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Spectrophotometric Method to Quantify Nitrite and Nitrate in Rat Nervous Tissue

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ABSTRACT

Nitric oxide (NO) is a gas present in most biological tissues, including nervous tissue. In nervous system, the many different physiological functions of NO include those of a neurotransmitter, neuromodulator and inductor of mitochondrial biogenesis. In addition, NO can be transformed into highly reactive and harmful molecules producing an impairment of DNA, lipids or proteins, and thus altering their function. This dual action of NO, through which it plays an important role both in homeostasis and the development of pathological processes, makes this molecule an interesting target for medical therapies. Because of this, NO tissue concentration measurement is important for evaluating various physiological and pathophysiological processes. NO is easily inactivated by oxidation, resulting in the formation of nitrite (NO_2^-) and nitrate (NO_3^-). The classic Griess method is widely used, simple and inexpensive, but is not sensitive enough to detect the low nitrite concentrations found in nervous tissue. This paper presents some modifications of classic Griess method. The introduced changes were chosen from literature in order to achieve the determination of low nitrite and nitrate concentrations in nervous tissue. Our studies showed that the modified Griess method is specific, sensitive, and has good linearity. Thus our results indicate that the changes introduced in the Griess method are useful, reliable and adaptable for low nitrate and nitrite concentration measures in nervous tissue.
KEYWORDS: Nitric Oxide, nitrite, nervous tissue.

INTRODUCTION

Nitric oxide (NO) is a lipophilic gas that easily crosses cell membranes. Due to the fact that NO has an unpaired electron, it acts as free radical with both a very high reactivity and very short half-life. Formation of NO from L-arginine is catalyzed by a diverse family of nitric oxide synthases (NOS) [1]. There are three NOS isoforms known as endothelial (eNOS), neuronal (nNOS) and of inducible type or macrophage (iNOS), depending on the tissue from which they were initially characterized. Regulation of these enzymes is dependent on the cellular environment, function, interaction with other molecules, and the required amount of NO [2]. The eNOS and nNOS are constitutively expressed in tissues producing nanomolar amounts of NO for a short time and its activity is calcium/calmodulin complex dependent [3]. The iNOS is not normally expressed, since it needs an immune/inflammatory stimulus. Once expressed, calcium independent iNOS produces NO in larger amounts (micromolar order), for extended periods [4, 5].

NO is easily inactivated by oxidation, resulting in the formation of nitrite (NO_2^-) and nitrate (NO_3^-); alternatively, it reacts with superoxide radical ($O_2^{\bullet}$) to form peroxynitrite ($ONOO^-$). This last reaction is extremely fast and depends only on existing concentrations of NO and $O_2^{\bullet}$.

The discovery of nitric oxide (NO) opened an important field of research because it is known to be involved in numerous processes of biological significance. Therefore, factors that alter its synthesis produce pathological changes as varied as hypertension, vascular complications, neurodegeneration [6] and development of pain, both acute and chronic, including neuropathic pain. Another important process in which NO is involved is cell death by apoptosis [7]. Finally, its action in the nervous system is noteworthy, where NO acts as a cellular messenger [8], neurotransmitter, neuromodulator and inductor of mitochondrial biogenesis [9].

Studying the levels of NO_2^- and NO_3^- in rat nerve tissue, without sophisticated equipment is very difficult due to the low concentrations at which these anions are found. The Griess method for the indirect determination of NO involves the spectrophotometric measurement of nitrite. Analysis of nitrate by this reaction requires chemical or enzymatic reduction of NO_3^- to NO_2^- and then NO_2^- specifically determined by the Griess reaction [10, 11]. Briefly, the Griess reaction is a two-step diazotization reaction in which the NO-derived nitrosating agent, dinitrogen trioxide (N_2O_3) generated from the acid catalyzed formation of nitrous acid from nitrite (or autoxidation of NO), reacts with

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sulfanilamide to produce a diazonium ion which is then coupled to N-(1-napthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 540 nm [12].

