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REVIEW

Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids

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Abstract

In human organism, the gaseous radical molecule nitric oxide (NO) is produced in various cells from L-arginine by the catalytic action of NO synthases (NOS). The metabolic fate of NO includes oxidation to nitrate by oxyhaemoglobin in red blood cells and autoxidation in haemoglobin-free media to nitrite. Nitrate and nitrite circulate in blood and are excreted in urine. The concentration of these NO metabolites in the circulation and in the urine can be used to measure NO synthesis *in vivo* under standardized low-nitrate diet. Circulating nitrite reflects consitutive endothelial NOS activity, whereas excretory nitrate indicates systemic NO production. Today, nitrite and nitrate can be measured in plasma, serum and urine of humans by various analytical methods based on different analytical principles, such as colorimetry, spectrophotometry, fluorescence, chemiluminescence, gas and liquid chromatography, electrophoresis and mass spectrometry. The aim of the present article is to give an overview of the most significant currently used quantitative methods of analysis of nitrite and nitrate in human biological fluids, namely plasma and urine. With minor exception, measurement of nitrite and nitrate by these methods requires method-dependent chemical conversion of these anions. Therefore, the underlying mechanisms and principles of these methods are also discussed. Despite the chemical simplicity of nitrite and nitrate, accurate and interference-free quantification of nitrite and nitrate in biological fluids as indicators of NO synthesis may be difficult. Thus, problems associated with dietary and laboratory ubiquity of these anions and other preanalytical and analytical factors are addressed. Eventually, the important issue of quality control, the use of commercially available assay kits, and the value of the mass spectrometry methodology in this area are outlined.

Keywords: *L-arginine, nitric oxide, nitrite, nitrate, mass spectrometry, quality control*

Abbreviations: *CE, capillary electrophoresis; CL, chemiluminescence; CZE, capillary zone electrophoresis; ECD, electrochemical detection or electron capture detection; EDNO, endothelium derived nitric oxide; EDRF, endothelium derived relaxing factor; FIA, flow injection analysis; FL, fluorescence; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GC–MS–MS, gas chromatography–mass spectrometry–mass spectrometry; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; m/z, mass-to-charge ratio; NICI, negative-ion chemical ionization; NO, nitric oxide; NOS, nitric oxide synthase; SIA, sequential injection analysis*

Introduction

The L-arginine/nitric oxide pathway

In 1980, the discovery of the endothelium-derived relaxing factor (EDRF) was reported [1]. Seven years later, it was suggested that EDRF and nitric oxide (NO) or a labile nitroso species are identical [2,3]. It was shown that L-arginine (L-Arg) is the precursor for nitrite/nitrate in macrophages [4]. Definite evidence that nitrite/nitrate are derived exclusively from the terminal guanidino N-atoms of L-Arg in activated macrophages was presented by means of gas chromatography–mass spectrometry (GC–MS) and L-[*guanidino*-$^{15}N_2$]-arginine [5]. By mass spectrometry (MS) studies using L-[*guanidino*-$^{15}N_2$]-arginine, it was

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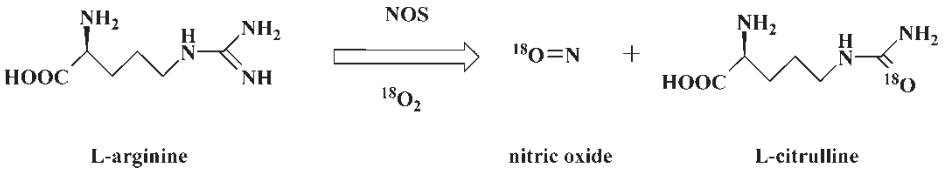


Scheme 1. Biosynthesis of nitric oxide (NO) from L-arginine. The enzyme NO synthase (NOS) catalyzes the oxidation of L-arginine to NO and L-citrulline by using molecular oxygen. For simplicity, other participants in this reaction including cofactors are not shown. More details are given in the text. Use of $^{18}O_2$ demonstrated that NOS incorporates O from dioxygen O_2 into both NO and L-citrulline [18–20].

demonstrated that vascular endothelial cells synthesize NO from L-Arg [6]. By means of the MS technology in combination with the use of L-[*guanidino*-$^{15}N_2$]-arginine, it was definitely demonstrated that L-Arg is a physiological precursor of endothelium-derived nitric oxide (EDNO) [7,8], and that NO is an intermediate in the oxidation of L-Arg to nitrite and nitrate in macrophages [9].

By means of GC–MS and orally administered L-[*guanidino*-$^{15}N_2$]-arginine to humans, its was demonstrated that ^{15}N-labelled nitrate was excreted in the urine, thus definitely demonstrating that L-arginine is a precursor for nitrate biosynthesis in humans [10,11]. Also, these studies showed that *in vivo* only a very small part of orally or intravenously administered L-[*guanidino*-$^{15}N_2$]-arginine is converted to ^{15}N-labelled nitrate [11]. In ferrets, only approximately 0.1% of the administered L-[*guanidino*-$^{15}N_2$]-arginine dose were found to be incorporated in urinary nitrate under basal conditions [11]. ^{15}N-Labelling experiments with intravenously or orally administered L-[*guanidino*-$^{15}N_2$]-arginine in patients and healthy subjects confirmed this order of magnitude of L-Arg involvement in the L-Arg/NO pathway in humans [12–14].

The enzyme responsible for the conversion of L-Arg to NO and L-citrulline (L-Citr), i.e. NO synthase (NOS, EC 1.14.13.39), is expressed in various cells including endothelial cells [15–17]. The constitutive NOS isozymes, i.e. endothelial and neuronal NOS, require various cofactors and prosthetic groups, i.e. NADPH, (6*R*)-tetrahydrobiopterin, Ca^{2+} and calmodulin, FAD and FMN and iron-protoporphyrin IX, whereas the inducible NOS has not shown a requirement for either Ca^{2+} or calmodulin [15–17].

The underlying mechanism of the unique oxidation of the guanidino group of L-Arg to NO was delineated by means of MS in combination with the use of ^{18}O-labelled water, i.e. $H_2^{18}O$, and ^{18}O-labelled molecular oxygen, i.e. $^{18}O_2$. GC–MS experiments clearly demonstrated that the O atom in the ureido group of the L-Citr product of macrophage NOS originates from dioxygen (O_2), not from water (H_2O) [18]. Also, it was unequivocally demonstrated that both constitutive and inducible NOS incorporate molecular oxygen into both NO and L-Citr, excluding reaction mechanisms that hinge on water to provide ureido oxygen (Scheme 1) [19,20]. Endogenous N^G-methylated analogues of L-Arg, namely N^G-monomethyl-L-arginine (NMMA) and N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA), may inhibit NOS-catalyzed production of NO [21,22]. Interestingly, purified neuronal NOS (nNOS) has been shown to produce the superoxide radical anion ($O_2^{\cdot-}$) by one-electron reduction of O_2 in a Ca^{2+}/calmodulin-dependent manner, with N^G-substituted L-arginine analogues having different effects on superoxide generation by nNOS [23].

Reactions and metabolic fate of nitric oxide

Chemistry and biochemistry of NO and its redox-activated forms have been reviewed in the past [24,25]. From the biological point of view, the most important reactions of NO are those with dioxygen in its various redox forms. In aqueous phase in the absence of biological material, NO exclusively autoxidizes to nitrite with the stoichiometry given in Scheme 2. The rate law obtained by Pogrebnaya et al. [26] and other goups [27] is $-\,d[NO]/dt = 4k_{aq}[NO]^2[O_2]$, i.e. it is second order with respect to [NO] with a third order rate constant $4k_{aq} \approx 8 \times 10^6\,M^{-2}\,s^{-1}$ at 25°C [26,27]. Like the gas-phase autoxidation of NO, it is assumed that autoxidation of NO in aqueous phase involves dimerization of NO to N_2O_2, the rate-limiting oxidation of which leads to formation of N_2O_4 that is finally converted to nitrite [26]. In aqueous buffered solutions in the absence of biological materials NO may exist several minutes. The half-life of NO in aqueous phosphate buffered solution of pH 7.4 was estimated to be 130 s [27]. By means of an NO electrode the half-life of NO released from *S*-nitrosocysteine in 50 mM phosphate buffer of pH 7.0 at 25°C was estimated to be 100 s (Figure 1). The decomposition of *S*-nitrosocysteine to NO under these conditions was first order with rate constant $k \approx 0.0166\,s^{-1}$, from which the half-life of

Scheme 2. Autoxidation of NO in aqueous phase in absence of biological material to nitrite.

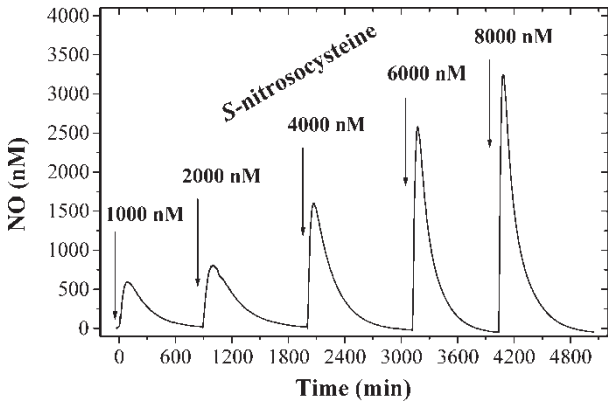
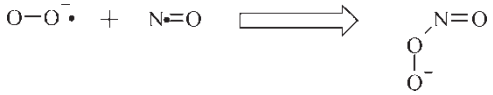


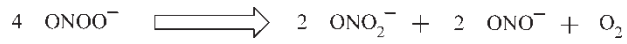
Figure 1. Release of NO from *S*-nitrosocysteine (SNC) and consecutive autoxidation of NO in aqueous 50 mM potassium phosphate buffer, pH 7, at 25°C. NO concentration was measured by means of an ISO-NO meter equipped with a 200-μm diameter shielded microsensor ISO-NOP200 and a Duo-18 data recording system from World Precision Instruments (Berlin, Germany). The NO electrode was inserted into a 1-ml aliquot of the buffer solution which was placed in a 1.5-ml glass vial and constantly mixed by a magnetic stirrer at 350 RPM. Arrows indicate the time points of consecutive addition of varying SNC concentrations to the same buffer solution from an aqueous stock solution of SNC (1 mM) in 20 mM HCl.

