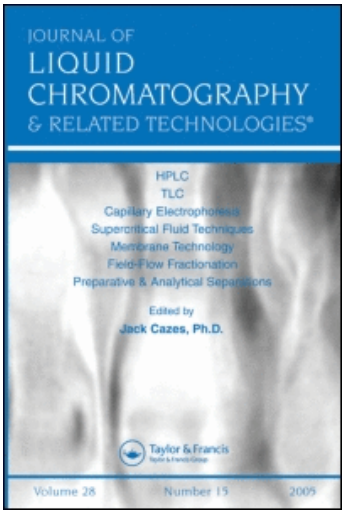

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Zivotije Radisavljevic[a]; Magdalena George[a]; David J. Dries[a]; Richard L. Gamelli[a]
[a] Burn-Shock Trauma, Institute Loyola University-Chicago School of Medicine, Maywood, IL

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DETERMINATION OF INTRACELLULAR AND EXTRACELLULAR NITRITE AND NITRATE BY ANION CHROMATOGRAPHY

Zivotije Radisavljevic, Magdalena George
David J. Dries, Richard L. Gamelli*

Burn-Shock Trauma Institute
Loyola University-Chicago
School of Medicine, Building 110/4th
2160 South First Avenue
Maywood, IL 60153

ABSTRACT

A highly sensitive method for the simultaneous direct detection as well as the ratio of intracellular and extracellular nitrite (NO_2^-) and nitrate (NO_3^-) in human macrophage (Mϕ), plasma and urine has been developed. Samples were deproteinized with the organic solvent acetonirile, lyophilized and reconstituted in buffer, determination and quantification of nitric oxide (NO) stable end products NO_2^- and NO_3^- was performed utilizing an isocratic high performance liquid chromatography (HPLC) with an anion exchange column. A mobile phase of 20 mM NaCl with 1mM mono sodium phosphate-NaH_2PO_4 at pH = 7.0 was used. Analyte anions were detected by direct UV-wavelength at 210 nm. Sensitivity in intracellular and extracellular fluids were 0.01 μmol/L for both anions with recovery rates of 99.6-99.4% for NO_2^- and NO_3^-. This method has successfully been applied to the determination of nitrite and nitrate in plasma and urine of normal human

volunteers as well as in cell line U-937 macrophage cytosol/supernatant. The mean concentration of NO_2^- in normal plasma (n=22) was 3.1 ± 0.4 μmol/L and NO_3^- 10.3 ± 0.3 μmol/Lwith ratio of NO_2^-:NO_3^- = 0.3; in normal urine (n=22) NO_2^- was 380 ± 61 μmol/L and NO_3^- 1345 ± 270 μmol/L with ratio of NO_2^-:NO_3^- = 0.3; in intracellular human macrophage fluid (n=21) NO_2^- was 0.66 ± 0.08 mol/L/10^6 Mϕ, and NO_3^- 0.68 ± 0.09 mol/L/10^6 Mϕ with ratio NO_2^-:NO_3 = 1; and in extracellular human macrophage fluid (n=21) NO_2^- was 0.03 ± 0.01 mol/L/10^6 Mϕ and NO_3^- 3.03 ± 0.33 mol/L/10^6 Mϕ with ratio NO_2^-:NO_3^- = 0.01.

INTRODUCTION

NO_2^- and NO_3^- are the stable metabolic products of NO, an intermediate in the metabolism of the amino acid L-arginine.[1-4] NO is synthesized by mammalian cells from the guanidino nitrogen atom of L-arginine and molecular oxygen (O_2) by the enzyme NO-synthase.[5,6] NO as an intermediate is rapidly oxidized in vivo to the nitrogen oxides, NO_2^- and NO_3^- by molecular O_2 or superoxide anion (O_2^-).[7]

Previously the nitrates present in biological fluids have been determined by colorimetric procedures and nitrite by diazotization and coupling reactions.[8] The reduction of nitrate to nitrite by a cadmium column and determination of NO_2^- by diazotization,[9] has been the more commonly employed method,[10-12] where NO_2^- reacts with the Griess reagent to form a purple azo dye and NO_2^- determined spectrophotometrically.[13-15] Determination of inorganic NO_2^- and NO_3^- by the kinetic cadmium-reduction methods is a refinement of this approach.[16] Other authors have detected NO_2^- and NO_3^- simultaneously, but with low sensitivity in plasma.[17-20]

We have developed a method for the simultaneous separation, determination and quantification of intracellular and extracellular NO_2^- and NO_3^- anions by an anion-exchange high performance liquid chromatography. This technique has proven to be a rapid and highly sensitive simple approach for the evaluation of the nitric oxide stable end products (NO_2^-, NO_3^-).

