Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 851 (2007) 71–82
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Analysis of nitrite and nitrate in biological samples using high-performance liquid chromatography ☆

Wenjuan S. Jobgen [a], Scott C. Jobgen [a], Hui Li [a], Cynthia J. Meininger [b], Guoyao Wu [a,b,*]

[a] *Department of Animal Science and Faculty of Nutrition, Texas A&M University, College Station, TX 77843, USA*
[b] *Department of Systems Biology and Translational Medicine and Cardiovascular Research Institute, Texas A&M Health Science Center, College Station, TX 77843, USA*

Received 27 March 2006; accepted 19 July 2006
Available online 14 August 2006

Abstract

Various analytical techniques have been developed to determine nitrite and nitrate, oxidation metabolites of nitric oxide (NO), in biological samples. HPLC is a widely used method to quantify these two anions in plasma, serum, urine, saliva, cerebrospinal fluid, tissue extracts, and fetal fluids, as well as meats and cell culture medium. The detection principles include UV and VIS absorbance, electrochemistry, chemiluminescence, and fluorescence. UV or VIS absorbance and electrochemistry allow simultaneous detection of nitrite and nitrate but are vulnerable to the severe interference from chloride present in biological samples. Chemiluminescence and fluorescence detection improve the assay sensitivity and are unaffected by chloride but cannot be applied to a simultaneous analysis of nitrite and nitrate. The choice of a detection method largely depends on sample type and facility availability. The recently developed fluorometric HPLC method, which involves pre-column derivatization of nitrite with 2,3-diaminonaphthalene (DAN) and the enzymatic conversion of nitrate into nitrite, offers the advantages of easy sample preparation, simple derivatization, stable fluorescent derivatives, rapid analysis, high sensitivity and specificity, lack of interferences, and easy automation for determining nitrite and nitrate in all biological samples including cell culture medium. To ensure accurate analysis, care should be taken in sample collection, processing, and derivatization as well as preparation of reagent solutions and mobile phases, to prevent environmental contamination. HPLC methods provide a useful research tool for studying NO biochemistry, physiology and pharmacology.

Keywords: Reviews; Nitric oxide; Plasma; Serum; Urine; Clinical and animal studies

Contents

☆ This paper is part of a special issue entitled "Analysis of the L-arginine/NO pathway", guest edited by D. Tsikas.

* Corresponding author at: Department of Animal Science, Texas A&M University, 2471 TAMU, College Station, TX 77843-2471, USA. Tel.: +979 845 1817; fax: +979 845 6057.
E-mail address: g-wu@tamu.edu (G. Wu).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.07.018

1. Introduction

1.1. Nitric oxide metabolism

Nitric oxide (NO), a free radical and a signaling molecule, plays an important role in regulating vascular tone, neurotransmission, host immunity, nutrient metabolism, and whole-body homeostasis [1]. NO is produced from L-arginine by NO synthase (NOS) in virtually all mammalian cells, including endothelial cells, macrophages, neuronal cells, muscle cells, adipocytes, enterocytes, and renal epithelial cells [1]. The environment provides another source of systemic NO. Airborne nitrogen oxides (NO_x) can enter humans and animals through inhalation, and their amounts depend on the extent of environmental pollution from the automobile emission, cigarette smoking, and other sources. In cells and blood, oxidation of NO via a variety of reactions results in the formation of nitrite and nitrate as the two major products, with nitrate being the predominant one [2]. For example, NO is readily oxidized to nitrite in aqueous solution (e.g., biological fluid) via autoxidation, and can also react with superoxide (O_2^-), an anion ubiquitously produced by cells, to yield peroxynitrite. Higher NO oxide (e.g., N_2O_3) and peroxynitrite can react with various biomolecules to generate *S*-nitrosothiols and nitrotyrosine. Nitrite is further oxidized by oxyhemoglobin [$HbFe(II)O_2$] to form nitrate and methemoglobin (MetHb). Importantly, NO directly reacts with oxyhemoglobin to yield nitrate and methemoglobin, and this reaction proceeds in both arterial and venous blood. The half lives of NO and nitrite in blood are <5 s and approximately 13 min [2], respectively. Because pH values of blood and cells are in the neutral range (approximately 7.4 and 7.1, respectively), nitrate does not undergo degradation in these compartments. Thus, NO is readily oxidized to its relatively stable metabolite nitrite and to the completely inert nitrate in the whole body (Fig. 1). Under physiological conditions, nitrite concentration in the plasma of fasted humans represents up to 80% of eNOS activity [4,5]. Nitrate accounts for >99% of all NO metabolites excreted into the urine of humans and animals [2,3].

The conventional diet may be the major source of nitrite and nitrate in the plasma of fed humans and animals (Fig. 1). Plant- and animal-origin foods, drinking water, and beverages generally contain much larger amounts of nitrite and nitrate than NO produced by NOS, and these two anions are readily absorbed by the small intestine into the portal vein [3]. For this reason, it is not appropriate to measure plasma or urinary nitrite/nitrate as an indicator of NO synthesis in fed subjects when their diets are not controlled for nitrite or nitrate. In addition, the metabolism of microbes in the intestinal lumen contributes nitrite and nitrate to the portal blood and thus the systemic circulation. The intestinal contribution of nitrite and nitrate can be minimized under fasting conditions. Further, oral medicines such as organic nitrates or supplements that contain nitro-substances may release nitrite and nitrate in the gastrointestinal tract and other tissues. The time required for the exogenous nitrite and nitrate to be completely excreted into urine ranges from 12 h to 3 days, depending on their amounts and renal function [3]. In healthy humans consuming a diet low in nitrite and nitrate (210 μmol/day), approximately 50% of the urine nitrate originates from the whole-body synthesis of NO from L-arginine [6]. Some evidence indicates that, after a 12-h fast, plasma concentrations of nitrite and nitrate can reach a steady-state level in healthy humans consuming a low nitrite/nitrate diet [4]. Under this condition and when the diet is poor in nitrite or nitrate, plasma concentration of nitrite may serve as a valid indicator of NO synthesis by the endothelial NOS, whereas urinary excretion of nitrate reflects whole-body NO synthesis [2]. The validity of measurement of nitrite and nitrate in plasma and urine as indicators of NO synthesis critically depends on the amounts of NO_x gases from the atmosphere