S-nitrosocysteine is estimated to be 42 s, i.e. considerably shorter than that of NO (Figure 1). However, it should be noted that both release of NO from *S*-nitrosocysteine and other endogenous *S*-nitrosothiols including *S*-nitrosoalbumin and consecutive autoxidation of NO may be influenced by transition metal ions such as Cu^{2+} as well as organic biomolecules notably cysteine at physiological concentrations [28]. It is worth mentioning that *S*-nitrosocysteine has been considered to be the EDRF [29], whereas *S*-nitrosoalbumin has been suggested to be a long-lived carrier and pool of endogenous NO [30]. The half-life of ^{15}N-labelled *S*-nitrosoalbumin *in vivo* in the rat has been estimated to be 10 min [31].

The superoxide anion radical is ubiquitous in mammalian cells and is also constitutively produced by all known NOS isozymes [23,32,33]. NO reacts very rapidly with superoxide in aqueous solution to form peroxynitrite ($ONOO^-$; Scheme 3). The rate constant for this reaction was determined to be $\approx 7 \times 10^9\,M^{-1}\,s^{-1}$ [34], which is much faster than the reaction of NO with haem compounds ($k < 10^8\,M^{-1}\,s^{-1}$) and comparable to the rate at which superoxide reacts with superoxide dismutase (SOD) enzymes ($\approx 2 \times 10^9\,M^{-1}\,s^{-1}$) [35]. This is of particular interest because SOD may compete with the reaction of superoxide with NO, which may result in



Scheme 3. Reaction of superoxide with NO to form peroxynitrite.

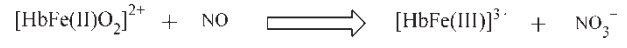


Scheme 4. Decomposition of peroxynitrite to nitrate, nitrite and dioxygen.

enhanced bioavailability of NO from L-arginine by NOS [36]. Prevention of peroxynitrite formation is not only from this standpoint of interest. The conjugate acid of peroxynitrite, i.e. peroxynitrous acid (ONOOH; $pK_a \approx 6.8$), is a strong oxidant and nitrating agent, and as such harmful to cells [37]. Reaction of peroxynitrite with biomolecules may lead to impaired function, toxicity and alterations in signaling pathways. The decomposition reactions of peroxynitrite are controversial. For long time, nitrate was considered to be the sole decomposition product of the peroxynitrite/peroxynitrous acid system in aqueous phase. Recent investigations using relatively pure preparations of peroxynitrite and improved analytical methods indicate, however, that the final decomposition products of peroxynitrite in aqueous phase within a large pH range comprise nitrite and dioxygen in addition to nitrate [38,39]. The finding that peroxynitrite decomposes at physiological blood pH to nitrite and dioxygen at a molar ratio of 2:1 [38] suggests that peroxynitrite decomposes to nitrate, nitrite and dioxygen in accordance with the reaction shown in Scheme 4. This issue is discussed below in more detail.

Oxidation of nitric oxide and nitrite by oxyhaemoglobin

By far the most important reaction of NO is that with iron in haem-containing proteins, particularly in guanylyl cyclase and haemoglobin. Binding of NO to the haem-moiety of guanylyl cyclase activates the enzyme that produces cGMP which causes the muscle to relax by decreasing intracellular concentration of free Ca^{2+} [25]. This reaction is mainly responsible for the NO-related biological actions. The ratio of rates of NO binding and release for Fe(II)-haemoglobin is 5 to 6 orders of magnitude greater than that of O_2. NO reacts with oxyhaemoglobin ($HbFe(II)O_2$) to produce methaemoglobin (MetHb) and nitrate (Scheme 5). The rate constant of this reaction was determined to be $k \approx 8.9 \times 10^7\,M^{-1}\,s^{-1}$ [40]. Interestingly, it has been suggested that oxidation of NO to nitrate by oxyhaemoglobin proceeds *via* intermediate peroxynitrito complexes such as $HbFe^{3+}OONO$, with nitrate being the sole quantitatively formed reaction product of NO without nitrating the globin moiety [40]. Wennmalm and colleagues investigated *in vitro* and



Scheme 5. Oxidation of NO to nitrate by oxyhaemoglobin. For simplicity the oxyhaemoglobin and haemoglobin molecules are shown charge-free.

in vivo metabolism of NO in humans [41,42]. In human plasma NO has been shown to quantitatively oxidize to nitrite and nitrate in a molar ratio of 1:5 [41]. In blood from healthy humans, NO metabolism has been shown to depend upon the oxygenation of red cell haemoglobin [41]. Thus, in arterial human blood (O_2 saturation of 94–99%) NO was found to quantitatively oxidize to nitrate and methaemoglobin, whereas no nitrite was detected and *Fe*-nitroso haemoglobin [HbFe(III)NO] formation was measured to be low [41]. In venous blood (O_2 saturation of 36–85%) more *Fe*-nitrosohaemoglobin and less nitrate was found to be formed, in comparison with venous blood [41]. In arterialized blood, nitrite was found to be converted semiquantitatively to nitrate and methaemoglobin [42]. Upon inhalation of NO by healthy subjects and patients, plasma nitrate and methaemoglobin concentrations were found to increase, whereas no changes were detected for plasma *Fe*-nitrosohaemoglobin. These studies by Wennmalm et al. suggested that the uptake of NO into the red blood cells with subsequent conversion to nitrate and methaemoglobin could be the major metabolic pathway for endogenously formed NO [42]. The occurrence of *Fe*-nitrosohaemoglobin *in vivo* has been assumed to indicate liberation of NO to partly deoxygenated blood (see below).

To avoid problems originating from the endogenous occurrence of nitrite and nitrate in human blood, we investigated by GC–MS [43,44] the oxidation of ^{15}N-labelled NO to ^{15}N-labelled nitrite ([^{15}N]nitrite) and ^{15}N-labelled nitrate ([^{15}N]nitrate) in human venous blood. As Figure 2 shows, ^{15}NO oxidized in human blood both to [^{15}N]nitrate and [^{15}N]nitrite, with [^{15}N]nitrate being by far the major oxidative metabolite of ^{15}NO. The initial formation rates were determined to be 17.5 μM/min of [^{15}N]nitrate and 0.038 μM/min for [^{15}N]nitrite. On the assumption that ^{15}NO oxidation to [^{15}N]nitrate is catalyzed by oxyhaemoglobin in red bloods cells and that [^{15}N]nitrite is formed exclusively by autoxidation of ^{15}NO in blood plasma, it can be estimated from the initial formation rates that oxidation by oxyhaemoglobin is approximately 450 times faster than NO autoxidation. The concentration-time course of [^{15}N]nitrite shows a maximum at 15 min suggesting further oxidation of [^{15}N]nitrite to [^{15}N]nitrate in red blood cells. Figure 2 shows that despite exposure of blood to a relatively high amount of ^{15}NO, erythrocytes possess a high oxidative capacity for NO. Because nitrate itself is biologically completely inactive, in contrast to nitrite, oxidation of NO in erythrocytes to nitrate by oxyhaemoglobin can be regarded as the most effective and definite inactivating metabolic fate of NO. Nevertheless, it should be mentioned that there is currently a controversial discussion about the physiological roles of other quantitatively by far minor, but biologically very potent reaction products/metabolites of NO such as *Fe*- and *S*-nitrosohaemoglobin [45,46].

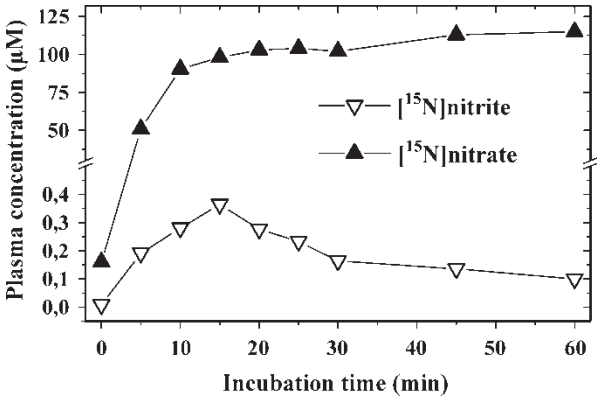


Figure 2. Oxidation of ^{15}N-labelled NO in human venous whole blood to ^{15}N-labelled nitrite ([^{15}N]nitrite) and ^{15}N-labelled nitrate ([^{15}N]nitrate). ^{15}N-Labelled NO, produced from the sodium salt of ^{15}N-labelled nitrite (0.4 mg, 5.7 μmol) by reduction with KI in aqueous acetic acid solution, was introduced into 20 ml venous human blood at 25°C as described elsewhere [43]. Plasma and plasma ultrafiltrate (cut-off, 20 kDa) were prepared by centrifugation from 0.5-ml aliquots of blood taken at the indicated time points, and the concentration of [^{15}N]nitrite and [^{15}N]nitrate was determined in plasma (100 μl aliquots) by GC–MS using the endogenous anions as internal standards, the concentration of which had been determined before to be 1.2 and 35 μM, respectively, by GC–MS [44].

We investigated the kinetics of nitrite oxidation in human whole blood by GC–MS using [^{15}N]nitrite (Figure 3). Application of the differential method revealed a reaction order of one ($n = 1$; slope of the straight line), a rate constant k of 0.054 min^{-1} (y-axis intercept) and a half-life ($t_{1/2}$) of 13 min ($t_{1/2} = \ln 2/k$) for [^{15}N]nitrite. The integral method resulted in similar data. Thus, the linearity between $\ln C$ and time suggests that the reaction order is one ($n = 1$). The slope of the straight line gives the value for the rate constant k which was determined to be 0.062 min^{-1}; from the k value the half-life of [^{15}N]nitrite is determined to be 11.2 min ($t_{1/2} = \ln 2/k$). In human blood stored on ice and in human plasma incubated at 37°C, [^{15}N]nitrite and [^{15}N]nitrate were found not to change with incubation time for at least 60 min as measured by the same GC–MS method (data not shown).

