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Is nitrate a good biomarker of the nitric oxide status?

Este ionul nitrat un bun biomarker al producţiei endogene de monoxid de azot?

Mircea Dumitru Croitoru[1], Ibolya Fülöp[2*], Erzsébet Fogarasi[3], Daniela-Lucia Muntean[4]

1. Department of Toxicology and Biopharmacy, University of Medicine and Pharmacy of Tîrgu Mureş 2. University of Medicine and Pharmacy of Tîrgu Mureş; 3. Department of Toxicology and Biopharmacy, University of Medicine and Pharmacy of Tîrgu Mureş; 4. Analytical Chemistry and analysis of medicines, University of Medicine and Pharmacy of Tîrgu Mureş

Abstract

A method of measuring in vivo nitric oxide (NO) levels is required to detect pathological conditions in which endogenous production is decreased or to identify agents able to release this biomolecule. Unfortunately, nitric oxide has a very short biological half-life and is very difficult to measure. Assay of the oxidative products' of NO levels, nitrite (NO_2^-) and nitrate (NO_3^-), measured as total amount, after the reduction of nitrate to nitrite, determined after conversion in an azo dye, is usually the used method, named NOx test. The NOx test is frequently used as a NO biomarker in human studies and also in animal experiments. The aim of this work is to evaluate the suitability of the NOx test for the detection of an instant release of nitric oxide.

Rabbits were used as experimental animals, a validated HPLC-UV/VIS method was used for speciation of nitrite and nitrate. The following substances were administered: blank; "negative blank": phenyl-N-tert-butylnitrone (PBN); "positive blank" (nitroglycerin); nitrite.

PBN administration significantly increased nitrate and decreased nitrite levels, nitrite administration excessively increased nitrate levels, while nitroglycerin (1 mg/kg) significantly increased both nitrate and nitrite levels.

Results show that NOx test cannot be considered accurate in acute nitric oxide status testing. Nitrite alone should be used as an in vivo released nitric oxide marker.

***Keywords**: nitrate, nitrite, nitric oxide, nitroglycerin, rabbit*

Rezumat

Determinarea concentraţiilor in vivo ale monoxidului de azot (NO) este necesară pentru identificarea condiţiilor patologice în care producţia endogenă a acestui compus este redusă sau pentru identificarea unor agenţi care pot genera in vivo acest compus. Instabilitatea crescută a oxidului de azot îl face extrem de greu de determinat. Ionii nitrat şi nitrit (produşi de oxidare ai NO) sunt de obicei determinaţi ca sumă prin reducerea nitratului la nitrit şi determinarea nitritului spectrofotometric ca azocolorant. Metoda se numeşte testul NOx şi este frecvent folosită

***Corresponding author**: Ibolya Fülöp, University of Medicine and Pharmacy of Tîrgu Mureş, Tîrgu Mureş, România, e-mail: fulop.ibolya@umftgm.ro, fulopibolya@yahoo.com

ca un biomarker al NO în studii experimentale pe om şi animale de experienţă. În lucrarea de faţă s-a evaluat utilitatea testului NOx în detectarea unei eliberări de moment a unor cantităţi crescute de NO.

Ca animale de experienţă au fost folosiţi iepuri, determinarea nitritului si a nitratului realizându-se cu o metodă HPLC validată. S-au făcut următoarele administrări: martor; "martor negativ": fenil-N-terţ-butilnitrona (PBN); "martor pozitiv" (nitroglicerina); ion nitrit.

Administrarea de PBN a crescut semnificativ nitratemia în paralel cu scăderea nitritemiei, administrarea nitritului a crescut excesiv concentraţiile nitratului, iar administrarea nitroglicerinei (1 mg/kg) a crescut semnificativ atât nitritemia cât şi nitratemia.

Rezultatele arată că testul NOx nu poate fi considerat ca având acurateţe corespunzătoare în estimarea producţiei endogene de NO. Ionul nitrit ar trebui folosit în locul testului NOx ca marker al eliberării NO.

***Cuvinte cheie**: nitrat, nitrit, monoxid de azot, nitroglicerină, iepure*

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Introduction

Nitric oxide (NO) is reported to play a significant role in many physiological functions in the human body. Its importance for biomedical research is owing to: (a) it is a free radical found in all tissues, (b) it is involved in a large number of physiological and pathological processes, (c) NO donors are used in clinical therapy and new molecules with the ability to release NO are under constant demand (1). NO concentrations are particularly important since, as with any messenger molecule, too little or too much of the substance can result in pathological events (2,3). Biological desired concentrations of NO are in the picomolar range, while nanomolar concentrations are produced under pathological conditions and are associated with pathological processes (4). The high interest in developing NO donors requires analytical techniques capable to accurately and precisely measure NO concentrations.