MATERIALS AND METHODS

Apparatus

An isocratic high performance liquid chromatography (HPLC) (Perkin Elmer, Norwalk, CT, USA) was used for simultaneous separation of NO_2^- and NO_3^- anions in intracellular and extracellular fluids such as human macrophage, plasma, and urine. The HPLC system included: a UV-785A programed absorbance detector, Perkin Elmer; a Diode Array Detector (DAD)-235C Perkin Elmer; an advanced LC sample processor ISS 200, Perkin Elmer; an isocratic-gradient IC-pump Perkin Elmer; an interface 600 series LINK PE-NELSON; a computer Digital DECpc LPv 466d2, linked to a printer (Hewlett-Packard Laser Jet 4 Plus); a pre-column (PRP-×100, guard 250×2.3mm) with an exchange capacity of 200 μeq/gr to provide an effective filter and coupled to a anion exchange post column (PRP-×100, 150×4.1 mm ID) containing a strong basic poly-styrene-divenylbenzene-trimethyleammonium exchanger (Hamilton, Reno, NE, USA).

Reagents

Inorganic salts used for standard analytes and mobile phase electrolyte were analytical reagent grade. NaCl and monosodium phosphate NaH_2PO_4 (Fluka Chem Co, Ronkonkoma, NY, USA) were used for the buffer solutions. The nitrogen standard used were NO_3^- (1mL =1mg) and NO_2^- (1mL =0.25 mg) (Ricca Chem. Co. Arlington, TX, USA).

The standards were prepared in Electrochemical-Millipore-Q-pure-water as a stock solution containing NO_2^-=125 μg/mL and NO_3^-=500μg/mL 10mL total volume, aliquotted in 10 vials (1 mL) and stored at -70 C. The Millipore-Q-water (18.2 megohm-cm) was double filtered by vacuum through Nylon 66-membrane filter, 47 mm diameter (Alltech Assoc. Co. Deerfield, IL, USA) and a Sep-Pak-Cartridges-C-18 from Millipore System, (Millipore, Milford, MA, USA) to yield Electrochemical-Millipore-Q-water.

The mobile phase was prepared with Electrochemical pure-water to which was added 20 mM NaCl and 1mM NaH_2PO_4. The final pH of the mobile phase was pH = 7.0. The resultant mobile phase buffer solution was vortexed and then filtered through a 0.2 μ Nylon 66-membrane filter and degassed under strong vacuum for 20 minutes using a Pierce filtration system (Pierce Chemical Co., Rockford, IL, USA).

Chromatography

The mobile phase (20 mM NaCl with 1mM NaH_2PO_4) was pumped through the isocratic HPLC-anion chromatography system with a flow rate of 1 mL/min, producing a background pressure of between 500-700 psi. Injection volumes that we used were between 20-100 µl. The isocratic HPLC-system was run at ambient temperature (20-24 C). Samples for analysis were thermostated at 4 C.

Detection of NO_2^- and NO_3^- anions was performed by direct UV-detection using a 210 nm wavelength. Detection by the DAD-system was performed with a spectrum of 205-300 nm as inorganic anions absorb less in the mid UV-wavelength than mobile phase anions. Anion retention times and run times were alternated by changing eluent strength and flow rate without overlapping other anions.

Cell Culture

Human monocyte/macrophage cell line U-937 (American Type Culture Collection-ATCC, Rockville, MD, USA) was grown in suspension culture at 37°C in a humidified atmosphere containing 5% CO_2 in a tissue culture flasks (75 cm^2/250 mL) in 20 mL in a culture medium of RPMI 1640 containing 10% FBS (fetal bovine serum), 30% bicarbonate, 5% penicillin/streptomycin, and 2% fungizone (Biologos, Naperville, IL, USA). Cell density was maintained between approximately 5 x 10^5 and 1 x 10^6/mL. Culture medium was changed on days 3 and 5 and every day there after by adding 10 mL of fresh medium and removing 10 mL of medium. When culture media was changed macrophages were centrifuged at 500 x g for 5 minutes.

Sample Preparation

Human blood, urine and the human macrophage cell line U-937 were used for study. Blood and urine was obtained from healthy adults. Samples were centrifuged at 3000 × g for 10 minutes at 4°C and the supernatant aliquotted in microtubes, 1.5 mL and stored at -70°C. Human macrophage cultures (cell line U-937) (1 x 10^6 cells/mL) were centrifuged at 500 × g for 5 minutes at 4°C and supernatants were aliquotted and stored at -70°C. The macrophage pellet was dissolved in Dulbeco's phosphate buffered saline,

(Sigma Chem. Co., St. Louis, MO, USA) and the cell membranes disrupted by sonicator (Sonifer 250 at cycle 3 for 60 sec.) (Fisher, Itasca, IL USA) and centrifuged at 3000 × g for 10 minutes at 4°C then the cytosol was aliquotted and stored at -70°C.

