrocytes and plasma of healthy volunteers under basal conditions after an overnight fasting of at least 8 h. Table 3 summarizes the nitrite concentrations measured in the freshly obtained and immediately analyzed whole blood, erythrocytes and plasma samples of the volunteers. In this table the calculated ratio of C_E to C_P is also listed. The C_E-to-C_P ratio can be used as a measure of the distribution of nitrite between erythrocytes and plasma. In the volunteers, the C_E-to-C_P ratio ranged between 0.65 and 1.34 with the mean value being close to 1.00. This indicates a fairly even distribution of endogenous nitrite in blood of healthy young volunteers with no statistically significant difference between 6 females and 4 males ($P = 0.22$, unpaired t-test).

3.3. Kinetics of exogenous nitrite in whole blood

Incubation of freshly obtained whole blood, spiked with nitrite at the relatively high initial concentration of 10 μM, at 37 °C and GC–MS determination of nitrite concentration in whole blood, erythrocytes and plasma revealed the kinetics shown in Fig. 5.

The following relationship exists between C_B, C_P, C_E and the hematocrite (Hct):

$$C_B = (1 - \mathrm{Hct}) \times C_P + \mathrm{Hct} \times C_E \quad (1)$$

Considering the mean Hct value of 40% measured in this experiment, i.e., Hct = 0.40, Eq. (1) is converted to Eq. (2):

$$C_B = 0.60 \times C_P + 0.40 \times C_E \quad (2)$$

Immediately after addition of nitrite to the blood sample and prior to incubate the ice-cold, nitrite-spiked blood in the water bath at 37 °C, the measured nitrite concentrations were (mean ± SD, $n = 2$): $C_B = 11.4 \pm 0.98$ μM, $C_P = 15.5 \pm 0.8$ μM and $C_E = 8.5 \pm 1.9$ μM (Fig. 5A). These findings indicate that: (1) the concentration of endogenous nitrite in the whole blood used in this experiment is about 1.4 μM, and that (2) the major fraction of nitrite added to the ice-cold blood remains apparently in the plasma; however, a considerable portion of nitrite reaches the erythrocytes immediately after nitrite addition to the ice-cold whole blood. After 5 min of incubation of blood at 37 °C, nitrite concentration fell slightly in whole blood ($C_B = 10.6 \pm 0.4$ μM) and markedly (by 30%) in plasma ($C_P = 10.8 \pm 0.14$ μM), whereas nitrite concentration in erythrocytes remained almost unchanged ($C_E = 7.7 \pm 0.64$ μM). After further 5 min of incubation, nitrite concentration fell in all matrices. Incubation times longer than 20 min resulted in constant but slight decreases in C_B, C_P and C_E (Fig. 5A).

Data analysis by the integral method revealed two regimes in which the course of C_B and C_P followed a first order exponential kinetics. This is indicated by the linear relationships ($R > 0.99$) found between $\ln[C_o/C]$ (y) for whole blood or plasma and incubation time (x) in the ranges 0–20 min (i.e., regime α) and 20–60 min (i.e., regime β), respectively (Fig. 5B). By contrast, the fall in C_E seems to follow a first order kinetics in the whole observation time-window ($R = 0.93$; Fig. 5B). The rate constant (k) and the half-life ($t_{1/2} = \ln 2/k$) of nitrite in the erythrocytes were calculated to be 0.016/min and 43.3 min, respectively. The rate constant values of nitrite in plasma were determined to be 0.056/min (0–20 min, regime α) and 0.014/min (20–60 min, regime β) corresponding to half-life values of 12.4 and 49.5 min, respectively. Analogous, the rate constant values of nitrite in whole blood were determined to be 0.055/min (0–20 min, regime α) and 0.008/min (20–60 min, regime β) corresponding to half-life values of 12.6 and 86.6 min, respectively. These results suggest that the transport of exogenous nitrite