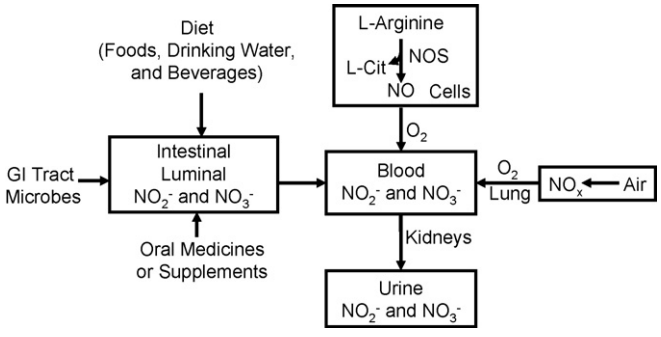

Fig. 1. Schematic of exogenous and endogenous sources of nitrite and nitrate in blood and urine. In animals and humans, the NO derived from cells and the environment is rapidly oxidized to nitrite and nitrate. Nitrite and nitrate are excreted almost exclusively in urine. Diet that is not controlled for nitrite or nitrate is the major source of blood nitrite and nitrate. In healthy humans consuming a diet low in nitrite and nitrate, approximately 50% of the urine nitrate originates from the whole-body synthesis of NO from L-arginine. GI, gastro-intestinal; NO_x, nitrogen oxides.

as well as nitrite and nitrate taken from the diet (including foods, drinking water, and beverages).

1.2. Current methods for nitrite and nitrate analysis

The availability of appropriate analytical techniques to quantify NO synthesis is crucial for studying its biochemistry as well as its role in physiology and pathophysiology. However, because of its very short half-life in circulation and cells (<5 s), the direct measurement of NO is extremely difficult, particularly in the complex physiological milieu. For this reason, various analytical techniques have been developed to determine nitrite and nitrate in biological samples. Batch and some automated methods include the Griess colorimetric assay, fluorometry, flow or sequential injection analysis with visible absorbance, chemiluminescence, and electrochemical detection (ECD). Separation-based methods include GC–MS, CE, and HPLC with a variety of detection systems that are also used in the batch methods [2,5]. The choice of an assay system largely depends on sample type, expertise, and facility availability. General methods of analysis for nitrite and nitrate have been reviewed recently by various groups [2,3,5,7,8]. Thus, our present paper focuses mainly on HPLC methods that are suitable for the quantitative determination of these two anions in various biological samples.

2. HPLC methods for analysis of nitrite and nitrate

2.1. Precautions in sample preparation

Because of low concentrations of nitrite and nitrate but high concentrations of interfering substances in biological samples, sample preparation (e.g., clean-up) with a high recovery rate is a necessary step prior to HPLC analysis. It is also important to minimize the blank values of nitrite and nitrate so that NO synthesis can be assessed with high accuracy. Contamination of samples with nitrite and nitrate can arise from many sources in the laboratory, including water, chemicals, glassware, plasticware, pipettes, ultrafiltration cartridges, and blood collecting tubes, as these materials all contain a certain amount of nitrite and/or nitrate [2,5,7] and, if possible, should be thoroughly washed with water of low nitrite/nitrate content before use. This is a simple but critical step for successful HPLC analysis of nitrite and nitrate in biological samples, because it efficiently removes the contaminations of these two ions and reduces their blank values [9]. Some culture media contain a large amount of nitrite/nitrate, and care should be taken to make a wise selection while not compromising cell viability or growth. In our experience, we have consistently found that the double-distilled and deionized water (DD-water) contains very low levels of nitrite (61 nM) and nitrate (130 nM) [9,10]. Such water is suitable for washing glassware, plasticware, and pipettes as well as for preparing reagent solutions and HPLC mobile phases. To further substantially reduce the blank value for nitrite, DD-water can be filtered using a nitrocellulose membrane [11] and commercially available nitrite-free water (molecular biology grade) can also be used.

Biological samples should be treated with appropriate means to stabilize nitrite and nitrate during and/or after their collection. For example, blood samples need to be centrifuged immediately at low temperature to remove red blood cells, because their oxyhemoglobin can oxidize nitrite [2]. Urine samples can be alkalinized to stabilize nitrite and prevent bacterial reduction of nitrate to nitrite [12]. Another simple and effective means to protect a 24-h urine collection is adding antibiotics to the collection bottle [13]. Biological samples are very diverse in their composition, including proteins, fatty acids, glucose, amino acids, biogenic amines, ammonia, urea, thiol molecules, N-nitroso compounds, and minerals. High levels of protein, some ions, notably chloride, and other substances may interfere with either the measurement or the derivatization of nitrite and nitrate, and thus should be removed before HPLC analysis. Indeed, high concentrations of chloride in biological samples (e.g., 100–110 mM in plasma) are the major interference in HPLC analysis with UV and conductivity detection for nitrite, due to the lack of resolution and column saturation. Thus, eliminating or reducing the high level of chloride from samples is a pre-requisite for UV/VIS or conductivity detection of nitrite [12]. For plasma samples, proteins can be removed by ultrafiltration or precipitation [8]. Diluted samples (e.g., plasma, urine, and culture medium) can be effectively filtered through a 10-kDa cutoff ultrafilter at 14,000 × *g* for 15 min [9]. We found that the recovery of nitrite and nitrate in these biological samples following passage through the ultrafilter was essentially quantitative, as determined with known amounts of standards. Since acidic conditions may cause the loss of nitrite partly via conversion to NO gas, alkaline or acetonitrile can be used to deproteinize blood samples [14]. Chloride can be removed from samples using cation-exchange chromatography, precipitation with silver reagents, or silver-based C_{18} solid-phase extraction [15]. When an appropriate, very sensitive method is used for nitrite analysis (e.g., fluorescence detection), a sample is diluted to such a great extent (e.g., 3- and 2000-fold for culture medium and urine, respectively) that removal of protein or chloride may not be necessary [9].

