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Rapid determination of nitrite by reversed-phase high-performance liquid chromatography with fluorescence detection

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Abstract

Measurement of nitrite and nitrate, the stable oxidation products of nitric oxide (NO), provides a useful tool to study NO synthesis in vivo and in cell cultures. A simple and rapid fluorometric HPLC method was developed for determination of nitrite through its derivatization with 2,3-diaminonaphthalene (DAN). Nitrite, in standard solution, cell culture medium, or biological samples, readily reacted with DAN under acidic conditions to yield the highly fluorescent 2,3-naphthotriazole (NAT). For analysis of nitrate, it was converted to nitrite by nitrate reductase, followed by the derivatization of nitrite with DAN to form NAT. NAT was separated on a 5-μm reversed-phase C_8 column (150×4.6 mm, I.D.) guarded by a 40-μm reversed-phase C_{18} column (50×4.6 mm, I.D.), and eluted with 15 m*M* sodium phosphate buffer (pH 7.5) containing 50% methanol (flow-rate, 1.3 ml/min). Fluorescence was monitored with excitation at 375 nm and emission at 415 nm. Mean retention time for NAT was 4.4 min. The fluorescence intensity of NAT was linear with nitrite or nitrate concentrations ranging from 12.5 to 2000 n*M* in water, cell culture media, plasma and urine. The detection limit for nitrite and nitrate was 10 pmol/ml. Because NAT is well separated from DAN and other fluorescent components present in biological samples, our HPLC method offers the advantages of high sensitivity and specificity as well as easy automation for quantifying picomole levels of nitrite and nitrate in cell culture medium and biological samples.

Keywords: Nitrite; nitrate

1. Introduction

The diverse physiological and pathological roles for nitric oxide (NO) in the cardiovascular, immune and nervous systems [1–3] have led to the development of various methods for determining NO synthesis [4,5]. Because NO is a free radical molecule released by cells in picomolar to nanomolar ranges and has a very short half-life [6], a direct measurement of its production is difficult. Thus, the analysis of nitrite and nitrate, the stable products of NO oxidation, is often performed to estimate NO synthesis in biological systems and cell cultures [4,7].

The commonly employed methods for nitrite determination have included the Griess colorimetric assay [8], the chemiluminescence analysis [9], and

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the fluorometric method [10]. For nitrate analysis, it is converted to nitrite either by reducing metals such as cadmium [11] or by nitrate reductase [8,12]. The Griess assay is based on the two-step diazotization reaction in which nitrite is chemically transformed into a colored azo dye. Although the Griess reaction is simple, the detection limit for nitrite and nitrate by a UV–VIS spectrophotometer is only 1–2 μ*M* [10–12]. The lack of sensitivity severely restricts the application of this colorimetric method for quantifying submicromolar levels of nitrite and nitrate in biological samples. The chemiluminescence assay was originally developed to measure NO in air samples, which involves the reaction of NO with ozone to generate light for detection by a photomultiplier tube, and fewer than 10 pmol NO can be detected [13]. This method can be adapted to analyze nitrite and nitrate in aqueous solutions after the release of NO from nitrite or nitrate under acid–iodide or stronger reducing conditions [9,14]. The chemiluminescence assay, however, requires an expensive and bulky apparatus, which is not available in most laboratory settings, and is interfered by N^G-nitro-L-arginine (a commonly used inhibitor of NO synthase) and some nitroso compounds (e.g., *S*-nitrosothiols and nitrosodiphenylamine) [9,14]. The batch fluorometric assay is based on the reaction of nitrite with 2,3-diaminonaphthalene (DAN) under acidic conditions to yield the highly fluorescent product 2,3-naphthotriazole (NAT) [15] (Fig. 1), and can be used to detect 10–20 n*M* nitrite and nitrate [10]. However, there is a great difficulty in employing the batch fluorometric method to detect picomole levels of nitrite and nitrate in cell culture medium and biological samples, because of high blank values as well as the fluorescence quenching and interference by biological components and colorimetric chemicals.