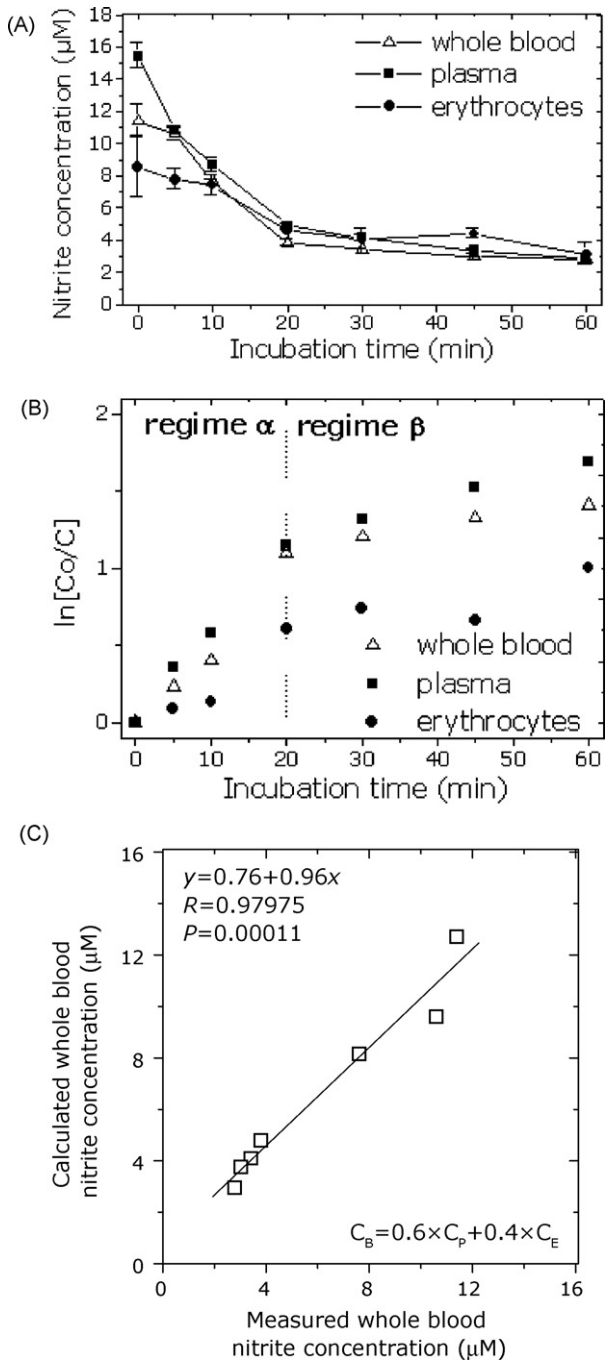

Fig. 5. Kinetics of nitrite in whole blood, erythrocytes and plasma. Whole blood of a healthy volunteer (female, 28 years of age) was spiked with nitrite at an added final concentration of 10 μM (with respect to the volume of the whole blood) and incubated at 37 °C for the indicated times. Nitrite was determined in all matrices using the same procedure (see Scheme 2). All data are shown as mean ± SD from duplicate analyses. (A) Concentration-time profile of nitrite in whole blood, plasma and erythrocytes. (B) Application of the integral method to determine the kinetics of nitrite. The natural logarithm (ln) of the nitrite concentration ratio at time zero and other incubation times (C_o/C), i.e., $\ln[C_o/C]$, is plotted versus the incubation time (t). In case of a first order kinetics, the following equation would be valid: $\ln[C_o/C] = k \times t$, with k being the rate constant of the reaction. (C) Correlation between calculated (by setting the measured C_P and C_E values into Eq. (2); see text and insertion) and measured nitrite concentration in whole blood at various incubation times. The hematocrite value in this blood was 40% (for more details see the text).

from plasma into the erythrocytes occurs considerably faster than the decrease of nitrite concentration within the erythrocytes.

Using Eq. (2) and the measured concentration of nitrite in plasma and in erythrocytes we calculated the concentration of nitrite in whole blood. Fig. 5C shows that the calculated nitrite concentrations in whole blood correlate well ($R = 0.98$) with those measured in whole blood.