Griess method is simple, inexpensive and widely used but has a low sensitivity (1–5 μM) [13, 14]. Because of the increased demand for the analysis of nitrite and nitrate in biological samples, many researchers have made Griess method changes in recent years to improve its sensitivity and some vendors have made a Griess reaction assay kit available. Considering others authors [15, 16], our team introduced 3 of those modifications to the classic spectrometric Griess method in order to achieve higher sensitivity and be able to measure very low NO_3^- and NO_2^- nervous tissue concentrations. The introduced changes were deproteinization of samples, the reduction of nitrate to nitrite with cadmium sulfate and working at low temperatures.

There are numerous papers describing measurements NO_3^- and NO_2^- in CNS using Griess reagent but there is no paper disclosing the methodology in details. In this paper it will find a protocol complete and several studies in order to show that the modified Griess method has a high sensitivity, is specific and has good linearity.

MATERIALS AND METHODS

Animals, surgical procedure and preparation of samples.

The experiments were performed according to the European Union directives (86/609/EEC) and the rules and recommendations for handling laboratory animals FESSCAL (Federation of South American Societies for Laboratory Animal Science).

Eight male Wistar rats, obtained from the Animal Central School of Medicine, National University of Tucuman, Argentina, weighing between 270 and 370g were used. They were kept under standard vivarium conditions.

Animals were sacrificed by ketamine- xylacine overdose and both sciatic nerves were extracted. The nerves were each separately homogenized by a cold mortar with 20% trichloroacetic acid (TCA 20%).

All animals used for this work were from control groups from other experiments carried out by our group.

Equipment

To quantify nitrite and nitrate we used a 330 Metrolab UV-Vis spectrophotometer (range 330-1000nm).

Changes in the Griess method

The Griess method was used for the determination of NO metabolites. This classic method entails formation of a chromophore from the diazotization of sulfanilamide by acidic nitrite followed by coupling with bicyclic amines such as N-1-(naphthyl) ethylene diamine. The resulting compound has a characteristic absorbance peak at 540nm. In order to improve the sensibility of Griess method we introduced deproteinization as previous step, reduction of nitrates into nitrites and working at low temperature.

Deproteinization

This step is very important because S-nitrosothioles and L- arginine derivatives were found to be potential interfering agent in the Griess method [17]. There are several methods of protein precipitation. In fact, there are combinations of some of these methods, such as the use of organic solvents with inorganic salts to help salt out the proteins [18].

The nerves were prepared with TCA 20% to obtain a homogenized sample and reduce their protein content. Although TCA is a good precipitant agent is not enough as sodium hydroxide and zinc sulfate [19] which can effectively remove all the proteins including high lipid content of nerves [20]. For these reason we carried out a second deproteinization step with $ZnSO_4$/NaOH after TCA samples treatment.

For deproteinization in alkaline medium, 100 μl of 1 M NaOH and 100 μl 30% $ZnSO_4$ were added to 1 mL of the homogenized nerve [15]. A white precipitate which was subsequently removed by centrifugation was obtained.

Reduction of Nitrate

Because Griess reagents react only with nitrites, 0.10 to 0.15 g of cadmium sulphate was used to reduce nitrates into nitrites [16]. In order to prevent nitrite oxidation, samples were protected from light and heat.

Working at low temperature

All materials in this study were used just after having been removed from the refrigerator and in a refrigerated environment (18ºC). The reaction temperature was lowered in order to increase the stability of the diazotization product [21].

Nitrite quantify

The samples were added to 100 μL of a mixture of Griess reagents A and B (1:1) after reduction and incubated for 30 minutes in a refrigerator (4º-8°C).

In short, the protocol used was:

- The nerves were each separately homogenized by a cold mortar with 20% trichloroacetic acid (TCA 20%).
- 100µl of 1M NaOH and 100µl 30% $ZnSO_4$were added to 1 mL of the homogenized nerve. The white precipitate obtained was subsequently removed by centrifugation.
- 0.10 to 0.15 g of cadmium sulphate was added and incubated for 20 minutes in a refrigerator (4º-8°C). In this time, samples were protected from light.
- 100 µL of a mixture of Griess reagents A and B (1:1) was added after reduction and incubated for 30 minutes in a refrigerator (4º-8°C).
- Absorbance reading at 540 nm.

