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ScienceDirect
Journal of Chromatography B, 851 (2007) 106–123
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Recent methodological advances in the analysis of nitrite in the human circulation: Nitrite as a biochemical parameter of the L-arginine/NO pathway☆

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Received 23 August 2006; accepted 1 February 2007
Available online 14 February 2007

Abstract

Nitric oxide (NO) plays a pivotal role in the modulation of multiple physiological processes. It acts as a messenger molecule within the cardiovascular system. NO is a highly unstable free radical in circulating blood and is oxidized rapidly to nitrite and nitrate. Recent studies suggest that nitrite has the potential to function as a surrogate of NO production under physiological and pathophysiological conditions and could therefore be of high relevance as a biochemical parameter in experimental and clinical studies. Under hypoxic conditions nitrite is reduced to bioactive NO by deoxyhemoglobin. This mechanism may represent a dynamic cycle of NO generation to adapt the demand and supply for the vascular system. Because of these potential biological functions the concentration of nitrite in blood is thought to be of particular importance. The determination of nitrite in biological matrices represents a considerable analytical challenge. Methodological problems often arise from pre-analytical sample preparation, sample contamination due to the ubiquity of nitrite, and from lack of selectivity and sensitivity. These analytical difficulties may be a plausible explanation for reported highly diverging concentrations of nitrite in the human circulation. The aim of this article is to review the methods of quantitative analysis of nitrite in the human circulation, notably in plasma and blood, and to discuss pre-analytical and analytical factors potentially affecting accurate quantification of nitrite in these human fluids.

Keywords: Reviews; Nitrite analysis; Human circulation; Clinical studies; Biochemical parameter; Validation

Contents

☆ This paper is part of a special issue entitled "Analysis of the L-arginine/NO pathway", guest edited by D. Tsikas.

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doi:10.1016/j.jchromb.2007.02.002

1. Introduction

Endothelium-derived nitric oxide (NO) is one of the most potent endogenous vasodilators [1,2] and has been viewed both as an autocrine [3,4] and a paracrine effector molecule [5]. It plays an important role in the local control of vascular homeostasis by regulating vessel tone and by inhibiting smooth muscle cell proliferation and blood cell adhesion and lipid peroxidation [2,3]. NO binds to the heme-moiety in guanylate cyclase and activates the enzyme to produce cGMP which causes the muscle to relax by decreasing intracellular concentration of free Ca^{2+} [6].

In the endothelium NO is synthesized from the amino acid L-arginine by the constitutive, Ca^{2+}/calmodulin-dependent endothelial NO synthase (eNOS) [7]. NO reacts with molecular oxygen and other reactive oxygen species such as superoxide, and the intermediate reaction products including peroxynitrite may react with biomolecules to form a variety of reaction products [2,8,9]. In aqueous solutions free of biological material, NO autoxidizes almost exclusively to nitrite [10,11], whereas in whole blood NO is oxidized by oxyhemoglobin almost completely to nitrate [12,13], but a minor part is oxidized to nitrite [14].

In humans, pioneer studies by gas chromatography–mass spectrometry (GC–MS) using guanidino-^{15}N-labeled L-arginine showed that under fasting conditions with a low intake of nitrite and nitrate, 90% of circulating nitrite endogenously originated from the L-arginine/NO pathway [15]. In plasma taken from overnight fasting humans the majority of the plasmatic nitrite has been shown to be generated by eNOS [16]. Plasma nitrite has been described as an index of eNOS activity in the regional [17] and systemic circulation in humans and various mammals [16]. Next to its important role in physiology, a reduced NO availability is generally assumed to be a hallmark of endothelial dysfunction occurring in early arteriosclerosis. By using a flow injection analysis (FIA) technique based on the Griess reaction it has been shown very recently that plasma nitrite concentration decreases with increasing numbers of cardiovascular risk factors, thus reflecting acute changes in eNOS activity [18]. In addition, a study using nitrite infusion into the forearm brachial artery of healthy humans before and during exercise evaluated that deoxyhemoglobin reduces nitrite to NO and dilates detector vessels in an oxygen sensitive manner [19]. Various studies give evidence that no other reaction product of NO than circulating nitrite reflects more reliably changes in endothelial function (see for detail the discussion in Section 3.2) [18]. The short half-life of nitrite in whole blood [20] represents a challenge for nitrite measurement in biological fluids.

Previous reviews compared quantitative analytical methods of analysis of nitrite and nitrate in human biological fluids [20,21] in the framework of their significance in clinical studies and their relevance from the clinical biochemistry perspective [22]. The present paper reviews and discusses the principles and techniques of direct and indirect quantitative analysis of nitrite in the circulating blood. It especially focuses on methodological issues and critically discusses potential advantages and disadvantages of reported analytical methods for circulating nitrite. An attempt is made to interpret the reported highly diverging nitrite concentrations in human plasma and blood from the analytical standpoint. In consideration of the very recent findings on the emerging relevance for nitrite as a storage form and donor of NO within the human vascular system and the potential relevance of nitrite as a new diagnostic and therapeutic tool for NO-related dysfunction [19,23], these issues are also discussed from the biological, clinical and analytical points of view.

2. Analytical approaches for circulating nitrite

Prior to the identification of nitrite as a metabolite of endogenous NO, this anion attracted attention because of its toxic and carcinogenic potential [24]. Because of this interest in nitrite, numerous analytical methods have been developed for the analysis of nitrite in various matrices. However, only a minor part of these methods is indeed applicable to human biological fluids, notably plasma, serum, blood or urine. Most of the analytical methods originally developed and validated for simple matrices such as drinking and surface water could not be adopted for blood or plasma, mainly because of the complexity of these matrices and their relatively low content of nitrite [25,26].

Nitrite in biological matrices can be measured directly, i.e. without any derivatization step, by capillary zone electrophoresis (CZE), high-performance capillary electrophoresis (HPCE) and high-performance liquid chromatography (HPLC) using UV absorbance, electrochemical or conductivity detection. Indirect analytical methods require a derivatization step such as the Griess reaction or the reaction with 2,3-diaminonaphthalene (DAN). Indirect methods include FIA, HPLC, GC–MS and chemiluminescence detection (CLD). Table 1 summarizes the

Table 1
Summary of reported analytical techniques for the determination of circulating nitrite

Method	Initial processing of the sample	Subsequent processing	Detection method	Reference
Direct measurements				
CIA	Ion separation		Diode array detection (214 nm)	[27]
CZE	ITP stacking and separation in fused-silica capillaries		UV absorbance detection (214 nm)	[28,29]
HPCE	ITP stacking and separation in a capillary electrophoresis		Conductivity detection	[30,31]
HPCE	Sample stacking in a fused silica capillary	Electromigration	UV absorbance detection (265 nm)	[32]
HPLC	Anion separation via anion exchanger		UV absorbance detection (210 or 214 nm)	[33,34]
Indirect measurements				
FIA with Griess	Reaction with sulfanilamide	Reaction with *N*-(1-naphthyl)ethylenediamine	Vis absorbance detection (540 nm)	[25]
Reversed phase HPLC	Conversion of nitrite by *N*-acetyl-L-cysteine	UV absorbance (333 nm)	[35]	
HPLC	Protein precipitation	High performance anion exchange chromatography	Amperometric detection	[36]
HPLC	On-line reduction of the anions in a cadmium column	Colorimetric reaction with the Griess reagent	Vis absorbance detection (540 nm)	[37]
HPLC with DAN	Protein precipitation	Pre-column reaction with dapsone or DAN	Fluorescence detection	[38,39]
GC–MS	Nucleophilic substitution of bromide in PFB bromide	Gas chromatography	Mass spectrometry	[40]
CLD	Acidic reduction	Reduction to NO, and NO reaction with ozone	Chemiluminescence reaction	[27]

CIA: capillary ion analysis, CZE: capillary zone electrophoresis, HPCE: high-performance capillary electrophoresis, HPLC: high-performance liquid chromatography, FIA: flow injection analysis, GC–MS: gas chromatography–mass spectrometry, CLD: chemiluminescence detection.

reported analytical techniques used for the determination of nitrite in biological samples. These methods are subdivided into direct and indirect methodologies. Unlike the indirect methods, in direct methods nitrite is analyzed and detected without any chemical conversion of derivatization.

Reported analytical methods for circulating nitrite based on different analytical techniques are summarized in Table 2, together with the reported limit of detection (LOD) and matrix investigated. These analytical methods are discussed in detail below. Reported nitrite concentration in plasma, red blood cells and whole blood are summarized in Table 3. As can be seen in Table 3, plasma is the most frequently analyzed matrix. Whole blood and erythrocytes represent problematic matrices and are rarely used for quantitative analyses, because nitrite is unstable in these matrices. However, recently an analytical solution has been provided for this particular difficulty, which now enables reliable quantitative determination of nitrite in whole blood and erythrocytes [23].