Sample Deproteinization

A) Assay for plasma deproteinization was performed by adding 250 µl of acetonitrile to 500 µl plasma in a 1.5 mL microcentrifuge tube and then vortexed for 10 sec. The protein sedimentation was performed by centrifugation for 15 minutes at 3000 × g. The supernatant was treated with an additional 250 µl acetonitrile [v:v], vortexed and centrifuged. Plasma supernatants were lyophilized in a vacuum system (vacuum = 839 u, heater = 70°C, time = 2 h) (AS 160 automatic speedvac, Savant, Inst. Inc. New York, NY, USA) and reconstituted in a volume or 100-500 µl of buffer pH = 7 (mobile phase) which yielded supernatants of consistent quality. The supernatant was transferred to insert assembled amber target vials with teflon/silicone/teflon septums (National Scientific Co., Lawrenceville, GA, USA) and placed in the autosampler processor and the simultaneous detection of nitrite and nitrate performed.

B) Assay for urine and macrophage cytosol and supernatant was performed by adding 250 µl of acetonitrile to 1mL of macrophage cytosol and supernatant or urine in 1.5 mL centrifuge tubes and vortexed and centrifuged as for plasma. The resultant supernatant was filtered through a disposable Anopop-IC syringe filter (0.2 µ, 10 mm) (Alltech Inc., Deerfield, IL, USA) and the purified supernatant was lyophilized and reconstituted in 100-1000 µl of buffer pH = 7 and transferred to insert vials of the autosampler to be injected in the HPLC column.

Validation of HPLC Methodology

Comparison of HPLC methodology with the Griess-Saltzman assay

Validation of our anion liquid chromatography methodology for the detection of NO_2^- and NO_3^- beyond that obtained from comparison of sample values to calibration curves of known standards was performed by a comparison of the HPLC methodology to the Griess-Saltzman reaction assay.

NO_2^- and total NO_3^-/NO_2^- were determined by Griess-Saltzman reaction.[13-15] NO_2^- was determined before NO_3^--reduction, and the total NO_3^-/NO_2^- was determined after reduction of NO_3^- to NO_2^- by nitrate reductase,[21] using nitrate/nitrite Cayman's Kit (Alexis Corporation, San Diego, CA, USA). The first step is measurement of NO_2^- by the Griess-Saltzman reaction before reduction in prepared samples. Standard $NaNO_2$ was made in concentrations of between 5 and 80 μM. Human plasma were deproteinized at a v:v of 1:1, lyophilized and reconstituted (concentrated 5 times) in Buffer pH = 7. The Griess reagent consisted of 1 part 0.1% naphthylethylenediamine dihydrochloride in distilled water and 1 part 1% sulfanilamide (or sulfanilic acid) in 5% concentrated H_3PO_4, the 2 parts being mixed together within 12 h of use and kept chilled. Addition of the Griess-Reagent to the samples converted NO_2^- yielding a deep purple azo compound. The second step was the stoichiometric reduction i.e. conversion of NO_3^- to NO_2^- utilizing 0.1 u/mL nitrate reductase, and addition of the Griess-Reagent which converted NO_2^- into a azo compound. Spectrophotometric measurement of absorbancy at 540 nm (spectrophotometer model MK-II Titertek Multy Skan-Plus, ICN Biomedical, Irvine, CA, USA) of the azo chromophore allowed determination of the nitrite concentration in the measured specimens.

Additionally, we obtained and analyzed plasma samples from 22-healthy adult volunteers. These samples were used for paired determination of nitrite and nitrate by the Griess-Saltzman reaction and compared to our method of anion-chromatography (HPLC) detection using the techniques as outlined above.

Recovery

The accuracy of our HPLC methodology was established by the "spiked" recovery approach. The recoveries for both anions were performed by spiking standards of the NO_2^- and NO_3^- into human control plasma. Intra-assay variation was estimated by calculating the mean and standard deviation of the concentration of six samples of two different spiked standards of NO_2^- and NO_3^-. Inter-assay variation was estimated from the concentration of spiked samples determined in six consecutive chromatographic assays. The precision of the assay was demonstrated by the coefficient of variation of the measured concentrations of the spiked NO_2^- and NO_3^- standards in human control plasma.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. NO_2^- and NO_3^- values are expressed as μmol/L. The ratio of NO_2^-:NO_3^- is presented as an index of relative partitioning of nitric oxide metabolism. Test statistics used include one factor and two factor analyses of variance (ANOVA). Results were considered significant at the .05 level.

RESULTS

Initial calibrations were performed by analyzing data from standard samples. The chromatography software program determined calibration levels for unknown component amounts in individual samples by comparing response peak areas with those responses obtained from known standard samples. In an attempt to improve the reliability of this approach, more than one standard was used. A standard calibration curve with five levels of concentration was used for NO_2^- (0.015-1.95 μg/mL) and NO_3^- (0.98-125 μg/mL) with a sample size of 20 μl. The area of the external standard curves generated linear correlation coefficients between 0.9986-1.0000 for NO_2^- and NO_3^-. The peak purity for NO_2^- and NO_3^- was 1.000. Detection limits were based on the ratio of peak analyte area to that of the external standard area.

NO_2^- and NO_3^- values were determined in 86 samples of control plasma (n=22) and urine (n=22) of healthy adults as well as in human macrophage cytosol (n=21) and supernatant (n=21). The peak for NO_2^- and NO_3^- anions appeared with different retention times and their recognition was by standard peak run times for both anions by UV-detection (Figures 1, 2 and 3). Each sample was calculated by software data acquisition and expressed as μmol/L.