Nitrite and nitrate can be directly measured by absorbance detection at 210–220 nm or by their conductivity. However, most of the methods for nitrite and nitrate analysis require a derivatization procedure, in which nitrite and nitrate are converted into products that absorb light at a specific wavelength or are highly fluorescent [5]. A majority of these derivatization reactions are specific to nitrite. Therefore, another step is often adopted to quantitatively convert nitrate to nitrite. The difference in total nitrite concentrations between pre- and post-nitrate conversion in the sample is used to calculate the amount of nitrate. The reduction of nitrate can be performed using either chemicals such as cadmium or the enzyme nitrate reductase with NADPH as a cofactor. In the latter method, NADPH should not be used at a high concentration, as it may interfere with the Griess assay and certain other derivatization reactions [16,17]. Of note, cadmium is a highly toxic metal, and does not yield a complete or satisfactory reduction of nitrate to nitrite in biological samples [16]. In contrast, the enzymatic method offers the advantages of high specificity, simplicity, rapidity, safety, and commonly complete conversion of nitrate into nitrite [9,18].

2.2. The HPLC system

HPLC methods generally provide excellent efficiency, precision and accuracy in biochemical analyses. Over the past 20 years, these advantages have been capitalized for determining nitrite and nitrate in biological samples [2,5]. Two major types of chromatography have been used in nitrite and nitrate assays: ion-exchange and ion-pair reversed-phase HPLC on the basis of the distinct chemical properties of their column packing materials and solvent gradients [19]. The HPLC system typically consists of an autosampler or injector, HPLC columns (a guard and an analytical column), a column heater (in some methods), solution-delivery pumps, a detector(s), and a computer workstation for peak integration and data processing (Fig. 2). The autosampler allows for automated sample analysis and high throughput. Columns and mobile phases are the two key elements for the satisfactory separation of nitrite and nitrate or their derivatives from other compounds in a biological sample. In addition, column regeneration after a run is necessary for the automation, precision, and accuracy of HPLC analyses. The detection principles of HPLC analyses of nitrite and nitrate are based on UV and VIS absorbance, electrochemistry, chemiluminescence, and fluorescence, providing different degrees of detection limits (defined as three times the baseline noise) and linear range. Both injection volume and the gain setting of a detector affect detection limit for nitrite and nitrate. The major advantages and disadvantages of these detection systems are summarized in Table 1.

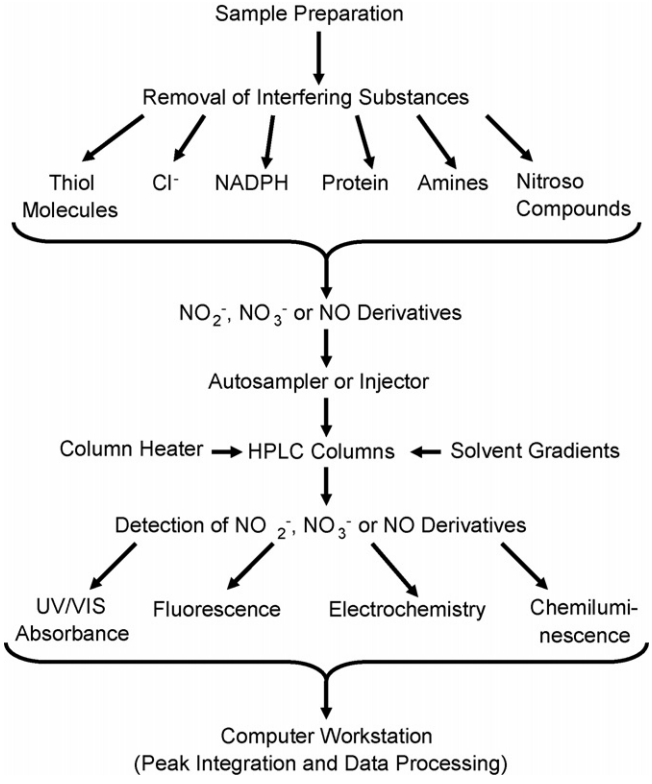

Fig. 2. Schematic of HPLC analysis of nitrite and nitrate in biological samples. The HPLC analysis requires several steps, including sample preparation, derivatization (if required, e.g., by 2,3-diaminonaphthalene), injection of sample into the column, elution with mobile phase, detection, and data processing.

2.3. Detection systems

2.3.1. UV/VIS absorbance

Nitrite and nitrate can absorb UV light at 200–220 nm. Thus, they can be detected directly by a UV detector without further derivatization after HPLC separation [20–24]. However, problems are frequently encountered in measuring nitrite in plasma or urine due to considerable interference by large amounts of chloride that immediately precedes nitrite in chromatographic elution and often masks the nitrite peak [2]. Recently, an HPLC method with UV absorbance at 333 nm of a nitrite derivative was reported for plasma nitrite and nitrate [25]. The principle of this method is based on the specific conversion of nitrite by *N*-acetylcysteine to *S*-nitroso-*N*-acetylcysteine under acidic conditions, with the product detected at a wavelength of 333 nm. An advantage of this method is the elimination of chloride interference of nitrite detection. Conversion of nitrate into nitrite is required for nitrate analysis using the *N*-acetylcysteine reagent [25].

