

# Protein Precipitation Methods Evaluated for Determination of Serum Nitric Oxide End Products by the Griess Assay

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## ABSTRACT

Deproteinization is a necessary step in the measurement of serum NO<sub>x</sub> (nitrite+nitrate) concentrations. This study aims at comparing different protein precipitation methods for measurement of serum nitric oxide end product concentrations by the Griess method. For this purpose, ten protein precipitation methods most of previously used for deproteinization in serum NO<sub>x</sub> determination, were applied for protein removal. Serum samples from healthy volunteer deproteinized with different methods, subsequently serum NO<sub>x</sub> concentrations were determined using the Griess assay. Pearson correlation coefficients and mean differences between values were determined for each method (using ultrafiltration as a reference method). P-values below 0.05 were considered significant. The nitrite standard curve had linearity up to 150 μmol/l ( $r_2=0.998$ ). Our results showed that acid solutions are not suitable for protein removal in serum NO<sub>x</sub> determination. Using methanol, ethanol, and diethylether/methanol resulted with higher serum NO<sub>x</sub> values. Acetonitrile and zinc sulfate showed good agreement with the ultrafiltration method for serum NO<sub>x</sub> determination, in terms of their mean differences from ultrafiltration method ( $-1.6 \pm 8.6$  and  $-2.3 \pm 7.4$  respectively). It can be concluded that zinc sulfate and acetonitrile are the methods of choice for protein removal in determining serum NO<sub>x</sub> concentrations using the Griess method.

**Key words:** Nitric oxide; Nitrite; Nitrate; Griess assay; Deproteinization

## Introduction

Nitric oxide (NO) is an important molecule synthesized from L-arginine [1]. Physiological processes including neuronal transmission, [2] reproduction, [3] apoptosis, [4] and pathological processes such as inflammation, [5] septic shock, and asthma [6] are among the many proc-

esses in which NO plays some roles. NO is a very unstable gas with a short half life, making its direct measurement difficult. Serum nitrite and nitrate (NO<sub>x</sub>) levels are measured as an indirect marker of in vivo NO formation [7]. Measurement of these NO end products provides a reliable estimate of NO output in vivo [7]. Numerous studies have measured NO<sub>x</sub> in serum [8-11] Interest in measurement of serum NO<sub>x</sub> is increasing because NO<sub>x</sub> concentrations are influenced by diseases including heart failure, sepsis, and liver cirrhosis [10].

Although there are various methods for determination of NO<sub>x</sub>, nevertheless simplicity, rapidity, and cheapness of the Griess assay have made this method more popular than others [7]. Among the other pre-analytical factors deproteinization is a necessary step in measurement of serum NO<sub>x</sub> concentrations mostly because of the turbidity resulting from protein precipitation in an acidic environment [12-14]. Based on our knowledge there is only one publication about protein precipitation methods in NO<sub>x</sub> measurement using the Griess assay and this study has not included all precipitation methods previously used for serum NO<sub>x</sub> determination [15]. The aim of the present study is to compare different protein precipitation methods for measurement of serum NO<sub>x</sub> concentrations using the Griess reaction.

## Materials and methods

### Materials

Sulfanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), hydrochloric acid, methanol, ethanol, acetonitrile, diethylether, zinc sulfate, trichloroacetic acid (TCA), Perchloric acid (PCA), sodium tungstate, and ammonium sulfate were obtained from Merck Company (Germany). Vanadium (III) chloride (VCl<sub>3</sub>) was purchased from Fluka (Swiss) and 10 KD filters from Sartorius Company (Germany).

### Sample preparation

Serum samples (n=42) were obtained from healthy volunteers and centrifuged at 1000 × g for 10 min; subsequently deproteinization was done. The proposal of this study was approved by the research council of the Research Institute for

Endocrine Sciences of Shaheed Beheshti University of Medical Sciences.

### Deproteinization methods

- Ultrafiltration:** Serums were passed through 10 KD filters [7].
- Methanol, acetonitrile, ethanol, methanol/diethylether:** Methanol (1:1, v/v), acetonitrile (1:1, v/v) (15, 16), ethanol (sample: ethanol, 1:2, v/v) (17), and methanol/diethylether (3/1, v/v) (sample: methanol/diethylether, 1:9, v/v) (12) were added to serums vortexed for 1 min and centrifuged at  $10000 \times g$  for 10 min at 4 °C and supernatant was removed for NOx determination.
- Zinc sulfate:** Six mg of zinc sulfate powder was added to 400  $\mu$ l of serum (15g/l) vortexed and centrifuged as mentioned above (14).
- TCA:** TCA (5%) was added to the serum (sample: TCA, 1:9, v/v), vortexed and centrifuged as mentioned above [18].
- PCA:** 0.6 molar PCA was added to the serum (sample: PCA, 1:9, v/v), vortexed and centrifuged as mentioned above [19].
- Sodium tungstate:** Sodium tungstate (1%, prepared in 0.67 M sulfuric acid) was added to the serum (sample: sodium tungstate, 1:10, v/v), vortexed and centrifuged as mentioned above [18].
- Amonium sulfate:** Six hundred mg of Amonium sulfate powder was added to 1 ml of serum (600g/l), vortexed and centrifuged as mentioned above [20].