Using direct UV-detection we found the plasma NO_2^- of 22 healthy controls to be 3.1 ± 0.4 μmol/L and NO_3^- 10.3 ± 0.7 μmol/L with a ratio of NO_2^-:NO_3^- = 0.3, and that urine NO_2^- was 380 ± 61 μmol/L and NO_3^- 1345 ± 270 μmol/L with a ratio of NO_2^-:NO_3^- = 0.3 (Table 1). Human macrophage cytosolic NO_2^- after washing and 2 h of incubation in media at 37°C was 0.66 ± 0.08 mol/L/10^6 Mϕ, and NO_3^- 0.68 ± 0.09 μmol/L/10^6 Mϕ with a ratio of NO_2^-: NO_3^- = 1, and extracellular (supernatant) NO_2^- was 0.03 ± 0.01 μmol/L/10^6 Mϕ and NO_3^- 3.03 ± 0.33 mol/L/10^6 Mϕ with a ratio of NO_2^-:NO_3^- = 0.01 (Table 2).

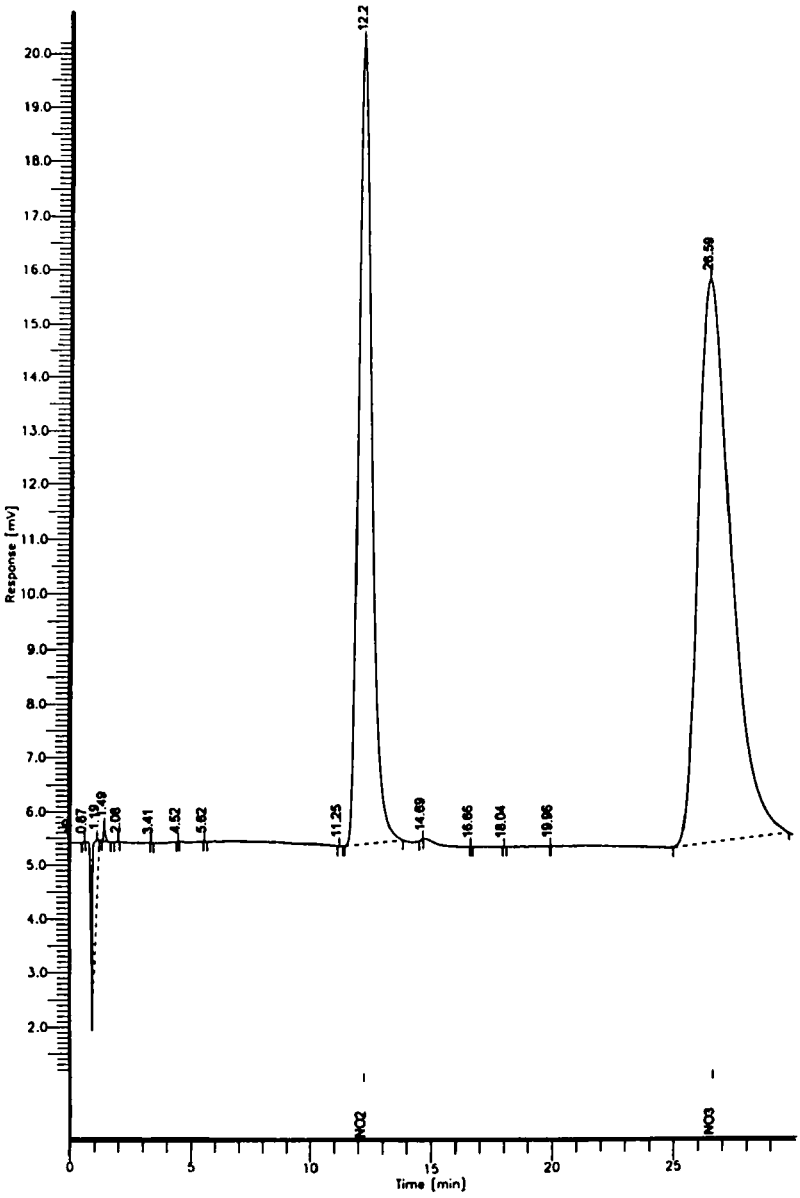

Figure 1. Chromatogram of HPLC Separation of NO_2^- and NO_3^- Anions: Standard.

