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Comparison of nitrite/nitrate concentration in human plasma and serum samples measured by the enzymatic batch Griess assay, ion-pairing HPLC and ion-trap GC–MS: The importance of a correct removal of proteins in the Griess assay☆

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Abstract

Mass spectrometry-based approaches are the reference techniques for the determination of nitrite and nitrate in plasma and serum. However, due to their simplicity and rapidity, assays based on the Griess reaction or HPLC are generally used in clinical studies, but they generate diverging values for nitrite/nitrate concentration. In this study, particular attention is paid to the optimization of the deproteinization procedure for plasma and serum samples prior to nitrite/nitrate analysis by an enzymatic batch Griess assay, HPLC and GC–MS. A method is reported to verify completeness of deproteinization and to correct for nonspecific contribution to the absorbance of the diazo dye at 540 nm. With the application of such optimized procedures, we were able to significantly improve the correlation between Griess and HPLC method or the GC–MS technique for nitrite + nitrate concentrations in human serum and plasma. Despite remaining potentially interfering pre-analytical and analytical factors, the procedures reported in the present study may be helpful in a critical evaluation of limits and possibilities of the enzymatic batch Griess assay as a large-scale method for nitrite/nitrate determination in human serum in clinical studies.

Keywords: Nitric oxide; Nitrite; Nitrate; Griess assay; Mass spectrometry; High-performance liquid chromatography

1. Introduction

Nitric oxide (NO) is a reactive molecule synthesized from L-arginine by at least three isoforms of the NO synthase (NOS). NO exerts a number of important biological functions such as vascular tone regulation, immune response modulation, and neurotransmission [1–3]. The half-life of NO, in vivo, in the circulation is most likely shorter than 0.1 s [4], therefore it is rather difficult to determine NO content in plasma and serum samples. NO reacts with oxygen species and biological molecules such as dioxygen, superoxide anion and oxyhemoglobin to form a variety of products, including nitrite and nitrate [3,5–7]. Nitrite and nitrate are the major stable metabolites of endogenous NO and are accessible to quantitative analysis. Determination of these inorganic NO metabolites in blood and urine turned out to be the most suitable method to assess indirect quantification of NO production in vivo.

Presently, nitrite and nitrate can be quantified in plasma, serum and urine of humans by various methods based on different analytical principles [8,9]. These methods have produced diverging values of nitrite and nitrate in the circulation of healthy humans. This variation can only partially be explained by differences in dietary intake of these compounds. The major reason for the discrepancies seems to be methodological problems [7,9]. Several authors have shown that accurate and interference-free quantification of nitrite and nitrate in plasma

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and serum may be difficult using the oldest method based on the Griess reaction. Many pre-analytical factors, such as anticoagulants and nitrite/nitrate contamination, may interfere with nitrite/nitrate quantification in blood samples [10–12]. Gas chromatography-mass spectrometry (GC–MS)-based methodologies are presently the most accurate quantitative methods of nitrate and nitrite in plasma and serum, but other analytical methods, such as gas and liquid chromatography, capillary electrophoresis and chemiluminescence, are also applied [8,9,13–15]. However, the simplicity, rapidity and cheapness of the batch Griess assay encourage attempts to improve its dependability especially on large population in clinical studies.

The aim of the present study was to identify, and possibly eliminate, specific factors linked to protein precipitation or protein removal procedure, on the basis of diverging values of nitrite and nitrate concentrations obtained by three different techniques. Nitrite and nitrate concentrations measured in plasma and serum samples by an enzymatic batch Griess method, GC–MS and a newly developed HPLC method were compared. Different protocols to eliminate proteins were used, and a simple method was developed to verify the efficiency of the deproteinization procedure and to correct for nonspecific colorimetric signal in the Griess assay. Sample treatment by acetonitrile and chloroform was found to be the most efficient method to remove proteins by reducing the potential of interferences by proteins. With the application of such procedures, we were able to significantly improve the correlation between Griess method and GC–MS technique for nitrite + nitrate concentrations in human serum and plasma in comparison to previously obtained correlation by our and other groups [7]. Finally, Griess reaction and photometrical analysis were performed on an automatic 96-well microplate analyzer allowing fast and reproducible measurements of about 100 samples per hour. The observations of the present study could be helpful in a critical evaluation of limits and possibilities of application of the enzymatic batch Griess method to determine nitrite + nitrate concentrations in plasma and serum.

2. Experimental

2.1. Chemicals

Filtering membranes (3 kDa and 10 kDa, cut-off) were obtained from Nanosep® Centrifugal Devices (Pall Gelman Laboratory, Ann Harbor, MI, USA). Tetrabutylammonium hydroxide was used as the ion-pairing agent for the HPLC separation of nitrite and nitrate and it was obtained as a 55 vol.% water solution from Nova Chimica (Milano, Italy). Far-UV HPLC-grade acetonitrile and HPLC-grade chloroform were purchased from LabScan (Dublin, Ireland). HPLC-grade methanol and diethyl ether were supplied by Carlo Erba Reagenti (Milan, Italy). Zinc sulfate, sodium hydroxide, sodium [^{15}N]nitrate (declared as 98 at.% at ^{15}N), ultrapure HPLC standards and KH_2PO_4 at the highest purity available were obtained from Sigma (St. Louis MO, Italy). Standard stock solutions (1 mM for HPLC and 6 mM for GC–MS) of unlabelled nitrite and nitrate and ^{15}N-labelled nitrate were prepared in double distilled water. 2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br) was obtained from Aldrich (Steinheim, Germany). Reagents used to perform the Griess assay were all included in a colorimetric commercial kit supplied by Roche (Penzberg, Germany). Other reagents were purchased by commercial sources.

2.2. Procedures for blood sampling and preparation of plasma and serum

Plasma (lithium heparin) and serum samples were obtained from blood withdrawal (antecubital veins), at the same time point, from healthy volunteers of our laboratories. Blood samples were treated taking in account previously described pre-analytical interfering factors [9]. Briefly, blood samples were centrifuged at 4 °C (1500 × *g*, 5 min) immediately after collection and supernatants were subjected to deproteinization procedure. If not immediately analyzed, deproteinized samples were aliquoted and stored at −80 °C.

2.3. Deproteinization methods

2.3.1. Methods A and B (acetonitrile–chloroform)

Far-UV HPLC-grade acetonitrile (ACN) was added (1:1, v/v) to 1-ml aliquots of the samples and vortexed for 60 s. After centrifugation (20690 × *g*, 10 min, 4 °C) the supernatants (about 1.8 ml) were saved, extracted by vigorous agitation with a double volume of HPLC-grade chloroform, and centrifuged again (20690 × *g*, 5 min, 4 °C). The upper aqueous phases (about 0.8-ml aliquots) were immediately collected (method B) or the above steps were repeated twice and samples subjected to chloroform washings for two more times (method A).

2.3.2. Method C (ultrafiltration)

Samples (100 μl) were diluted with double-distilled water (1:5, v/v). Aliquots of each sample were transferred in Eppendorf tubes equipped with a filtering membrane (10 kDa cut-off) for a first centrifugation (10500 × *g*, 20 min, 4 °C). Successively, samples were collected in tubes with a filtering membrane (3 kDa, cut-off) and a second centrifugation was performed (10500 × *g*, 20 min, 4 °C). The filters were rinsed three times each with 400-μl aliquots of HPLC-grade water prior to ultrafiltration of serum or plasma.