A number of ion-exchange [7,16,17] and reversed-phase ion-paired [18,19] HPLC methods have been developed for measuring nitrite and nitrate in biological systems, with detection by either UV–VIS absorbance [7,17–19] or conductivity [16]. Most HPLC methods require several purification steps to remove interfering substances such as chloride [16] and biogenic amines [17]. These additional preparative steps may cause variable recovery and introduce contamination by environmental nitrite and nitrate. In addition, the HPLC methods with UV–VIS or conductimetric detection suffer from low sensitivity [7,16–19] compared with fluorescence and chemiluminescence assays [10,9,13,14]. Thus, quantification of picomole levels of nitrite and nitrate in cell culture medium and biological samples is still a challenge.

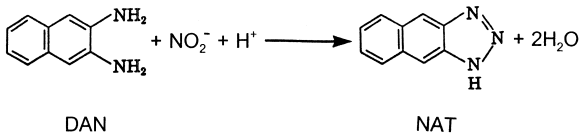

Fig. 1. Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NAT) under acidic conditions.

The objective of this study was therefore to develop a rapid, sensitive and specific HPLC method for measuring nitrite and nitrate. This method involves the reaction of nitrite with DAN to form NAT, the chromatographic separation of NAT, and the fluorescence detection of NAT. Thus, our HPLC method offers high sensitivity and specificity as well as easy automation for determining picomole levels of nitrite and nitrate in cell culture medium and biological samples.

2. Experimental

2.1. Chemicals

HPLC-grade methanol and water were purchased from Fisher Scientific (Houston, TX, USA) and were used for preparation of mobile phase solution. Double-distilled and deionized water (DD-water) was used for preparing other solutions. DAN, sodium phosphate, sodium nitrite, sodium nitrate and lipopolysaccharide (LPS) (from *Escherichia coli* serotype 0127:B8) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and amphotericin B were obtained from Gibco (Grand Island, NY, USA). Dialyzed fetal bovine serum (FBS) was obtained from Summit (Greeley, CO, USA). Nitrate reductase and NADPH were obtained from Boehringer Mannheim (Indianapolis, IN, USA).

2.2. Culture medium and biological samples

2.2.1. Bovine venular endothelial cell culture medium

Bovine venular endothelial cells were isolated from coronary venules (15 μm in diameter) using a bead perfusion technique as previously described [20,21]. Endothelial cells (2×10^6) were cultured at 37°C in 6 ml of phenol red-free DMEM containing 0.4 m*M* L-arginine, 0.5 m*M* L-glutamine, 11 m*M* D-glucose, 20 m*M* Hepes (pH 7.4), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2% dialyzed FBS, and 10 units/ml heparin in a humidified CO_2/air atmosphere. After a 48-h culture period, the conditioned medium was stored at −80°C until analyzed. Culture medium without cells was used as a blank.

2.2.2. Rat plasma

Rats (325–350 g), which had free access to DD-water and a nitrite- and nitrate-free semipurified diet [22], were anesthetized with CO_2. Blood samples (3 ml) were then withdrawn from the heart into heparinized tubes. Blood was immediately centrifuged at 3000 *g* for 10 min. The plasma (supernatant) was collected and stored at −80°C until analyzed.

2.2.3. Rat urine

Rats (325–350 g), which had free access to DD-water and a nitrite- and nitrate-free semipurified diet [22], were placed individually in metabolism cages. Urine was collected for 24 h from rats following intraperitoneal administration of saline or LPS (1 mg/kg body wt), as previously described [22]. After centrifugation at 3000 *g* for 10 min to remove solid matter, urine was stored at −80°C until analyzed.