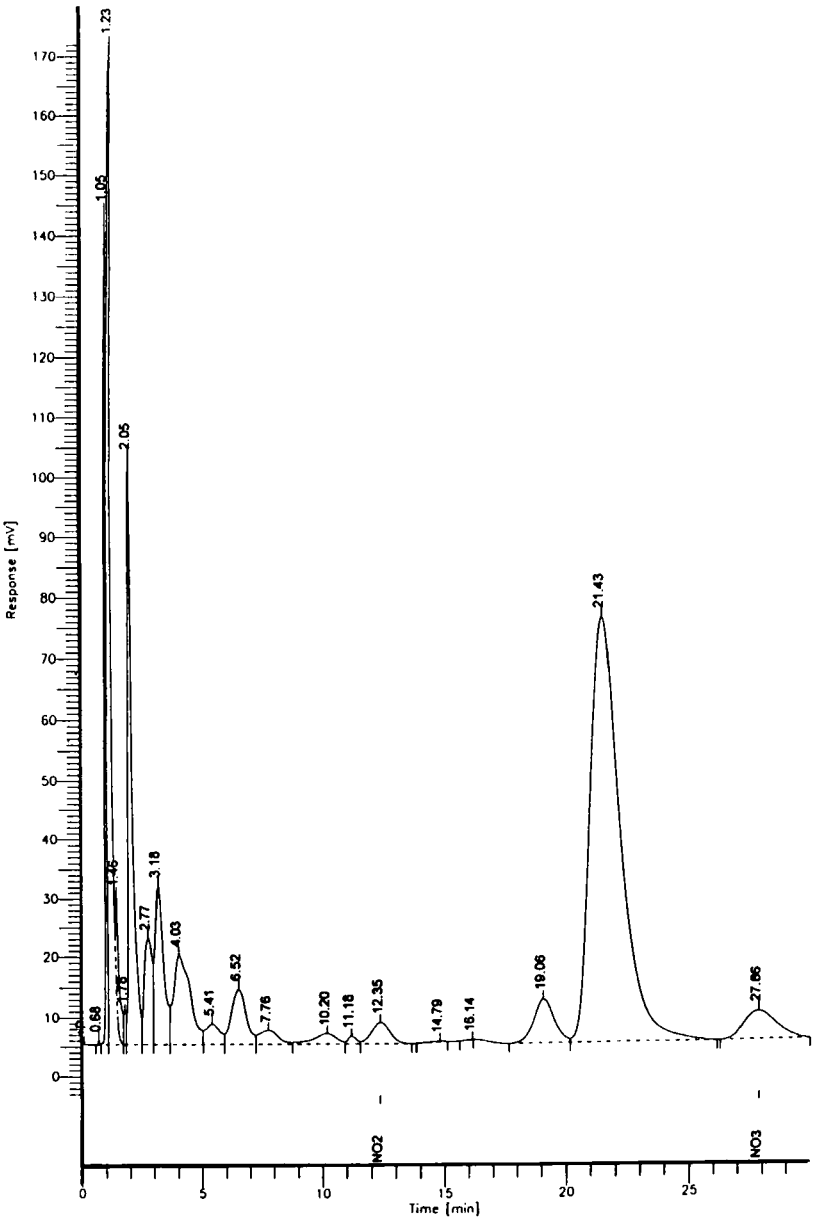

Figure 2. Chromatogram of HPLC Separation of NO_2^- and NO_3^- Anions: Control Human Plasma.