Nitrite and nitrate do not absorb visible light, and thus cannot be detected directly by VIS absorbance without their chemical derivatization. Techniques involving the post-column Griess reaction have been developed for the detection of nitrite and nitrate after their separation by HPLC [26,27]. In this assay, nitrate is reduced to nitrite on a copper-plated column filled with cadmium powder. Finally, nitrite (the original nitrite and the nitrate-derived nitrite) reacts sequentially with the Griess reagents sulfanilamide and *N*-(1-naphthyl)ethylenediamine to form a diazo-compound which is detected at 540 nm.

The UV/VIS detector is the most versatile and least expensive detector used in HPLC analysis due to its simplicity and wide linear range (Table 2). However, commonly used UV and VIS absorbance detectors suffer from low sensitivity, with a detection limit for nitrite or nitrate usually being only approximately 30 to 500 nM depending on the apparatus used, and thus require a relatively large amount of samples for analysis. For example, the VIS detection limit for both nitrite and nitrate was 75 nM when an injection volume was 400 μl [26]. This introduces higher blank values and increases the amounts of interfering substances. In addition, the post-column conversion of nitrate to nitrite is far from complete. UV detection requires a good resolution of sample peaks due to its low specificity. In addition, the methods involving the direct absorbance of nitrite and nitrate around 210 nm are vulnerable to severe interference from chloride that is usually present in large amounts in biological samples. Therefore, this method needs careful clean-up procedures to remove interfering substances. Solid-phase extraction is one commonly used method. For instance, the C_{18} SPE column removes most of the particles and undesirable biomolecules (but not chloride), while the IC–Ag^+ column can specifically remove chloride ions [15]. However, these cartridges are not suitable when the sample volumes are small. To circumvent this shortcoming, Stratford et al. [28] have proposed direct addition of Ag^+-resin to the sample to effectively reduce the chloride level.

Table 1
Advantages and disadvantages of the detection systems used for HPLC analyses of nitrite and nitrate in biological samples

Detection system	Advantages	Disadvantages
UV absorbance-direct detection	Simultaneous detection of NO_2^- and NO_3^-, simplicity, wide linear range, versatility, and low cost	Low sensitivity, interference by Cl^-, low specificity, and requirement of a large amount of sample
UV absorbance-indirect detection (nitrite derivatization with NAC)	Easy derivatization, good separation of NO_2^- from Cl^-, simplicity, wide linear range, versatility, low cost, and elimination of Cl^- interference	Low sensitivity, requirement of a large amount of sample, and inability to directly detect NO_3^-
VIS-indirect detection (nitrite derivatization with Griess reagents and post-column reduction of NO_3^-)	Simultaneous detection of NO_2^- and NO_3^-, simplicity, wide linear linear range, versatility, and low cost	Low sensitivity, interference by substances, low specificity, and requirement of a large amount of sample
ECD-conductimetry (ECDC)	Simultaneous detection of NO_2^- and NO_3^-, simplicity, versatility, and higher sensitivity than UV/VIS	Interference by ionic substances, low specificity, and extensive sample preparation
ECD-amperometry (ECDA)	More sensitive, higher selectivity, and wider linear range than the ECDC method, simplicity, and versatility	Interference by oxidizable anions, low specificity, extensive sample preparation, and inability to detect nitrate
ECDA–UV in line (a strong base anion-exchange column and low-pH eluent solution)	Simultaneous detection of NO_2^- and NO_3^-, offering the same advantages as ECDA and direct UV absorbance, minimizing sample pre-treatment, and preventing interference by organic substances	The same disadvantages as ECDA and direct UV absorbance, and complex detection instrumentation
Chemiluminescence	High sensitivity, rapidity, simple sample preparation, and requirement of a small amount of sample	Low specificity, false signal from organic nitrites, bulky apparatus, and inability to detect directly NO_3^-
Fluorescence (NO_2^- reacts with DAN to yield NAT)	High sensitivity, high specificity, simplicity in both sample preparation and derivatization, stable derivatives, lack of interference when sufficient dilution of a sample is made, linearity with a wide range of NO_2^- and NO_3^- levels, low cost, and universality	Inability to directly detect NO_2^- and NO_3^-

DAN, 2,3-diaminonaphthalene; ECD, electrochemical detection; NAC, *N*-acetyl-L-cysteine; NAT, 2,3-naphthotriazole.

2.3.2. Electrochemistry

There are three types of electrochemical detection: conductimetry, amperometry and potentiometry [8]. Conductimetry is universal for ionic substances, whereas amperometry is selective for oxidizable anions. Conductimetry is used for the detection of all those compounds that ionize. It is simple and is applicable to the direct analysis of nitrite and nitrate in biological fluids, such as urine [29], serum [30], and saliva [31]. Compared to the conductimetric detector, the amperometric detector is more sensitive, provides higher selectivity, and has a wider linear range [8]. Amperometric detection is based on the electrochemical activity of the compounds that can be oxidized. Oxidation occurs on the surface of the electrodes, which generates an electron flow. Nitrite, not nitrate, can be oxidized, and, therefore, nitrite can be detected by amperometry, simply referred to ECD in most of the literature. Due to its higher sensitivity compared to UV, amperometry is sometimes used to replace UV absorbance to measure low levels of nitrite in biological samples. Some researchers have used a two-detector system (ECD and UV) in line for the simultaneous analysis of nitrite and nitrate, which improves the detection limit for nitrite while permitting nitrate detection [12,14,32,33]. Notably, the use of an analytical column containing strong base anion-exchange groups and of a stable low-pH mobile phase solution minimizes sample pre-treatment, prolongs the column's life, and eliminates interference by organic substances.