The finding that NO oxidizes in human plasma mainly to nitrite [41] suggests that NO may autoxidize in plasma to nitrite in a similar manner to its autoxidation in pure water or aqueous buffered solutions [26,27]. To test this hypothesis we investigated by GC–MS the autoxidation of NO (supplied as a gas) in freshly prepared human native plasma which was diluted with $H_2^{18}O$ by 2:1 (v/v). The mass spectrum of the pentafluorobenzyl (PFB) derivatives of plasma nitrite shows two pairs of ions with a difference of 2 Da each with an intensity ratio of approximately 2:1 each (Figure 4). This difference corresponds to the mass difference of the oxygen isotopes O^{18} (18 Da) and O^{16}

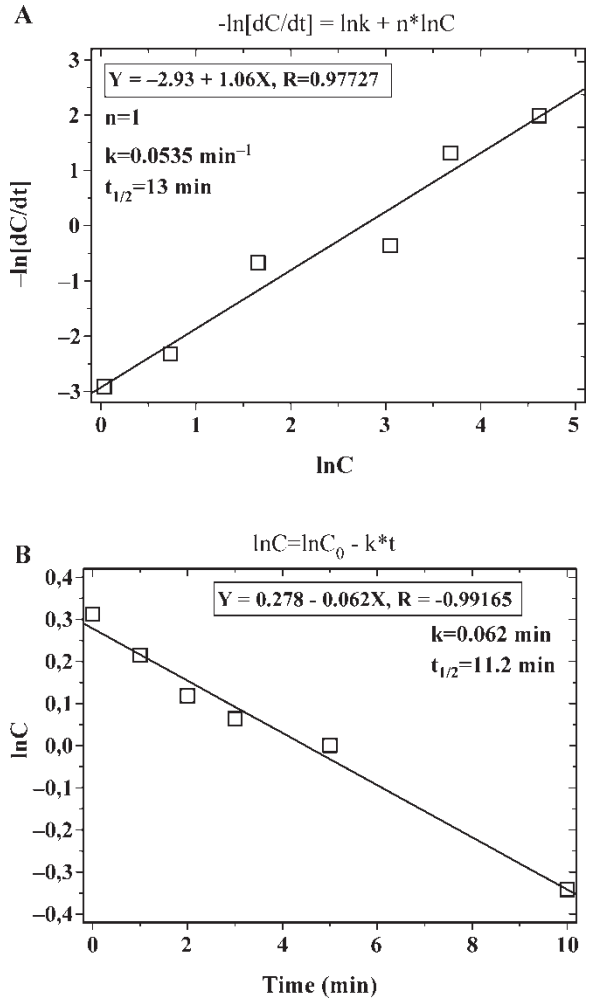


Figure 3. Application of the differential (A) and integral method (B) to study the oxidative metabolism of ^{15}N-labelled nitrite ([^{15}N]nitrite) in human venous whole blood. (A) [^{15}N]Nitrite at varying concentrations of 1.09, 2.17, 5.7, 21.7, 43.5 and 109 μM was incubated in blood at 37°C. The reaction was stopped each 1 min after addition of [^{15}N]nitrite by treating whole blood (100 μl) with acetone (400 μl). The concentration of [^{15}N]nitrite in blood before and 1 min after addition of [^{15}N]nitrite was determined by GC–MS [44] using the endogenous nitrite (1.6 μM) as internal standard. The natural logarithm of the initial reaction rate obtained (i.e. $-\ln[dC/dt]$) was plotted versus the initial concentration of [^{15}N]nitrite (i.e. $\ln C$). (B) [^{15}N]Nitrite at 1.48 μM was incubated in blood (2 ml) at 37°C. At the indicated time points blood samples (100 μl) were taken and treated with acetone (400 μl). The concentration of [^{15}N]nitrite in blood was determined by GC–MS [44] using the endogenous nitrite (1.6 μM) as internal standard. The natural logarithm of the [^{15}N]nitrite concentration ($\ln C$) was plotted versus the incubation time. Results are discussed in the text.

(16 Da). The ions in the mass spectrum have mass-to-charge (m/z) values of 46 due to $[ONO]^-$ and m/z of 48 due to $[^{18}ONO]^-$, and m/z 226 due to $[PFB\text{-}ONO^- - 1]^-$ and m/z 228 $[PFB\text{-}^{18}ONO - 1]^-$. The mass spectrum shown in Figure 4 and the intensity ratios of both ion pairs of ≈2:1, which closely corresponds to the volume ratio of 2:1 for H_2O (from plasma) to externally added $H_2^{18}O$, clearly indicates that ^{18}O from water is incorporated into autoxidized NO. Moreover, this finding supports the suggestion by Pogrebnaya et al. that incorporation of O from H_2O into NO takes place during hydrolysis of an intermediate such as N_2O_4 [26].

Definite evidence that the L-Arg/NO pathway is the major source of plasma nitrite in humans was presented by Rhodes et al. by means of ^{15}N-labelling GC–MS experiments [47]. By infusing L-[*guanidino*-$^{15}N_2$]-arginine into fasted humans and GC–MS analysis of plasma nitrite as PFB derivative this group unequivocally demonstrated that as much as 90% of circulating nitrite is derived directly from the L-Arg/NO pathway in fasted humans. The results of Moncada's group endorse the use of plasma nitrite as quantitative indice of NO production in fasted humans [47]. Also, ^{15}N-labelling experiments in the mouse with orally administered L-[*guanidino*-$^{15}N_2$]-arginine and GC–MS analysis of nitrite as PFB derivative definitely revealed that urinary nitrite is derived from the L-Arg/NO pathway [48].

Assessment of nitric oxide synthesis in vivo *by measuring nitrite and nitrate*

Although only a minor part of L-arginine of ≈0.1% is converted to NO by NOS in healthy humans (see above), endogenous NO production rate from L-arginine is obviously sufficient to maintain important physiological functions such as modulation of vascular tone, platelet function, and neurotransmission. Assessment of NO synthesis and quantification of other relevant members of the L-Arg/NO family, such as the endogenous NOS inhibitor ADMA, is of particular interest, as it may characterize the status of this pathway in health and disease as well as to monitor the progress of pharmacological interventions.

Authentic NO can be directly measured in human circulation upon stimulation [49]. However, endogenous basal authentic NO has not been reliably quantified in the blood, thus far. The half-life of NO *in vivo* in the circulation is most likely shorter than 0.1 s [50]. This circumstance offers certain analytical problems, in particular insufficient sensitivity. Thus, circulating basal L-Arg-derived NO could not be detected at concentrations below the detection limit of 5 nM of sensitive analytical methods such as the electrochemical detection by means of porphyrinic sensors [49]. The presence of endogenous NO in the exhaled air of animals and humans (at a basal concentration of ≈8 ppb) has been demonstrated by chemiluminescence, diazotization and mass spectrometry [51]. However, measuring of exhaled NO is limited to the pulmonary physiology and pathophysiology. Other potential reaction products and/or metabolites of endogenous NO such as the nitroxyl anion (NO^-) and dinitrogen oxide (N_2O), nitrogen dioxide (NO_2) and peroxynitrite (Table I), have not been shown to allow assessment of NO synthesis

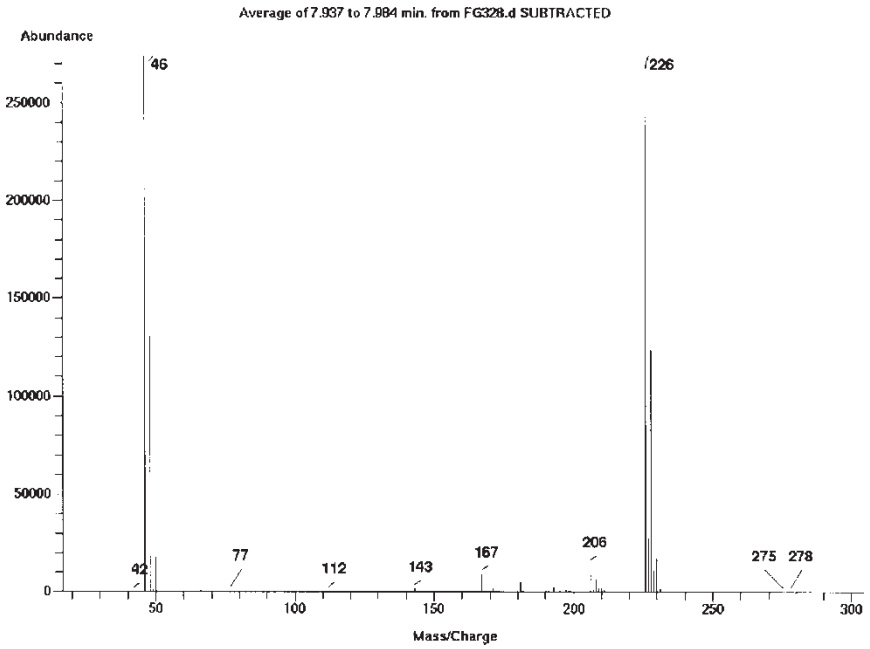


Figure 4. Mass spectrum from the GC–MS analysis of nitrite as pentafluorobenzyl (PFB) derivative. Native human plasma (400 μl) was diluted with 200 μl of ^{18}O-labelled water ($H_2^{18}O$, 99 atom % of ^{18}O; Campro Scientific, Berlin). The diluted plasma was bubbled with NO which was produced from nitrite as described elsewhere [43]. Plasma pH did not change upon NO bubbling. An 100-μl aliquot of the diluted plasma was derivatized with PFB bromide and reaction products were extracted by toluene as described [44]. An aliquot of 1 μl of the toluene phase was injected into the GC–MS apparatus (MS Engine Hewlett-Packard), and a full scan between mass-to-charge ratio (*m/z*) of *m/z* 20 and *m/z* 300 was derived from the GC peak eluting with the retention time of the PFB derivative of synthetic nitrite in the negative-ion chemical ionization mode. This mass spectrum corresponds to a mixture of nitrite, i.e. ONO^-, and simply ^{18}O-labelled nitrite, i.e. $^{18}ONO^-$, of a molar ratio of ≈2:1. The ions at *m/z* 46 and 48 correspond to ONO^- and $^{18}ONO^-$, respectively; the ions at *m/z* 226 and 228 correspond to $[PFB\text{-}ONO - 1]^-$ and $[PFB\text{-}^{18}ONO - 1]^-$, respectively. Note that the natural abundance of the ^{18}O isotope is 0.2%.