### Total nitrite and nitrate (NOx) determination

Before comparing deproteinization methods we validated measurement of serum NOx levels reported by Miranda et al. [7] in our laboratory. To do this, we first ran a standard curve for 0-150  $\mu$ mol/l nitrite ten times, and then calculated sensitivity, precision (inter and intra-assay coefficient of variations), and accuracy (parallelism and recovery) of serum NOx determination. Deproteinized serum samples were then used for NOx determination. In brief, 100  $\mu$ L of supernatant was applied to a micotiter plate well, 100  $\mu$ L vanadium (III) chloride (8mg/ml) was added to each well (for reduction of nitrate to nitrite) and this was followed by addition of the Griess reagents, 50  $\mu$ L sulfanilamide (2%) and 50  $\mu$ L N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1%). After 30 min incubation at 37 °C, absorbance was read at 540 nm using the ELISA reader (Sunrise, Tecan, Austria). Concentration of NOx in serum samples was determined from linear standard curve established by 0-150  $\mu$ mol/l sodium nitrite.

### Determination of serum NOx in healthy subjects

Serum NOx levels were determined in 60 healthy subjects (age range: 32-67 years) after overnight fasting. For this, we used zinc sulfate for protein precipitation.

### Statistical analyses

Data were presented as mean  $\pm$  SD or SEM. Pearson correlation coefficients and mean differences were determined be-

tween different deproteinization methods with the ultrafiltration method as references. SPSS program (SPSS Inc., Chicago, IL, USA; Version 15) was used for data analysis and 2-sided p-values below 0.05 were considered significant.

## Results

Typical standard curves for serially diluted nitrite concentrations are presented in Fig 1. Assay linearity was maintained until 150  $\mu$ mol/l; this assay had sensitivity of 2  $\mu$ mol/l. Intra-assay coefficient of variations for 25, 75, and 125  $\mu$ mol/l NOx were 5.5, 3.3, and 3.8 % respectively. Inter-assay coefficient of variations for 25, 75, and 125  $\mu$ mol/l NOx were 5.8, 4.5, and 4.6 % respectively. Recovery of serum NOx assay in the present study was  $93 \pm 1.5$  % and the mean of the parallelism test was  $102 \pm 3.3$  %.

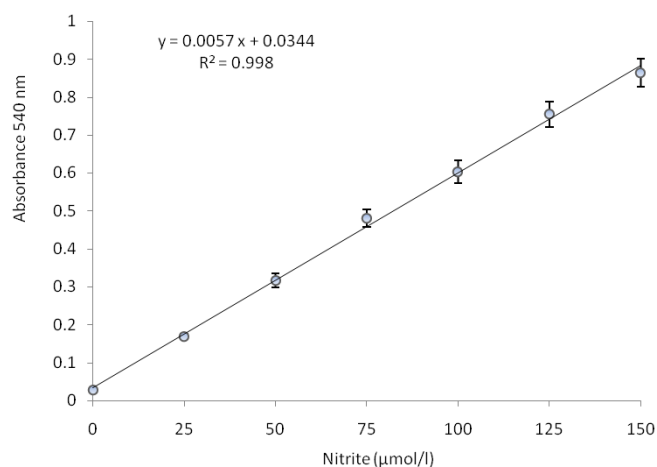


Figure 1. Nitrite standard curve.

Concentrations used for standards range between 0 to 150  $\mu$ mol/l. Each point represents mean  $\pm$  SEM of ten times repeat.

Table 1 shows mean serum NOx concentrations, regression equation, Pearson correlation coefficients, and mean differences (with ultrafiltration method as references) for each deproteinization method. Range of correlation is from 0.53 (for sodium tungstate) to 0.93 for PCA. Minimum and maximum mean differences belonged the acetonitrile and ammonium sulfate ( $-1.6 \pm 8.6$  and  $-24.8 \pm 13.9$  respectively).

The mean  $\pm$  SD of serum NOx concentrations determined in healthy subjects was  $33 \pm 10$   $\mu$ mol/l (range: 14-57).

## Discussion

We evaluated ten protein precipitation methods for determining serum NOx levels by the Griess reaction. This is the first study comparing deproteinization methods for serum NOx concentrations using vanadium (III) chloride for reduction of nitrate to nitrite.