An advantage of ECD is its greater sensitivity compared to UV/VIS detection. For example, Jedlickova et al. [34] have reported that under the same conditions (i.e., the same column, mobile phase and flow rate), the detection limit of nitrite by ECD is 100 times lower than with UV absorbance detection (1 nM by ECD versus 100 nM by UV). However, the ECD–UV in-line detection system requires two detectors, complicates the instrumentation, and is not commonly available in laboratories. Both UV and ECD detection is susceptible to the interference of chloride, which is present in biological samples at high levels. Therefore, removing chloride or substantially reducing its level is an important step in sample preparation. The requirement of these clean-up procedures may greatly increase the possibility of contamination. In addition, the cost is another issue when silver resin is used to remove chloride. These disadvantages can be partially overcome by using a styrene-divinylbenzene-based ion-exchange analytical column and an acidified 20-mM $NaClO_4$ eluting solution [34].

2.3.3. Chemiluminescence

The principle of chemiluminescence detection is based on the reduction of nitrite to NO by potassium iodide or a stronger reductant and the subsequent reaction of NO with ozone to form NO_2^*. The latter is an electronically excited molecule and falls to a ground state with the emission of light in the near infra-red region (600–3000 nm). The emitted photon is detected by a photomultiplier tube [35]. Chemiluminescence detection offers high sensitivity, which can reach 1 nM for nitrite [35]. This advantage has been utilized for HPLC analysis of nitrite in biological samples. For example, Sen et al. [12] have developed a useful HPLC method with chemiluminescence detection for determining nitrite in human saliva and human urine. This method uses

Table 2
Comparison of detection limits and linear ranges in HPLC analyses of nitrite and nitrate

Reference	Column	Detector	Mobile phase	Detection limit (injection volume)	Linear range
Smith et al. [20]	AE	UV (214 nm)	5 mM K_2HPO_4 and 25 mM KH_2PO_4 (pH 3.0)	30 nM (100 μl)	31 nM–1 mM
Everett et al. [21]	AE	UV (214 nm)	5 mM K_2PO_4 and 25 mM KH_2PO_4 (pH 3.0)	100 nM for NO_2^- (IJV not reported)	0.2–100 μM
Radisavljevic et al. [24]	AE	UV (210 nm)	20 mM NaCl and 1 mM NaH_2PO_4 (pH 7.0)	10 nM (20–100 μl)	0.33–42 μM for NO_2^-; 15.8–20 μM for NO_3^-
Monaghan et al. [55]	AE	UV (214 nm)	Chloride gradient (pH 7.5)	250 nM (30 μl)	Up to 30 μM
Tsikas et al. [25]	RP	UV (333 nm)	10 mM NaH_2PO_4 in acetonitrile-water (15:85, v/v) (pH 2.0)	50 nM for NO_2^- (200 μl)	0–50 μM for NO_2^-; 0–100 μM for NO_3^-
Muscara and de Nucci [26]	AE	VIS (540 nm)	0.06 M NH_4Cl (pH 2.8)	75 nM (400 μl)	Up to 150 μM
Yamada and Nabeshim [27]	RP	VIS (540 nm)	10% methanol containing 0.15 M NaCl/NH_4Cl and 0.5 g/L Na_4-EDTA	30 nM (IJV not reported)	N.R.
Helaleh and Korenaga [31]	AE	Conductivity	2.7 mM Na_2CO_3-0.3 mM $NaHCO_3$	326 nM for NO_2^-; 540 nM for NO_3^- (25 μl)	0.65–652 μM for NO_2^-; 0.81–645 μM for NO_3^-
Preik-Steinhoff and Kelm [14]	AE	UV (220 nm) for NO_3^- and ECD for NO_2^-	40 mM NaCl in acetonitrile-methanol-water (70:10:20, v/v)	3 nM for NO_2^- (ECD); 500 nM for NO_3^- (UV) (20 μl)	10–1000 nM
Rizzo et al. [33]	RP	UV (220 nm) and ECD	10 mM n-Octylamine (pH 6.0)	0.9 nM for NO_2^- (ECD); 4.4 nM for NO_3^- (UV) (10 μl)	1–1000 μM
Jedlickova et al. [34]	AE	UV (212 nm) and ECD	0.02 M $NaClO_4$ (pH 3.9)	1 nM for NO_2^- (ECD); 100 nM for NO_2^- (UV); 200 nM for NO_3^- (UV) (IJV not reported)	1–20 μM for NO_2^-; 1–100 μM for for NO_3^-
Sen et al. [12]	RP	Chemiluminescence	0.05 M KH_2PO_4 (pH 6.0) and 5 mM tetrabutyl-ammonium hydrogen sulphate	14.5 nM for NO_2^- (50–100 μl)	0.43–435 μM
Li et al. [9]	RP	Fluorescence	15 mM sodium phosphate buffer (pH 7.5) containing 50% methanol (v/v)	10 nM at a submaximal gain setting (15 μl)	12.5–2000 nM
Gharavi and El-Kadi [40]	RP	Fluorescence	15 mM sodium phosphate buffer (pH 7.5)/methanol (60:40, v/v)	10 pM for NO_2^- (30 μl)	10–200 pM
Woitzik et al. [41]	RP	Fluorescence	10 mM borate buffer (pH 9.0) and 25% acetonitrile	0.27 nM for NO_2^-; 2.25 nM for NO_3^- (IJV not reported)	0.01–50 μM

AE, anion exchange; IJV, injection volume; N.R., not reported; RP, reversed-phase.

a reversed-phase column as well as 0.05 M KH_2PO_4 (pH 6) and 5 mM tetrabutylammonium hydrogensulfate as the mobile phase. A post-column reactor is equipped to convert nitrite to NO. Like the batch chemiluminescence method [35], the HPLC analysis with chemiluminescence detection provides high sensitivity and rapidity for nitrite analysis. The HPLC method reported by Sen et al. [12] is more rapid and 200 times more sensitive than the traditional Griess method, while requiring only minimal sample preparation and a small amount of sample. However, the chemiluminescence detection suffers from the disadvantage of complexity in post-column reactions and an inability to directly detect nitrate. Also, organic nitrites can be reduced under acid-iodide or stronger reducing conditions to produce NO, and the measurement of nitrite by chemiluminescence is highly susceptible to interference by experimental inhibitors of NO synthase (N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine) (discussed in ref. [36]) and some *N*-nitroso compounds (e.g., *S*-nitrothiols and nitrosodiphenylamine) [35] present in biological samples. Thus, these interfering substances should be separated satisfactorily from nitrite on an HPLC column. In addition, pre-column treatment (e.g., incubation) of sample with sulfanilamide can provide an option to differentiate chemiluminescence signals from nitrite and other substances [37]. Further, different reducing agents have different efficiencies for reducing nitrite and other compounds [38], and can be used to minimize chemiluminescence signals from compounds other than nitrite.