2.2.4. RAW 264.7 cell culture medium

It is desirable to compare our HPLC method with the batch fluorometric method [10] for determining both nitrite and nitrate. Because the batch fluorimetric method failed to detect nitrite in endothelial cell culture medium, plasma and urine, or nitrate in endothelial cell culture medium, due to particularly high blank values and interference, we used the LPS-activated RAW 264.7 cell (a murine macrophage cell line) which is known to produce large amounts of both nitrite and nitrate [23]. RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells (2×10^5) were cultured at 37°C in 1.0 ml of phenol red-free DMEM containing 25 m*M* Hepes (pH 7.4), 0.4 m*M* L-arginine, 0.5 m*M* L-glutamine, 10 m*M* D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 1% dialyzed FBS, and 0.5 μg/ml LPS in a humidified CO_2/air atmosphere. After a 48-h culture period, the conditioned medium was stored at −80°C until analyzed. Culture medium without cells was used as a blank. RAW 264.7 cells produce little nitrite or nitrate in the absence of LPS [23].

2.3. Dilution and filtration of culture media and biological samples

The dilution of cell culture media and all biological samples was made with DD-water. For nitrite analysis, endothelial cell culture medium, plasma, urine (from rats not treated with LPS), and RAW 264.7 cell culture medium were diluted 1:3, 1:10, 1:10 and 1:50, respectively. For nitrate analysis, endothelial cell culture medium, plasma, urine (from rats not treated with LPS), and RAW 264.7 cell culture medium were diluted 1:6, 1:100, 1:2000 and 1:50, respectively. For analysis of nitrite and nitrate in urine from rats treated with LPS, urine samples were diluted 1:40 and 1:8000, respectively. All of these diluted samples (culture media, plasma and urine) were filtered through a 10-kDa cutoff ultrafilter (Pall Filton, Northborough, MA, USA) at 14 000 *g* for 15 min to remove high-molecular weight proteins. Because some filters may contain nitrite/nitrate, all ultrafilters were washed four times with DD-water (0.5 ml for each wash) before use to avoid contamination. Nitrite concentrations in the first, second, third and fourth 0.5-ml fractions from the filters were 3931±316, 282± 24, 159±11, and 62± 3.2 pmol/ml (means±SEM, $n=4$), respectively. The latter value was similar to nitrite concentration (61 pmol/ml) in DD-water. Nitrate concentrations in the first, second, third and fourth 0.5-ml fractions from the filters were 0.14±0.01 nmol/ml (means±SEM, $n=4$), similar to nitrate concentration in DD-water (0.13 nmol/ml). These data indicate substantial amounts of nitrite but little nitrate in the

ultrafilters used. The recovery of nitrite and nitrate from the ultrafilters was 100%, as determined with known amounts of sodium nitrite and sodium nitrate standards.

2.4. Conversion of nitrate into nitrite

Nitrate was converted to nitrite enzymatically by nitrate reductase as previously described [12], with some modifications in the concentrations of enzyme and NADPH used. A solution, which consisted of 200 μl of nitrate standard (0–2 μ*M*) or sample (ultrafiltered culture medium or biological samples), 10 μl of 1 U/ml nitrate reductase and 10 μl of 120 μ*M* NADPH, was incubated at room temperature for 1 h. After the conversion of nitrate to nitrite, this solution was used directly for nitrite analysis. The rate of conversion of nitrate to nitrite was 98%, as determined with known amounts of sodium nitrate and sodium nitrite standards.

2.5. HPLC analysis of the NAT formed from nitrite and DAN

2.5.1. Reaction of nitrite with DAN to yield NAT

DAN reacts rapidly with nitrite under acidic conditions to form the highly fluorescent product NAT [15] (Fig. 1), which is stable in alkaline solution [24]. Here we used the procedure reported by Misko et al. [10] to convert nitrite and DAN to NAT. For HPLC analysis of nitrite, 100 μl of nitrite standard (0–2 μ*M*) or sample (ultrafiltered culture medium or biological samples, or nitrate-derived nitrite solution) were incubated at 24°C with 10 μl of 316 μ*M* DAN (in 0.62 *M* HCl) for 10 min, followed by addition of 5 μl of 2.8 *M* NaOH. This reaction mixture was directly used for the chromatographic separation of NAT.