Unfortunately, NO has an extremely short half life of the order of seconds in biological systems. This makes the detection and quantitative measurement of this compound an extremely difficult task (5). Several detection methods have been developed in order to evaluate the nitric oxide status. Both direct and indirect detection techniques of NO are widely used. Direct detection methods as chemiluminescence, electron paramagnetic resonance and electrochemical sensors have been used to detect NO in various biological environments (5,6). Indirect methods usually detect redox related species of NO (nitrite or nitrate) or products formed during the interaction of NO derived species with the biological matrix (S-nitrosothiols, nitrated proteins) (7).

Frequently used for estimation of nitric oxide status, both in animal and human experiments, is the determination of nitrite and nitrate as a total amount, called the NOx test. This is based on the reduction of nitrate to nitrite by various reagents, followed by the well known Griess reaction when a colored azo dye is formed after the reaction of nitrite with sulphanilic acid and 1-naphtylamine (8-12).

The purpose of this work is to carefully asses, using a rabbit-based animal model, the utility of the NOx test for the evaluation of an acute nitric oxide release.

Material and methods

All common chemicals and reagents (ethanol pa, gradient-grade methanol and gradient-grade acetonitrile, tetrabutylammonium hydroxide) were purchased from local providers and were used without any further purification. Ultra-pure water was obtained using a Mili-Q purification system (Millipore Corporation, USA). All animal experiments were carried out with institutional approval received from our university's

Ethics Committee and in accordance with international regulations. Ten rabbits with an average weight of 4.62±0.17 kg were used for administration of the following treatments:

- nitroglycerin as "positive blank" in therapeutic dose 0.1 mg/kg (13) and high dose 1.0 mg/kg was intraperitoneally administered. Because pure nitroglycerin was not available, nitroglycerin tablets containing 0.5 mg nitroglycerin/tablet (Zentiva S.A.) were used. They were dissolved in the solvent, followed by filtration through a 0.5 μm filter.
- blank administration (20% hydro-alcoholic solution in dose of 1.5 ml/kg was intraperitoneally administered). Blank administration was done two times (Blank 1 and Blank 2 – Figure 1 and Figure 2);
- phenyl-N-tert-buylnitrone (PBN) as "negative blank" was intraperitoneally administered in dose of 100 mg/kg. The substance was prepared by a method described in detail elsewhere (14), in order to ensure substance identity and purity (MS spectra, and thin layer chromatographic separation were performed);
- nitrite 0.25 mg/kg (in the form of sodium nitrite, Merck Germany) was intravenously administered;

With all treatments, substances were dissolved in the same volume of solvent used for blank administration.

Measurements of nitrite and nitrate were done using a validated method published in detail elsewhere (15).

In order to evaluate the statistical significance of the results, ANOVA and paired two tail "t" test were performed. Statistical significance was considered for $p<0.05$.

Results

Figure 1 and 2 show the modifications recorded in the concentrations of nitrate and nitrite during 7 hours after the blank administration and readministration. Table I shows the statistical significance of the observed modifications and the statistical significance of the differences