in vivo. By contrast, nitrite and nitrate are stable metabolites of NO, present both in blood and urine, and accessible to quantitative analysis. Therefore, measurement of nitrite and nitrate in various biological fluids, notably plasma or serum and urine, tourned out to be the most suitable and practical method to assess NO synthesis *in vivo*. Studies *in vivo* in humans and mammals indicate that circulating nitrite rather than nitrate reflects endothelial-dependent NO synthesis in humans and mammals [52–54]. These studies suggest that in particular short-term changes in endogenous endothelial NO synthesis upon pharmacological intervention are best assessed by measuring plasma nitrite concentration [52–54]. It is clearly established that the major urinary metabolite of NO is nitrate (see references cited above). In general, the excretion rate of nitrate in the urine is a reliable non-invasive method to assess gradually, long-lasting changes in whole body NO synthesis under basal conditions as well as upon pharmacological or physical treatment [55–58].

Table I. Nitric oxide and its metabolites/reaction products (NO_x).

NO_x	Formula	Oxidation number of N
Dinitrogen oxide	N_2O	+ 1
Nitroxyl anion	NO^-	+ 1
Nitric oxide	NO	+ 2
Nitrite	NO_2^-	+ 3
Nitrosyl cation	NO^+	+ 3
Peroxynitrite anion	$ONOO^-$	+ 3
Dinitrogen trioxide	N_2O_3 (ON-NO_2)	+ 2/+4
Nitrogen dioxide	NO_2	+ 4
Nitrate	NO_3^-	+ 5
Nitryl cation	NO_2^+	+ 5
Peroxynitrate anion	O_2NOO^-	+ 5

Besides choice of the proper analytical method, choice of the suited biological matrix may be the deciding factor. Thus, measurement of nitrite + nitrate in plasma by the Griess assay (the method is described below in detail) showed only a borderline trend for circadian variation of endogenous NO synthesis [59], whereas quantification of nitrate in eight consecutive 3-h urine collection periods by GC–MS (the method is described below in detail) revealed a statistically significant circadian rhythm of NO synthesis in healthy and diseased subjects, with the maximum and minimum excretion rates being measured in the urine samples collected from 5 to 8pm and 5 to 8am, and a 24-h amplitude of approximately 25% [58].

Unlike plasma, urinary concentration of nitrate is corrected for creatinine excretion in particular for urine collected by spontaneous micturition, and is expressed, e.g. as μmol nitrate per mmol creatinine; in case of 24-h collection periods, urinary nitrate excretion is presented as μmol per 24 h [44].

L-Arg/NO-derived nitrite not only circulates in blood [47], but it is also excreted into the urine [48]. Unlike circulating nitrite, however, the significance of urinary nitrite as a biomarker of endogenous NO synthesis from L-Arg in the kidney and/or in the whole organism is still unclear. In healthy humans with uncontrolled nitrate diet, urinary excretion rates of nitrate and nitrite are of the order of 110 μmol/mmol creatinine (or 1200 μmol/24 h) and 0.5 μmol/mmol creatinine (or 6 μmol/24 h), respectively [44]. In the same subjects being on standardized low-nitrate diet, urinary nitrate excretion rate was of the order of 50 μmol/mmol creatinine [44], strongly indicating that diet may be a considerable contributor to urinary nitrate. In healthy young non-smoking volunteers we found a weak correlation between urinary nitrite and nitrate at the basal state by GC–MS (Figure 5) [60,61]. Interestingly, both serum nitrite concentration and urinary nitrite excretion into the urine significantly increased after oral administration of therapeutically relevant doses of the organic nitrate drugs isosorbide dinitrate and pentaerythrityl tetranitrate to healthy humans [60]. In conclusion, accurate assessment of NO synthesis *in vivo* by measuring nitrite and nitrate in plasma and urine is possible when effects of diet and NO-donating drugs are taken into account.

Methods of quantitative analysis of nitrite and nitrate in biological fluids

Prior to identification of nitrite and nitrate as the major metabolites of endogenous NO, these anions have attracted attention because of their toxic and cancerogenic potential. To date, more than 200 analytical methods for the analysis of nitrite and nitrate have been reported in chemical analysis-oriented journals. However, only a minor part of these methods are applicable to human biological fluids such as plasma, serum and urine. Analytical methods originally developed and validated for simple matrices such as drinking and surface water could not be adopted for circulating and excretory nitrite and nitrate, mainly because of the complexity of these matrices and their relatively low content of nitrite and nitrate particularly in plasma.

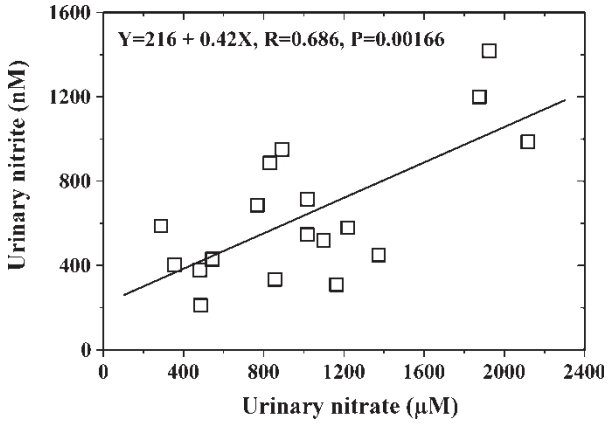


Figure 5. Correlation between urinary nitrite and urinary nitrate in healthy young non-smoking volunteers at the basal state. The figure was constructed by using the data of the study by Keimer et al [60]. Nitrite and nitrate concentrations in urine were determined simultaneously by GC–MS (on MS Engine from Hewlett-Packard) [61].

Current methods available for the analysis of nitrite and nitrate in human biological fluids in the frame of experimental and clinical studies in the field of NO research include colorimetric and ultraviolet spectrophotometric methods, fluorometric assays, chemiluminescence (CL), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), gas chromatography (GC), and gas chromatography–mass spectrometry (GC–MS) [62]. To the best of the knowledge of the author, no liquid chromatography–mass spectrometry (LC–MS) methods for nitrite and nitrate in biological fluids have been reported so far. Among the various analytical methods currently available for the analysis of nitrite and nitrate as well as of other members of the L-Arg/NO family, mass spectrometry-based approaches emerged as indispensable analytical tools for the reliable quantitative analysis of the whole NO family and form the basis of reference methods for nitrite and nitrate [62] and other NO metabolites including 3-nitrotyrosine [63,64].

Reported basal levels for circulating and excretory nitrite and nitrate obtained by the use of various analytical methods are summarized in Tables II and III, respectively. These methods have produced diverging values of nitrite and nitrate in particular in plasma and serum (Table II). The relatively great differences regarding nitrite and nitrate levels in the circulation of healthy humans can only in part be explained by differences in dietary intake of these salts. The major reason for the discrepancies seems, however, to be methodological problems [65]. Below, the analytical methods listed in Tables II and III are discussed and compared among each other.

Important preanalytical factors

Reliable quantitative determination of nitrite and nitrate *in vivo* in a certain biological matrix and a given clinical condition requires the use of specific, selective, interference-free, sensitive and accurate analytical approaches. However, independently of the analytical method to be used for the final detection, the ubiquity of nitrite and nitrate and the short life of nitrite in whole blood makes it necessary to take precautionary measures prior to analysis.

Table II. Reported batch and instrumental methods of analysis of nitrite and nitrate and their basal concentrations (in μM) in plasma or serum of healthy humans.

Nitrite	Nitrate	Method	Reference
Batch methods			
32 (nitrite + nitrate)		Griess	Hibbs et al. [12]
N.R.	N.R.	Fluorometry	Misko et al. [66]
1–13	4–45	Griess	Moshage et al. [67]
34 (nitrite + nitrate)		Fluorometry	Marzinzig et al. [68]
Instrumental methods			
N.R.	N.R.	GC-ECD	Tesch et al. [69]
N.R.	15–60	Griess (automated)	Green et al. [70]
N.R.	22	Griess (automated)	Kanno et al. [55]
0.14	N.R.	CL	Farell et al. [71]
1.3	26	HPLC-UV	Wennmalm et al. [42]
0.45	41	CE	Leone et al. [72]
1.8	38	GC–MS	Tsikas et al. [44]
0.6	37	GC–MS (?)	Rhodes et al. [47]
3.3	52	CZE	Ueda et al. [73]
0.58	25	HPLC-ECD	Preik-Steinhoff, Kelm [74]
1.1 (nitrite + nitrate)		HPLC-UV	El Menyawi et al. [75]
N.R.	8–81	GC–MS	Smythe et al. [76]
N.R.	27	GC–MS–MS	Tsikas et al. [77]
0.55	27	HPLC-UV	Tsikas et al. [78]
0.3	25	FIA-Griess	Lauer et al. [53]
0.3	24	FIA; CL; HPLC	Kleinbongard et al. [54]
5–20	19–52	SIA	Pinto et al. [79]

N.R., not reported.