3.4. Kinetics of endogenous nitrite in whole blood and plasma during reactive hyperemia

We applied the GC–MS method to measure nitrite in whole blood and plasma of a healthy volunteer (being author of this article) under basal conditions and during a 60-s lasting forearm reactive hyperemia, following a 5-min ischemia period. The reperfusion-induced shear stress triggered production of NO, resulting in maximum nitrite concentrations at 60 s after cuff release for both whole blood and plasma (Fig. 6A). Fig. 6B shows the course of the calculated erythrocytic nitrite concentration and of the C_E-to-C_P ratio during reactive hyperemia. In whole blood we measured 691 ± 38 nM nitrite at baseline and 883 ± 53 nM (increase by 28%) after 60 s. In plasma we measured 656 ± 37 nM nitrite at baseline and 828 ± 62 nM (increase by 26%) after 60 s. The increase of whole blood nitrite concentration was matched by an increase in forearm blood flow of 67% of the baseline values at 60 s after cuff release (Fig. 6C).

4. Discussion

4.1. Methodological issues

Measuring nitrite by a GC–MS method based on the analysis of nitrite as PFB derivative (Scheme 1) in serum of fasted humans who received [*guanidino*-$^{15}N_2$]-L-arginine revealed that about 90% of circulating nitrite derives from L-arginine [16]. Findings of more recent studies suggest that under certain conditions the concentration of nitrite in human circulation may be a useful indicator of endothelial NO synthase (eNOS) activity (discussed in Refs. [2,3]). A recent study suggested that endogenous nitrite accumulates in erythrocytes, i.e., nitrite is distributed unevenly in blood compartments, and that erythrocytic nitrite may serve as a storage of NO and NO-related bioactivity [5], a role that has been originally assigned to *S*-nitrosothiols (discussed in Refs. [2,12,17,18]). However, this information largely derives from a single investigator group that uses a chemiluminescence method for measuring nitrite in blood. Yet, concentrations and roles of *S*-nitrosothiols in human blood are controversial [17]. A major portion of uncertainty comes from the analytical difficulty to accurately measure nitrite and *S*-nitrosothiols in plasma, serum, and in particular in erythrocytes and whole blood. Nitrite and *S*-nitrosothiols are unstable in plasma, whole blood and erythrocytes, and they may undergo manifold reactions including oxidation to nitrate [2]. All these factors together may alter the original concentration of nitrite and *S*-nitrosothiols until final analysis and detection and may lead to conclusions that are based on erroneous measurements.

We speculate that nitrite's instability/reactivity in whole blood and in erythrocytes is likely the reason that nitrite is commonly measured in plasma or serum but not in whole blood or erythrocytes. In addition to nitrite's chemical instability/reactivity towards certain metalloproteins and oxyhemoglobin, many other method-specific analytical issues and pre-analytical factors such as blood sampling and storage are likely to prevent investigators from measuring nitrite in whole blood or erythrocytes. Nitrite loss due to oxyhemoglobin-catalyzed oxidation to nitrate and many of the pre-analytical and analytical difficulties related to whole blood or erythrocytes become irrelevant if nitrite is analyzed in plasma or serum [2,3,5,12].