2.3.3. Method D (zinc sulfate)

Serum samples (0.4 ml) were diluted with distilled water (1:4, v/v) and deproteinized by adding 1/20th volume of zinc sulfate (300 g/l) to reach a final concentration of 15 g/l. After centrifugation (10000 × *g* for 5 min at room temperature or 1000 × *g* for 15 min), samples were analyzed by the Griess assay. This method was performed as described by Moshage et al. [16].

2.3.4. Method E (zinc sulfate–sodium hydroxide)

Serum samples were deproteinized as described by Navarro-Gonzalves [17] with slight modifications. Briefly, 10 μl of 10 M NaOH and 600 μl of 0.15 M $ZnSO_4$ were added to the samples (1 ml). After incubation on ice for 15 min, samples were centrifuged (12000 × *g*, 5 min).

2.3.5. Method F (methanol–diethyl ether)

We applied the deproteinization procedure described by Guevara et al. [18]. Briefly, 0.1-ml aliquots of serum samples were incubated overnight with 0.9-ml aliquots of methanol–diethyl ether (3:1, v/v) and, after incubation, the samples were centrifuged (20690 × *g*, 10 min, 4 °C). However, in contrast to Guevara et al. [18], reduction and Griess reagents addition were performed after deproteinization.

Total protein concentration was measured in each sample before and after deproteinization on OLYMPUS AU2700 instrument for routine determination of patient protein levels. The principle of this method is based on the reaction of cupric ions in an alkaline solution with proteins and polypeptides that contain at least two peptide bonds to produce a violet colored complex in a manner depending on protein concentration in the sample. Organic solvents and reagents used in all deproteinization methods do not to interfere with protein determination by this method.

2.4. Absorbance correction for non-specific scattering signal

Nitrate and nitrite concentrations were determined photometrically on microplate using a reagent kit (Nitric Oxide Colorimetric Assay, Roche). Addition of reagents and absorbance reading (at fixed wavelengths of 405, 450, 492, 540, 600 and 620 nm) were automatized on the microtiter-plates automatic analyzer TRITURUS (Grifols Italia S.p.A, Ghezzano (Pisa)). In parallel, the Griess reaction was performed with five samples in the spectrophotometer cell, and spectra (400–620 nm) were recorded on a Cary 3 dual-beam spectrophotometer (Varian Australia Pty. Ltd., Mulgrave, Australia) after each reaction step to compare the absorbance values of the two instruments. Fig. 1A shows representative spectra of a deproteinized serum sample before and after incubation with the Griess reagents and the fixed-wavelength absorbance values of the same sample by the automatic analyzer photometer.

In order to access the efficacy of protein removal by deproteinization processes, protein concentration was measured in 20 serum samples before and after protein precipitation by each deproteinization method. In addition, the absorbance spectra of these solutions were recorded from 405 to 620 nm to determine the non-specific contribution of each sample to absorbance values at 540 nm before addition of Griess reagents.

Particles in solution, principally aggregating/precipitating proteins, increase absorbance values inversely with wavelength [19] due to scattering phenomenon. However, even after complete removal of proteins, a non-specific low contribution to absorbance values derives from other serum constituents (<0.1 A.U. for the 405–620 nm range). Therefore, the actual sample signal at 540 nm, derived from dye production, must be corrected for the non-specific contribution that can be measured in two ways: (1) A "blank" reading sample, to which all the Griess reagents except for *N*-(1-naphthyl)ethylenediamine dihydrochloride are added to avoid diazo dye production, has to be recorded for each sample; this procedure doubles time and costs of the determination; (2) An extrapolated value of the non-specific contribution at 540 nm can be easily calculated by an analysis program as follows. Although the scattering signal is a complex function of the wavelength and particles concentration and size [19], it was possible to extrapolate the contribution at each wavelength by a phenomenological linear fitting of TRITURUS fixed-wavelength (λ) data. We observed the equation: absorbance = $a \times \lambda + b$ ($r^2 = 0.97 \pm 0.01$; $n = 320$), where a and b are fitted parameters specific for each sample. A perfect agreement between experimentally measured absorbance at 540 nm and extrapolated value, not including the 540–492 nm points in the fit, was obtained (difference <0.005 A.U.). As an alternative, a simpler correction could be applied by just subtracting the weighed average of absorbance values at 450 and 600 nm. However, in this case slight differences (≤0.03 A.U., corresponding to about ≤0.9 μM of nitrite) can be observed. For samples deproteinized by method B (n=20), experimental absorbance values were 0.06 ± 0.02 A.U., extrapolated absorbance values were 0.06 ± 0.02 A.U., and weighted average absorbance values in the range 450–600 nm were 0.07 ± 0.03 A.U.

Finally, in order to measure the TRITURUS instrument's photometer repeatability, resolution and low detection limit, absorbance was recorded 10 times at 540 nm for five repeated measures on distilled water (also used as "blank" sample) and five different serum samples, with absorbance values ranging from 0.1 to 2 A.U. The mean RSD of absorbance values was <0.5% (n = 250), the mean RSD of repeated measures was 3.1 ± 1.1%. Instrument low detection limit (i.e., the water reading at 540 nm, n = 10) and resolution were 0.006 ± 0.002 and 0.003 A.U., respectively.

2.5. Determination of nitrite + nitrate by the Griess assay

Determination of nitrite + nitrate (NO_x) concentrations by the enzymatic batch Griess assay in samples treated by the deproteinization methods described above was performed using a reagent kit (Nitric Oxide Colorimetric Assay, Roche). This procedure consists of the following steps: (1) dilution (1:1, v/v) of deproteinized samples (0.3 ml) with 100 mM potassium phosphate buffer, pH 7.5; (2) incubation (30 min, 25 °C) with 6.6 U/l nitrate reductase (50 μl) and a mixture (20 μl) of 1.5 mg/ml NADPH and 0.03 mg/ml FAD; (3) successive addition (75 μl) of 1 wt.% sulfanilamide and 0.1 wt.% acidified *N*-(1-naphthyl)ethylenediamine dihydrochloride to a 150-μl aliquot of the incubation solution (step 2); incubation for 5 min at 25 °C; (4) quantitative estimation of NO_x concentration by spectrophotometric measurement of the absorbance at 540 nm.

A calibration curve was performed with 80 mM potassium nitrate standard aqueous solution serially diluted from 80 to 0 μM. In parallel, the same standard solution samples were treated by method B (acetonitrile–chloroform) to generate a second calibration curve. No significant differences were found in the final absorbance values in the calibration curves in the concentration range investigated.