Modification Contributions

To know whether the modifications made in the method significantly improved detection of nitrite, sample determinations were performed with cadmium/without deproteinization (Cd/NoD), with cadmium/with deproteinization (Cd/D), without cadmium/without deproteinization (NoCd/NoD) and without cadmium/with deproteinization (NoCd/D). All determinations were carried out under cold conditions in triplicate (n=12).

Determining linearity

For the linearity study, two calibrations curves were prepared: nitrite and nitrate. Both curves covered the range 0-12 µM and were prepared respectively with $NaNO_2$ (Merck) (n=8) and $NaNO_3$ (Merck) (n=7) with bidistilled water. The linearity of the curve of the method was performed using concentrations between 2-12 µM. These values were chosen in order to set the mean rat sciatic nerve concentration of NO_2^- and NO_3^-at the middle of the curve.

Specificity: Preparation of forced test degradations

For specificity demonstration, acidic, basic, oxidative and thermal stresses were applied following the standards recommended by AOAC (The Association of Official Analytical Chemists and Peer-Verified Methods Chemists).

For the oxidative degradation study, 10 mL 5 µM $NaNO_2$ solution was mixed with 1 mL 30% H_2O_2, refluxed for 30 minutes, cooled and then diluted up to 50 with bidistilled water. For thermal degradation, 10 mL of 5 µM $NaNO_2$ solution and 0.69 g $NaNO_2$ (solid) were dried in an oven at 100°C for 90 minutes. For acidic degradative study, equal volumes of solution of 5 µM $NaNO_2$ and 1 N HCl were mixed at room temperature, refluxed, cooled and neutralized with 1 M NaOH after one hour and then diluted to 50 mL with bidistilled water. For basic degradation, equal volumes of 5 µM $NaNO_2$ and 1 N NaOH were mixed at room temperature, refluxed, cooled and neutralized with 1 M HCl for one hour and then diluted to 50 mL with bidistilled water. To study the forced degradation by photolysis, 10 mL 5 µM $NaNO_2$ solution was placed into direct sunlight for four days.

The stressed samples were analyzed using the modified Griess method.

Precision

To evaluate the precision of this method, six samples of the same sciatic nerve were studied by triplicated determinations. In another test, three different samples were measured by two different people using the same reagents and equipment.

Accuracy

The most common technique for determining accuracy is the spike recovery method, in which the amount of a target compound is determined as a percentage of the theoretical amount present in the matrix. In a spike recovery experiment, a measured amount of the constituent of interest is added to a matrix (spiked) and then the analysis is performed on the spiked material, from the sample preparation through spectrophotometric determination. A comparison of the amount found versus the amount added provides the recovery of the method, which is an estimate of the accuracy of the method. Recovery is frequently concentration dependent; the FDA guidance for drugs [22] suggests that matrices be spiked at 80, 100, and 120% of the expected value, and that the experiment be performed in triplicate. To do this, 1 mL 5 µM $NaNO_2$was added to a sample, which should correspond to the theoretical amount of analyte present in the sciatic nerve.

Statistical Analysis

All statistical procedures were performed with SPSS (15.0). The significance of differences was calculated with repeated analysis of variance (ANOVA) or Student's T test. Results were considered significant when $p<0.05$.

RESULTS

Modification Contribution

Determining changes in the sensitivity due to the changes introduced

The modified Griess method was performed on four groups: with cadmium/without deproteinization (Cd/NoD), with cadmium/with deproteinization (Cd/D), without cadmium/without deproteinization (NoCd/NoD) and without cadmium/with deproteinization (NoCd/D). Three homogenates nerve samples were used in each group (n=12). Each sample was prepared with 1 mL of rat sciatic nerve in 100% nitrite standard solution (corresponding approximately to the amount of nitrite and nitrate contained in the sample). In Table 1, the absorbance values obtained for each group are shown. As it can be seen there, groups NoCd/NoD and NoCd/D were recovery rates less than 60%. The group Cd/NoD were recovery rates less than 65%. Only in the Cd/D group were recovery rates near 100% values (98.83%).