One important precaution is minimization of blank nitrite and nitrate present in laboratory chemicals and materials including glass and plastic ware, ultrafiltration cartridges for plasma or serum ultrafiltration, and monovettes used to take blood samples [65,83–85]. Commercially available monovettes containing EDTA or citrate but not lithium heparin as anticoagulants may contain considerable amounts of nitrite and nitrate, which may significantly contribute to endogenous values, e.g. by about 1 μM [48], with the extent of contribution being dependent upon the blood volume taken [48]. Monovettes routinely used to generate blood serum were found to contain very low nitrite amounts [48]. On the other hand, when preparing serum, blood should be centrifuged immediately at low temperature, e.g. at 2°C, to avoid nitrite oxidation by oxyhaemoglobin (Figure 3) [48]. It should also be taken into account that some preanalytical factors such as anticoagulants may interfere with the quantification of nitrite in some methods such as the Griess assay [84].

Table III. Reported batch and instrumental methods of analysis of nitrite and nitrate and their basal concentrations (μM, μmol/mmol creatinine or μmol/24 h) in urine of healthy humans.

Nitrite	Nitrate	Method	Reference
Batch methods			
768 μM (nitrite + nitrate)		Colorimetry	Radomski et al. [80]
690 μmol/24 h (nitrite + nitrate)		Griess	Hibbs et al. [12]
990 μM (nitrite + nitrate)		Griess	Moshage et al. [67]
Instrumental methods			
250–2000 μM		Griess (automated)	Green et al. [70]
124 μmol/mmol		Griess (automated)	Kanno et al. [55]
20–170 μM		HPLC-UV	Wennmalm et al. [42]
104 μmol/mmol		GC-ECD	Bode-Böger et al. [81]
49–109 μmol/mmol		GC–MS	Tsikas et al. [44]
23–1865 μM		GC–MS	Smythe et al. [76]
178–2889 μM		GC–MS–MS	Tsikas et al. [77]
1100 μM		HPLC-UV	Tsikas [82]

In case of performing studies with chemicals and drugs that contain nitro groups, such as the organic nitrates [60] and the exogenous NOS inhibitor N^G-nitro-L-arginine [85,86], possible contribution by these compounds to nitrite and/or nitrate during analysis, e.g. reduction of nitrate to nitrite by cadmium, should be examined and the extent of contribution quantified. Alternatively, potential interferences may be eliminated by using appropriate procedures such as solid-phase extraction [86].

In addition to nitrite-loss by oxidation in red blood cells which may occur until blood centrifugation, loss of endogenous nitrite may also occur in analytical methods requiring acidification of plasma or serum for purpose of protein precipitation and/or sample derivatization (see below). In such cases the relatively chemically inert nitrite is converted to the much higher reactive nitrous acid ($pK_a = 3.4$), which is a strong nitrosating agent of free and protein-associated amino acids including tyrosine and cysteine [65].

While interference by thiols can be effectively eliminated by using the highly reactive, thiol-specific agent *N*-ethylmaleimide (NEM), avoidance of acid-catalyzed nitrosation of aromatic amino acids such as tyrosine is practically not possible without chromatographic separation [64].

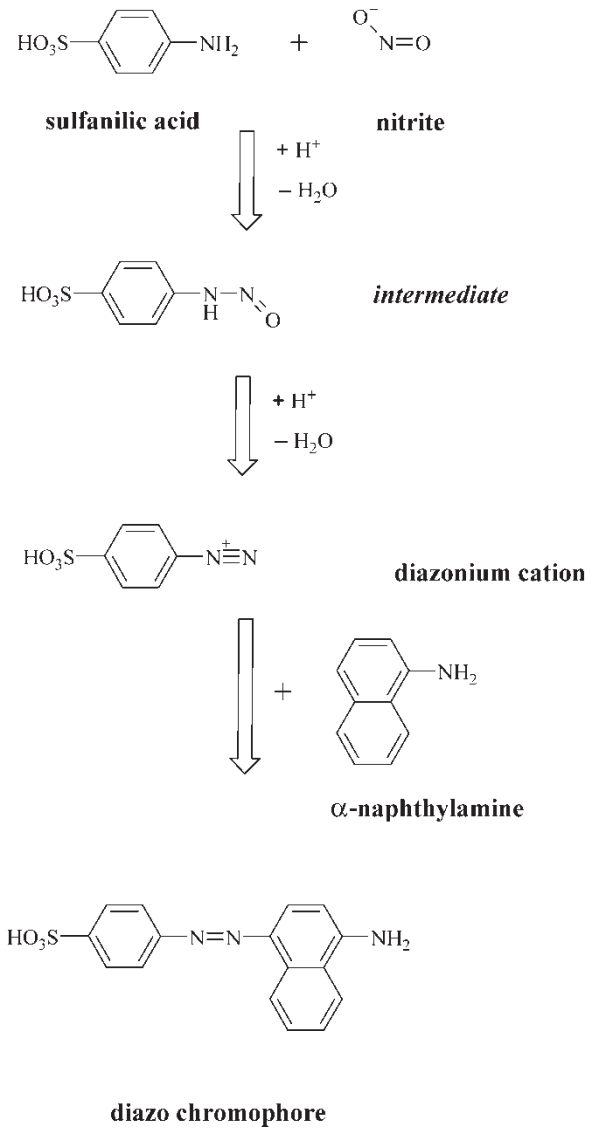
Derivatization reactions

In most analytical methods presently available for nitrite and nitrate, derivatization and/or inter-conversion of these anions is an absolute requirement. Analytical approaches not requiring any chemical modification of nitrite and nitrate are based on detection of the native anions by measuring ultraviolet absorbance detection around 210 nm, conductivity detection and electrochemical detection. In batch and instrumental methodologies based on colorimetry and fluorometry, derivatization aims at converting nitrite and nitrate into derivatives that absorb light in the ultraviolet/visible range or are fluorescent, mainly in order to overcome the unspecific low-wavelength ultraviolet absorbance detection around 210 nm. The main goal of derivatization of nitrite and nitrate in methods based on GC is generation of volatile and thermally stable derivatives. The choice of the chemical derivatizing agent in GC is also directed to the method of detection. Maximum sensitivity can be achieved by using derivatizing agents possessing electron-capturing elements such as fluorine for electrochemical and mass spectrometric detection. Fluorine-containing derivatizing agents such as pentafluorobenzyl bromide, e.g. for analysis of nitrite and nitrate by GC–MS, have found wide application to the NO field of research (see below) [63]. Detection of nitrite and nitrate by chemiluminescence also requires reduction of these anions to NO [87–89].

It should be noted that the majority of the derivatization reactions are specific for nitrite, e.g. the diazotization reaction, or nitrate, e.g. acid-catalyzed nitration of aromates, so that determination of both ions may require a second derivatization reaction for reduction of nitrate to nitrite or oxidation of nitrite to nitrate. In these cases, the second ion is determined by difference. The requirement of a second derivatization reaction, e.g. reduction of nitrate to nitrite in the Griess assay, usually involves severe analytical problems (see below) [65].

The diazotization reaction

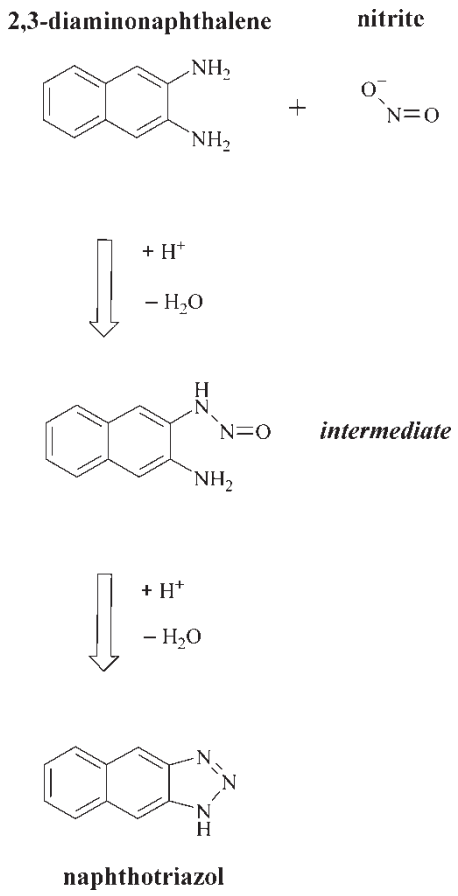
The most famous and most frequently used method to measure nitrite and nitrate in the area of NO research is based on the Griess reaction which is a diazotization reaction and is known since 1864 [90], and should be discussed in detail. In the original work reported by Griess sulfanilic acid reacts with nitrite under acidic conditions to form a diazonium cation which couples with α-naphthylamine to form a diazo compound, which is stable for a few minutes in aqueous solution and absorbs light in the visible range around 540 nm (Scheme 6). This and other diazotization reactions are specific for nitrite. Thus, analysis of nitrate by assays based on the Griess reaction requires preceding reduction to nitrite. Instead of sulfanilic acid and α-naphthylamine, derivatives of these compounds such as sulfanilamide and *N*-(1-naphthyl)ethylendiamine, respectively, can also be used [65]. Also, sulfanilic acid can be replaced by 4,4′-diaminodiphenylsulfone (i.e. dapsone) [68]. The diazotization reaction can also occur intramolecularly, i.e. by using a single compound that contains two amino groups in *ortho*-position such as 2,3-diaminonaphthalene [66,91], or one amino group and a nitrosable nitrogen



Scheme 6. The Griess reaction is a diazotization reaction. Under acidic conditions sulfanilic acid reacts with nitrite to form a diazonium cation which consecutively reacts with α-naphthylamine to form a diazo compound which absorbs light around 540 nm.

atom such as hydralazine [92]. Interestingly, the diazotization of 2,3-diaminonaphthalene yields 2,3-naphthotriazole, which is a stable highly fluorescent compound (Scheme 7). Methods based on the diazotization reaction have been utilized to measure nitrite and nitrate both in batch and automated assays (Tables II and III). Batch assays of nitrite and nitrate based on diazotization reactions may be associated with problems resulting from unspecificity [65,66]. Therefore, automated assays such as those described by Green et al. [70], in which interfering compounds are eliminated by appropriate measures, should be preferably used. Alternatively, interfering compounds may be eliminated by HPLC analysis and post-column [54] or pre-column [93] derivatization of appropriately treated plasma samples.