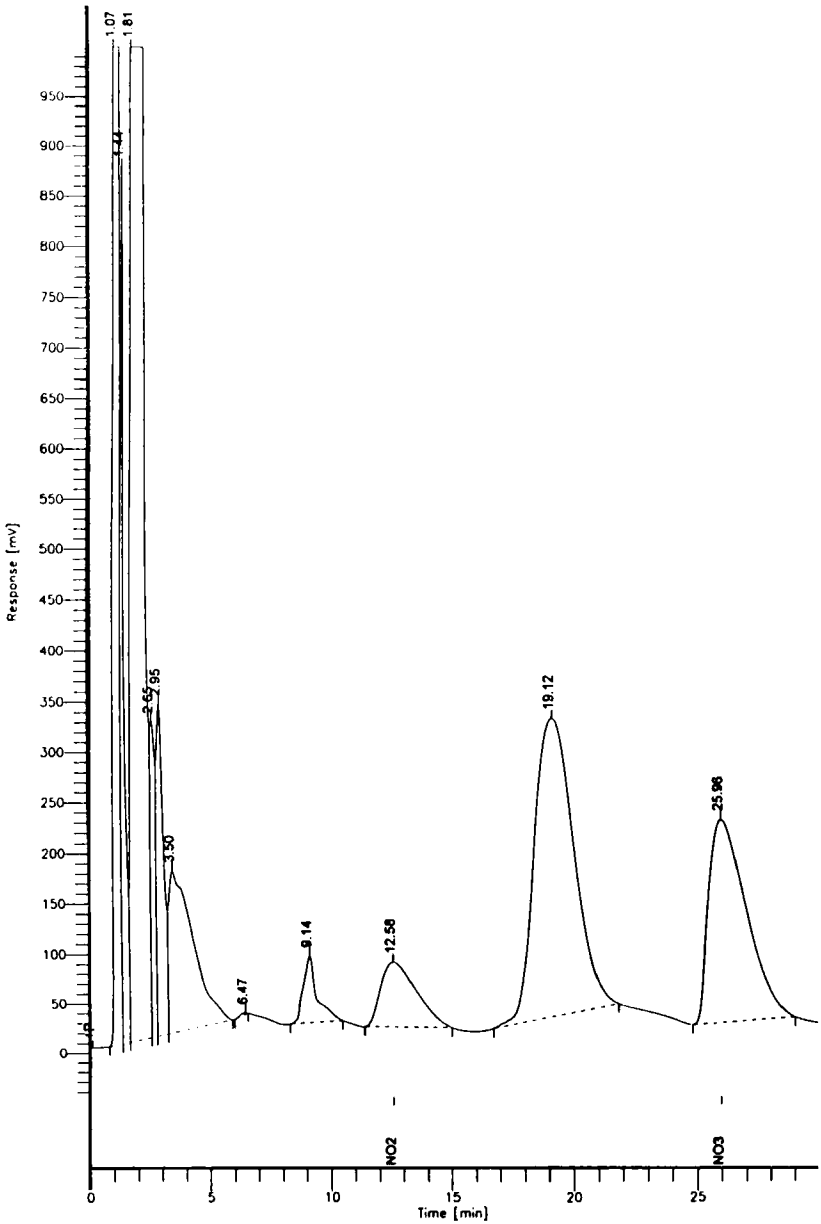

Figure 3. Chromatogram of HPLC Separation of NO_2^- and NO_3^- Anions: Control Human Plasma.

Table 1

Nitric Oxide Stable End Products Nitrite and Nitrate in Normal Human Plasm and Urine

	NO_2^- μmol/L	NO_3^- μmol/L	NO_2^-:NO_3^- Ratio
Plasma	3.1 ± 0.4	10.3 ± 0.7	0.3
Urine	380 ± 51	1345 ± 270	0.3

Table 2

Intercellular and Extracellular Nitrite and Nitrate in Human Macrophage Cell Line U-937

NO_2^- μmol/L/10^6Mϕ	NO_3^- μmol/L/10^6Mϕ	NO_2^-:NO_3^- Ratio	NO_2^- μmol/L/10^6Mϕ	NO_3^- μmol/L/10^6Mϕ	NO_2^-:NO_3^- Ratio
0.66 ± 0.08	0.68 ± 0.09	1	0.03 ± 0.01	3.03 ± 0.33	0.01

The average of recovery was 99.6 ± 0.9 % for NO_2^- and 99.4 ± 2.3% for NO_3^-. The precision of the assay was estimated with the coefficient of variation (Table 3). The precision of the assay over three consecutive days is shown on Table 4.

The intra-assay variabilities was calculated from concentrations of 31.2 μg/mL and 62.5 μ/mL of spiked plasma samples of NO_2^- and NO_3^- standards using the concentration determined for 6 samples that were analysed during the same analysis. The mean calculated concentration was 30.75 ± 0.25 μg/mL for NO_2^- and 30.28 ± 0.74 μg/mL for NO_3^- for the 31.2 μg/mL spiked samples, and 60.89 ± 0.21 μg/mL for NO_2^- and 59.61 ± 0.71 μg/mL for NO_3^- for the 62.5 μg/mL spiked sample. The percent coefficient of variation was 1.6%-6.0% and 0.8%-2.9% for the 31.2 μg/mL and 62.5 μg/mL spiked sample respectively (Table 5).

The inter-assay variability were calculated from the concentration of 15.6 μg/mL spiked samples of NO_2^- and NO_3^- determined during six assays run on consecutive day. The mean calculated concentration of 15.6 μg/mL spiked

Table 3

Recovery and Precision of NO_2^- and NO_3^- in Spiked Human Plasma Samples from Healthy Adults

	Concentration Spiked in Plasma μg/mL	Conc. Found in Plasma μg/mL	Coefficient of Variation %	Recovery %	Average Recovery %
NO_2^-	1.9	1.85 ± 0.02	2.16	97.37	
	3.9	3.92 ± 0.01	0.76	100.50	
	7.8	7.86 ± 0.03	1.02	100.77	
	15.6	15.31 ± 0.46	7.38	98.14	99.56 ± 0.94
	31.2	32.18 ± 0.59	4.54	103.14	
	62.5	60.89 ± 0.21	0.90	97.42	
NO_3^-	3.9	3.95 ± 0.05	0.23	101.28	
	7.8	8.44 ± 0.33	6.03	108.21	
	25.0	25.65 ± 0.62	3.48	102.60	
	62.5	59.95 ± 4.23	9.57	95.92	99.40 ± 2.30
	125.0	118.95 ± 4.05	4.58	95.08	
	225.0	210.00 ± 6.00	3.77	93.33	

Baseline amount in plasma samples before summplementation NO_2^- = 0.28 μg/mL; NO_3^- = 1.34 μg/mL.