Table 2
Comparison of reported detection limits in methods of analysis of nitrite

Methodology	Nitrite detection	Matrix	LOD	Reference
Electrophoresis				
CZE	Direct	Aqueous solution	150 nM	Szöko et al. [41]
HPCE	Direct	Plasma	8.9 nM	Boudko et al. [42]
Flow injection analysis (FIA)				
FIA with Griess	Indirect	Aqueous solution, plasma	12.5 nM	Kleinbongard et al. [25]
FIA with Griess	Indirect	Aqueous solution (1:2), plasma (1:2)	2 nM, 8 nM	Schulz et al. [43]
FIA with DAN	Indirect	Blood	10 nM	Misko et al. [44]
High-performance liquid chromatography (HPLC)				
HPLC	Direct	Plasma	8 nM	Preik-Steinhoff and Kelm [36]
HPLC with Griess	Indirect	Plasma	1 nM	Rassaf et al. [37]
HPLC with DAN	Indirect	Aqueous solution, plasma	10 nM	Li et al. [39]
Gas chromatography–mass spectrometry (GC–MS)				
GC–MS	Indirect	Plasma	22 fmol	Tsikas et al. [40]
Chemiluminescence (CLD)				
CLD	Indirect	Aqueous solution	50 nM	Cox et al. [45]
CLD	Indirect	Blood, plasma, tissue	5 nM	Feelisch et al. [26]

Table 3
Reported basal nitrite concentrations in plasma, serum, blood or erythrocytes of healthy humans

Method	Nitrite (μM)	Biological matrix in humans	Reference
Electrophoretic methods			
CZE	3.26	Plasma	Ueda et al. [29]
HPCE	0.450	Plasma	Leone et al. [46]
Griess (batch)			
Griess	0.221	Plasma	Giustarini et al. [47]
Griess	3.3	Serum	Phizackerley et al. [48]
Flow injection analysis			
FIA-Griess	0.3	Plasma	Lauer et al. [17]
FIA-Griess	0.305	Plasma	Kleinbongard et al. [16]
High-performance liquid chromatography			
HPLC-Griess	0.13	Plasma	Ishibashi et al. [49]
HPLC-Griess	0.2	Plasma	Rassaf et al. [37]
HPLC	N.D.	Plasma	Meulemans et al. [50]
HPLC	0.2	Plasma	Michigami et al. [51]
HPLC	0.578	Plasma	Preik-Steinhoff et al. [36]
HPLC	1.3	Plasma	Wennmalm et al. [52]
HPLC	1.4	Plasma	Jedlickova et al. [53]
HPLC-UV	0.55	Plasma	Tsikas et al. [35]
HPLC-DAN	1.13	Plasma	Hata et al. [54]
HPLC	26	Plasma	Gorenflo et al. [55]
Gas chromatography–mass spectrometry			
GC–MS	1.8	Plasma	Tsikas et al. [40]
Ozone-based chemiluminescence			
CLD	0.114	Plasma	Pelletier et al. [56]
CLD	0.121	Plasma	Dejam et al. [57]
CLD	0.29	Plasma	Bryan et al. [58]
CLD	5.5	Plasma	Akiyama et al. [59]
CLD	0.176	Whole blood	Dejam et al. [57]
CLD	0.288	Erythrocytes	Dejam et al. [57]
CLD	0.29	Erythrocytes	Rogers et al. [60]
CLD	0.68	Erythrocytes	Bryan et al. [58]

N.D.: not detectable.

2.1. Electrophoretic methods

Capillary electrophoresis (CE) encompasses a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, mass and size.

2.1.1. Capillary ion analysis (CIA)

CIA is an analytic separation technique characterized by high resolving power, minimal sample preparation requirements, the ability to operate in aqueous media with short analysis time and low sample consumption (1–50 nl injected). Sensitivity, however, is not a particular strength of this technique [61], and recently there have been some important advances towards improving this aspect of CIA technology. These include high-sensitivity Z-cell capillaries, dedicated diode-array detectors and extended light-path capillaries. Leone and colleagues [46] have measured basal plasma nitrite using both a Waters Quanta 4000 capillary electrophoresis system fitted with a high-sensitivity Z-cell capillary, and an HP 3D capillary electrophoresis system with diode-array detector fitted with an extended light-path capillary [46]. The Z-cell capillary has an extended optical path length of 3 mm, a 40-fold increase compared with a standard 75-μm internal diameter (i.d.) capillary. The extended light-path or "bubble" capillary used in the HP 3D system contains a three-time expanded i.d. section (225 μm) at the point of detection, giving a three-time extended optical path-length and a similar 10-fold increase in sensitivity for basal plasma anions [62]. The intra-assay coefficient of variation (CV) is 10% for basal nitrite; inter-assay CV for basal and spiked nitrite are in the range of 9–11% ($n = 14$) [62].

The presence of anions in plasma at much higher concentrations than nitrite and nitrate, such as chloride and sulfate, is a cause of direct interference in many electrophoretic assays. However, the use of direct detection in CIA is advantageous because neither chloride nor sulfate hinder the detection. Signal-to-noise (S/N) was maximal at 214 nm and therefore a small bandwidth value around this wavelength for the diode-array detector was chosen. Specificity as well as sensitivity was enhanced by the selective nature of the electromigration step. This, coupled with the high-resolution power of the technique, made it possible to keep sample handling to a minimum [62].

Care must be exercised when using electromigration, since many ions will effectively compete with nitrite in biological samples. In water, where other ions are present at very low concentration, sensitivity for nitrite is similar to that of GC–MS. In plasma, however, chloride and sulfate are present at far higher concentrations than nitrite and so effectively compete with these anions during the injection. The sensitivity achieved is therefore reduced compared to aqueous matrix but is still significantly higher than in conventional pressure or gravity injection methods [63].

2.1.2. Capillary zone electrophoresis

CE was developed for the detection of inorganic anions [64]. But it still seemed difficult to analyze nitrite in blood plasma, because the plasma contains approximately 3500 mg/l chloride, and this high ion concentration severely interferes with the quantification of nitrite by CZE. Leone and colleagues [46] reported the CE analysis of nitrite using a detection wavelength of 214 nm, at which chloride does not interfere with nitrite detection. However, in this method, samples were introduced in the capillary tube by electrokinetic injection. There might be two biases in quantitative CE caused by electrokinetic sample injection [28]. One is brought about by the different mobilities of nitrite in the sample solution. In the electrokinetic injection, the amount of ions introduced into the capillary is determined not only by the concentration of ions in the sample solution, the cross-sectional area of the capillary and the injection time, but also by ion mobility. Another bias is related to the electrolyte concentration of the medium in which the ions are dissolved. In the electrokinetic injection, mobility of the ions and electroosmotic flow rate of the sample both increase linearly with decreasing electrolyte concentration so that the absolute amount of the same ion will vary between the different samples. Alternatively, these biases can be avoided entirely by using hydrostatic injection. Therefore, Huang and colleagues [28] performed anal-

ysis of diluted and ultrafiltered human plasma samples (cut-off molecular weight 10 kDa) on a CIA connected to Model 805 data station. The separation was performed on a polyimide-coated, 60 cm × 75 μm i.d. fused silica capillary, applying a constant voltage of 20 kV. During the run, the cathode and capillary were immersed in the running buffer at the injection site, and anode and capillary were immersed in the buffer at the receiving site. Under these conditions a current of 312 μA was encountered. On-column detection was at 214 nm. The CVs for within-day precision of retention time are less than 0.6% and those with peak area are less than 5%. CVs for between-day precision of these factors are less than 0.5% and 5%, respectively. Measured plasma nitrite levels are 0.15 ± 0.07 mg/l (corresponding to 3.26 ± 1.5 μM) [29].

The above-described method allows separation and analysis of the analyte within 3 min [46]. However, the detection sensitivity needs to be improved. Strategies to improve the detection limit in CZE have been described and include: injection of low-conductivity sample matrices in which the diluted sample components are preconcentrated during the stacking across the stationary concentration boundary between the sample and the background electrolyte [65–67], use of an on-line packed precolumn [68], variation of pH [69-71], removal of chloride from the sample matrix (either by solid-phase extraction or reversed pre-electrophoresis) [72], and the use of on-line coupled column isotachophoresis-CZE [66,70,71].

The current capillary isotachophoresis (ITP) arrangement is capable of separating and concentrating a sample having a volume of several microliters into the narrow zone of nanoliter volume. The sample introduction procedure is as follows. First, both capillaries are filled with the leading electrolyte, which contains an ion that has an electrophoretic mobility higher than any sample component of interest. Then the sample mixture is injected through the septum into the volume between the injection port and the electrode chamber. The electrode chamber is then filled with the terminating electrolyte, which contains an ion that has an electrophoretic mobility lower than any sample component of interest. When the separation current is switched on, the sample species separate and concentrate into narrow ITP zones. The concentrations of these zones do not vary over time and are given by the Kohlrausch's law [73].

On-line ITP preconcentration can be performed either on-column or in a coupled-column arrangement [74]. In the on-column arrangement method, analysis is conducted such that both ITP preconcentration and CZE separation proceed in the same capillary. Two basic electrolyte systems can be envisioned as follows:

Method A1: If the background electrolyte (BGE) is selected with the co-ion having a higher effective mobility than the sample ions, e.g. nitrite, ITP migration is achieved by using a suitable terminating electrolyte behind the sample zone. Method A2: If the capillary contains a BGE with a lower effective mobility co-ion C (running buffer), the sample itself must be supplemented by a leading ion (such as NH_4^+, K^+ or Na^+ for cationic solutes) to maintain transient ITP migration.

Method B: In a coupled-column arrangement the sample migrates isotachophoretically in the preconcentration capillary between the leading (L) and the terminating (T) ions. The capillary is connected on-line at the end of the preconcentration capillary as described by Foret et al. [71]. After the sample zones have entered the analytical capillary, the separation continues in either the ITP or CZE mode. This method will work well for small molecules.

Method A1 involves the change of the terminating electrolyte in the electrode vessel to the leading electrolyte after ITP preconcentration, and is the most universal approach as no sample pretreatment is necessary. Disadvantages from electrolyte change are minor when an automated instrument is used, where the change can easily be programmed. The duration of the ITP step depends on the sample composition and usually ITP will be completed within 1–5 min. Method A2, where the BGE electrolyte itself serves as the terminating electrolyte for the transient ITP preconcentration, is useful mainly for samples containing salts of highly mobile ions which can serve as a source of leading ions. Moreover, the addition of a salt can be also expected to narrow the differences in conductivity and ionic strength between various samples. The optimum concentration of the leading electrolyte in the sample should generally be around 0.01 M.