Table 1. Correlation and agreement parameters of NO<sub>x</sub> concentrations measured by the Griess assay performed in 42 serum samples after protein precipitation with different methods

Deproteinization method	Mean ± SD of serum NO <sub>x</sub> [μmol/l]	Regression equation	Pearson correlation coefficient	P value	md ± SD [μmol/l]
Ultrafiltration	36 ± 18	N.A.	N.A.	N.A.	N.A.
Methanol	49 ± 25	y= 1.18 x+ 6.78	0.84	<0.001	13 ± 14
Acetonitrile	34 ± 16	y=0.79x + 5.6	0.88	<0.001	-1.6 ± 8.6
Ethanol	52 ± 26	y= 1.34 x+ 4.31	0.92	<0.001	16 ± 12
Zinc sulfate	33 ± 14	y= 0.73 x+ 4.1	0.92	<0.001	-2.3 ± 7.4
Methanol/diethylether	40 ± 22	y= 0.96 x+ 5.89	0.79	<0.001	4.4 ± 13.3
PCA	23 ± 10	y= 0.54 x+ 3.77	0.93	<0.001	-12.6 ± 9.1
TCA	31 ± 18	y= 0.89 x+ 0.92	0.85	<0.001	-4.9 ± 9.8
Sodium tungstate	24 ± 18	y= 0.52 x+ 5.88	0.53	<0.01	-10.7 ± 17.5
Ammonium sulfate	10 ± 6	y= 0.52 x+ 1.43	0.78	<0.001	-24.8 ± 13.9

md: mean differences, N.A.: not applicable, PCA: perchloric acid, TCA: trichloroacetic acid

In the validation section of our study, we found that the sensitivity of the assay was 2 μmol/l; a finding that is in agreement with others reporting sensitivity values of 1.5-2 μmol/l [14, 21]. Recovery found in this study (93%) was comparable to that found by Moshage *et al.* [14] (84-90%) and Guevara *et al.* [12] (88%). Among the precipitation methods used in the present study methanol (16), Acetonitrile [15], Ethanol [17], Zinc sulfate [14], Methanol/diethylether (12), PCA [19], and ultrafiltration [7] were used previously, at least once, for protein precipitation in serum NO<sub>x</sub> determination. TCA, Sodium tungstate, and Ammonium sulfate although used for protein precipitation [18, 20], were not used for NO<sub>x</sub> determination. Some other studies have suggested that deproteinization with acid solutions should be avoided for serum NO<sub>x</sub> determination due to rapid conversion of nitrite to nitric oxide and nitrogen dioxide gases in acidic environment and loss of nitrite [7, 13]. Our results in case of protein precipitation with PCA and TCA although supported this suggestion, the correlation coefficient between PCA and ultrafiltration methods was high ( $r=0.93$ ), protein removal with PCA has been caused lower values of serum NO<sub>x</sub>. Deproteinization with PCA for measurement of serum NO<sub>x</sub> has been used in one study [19].

Ethanol, methanol, and methanol/diethylether resulted in higher serum NO<sub>x</sub> values. Recently Romitelli *et al.* [15] have reported that samples deproteinized by methanol/diethylether resulted in higher values for NO<sub>x</sub>. They believe that this issue is related to the high residual protein concentration in these samples [15], and have also suggested that higher NO<sub>x</sub> concentrations using methanol/diethylether could be due to interferences with the Griess reagents by the organic solvents. Although we have not determined interferences of organic solvents with the Griess assay in this study, it could be concluded that this possible interference may be the cause of observed higher serum NO<sub>x</sub> values using methanol, ethanol, and methanol/diethylether for protein removal.

Sodium tungstate and ammonium sulfate had the weakest correlations with ultrafiltration method and both of these methods resulted in lower serum NO<sub>x</sub> concentrations. Therefore these methods are not recommended for protein removal in serum NO<sub>x</sub> measurement.

Protein removal with acetonitrile and zinc sulfate had

relatively good agreement with the ultrafiltration protein precipitation method as shown by their least mean differences. Romitelli *et al.* [15] have reported that serum treatment with acetonitrile is the most efficient method to precipitate proteins in the Griess assay, a finding supported with our results. Despite this, Sakuma *et al.* [18] have challenged the evaporation of acetonitrile leading to loss of volume, which may be conducted to higher recovery in determination of uric acid in serum. We centrifuged samples at 4 °C which can prevent potential evaporation. In case of protein precipitation with zinc sulfate, our results are relatively comparable with those of acetonitrile. Romitelli *et al.* [15] have reported that although zinc sulfate removes 85% of serum proteins, it nevertheless yields higher serum NO<sub>x</sub> concentrations. On the other hand, Sun *et al.* [13] have reported that although zinc sulfate removes about 50% of proteins from samples it is however effective in preventing the formation of precipitate/turbidity in subsequent Griess reactions even in samples with high protein content. Preventing sample dilution has been mentioned as an advantage in protein removal for the ultrafiltration method [13]; in this study, we used zinc sulfate powder, which allows protein removal without dilution of samples. Cortas *et al.* [22] have reported that using zinc sulfate for protein precipitation decreases interference with ascorbate and phosphate in the Griess method. Based on all these findings, it can be suggested that deproteinization using zinc sulfate is the method of choice for serum NO<sub>x</sub> determination with the Griess method.