Table 1 Changes in the sensitivity due to changes introduced

		DEPROTEINIZATION			
		YES		NO	
		Absorbance	% Recovery	Absorbance	% Recovery
REDUCTION	YES	0.039 ± 1.53E-03 (3)	98.83	0.053 ± 4.36E-03 (3)	61.24
	NO	0.032 ± 0.00 (3)	58.75	0.026 ± 5.77E-04 (3)	53.72

The modified Griess method was performed on four groups: with cadmium/without deproteinization (Cd/NoD), with cadmium/with deproteinization (Cd/D), without cadmium/without deproteinization (NoCd/NoD) and without cadmium/with deproteinization (NoCd/D). Three homogenates nerve samples were used in each group (n=12). Each sample was prepared with 1 mL of rat sciatic nerve in 100% nitrite standard solution (corresponding approximately to the amount of nitrite and nitrate contained in the sample). The absorbance values obtained (at 540nm) for each group are shown. Only in the Cd/D group were recovery rates near 100% values.

Goodness of changes

Linearity Studies

The **Figure 1** shows the nitrite curve and it regression study. As it can see, almost all variations observed in absorbance are consequence of the variations in nitrite concentration. As matter of facts, the equation obtained indicates that 96.4% of the variation is due to the influence of the nitrite concentration in the tissue studied.

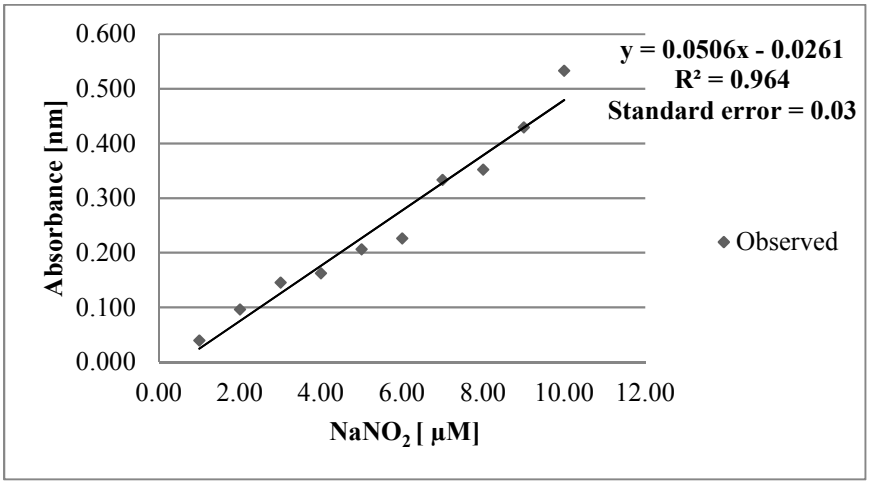

Figure 1 Nitrite Curve

Linearity study was carried out with solutions from a 10 mM $NaNO_2$ stock solution. The curve covered the range 2-12 µM. The equation indicates that 96.4% of the variation of the absorbance is due to the influence of the nitrite concentration in the tissue studied.

The **Figure 2** shows a nitrate curve. In the same way to the nitrite curve, the equation obtained from the regression study, indicates that 91.7 % of the variation is due to the influence of the nitrate concentration in the tissue studied.