The coupled-column approach (Method 2) has several advantages, including the possibility of injecting large sample volumes, effective sample clean-up, and, with controlled current switching, selected ion analysis [75].

Melanson and Lucy [76] used a Prince capillary electrophoresis system equipped with a UV detector, set at 214 nm for the nitrite measurement in aqueous solutions. Separation was carried out in uncoated fused silica capillaries of 75 μm i.d., 365 μm outer diameter (o.d.), 70 cm total length, 55 cm to the detector (Polymicro Technology, Phoenix, Arizona, U.S.A.). Thirty millimolar sulfate—β-alanine pH 3.8 buffer was used for the separation which was performed at a constant current of 75 μA.

At lower pH, the resolution of the analytes could be increased, because of the selective retardation of nitrite [76]. Low separation pH allows adjustment of the resolution of nitrite. Furthermore rapid analysis time was achieved even without using electroosmotic flow modifier. On-capillary sample concentration with field amplified stacking was achieved by dilution of the sample with deionized water. Dilute, low conductivity sample could be injected hydrodynamically into the capillary filled with the separation buffer of higher conductivity. After high voltage was applied, the ions in the low-conductivity sample plug experience a higher field strength than those in the high-conductivity separation buffer, thus the sample ions move quickly through the sample matrix and slow down when enter the separation electrolyte, resulting in a narrow zone of analytes at the boundary of the two solution [32].

The separation efficiency was gained when 90% of the capillary was loaded with the sample after its 100-times dilution, since the low-conductivity sample matrix was removed by the electroosmotic flow through the capillary inlet. This procedure provides considerable sample concentration. Estimated LOD achievable in the undiluted biological specimens is about 2 μM nitrite (in tissue). The rather long analysis time because of the lengthy procedure of the sample stacking is a disadvantage of

this method [41]. On-capillary transient ITP migration permits injection of large volumes (30–50% of capillary volume) of sample into the capillary, without the restriction of having low sample conductivity, characteristic of the field amplified sample stacking [74]. The LOD (S/N of 3:1) in aqueous solution of nitrite (prepared in acetate buffer containing sodium chloride) was found to be 150 nM. The lower limit of quantification (LLOQ) was reported to be 300 nM for nitrite. The intra- and inter-day precision and accuracy were determined at three concentration levels of nitrite. Accuracy was between 101 and 114% for nitrite. Between-day precision was 10% and accuracy was 115% for the analyte [41].

2.1.3. High-performance capillary electrophoresis (HPCE)

The further development of the CZE technique to a HPCE technique allows analysis of nitrite in subnanoliter sample volumes from mammalian cells. The sample is introduced into the capillary by ITP-stacking injection (−6 kV for 20 s) to a preloaded plug of high-mobility electrolyte (pressure-loaded 12 mM LiOH, 50 mbar for 12 s). The separation conditions are as followed: −28 kV, 3–3.5 μA, 22 ± 0.5 °C, running time 15 min [30,31,77]. The measurements were performed using a computerized CE system with a Crystal 1000 contact conductivity detector. The method includes an efficient chloride removal, sampling dilution, and ITP stacking injection of high-diluted samples which had been generated by using a custom-designed solid-phase microextraction cartridge. This method was employed to measure nM- to μM-concentrations of nitrite in the presence of high chloride concentrations in many biological samples such as plasma. An advantage of this HPCE method is the high sensitivity, with the reported LOD values being 8.9 nM (0.41 ppb) for nitrite [42]. The HPCE technique also allows the simultaneous determination of nitrate at biologically relevant concentrations not only in plasma but also in other matrices like tissue so that it can also be applied to single-cell analysis [29].

Leone et al. [46] developed a method based on a modification of the CIA technique (see above) by Wildman [64] that uses improved detection and sample introduction techniques allowing reliable measurement of basal plasma nitrite in a short run. The 1:10-diluted ultrafiltered plasma samples were analyzed in an HP 3D capillary electrophoresis system (Hewlett-Packard Ltd.), using 72 cm fused silica capillaries of 75 μm i.d. (extended light path, 225 μm). Samples were injected by electromigration for 20 s at −6 kV and analyzed at an applied negative potential of 300 V/cm. The capillary was purged with electrolyte for 1 min between runs. Data was acquired at a response time of 0.1 s, with 0.01 peak width selection at 214 nm onto an HP 3D CE Chem Station data system. The intra-assay CV was 10% for basal nitrite and 4.6% for 50 μM nitrite. The inter-assay CV for basal and spiked nitrite was 9%. The normal basal plasma nitrite was determined to be 450 nM (range 250–650 nM) by this method [46].

A significant interference seen in this assay was due to plasma protein effects on the capillary wall. After injection of only 30 samples the migration times of nitrite started to increase, limiting its use as a routine assay. With the introduction of the ultrafiltration step, which removed all compounds with a molecular weight greater than 5 kDa in that method, capillaries were stable for over 300 analyses. The assay also allowed the simultaneous determination of nitrate within a run [46].

Another area of concern in HPCE since its inception has been the short optical path associated with on-column detection [78]. Conventional UV detectors have detection limits between 1 and 10 μM analyte for a 75-μm i.d. column. An increase in sensitivity usually is obtained through improvement of the detector [70,71,79–81].

A simpler concentration technique, called sample stacking, is well-known in electrophoresis [65,82,83] and can also be used to improve nitrite detection. An increase by a factor of 10 in detectability using sample stacking in HPCE was reported by Lauer and colleagues [80]. In the application of sample stacking by Burgi and Chien [32], the composition of the buffer in the sample plug and in the column are the same. A long plug of sample, dissolved in a lower concentration buffer, is introduced hydrostatically into the capillary containing the same buffer but with a higher concentration. Then the separation voltage is applied across the column. Because the buffer in the sample plug contains ions at low concentration, the resistivity in the sample plug region will be higher than the resistivity of the rest of the column. Consequently, a high electric field is set up in this region. As a result, the ions will migrate rapidly under this high field toward the steady-state boundary between the lower concentration plug and the support buffer. Once the ions pass the concentration boundary between the sample plug and the rest of the column, they immediately experience a lower electric field and slow down, thus causing a narrow zone of analyte to be formed in the support buffer region. The thin zone of ions then moves through the support buffer and separates into individual zones according to conventional free zone electrophoresis. The stacking mechanism occurs for both positively and negatively charged species. Cations stack up in front of the sample plug, and anions stack up in back of the sample plug. Neutral compounds are left in the sample plug and co-elute. Theoretically, the amount of stacking is proportional to the ratio of resistivities between the buffer in the sample plug and the buffer in the rest of the column. If the two different buffer regions have similar compositions, this ratio of resistivities is simply the inverse of the ratio of concentrations.

Burgi and Chien [32] used a CE instrument similar to the one reported by Jorgenson and Lukacs [78]. The high-voltage power supply delivered 39 kV in 5 kV increments. The column was a 100-cm long, 75-μm i.d., 365-μm o.d. fused silica capillary with a detector window at 65 cm from the injection end. The UV absorbance detector was a Varian 2550 with a 100-μm slit in a modified microcell holder. The wavelength of analysis was 265 nm.

The optimal condition for the sample stacking is the sample preparation in a buffer with a concentration that is about 10 times less than that used for electrophoretic separation and a sample plug length up to 10 times the diffusion-limited peak width. Less concentrated buffer in the sample plug than the optimal conditions will generate laminar flow, thus causing peak broadening. Longer plug lengths than the optimal conditions lead to a

dramatic decrease in resolution due to laminar broadening and a reduction of the electric field in the high-concentrated-buffer region [32].

2.2. Flow injection analysis (FIA)

FIA is an analytical technique based on microfluidic manipulation of samples and reagents. This method is known since the early 1970s and is characterized by its especially simple design and great versatility [84,85]. Samples are injected into the system through which they move until the whole analysis is completed. Samples become a part of a continuous, non-segmented flow of a carrier solution, into which, at predetermined points, reagents are added at fixed flow rates. The processed stream finally flows through the reaction coil and the detector cell where the quantitative measurement is executed and the signal is continuously recorded. The movement of all liquids within the conduits of the unit is controlled by low-pulsation pumps with minimal flow variation to obtain a very stable baseline. An appropriate chemical reaction takes place during the transport [84,43].

The combination of the FIA technique with the Griess reaction can be used to determine nitrite in deproteinized biological fluids. Within the reaction coil nitrite reacts with the carrier solution containing sulfanilamide (40 g/l dissolved in a 1% HCl solution; stable at room temperature in a lightproof container) and *N*-(1-naphthyl)ethylenediamine (2 g/l dissolved in water; stable at 4 °C) under acidic conditions to form a red-colored azo compound [25]. The detector measures the absorbance at the characteristic wavelength of 540 nm.

The complete reaction of nitrite with the Griess reagent, which may take about 20 min [86], is not necessary for the measurement of this anion by FIA. Because external conditions such as flow, capillary length, and temperature are standardized, it is possible to measure nitrite samples that have incompletely reacted with the Griess reagent [87].

For nitrite determination, aqueous samples can be used directly, protein-containing samples like human plasma have to be transferred to ultrafiltration tubes with a pore size of 10 kDa (e.g. Centricon, Millipore) and centrifuged at 4 °C for 120 min at 6000 × *g* prior to FIA. For detection, a sample volume of 20 μl has to be injected [25]. LOD values for nitrite were found to be 2 nM in aqueous solution and 8 nM in 1:2-diluted (v/v) ultrafiltered plasma samples [25,43]. Plasma nitrite concentrations usually measured by this technique amount to 305 nM [16].