Values obtained from the Griess assay represent the sum of nitrite and nitrate (NO_x). Because of the insensitivity of the present Griess assay, we were not able to measure basal nitrite levels in serum samples. Mean accuracy (recovery), imprecision

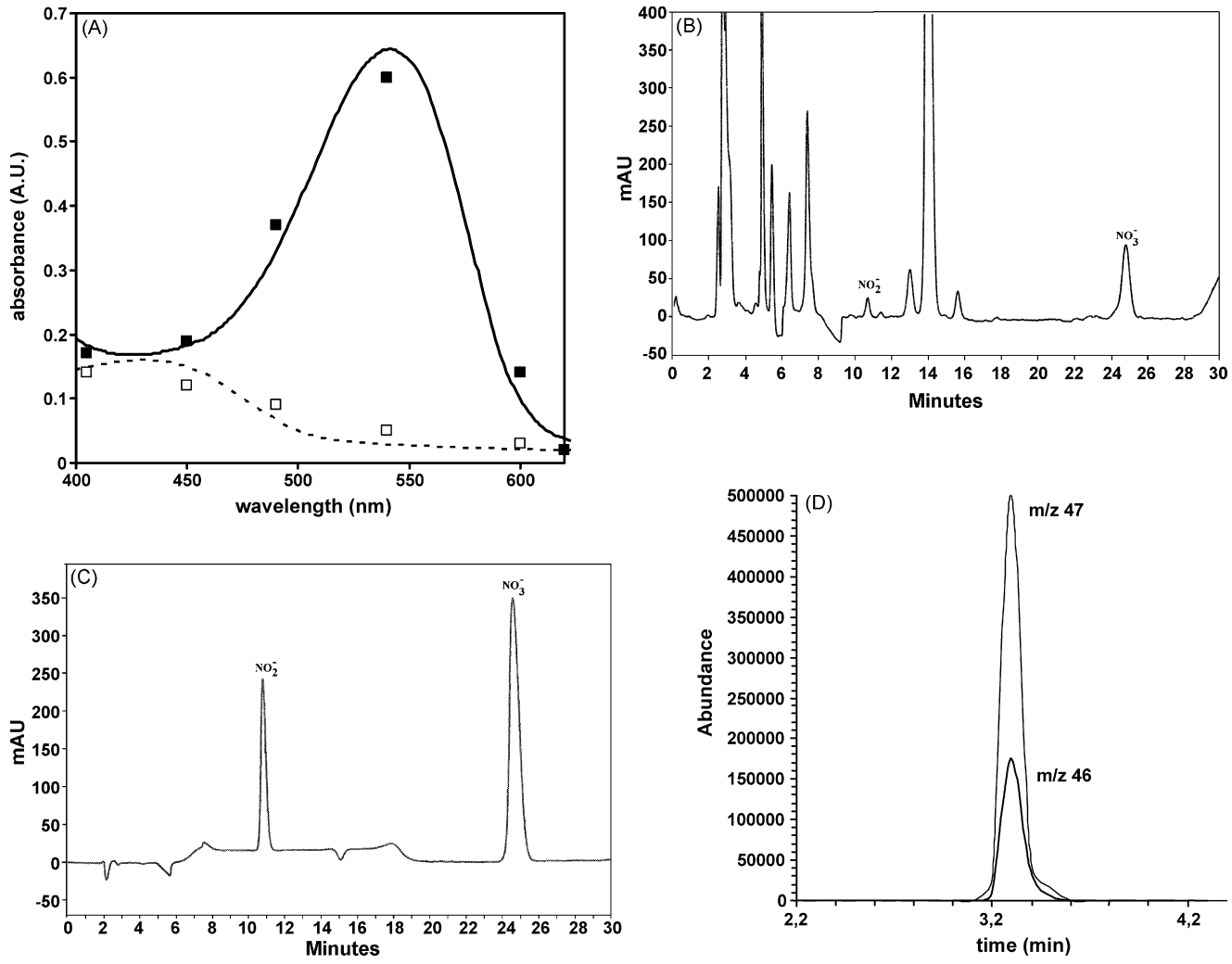

Fig. 1. Original representative tracings from the analysis of nitrite and nitrate in a sample from an adult healthy subject by the methods applied in the present study. (A) Griess assay (nitrite + nitrate) using deproteinization method B. Scaled spectra of the deproteinized serum sample before (dotted line) and after Griess reagents addition (continuous line), and fixed-wavelength absorbance readings before (□) and after (■) Griess reagents addition of the same sample by the automatic photometer. (B) HPLC analysis (206 nm) of nitrite and nitrate using deproteinization method C in plasma sample. (C) HPLC analysis (206 nm) of a standard mixture of nitrite (25 μM) and nitrate (25 μM). (D) GC–MS analysis of nitrite + nitrate using deproteinization method C in plasma sample. Endogenous nitrite + nitrate concentrations in this sample were measured as 20.2 μM by Griess, 24.3 μM by HPLC (sample diluted 1:5, v/v) and 28.0 μM by GC–MS.

(RSD) and limit of quantification (LOQ) of the Griess assay in serum samples (deproteinized with method B) were determined to be 95.5%, 5.1% and 5 μM, respectively, in the range of 0–80 μM for added nitrate.

2.6. Determination of nitrate and nitrite by HPLC and by GC–MS

Comparison between NO_x concentrations measured by Griess assay, GC–MS and HPLC was performed on 50 samples from healthy volunteers.

2.6.1. Analysis of plasma nitrite and nitrate by HPLC and method C

The ion-pairing HPLC method used in this study for the determination of nitrite and nitrate in plasma samples deproteinized by method C is an extension of the method by Tavazzi et al. previously reported for *N*-acetylated amino acids, purines, pyrimidines, GSH, GSSG and dicarboxylic acids [20]. The HPLC apparatus consisted of a SpectraSystem P4000 pump and a highly sensitive UV6000LP diode array detector (ThermoElectron Italia, Rodano, Milan, Italy), equipped with a 5-cm light-path flow cell. Data were acquired and analyzed by a PC using the ChromQuest® software package provided by the HPLC manufacturer. Chromatography was carried out using a Hypersil (250 mm × 4.6 mm i.d.) column, 5-μm particles size, which was provided with its own guard column (ThermoElectron Italia, Rodano, Milan, Italy).

Two mobile phases with the following composition were used: mobile phase A consisted of 12 mM tetrabutylammonium hydroxide, 0.125 vol.% methanol, 10 mM KH_2PO_4, pH 7.00. Mobile phase B consited of 2.8 mM tetrabutylammonium hydroxide, 30 vol.% methanol, 100 mM KH_2PO_4, pH 5.50. Mobile phases were properly filtered through a 0.22-μm HV-Millipore membrane and degassed. The chromatographic column was conditioned for 20 min with mobile phase A at a flow

of 1.2 ml/min. A step gradient from mobile phase A to mobile phase B was formed as follows: 23 min at 100% mobile phase A; 8 min to 80% mobile phase A; 10 min to 70% mobile phase A; 12 min to 55% mobile phase A; 11 min to 40% mobile phase A; 9 min to 15% mobile phase A; 10 min to 0% mobile phase A; hold for column washing 0% buffer A for additional 20 min. Flow rate of 1.2 ml/min and column temperature of 10 °C were maintained constant throughout the analysis. Concentration was calculated from the standard run data at a wavelength of 206 nm for nitrite and nitrate. The reproducibility of the present HPLC method was demonstrated by the low variability of the retention times and of the peak areas of nitrite and nitrate determined on five chromatographic runs referring to five standard solutions prepared and analyzed on five consecutive days. The retention time of nitrite and nitrate determined in the standard mixtures was (mean ± SD) 11.1 ± 0.07 min and 24.74 ± 0.06 min, respectively. The coefficient of variation of the peak areas was 0.75 ± 0.07% for nitrite and 0.56 ± 0.04% for nitrate.

Lower limit of detection (LLOD), upper limit of detection (ULOD), linearity and the reproducibility were evaluated by injecting 200-μl aliquots of standard mixtures in quintuplicate. The LLOD values (signal-to-noise ratio of 2:1) of the method were determined to be 0.25 μM for nitrite and 0.1 μM for nitrate. The ULOD was determined to be 500 μM for nitrite and to 100 μM for nitrate. Linearity was determined by assaying standard aqueous solutions of nitrite and nitrate with the following concentrations: LLOD, 10 × LLOD, 50 × LLOD, 100 × LLOD, 500 × LLOD. The correlation coefficient obtained was 0.994 for nitrite and 0.999 and nitrate.