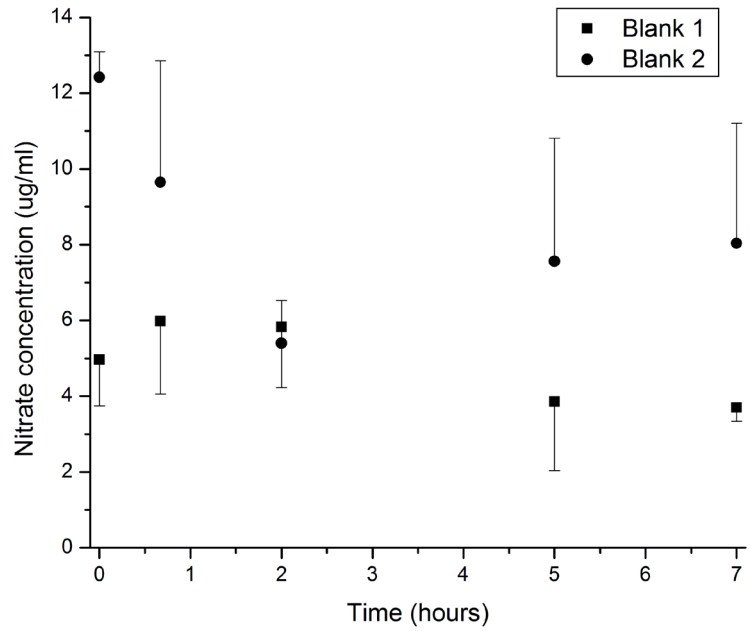

Figure 1. Blood nitrate concentration after blank administration to rabbits

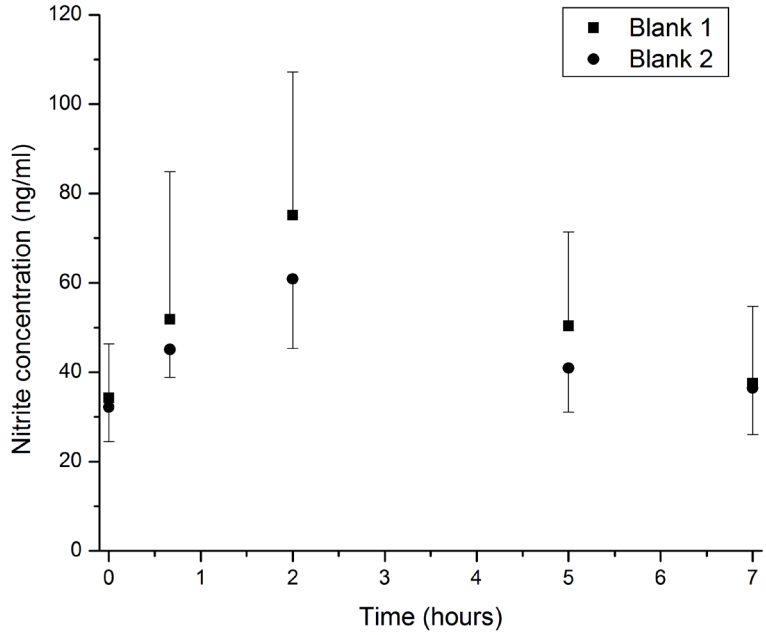

Figure 2. Blood nitrite concentration after blank administration to rabbits

recorded between the two blank (Blank 1 and Blank 2) administrations.

Administration of the nitroglycerin in high dose (1 mg/kg) resulted in an impressive (statistically significant) increase in nitrate and nitrite concentrations. In the case of the therapeutic dose of nitroglycerin (0.1 mg/kg), with a small increase in nitrite concentrations, no statistical significance compared with the blank was observed. This was followed by a rapid decrease in nitrite concentrations (Table II).

Administration of PBN (an inhibitor of the nitric oxide production) was accompanied by an increase in nitrate concentration. At the same time, nitrite concentrations significantly decreased (Table II).

In order to track short-term changes of nitrite resulted as decomposition product of nitric ox-

Table I. Statistical analysis (p values) of the results obtained in the case of the blank administration

Time (hours)	0	0.666	2	5	7
			Nitrate		
blank 1[a]		0.0900	0.0935	0.1602	0.0107*
blank 2		0.0188*	<0.0001***	0.0008***	0.0015**
blank 1 vs blank 2[b]	<0.0001***	0.0012**	0.4420	0.0369*	0.0017**
			Nitrite		
blank 1		0.0469*	0.0014**	0.0414*	0.5202
blank 2		0.0023**	0.0008***	0.0227*	0.3461
blank 1 vs blank 2	0.6735	0.4792	0.1404	0.1129	0.7977

[a] in order to evaluate the statistical significance concentrations at every time point were compared with those measured just before the administration of the blank (time 0)

[b] comparison was made using the concentrations recorded at a certain time point after the blank administration

Table II. Changes[a] in the nitrite and nitrate concentrations following PBN, nitroglycerin and nitrite administration to rabbits

Administration	NOX	TIME (h)			
		0.666	2	5	7
PBN	nitrate[c]	4.97	6.19	8.98	ND[b]
	p	0.0648	0.0117*	0.0102*	
	nitrite[d]	-73.3	-38.1	-33.4	
	p	0.0134*	0.0119*	0.0576	
NG 1 mg/kg	nitrate	1.37	1.97	3.36	ND
	p	0.0005***	0.0006***	0.0003***	
	nitrite	83.2	37.3	40.9	
	p	0.0011**	0.0048**	0.0017**	
NG 0.1 mg/kg	nitrate	4.42	8.92	5.83	4.69
	p	0.0002***	0.0002***	< 0.0001***	0.0003***
	nitrite	3.0	-33.2	-22.7	-22.8
	p	0.3734	0.0045**	0.0229*	0.0176*
Nitrite 0.25 mg/kg	nitrate	1.67	7.14	-1.55	1.71
	p	0.3674	0.007**	0.2189	0.1572
	nitrite	313.8	4.4	-44.0	-45.7
	p	0.0007***	0.3906	0.0427*	0.0143*