The FIA method is suitable for the measurement of nM-concentrations of nitrite with only low susceptibility to interferences in comparison to the commercially available colorimetric batch Griess assay [88]. The FIA technique allows a high-throughout analysis of nitrite when coupled to an autoinjector. Samples are injected precisely at 55-s intervals at which the sample is introduced, the Griess reaction takes place and the signal is displayed. The signal then reaches the baseline and another sample can be introduced. This configuration yields a maximum throughput of 65 analyses per hour [87].

The sensitivity and the sample throughput can be adjusted by varying the capillary diameter and the length of the reaction loop. All capillaries, which are made of polyether ether ketone (PEEK), should have an internal diameter of 0.5 mm. The length of the capillary tubes has to be kept as short as possible. The optimum dimension of the reaction loop is 90 cm from the autosampler to the detector [25].

Hemoglobin, ethanol and citrate may interfere with the FIA-Griess measurement of nitrite [25]. These interactions should be kept in mind when choosing the dilutor and anticoagulant for the sample. Heparin is a non-interacting anticoagulant that can be used for plasma generation. Other substances that may lead to interferences in the Griess reaction have been reported to include biogenic amines, zinc sulphate, cadmium, manganese, iron, zinc, urea, thiols, proteins and other plasma constituents [89,90]. To identify possible interferences, calibrations curves with aqueous nitrite solutions should be routinely performed [43].

2.3. High-performance liquid chromatography

HPLC is a useful separation-based analytical technique for the simultaneous determination of nitrite and nitrate. An injector or autosampler, two-piston pumps, a guard column as well as an analytical column and a detector built the basis of this chromatographic system. The analytes permeate through the analytical column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase. Two different types of chromatography, the anion-exchange and ion-pairing reversed-phase, were used to determine nitrite and nitrate by HPLC. The columns differ in their distinct chemical properties of the packing material [91]. Detection is performed by UV or VIS absorbance, electrochemistry, fluorescence or chemiluminescence.

Nitrite and nitrate absorb UV light at 200–220 nm. Therefore, a direct detection after separation by using an anion exchanger and a UV detector set at 210 or 214 nm is possible [33,34]. This method shows a LOD for nitrite of 30 nM [33] and 10 nM [34], respectively. The simplicity of this chromatographic system is an advantage, certainly, but the major problems result from the interferences of chloride with UV absorbance detection for nitrite, due to the lack of resolution and column saturation [92].

Tsikas et al. described a reversed-phase HPLC method with UV absorbance detection at 333 nm for the determination of nitrite and nitrate in ultrafiltered plasma samples [35]. The principle of this method is based on HCl-catalysed conversion of nitrite by *N*-acetyl-L-cysteine to *S*-nitroso-*N*-acetyl-L-cysteine. Plasma nitrite levels in healthy volunteers were determined to be 0.55 ± 0.16 μM by this method. The LOD of this method was reported to be 50 nM nitrite (in aqueous solutions) [35]. The elimination of chloride interference represents an advantage of this HPLC method.

Preik-Steinhoff and Kelm developed a sensitive method for the quantification of nitrite and nitrate in human blood with high-performance anion-exchange chromatography and amperometric detection for nitrite and UV absorbance detection for nitrate [36]. The amperometric detection system uses the electrochemical activity of nitrite after oxidation on the surface of the electrodes. An advantage of the electrochemical detection is its greater selectivity and sensitivity compared to methods based on UV absorbance and chemiluminescence [93]. The interfer-

ence by oxidizable substances represents a major disadvantage of this method. In comparison to commonly used acid-catalyzed deproteinization, which may result in considerable loss of nitrite due to nitrosation reactions and due to reduction of nitrite to NO, blood can be alkalinized with aqueous sodium hydroxide to precipitate proteins and to prevent oxidation of nitrite. This method has an LOD value of 8 nM [36]. Jedlickova et al. analyzed nitrite in human plasma without deproteinization using 0.02 M sodium perchlorate monohydrate as a mobile phase at pH 3.9, at which organic substances do not affect the analysis. The LOD of this method was reported to be 1 nM (with electrochemical detection) [53], but it does not take into account that nitrite can be possibly reduced. A column filled with an ion exchanger based on styrene-divinylbenzene with quaternary amine in the Cl^--form should be used to avoid the interference of chloride [53].

Another HPLC technique couples ion chromatography with on-line reduction of nitrate to nitrite in a cadmium reduction column, subsequent colorimetric reaction with the Griess reagent and VIS absorbance at 540 nm. In order to minimize interferences with proteins, plasma was subjected to methanol precipitation [37]. An advantage of this method is the elimination of chloride interference because chloride does absorb visible light. With LOD values of 100 nM [49] and 1 nM [37,49] for nitrite, two distinct LOD values were reported for this method. We can only guess how these differences appeared. Differences in reported LOD values can be caused by the use of different detectors or by differences in carrier solution, just to mention some possibilities.

In their reversed-phase HPLC method Marzinzig et al. [38] and Li et al. [39] replaced the post-column colorimetric Griess reaction by a pre-column fluorimetric reaction by using dapsone (4,4′-diamino-diphenylsulfone) and 2,3-diaminonaphthalene (DAN), respectively. DAN is a weakly fluorescent agent that reacts with nitrite under acidic conditions to form the strong fluorescent 2,3-naphthotriazol [94]. This method has a LOD of 10 nM at a submaximal gain setting [39]. Simple sample pre-treatment using ultrafiltration to remove proteins and the lack of interferences when sufficient dilution of a sample is made, are the advantages of using fluorescence detection in HPLC methods [39].

2.4. Gas chromatography–mass spectrometry

With respect to the composition of GC–MS instruments and the gas chromatographic and mass spectrometric principles of this particular technique is referred to the literature [95]. A GC–MS method for the analysis of nitrite in biological fluids uses pentafluorobenzyl (PFB) bromide as the derivatization agent. Nucleophilic substitution of bromide in PFB bromide by nitrite and nitrate leads to the formation of the nitro PFB derivative (PFB-NO_2) [96] and nitric acid ester PFB derivative (PFB-ONO_2) [97], respectively. This is the sole derivatization reaction that allows for the simultaneous analysis of nitrite and nitrate by GC–MS [97].

^{15}N-Labeled nitrite ([^{15}N]nitrite) is added as the internal standard to the respective biological matrix at appropriate final concentrations. Endogenous nitrite ([^{14}N]nitrite) and [^{15}N]nitrite undergo all chemical and physical changes in an almost identical manner during the whole analytical process, i.e. derivatization with PFB bromide, extraction with toluene and GC analysis. Negative-ion chemical ionization of the PFB derivatives of [^{14}N]nitrite and [^{15}N]nitrite produces the [^{14}N]nitrite and [^{15}N]nitrite anions, which are separated in the mass spectrometer due to their different mass-to-charge (m/z) ratios of m/z 46 for [^{14}N]nitrite and m/z 47 for [^{15}N] nitrite. The LOD of this method was reported to be 22 fmol for [^{14}N]nitrite. The concentration of nitrite in the plasma of healthy young volunteers with standardized nitrate diet was determined with this method to be 1.8 μM [40].

Mass spectrometry-based approaches emerged as indispensable analytical tools for the reliable quantitative analysis of the whole NO family and represent a reference method for nitrite and nitrate [22]. Quantification of nitrite and nitrate in human biological fluids by GC–MS as PFB derivatives has been shown to be free of any interference [20,97]. GC–MS offers the extra benefit of allowing isotopic analyses so that in vitro and in vivo studies can be performed with stable-isotope labeled substances including [^{15}N]nitrite [98].

GC–MS allows a highly specific simultaneous derivatization and accurate and precise quantification of nitrite and nitrate in any biological sample, but its use is restricted to very few investigator groups, so far. GC–MS equipment is relatively expensive in both purchase price and running costs [62].

2.5. Ozone-based chemiluminescence detection

The reaction of NO with ozone (O_3) results in the emission of light, and this light (emitted in proportion to the NO concentration) is the basis for one of the most accurate NO assays available [99,100]. The need to understand the technical aspects of the chemiluminescence NO assay has increased with the recent discovery of the importance of biologically synthesized NO [2].

In addition to measuring NO itself, the NO chemiluminescence assay can be used to measure the intermediates and end-products of NO oxidation, e.g. nitrite [45] which is useful as the oxidation of NO is very rapid in the presence of oxygen and oxygen-derived species, making measurements of NO or nitrite important for estimation of NO synthesis in many biological systems [62].

Light emission accompanying the reaction of O_3 or singlet oxygen with gases such as NO, NO_2, CO and SO_2 was described in the early 1960s. It was found that singlet oxygen reacts with all of these gases, while O_3 reacts most readily with NO. This is the basis of the O_3 chemiluminescence assay's selectivity for NO. The reaction of NO with O_3 yields nitrogen dioxide, some of which is in the excited state (NO_2^*) [101]:

$$NO + O_3 \rightarrow NO_2^* + O_2$$

In the excited state, the electrons dissipate energy as they regain their original stable "ground" state. The excess energy of the excited nitrogen dioxide can either be quenched by interaction with other gas molecules (M) or released as a photon

(*hv*) [101]:

$$NO_2^* \rightarrow NO_2 + hv$$

$$NO_2^* + M \rightarrow NO_2 + M$$

The light is emitted in the red and infrared region of the spectrum (~640–3000 nm) with peak intensity at ~1100 nm [102]. Photomultipliers, used to detect the emitted light, are sensitive only to wavelengths below 900 nm. Nevertheless, the amount of light emitted by the $NO + O_3$ reaction in the 640–900 nm-range is still sufficient to make chemiluminescence one of the most sensitive NO assays available [100].