2.5.2. HPLC apparatus and chromatographic procedure

The Waters HPLC apparatus consisted of a Waters Model 600E Powerline delivery system with 100-μl heads, a Model 712 WISP autosampler, a 5-μm reversed-phase C_8 column (150×4.6 mm, I.D.) guarded by a 40-μm reversed-phase C_{18} guard column (50×4.6 mm, I.D.), a Model 474 fluorescence detector, and a Model 810 baseline Workstation (Waters, Milford, MA, USA). Both columns were obtained from Supelco (Bellefonte, PA, USA). The C_{18}-guard column was employed to achieve better separation of NAT from DAN and also greatly extended the C_8-column lifetime. An aliquot (0.1 ml) of the derivatized nitrite-DAN solution was transferred to a plastic insert tube placed in a 5-ml brown glass vial. This vial was placed onto the autosampler, which was programmed to inject 15 μl sample into the HPLC column. The mobile phase (1.3 ml/min) was 15 m*M* sodium phosphate buffer (pH 7.5) containing 50% methanol (0.0–3.0 min), followed sequentially by 100% HPLC-grade water (3.1–5.0 min), 100% methanol (5.1–8.0 min), 100% HPLC-grade water (8.1–10.0 min), and the initial 15 m*M* sodium phosphate buffer (pH 7.5)–50% methanol solution (10.1–15.0 min). The use of 100% HPLC-grade water before and after 100% methanol was necessary to prevent an abrupt marked increase in column pressure, and was sufficient to regenerate the columns for automatic analysis of multiple samples. All chromatographic procedures were performed at room temperature. Fluorescence was monitored with excitation at 375 nm and emission at 415 nm. Peak integration was performed by a Model 810 baseline Workstation.

2.6. Batch fluorometric analysis of the NAT formed from nitrite and DAN

For batch fluorometric analysis of nitrite, which requires a minimum volume of 1.2 ml in a 1-cm pathlength cuvette, 1.2 ml of nitrite standard or sample (ultrafiltered culture medium or biological samples, or nitrate-derived nitrite solution) were incubated at 24°C with 120 μl of 316 μ*M* DAN (in 0.62 *M* HCl) for 10 min, followed by addition of 60 μl of 2.8 *M* NaOH. This reaction mixture was directly used for NAT analysis by a Model 450 Turner fluorometer (Dubuque, IA, USA) with excitation at 375 nm and emission at 415 nm.

2.7. Statistical analysis

Results were analyzed by unpaired *t*-test [25]. Probability values <0.05 were taken to indicate statistical significance.