2.3.4. Fluorescence

Neither nitrite nor nitrate itself yields fluorescence. Thus, these two anions must be derivatized with an appropriate reagent to yield a highly and stable fluorescent derivative. We recently developed a fluorescence HPLC method for analysis of nitrite and nitrate in biological samples [9], on the basis of a batch fluorescence method which involves the reaction of nitrite with 2,3-diaminonaphthalene (DAN) for 10 min under acidic conditions to yield 2,3-naphthotriazole (NAT) (Fig. 3). The fluorescence intensity of NAT with excitation at 375 nm and emission at 415 nm is enhanced markedly when the assay solution is neutralized with NaOH at the end of the derivatization reaction [17].

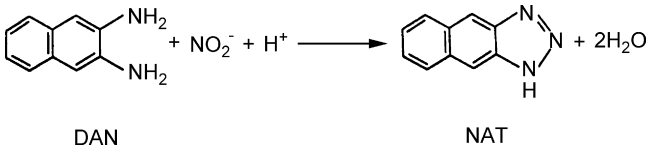

Fig. 3. Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NAT) under acidic conditions. Reprinted from Journal of Chromatography B 746, H. Li, C.J. Meininger, G. Wu, Rapid determination of nitrite by reversed-phase high-performance liquid chromatography with fluorescence detection, 199–207, 2000, with permission from Elsevier.

NAT is stable in the alkaline solution and at room temperature (20–25 °C) for at least 24 h [9]. The fluorescence spectra of DAN and NAT are illustrated in Fig. 4. At an emission wavelength of 415 nm and an excitation wavelength of 375 nm, NAT exhibits relatively high fluorescence intensity but DAN does not (Fig. 4, panel A). Notably, at an excitation wavelength of 375 nm and an emission wavelength of 415 nm, NAT yields the highest fluorescence intensity but DAN exhibits very weak fluorescence intensity (Fig. 4, panel B).

Our motivation to originally develop the fluorescence HPLC method for nitrite analysis arose from our inability to detect picomole levels of nitrite and nitrate in cell culture medium and certain biological samples using the DAN batch fluorescence method due to the lack of specificity and chemical quenching [9]. Specifically, this failure results from high blank values and the DAN's intrinsic fluorescence property as well as the fluorescence quenching and interference by inherent biological substances and colorimetric chemicals. Employing a 5-μm reversed-phase C_8 column (150 mm × 4.6 mm I.D.) guarded by a 40-μm reversed-phase C_{18} column (50 mm × 4.6 mm I.D.) and a mobile phase consisting of 15 mM sodium phosphate buffer (pH 7.5) and methanol, the nitrite-DAN derivative (NAT) is rapidly and satisfactorily separated from DAN and fluorescent substances present in cell culture medium and biological samples [9]. Nitrate can be reduced to nitrite using nitrate reductase with 98–100% reduction recovery [9,18], and the enzymatic reaction conditions do not interfere with the subsequent derivatization of nitrite with DAN to form NAT or the chromatographic separation of NAT [9]. The fluorescence of NAT is linear with nitrite and nitrate concentrations range from 12.5 nM to 2 μM in water and biological samples (including cell culture medium, cell extracts, plasma, and urine) [9]. The detection limit for nitrite and nitrate is 10 nM when the Waters 474 fluorescence detector is set at a gain of 100 (maximum setting of up to 1000), and can be further reduced when a higher gain setting is applied. This high detection sensitivity is achieved at an injection volume of only 15 μl. A representative HPLC chromatogram for analysis of nitrate in biological samples via its enzymatic conversion to nitrite is illustrated in Fig. 5. Thus, our HPLC–DAN method offers greater sensitivity and higher specificity than other methods with UV/VIS absorbance, electrochemistry, and chemiluminescence detection.

Because of the greatly improved sensitivity, cell culture medium and biological samples are routinely diluted with DD-water prior to derivatization with DAN. For example, for nitrite analysis, endothelial cell culture medium, activated-macrophage culture medium, plasma, and urine (from healthy subjects) are routinely diluted 3, 50, 10, and 10 times with DD-water, respectively. For nitrate analysis, endothelial cell culture medium, activated-macrophage culture medium, plasma, and urine (from healthy subjects) are routinely diluted 6, 50, 100, and 2000 times with DD-water, respectively. Such a high degree of dilution effectively eliminates the need for sample clean-up, and prevents the effect of the complex matrix of the biological sample on derivatization reactions, chromatographic separation, and detection [9]. Using our HPLC method, we have successfully determined nitrite and nitrate in a variety of biological samples [9,39]. This technique has also been adapted to analyze nitrite and nitrate in hepatoma cells [40] and brain microdialysate samples [41]. A disadvantage of all HPLC methods with fluorescence detection is the inability to directly detect nitrite and nitrate. However, in the case of nitrite, this shortcoming can be readily overcome in all laboratories due to the simplicity of the reaction between nitrite and DAN, the rapidity of sample preparation, and easy automation. Additionally, nitrate can be efficiently reduced to nitrite by nitrate reductase in the presence of NADPH as a co-factor [9]. Thus, because fluorescence detectors are readily available at relatively low cost in laboratories, the DAN-based fluorescence HPLC method enjoys universal use for the analysis of nitrite and nitrate in all biological samples.