In 1970, Fontijn et al. constructed the first NO chemiluminescence analyzer [99]. They showed that the emitted light is linearly proportional to the NO content of the specimen over a broad range and is not interfered by NO_2, CO_2, CO, C_2H_4, NH_3, SO_2 or H_2O [99]. The NO chemiluminescence analysers can be obtained from a number of manufacturers, e.g. ECO Physics (Switzerland), ChemLab Instruments Ltd. (UK) or Sievers Instruments Inc. (USA). The NO chemiluminescence analyser belongs to the group of luminometers. In contrast to other light-measuring analytic devices, such as spectrophotometers or fluorometers, luminometers do not need a light source. Consequently, the basic scheme of a luminometer is very simple. It consists of a reaction chamber, where the chemiluminescence reaction takes place, a light detector that measures the emitted light, and a recorder. A vacuum pump draws O_3 and sample into the chemiluminescence reaction chamber. In the reaction chamber, NO from the sample reacts with O_3 and the emitted light is detected by a cooled photomultiplier and recorded. O_3 is created by electrical discharge in the O_3 generator. The inflow rate of the sample gas is regulated by the needle valve with the aid of an optional flowmeter. A red cut-off filter increases the specificity of NO detection (Fig. 1) [62].

As NO_2^* emits relatively weak red and infrared light, the detector of choice for the $NO + O_3$ chemiluminescence is a photomultiplier because of its sensitivity to low levels of light at the red end of the spectrum. In the photomultiplier, photons strike a photosensitive surface and the impact releases electrons which are accelerated toward an electron-sensitive surface (the first dynode) by an electric field. Each electron's impact causes emission of several electrons from the first dynode, and these are accelerated towards a second dynode. This step is repeated several times and finally the electrons are attracted to the terminal electrically charged element, the anode. The resulting current is measured. The amplification of this cascade is in the order of millions of electrons at the anode for each electron emitted from the photosensitive surface [103].

Besides high sensitivity, another advantage of the photomultiplier over alternative light-detection devices is the stability of dark current. Nonetheless, a number of factors can affect dark current, including temperature, light and voltage history. For this reason it is desirable not to expose the analyser to excessive amounts of NO, as this leads to enormous light emissions and increased dark current. Disturbances of dark current may take days to stabilize [103]. The photomultiplier should be separated from the reaction chamber by a red cut-off filter. Its purpose is to prevent the photomultiplier from detecting light with wavelengths below those emitted by the $NO + O_3$ reaction, such as the blue and ultraviolet emission of alkenes and sulphur-containing species [104]. It also removes part of the background signal due to the poorly understood interactions between O_3 and the reaction chamber wall (Sievers nitric oxide analyzer NOA^{TM} 270B 1992).

With certain reducing agents in an acidic environment, nitrite can be converted to NO. In the presence of an acid (such as HCl, citric acid or glacial acetic acid), nitrite is converted to the nitrosonium ion (NO^+):

$$NO_2^- + 2H^+ \rightarrow NO^+ + H_2O$$

NO^+ associates rapidly with anions and other nucleophiles. For several reasons (such as the speed of the reaction and

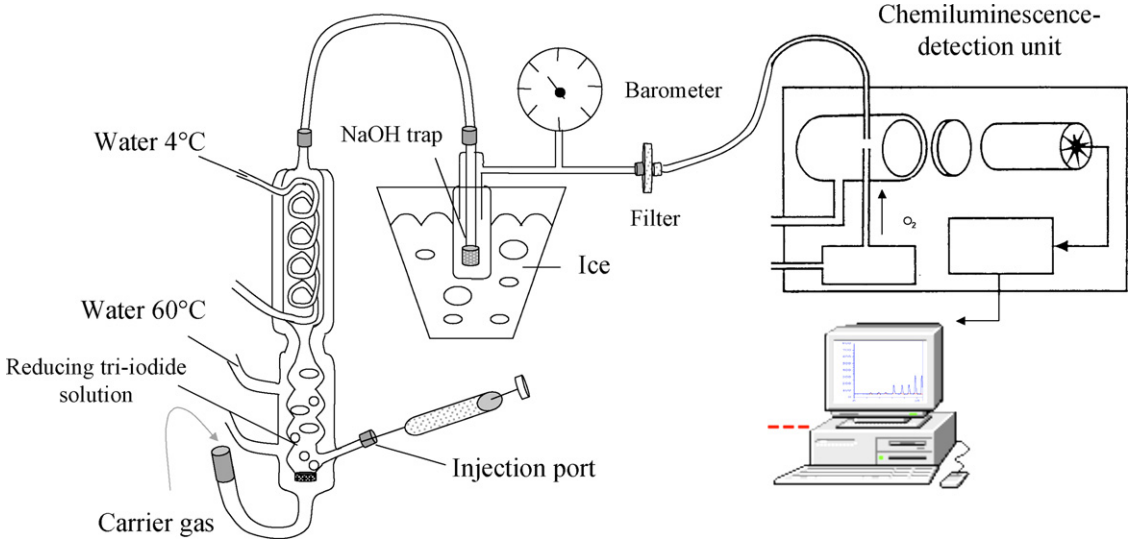

Fig. 1. Schematic of a chemiluminescence apparatus for the analysis of nitrite in biological fluids. The sample is directly injected into the reaction chamber (held at 60 °C) which contains the reducing agent (e.g. tri-iodide solution). Here nitrite is reduced to NO. The chamber, which is constantly purged with a carrier gas stream, e.g. helium, is in-line connected with a NaOH-trap to scavenge higher nitrogen oxides, and with a NO chemiluminescence and detection unit.

non-volatility), iodide (NaI or KI), 1,1′-dimethylferrocene or vanadium(III) (at room temperature) are advantageous for the nitrite chemiluminescence assay [45,105]. If iodide is used, NO^+ is converted to NO via a nitrosyl iodide (ONI) intermediate:

$$NO^+ + I^- \rightarrow ONI$$

$$2ONI \rightarrow 2NO + I_2$$

In the original description of the method [45], a 20-ml sample volume was mixed with 1 ml of 0.2 M NaI and 3 ml of acetic acid. The disadvantage of this approach is that all reagents must be replaced after each specimen. It consumes additional time, as one must wait for the signal produced by KI and acid alone [45,106] to disappear. The origin of this large spike is unknown, but it is dependent upon O_3 as turning the ozone generator off prevents its occurrence. It is more practical to use a large excess of reagents, which allows measurements of many samples before reagents must be replaced, KI and acid should be replenished, though, before the pH of the reagent mixture rises by more than one unit [105].

The LOD values of chemiluminescence-based methods for nitrite improved from 50 nM in the early 1980s [45] to presently 5 nM [26], possibly by improvement of the sample and solution preparation, and improvement of the NO chemiluminescence analyzers or experimental set up (e.g. improved flow rate). Typically measured plasma nitrite concentrations in healthy humans are in the range of 114 nM [56] to 5.5 μM [59]. The majority of publications provide plasma nitrite concentrations in the nM-range, although this range varies over several magnitudes (Table 3). Whole blood nitrite concentration is reported to be 176 nM [57] (Table 3). Fig. 2 shows original tracings of nitrite measurement in plasma and whole blood with good reproducibility of the peak areas. The measured nitrite concentrations of 210 nM in plasma and 180 nM in whole blood (unpublished data) reflect the so far obtained nitrite concentrations in biological fluids as presented in Table 3.

The CLD technique allows the detection of nitrite in biological fluids, such as blood, plasma, urine or serum, without extensive sample preparation. Neither a deproteinization step nor the removal of blood cells or cellular constituents is necessary [26]. The CLD also allows the detection of other NO metabolites, e.g. *S*- and *N*-nitrosated proteins or nitrate, but their measurement requires sample preparation prior to analysis, i.e. reaction with nitrate reductase for the measurement of nitrate or the treatment of the plasma sample with EDTA, sulfanilamide and *N*-ethylmaleimide for the determination of total protein-bound NO except for nitrosyl hemoglobin (*Fe*-NO). Typical analysis time in CLD is approximately 5 min per sample [45].

3. Discussion

Nitrite is a marker of NO synthesis with an undisputed relevance as a biochemical parameter established both in experimental studies and in clinical studies [16,17,107]. Accumulating evidence suggests that nitrite is also a vascular endocrine NO reservoir that contributes to hypoxic signaling [19] and physiological vasodilation [108–110]. In these studies a vasodilation had been observed at nearly physiological concentrations of nitrite (<5 μM). These important findings call to evaluate sensitive methodologies for the measurement of nitrite in biological samples and to detect nitrite concentrations in relevant biological fluids of healthy humans. But up to date there is no consensus concerning the real nitrite concentration in human plasma or blood. A wide range of nitrite concentrations in plasma, erythrocytes and whole blood in healthy humans has been observed, as summarized in Table 3 and illustrated in Fig. 3. Published concentrations for circulating nitrite range from "non detectable" to 26 μM. The insertion of Fig. 3 shows a categorization of the most frequently published nitrite concentrations being below 1 μM. In accordance with Fig. 3, it appears that most investigator groups measured basal plasma nitrite concentrations in healthy humans ranging from 100 nM to 600 nM, however, this range is not shared by all investigator groups [33,52–55,59,97].

Pre-analytical factors as well as analytical problems can lead to such great differences in nitrite concentrations. Therefore, it is important to thoroughly discuss potential reasons for this discrepancy.