Intra-assay accuracy, precision and LLOQ of the HPLC method using deproteinization method C were determined by spiking in triplicate 10 non-pathological plasma samples with nitrite and nitrate at final concentrations of 0.5, 1, 2, 5 and 20 μM each. Accuracy (recovery) ranged from 97.2 to 99.8% for nitrite and from 98.7 to 101.3% for nitrate. Mean imprecision (RSD) was 2.9% for nitrate and 3.1% for nitrite. The LLOQ of the HPLC method was determined to be each 1 μM for nitrite and nitrate.

By this HPLC method, nitrite and nitrate were found to be present in all plasma samples analyzed in this study. Plasma samples obtained from 25 healthy adults (aged 25–50 years) were deproteinized by method C, diluted (1:5, v/v) with double distilled water immediately prior to injection, and analyzed by HPLC. Basal nitrite and nitrate concentrations were determined to be (mean ± SD) 2.17 ± 0.34 and 44.1 ± 11.3 μM, respectively. In Fig. 1B, a chromatogram from the HPLC analysis of nitrite and nitrate in a human plasma sample is shown.

2.6.2. Analysis of plasma nitrite and nitrate by GC–MS and method C

A Finnigan PolarisQ Ion Trap GC/MS system consisted of a PolarisQ mass spectrometer and a TRACE gas chromatograph (ThermoElectron Italia, Rodano, Milan, Italy) was used for GC–MS analyses. A fused-silica capillary column DB-5 MS (30 m × 0.25 mm i.d., 0.25-μm film thickness) from J & W Scientific (Rancho Cordova, CA) was used. Helium (70 kPa) and methane (200 Pa) were used as the carrier and the reagent gases, respectively, for negative-ion chemical ionization (NICI). Electron energy and electron current were set to 230 eV and 300 μA, respectively. The following oven temperature program was used: 1 min at 70 °C, then an increase to 280 °C at a rate of 30 °C/min. Constant temperatures of 180, 280 and 200 °C were kept at the ion source, interface and injector, respectively.

The sum of nitrite and nitrate was determined by GC–MS using reduction and derivatization procedures described by Tsikas et al. [21], with minor modifications. Briefly, to a 100-μl aliquot of plasma ^{15}N-labeled nitrate was added at a final concentration of 80 μM. After addition of cadmium and acetic acid, reduction of nitrate to nitrite was performed by incubation for 15 min. Subsequently, samples were ultrafiltered (method C) and derivatized as described [21].

After derivatisation, samples were cooled to 25 °C, acetone was evaporated under a nitrogen stream, reaction products were extracted by vortex-mixing with toluene (1 ml) for 1 min, and 1-μl aliquots were injected into the GC–MS system in the splitless mode. Quantification of the sum of nitrite + nitrate was performed in the NICI mode by selected ion monitoring (SIM) of the ions *m/z* 46 for nitrite and nitrate-derived nitrite, and of *m/z* 47 for [^{15}N]nitrate-derived [^{15}N]nitrite. The dwell-time was 50 ms for both ions.

The GC–MS method was validated for plasma nitrate + nitrite within the range of 10 μM–100 μM. Intra-assay accuracy (recovery) and imprecision (RSD) were of the order of 97 and 2%, respectively. A typical GC–MS chromatogram from the analysis of nitrite + nitrate in plasma of a healthy volunteer after reduction to nitrite and derivatization is shown in Fig. 1D.

2.7. Statistical analysis

SPSS version 12.0 and Microsoft Excel 2003 were used for data analysis. Correlation and agreement between different deproteinization methods and techniques applied to measure nitrite and nitrate in plasma and serum samples was tested both by ordinary and Deming linear regression and paired *t*-test or ANOVA as appropriate. The evaluation of fit and correlation goodness was accessed by r^2 calculation and by the method of Bland and Altman [22]. In this graphical method the differences between values obtained by two methods are plotted against the averages of the values of the techniques. The mean difference (md) ± SD was used to describe agreement of analysis. In addition, the correlation of the differences between methods upon the average of the methods across the range of measurement can be used to check for bias in the methods tested [22,23].

3. Results

3.1. Comparison of photometric fixed-wavelength absorbance readings and absorbance spectra

The absorbance readings at different wavelengths and the absorbance spectra, recorded on an automatic microplate analyzer and on a spectrophotometer, respectively, were compared. The values were found to correlate, with the microplate analyzer absorbance values being about twice the

Table 1
Mean total residual protein concentration and absorbance values at 540 nm in 20 serum samples before addition of the Griess reagents using different deproteinization methods

Deproteinization method	Total protein [g/dl, mean ± SD]	Absorbance at 540 nm [A.U., mean ± SD]
Non-deproteinized original sample	7.1 ± 0.9	0.55 ± 0.11
Acetonitrile reference method (A)	<0.1 (LOD)	0.05 ± 0.02
Acetonitrile rapid (B)	<0.1 (LOD)	0.06 ± 0.02
Ultrafiltration (C)	0.6 ± 0.9	0.20 ± 0.13
Zinc sulfate (D)	1.1 ± 0.4	0.29 ± 0.04
Zinc sulfate/sodium hydroxide (E)	0.8 ± 0.2	0.23 ± 0.03
Methanol/diethyl ether (F)	3.5 ± 0.9	0.42 ± 0.09

A.U.: absorbance units.

values recorded by the spectrophotometer (regression equation: $Abs_{TRITURUS} = 1.98 Abs_{Spectrophotometer} + 0.01$; $r^2 = 1.00$), depending upon different optical geometry and cell optical length of the two instruments. Moreover, the comparison of extrapolated values by linear fit at different wavelengths (as described in Section 2.4, including or not including the 492-nm and 540-nm points in the fit) and actual readings before addition of the Griess reagents to the sample showed only slight differences in the 450–620 nm-range (<0.005 A.U., data not shown).

3.2. Protein interference in nitrite and nitrate determination by the Griess assay

The removal of proteins from plasma and serum samples is a critical step in the determination of nitrite and nitrate concentration by assays based on the Griess reaction [9,18,24]. The results from the comparison of different deproteinization procedures are summarized in Table 1. The mean total residual protein concentration and the respective absorbance contribution at 540 nm before addition of the Griess reagents are reported. The most effective deproteinization method is the triple extraction of samples with ACN–chloroform (method A; residual protein concentration <0.1 g/dl to <1% of original protein concentration). Similar results were obtained with the single ACN–chloroform treatment (method B). Methods C, D and E were able to remove 85–92% of the originally present proteins. However, ultrafiltration method (method C), still removing almost all protein content in 17 samples (final protein concentration <0.2 g/dl), left over more than 1.0 g/dl in 3 samples, probably due to membrane breaking. On the other hand, application of method F resulted in a mean residual protein concentration of about 50% of the initial level (Table 1).

The non-specific absorbance contribution of each sample at different wavelengths (from 405 to 620 nm) was measured and the mean values at 540 nm are shown in Table 1. The lowest absorbance value was obtained by methods A and B (i.e., 0.05 and 0.06 A.U., respectively). This contribution was interpreted as a non-specific signal due to scattering phenomenon caused by aggregated/precipitated particles in each sample.