Table 4

Precision Data for the NO_2^- Nno_3^- In Plasma Samples Spiked with 62.5 μg/mL of NO_2^- and NO_3^- Standards During Three Consecutive Days

Anion	Day	Mean Concentration μg/mL	Coefficient Variation %
NO_2^-	1	61.06 ± 0.22	0.87
	2	61.28 ± 0.19	0.70
	3	61.00 ± 0.21	0.87
NO_3^-	1	59.61 ± 0.71	2.90
	2	59.67 ± 0.69	2.85
	3	59.57 ± 0.70	2.87

Table 5

Intra-assay Variation (n = 6)

Spiked Concentration μg/mL	Mean Concentration	SD	Coefficient of Variation, %
NO_2^- 31.2 μg/mL	30.75 ± 0.25	0.50	1.63
NO_3^- 31.2 μg/mL	30.28 ± 0.74	1.82	6.01
NO_2^- 62.5 μg/mL	60.89 ± 0.21	0.52	0.85
NO_3^- 62.5 μg/mL	59.61 ± 0.71	1.73	2.90

samples was 15.22 ± 0.56 μg/mL for NO_2^- and 15.33 ± 0.37 μg/mL for NO_3^-. The percent coefficient of variation was 8.15 % and 5.87% for NO_2^- and NO_3^- respectively (Table 6).

Anion retention time and run times were alternated by changing eluent strength and flow rate without overlapping other anions. The individual specimen values of NO_2^- and NO_3^- were quantified by comparison to the external standard area. The added NO_2^-/NO_3^- standards in plasma appeared at the expected retention time and were 12.26 ± 0.09 min for NO_2^- and 27.01 ± 0.27 min for NO_3^- (Table 7).

The comparative analysis of the control plasma specimens revealed that there were no difference (p=0.1) for NO_2^- as detected by the Griess reaction [5.63 ± 1.02 μmol/L] and by our HPLC method [3.09 ± 0.36) μmol/L]. Also, there were no difference (p=0.2) for total NO_3^- /NO_2^- concentration detected by Griess-Saltzman reaction [17.28 ± 2.69 mol/L] and by HPLC [13.44 ● 0.63 mol/L] (Table 8). The HPLC technique had a sensitivity of 0.01 μmol/L separately for both anions.

DISCUSSION

High performance anion-exchange liquid chromatography in conjunction with a specific mobile phase of sodium chloride and sodium phosphate appears to represent a powerful and very sensitive method for the simultaneous separation and detection of NO_2^- and NO_3^- anions. This approach offers the advantages of rapid, simultaneous chromatographic detection, determination

Table 6

Inter-assay Variation (n = 6)

Spiked Concentration 15.6 μg/mL	Mean Concen. μg/mL	SD	Coefficient of Variation, %
NO_2^-	15.22 ± 0.56	1.24	8.15
NO_3^-	15.33 ± 0.37	0.90	5.87

Table 7

Retention Times of Spike NO_2^- and $NO3^-$ in Controlled Human Plasma

Retention Times (RT) in Minutes

	Mean Standard RT ± SE	Mean Plasma RT ± SE
NO_2^-	12.25 ± 0.04*	12.26 ± 0.09
NO_3^-	26.39 ± 0.15	27.01 ± 0.27

*p = ns for standard vs. plasma retention times.

Table 8

Comparison of the Griess Reaction to HPLC Determination of NO_2^- and Total NO_3^-/NO_2^- in Human Control Plasma

Anions	Griess	HPLC
NO_2^- μmol/L	5.63 ± 1.02^*	3.09 ± 0.36
Total NO_3^-/NO_2^- μmol/L	17.28 ± 2.69*	13.44 ± 0.63

^ = mean ± SE

*p = ns for Griess Reaction vs. HPLC

and the quantification of NO_2^- and NO_3^- anions at ambient temperature in biological fluids. We have found that this method worked well for intracellular and extracellular NO_2^- and NO_3^- detection in human biological samples. We were able to clearly separate simultaneously both anions in plasma, urine and macrophage cytosol/supernatant. It was necessary to develop this method for simultaneous determination of nitrite and nitrate anions, as present methods can not measure individually NO_3^- or NO_2 anions with high sensitivity.

Monitoring nitrite and nitrate generation could be of use in the monitoring of NO production,[22] or the status of NO-oxidation.[7] NO-production as well as NO-oxidation is associated with membrane receptor regulated oxygen consumption pathways.[23,24] An alternate pathway of oxygen consumption is for oxidative phosphorylation of ADP and P_i to yield ATP, where the electron-transport chain transfers electrons to oxygen in a series of exergonic reactions from cytochrome-c to cytochrome-aa_3 (cytochrome oxidase) and catalyzes the reaction of electrons and protons with molecular oxygen to produce water.[25] Nitric oxide generation,[22] and NO-oxidation which occurs when NO reacts with molecular oxygen producing NO_2 and NO_3^- [$2NO + O_2 \rightarrow 2NO_2$; $2NO_2$ 8 N_2O_4 $\xrightarrow{-H2O}$ $NO_2^- + NO_3- + 2H^+$] or with superoxide anion generating NO_3^- [$NO + O_2^- \rightarrow NO_3^-$],[7] both are important steps in cell respiration receptor regulated pathways.[23,24] The production of NO by human monocyte in this paper represent basal value of NO release. The human monocytes can be classified into two different populations: a low-NO-producing and high-NO-producing monocytes. These differences likely result from the differential in vivo activation of these cells.[26]