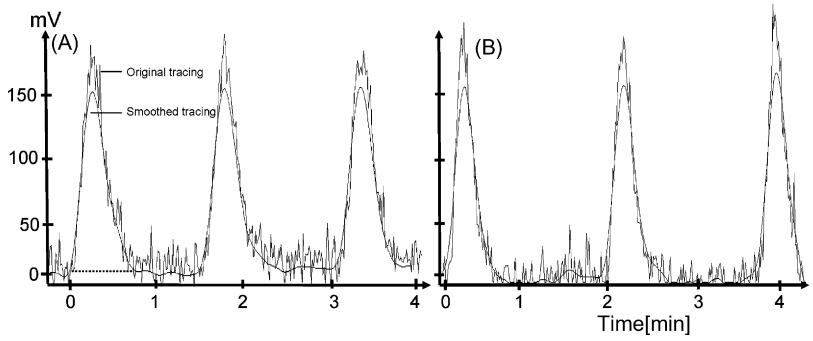

Fig. 2. Original and smoothed tracings of triple measurement of nitrite in plasma (A) and whole blood (B) of a subject by tri-iodide chemiluminescence as described elsewhere [26,56,57]. For data analysis we integrated the tracing peak by peak to obtain the area under the curve (measured in mV). (A) Whole blood was spun down at 800 × g for 15 min and 100-μl aliquots of the supernatant were injected. The peak area corresponds to a nitrite concentration being 210 nM (data not published). (B) Whole blood was diluted with a nitrite preservation solution containing 0.8 M ferricyanide, 10 mM *N*-ethylmaleimide (NED), and 1% NP-40 (blood-preservation solution, 5:1, v/v). The sample was deproteinized with methanol (1:1, v/v), spun down at 15,000 × *g* for 10 min, and 100-μl aliquots of the supernatant were injected. The peak area corresponds to a nitrite concentration being 180 nM (data not published).

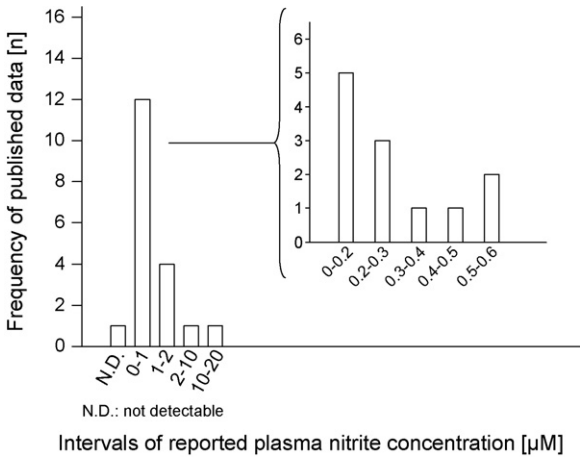

Fig. 3. Frequency of reported basal plasma nitrite concentrations in healthy humans. The basal plasma nitrite concentrations reported in Table 3 are summarized and presented with respect to the frequency of detected levels in humans; a total 19 studies was considered. Insertion shows the frequency of studies having reported plasma nitrite levels below 1 μM. This Figure shows that most investigator groups found plasma nitrite levels between 100 and 600 nM.

3.1. Importance of pre-analytical and analytical factors

3.1.1. Pre-analytical factors

Analytical methods for circulating nitrite have produced a wide range of basal nitrite concentrations in healthy volunteers (Table 3, Fig. 3). It is conspicuous that reported nitrite concentrations do not only vary significantly between methods based on different principles, but also within the same analytical approach used by different investigator groups (Table 3). For example, by using the HPLC technique, nitrite levels in plasma from healthy volunteers vary from non detectable [50] to 26 μM [55].

Reliable quantitative determination of nitrite requires a sophisticated experimental set-up, including appropriate storage conditions of internal nitrite standards and chemicals, careful sample preparation, elimination of interferences, chemical derivatization, and improvement of detection. Influencing factors such as nutrition, age, physical conditions and physical fitness represent additional important pre-analytical factors. In clinical studies blood is usually taken from overnight fasting volunteers to exclude short-term effects of recently consumed food, but the general dietary intake differs epidemiologically and may affect nitrite concentration. The influence of flavanol-rich food on the circulating bioactive NO pool was shown by Heiss and colleagues who observed an increase in nitrite after ingestion of flavanol-rich food in volunteers with endothelial dysfunction [111]. Next to flavanols, other dietary compounds like virgin olive oil [112], fish oil [113], aged garlic extract [114,115], or a soy-protein rich diet [116] may positively influence the cardiovascular homeostasis by increasing eNOS activity. Physical activity also positively influences the eNOS activity [117,118]. Therefore, it is difficult to compare studies when eating habit and physical conditions of study participants is not known. These points lead to the conclusion that blood drawn should be performed under the same conditions and should preferably be done at rested and overnight fasting volunteers and the eating habit should be noted, if possible.

3.1.2. Analytical factors

3.1.2.1. Nitrite contamination. The major source of nitrite contamination has been reported to include conical tubes and pipette tips used to transfer solution [119]. Other laboratory ware was also found to contain nitrite which potentially contributes to false results. Especially soft glass ware and ultrafiltration units for protein precipitation were found to contain considerable amounts of nitrite even after extensive washing procedures which may contribute to elevated nitrite concentration in the sample [25,33,56,120–122]. It should be noted that it is impossible to wash laboratory ware like pipette tips or ultrafiltration cartridges prior to use. Thus, the membrane of ultrafiltration cartridges is very sensitive and may burst during the washing procedure. Prior to use, ultrafiltration cartridges and other laboratory ware should be tested concerning their nitrite content. Fig. 4 shows the nitrite concentration of ultrafiltration cartridges from two different companies (Millipore and Sartorius). The measurement shows that differences in the nitrite content appear both between companies and between different charges of the same product. It is recommended to avoid the use of soft glass, ultrafiltration cartridges or vacuum blood sampling tubes showing high nitrite concentrations after testing.

Protein precipitation, which is absolutely necessary for the nitrite measurement in many matrices, notably plasma, by HPLC- and FIA-based techniques, should be performed using deproteination agents such as methanol or acetonitrile. Acid-induced deproteinization should be avoided because it may result in rapid and abundant loss of nitrite due to nitrosation reactions

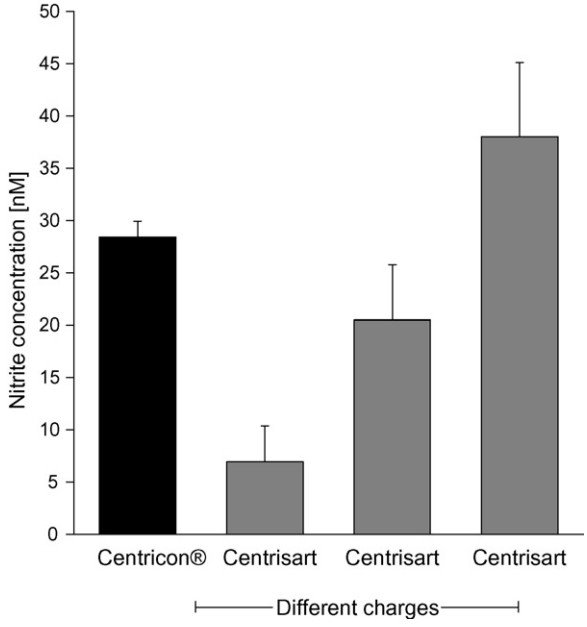

Fig. 4. Nitrite concentration measured in ultrafiltration cartridges purchased from two different companies, i.e. Centricon® from Millipore (cut off 10 kDa) and Centrisart from Sartorius (three different charges; cut off 10 kDa each). Cartridges were filled with phosphate buffered saline (PBS) from PAA – The Cell Culture Company (2 ml) and nitrite was determined by CLD. Data are presented as mean + SD ($n = 10$ each) difference before and after filtration of PBS.

and in some conditions due to reduction of nitrite to NO [36]. Conclusively, the less sample preparation is needed, the less nitrite contamination contributes to the measurement of nitrite.

All methods discussed in this review allow for nitrite analysis in small volumes of native and diluted samples. Thus, samples were diluted tenfold for the use in the HPCE method [46] before nitrite measurement. The CZE allows 100-fold dilution of the samples [41]. Measurement with the FIA method is performed at 1:2 (v/v) diluted samples [16]. Also, samples measured by the HPLC technique are diluted up to 4-fold [53]. For the nitrite measurement using CLD the whole blood is usually diluted 5-fold [111,123]. Regularly, sample dilution is not required in GC–MS methods of nitrite [40,97]. Sample dilution may be required if nitrate has to be reduced to nitrite under alkaline conditions [124]. Sample dilution reduces the amount of blood that has to be taken from the volunteer. However, sample dilution represents a risk factor for the nitrite measurement due to contaminating nitrite present in the dilutor. In our laboratory we consistently measure low concentrations of nitrite in dilution buffers (e.g. phosphate buffered saline) and Millipore water (about 10 nM).

3.1.2.2. Comparison of methods. For reliable quantitative determination of nitrite in a certain biological matrix and a given clinical condition it is imperative to use specific, selective, interference-free, sensitive and accurate analytical methods. HPLC, FIA, GC–MS and electrophoresis-based systems are generally fully automated, and external factors potentially leading to unspecific reactions once the sample is injected into the system are very rare in these techniques. Chloride in plasma interferes with the quantification of nitrite by CZE [64]. The electrokinetic injection of the sample is associated with two problems [28], i.e. different mobility of nitrite in the sample solution and the electrolyte concentration of the medium in which the ions are dissolved. These problems can be avoided by using hydrostatic injection [28]. The sample stacking led to a LOD of 150 nM [41]. Another analytical issue that has to bear in mind is the running time, being 15 min for one sample when using HPCE [42]. This method does not possess the sensitivity to allow a reliable measurement of nitrite in human biological fluids because its LOD is higher than commonly determined plasma nitrite concentrations (see Table 3).

One specification of the FIA (and HPLC) technique is that the samples have to be deproteinized to guarantee continuous sensitive and selective nitrite detection. In the FIA method hemoglobin, alcohol and citrate may interfere [25]. Using a UV/VIS detector set at 540 nm ensures specific detection of nitrite and enables a LOD of 8 nM for plasma nitrite [43] (see Table 2). The FIA equipment is affordable, and most of the reagents used are standard chemicals in the laboratory. FIA instruments are equipped with an autoinjector and appropriate software that allow high-throughput analysis, e.g. 65 samples per hour [87].