Numerous methodological problems in the analysis of nitrate by assays based on the diazotization reactions, e.g. the Griess assay, may occur during the preceding reduction of nitrate to nitrite and the diazotization reaction. Thus, even when nitrate is analyzed in freshwater, many inorganic compounds (e.g. chlorides, hydrogen carbonate, phosphates, sulphate) and organic compounds (e.g. EDTA, alanine, cysteine) may interfere positively or negatively in the reduction of nitrate to nitrite with cadmium [94]. In the Griess assay and other assays based on the diazotization reaction, quantitative determination of nitrite and nitrate in biological fluids is usually performed by means of calibration curves prepared in distilled water (see below). It is very likely that the yield of nitrite from nitrate by cadmium in a biological fluid is different from that in distilled water [61,65]. Therefore, variation of nitrite yield due to incomplete reduction of nitrate would generate inaccurate values for nitrate, unless reduction yield is determined in each biological sample by means of a calibration curve. Cadmium-related methodological problems, in particular incomplete reduction of nitrate to nitrite, have been reported to be effectively eliminated by reducing nitrate enzymically, e.g. by nitrate reductase [95]. Nevertheless, even in these cases interferences, such as by anticoagulants and other preanalytical factors, in assays based on the Griess reaction or related diazotization reactions can not be completely excluded [84]. Generally, reduction of nitrate to nitrite by nitrate reductase is assumed to be quantitative. In practice, however, enzymic reduction of nitrate to nitrite may be incomplete and may vary from sample to sample. Quantitative yield may require optimization of enzyme activity and cofactor concentration for each biological sample [84], like cadmium-catalyzed reduction, thus making the assay time-consuming.

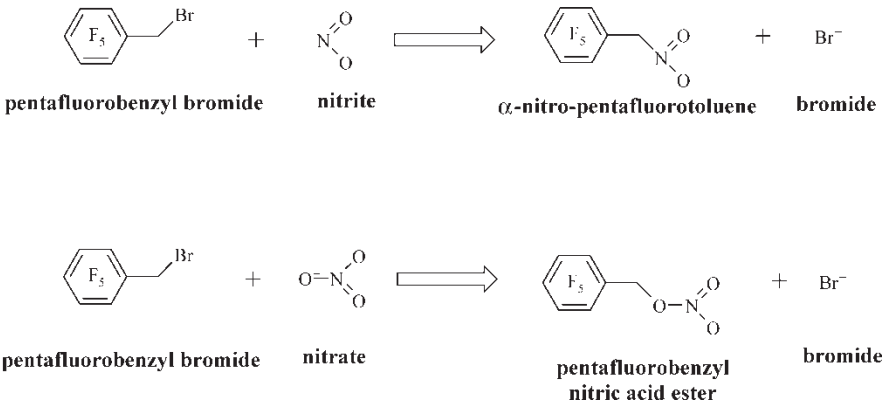


Scheme 7. Intramolecular diazotization of 2,3-diaminonaphthalene produces the highly fluorescent 1(*H*)-naphthotriazol.

Derivatization reactions in GC and GC–MS

Two main derivatization reactions have been described and used for the analysis of nitrite and nitrate by GC-based techniques such as GC–MS, thus far. The first method utilizes an aromatic compound such as benzene or trimethoxybenzene which serves as electrophile, requires concentrated sulfuric acid as the catalyst, and yields volatile, thermally stable, and electron-capturing nitro-aromates [69,70,96,97]. Under the strong acidic conditions of the nitration reaction, nitrite can also be converted to the same nitroaromatic derivative, but maximum recovery requires preceding oxidation of nitrite to nitrate, for instance by means of H_2O_2 [97]. More recently, nitration of aromates has been performed by using trifluoroacetic anhydride instead of concentrated sulfuric acid as the catalyst and toluene as the electrophile; this nitration reaction produces three nitrotoluene isomers [76].

The second derivatization reaction uses pentafluorobenzyl (PFB) bromide (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) [44,98] and nitric acid ester PFB derivative (PFB-ONO_2) [61], respectively (Scheme 8). To date, this is the sole derivatization reaction that allows for the simultaneous analysis of nitrite and nitrate by GC–MS [61]. For the



Scheme 8. Derivatization of nitrite and nitrate by pentafluorobenzyl bromide. This derivatization reaction is usually performed by treating a 100-µl of the biological sample (e.g. blood, plasma, serum, urine, saliva, tissue homogenate) with acetone (400 µl) and 10 µl pentafluorobenzyl bromide and heating at 50°C. Derivatization of nitrite requires an incubation time of 5 min. Analysis of nitrate and simultaneous analysis of nitrite and nitrate require a reaction time of 60 min [61]. For simplicity the charge of N in nitrite and nitrate and that of N and O in the derivatives is not shown.

simultaneous determination of nitrite and nitrate in human plasma by GC–MS extractive alkylation with PFB bromide using tetradecyldimethylbenzylammonium chloride as the phase-transfer catalyst with 1,3,5-tribromobenzene as the internal standard (see below) has also been reported [99].

The major shortcoming of the nitration reaction is that nitrite can not be accurately determined in the presence of high excess (e.g. 40-fold or higher) of nitrate over nitrite, i.e. in human plasma and serum (Table II) and in particular in urine (Table III). Moreover, in contrast to the method involving use of PFB bromide, the nitration reaction can not be used for the simultaneous analysis of nitrite and nitrate. Derivatization of nitrite and nitrate by PFB bromide proceeds equally effective in every biological fluid and tissue and does not require any sample pretreatment [61].

Methods of quantification

The mass spectrometry methodology is the single technology in which quantification is carried out by using stable-isotope labelled analogues of the endogenous compounds which serve as internal standards. The element nitrogen (N) naturally occurs in two isotopes, i.e. ^{14}N and ^{15}N with a natural abundance of 99.635 and 0.365%, respectively. Commercially available are sodium and potassium salts of ^{15}N-labelled nitrite and nitrate, i.e. $^{15}NO_2^-$ and $^{15}NO_3^-$, respectively, of high isotopic purity. For the quantification of endogenous nitrite and nitrate (i.e. NO_2^- and NO_3^-, respectively) the internal standards $^{15}NO_2^-$ and $^{15}NO_3^-$ are added to the respective biological matrix at appropriate final concentrations and undergo all chemical and physical changes during the whole analytical process, i.e. derivatization, extraction and chromatographic separation. Endogenous anions and their respective stable-isotope labelled analogues in their native and derivatized forms behave almost identically until their separation in the mass spectrometer according to their distinctly different mass-to-charge ratios (*m/z*). Figure 6 shows mass spectra generated from aqueous mixtures containing $^{14}NO_2^-$ and $^{15}NO_2^-$ (upper panel) and $^{14}NO_3^-$ and $^{15}NO_3^-$ (lower panel), respectively, after derivatization to their PFB derivatives. The PFB derivatives PFB-$^{14}NO_2$ and PFB-$^{15}NO_2$ ionize in the ion-source of the mass spectrometer to produce $^{14}NO_2^-$ (*m/z* 46) and $^{15}NO_2^-$ (*m/z* 47), respectively, whereas PFB-O$^{14}NO_2$ and PFB-O$^{15}NO_2$ ionize to form $^{14}NO_3^-$ (*m/z* 62) and $^{15}NO_3^-$ (*m/z* 63), respectively. For quantification the ions with *m/z* 46 for $^{14}NO_2^-$, *m/z* 47 for $^{15}NO_2^-$, *m/z* 62 for $^{14}NO_3^-$, and *m/z* 63 for $^{15}NO_3^-$ are selected and monitored in the selected-ion monitoring (SIM) or multiple-ion detection (MID) mode. Calculation of endogenous ion concentration is simply performed by multiplying the measured ratio of the peak areas or peak heights of the endogenous anions to those of the internal standards with the known added concentration of the internal standard [48,61]. Quantification of nitrite and nitrate in human biological fluids by GC–MS as PFB derivatives has been shown to be free of any interferences [61].

In all the other non-mass spectrometry methodologies, quantification of nitrite and nitrate must be performed by using calibration curves. Because of the physiological occurrence of nitrite and nitrate in every biological sample, calibration curves for nitrite and nitrate have to be generated in matrices distinctly different from the biological matrices, e.g. in aqueous solution. Unlike the use of stable-isotope labelled nitrite and nitrite, this proceeding does not consider at all the well-known matrix-associated effects discussed above, including unknown recovery of the final species to be detected. This circumstance is the most likely reason for the great discrepancies between different analytical methods with respect to endogenous