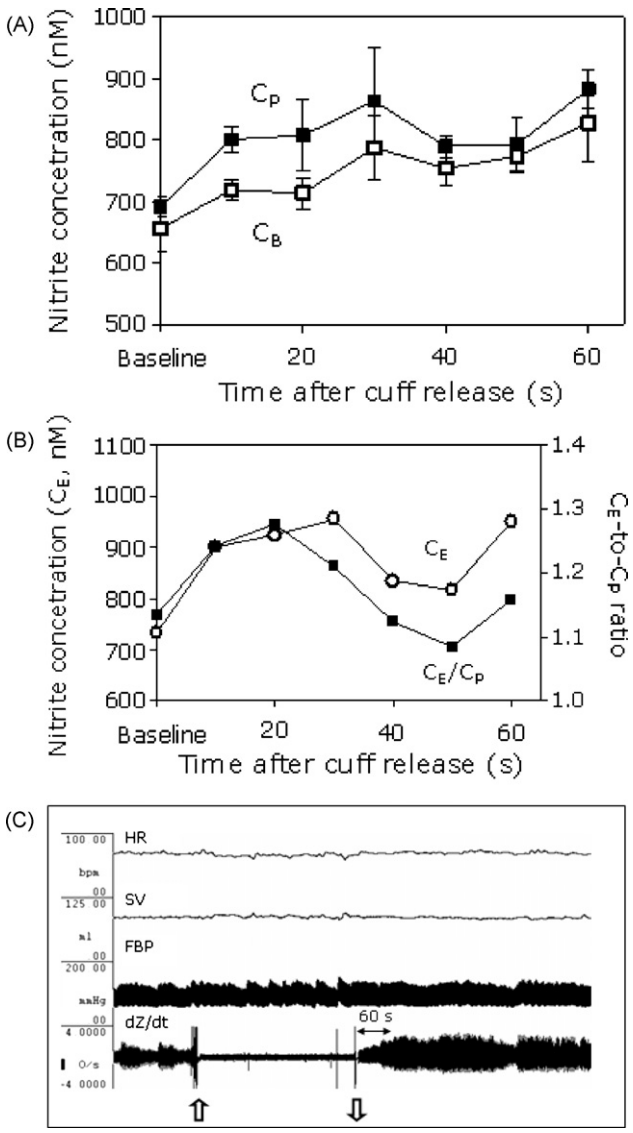

Fig. 6. Kinetics and dynamics of endogenous nitrite concentration during reactive hyperemia. (A) Nitrite concentrations in whole blood and plasma were measured (in triplicate) in antecubital vein blood in a healthy volunteer (being an author of this article) before and after a 5-min ischemia period. The reperfusion-induced shear stress triggered production of NO, resulting in peak nitrite concentrations at 60 s after cuff release. Symbols represent mean ± SE. (B) Calculated mean nitrite concentration in erythrocytes after transformation of Eq. (1), i.e., $C_E = (C_B - 0.55 \times C_P)/0.45$, with Hct being 0.45 for this subject, and calculated mean C_E-to-C_P ratio (C_E/C_P). (C) Reactive hyperemia measured by impedance plethysmography in the left forearm. Upper and lower arrows indicate inflation and release of the cuff, respectively. Note the increase in dZ/dt compared to baseline after cuff release (suprasystolic occlusion of the brachial artery for 5 min). Systemic hemodynamic parameters did not change. HR, heart rate; SV, stroke volume calculated from thoracic impedance cardiography; FBP, finger arterial blood pressure measured on the right hand; dZ/dt (Ω/s), first time-derivative of the impedance plethysmogram.

Previously, we showed that the derivatization of nitrite by PFB-Br is feasible in plasma, serum, urine and saliva, and that analysis of nitrite as PFB derivative by GC–MS is a useful method for the accurate and interference-free quantitative determination of nitrite in these biological samples [13]. Extension of that method to nitrite in whole blood and erythrocytes appeared difficult despite the use of ^{15}N-labelled nitrite as the internal standard, which should actually compensate for all changes of nitrite during the whole analytical process. It is noteworthy that whole blood is an inhomogeneous

matrix and that labile internal standards such as [^{15}N]nitrite may be distributed differently in blood compartments and may undergo different changes and reactions than their endogenous congeners. The recent findings on the potential role of nitrite as a storage form of NO and NO-bioactivity prompted us to re-investigate the potential usefulness of the PFB-Br-based GC–MS method for the quantitative determination of nitrite in whole blood and erythrocytes.

By measuring [^{15}N]nitrite in blood plasma we observed that nitrite is stable in blood for several hours if blood is stored in an ice bath (unpublished data), unlike in blood that is stored at higher temperatures (e.g., 25 or 37 °C). By measuring nitrite in plasma, the half-time of nitrite in blood (at 37 °C) was determined by us to be about 11–13 min [1]. Others have reported similar values for the half-life of nitrite in human blood by using other analytical techniques [5,19]. Therefore, cooling of blood in an ice bath throughout the analytical procedure, where applicable, was considered a promising approach in our investigations (steps 1–3 in Scheme 2) towards nitrite loss.

In addition, the reportedly successful prevention of Hb-catalyzed oxidation of nitrite by ferricyanide, for instance supplied as $K_3Fe(CN)_6$, and the feasible measurement of nitrite in whole blood by means of a chemiluminescence method [5,12], offered us an alternative approach to be tested for its usefulness in our GC–MS method (step 1 in Scheme 2).