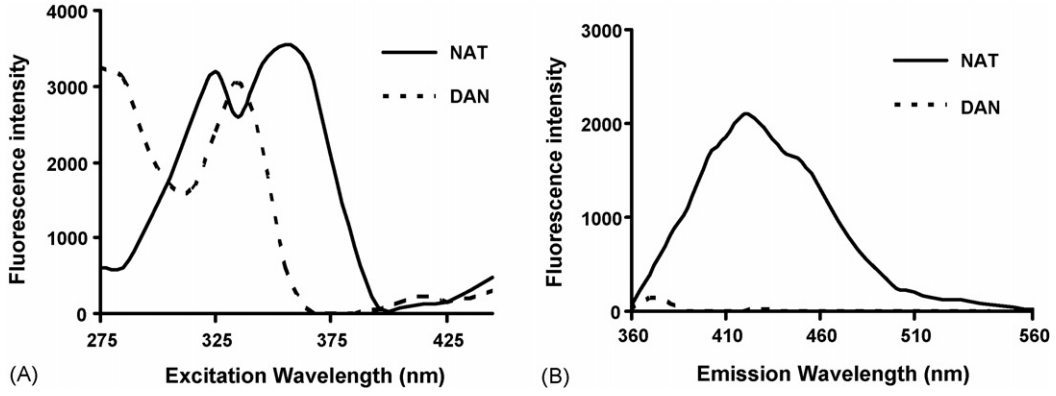

Fig. 4. Fluorescence spectra of 2,3-diaminonaphthalene (DAN) and 2,3-naphthotriazole (NAT) at an emission wavelength of 415 nm and various excitation wavelengths (panel A) and at an excitation wavelength of 375 nm and various emission wavelengths (panel B). The fluorescence intensity of 90 μM DAN and 3 μM NAT, which were prepared with 15 mM sodium phosphate buffer (pH 7.5) containing 50% methanol (the mobile phase used for the HPLC separation of DAN and NAT), was recorded in a 1-cm path-length cuvette at 25 °C using Molecular Devices SpectraMax M2 (Sunnyvale, CA).

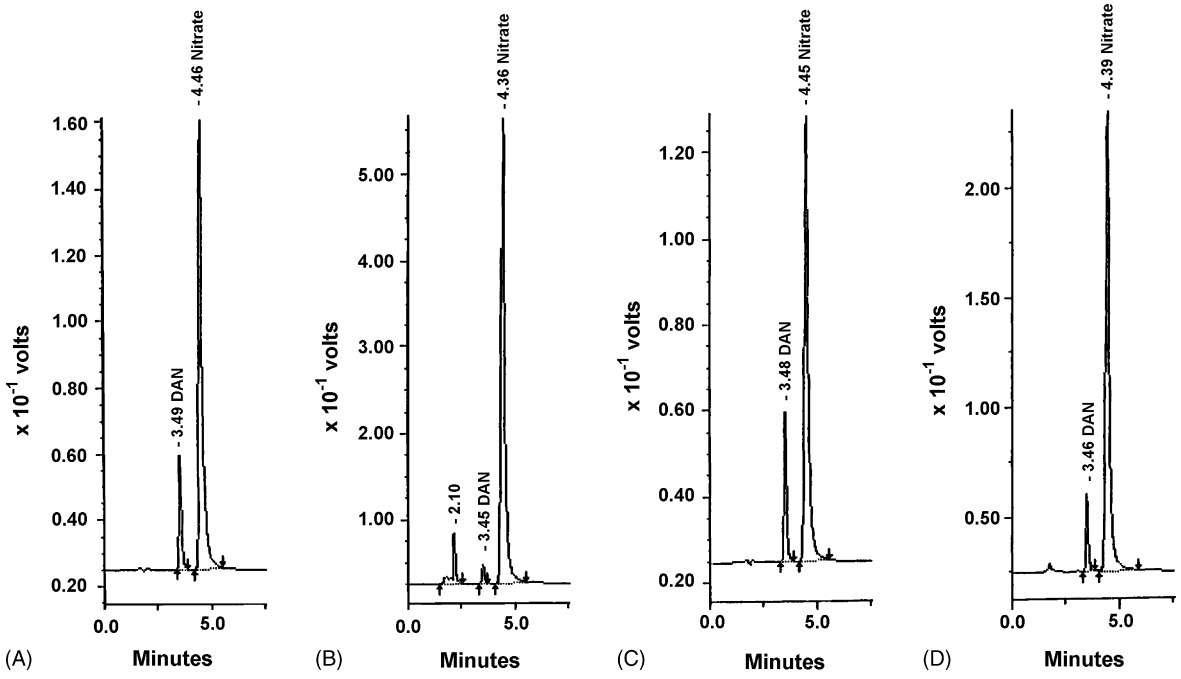

Fig. 5. Analysis of nitrate in biological samples using the HPLC–DAN method. Nitrate was reduced to nitrite by nitrate reductase. Nitrite reacts with 2,3-diaminonaphthalene (DAN) to yield 2,3-naphthotriazole (NAT) under acidic conditions. DAN was separated from NAT by reversed-phase HPLC followed by fluorescence detection at 375 nm excitation wavelength and 415 nm emission wavelength. (A) 200 nM nitrate standard; (B) endothelial cell culture medium; (C) plasma; (D) urine. Reprinted from Journal of Chromatography B 746, H. Li, C.J. Meininger, G. Wu, Rapid determination of nitrite by reversed-phase high-performance liquid chromatography with fluorescence detection, 199–207, 2000, with permission from Elsevier.