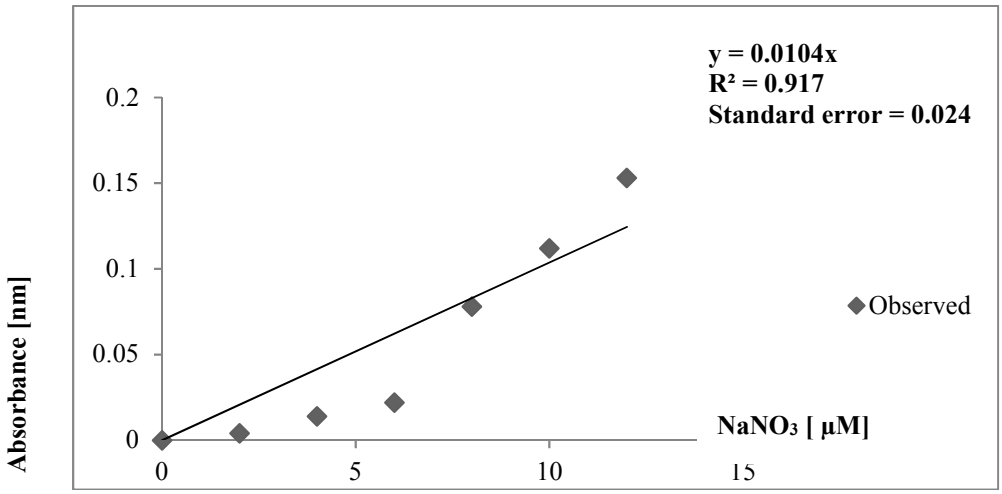

Figure 2 Nitrate Curve

Linearity Study was carried out with solutions from a 10 mM $NaNO_3$ stock solution. The curve covered the range 2-12 µM. The equation indicates that 91.7% of the variation of the absorbance is due to the influence of the nitrate concentration in the tissue studied.

Determining specificity: Forced tests degradations

All forced test degradations measurements were made at different wavelengths to study the possible degradation products.

Acidic Condition: the samples subjected to acid stress showed a drop in absorbance at 540 nm. The high absorbance levels that could be due to degradation products decrease from 480 to 530 nm (**Figure 3**, squared line). In other words, the absorbance due to degradations could not interfere with the assay because they are not at the wavelength used in Griess method (540 nm).

Alkaline Conditions: the curve obtained shows high absorbance levels which could be due to degradation products in every wavelength studied (**Figure 3**, rounded line). However, this should not interfere because the Griess method is performed in acid medium (5 N acetic acid).

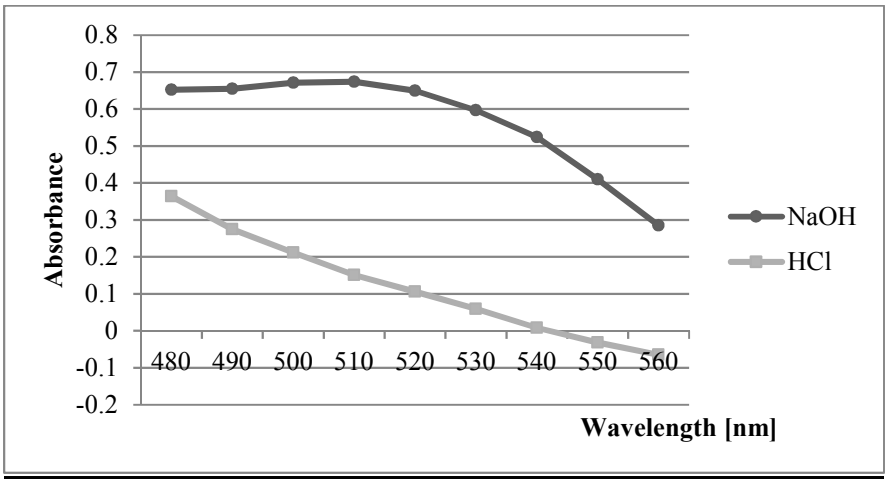

Figure 3 Specificity Curve

Alkaline hydrolysis: 5 µM $NaNO_2$ and 1 M NaOH were mixed, refluxed, cooled and neutralized with 1M HCl.
Acid hydrolysis: 5 µM $NaNO_2$ and 1 M HCl were mixed, refluxed, cooled and neutralized with 1M NaOH.

Influence of heat: As it can be observed in the **figure 4**, the curve of 5 µM $NaNO_2$ seems to be similar to alkaline stress with high absorbance levels at other than 540nm, which could be due to degradation products (squared line). Furthermore this thermal degradation should not occur because our modified Griess is carried out at low temperatures (4-8°C). The curve showing the influence of thermal degradation on the solid drug is similar to the solution but with higher absorbance, and it shows a fall in absorbance at 540nm (rounded line).