The PFB-Br-based GC–MS method for nitrite was found not to be affected by plasma proteins, because nitrite does not react with precipitated plasma proteins including human serum albumin at neutral pH [13]. Also, because *S*-nitrosothiols in plasma are rather in the lower nM range [17], their contribution to nitrite due to thermal decomposition is considered rather low, notably in the absence of reagents such as $HgCl_2$ and in the presence of chelators such as the anticoagulant EDTA. Therefore, in routine plasma nitrite analysis by the original protocol, plasma proteins are regularly not removed prior to PFB-Br derivatization. By contrast, there are serious reasons to assume that erythrocytic proteins may interfere with nitrite analysis. On the one hand, nitrite may undergo reaction(s) with the haem group of erythrocytic Hb during PFB-Br derivatization, even if Hb is precipitated. Decreased peak areas of the internal standard [^{15}N]nitrite and concomitantly increased peak areas of [^{15}N]nitrate in the Hb-containing derivatization mixtures are supportive of such an interference. The most abundant source for nitrite is likely to be decomposed *S*-nitrosohemoglobin (SNOHb) and *Fe*-nitrosohemoglobin (HbFeNO). However, their concentration in the blood is highly questionable. Thus, in rat and human blood SNOHb concentration has been reported to range by three orders of magnitude, i.e., between 25 nM and 22 μM [15]. Therefore, we reasoned that removal of acetone-precipitated Hb prior to PFB-Br derivatization (step 4 in Scheme 2) would also be an appropriate measure to minimize contribution of other NO-containing species to nitrite in this method. Whether a modification of the GC–MS method presented here can be utilized to measure SNOHb and/or HbFeNO in whole blood or erythrocytes remains to be investigated by using ^{15}N-labelled internal standards.

Nitrite derivatization by PFB-Br in aqueous acetone is dependent upon various experimental conditions including temperature, time and amount of the derivatization agent PFB-Br [13,14]. The same derivatization procedure can be applied for the simultaneous quantitative determination of nitrite and nitrate in biological matrices such as plasma and urine. However, the kinetics of the formation of the PFB derivatives of nitrite (PFB-NO_2) and nitrate (PFB-ONO_2) are considerably different [13]. Thus, maximum PFB-NO_2 formation occurs after 5 min incubation time, whereas PFB-ONO_2 formation at an analytically satisfactory yield requires much longer derivatization times, commonly 60 min [13]. Because of our special interest in nitrite in the present study we applied the optimum derivatization time of 5 min for nitrite (step 7 in Scheme 2).

Finally, in order to optimize the PFB-Br-based GC–MS method for whole blood and erythrocytic nitrite, we investigated the effect of the order of addition of the required reagents and solvents to the blood samples. Because acetone and water are very well miscible and in order to save time, internal standard and ferricyanide were supplied in ice-cold acetone at proper concentrations (step 1 in Scheme 2).

The present study shows that the modifications made to the original PFB-Br-based GC–MS method [13] improved the quantitative determination of nitrite in human whole blood and erythrocytes. The most important modifications are: (1) quick working at temperatures near 0 °C, where possible; and (2) quantitative removal of precipitated Hb by centrifugation of blood or erythrocytes samples after addition of the ice-cold acetone solution and by decantation of the supernatant (steps 4 and 5 in Scheme 2). Analogous to plasma or serum and other protein-rich samples, addition of 100-μl aliquots of ice-cold blood or erythrocytes to 400-μl aliquots of ice-cold acetone leads to instantaneous and complete precipitation of proteins including Hb. Short centrifugation of acetone-treated blood samples in the cold (e.g., at 4 °C; step 4 in Scheme 2) readily produces a well-formed solid precipitate, and a clear and colorless supernatant which is easily decanted, without any protein carry off. Thus, the subsequent derivatization occurs in an Hb-free matrix, with Hb-dependent loss of nitrite being completely prevented.