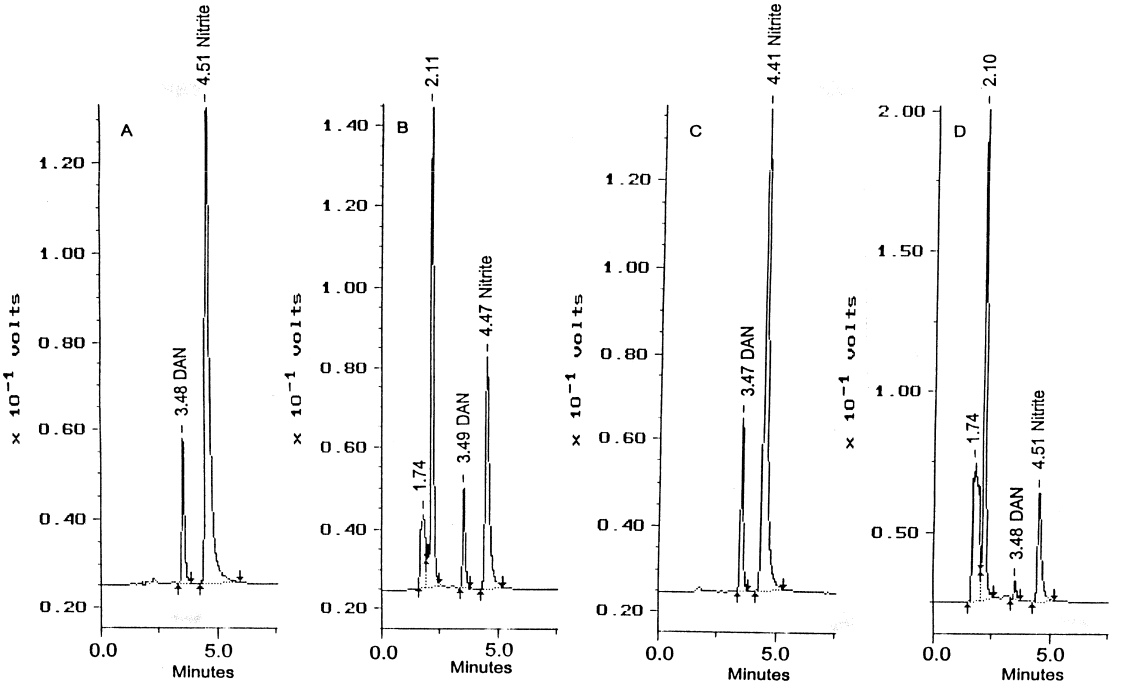

Fig. 2. HPLC analysis of nitrite. Nitrite reacted with 2,3-diaminonaphthalene (DAN) to yield 2,3-naphthotriazole under acidic conditions. The nitrite-DAN derivative was separated by reversed-phase HPLC, followed by fluorescence detection: (A) 200 n*M* nitrite standard; (B) endothelial cell culture medium; (C) plasma; (D) urine.

3. Results and discussion

3.1. Chromatographic separation of NAT

Figs. 2 and 3 show typical HPLC chromatograms for the analysis of nitrite and nitrate, respectively, in DD-water, endothelial cell culture medium, plasma and urine. Under the experimental conditions used, the nitrate reduction step did not interfere with the subsequent reaction of nitrite with DAN to form NAT or the chromatographic separation of NAT. The retention times for DAN and the nitrite-DAN derivative (NAT) were 3.48±0.001 and 4.43±0.012 min (means±SEM, $n=14$), respectively. The fluorescence intensity for NAT is at least 90–100-fold higher than that observed for an equimolar concentration of DAN when the solution is excited at 375 nm and photon emission is monitored at 415 nm [23]. However, the excess of DAN employed in the derivatization reaction still significantly contributes to high blank values when picomole levels of nitrite and nitrate are determined with a conventional batch fluorometer. It is noteworthy that DAN and fluorescence substances present in cell culture medium and biological samples were well separated from NAT on the HPLC system and did not interfere with detection of NATs (Figs. 2 and 3). Thus, our HPLC method offers greater specificity for determining nitrite and nitrate in cell culture medium and biological samples than all the batch fluorometric, colorimetric and chemiluminescence methods.

3.2. Stability, linearity and detection limit of NAT

At room temperature, NAT concentrations in the assay mixtures of DD-water, cell culture media, plasma and urine at 24 h after the derivatization of nitrite with DAN were 102.1±0.04, 99.5±0.79, 101.7±0.22 and 102.3±0.21% (means±SEM, $n=5$), respectively, of the initial corresponding values obtained immediately after the derivatization of nitrite with DAN. These data indicate that NAT was stable in alkaline solution for at least 24 h at room temperature. Using our HPLC method, the linearity