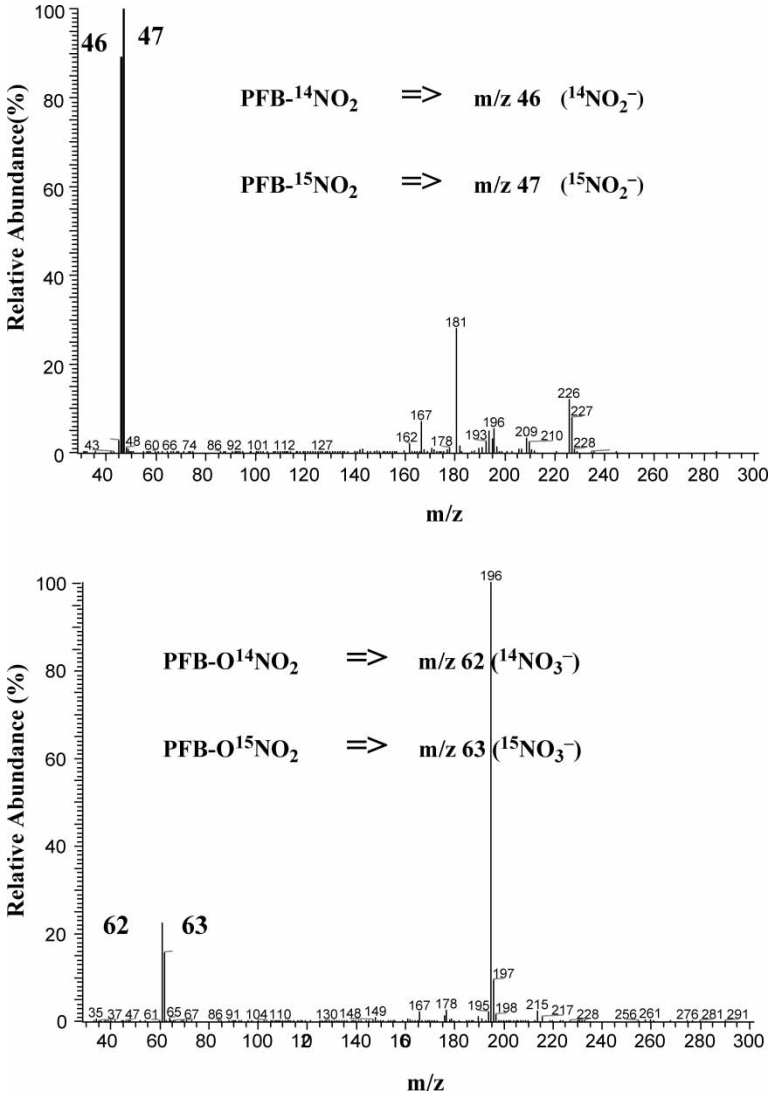


Figure 6. Mass spectra of the PFB derivatives of unlabelled and ^{15}N-labelled nitrite (upper panel) and unlabelled and ^{15}N-labelled nitrate (lower panel). Derivatization was performed by PFB bromide as described [61]. The TSQ 7000 instrument from ThermoQuest was used in the negative-ion chemical ionization mode. Please note that the intensity of ions in the mass spectra may depend upon the mass spectrometer instrument, e.g. see Figure 4.

basal levels of nitrite and nitrate, particularly in comparison to GC–MS (Tables II and III; see also below).

Quality control—precision and accuracy

In the field of NO clinical research quality control (QC) is not yet an integral and routine part of clinical studies. Moreover, the commercial availability of "ready-to-use" assay kits and even complete HPLC instruments for the quantitative analysis of nitrite and nitrate leads to an uncritical proceeding with the use of this material on the part of the investigators, regardless of the well recognized analytical problems in this area. The commercial availability should not be regarded as a guarantor for achieving reliable quantitative analysis, because commercially available assay kits and instruments are not peer-reviewed by external referees, but merely underwent internal validation, which is usually restricted to demonstrating linearity of the assay. Nevertheless, it should be pointed out that this important issue touches on further members of the L-Arg/NO family such as *S*-nitrosothiols and 3-nitrotyrosine [100–102] as well as other areas of research [103,104], and recommendations were done to overcome this problem [100–104]. Two major recommendations include: (1) the use in clinical studies of only validated analytical methods; and (2) incorporation of a QC system for the methods of analysis of nitrite and nitrate. The QC system should involve concomitant analysis of QC samples in addition to study samples and report of analytical data on the method's accuracy and precision generated from the analysis of the QC samples. We have developed such a QC system for the quantitative analysis of

nitrite and nitrate in human plasma and urine in the frame of clinical studies [61]. This QC system is an integral part of our clinical studies on NO [60,105,106] and is described below for urinary nitrate.

Study urine samples are analyzed alongside each three QC samples, i.e. QC1, QC2 and QC3, in duplicate. For this purpose a pooled urine is collected for 24 h, aliquoted into 1-ml portions which are stored at − 20°C. The internal standard [^{15}N]nitrate is added to 100-μl aliquots of all QC samples at a final concentration of 400 μM. QC1 is analyzed without external addition of nitrate. QC2 and QC3 are spiked with 200 and 400 μM of unlabelled nitrate, respectively. From the QC samples the accuracy (recovery, in %) and precision, i.e. more correctly imprecision (relative standard deviation, RSD, in %), are calculated. *Accuracy* measures the closeness with which an individual measurement approaches the true value. *Precision* is a measure of the ability to reproducibly return the same value for a sample that is repeatedly analyzed [102,107]. Accurate and precise analytical methods are characterized by high recovery values, e.g. close to 100%, and low RSD values, e.g. $<10\%$, with the highest allowable RSD value being 20% for very low analyte concentrations, i.e. of the order of the limit of quantitation [107]. Figure 7 shows the QC data for urinary nitrate which was measured by GC–MS as PFB derivative [61]. The mean recovery representing accuracy was 95% for QC2 and 98% for QC3. Method's mean precision representing reproducibility was above 98% for all QC samples.

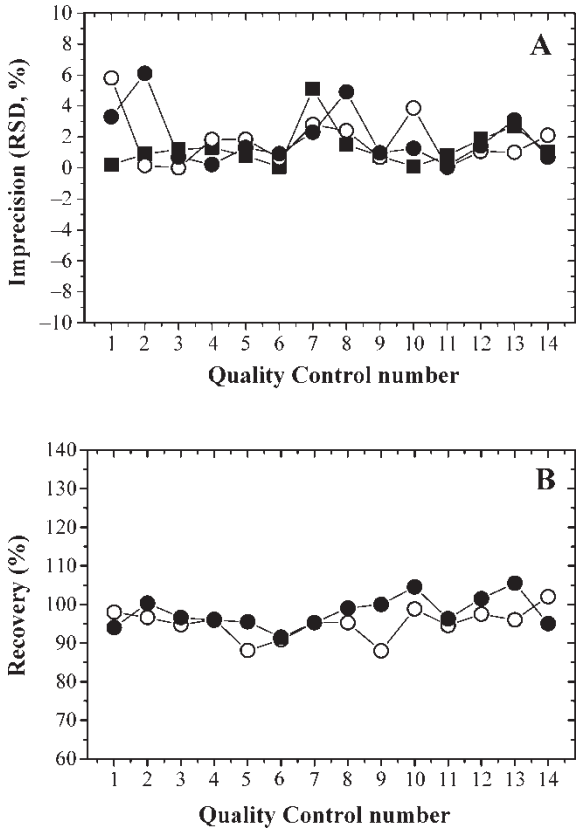


Figure 7. Quality control data on the imprecision (A) and recovery (B) of the GC–MS method for the quantitative determination of nitrate as PFB derivative in human urine. A set of 14 quality control samples were analyzed in parallel with samples from various clinical studies within a time period of one year. Three laboratory assistants worked up the samples in accordance with an internal Quality Control Standard Operation Procedure. The MS Engine instrument from Hewlett-Packard was used throughout. QC1, closed squares; QC2, open circles; QC3, closed circles.

Comparison of assays with mass spectrometry methods

The most frequently used statistical method to compare analytical methods is testing of correlation by simply plotting the concentrations measured by method A versus those measured by method B. However, it has been shown that neither the correlation coefficient nor techniques such as regression analysis are appropriate [108]. Errors relating to the use of the correlation coefficient and bivariate linear regression are often to be found in medical publications [109]. A more appropriate statistical method to compare two methods, e.g. of clinical measurement, was suggested by Bland and Altman [108]. In accordance with this method, two methods are compared in that way that the difference in the concentrations measured by methods A and B is plotted versus the average of the concentrations measured by the methods. Good agreement between two methods exists when the mean of the difference is close to zero and the individual differences are within the limits of agreement, i.e. within the range mean ± 2 SD, and differences do not vary in any systematic way over the relevant range of measurement [108].

Below some results from comparison studies are presented by using the method by Bland and Altman as well as by comparing correlation coefficients (R). Because comparison should be based on measurements from a single laboratory or by various laboratories which analyzed the same samples, the examples illustrated below come from the author's laboratory and his cooperating groups who had used established analytical methods for nitrite and nitrate measurement in human biological fluids. In these comparison studies, the mass spectrometry-based methods, i.e. GC–MS and GC–MS–MS, served as the reference methods.

Measurement of urinary nitrate by GC–MS and GC–MS–MS

Urinary nitrate was derivatized by PFB bromide after reduction to nitrite by cadmium in urine samples diluted with borate buffer (pH 8); aliquots of the toluene extracts of the same samples were analyzed by GC–MS and GC–MS–MS as described elsewhere [77].

For comparison the GC–MS–MS method, i.e. method B, was used as the reference method for the GC–MS method, i.e. method A.

Linear regression analysis of the urinary nitrate concentrations (in μM, range 100–5000 μM) measured by GC–MS (y) versus those measured by GC–MS–MS (x) revealed a straight line with the regression equation $y = 1.14 + 0.988x$, and a correlation coefficient $R = 0.99889$. The low level of the y-axis intercept, the slope of 0.988 which is very close to the unity (a slope of 1 represents complete agreement), and the R value of 0.99889 suggest that the GC–MS and GC–MS–MS methods are highly comparable regarding quantification of nitrate in human urine within a relevant concentration range. This is also supported by the paired t-test analysis which showed that the means of urinary nitrate (1012 vs. 1024 μM) are not statistically significantly different ($P = 0.265$). Analysis of the data by the method of Bland and Altman revealed the picture shown in Figure 8. The difference in urinary nitrate concentration between the methods was -11 ± 47 μM (mean ± SD, $n = 23$) which is very low in regard to the concentration range. With exception of a single concentration, all other data points are within the limits of agreements, i.e. within the range mean ± 2 SD.