The present study also suggests that $K_3Fe(CN)_6$ addition is not mandatory in this GC–MS method. On the other hand, $K_3Fe(CN)_6$ does not affect quantitative determination of nitrite in whole blood and erythrocytes by the same method. Furthermore, $K_3Fe(CN)_6$, when added at 800 mM to EDTA plasma, did not to interfere with the quantitative determination of nitrite in plasma. Therefore and for the sake of better comparison with nitrite concentrations measured by others, $K_3Fe(CN)_6$ was routinely used in the present study. Hb precipitates immediately upon addition of the ice-cold blood sample to the $K_3Fe(CN)_6$-containing ice-cold acetone solution. Possibly, $K_3Fe(CN)_6$ does not react at all with Hb, despite the about 80-fold molar excess of $K_3Fe(CN)_6$ over Hb. Hence, Hb removal prior to PFB-Br derivatization seems to be more effective than the use of a nitrite-stabilizing aqueous solution composed of $K_3Fe(CN)_6$, the detergent Nonidet-40 (NP-40) to solubilize red blood membranes and *N*-ethylmaleimide (NEM) to alkylate sulfhydryl groups in other methods [5,12].

Differences in the analytical procedures used by us in the present GC–MS method and in chemiluminescence-based methods used by others could account for discrepant basal nitrite measurements in whole blood, in erythrocytes and in plasma of healthy humans. Another discrepancy between the chemiluminescence-based method [5] and our GC–MS method concerns the distribution of nitrite between blood compartments, i.e., plasma and erythrocytes. Our results reveal a fairly even distribution of nitrite between plasma and erythrocytes, whereas the chemiluminescence-based method suggests a clearly uneven distribution, i.e., an appreciable accumulation of nitrite in erythrocytes [5].

In our GC–MS method we noted considerable imprecision for low, biologically relevant nitrite concentrations in whole blood and erythrocytes samples compared with plasma samples (Tables 1 and 2). This observation may be related to imprecise sampling of 100-μl aliquots of whole blood and erythrocytes because of the considerably higher viscosity of these matrices compared to plasma. Blood and erythrocytes sampling is unlikely to be the sole reason for imprecision, even when method imprecision is entirely within generally acceptable ranges. When analyzed whole blood for nitrate, i.e., by heating blood for 60 min at 50 °C, we always observed formation of [^{15}N]nitrate at very low levels due

to oxidation of the internal standard [^{15}N]nitrite (used at 5 μM). Nitrite oxidation seems to be not entirely avoidable. However, the low extent of nitrite oxidation cannot explain the order of imprecision in quantifying endogenous nitrite in whole blood. An additional factor could be the absorption of nitrogen gases from the laboratory atmosphere [20,21], which could be more abundant in blood and erythrocytes as compared to urine and plasma, and more serious for the lower nitrite concentrations. Finally, contaminating nitrite from equipment such as glassware may be another factor that contributes to nitrite concentration in biological samples to a varying extent [14]. The observation of nitrite oxidation in our method and our results from method validation experiments indicate that accurate and precise quantitative determination of nitrite in whole blood and erythrocytes is an analytical challenge.

4.2. Biologically relevant issues—kinetics and distribution of nitrite in human blood

The present GC–MS method was applied in the frame of small pilot studies. The first study focused on the distribution of endogenous nitrite between plasma and erythrocytes of blood of healthy young volunteers who had fasted overnight for at least 8 h. The second study investigated the distribution of endogenous nitrite between plasma and erythrocytes of blood of the same healthy young volunteers. In both studies nitrite was quantified in freshly obtained whole blood, erythrocytes and plasma samples. Blood was anticoagulated with EDTA and stored in an ice bath until use in the respective experiments in order to maintain as intact as possible the redox status of the blood, notably to avoid oxidation of plasmatic and erythrocytic thiols.

Our results show that endogenous nitrite is distributed quite evenly between plasma and erythrocytes of healthy humans. This finding confirms results from another group that administered radiolabelled nitrite and nitrate to animals [4]. By contrast, another group found that nitrite accumulates in erythrocytes [5]. The results by Parks et al. [4], Ishibashi et al. [7] and our present findings do not support the hypothesis by Gladwin and co-workers [5] that based on higher nitrite concentrations in erythrocytes than in plasma, erythrocytes may serve as a storage and vehicle form for circulating nitrite. From the kinetic perspective, however, our findings are supportive of a function of erythrocytes as storage for nitrite from endogenous and exogenous sources.

