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Original article

Blood collection vials and clinically used intravenous fluids contain significant amounts of nitrite☆

Luis E.F. Almeida[a,b], Sayuri Kamimura[a,b], Margaret Y. Nettleton[a,b], Celia M. de Souza Batista[c], Elizabeth Walek[a], Alfia Khaibullina[a], Li Wang[a], Zenaide M.N. Quezado[a,b,*]

[a] *The Sheikh Zayed Institute for Pediatric Surgical Innovation, Children's Research Institute, School of Medicine and Health Sciences, George Washington University, Washington, DC 20010, USA*

[b] *Department of Perioperative Medicine, National Institutes of Health Clinical Center, National Institutes of Health, Bethesda, MD 20892, USA*

[c] *Department of Nutritional Sciences, Howard University, Washington, DC 20059, USA*

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ABSTRACT

The biology of the inorganic anion nitrite is linked to nitric oxide (NO) as nitrite can be reduced to NO and mediate its biological activities. Thus, studies of nitrite biology require sensitive and selective chemical assays. The acetic and ascorbic acids method is selective for nitrite and measures it in biological matrices. However, one of the pitfalls of nitrite measurements is its ubiquitous presence in sample collection tubes. Here, we showed high levels of nitrite in collection tubes containing EDTA, sodium citrate or sodium heparin and smaller amounts in tubes containing lithium heparin or serum clot activator. We also showed the presence of nitrite in colloid and crystalloid solutions frequently administered to patients and found variable levels of nitrite in 5% albumin, 0.9% sodium chloride, lactated ringer's, and dextrose-plus-sodium chloride solutions. These levels of nitrite varied across lots and manufacturers of the same type of fluid. Because these fluids are administered intravenously to patients (including those in shock), sometimes in large volumes (liters), it is possible that infusions of these nitrite-containing fluids may have clinical implications. A protocol for blood collection free of nitrite contamination was developed and used to examine nitrite levels in whole blood, red blood cells, plasma and urine from normal volunteers. Nitrite measurements were reproducible, had minimal variability, and did not indicate sex-differences. These findings validated a method and protocol for selective nitrite assay in biological fluids free of nitrite contamination which can be applied for study of diseases where dysfunctional NO signaling has been implicated.

1. Introduction

The inorganic ion nitrite has emerged as one of the most important nitric oxide (NO)-derived species. Evidence from human and animal studies suggest that nitrite plasma levels reflect the activity of endothelial nitric oxide synthase (eNOS) [1–3]. For example, pharmacological manipulations of eNOS produce acute changes in plasma nitrite levels and endothelial dysfunction is associated with decreased plasma nitrite [4,5]. Nitrite has also been shown to serve as a NO storage pool and enzymatic nitrite reductase function (reduction of nitrite into NO) has been described for several human enzymes including deoxyhemoglobin, xanthine oxidoreductase (XOR), eNOS, cytochrome C and others [5]. Importantly, several of these enzymes operate under physiological and pathological conditions, particularly those associated with hypoxia and acidosis, to generate NO. For instance, nitrite supplementation increases nitrite levels in wild-type and eNOS knockout mice and has protective effects against ischemia/reperfusion injury [6,7] and against right ventricular hypertrophy in a model of pulmonary hypertension [8]. Thus, combined, these nitrite-reductases form a NOS-independent pathway for NO production and NO-mediated biological effects, especially under conditions when eNOS-derived NO synthesis from arginine is compromised. Therefore, an accurate measurement of nitrite levels will contribute to a better understanding of nitrite and NO biology and shed light onto the pathobiology of diseases associated with alterations in endothelial function and blood flow such as hypertension, diabetes, or sickle cell disease.

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* Correspondence to: Department of Perioperative Medicine, National Institutes of Health Clinical Center, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892.
E-mail address: zquezado@nih.gov (Z.M.N. Quezado).

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However, characteristics of NO biology pose technical challenges for its accurate measurement and understanding of its function. First, NO has a markedly brief half-life (≈100 msec) [9] and its levels cannot be measured directly in biological fluids. Second, NO can decay into a number of chemical species depending upon the biological environment where it is present. For example, in inflammatory environments, NO can react with superoxide and generate peroxynitrite, which can react with tyrosine residues and produce nitrotyrosine. In red blood cells, NO can react with oxyhemoglobin producing nitrate and methemoglobin or bind to the heme-iron producing nitrosyl-hemoglobin, among many other possible chemical pathways (for review see [10]). Therefore, given the technical challenges and diversity of NO-related chemical reactions, chemical assays selective for each NO-derived chemical species are needed.

Nitrite measurement in biologic samples can also be associated with technical difficulties. One such difficulty results from the ubiquitous presence of nitrite in vials used to collect biological samples [11,12] and in chemicals and laboratory glassware used for sample preparation. We have previously shown [13] that potassium cyanide, a reagent used as a component of an “enhanced” nitrite stabilization solution [14,15], can have high nitrite contamination levels. Therefore, meticulous care must be taken when attempting to measure nitrite in biological samples. Here we used the acetic and ascorbic acids method, which is selective for nitrite over several other nitroso species [16,17], to investigate the presence of nitrite contamination in tubes used for blood or urine collection. Additionally, we examined whether nitrite was present in colloid and crystalloid solutions, which are routinely administered in clinical settings such as surgery and trauma resuscitation. Lastly, we developed and validated a protocol that avoids sample contamination and can be used for the collection of human biological fluids.