[a] the changes were calculated using the formula $[(T_{xN} - T_{0N})-(T_{xB} - T_{0B})]$ where T_{xN} = concentration of nitrite or nitrate at a time point after nitrone administration; T_{0N} = concentration of nitrite or nitrate before nitrone administration (zero time concentration for nitrone); T_{xB} = concentration of nitrite or nitrate at a time point after blank administration; T_{0B} = concentration of nitrite or nitrate before blank administration (zero time concentration for blank)

[b] ND – not determined

[c] nitrate concentration is given in µg/ml

[d] nitrite concentration is given in ng/ml

ide, nitrite was administered in dose of 0.25 mg/kg intravenously to rabbits, and its kinetic parameters were measured (Figure 3). As expected, there was an abrupt rise in nitrite concentrations followed by an exponential decline. In this context, nitrate yielded a significant increase compared with the blank at only 2 hours time point (Table II). However, the increase was about 6 times higher than that expected, based on the volume of distribution of nitrate (approximately 0.2 l/kg in humans and dogs) (16,17).

Discussions

Theoretically, it is expected that nitrite concentrations would follow an instant nitric oxide status better than nitrate because: (a) nitrite is usually the first decomposition product of nitric oxide, (b) nitrite is not frequently present in food or water, (c) nitritemia is low (ppb level) and could be significantly altered when moderate changes are brought to nitric oxide production. The disadvantages of nitrate as NO marker are: (a) high nitrate amounts are present in blood

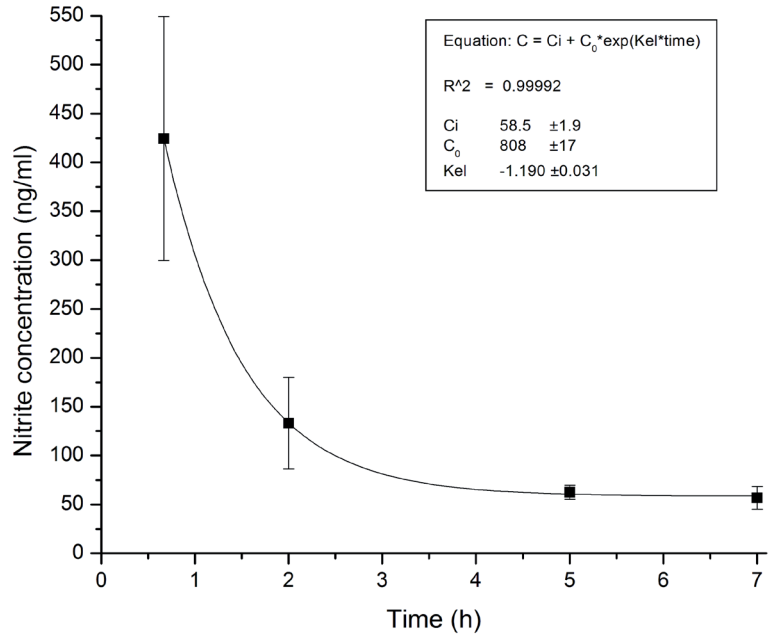

Figure 3. Modifications of nitrite blood concentrations after nitrite administration

(ppm level), so an instant change in the concentrations of nitric oxide would be difficult to spot; (b) since nutrients are important sources of nitrate, food cessation may lead to a physiological decrease in nitrate concentrations without a decrease in nitric oxide production; (c) the elimination through urine may be increased by the vasodilating effect of nitric oxide.

Our results show that concentrations of nitrate changed in an erratic manner following a blank administration to the experimental animals. These statistically significant differences were both qualitative and quantitative. Regarding the nitrite concentrations, it can be observed that following the administration of the two blanks, animals behave exactly the same way. Minor differences that were recorded between the two blanks are of no statistical significance at any time point and the modification pattern of nitrite concentrations is exactly the same in the case of the two administrations. The experiments made with the blank suggest that the changes in nitrite concentrations are more predictable than those of nitrate, and should, therefore, be used preferentially when an spontaneous change in the nitric oxide status is tracked. In this case, NOx concentrations measured as a sum of nitrite and nitrate, as it is done frequently in scientific literature for assessment of human nitric oxide status (8-12), could lead to misinterpretation of data. Literature data shows that epilepsy, multiple sclerosis (9), changes in the mesenteric arteries of diabetic patients (10), were correlated with the concentration of NOx. On the other hand, postmenopausal hot flushes intensity did not correlated with NOx concentrations (12). In an in vivo test designed to measure brain nitrate concentrations as nitric oxide marker in paraquat intoxication, it was observed that administration of N-nitro-L-arginine methyl ester, a NO synthesis inhibitor, is not followed by the expected reduction in nitrate concentrations (18). Acute pediatric kidney injury was associated with low urinary nitrate concentrations, but differences in nitrite concentrations did not attained statistical significance (probably due to the fact that