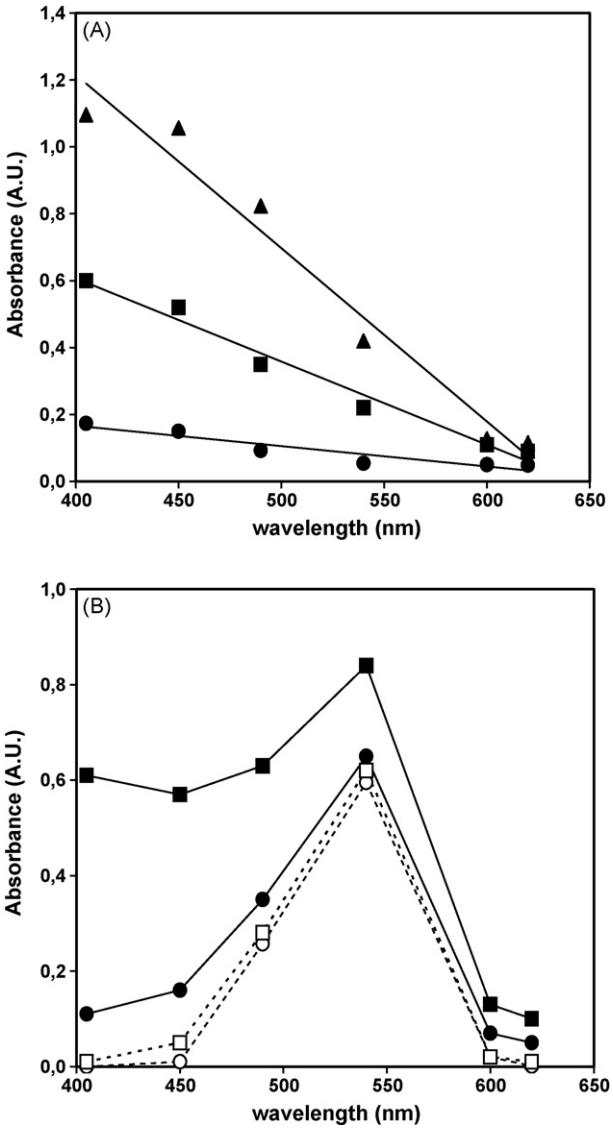

Fig. 2. (A) Representative photometric fixed-wavelength absorbance readings of a serum sample before (▲) and after deproteinization by methods A (●) and E (■) in the absence of Griess reagents. (B) Absorbance values of the same sample after Griess reaction without (solid lines, method A (●) and E (■)) and with absorbance correction (doted lines, method A (○) and E (□)) for the straight line fitting points 405, 450, 600 and 620.

Fig. 2A shows representative spectrophotometric fixed-wavelength absorbance readings of a serum sample before and after deproteinization by method A or E in the absence of the Griess reagents. Reagents used in different deproteinization procedures did not show specific spectrophotometric properties that could influence absorbance of samples. Therefore, apparent solution turbidity should derive from scattering of particles and should be principally dependent upon protein concentration. As reported in Section 2.4, it was possible to describe the spectra of samples from 405 to 620 nm, before addition of the Griess reagents, by a simple linear equation ($r^2 = 0.97$) (Fig. 2A).

Fig. 2B shows representative spectrophotometric fixed-wavelength absorbance values of the same serum sample deproteinized by methods A and E after performance of the

Griess reaction without (solid lines) and with absorbance correction (doted lines) using straight line fitting points 405, 450, 600 and 620 nm. The absorbance values at 540 nm before correction were 0.65 A.U. (method A) and 0.84 A.U. (method E), corresponding to a difference in NO_x concentration of about 22% (21.6 versus 27.7 μM). After subtracting the extrapolated non-specific absorbance contribution at 540 nm the difference was reduced to less than 3% (19.8 and 20.5 μM, respectively). However in about 25% of the samples analyzed, the application of this procedure was not able to reduce the difference (below 10%) between NO_x values in the same sample prepared by different methods. Therefore, absorbance measurements at different wavelengths allows for the evaluation of effectiveness of the deproteinization process. We propose that, if the absorbance value at lower wavelengths is >0.2 A.U., the possibility of the presence of aggregated particles needs to be evaluated; eventually, the deproteinization process has to be revised. Application of absorbance correction procedure is suitable only if the absorbance value at low wavelength is about 0.2–0.3 A.U. corresponding to a low residual protein concentration (<0.6 g/dl). Finally, absorbance values at 540 nm <0.08 have to be carefully evaluated considering possible non-specific contribution deriving from scattering phenomenon. Reliable measurement of serum nitrite concentrations by the batch Griess method is generally difficult. The present enzymatic batch Griess assay, which usually yields final absorbance values smaller than 0.1 units – this was the case in more than 90% of the 25 samples analyzed in the present study, can not be applied to measure nitrite in human serum at the basal state.

3.3. Comparison between different deproteinization methods using the Griess assay

Fig. 3 shows the results from comparison of NO_x basal values measured by the Griess assay in serum samples ($n = 31$) treated with different deproteinization methods (without any absorbance correction). Correlation and agreement parameters between NO_x concentrations measured by each method (y) and those measured by method A (x) serving as the reference method are shown in Table 2.

Griess reaction and calibration curves were performed in standard solutions of nitrate (range 0–80 μM) before and after treatment by methods A and B. No significant differences in the measured NO_x concentrations were found. These findings suggest that acetonitrile present in the reaction mixtures at about 10 vol.% did not interfere with the enzymatic reduction of nitrate to nitrite and the subsequent Griess reaction under the conditions of the present study.

Analysis of method comparison is described by ordinary and Deming linear regression analyses (regression equation and r^2) and by the Bland–Altman method {mean difference ± SD}. The significance of differences between methods determined by paired t-test analysis is also reported in Table 2.

Linear regression analysis of NO_x concentrations (range 8–68 μM) measured in serum samples deproteinized by method B versus reference deproteinization method A resulted in an excellent correlation ($r^2 = 0.99$, Table 2). Bland–Altman