2. Material and methods

2.1. Nitrite analysis in blood collection tubes and clinically used colloid and crystalloid solutions

Vacuette® vacuum blood collection tubes (Greiner Bio-One, Monroe, NC) containing EDTA (ethylenediaminetetraacetic acid tripotassium salt), sodium citrate (0.3 ml of a 3.2% solution), lithium heparin, sodium heparin or serum clot activator (procured from clinical areas of the hospital) were washed with 1 ml of nitrite stabilization solution (“stop solution” [SS] see below). At least 3 tubes from multiple lots for each tube type were assayed. Detailed lot number information is shown in Table 1. A butterfly collection line (23 G x ¾ inch long needle, BD Diagnostics, Franklin Lakes, NJ) attached to a 10 ml syringe (BD Diagnostics) coated with sodium heparin (1000 units/ml, Fresenius Kabi, USA) was also washed with 1 ml SS. Nitrite contamination in urine collection cups was investigated by washing the interior of the cups with 1 ml SS.

For nitrite assays in colloid solutions (5% human albumin), 1 ml from each container was mixed with 200 µl concentrated SS, so that the final SS concentration was equal to the one used in all other experiments. A sample from clinically used intravenous crystalloid solutions was collected sterilely from respective bags and injected into the NO reaction vessel without addition of stop solution. Lot numbers and manufacturers for colloid and crystalloid solutions analyzed are listed in Table 2. All fluid bags were obtained from the hospital's operating rooms, were kept at room temperature, and fluids were assayed before their expiration dates.

2.2. Human biological fluid sample collection and processing

The Children's National Health System (CNHS) Institutional Review Board approved the experimental protocol and consent was obtained from all healthy human volunteers. Blood and urine were collected from non-fasting volunteers between 10AM and 12 noon.

Table 1
Reference and lot numbers for blood collection tubes[a].

Clinical tubes	Reference #	Lot #
Potassium EDTA	454222	B14061IY
	454222	B16073DM
	454021	B160635S
Sodium Citrate (3.2%)	454334	B121324
	454334	B15123FG
	454334	B1607386
Lithium Heparin	454244	B071306
	454244	B1606364
	454244	B15083DV
Sodium Heparin	456028	B061301
	367871	6064579
	456028	B16013K9
Serum Clot Activator	454236	B121307
	454236	B15093FA
	454236	B16053FM
	456089	B15083DL

[a] Blood collection tubes were procured from various sites in a hospital (operating rooms, phlebotomy area, recovery room) and stored at room temperature until processed with nitrite stabilization solution. All experiments were carried out before lot expiration dates.

Table 2
Crystalloid and colloid intravenous solutions assayed for nitrite content.[e]

Intravenous fluid	National Drug Code	Lot number	Nitrite (nmols/100ml)
0.9% NaCl	0338-0049-03	C972992	5.8 ± 1.9
		C969477	8.2 ± 0.49
0.45% NaCl	0338-0043-03	C933978	20.4 ± 0.5
		C957050	6.2 ± 0.61
D5W + 0.9% NaCl	0338-0089-03	C971705	Undetectable
		C972984	0.9 ± 0.07
D5W + 0.45% NaCl	0338-0085-03	C971069	1.3 ± 0.54
D5W + 0.33% NaCl	0338-0081-03	C944504	Undetectable
LR	0338-0117-03	C974592	5.0 ± 0.71
		C971465	9.0 ± 0.92
D5W + LR	0338-0125-03	C967331	2.5 ± 0.58
	0409-7979-03[a]	45-702-FW	3.3 ± 0.72
Human 5% Albumin	68516-5214-3	C3NA5TVTW1[b]	7.5 ± 0.308
	76125-790-26	A1EB5NFP1[c]	
		EKEB500221[d]	

[a] Hospira, Inc (Lake Forest, IL).
[b] Grifols Biologicals, Inc (Los Angeles, CA).
[c] Plasbumin®-5 (Grifols Biologicals, Inc, Research Triangle Park, NC).
[d] Albuked™-5 (Kedrion Biopharma, Inc, Fort Lee, NJ). Nitrite levels are shown as mean ± standard error of the means.
[e] For each fluid analyzed, the national drug code and lot numbers are shown. All solutions were manufactured by Baxter Healthcare Corporation (Deerfield, IL, 60015, USA) except for those indicated otherwise. NaCl indicates sodium chloride, D5W indicates 5% dextrose, and LR indicates lactated ringer's.

Blood was collected from an antecubital vein using butterfly 23 G needle attached to a syringe coated with sodium heparin (see below). After collection, blood was transferred into a centrifuge tube and immediately placed on ice. After 15 min for cell stabilization, red blood cell count, hematocrit and hemoglobin content were measured with an automated cell counter (Drew Scientific, Miami Lakes, FL). Urine was collected by direct void into urine collection cups and was immediately processed for creatinine and nitrite assays (see below).

Stop solution was used to block nitrite metabolism by hemoglobin and other iron-containing enzymes and to increase the reliability of the nitrite chemiluminescence assay by avoiding NO capture and inactiva-

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