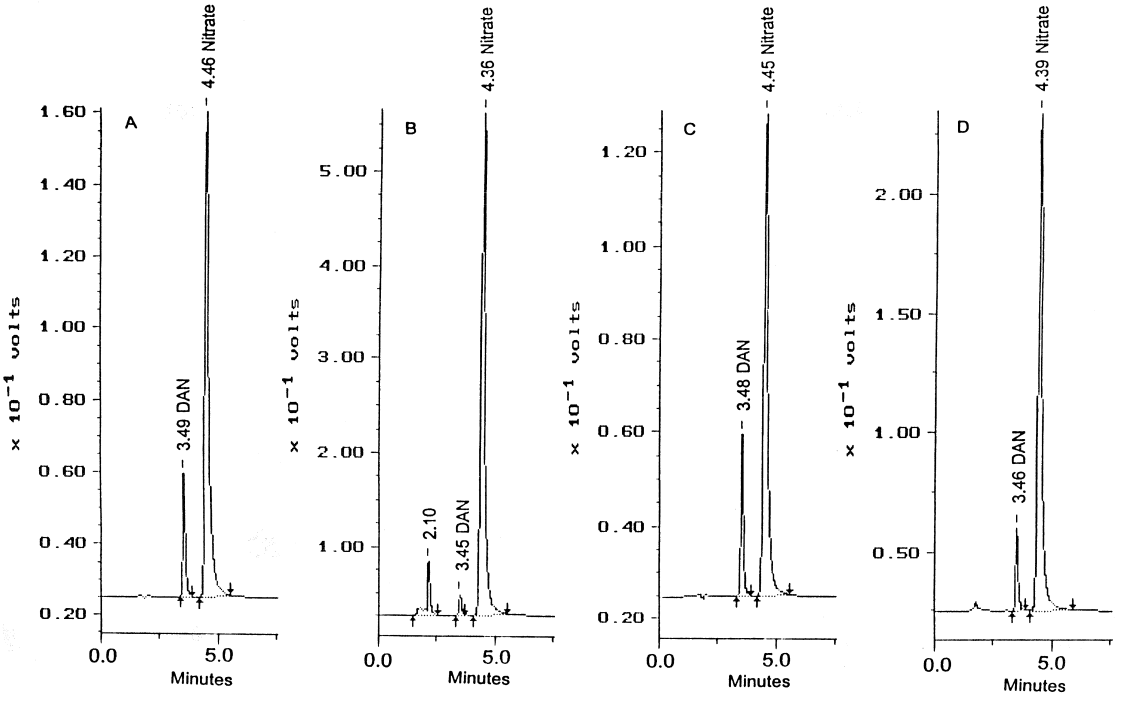

Fig. 3. HPLC analysis of nitrate. Nitrate was converted to nitrite by nitrate reductase. Nitrite then reacted with 2,3-diaminonaphthalene (DAN) to yield 2,3-naphthotriazole under acidic conditions. The nitrite-DAN derivative was separated by reversed-phase HPLC, followed by fluorescence detection: (A) 200 n*M* nitrate standard; (B) endothelial cell culture medium; (C) plasma; (D) urine.

of NAT fluorescence intensity was obtained for nitrite and nitrate solutions ranging from 12.5 to 2000 n*M* in DD-water, cell culture medium, plasma and urine, as indicated by correlation coefficients of 0.998, 0.999, 0.999 and 0.991, respectively, for nitrite analysis, and by correlation coefficients of 0.996, 0.989, 0.994 and 0.994, respectively, for nitrate analysis. The detection limit for nitrite and nitrate was 10 pmol/ml, based on a signal-to-noise ratio of 3, when using the Waters 474 Fluorescence Detector at a gain setting of 100 (maximum setting of up to 1000). The sensitivity of our HPLC method for nitrite and nitrate analysis even at a submaximal gain setting is similar to that of the batch fluorometric method (detection limit, 10–20 pmol/ml) [10] and is much greater than that of the Griess colorimetric assay (detection limit, 1–2 nmol/ml) [10–12] and the HPLC methods with UV–VIS or conductimetric detection (detection limit, 0.1–0.5 nmol/ml) [7,16]. The detection limits of the chemiluminescence method for nitrite and nitrate have been reported to be 1 and 80 pmol/ml, respectively [9], and thus this assay is less sensitive than our HPLC method for nitrate analysis.