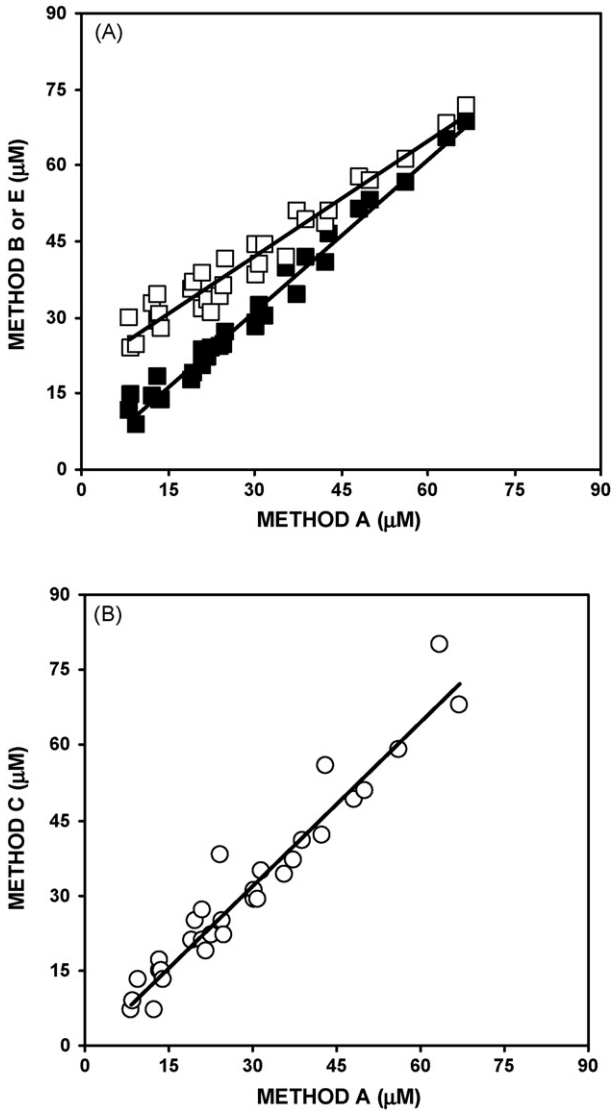

Fig. 3. Comparison of NO_x (nitrate + nitrite) basal values measured by the Griess assay in serum samples ($n = 31$) treated with different deproteinization methods without absorbance correction. (A) Correlation between method A (acetonitrile-chloroform) and method B (rapid acetonitrile-chloroform, ■) or method E (zinc sulfate-sodium hydroxide, □). (B) Correlation between method A and method C (ultrafiltration). Continuous lines were observed from linear regression analysis (see also Table 2).

analysis confirmed a very good agreement between methods B and A (md, 1.2 ± 2.3 μM). A satisfying correlation ($r = 0.97$) was observed between data obtained by methods D and E. However, methods D and E values were significantly higher than the method A values at concentrations <40 μM (md, 14.7 ± 6.8 μM); at higher concentrations (>40 μM) values tend to converge (md, 5.6 ± 4.3 μM). No correlation was found between methods A and F. Samples deproteinized by method F resulted in higher values for NO_x (md, 93.2 μM), most likely due to the high (3.5 g/dl) residual protein concentration as suggested by the high non-specific absorbance recorded within the range 405–620 nm for these samples (data not shown). However, because reduction of nitrate to nitrite and the Griess reaction were performed in the presence of methanol (and

Table 2
Correlation and agreement parameters of NO_x concentration measured by the Griess assay performed in 31 serum samples after deproteinization with different methods

Deproteinization method	Regression equation	r^2	*t*-test (*P*)	A md ± SD [μM]	B md ± SD [μM]
Acetonitrile reference method (A)	N.A.	N.A.	N.A.	N.A.	N.A.
Acetonitrile rapid (B)	$y = 0.99x + 1.38$	0.98	0.009	1.2 ± 2.3	0.8 ± 2.0
Ultrafiltration (C)	$y = 1.09x - 0.78$[a]	0.93[a]	0.035	1.9 ± 4.8	−0.3 ± 4.6
	$y = 0.99x + 0.63$[b]	0.98[b]			
Zinc sulfate (D)	$y = 0.81x + 27$	0.94	<0.001	21.8 ± 4.5	14.2 ± 4.6
Zinc sulfate/sodium hydroxide (E)	$y = 0.75x + 19.4$	0.95	<0.001	12.3 ± 4.9	8.0 ± 4.5
Methanol/diethyl ether (F)	$y = -0.13x + 126$	0.34	<0.001	93.2 ± 18.3	80.9 ± 18.6

Comparison was performed between each method (*y*) and method A (*x*) serving as the reference method. Regression equation, r^2, *t*-test and mean difference were calculated before (column A) and after (column B) application of the absorbance correction procedure. md: mean difference. N.A.: not applicable.
[a] Values from all samples were included in the regression (i.e., $n = 31$).
[b] Samples with residual protein concentration >1.0 were excluded (i.e., $n = 27$).

diethyl ether) in method F, the considerably higher NO_x concentrations measured using deproteinization method F could be due interferences with the Griess assay by the organic solvents used.

Finally, ultrafiltration method (method C) showed a good agreement and correlation with reference method A. However, in four serum samples significantly different concentrations were measured. In one sample, the NO_x concentrations measured with method A and method C were 12.4 and 7.0 μM, respectively. In three samples, NO_x concentrations measured by method C were higher than those measured by method A (differences >12 μM). Deproteinization by method C showed residual unspecific absorbance (at 540 nm) values >0.2 A.U in these samples, reflecting incomplete removal of proteins (total protein >1.0 g/dl).

Table 2 (column B) shows the mean difference of the concentration values obtained after application of absorbance correction procedure. Values measured in samples treated by methods B and C show a better agreement with reference method A. The high standard deviation of method C is presumably due to the four incompletely deproteinized samples (see above); non-consideration of these samples improves significantly the agreement of the methods (md, −1.4 ± 2.2 μM; data not shown).

On the basis of these results, and because method B is faster than method A, we used deproteinization method B in subsequent quantitative analyses by the Griess assay.

3.4. Correlation between different methods used to determine nitrite and nitrate concentrations

Circulating nitrate + nitrite (NO_x) basal concentrations were measured in parallel in 50 healthy subjects by the Griess assay in serum (deproteinization method B, no absorbance correction procedure), and by HPLC and GC–MS in plasma (deproteinization method C). Usually, vacutainer tubes are contaminated with nitrite and nitrate [9]. To measure potential contamination with nitrite and nitrate of the monovettes used in the present study to generate serum and heparinized plasma we filled the monovettes with distilled water and measured by the Griess assay the nitrite + nitrate concentration. In all monovettes tested by this way nitrite + nitrate concentration was below 5 μM.

From HPLC analysis, the individual values for nitrite and nitrate were obtained, and their sum was used for comparison. GC–MS analysis provided the sum of nitrite and nitrate which was used for comparison. In Table 3 we reported mean and range of NO_x concentrations obtained by different methods of determination and correlation analysis data. Inter-method correlation of NO_x concentrations measured by the above mentioned techniques are shown in Fig. 4. Also, Bland–Altman plots from these comparisons are shown in Fig. 5.

The best correlation (r^2=0.96) and agreement (md = 2.4 ± 3.5 μM) were found between HPLC and the Griess assay (method B). Even if a good correlation ($r^2 = 0.95$) was observed between GC–MS and the Griess assay (method B), NO_x concentrations measured by GC–MS were higher (md = −12.8 ± 4.1 μM) than those measured by the Griess assay confirming previous findings [9,23]. However, when comparing GC–MS with the Griess assay (method C) correlation and agreement were moderate ($r^2 = 0.91$, md = 10.7 ± 6.2 μM). Finally, NO_x concentrations obtained by HPLC were lower than those measured by GC–MS data ($r^2 = 0.93$, md = −10.4 ± 4.8 μM).

Table 3
Mean and range of NO_x concentrations measured by Griess (serum, deproteinization method B or C), HPLC (plasma) and GC–MS (plasma) in 50 samples

Detection technique	NO_x range [μM]	NO_x concentration [μM] (mean ± SD)	Correlation and agreement				
			Methods compared	Regression equation	r^2	md ± SD	*P*
Griess (B)	8.2–75	31.0 ± 17.0	GC–MS vs. Griess (B)	$y = 1.01x + 12.5$	0.95	12.8 ± 4.1	<0.001
Griess (C)	5.0–100.0	33.1 ± 20.2	GC–MS vs. Griess (C)	$y = 0.83x + 16.2$	0.91	10.7 ± 6.2	<0.001
HPLC	10.0–80.6	33.4 ± 17.5	HPLC vs. Griess (B)	$y = 1.01x + 2.15$	0.96	2.4 ± 3.5	<0.001
GC–MS	19.9–85.0	43.8 ± 17.6	HPLC vs. GC–MS	$y = 0.96x - 8.4$	0.93	−10.4 ± 4.8	<0.001

Results from correlation (Deming linear regression analysis) and agreement of analysis (Bland–Altman) of the methods compared are presented. md, mean difference.