To the best of our knowledge the present study is the first to demonstrate the time course of measured nitrite concentration in the erythrocytes in addition to the nitrite course in whole blood and plasma. Unlike endogenous nitrite, our study suggests that nitrite, when added externally to blood at supra-physiological but pharmacologically relevant concentrations, is unevenly distributed in human blood in vitro between erythrocytes and plasma. Nitrite is transported into the erythrocytes where it is mainly oxidized to nitrate [1]. Our previous and present results, however, do not exclude additional reactions of nitrite in erythrocytes, such as reduction to NO by hemoglobin species and carbonic anhydrases [10,11].

4.3. Kinetics and distribution of nitrite in human blood during reactive hyperemia

In our third pilot study, we demonstrated the usefulness of the proposed method for detection and quantification of rapidly occurring changes in endogenous nitrite concentration in whole blood and plasma during a 60-s lasting reactive hyperemia (Fig. 6A). The observed maximal increase in nitrite concentration was similar for both whole blood and plasma (28% versus 26%). These values are considerably lower than the values reported by others, e.g., 52% in blood [5] or 94% in plasma [22]. The acute changes in nitrite concentration seen in our volunteer during reactive hyperemia were different in plasma and erythrocytes. These changes led to a temporary increase in the C_E-to-C_P ratio after 10 and 20 s followed by a fall to the baseline value (Fig. 6B). These findings suggest that reactive hyperemia induces nitrite generation, presumably by shear stress-mediated NO formation in the endothelium. In hyperemia conditions, nitrite distribution between plasma and erythrocytes seems to be shifted in favor of the erythrocytes. Yet, a study involving a proper number of volunteers is warranted to investigate endogenous nitrite kinetics during reactive hyperemia by the present method.

4.4. Measuring nitrite in plasma, erythrocytes or whole blood?

Quantitative determination of nitrite and nitrate in whole blood, erythrocytes and plasma provides maximum information about the status of circulating nitrite in various conditions. In the frame of clinical studies, nitrite and other related, short-lived species such as *S*-nitrosothiols should be preferably analyzed "at the bedside" [3]. Should whole blood or erythrocytes turn out the most appropriate matrices in studies on NO, blood samples ought to be analyzed immediately after sampling. However, should analysis of nitrite in whole blood or erythrocytes not add important information to nitrite biochemistry, physiology and pharmacology than nitrite analysis in plasma or serum, there would be no pressing need to measure nitrite in whole blood, much less in erythrocytes. This subject is worthy of investigation in future studies.

5. Conclusions

We report here a stable-isotope dilution GC–MS technique for the quantitative determination of nitrite in whole blood, erythrocytes and plasma. The basis for the application of this method to whole blood and erythrocytes is immediate cooling of whole blood and erythrocytes in an ice bath, immediate and complete removal of erythrocytic Hb by acetone-precipitation and centrifugation, and derivatization with PFB-Br in the absence of Hb. Despite these effective measures and use of ^{15}N-labelled nitrite as the internal standard, accurate and precise quantitative determination of nitrite in whole blood and erythrocytes represents an analytical challenge. In particular contaminating nitrite from laboratory equipment and absorption of nitrogen gases from the atmosphere may considerably contribute to endogenous nitrite in blood.

By means of this GC–MS method we observed uneven distribution of nitrite between plasma and erythrocytes under basal conditions. However, in reactive hyperemia nitrite distribution in blood seems to change upon time leading to a temporary nitrite accumulation in erythrocytes. By contrast, exogenous nitrite accumulates in plasma. This information could be of particular importance in the context of pharmacological treatment of NO-related dysfunctions by inorganic nitrite and possibly by inorganic nitrate salts.

The GC–MS methodology described in the present article allows for quantification of nitrite and nitrate in whole blood, erythrocytes and plasma. This analytical feature should be of great help in delineating the complex biochemistry of nitrite, both from endogenous and exogenous sources such as nutrition and pharmaceuticals.

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