3.3. Precision and accuracy of the fluorometric HPLC method for nitrite and nitrate analysis

The precision (agreement between replicate measurements) of our HPLC method for the analysis of nitrite or nitrate was evaluated by the relative deviation (mean of absolute deviation/mean of replicate measurements×100%) [26]. The values of relative deviation for DD-water, cell culture medium, plasma and urine samples were 0.42±0.02, 1.1±0.08, 0.90±0.03, and 1.5±0.09% (means±SEM, $n=4$), respectively, for nitrite analysis, and were 0.56±0.03, 1.6±0.08, 0.43±0.02, and 1.2±0.11% (means±SEM, $n=4$), respectively, for nitrate analysis. The accuracy (the nearness of an experimental value to the true value) of our HPLC method for the analysis of nitrite and nitrate was

evaluated, with known amounts of sodium nitrite and sodium nitrate standards (0.2–2 μM), by the relative error [(measurement value−true value)/true value× 100%)] [26]. The values of relative errors for DD-water, cell culture medium, plasma and urine samples were 0.12±0.01, 2.4±0.13, 1.5±0.08, and 3.3±0.21% (means±SEM, n=4), respectively, for nitrite analysis, and were 0.19±0.02, 2.8±0.15, 1.7±0.14, and 1.2±0.10% (means±SEM, n=4), respectively, for nitrate analysis. These data indicate the high reproducibility and reliability of our HPLC method for determining nitrite and nitrate in water, culture medium and biological samples.

3.4. Quantification of nitrite and nitrate in cell culture medium and biological samples

Using the batch fluorometric method [10], we failed to detect nitrite and nitrate in endothelial cell culture medium, or nitrite in urine and plasma, due to particularly high blank values and interference. In contrast, NAT was well separated from DAN and other fluorescent components on the HPLC system (Figs. 2 and 3), thereby improving the specificity and sensitivity of the HPLC method for analysis of nitrite and nitrate in cell culture medium and biological samples. Thus, our HPLC method had been used successfully to quantify picomole levels of nitrite and nitrate in DD-water and endothelial cell culture medium, as well as nitrite and nitrate in urine and plasma (Table 1). Because NO synthesis (estimated on the basis of nitrite plus nitrate production) by cultured bovine venular endothelial cells is low (156 pmol/10^6 cells/h), our HPLC method is necessary for quantifying basal NO generation by constitutive NO synthase particularly when blank culture medium contained picomolar to nanomolar levels of nitrite and nitrate. We also noted that 40 μM of phenol red, which is commonly present in cell culture media, did not interfere with the derivatization of nitrite with DAN to form NAT or the chromatographic separation of NAT. As a result, our method can be employed for nitrite and nitrate analysis in cell culture media containing phenol red.

Either our HPLC method (Table 2) or the batch fluorometric method [10] can be used to determine the production of relatively large amounts of nitrite and nitrate by inducible NO synthase. These two methods yielded similar values of nitrite and nitrate concentrations in the culture medium of LPS-activated RAW 264.7 cells (Table 2), further indicating the reliability of our HPLC method for nitrite and nitrate determination. On the basis of nitrite and nitrate concentrations in blank and conditioned culture media (Table 2), NO production by LPS-activated RAW 264.7 cells is estimated to be 2.6 nmol/10^6 cells per h, approximately 20 times that by unstimulated bovine venular endothelial cells. Although both the HPLC and the batch fluorometric methods are applicable to the quantification of large amounts of nitrite and nitrate, it should be recognized that an additional advantage of our HPLC method is its easy automation, which allows for the analysis of large numbers of samples during daytime and at night.