Problems with anion exchange or ion-pair reversed-phase HPLC techniques may result from interference by chloride with UV detection for nitrite due to insufficient resolution and column saturation [92]. The HPLC equipment is relatively cost intensive when used for nitrite analysis in clinical studies [53], but the HPLC is the only reported method so far that allows the detection of nitrite and nitrate simultaneously without prior sample preparation. It also allows high-throughput analysis of nitrite, up to 65 samples per hour [87], and has the ability of measuring other members of the L-arginine/NO pathway. These characteristics compensate costs-related disadvantages of the HPLC methodology. For a detailed discussion of HPLC methods used in the analysis of nitrite and nitrate in biological fluids see the review by Jobgen et al. [125].

One disadvantage of the GC–MS methodology is the relatively high purchase and running cost. However, the method involves simultaneous derivatization and quantification of nitrite and nitrate as PFB derivatives and allows accurate, precise, interference-free and sensitive quantitative analysis of nitrite and nitrate in 100-μl aliquots of native human plasma or urine [40]. These characteristics make this GC–MS method to a reference method for the quantitative determination of nitrite (and nitrate) [126] and numerous other members of the L-arginine/NO family [98] in various biological fluids.

Cox [45] tested several reducing agents in the CLD technique for the measurement of nitrite and found that the iodide anion was the most efficient reducing agent in a weakly acidic medium, being interference free. Further sample preparation is needed for the measurement of other anions, notably nitrate, or other NO-containing compounds. The method allows high throughput analysis of nitrite (3 min per sample).

3.1.2.3. Bioanalytical method validation. The bioanalytical method validation is essential to achieve reliable results from quantitative analyses in biological samples. Analytical methods and techniques are constantly undergoing changes and improvements. Specific validation criteria are therefore needed for methods intended for analysis of a certain analyte.

In the last decade there has been made tremendous progress in the field of nitrite analysis. Thus, multi-well plates, automated robotic sample processing and electronic data evaluation are common practice. Given the certainty of continued technological advances, the future will very likely bring improved and more powerful bioanalytical approaches, particularly in terms of high-throughput analysis and increased sensitivity [127]. Despite these widespread advances, thorough validation of analytical methods intended for quantitative analyses in biological matrices remains an indispensable requirement, a must.

Bioanalytical method validation aims to demonstrate that a particular analytical method for the quantitative determination of a certain analyte in a particular biological matrix is reliable for the intended application [127]. The analytical parameters essential to ensure the acceptability of the performance of a bioanalytical method include accuracy, precision, selectivity, sensitivity and reproducibility. Accuracy (recovery %) provides information about the degree of closeness of the determined value to the known true value under prescribed conditions. Precision (relative standard deviation, RSD) describes the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Selectivity is the ability of the bioanalytical method to unequivocally measure a particular analyte in

the presence of other analytes which may be expected to be present in the sample. Reproducibility is defined as the precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period of time [127].

A difficulty in evaluating and comparing different analytical methods for a particular analyte, e.g. nitrite, is that the nomenclature regarding the main analytical parameters in method validation is not uniformly used throughout the literature. This especially applies to the LOD and LLOQ parameters which are confused. LOD is defined as the lowest amount of an analyte that the bioanalytical procedure can reliably differentiate from the background noise. Typically, LOD is defined as the analyte amount that produces a signal at least three times the noise, i.e. S/N ≥ 3:1. Frequently, LOD values are reported in concentration units. By contrast, LLOQ is defined as the lowest concentration of an analyte that can be quantitatively determined with satisfactory precision (usually with a RSD of ≥20%) and accuracy (usually with a recovery of 100 ± 20%). Usually, the lowest concentration on the standard curve prepared in a particular biological matrix is chosen as the LLOQ value of the method.

In this review it is not possible to compare the reviewed methods of nitrite on the basis of the main method validation parameters discussed above, because the majority of investigators did not thoroughly validate their methods or at least they did not report characteristic validation parameters. All methods evaluated in this review reported precision being below 10%. The CZE method provided precision of 9, 3, 2% for nitrite added at concentrations of 0.5, 1 and 2 μM, and an accuracy of 101–114% for nitrite in biological samples [41]. The HPCE technique has been reported to provide high precision (1%) and accuracy (99%) for the measurement of nitrite [42]. The CLD method provides good precision. Thus, intra-day precision was reported to range from 2 to 7% with nitrite standards and from 5 to 7% in biological samples, and inter-day precision was reported to amount to 5% [26]. The FIA-Griess shows similar characteristics with a precision calculated to be 7% (unpublished data). The precision of the reversed-phase HPLC method by Li and colleagues [39] was reported as 0.9% for the nitrite in plasma. For the GC–MS analysis of nitrite as PFB derivative precision and accuracy were reported to be 3% and 102%, respectively, for serum nitrite in quality controls samples [128]. The LOD values of the nitrite methods summarized in Table 2 and compared here differ significantly between the methods, with the GC–MS method having the lowest LOD (22 fmol) [40] and the CZE showing the highest LOD (150 nM) [41]. All other methods have reported LOD values being below 10 nM.

Validation parameters such as precision, accuracy and LLOQ are insufficiently investigated or reported so far. In this context it is necessary that a validation is performed when bioanalytical methods are transferred between laboratories or analysts, instrument and/or software platform changes, changes in species within a matrix or changes in the matrix within a species occur [127], and that these data are constantly published every time the method is used or changed. This proceeding would consequently allow the comparison of the methods on the basis of validation data including precision, accuracy, LOD and LLOQ.

To estimate the potentially most reliable method among different analytical methods available for the determination of nitrite in biological fluids one has to compare these analytical methods using the same biological matrix under the same experimental conditions. Kleinbongard et al. [16] applied CLD, FIA-Griess and HPLC-Griess techniques for the nitrite measurement in human plasma. The study showed that all three techniques provided similar results for nitrite concentration in human plasma with a mean of 345 ± 17 nM [16]. Plasma nitrite measured in that study correlated significantly between the three methods. The measurements showed close correlations: FIA versus CLD: $r = 0.96$; $P > 0.001$; CLD versus HPLC: $r = 0.97$; $P > 0.001$ [16]. This can also be seen in Fig. 5 which presents another data set of measured nitrite concentrations in healthy humans, obtained by the CLD, FIA-Griess and HPLC-Griess method (unpublished data). Besides intra-laboratory comparison, participation of several investigator groups in an inter-laboratory comparison study on nitrite concentrations in plasma or serum samples is highly desirable and would help define reference values and intervals for circulating nitrite in humans [129]. Our progress in understanding the NO biology hinges on the accuracy and specificity of the methods used. The methodological improvements in terms of the sensitivity and selectivity of analytical techniques that were introduced in part in the last decade have helped uncovering some of the complexity underlying NO signaling. As we continue to make progress in our understanding of the biological chemistry and physiology of NO we should not ignore the subtleties nor lose respect for the field of analytical chemistry, which provides an important basis for much of our research efforts in this field [129].

3.2. Nitrite as a biochemical parameter in experimental and clinical studies

Upon formation from L-arginine, NO undergoes multiple reactions leading both to biological activity and to its inactivation to nitrate via oxyhemoglobin-catalyzed oxidation in red

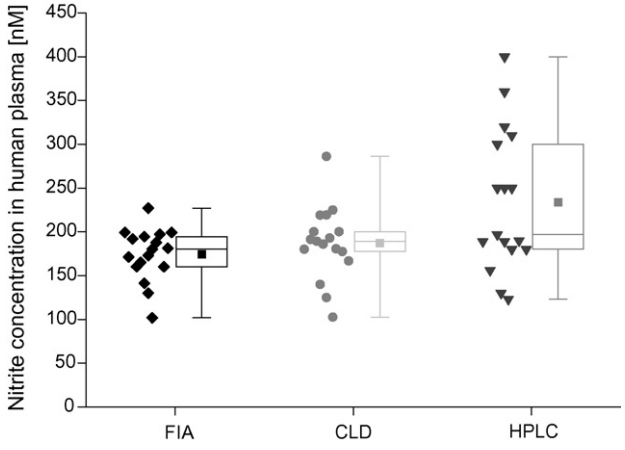

Fig. 5. Concentration of nitrite in plasma of healthy humans ($n = 17$) newly measured by three methods, i.e. FIA-Griess, CLD and HPLC-Griess. Obtained single data were summarized in intervals of five each. Individual values, intervals, mean, standard deviation and meridian values are shown.

blood cells and to nitrite by autoxidation [7,14,130]. These reactions keep the half-life of NO in the circulation far below 1 s [131] and the basal NO concentration in blood in the low nM-range [132]. Therefore, circulating NO at the basal state is not accessible to analytical determination [132]. By contrast, with a half-life of about 10 min in whole blood [57] nitrite has a considerably longer half-life than NO, while nitrate seems to be completely inert. Nitrite and nitrate circulating in blood are potential indices of NO production, at least from the analytical point of view. Nitrate concentrations are influenced by a variety of NOS-independent factors, including dietary nitrate intake, bacterial nitrate synthesis within the bowel, inhalation of atmospheric gaseous nitrogen oxides, and renal function [14,133]. Because of these factors and the relatively high basal levels, small changes in plasma nitrate concentrations may not sensitively reflect acute changes in the activity of NOS [17].