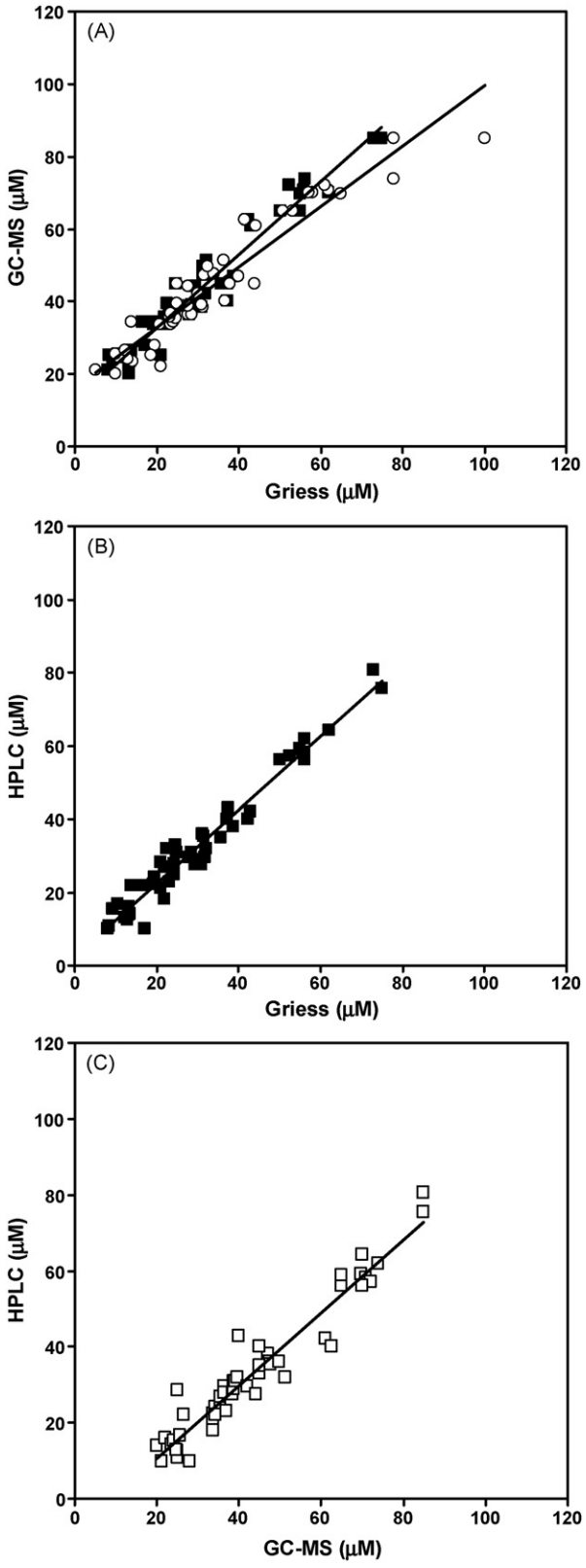

Fig. 4. Correlation between NO_x (nitrate + nitrite) concentrations measured in plasma and serum samples ($n = 50$) by different techniques. (A) GC–MS (plasma) vs. Griess (serum) using deproteinization method B (rapid acetonitrile-chloroform, ■) or deproteinization method C (ultrafiltration, ◯); (B) HPLC (plasma) vs. Griess using deproteinization method B; C) HPLC vs. GC–MS. Continuous lines were obtained from linear regression (see also Table 3).

4. Discussion

GC–MS is considered the most accurate technique for the quantitative determination of nitrite and nitrate in plasma, serum and urine samples [8,25,26]. Nevertheless, potentially less reliable methods based on the Griess reaction are widely used largely due to assay simplicity, rapidity and cheapness [16,24,27,28]. Many studies have shown that parallel comparison of nitrite and nitrate quantification by different methods, including the Griess assay, GC–MS, HPLC, produced diverging values and discrepancies, principally due to methodological problems [7,29]. One of the most important factors interfering with the Griess reaction is the protein content of plasma and serum samples. Turbidity resulting from aggregated/precipitated proteins, loss of nitrite and nitrate due to conversion of nitrite to *S*-nitroso compounds by reduced thiols, or due to other reactions such as nitrosation/nitration of tyrosine and tryptophan residues in proteins, may lead to inaccurate determination of nitrite and nitrate concentrations [7,12,16,18,30]. Thus, removal of proteins from plasma and serum samples may be a decisive factor for the reliable measurement of these NO metabolites by the classical Griess method. Therefore, in the present work we compared the efficiency of six different deproteinization methods and we evaluated the agreement and correlation of NO_x concentrations measured by an enzymatic batch Griess assay and two different techniques, i.e., by ion-pairing HPLC and ion-trap GC–MS.

Previously, many studies dealt with the optimization of the deproteinization procedure and the turbidity coming from proteins aggregation/precipitation, but measurement of efficiency of protein removal and final absorbance contribution of deproteinized samples had not always been checked [16–18]. The present study investigated this important issue in detail and proposes a simple method to verify removal of proteins and to correct the absorbance value at 540 nm for the extrapolated non-specific contribution. The correction procedure is useful for absorbance values <0.3 A.U. at wavelengths lower than 500 nm. A higher absorbance value indicates a residual protein contents >0.6 g/dl. In such cases, protein interference with the NO_x determination by the Griess assay may be considerable, and the absorbance correction should not be performed. Absorbance measurement at different wavelengths allows identify samples with incomplete deproteinization or the presence of non-specific spectrophotometric signal due to aggregating/precipitating particles. The absorbance correction procedure permits to obtain a better agreement between concentrations measured by the Griess assay in samples treated with different deproteinization methods.

Comparison of NO_x concentrations by linear regression analysis revealed a good correlation between GC–MS, HPLC and the Griess method (Table 3). However, GC–MS values are significantly higher (by 5–15 μM in the studied range) than the respective Griess and HPLC values. By contrast, a good agreement was observed between HPLC and Griess values, even if a statistically significant difference was found evaluating data with the paired *t*-test.