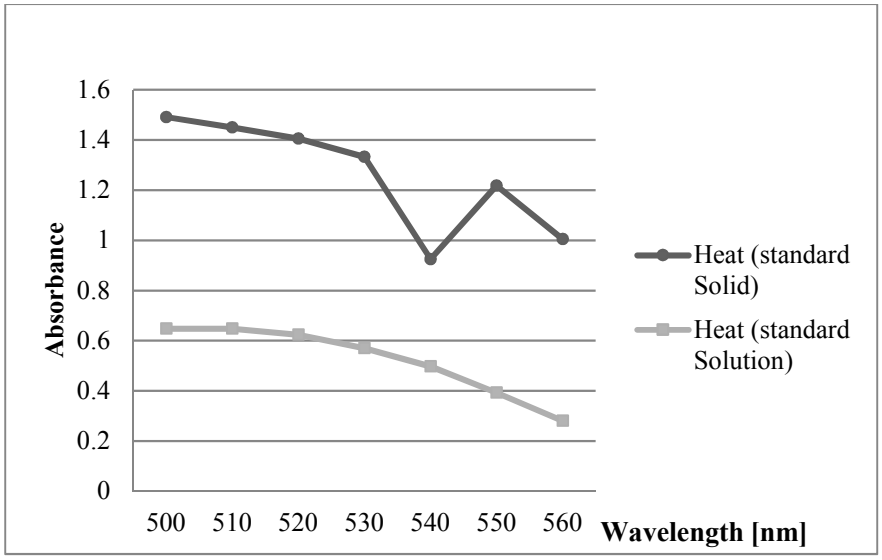

Figure 4 Thermal degradation

$NaNO_2$ standard (drug solution and solid) dry heat exposure for 90 minutes.

The **figure 5** shows the *Oxidative Action of H_2O_2.* The curve obtained, has the higher level at 460 nm and starts to drop in each wavelength studied and significantly falls at 540 nm, so that if a degradation compound should occur, it would not interfere with the reaction studied, because Griess method use 540 nm as wavelength.

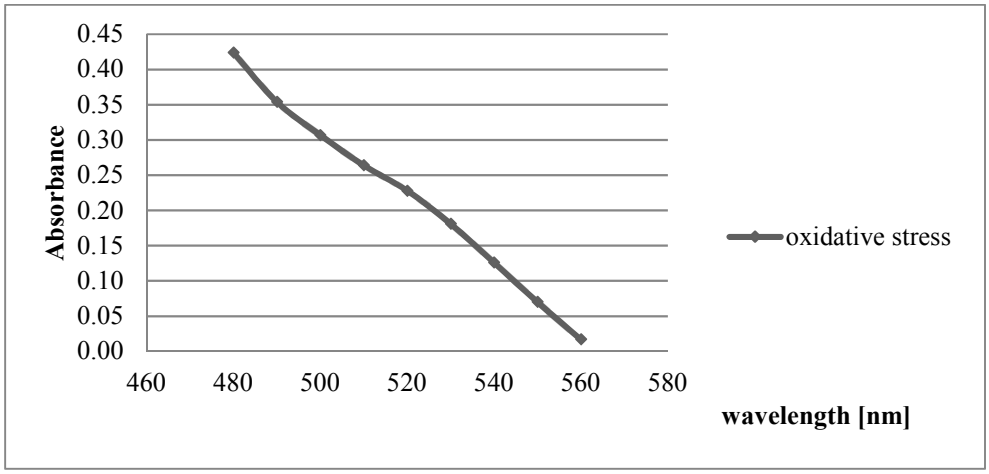

Figure 5 Oxidative Action of H_2O_2

10 mL 5 µM $NaNO_2$ was mixed with 1 mL 30% H_2O_2, refluxed and cooled. The curve obtained significantly falls at 540 nm, so that if a degradation compound should occur, it would not interfere with the Griess method.

The **figure 6** shows the *Influence of photolysis.* The curve has the higher absorbance at 480 nm and then the significant drop at 540 nm. These results indicate the importance of working with low light in this Griess method.