Measurement of urinary nitrate by HPLC and GC–MS

Urinary nitrate was analyzed by means of an anion-pairing HPLC method with UV absorbance detection without preceding derivatization [82] and by GC–MS after derivatization with PFB bromide [61]. Linear regression analysis of the urinary nitrate concentrations (in μM, range ≈100–3600 μM) measured by HPLC (y) versus those measured by GC–MS (x) revealed a straight line with the regression equation

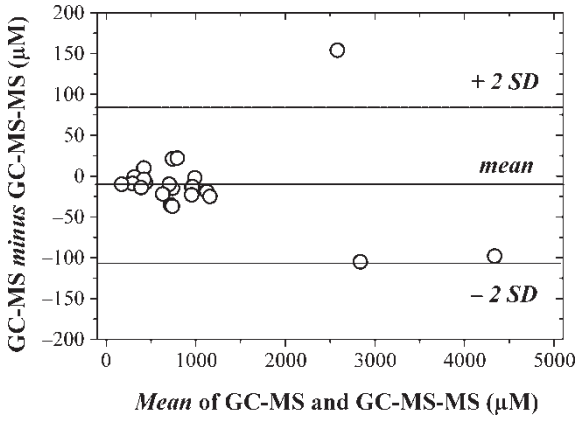


Figure 8. Comparison of GC–MS with GC–MS–MS regarding quantification of nitrate in human urine by the method of Bland and Altman [108]. Nitrate was determined in urine of 23 healthy humans after reduction to nitrite by cadmium and derivatization by PFB bromide as described [77]. The difference in the nitrate concentrations measured by GC–MS and GC–MS–MS are plotted versus the mean concentration. Horizontal lines show the mean difference and the ± 2 SD range.

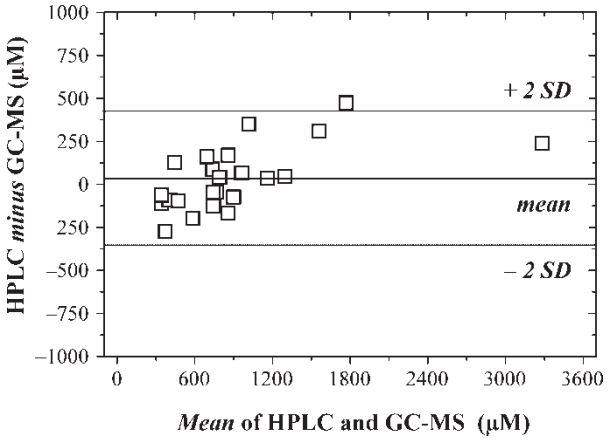


Figure 9. Comparison of HPLC with GC–MS regarding quantification of nitrate in human urine by the method of Bland and Altman [108]. Nitrate was determined in urine of 24 patients suffering from various diseases by HPLC as described elsewhere [82] and by GC–MS after derivatization by PFB bromide as described [61]. The difference in the nitrate concentrations measured by HPLC and GC–MS is plotted versus the mean concentration measured by the methods. Horizontal lines show the mean difference and the ± 2 SD range.

$y = -121 + 1.16x$, and a correlation coefficient $R = 0.97327$. Paired t-test analysis showed that the means of urinary nitrate (907 vs. 884 μM) are not statistically significantly different ($P = 0.554$). Data analysis by the method of Bland and Altman is showed in Figure 9. The difference in urinary nitrate concentration between the methods was -30 ± 183 μM (mean ± SD, $n = 24$) which is low with respect to the concentration range, but the difference varies much more than the difference between GC–MS and GC–MS–MS (see above). With exception of a single concentration, all other data points are within the limits of agreements, i.e. within the range mean ± 2 SD. Nevertheless, consideration of all comparison data suggests that the GC–MS method [61] is superior to the HPLC method [82] and should be used preferably when possible.

Measurement of plasma nitrate by the Griess assay and by GC–MS

As mentioned above assay methods based on the Griess reaction, in particular the so-called batch Griess assays, are frequently used to measure nitrite and nitrate in urine, plasma, serum and other biological fluids. We have thoroughly compared the most frequently used batch Griess assay with a GC–MS method [65]. This comparison showed that the batch Griess assay was not applicable to whole human plasma and urine samples. Thus, in human plasma the batch Griess assay revealed constantly much lower nitrate basal levels than GC–MS, while added nitrate was recovered from plasma with considerably higher recovery rate of the order of 160% by the Griess assay as compared to 110%

by GC–MS [65]. Also, the mean recovery rate of nitrate from urine samples was quantitative in the GC–MS method but amounted to only 30–80% in the Griess assay. Eventually, measurement of urinary nitrate resulted in an excellent correlation between two GC–MS methods ($R = 0.989$) but only in a weak correlation ($R = 0.8$) between the Griess assay and the GC–MS method [65].

In the frame of a clinical study [105] nitrate was determined in aliquots of plasma from cavernous and systemic blood by an automated Griess assay based on the method of Green et al. [70] and by GC–MS after reduction of nitrate to nitrite by cadmium and derivatization with PFB bromide [65]. Linear regression analysis of the plasma nitrate concentrations (in μM, range 15–60 μM) measured by the automated Griess assay (y) versus those measured by GC–MS (x) revealed a straight line with the regression equation $y = -21 + 1.37x$, and a correlation coefficient $R = 0.89594$. Paired t-test analysis showed that the means of plasma nitrate (36 vs. 41 μM) are statistically significantly different ($P = 0.00025$). Data analysis by the method of Bland and Altman is shown in Figure 10. The difference in the plasma nitrate concentrations between the methods was -5.5 ± 8.7 μM (mean ± SD, $n = 40$) which is relatively high in regard to the concentration range, and varies relatively strongly. The Bland–Altman-plot also clearly shows considerable discrepancies between the methods, despite the observation that only one data point is outside the range mean ± 2 SD. Taken into consideration the numerous analytical problems of the diazotization reaction and the lack of satisfactory agreement between Griess assays and GC–MS methods, it may reasonably be assumed that assays based on the diazotization reaction would provide by far less accurate values for nitrite and nitrate in human plasma and urine. Despite the great popularity of the batch Griess assay, in particular of commercially available kits, it is highly recommended that Griess assays are thoroughly validated by reliable analytical methods, preferably by methods based on the GC–MS methodology.

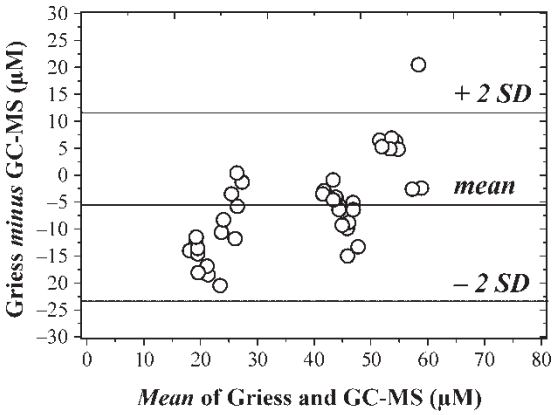


Figure 10. Comparison of an automated Griess assay with a GC–MS method regarding quantification of nitrate in human plasma by the method of Bland and Altman [108]. Nitrate was determined in plasma of males in the frame of a clinical study [105] by an automated Griess assay [70] and by GC–MS after reduction of nitrate to nitrite by cadmium and derivatization with PFB bromide [65]. Fourty plasma samples were considered. The difference in the nitrate concentrations measured by the Griess assay and by the GC–MS method is plotted versus the mean concentration measured by the methods. Horizontal lines show the mean difference and the ± 2 SD range.

Conclusions and future prospects

Upon formation from L-arginine, NO undergoes multiple reactions leading both to biological activity and to its inactivation to nitrate by oxyhaemoglobin-catalyzed oxidation in red blood cells and to nitrite by autoxidation. These reactions keep the NO's half-life in the circulation far below 1 s. Therefore, authentic NO is not accessible to analytical determination. Unlike exhaled NO, circulating NO cannot be used to assess NO synthesis *in vivo*. Nitrate and nitrite produced from NO oxidation circulate in the blood and are excreted in the urine. The concentration of nitrite and nitrate in blood plasma and serum and in urine is accessible to quantitative analysis and can be used to measure NO synthesis in different conditions. Circulating nitrite seems to reflect constitutive endothelial NO synthase activity, whereas urinary nitrate is regarded as an integral indicator of systemic NO production.

Nitrite and nitrate in biological fluids can rarely be measured in their native forms, e.g. by HPLC and CE using UV absorbance and conductivity detection. For the most part, however, reliable quantitative determination of these anions requires considerable sample work-up to eliminate interferences as well as chemical derivatization to improve detection. As a result of this need dozens of analytical methods have been reported to date. They include colorimetry, spectrophotometry, fluorometry, chemiluminescence, GC and GC–MS. The oldest and currently most frequently used assays are based on the Griess reaction which dates from 1864.

Application of these methods to circulating and excretory nitrite and nitrate provided greatly differing values at the basal state. Many preanalytical factors, e.g. anticoagulants, and analytical factors, e.g. lack in specificity and varying recovery of nitrate reduction to nitrite, and the ubiquity of nitrite and nitrate were found to considerably contribute to these divergences. Mass spectrometry-based methodologies are presently the most accurate quantitative methods of nitrite and nitrate in biological fluids and tissue. Among the mass spectrometric approaches, GC–MS analysis of nitrite and nitrate as pentafluorobenzyl derivatives is the single method

that allows simultaneous derivatization and quantification of these anions in any biological sample.

In clinical studies stepmotherly attention has been paid to the important issue of quality control and to the use of commercial assays not only by investigators but also by editors of clinical analysis-oriented journals. Thus, choice of analytical quantitative methods for nitrite, nitrate and other members of the L-Arg/NO family is usually directed to simplicity, rapidity and commercial availability of assays rather than to reliability. Also, there is an implicit but not justified belief in the reliability of commercially available nitrite and nitrate assays. This leads to an uncritical use of such assays. This practice does not promote progress in the area of NO research. Therefore, in the frame of clinical studies only validated analytical methods should be used, which should also be subject of critical review in addition to the results obtained by the methods, and quality control should be an indispensable part of the whole analytical process.

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