In addition to methods comparison by statistical means, the methods of analysis of nitrite and nitrate used in the present study

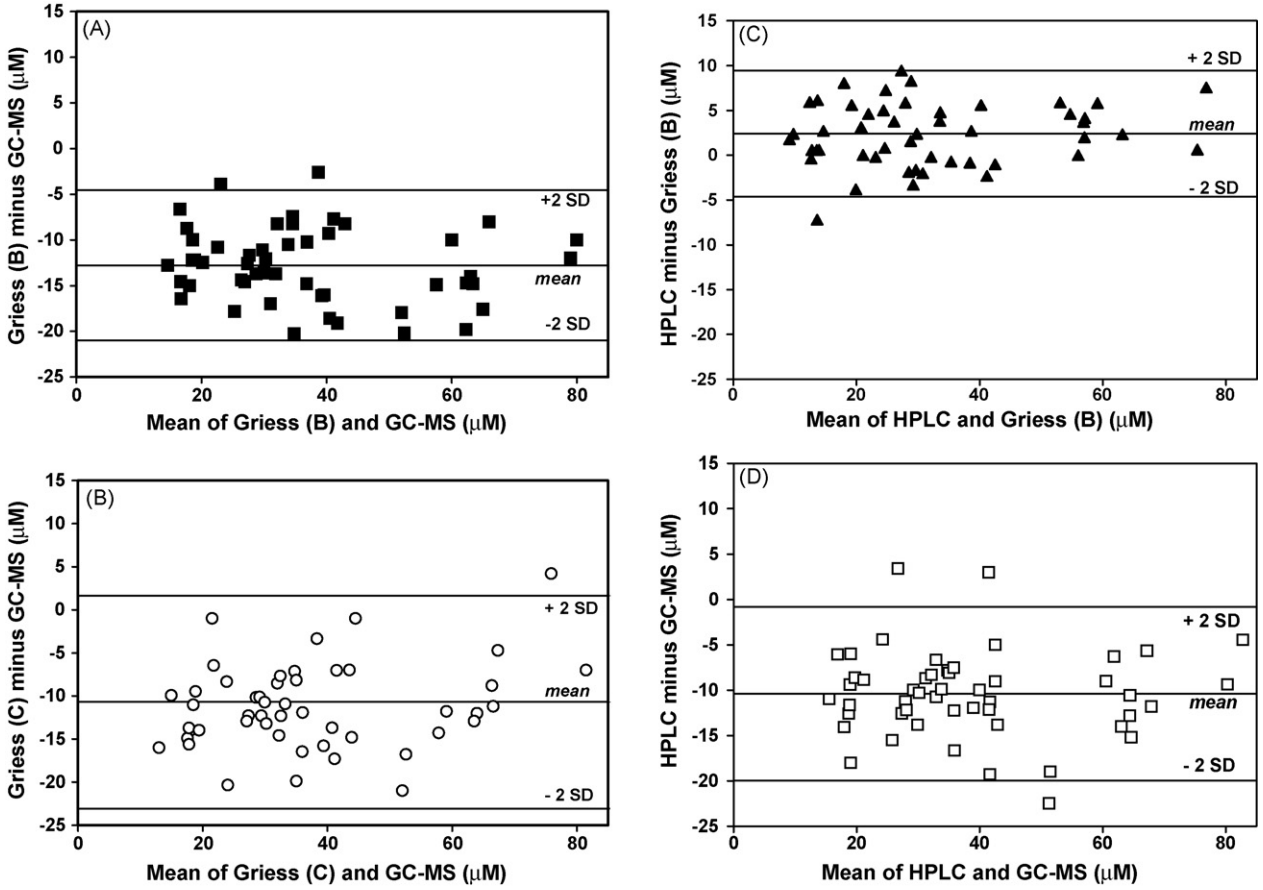

Fig. 5. Bland–Altman plots from comparison of circulating NO_x (nitrate + nitrite) concentrations measured in samples ($n = 50$) by Griess assay (serum), HPLC (plasma) and GC–MS (plasma). (A) Griess using deproteinization method B vs. GC–MS. (B) Griess using deproteinzation method C vs. GC–MS. (C) Griess using deproteinization method B vs. HPLC. (D) HPLC vs. GC–MS. See also Fig. 4.

should also be compared on the basis of other analytically relevant issues including throughput analysis and versatility. The Griess assay was performed in the present study on automatic microplate analyzers allowing fast (about 100 determinations per hour) and reproducible measurements, unlike the more time-consuming HPLC and GC methods. Recent results suggest that nitrate is largely less important than nitrite in human circulation in studying NO physiology [31]. HPLC and GC–MS can accurately and simultaneously measure nitrite and nitrate, while determination of basal nitrite concentrations by the present Griess assay is quite difficult due to lack of sensitivity. Thus, in the present study circulating nitrite concentrations could not be detected above 4 μM, the LOQ value of the Griess assay for circulating nitrite (data not shown). It is noteworthy that the absorbance value for 4 μM nitrite in plasma would be about 0.1 without any correction for non-specific scattering signal.

We would like to discuss some limitations of the present study both with regard to the comparison of the Griess assay with the HPLC and GC–MS methods and to the deproteinization methods used. The commercial Griess assay kit utilized in the present study is recommended by the supplier for use in serum samples, presumably because anticoagulants such as heparin may interfere with the enzymatic reduction of nitrate to nitrite [32]. On the other hand, accurate measurement of nitrite and nitrate by HPLC and GC–MS are usually performed in plasma samples [7,21,25,33]. In planning our study, we referred to previous work and chosen serum as the matrix in the Griess assay, but plasma as the matrix in the HPLC and GC–MS methods. In consideration of the known potential effects of the vacutainer tubes both on nitrite and nitrate concentration and other matrix-related factors [24], differences seen in the present study between the Griess assay and the HPLC or GC–MS method may be, at least in part, due to use of different matrices. To minimize matrix-related effects, serum and plasma samples were generated from blood of the same volunteers under very similar experimental conditions. Differences in the NO_x concentrations measured by HPLC and GC–MS could also originate, at least in part, from the use of different matrices subjected to analysis, i.e., plasma ultrafiltrate in HPLC and native plasma in the GC–MS method which actually does not require any deproteinization step [21,25]. Eventually, differences between the Griess assay and the HPLC or GC–MS method may also be due to the considerable higher LOD and LOQ values of the Griess assay as compared with the HPLC and GC–MS methods. This issue may become especially relevant for low NO_x concentrations.

The deproteinization procedures used in the present study have been described previously [16–18]. However, many of these procedures were performed in the present study after

modification of the originally published procedures. These methodological deviations should be considered when comparing and discussing the deproteinization methods used with respect to the deproteinization efficiency as well as to the Griess assay. Thus, in the original method by Guevara et al. [18] samples were first reduced, then deproteinized and subjected to the Griess reaction. By contrast, in the present work we adopted and modified this method, i.e., method F, in that way that serum samples were first deproteinized and then subjected to the Griess reaction including the reduction step. Nevertheless, the present study focused on the deproteinization procedure involving acetonitrile and chloroform, i.e., methods A and B. Beside simplicity and rapidity, these procedures were found to be the most effective methods in serum protein removal and found not to contribute by non-specific absorbance in the Griess assay. Although not thoroughly investigated, we have no evidence of interference by acetonitrile in the enzymatic batch Griess assay as performed in our study. Because of considerable sample dilution in methods A and B, the acetonitrile content in the Griess reaction mixture of the order of 10 vol.% seems not to interfere with nitrate reductase-catalyzed reduction of nitrate to nitrite or with the formation of the diazo dye.

5. Conclusion

Accurate analytical methods for the quantitative determination of relevant members of the L-arginine/NO family such as circulating nitrite and nitrate are required for the characterization of the status of this pathway in health and disease. In this context, the commercial availability of "ready-to-use" batch Griess assays is very tempting. However, it is well-recognized that pre-analytical and analytical factors may interfere with the analysis of nitrite and nitrate by batch Griess assays, presently the most frequently used nitrite/nitrate assays in experimental and clinical studies. Our results suggest that incomplete removal of proteins in serum samples may be the cause for considerable interferences in the enzymatic batch Griess assay. Serum treatment with acetonitrile is the most efficient method to precipitate proteins and to eliminate protein-associated interferences in the Griess assay. A method is reported to verify the extent of protein elimination and to correct for non-specific contribution to the absorbance of the diazo dye at 540 nm, if needed.

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