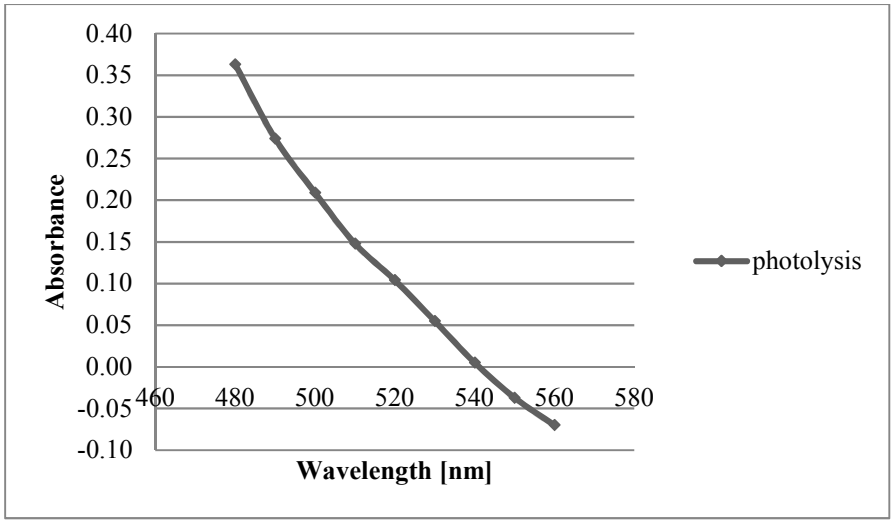

Figure 6 Photolysis

10 mL of 5 μM $NaNO_2$ solution was placed into direct sunlight for four days.

In overall these last results show that standard $NaNO_2$ exposed to extreme conditions may produce degradation products. However, these products will not interfere with the assay because they produce absorbance levels at other wavelengths different than 540nm or the products are consequence of conditions that are not used in this study.

Determining the Precision

Test-retest: 6 standard $NaNO_2$ solutions were prepared, and 3 determinations with the modified Griess method were carried out. As shown in **Table 2**, the differences within all assays are not significant ($F=1.46$; $p<0.3$).

Interobserver: repetition of the same procedures by two different analysts was also evaluated using the same reagents and equipment. Results are shown in **Table 3**. No significant differences were found within the results of the two analysts ($t=0.605$; $p<0.558$). Therefore, a good reproducibility of results is supported.

Table 2 Precision (test - retest)

	Mean	SD	N
meas1	0.0755	0.00731	6
meas2	0.0738	0.00781	6
meas3	0.0743	0.00739	6

meas = measurement / SD = standard deviation / F (2) = 1.46 p < 0.3

Six samples of the same sciatic nerve were studied by triplicated determinations. The differences within all assays are not significant ($F=1.46$; $p<0.3$).

Table 3 Precision (Interobserver)

AnalystI	Absorbance [nm]	SD	AnalystII	Absorbance [nm]	SD
1	0.006	1.18E-04	**1**	0.010	1.30E-03
2	0.006	1.18E-04	**2**	0.009	4.86E-03
3	0.006	1.18E-04	**3**	0.008	1.18E-04
4	0.008	1.30E-03	**4**	0.009	5.89E-04
5	0.006	1.18E-04	**5**	0.007	8.25E-04
6	0.005	8.25E-04	**6**	0.006	1.53E-03
Mean	**0.006**		**Mean**	**0.008**	
SD	**9.83E-04**		**SD**	**1.34E-03**	

Three different samples were measured by two different analysts using the same reagents and equipment. No significant differences were found within the results of the two analysts.

Determination of Accuracy

Three samples for each known concentration level of the analyte (80, 100 and 120%) were prepared and NO_2^- and NO_3^- determination were carried out. The data obtained are shown in **Table 4**, where it can be seen that the modified Griess method allows for recovery of almost 100% of the analyte in all concentrations.

Table 4 Accuracy

% Sciatic Nitrite	Experimental value [µM]	Theoretical value [µM]	% Recovery
80%	8.80	9.00	97.80
100%	9.90	10.00	99.00
120%	11.10	11.00	100.90
Mean			99.23
SD			$1.57 .10^{-2}$

Three samples for each known concentrations level analyte (80, 100 and 120%) were prepared and NO_2^- and NO_3^- determination were carried out. The modified Griess method allows for recovery of almost 100% of the analyte in all concentrations.