Kelm et al. determined the serum nitrite concentration in blood samples taken from the antecubital vein of healthy human volunteers. The results showed that the nitrite concentration sensitively reflects changes in endothelial NO formation in human forearm circulation. The infusion of acetylcholine, an endothelium-dependent vasodilator, led to an increase in serum concentration of nitrite by 240% as measured using a HPLC-Griess method. This result significantly correlated with the increasing forearm blood flow (by 347%). The eNOS inhibitor L-NMMA (i.e. L-N^G-monomethylarginine) reduced the forearm blood flow and endothelium-dependent vasodilation by 30%, and this was paralleled by a significant reduction in serum nitrite concentration (by 42%) at the highest dose of acetylcholine used, whereas infusion of L-arginine significantly increased both forearm blood flow (by 75%) and nitrite (by 322%) [107].

In humans and other mammals about 90% of circulating plasma nitrite is derived directly from the L-arginine/NO pathway [15,16]. Plasma nitrite levels are reduced up to 70% in eNOS knock-out mice and upon acute NOS inhibition in wild type mice [16]. Therefore, plasma nitrite seems to derive to a large portion from eNOS, and circulating nitrite could be a specific indicator of eNOS activity in vivo in humans. The correlation between the decrease in NOS-activity and plasma nitrite concentration across species was found to be independent of the applied analytical method, i.e. CLD, HPLC-Griess or FIA-Griess, the different NOS-inhibitors used and the distinct doses applied [17]. Boucher and colleagues [134] observed that the inhibitory constants of the employed inhibitors range from 40 nM for L-NNA (i.e. L-N^G-nitro-L-arginine) to 400 nM L-NMMA. This finding demonstrates that the majority of the basal nitrite concentration in all tested mammals was derived from NOS activity, corroborating the results by other described studies [15,16]. The study of Bode-Böger et al. additionally showed by GC–MS that L-arginine-stimulated NO-dependent vasodilation in healthy humans was accompanied by an increase in plasma nitrite concentrations [135].

Dejam et al. attempted to measure nitrite levels in whole blood (see Table 3) [57]. Nitrite measurement in whole blood or erythrocytes is possible by using a stabilization solution that contains ferricyanide which inhibits nitrite oxidation by oxyhemoglobin. Up to now the utility of nitrite in whole blood as an indicator of eNOS activity has not been investigated satisfactorily. The first investigation by Dejam et al. [57] showed that stimulation of the eNOS by acetylcholine and shear stress increase significantly nitrite concentrations in whole blood as measured using tri-iodide based reductive CLD. Measurement of nitrite in whole blood or erythrocytes would be advantageous over measurement in plasma or serum, if nitrite would be indeed non-uniformly distributed in blood [57].

In recent years, numerous clinical studies were performed aiming to detect nitrite in biological matrices under various conditions including therapeutic treatment. This reflects the increasing importance of nitrite in NO-related pathological conditions and in developing new therapeutic strategies including nitrite itself as a therapeutic mean.

For example, plasma nitrite concentration has been shown to constantly decrease with increasing numbers of cardiovascular risk factors. Thus, basal plasma nitrite concentration in healthy volunteers without cardiovascular risk factors was determined to be around 300 nM [18,57], but decreased to only 171 nM in volunteers with four cardiovascular risk factors [18]. Because of great inter-individual variation, circulating nitrite appears not to be a suitable diagnostic tool, however circulating nitrite could be a sensitive marker for intra-individual monitoring of disease states associated with alterations in NO-dependent endothelial function [18]. Endothelial dysfunction is an early stage of arteriosclerosis and has been attributed to impaired NO bioactivity and enhanced formation of reactive oxygen species and other types of free radicals [136]. In another study on healthy young subjects investigating reactive hyperemia of the forearm, mean plasma nitrite concentrations, which were detected using reductive CLD [137], were found to increase by 53% [138] and 54% [137], whereas in subjects with endothelial dysfunction no significant increase in plasma nitrite concentration was observed [137,138].

3.3. Nitrite as a source of NO in human circulation

For a long time nitrite was deemed to be a biologically inactive reaction product of NO. But in early studies Furchgott and Bhadrakom [139] reported in 1953 that sodium nitrite added at 100 μM caused vessel relaxation and therefore was believed to be vasoactive. Later in 1981, Doyle et al. reported on the non-enzymatic reaction of nitrite with deoxyhemoglobin to produce NO and methemoglobin [140]. Beside this reaction nitrite may be reduced to NO under physiologic levels of acidity, as occurs during cardiac ischemia and infarction, and can form dinitrogen trioxide (N_2O_3). This reactive nitrogen species can then nitrosate thiols to *S*-nitrosothiols which are vasoactive, or produce NO gas in the presence of an electron donor [5,141]. Alternatively, the conversion of nitrite to NO gas could be catalyzed by xanthine oxidoreductase [142,143]. It was also observed that respiring mitochondria readily reduce nitrite to NO which was made visible by nitrosylation of deoxyhemoglobin [144]. Gladwin et al. observed a significant arterial-venous gradient in humans under basal conditions, exercise, and conditions involving NOS inhibition [142]. Moreover, it was shown that infusion of 36 and 0.36 μM of nitrite into human brachial artery

caused vasodilation which was associated with the formation of erythrocytic *Fe*-NO hemoglobin and, to a lesser extent, *S*-nitroso-hemoglobin [19]. Low pO_2 levels together with these concentrations of nitrite may allow deoxyhemoglobin to act as a nitrite-reductase [19] and as a vasodilator [145,146]. The reaction of nitrite with deoxyhemoglobin produces NO and possibly other NO intermediates which may exert NO-related bioactivity and export them from the erythrocyte to the tissue [147]. These erythrocytic reactions are emerging as important modulators of intravascular NO bioactivity, especially under physiologic hypoxia [19,108,148]. It has been shown that the NO produced by the conversion of nitrite causes vasodilation by activating the soluble guanylate cyclase [149]. Recently, an eNOS-like protein was localized in the cytoplasm leaflet and in the cytoplasm with activity and regulatory mechanisms resembling those of endothelium derived eNOS. This NOS is regulated by its substrate L-arginine, by calcium, and by phosphorylation via PI3 kinase. The NOS-dependent conversion of L-arginine in red blood cells is comparable to that of cultured human endothelial cells [150]. All these observations shed new light on nitrite and hemoglobin-rich erythrocytes in the human circulation.

4. Conclusion

The purpose and intention of this review was to discuss the methods of analysis of nitrite and to sensitize the reader to the manifold specific methodological problems and pitfalls associated with the quantitative analysis of nitrite in the human circulation. Today, we have a rich found of analytical techniques and methods based on distinctly different principles for the quantitative determination of nitrite in human circulation, notably in plasma or serum. Also, we have a solid knowledge of the multiple pre-analytical factors that may decidedly determine the quality of the analytical result. These issues were thoroughly discussed in the present article.

In principle, all analytical techniques and methods of nitrite analysis reviewed in this article are suitable for the quantitative determination of nitrite in human circulation. A reliable methods comparison on the basis of important validation data, notably accuracy, precision and LLOQ is, however, not possible because of lack of thorough validation in the majority of the articles discussed. The reader's selection of a particular analytical method for use in clinical studies for the quantification of nitrite in the field of the L-arginine/NO pathway may be directed to various analytical and non-analytical parameters and specific requirements. Eventually, purchase price, running costs and the need of skilled personnel may also be of particular importance. However, regardless of the analytical methodology to be used, reliable quantitative determination of circulating nitrite requires thorough validation prior to use, as well as application of sophisticated and standardized procedures for blood sampling, generation of plasma or serum, and preparation of a suitable analytical matrix. Most importantly, reliable quantification of circulating nitrite also requires avoidance of nitrite loss due to oxidation or reduction as well as minimization of sample contamination by ubiquitous nitrite by taking appropriate and specific precautions. Control of nitrite and nitrate intake by diet is also essential for the generation of reliable results in clinical studies.

To date, reference values and intervals for circulating nitrite have evaded definition because of the greatly diverging values (i.e. from non-detectable to 26 μM) reported by many investigator groups using different analytical approaches. However, there is increasing evidence in recent years that the nitrite concentration in plasma of healthy humans at the basal state most likely ranges from 100 nM to 1 μM. In consideration of the potential importance of nitrite in human circulation, a general consensus of reference values and intervals for circulating nitrite is an urgent need. From the particular analytical point of view, availability of such a consensus may also be utilized as an important additional criterion for the reliability of an analytical method.

Nomenclature

BGE	background electrolyte
CE	capillary electrophoresis
CIA	capillary ion analysis
CLD	chemiluminescence detection
CV	coefficient of variation
CZE	capillary zone electrophoresis
DAN	2,3-diaminonaphthalene
Fe-NO	nitrosyl hemoglobin
FIA	flow injection analysis
GC–MS	gas chromatography–mass spectrometry
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
i.d.	inner diameter
ITP	isotachophoresis
LOD	limit of detection
LLOQ	lower limit of quantification
L-NMMA	L-N^G-monomethylarginine
L-NNA	L-N^G-nitro-L-arginine
m/*z*	mass-to-charge
N_2O_3	dinitrogen trioxide
NO	nitric oxide
NO^+	nitrosonium ion
NO_2	nitrogen dioxide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
o.d.	outer diameter
O_3	ozone
ONI	nitrosyl iodide
PEEK	polyether ether ketone
PFB	pentafluorobenzyl
PFB-NO_2	nitro pentafluorobenzyl derivative
PFB-ONO_2	nitric acid ester PFB derivative
RSD	relative standard deviation
S/N	signal-to-noise

Acknowledgements

Part of the authors work was supported by the Deutsche Forschungsgemeinschaft (DFG): Malte Kelm (Ke405/4-3),

Tienush Rassaf (Ra969/1-1 and GRK 1089/01 Part project 3), Petra Kleinbongard and Christine Drexhage (both GRK 1089/01 Part project 3). Thomas Lauer was supported by the Hans and Gerti Fischer-Foundation (TL). The authors would like to thank the guest editor Dr. Dimitrios Tsikas for his fruitful discussion.

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