We measured levels of NO<sub>x</sub> in 60 normal human serums using zinc sulfate for protein precipitation. Mean ± SD of these values (33±10 μmol/l, Range: 14-57) were similar to those reported by others. Green *et al.* [23] have reported a range of 15-60 μmol/l for plasma NO<sub>x</sub> and Guevara *et al.* [12] have reported a mean value of 34.9 ± 9.7 μmol/l (range 25-51.9). A limitation of this study is that we did not determine residual protein concentration for different deproteinization methods.

In conclusion, the results of this study revealed that zinc sulfate and acetonitrile are the methods of choice for protein removal in determining serum NO<sub>x</sub> concentrations using the Griess reaction.

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## REFERENCES

- Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;87:315-424.
- Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 1988;336:385-8.
- Inoue T, Kaibara M, Sakurai-Yamashita Y, et al. Increases in serum nitrite and nitrate of a few-fold adversely affect the outcome of pregnancy in rats. *J Pharmacol Sci* 2004;95:228-33.
- Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* 1994;63:175-95.
- Kone BC, Baylis C. Biosynthesis and homeostatic roles of nitric oxide in the normal kidney. *Am J Physiol* 1997;272:F561-78.
- Singh S, Evans TW. Nitric oxide, the biological mediator of the decade: fact or fiction? *Eur Respir J* 1997;10:699-707.
- Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* 2001;5:62-71.
- Choi JW, Pai SH, Kim SK, et al. Increase in nitric oxide concentrations correlate strongly with body fat in obese humans. *Technical Briefs* 2001;47:1106-9.
- Di Massimo C, Scarpelli P, Di Lorenzo N, et al. Impaired plasma nitric oxide availability and extracellular superoxide dismutase activity in healthy humans with advancing age. *Life Sci* 2006;78:1163-7.
- Watanabe T, Akishita M, Toba K, et al. Influence of sex and age on serum nitrite/nitrate concentration in healthy subjects. *Clin Chim Acta* 2000;301:169-79.
- Kondo T, Ueyama J, Imai R, et al. Association of abdominal circumference with serum nitric oxide concentration in healthy population. *Environmental Health and Preventive Medicine* 2006;11:321-5.
- Guevara I, Iwanejko J, Dembinska-Kiec A, et al. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clin Chim Acta* 1998;274:177-88.
- Sun J, Zhang X, Broderick M, et al. Measurement of nitric oxide production in biological systems by using griess reaction assay. *Sensors* 2003;3:276-84.
- Moshage H, Kok B, Huizenga JR, et al. Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* 1995;41:892-6.
- Romitelli F, Santini SA, Chierici E, et al. 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. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;851:257-67.
- Himeno M, Ishibashi T, Nakano S, et al. A practical procedure for achieving a steady state of NOx concentration in plasma: with special reference to the NOx content of Japanese daily food. *Tohoku J Exp Med* 2003;199:95-110.
- Vaziri ND, Ni Z, Oveisi F. Upregulation of renal and vascular nitric oxide synthase in young spontaneously hypertensive rats. *Hypertension* 1998;31:1248-54.
- Sakuma R, Nishina T, Kitamura M. Deproteinizing methods evaluated for determination of uric acid in serum by reversed-phase liquid chromatography with ultraviolet detection. *Clin Chem* 1987;33:1427-30.
- Tanaka S, Yashiro A, Nakashima Y, et al. Plasma nitrite/nitrate level is inversely correlated with plasma low-density lipoprotein cholesterol level. *Clin Cardiol* 1997;20:361-5.
- Jiang L, He L, Fountoulakis M. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J Chromatogr A* 2004;1023:317-20.
- Ricart-Jane D, Llobera M, Lopez-Tejero MD. Anticoagulants and other preanalytical factors interfere in plasma nitrate/nitrite quantification by the Griess method. *Nitric Oxide* 2002;6:178-85.
- Cortas NK, Wakid NW. Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. *Clin Chem* 1990;36:1440-3.
- Green LC, Ruiz de Luzuriaga K, Wagner DA, et al. Nitrate biosynthesis in man. *Proc Natl Acad Sci U S A* 1981;78:7764-8.

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