DISCUSSION

In order to quantify less than micromolar amounts of NO_2^- and NO_3^- in nervous tissue, some modifications of classical Griess method were studied. These changes included: reduction of nitrate to nitrite, deproteinization and working at low temperatures.

This colorimetric modified Griess reaction for the detection and quantification of nitrite (NO_2^-) and nitrate (NO_3^-) showed that the reduction step is necessary since in control samples (unreduced) NO_2^- concentration decreased significantly (table 1). We choose cadmium powder reduction because it has certain advantages compared with other methods of reduction. Cadmium has a reduction potential of -0.403V. Furthermore, the redox potential pair $NO_3^-/NO_2^-$is pH dependent (0.94V in acid solution to 0.015V in basic solution). Therefore, the reduction reaction of NO_3^-into NO_2^- by cadmium is thermodynamically favorable [23]. $CdSO_4$ was used in powder because it reduces approximately 100 nmol NO_3^- in several minutes and does not require a subsequent step to separate, compared to granulate cadmium that requires several hours to reduce the same amount nitrate and it is necessary to separate it later by centrifugation. Other authors used copperized cadmium (obtained by exposing it to a solution of $CuSO_4$) and metallic zinc [16]. However, they both are too reactive and lead to a reduction of the resultant NO_2^- and therefore result in low performance of the Griess assay method [15]. Zinc also involves a very prolonged incubation time (about 2 hours) [15], with the difficulties that any extensive technique involves.

Recently the use of vanadium (III) (V^{3+}) as the reducing agent was described [17]. The cadmium advantage is that it is stable in solution and does not require the frequent monitoring needed when V^{3+} is used.

Other reduction methods found in the literature are based on the bacterial nitrate reductase (NR) enzyme [14, 24]. Bacterial NR enzyme catalyzes the nitrate reduction through NADPH. While this method has been widely used in the determination of NO metabolites in biological samples, it was discovered that NADPH interferes with the Griess reaction [24]. Our results show, however, that cadmium does not produce interfering substances (Figure 2-4).

Biological samples such as plasma or tissues have a high protein content and high turbidity and also can convert NO_2^- into NO and NO_2 [25]. In this study it was shown that the Griess reaction is more sensitive in deproteinized samples and that, in samples without this treatment, approximately half of the NO_2^- and $NO_3^-$was quantified (Table 1). Deproteinization was performed with $ZnSO_4$/NaOH because protein precipitation methods in acid medium can promote loss of NO_2^-. In the literature [26], precipitation was performed using zinc acetate + potassium ferrocyanide (Carrez Method) for deproteinization of samples. In terms of the removal of proteins, $ZnSO_4$ removes about 50% of the protein in samples, compared to zinc acetate + potassium ferricyanide, which removes approximately 85% [21]. However, the method of $ZnSO_4$ is more effective in preventing the formation of precipitate/turbidity in the Griess reaction even in samples with high protein content, such as plasma [25, 27].

The third modification was adding Griess reagents sequentially at low temperatures (4-8°C). This procedure not only increases the limit of quantification, but also prevents the occurrence of degradation products which interfere with the assay (Figure 4).

CONCLUSIONS

There are numerous papers describing measurements NO metabolites in nervous tissue using Griess reagent but there is no paper disclosing the methodology in details, and this issue is considered to be important for neuroscience.

The applicability and adaptability of the method developed for the determination of the concentration of nitrites and nitrates in the Wistar rat sciatic nerve was demonstrated. Thus the method can be used to indirectly measure the amount of NO in this type of tissue and to study a wide range of functions in a simple, inexpensive and reliable way. It could also be applied in other tissues (kidney, liver) and plasma or serum.

In order to show the goodness of introduced changes we carried out several validation studies. The results indicated that modified Griess method allow obtaining specific, precise and accurate data. The method is specific as there is no interference from the degradation products. The method is linear in the range of concentrations 2-12 μM. The method is precise because repeatable results were obtained and it is reproducible by different analysts. The method is accurate because there is no difference between the mean recovery (99.23%) and 100%.

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