The dilution of cell culture medium, plasma and urine with DD-water greatly minimizes the influences of some compounds (e.g., NADPH, glutathione and dithiothreitol) present in these samples on the derivatization of nitrite with DAN to yield NAT

Table 1
Determination of nitrite and nitrate in distilled water, culture medium, plasma and urine by the fluorometric HPLC method[a]

	Nitrite (pmol/ml)	Nitrate (nmol/ml)
DD-water	61±0.17	0.13±0.001
Effluent from ultrafilter[b]	62±3.2	0.14±0.001
Blank culture medium[c]	144±1.2	3.1±0.48
EC culture medium[d]	415±20	5.3±0.29
Rat plasma	690±119	13.6±0.80
Rat urine (−LPS)[e]	682±66	605±69
Rat urine (+LPS)[f]	12 682±2892*	12 233±1586*

[a] The fluorometric HPLC method described in this study was used to analyze nitrite and nitrate in DD-water, endothelial cell (EC) culture medium, plasma and urine. Data are means±SEM, n=5. *P<0.01: different from the rats not treated with LPS.

[b] Ultrafilters (10-kDa cutoff) were washed four times with DD-water (0.5 ml for each wash), and the fourth 0.5-ml effluent from the filter was collected for nitrite and nitrate analysis.

[c] Phenol red-free Dulbecco's modified Eagle's medium (DMEM).

[d] Bovine venular EC (2×10^6) were cultured for 48 h at 37°C in 6 ml of phenol red-free DMEM for 48 h, and conditioned media were used for nitrite and nitrate analysis.

[e] Urine samples were obtained from rats not treated with LPS.

[f] Urine samples were obtained from rats treated with LPS (1 mg/kg body weight).

Table 2
Comparison of the fluorometric HPLC method and the conventional batch fluorometric method for determining nitrite and nitrate in the culture medium of activated RAW 264.7 cells[a]

Analytical method	Blank culture medium[b]		RAW 264.7 cell culture medium[c]	
	Nitrite (nmol/ml)	Nitrate (nmol/ml)	Nitrite (nmol/ml)	Nitrate (nmol/ml)
Fluorometric HPLC method	0.14±0.001	2.9±0.42	12.3±0.20	15.7±0.79
Batch fluorometric method	ND	ND	12.1±0.36	15.3±0.92

[a] The fluorometric HPLC method described here and the conventional batch fluorometric method [10] were employed for nitrite and nitrate analysis. Both methods are based on the same chemical reaction of nitrite with 2,3-diaminonaphthalene to form 2,3-naphthotriazole under acidic conditions. Data are means±SEM, $n=8$. ND, not detected.

[b] Phenol red-free Dulbecco's modified Eagle's medium (DMEM).

[c] RAW 264.7 cells (2×10^5) were cultured for 48 h at 37°C in 1.0 ml of phenol red-free DMEM containing 0.5 μg/ml lipopolysaccharide, and conditioned media were used for nitrite and nitrate analysis.

[10,23]. In our routine assays, urine samples were diluted 1:2000 or 1:8000 with DD-water for nitrate analysis, and endothelial cell culture medium was diluted 1:3 or 1:6 with DD-water for nitrite and nitrate determination. We found that such diluted urine and cell culture medium samples could be used directly for converting nitrate to nitrite by nitrate reductase and for nitrite analysis, without the need of prior filtration through 10-kDa cutoff ultrafilters. However, to accurately calculate the nitrite and nitrate concentrations in biological samples and cell culture media, it is still important to include appropriate background controls such as blank culture medium when constructing the standard curves for nitrite and nitrate. Determination of nitrite and nitrate is preferred to the measurement of citrulline (a product of NO synthase) for quantifying NO synthesis by mammalian cells, because citrulline can be recycled into arginine in various cell types including macrophages, endothelial cells, smooth muscle cells and neurons [27].

In conclusion, the simple and rapid fluorometric HPLC method described here offers high sensitivity and specificity as well as easy automation for determining picomole levels of nitrite and nitrate in small volumes of cell culture media and biological samples. This new analytical method may provide a useful tool for investigating NO synthesis in vitro and in vivo under physiological and pathophysiological conditions. As there is growing interest in the arginine-dependent NO pathway [27], our HPLC method for nitrite and nitrate analysis may greatly facilitate research in this ever-expanding field.

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