nitrite concentration in some samples was under the limit of detection) (19). In acute respiratory infections, nitrate concentration in nasal wash were higher in the presence of respiratory syncytial virus subtype B compared with other viruses. In this case too, many samples contained nitrite in undetectable concentrations and due to this fact correlations were not possible to be observed (20). NOx test was of no use in predicting cardiopulmonary bypass inflammatory response, even if NO concentrations are known to increase in inflammatory conditions (21). It can be seen that our results correlates with literature data in showing that nitrate concentrations are not always a reliable predictor of the nitric oxide status. Unfortunately, in many studies available in the scientific literature, nitrite is not determined (probably due to the lack of a proper analytical method), therefore failure of nitrate as nitric oxide marker can not be correlated with the successful use of nitrite for this purpose.

It is important to note that low amounts of nitroglycerin generated only statistically insignificant increase in nitrite concentrations, compared with the blank, while high doses of nitroglycerin strongly increased all NOx concentrations at all time points. The low detectability of nitric oxide production, observed at low doses of nitroglycerin, can be explained by the fact that a single administration of a therapeutic dose of nitric oxide donor would not dramatically increase nitrite concentrations, probably due to the low amount of nitric oxide needed to provide a vasodilating activity. Furthermore, moderate vasodilatation could increase the blood flow in the kidneys, leading to an enhanced elimination of nitrite through urine. Also, chronic administration of nitroglycerin was found to decrease endothelial nitric oxide production (22). It is a possibility that this decreasing effect can occur even at the first administration, this way reducing the endogenous production of nitric oxide, thus yielding less endogenous nitrite after the initial moderate increase.

When nitrite, at a low dose of 0.25 mg/kg, was administered to animals in order to track its fate and to analyze its influence on nitrate concentrations, significant increases in nitrite and nitrate concentrations were observed. The decline in the nitrite concentrations after the first increase was not fitted properly by the formula:

$$\mathbf{C = C_0 * exp(Kel * time).}$$

But it was excellently fitted ($R>0.9999$, residuals < 4%) by an equation of the type:

$$\mathbf{C = C_i + C_0 * exp(Kel * time),}$$

specific for the excretion of compounds that are endogenously generated (Figure 3). An exaggerate increase (about 6 times higher than expected) was seen in nitrate concentrations after the administration of a low dose of nitrite. Such large increase of nitrate concentration, when administering small amounts of nitrite, cannot be linked with the oxidation of nitrite to nitrate. This finding further substantiates the need of an accurate nitrite detection method for the investigation of the acute nitric oxide release.

There are several cases described in the literature, in which NOx concentrations measured as a sum are linked to several diseases (8-12), but in these cases long term changes in nitric oxide formation is tracked. In such cases, nitrate concentrations increase if high nitrite amounts are generated continuously, due to its far higher half life (5.8 hours) compared with that of nitrite (0.58 hours).

Literature data suggest that PBN is able to reduce nitric oxide synthesis in vivo by blocking the nitric oxide synthase activity (23-25). Our results confirm the ability of PBN to reduce nitric oxide production, since in this experiment, PBN not only completely abolished the increase of nitrite concentration induced by the blank administration, but reduced even the nitritemia of

the experimental animals. With its well-known antioxidant properties, an enhanced oxidation of nitrite due to PBN administration can be ruled out as explanation for this effect. Contrary to expectations, the nitrate concentration increased after the administration of PBN. This increase, certainly can not be attributed to an in vivo NO release.

All the results of this experiment indicate that nitrate concentrations can change in unexpected ways when substances known to modify nitric oxide production are administered to experimental animals. For this reason, NOx test could not be considered suitable for nitric oxide status evaluation, especially in short term experiments. Nitrite concentrations, however, are modified as expected and modifications are reproducible, therefore it should be used as a preferred nitric oxide marker. Best results could be obtained by a speciation of nitrite and nitrate, using a suitable analytical technique (15). This way both nitrite and nitrate concentrations could be followed and evaluated.

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Disclosure

The authors state that they have no conflict of interest.

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