Previous methods, have determined nitrate by colorimetric techniques where nitration of phenol-2,4-disulfonic acid or nitration of brucine yields an orange-brown solution, which can be determined colorimetrically . NO_2^- has been determined by the formation of 5-nitro-2,4-xylen-1-ol which with dilute sodium hydroxide forms a red salt. This involves, first, the reaction in acid solution of a primary amine such as sufanilic acid or sulfanilamide with nitrite to form a diazonium salt. The latter is then coupled to an aromatic amine to yield the red azo-die (diazonium salt) whose concentration can be determined in a colorimeter.[8]

Automated procedures for the analysis of nitrate via the reduction of nitrate to nitrite with a high-pressure cadmium column, are based on the Griess reaction. In the Griess reaction NO_2^- reacts with 1% sulfanilamide in 5% H_3PO_4 / 0.1% naphthalene-ethylenediamine dihydrochloride forming a purple azo dye. Using high performance liquid chromatography with a cadmium column all NO_3^- reduces to NO_2^- at high pressures through a column packed

with fine particles of copper-plated cadmium metal. The color of the product dye is developed by a 60°C water bath and followed by cooling at 0°C absorbance at 546 nm is detected by flow through a spectrophotometer and nitrate quantification determined.[9] Other methods use conversion of nitrate to nitrite with reductase and then nitrite is transformed to a chromophore via the Griess reagent with detection at 543nm and quantified spectrophotometrically.[27] Recently a kinetic cadmium reduction method has been developed, where NO_3^- is reduced to NO_2^- by copper-coated cadmium granules and nitrite determined by diazotization with sulfanilamide solution followed by N-naphthylethylene diamine.[16] Using ion-exchange HPLC column and a mobile phase of 50 mM H_3PO_4 with 2% (v/v) tetrahydrofuran at pH = 1.9 Romero and coworkers,[28] have been able to determine only NO_3^- anions in cytosol of Anacystis cells, but they could not determine NO_2^- anion by ion chromatography.

Previous investigators,[13] using ion chromatographic methods and an eluent solution of 0.75 mM $NaHCO_3$ and 2.2 mM Na_2CO_3 have been able also to determine only NO_3^- anions in serum and ocular fluid of healthy cattle, but nitrite could not be determined, and the detection limits was 0.01 mM/L Similar sensitivity limits have been reported in human plasma of 0.01 mM/L for NO_3^- anion which was in the concentration ranged from 0.03-0.12 mM/L (mean=0.06), but NO_2^- concentration were not detecable using a mobile phase of 50 mM NaH_2PO_4, 3 mM NaCl and 4 mM acetic acid at pH = 3.95.[17] Moreover, the sensitivity limits have been increased to 8 µmol/L for NO_3^- and 2 µmol/L for NO_2^- using ion-pair chromatography in rat plasma.[18]

Using anion exchange chromatography and direct UV-detection at a 210 nm wavelength we were able to determinate NO_2^- in our samples, but previous methods could not determine this anion in intracellular fluid and in plasma.[9,10,16,17,29] Human macrophages generates nitric oxide and the stable end products NO_2^- and NO_3^- anions, which primarily released immediately outside of cell to the intercellular space,[3-5,26] in that reason we found four times higher concentration of nitrate in extracellular fluid.

The novel aspect of our method is the use of the anion exchange chromatography with a specific mobile phase of an aqueous solution of 20 mM sodium chloride with 1 mM mono sodium phosphate at pH = 7, ambient temperature and a UV-wavelength of 210 nm, which allowed the simultaneous direct detection of intracellular and extracellular nitrite and nitrate with a sensitivity of 0.01 mol/L for both anions.

Previous investigators,[13] using ion chromatographic methods and an eluent solution of 0.75 mM $NaHCO_3$ and 2.2 mM Na_2CO_3 have been able also to determine only NO_3^- anions in serum and ocular fluid of healthy cattle, but nitrite could not be determined, and the detection limits was 0.01 mM/L Similar sensitivity limits have been reported in human plasma of 0.01 mM/L for NO_3^- anion which was in the concentration ranged from 0.03-0.12 mM/L (mean=0.06), but NO_2^- concentration were not detecable using a mobile phase of 50 mM NaH_2PO_4, 3 mM NaCl and 4 mM acetic acid at pH = 3.95.[17] Moreover, the sensitivity limits have been increased to 8 µmol/L for NO_3^- and 2 µmol/L for NO_2^- using ion-pair chromatography in rat plasma.[18]

Using anion exchange chromatography and direct UV-detection at a 210 nm wavelength we were able to determinate NO_2^- in our samples, but previous methods could not determine this anion in intracellular fluid and in plasma.[9,10,16,17,29] Human macrophages generates nitric oxide and the stable end products NO_2^- and NO_3^- anions, which primarily released immediately outside of cell to the intercellular space,[3-5,26] in that reason we found four times higher concentration of nitrate in extracellular fluid.

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