3. Reference values for nitrite and nitrate in biological samples

Reported concentrations of nitrite and nitrate in biological samples determined using HPLC methods are summarized in Table 3. The samples were obtained from humans under various nutritional, physiological, and pathological conditions, where diet might not have been routinely controlled for nitrite or nitrate. These published studies illustrate the popularity and usefulness of HPLC methodology in quantifying both nitrite and nitrate in various biological samples. They include plasma, serum, urine, saliva, cerebrospinal fluid,

Table 3
Mean basal concentrations of nitrite and nitrate in biological matrices determined by HPLC methods

Reference	Detection	Species	Samples	Nitrite (μM)	Nitrate (μM)
Smith et al. [20]	UV	Human	Plasma	3.5	46.8
Radisavljevic et al. [24]	UV	Human	Plasma	3.1	10.3
Tsikas et al. [25]	UV	Human	Plasma	0.55	27.4
Kleinbongard et al. [54]	UV	Human	Plasma	0.31	N.R.
Wennmalm et al. [57]	UV	Human	Plasma	1.3	27
Preik-Steinhoff et al. [14]	UV–ECD	Human	Plasma	0.58	25
Stratford et al. [28]	UV–ECD	Human	Plasma	0.71	47.8
Jedlickova et al. [34]	UV–ECD	Human	Plasma	1.3	19.2
Monaghan et al. [55]	UV	Human	Serum	4.2	39.9
Kleinbongard et al. [56]	UV	Rat	Plasma	0.19	N.R.
Li et al. [9]	Fluorescence	Rat[a]	Plasma	0.69	13.6
Tsikas [43]	UV	Human	Urine	N.R.	1100
Wennmalm et al. [57]	UV	Human	Urine	N.R.	470–1130
Li et al. [9]	Fluorescence	Rat[a]	Urine	0.68	605
Present study	Fluorescence	Mouse	CSF[b]	1.31	23.6
Present study	Fluorescence	Rat	CSF[c]	0.75	14.9

N.R., not reported; CSF, cerebrospinal fluid.

[a] Rats were fed a nitrite- and nitrate-free diet.

[b] Cerebrospinal fluid was obtained from adult healthy mice fed a standard rodent chow and analyzed for nitrite and nitrate using the HPLC–DAN method [9]. S.E.M. values for nitrite and nitrate were 0.17 and 1.52 μM, respectively ($n = 5$).

[c] Cerebrospinal fluid was obtained from adult healthy rats fed a nitrite- and nitrate-free diet and analyzed for nitrite and nitrate using the HPLC–DAN method [9]. S.E.M. values for nitrite and nitrate were 0.09 and 1.06 μM, respectively ($n = 7$).

tissue extracts, and fetal fluids, as well as meats and cell culture medium.

In healthy humans, reported plasma or serum concentrations of nitrite and nitrate are in the ranges of 0.3–4 μM and 20–70 μM, respectively (Table 3). These ranges are confirmed by sensitive, accurate, and interference-free GC–MS methods [2,42]. Note that serum concentrations of nitrite plus nitrate in healthy humans of 1.1 ± 0.05 μM reported by Menyawi et al. [22] are abnormally low, likely due to methodological shortcomings [36]. In healthy rats fed a nitrite- and nitrate-free diet, plasma concentrations of nitrite and nitrate are 0.69 ± 0.12 and 13.6 ± 0.80 μM (means ± S.E.M., $n = 5$), respectively, as measured by the HPLC–DAN method [9]. Concentrations of nitrite and nitrate in the urine of healthy humans are in the ranges of 4 to 8 μM [24] and 1100 to 1500 μM [24,43], respectively. Lower values have been reported for the urine of rats fed a nitrite- and nitrate-free diet (0.68 μM nitrite and 605 μM nitrate) [9]. Accordingly, urinary excretion of nitrite and nitrate by adult healthy humans consuming nitrate-uncontrolled diets averages approximately 0.085 and 17 μmol/kg body weight per 24 h, respectively [2]. Urinary excretion of nitrite and nitrate by rats fed a nitrite- and nitrate-free diet is 0.017 and 15 μmol/kg body weight per 24 h, respectively [9]. It is noteworthy that, using the HPLC analysis with conductivity detection, Calo et al. [29] reported that urinary concentration or excretion of nitrite in healthy humans was 66% higher than that of nitrate. This data might have resulted from errors and problems of the assay, such as possible contamination of nitrite from the glassware and pipettes used as well as incomplete removal of chloride present in the urine sample that might be co-eluted with nitrite. The same reasons may also explain, in part, the previous observation that the concentration of nitrite in the cerebrospinal fluid of healthy adult humans was 2.6-fold greater than that of nitrate, when an HPLC method with UV detection was employed to determine these two anions [44].

4. HPLC methods in clinical and animal studies

4.1. Diabetes

Humans and animals with insulin-dependent (type-1) or non-insulin-dependent (type-2) diabetes exhibit impaired NO synthesis and reduced NO bioavailability in endothelial cells [45–47]. In our study involving non-diabetic and streptozotocin-induced diabetic rats fed a nitrite- and nitrate-free 20%-casein diet [48], analysis with the HPLC–DAN method [9] indicated reduced plasma levels of nitrite in diabetic rats (0.71 ± 0.08 μM in nondiabetic rats versus 0.53 ± 0.05 μM in diabetic rats; mean ± S.E.M., $n = 8$; $P < 0.05$). Using the same HPLC technique [9], we found that venous plasma levels of nitrite were (mean ± S.E.M., $n = 20$) 0.50 ± 0.04 and 0.74 ± 0.06 μM ($P < 0.01$) in diabetic BB rats (an animal model of type I diabetes [49]) at 30 days post-onset of diabetes and age-matched nondiabetic-prone BB rats, respectively. Using an HPLC method with a post-column Griess reaction and VIS absorbance detection, Maejima et al. [50] measured the plasma level of nitrite and nitrate in 129 type-2 diabetic humans and 76 age-matched nondiabetic controls. Their results indicate that plasma levels of nitrite did not differ between these two groups of subjects (0.16 ± 0.19 μM in diabetics versus 0.21 ± 0.22 μM in non-diabetics; $P > 0.05$), while plasma nitrate concentrations were higher in the diabetic group (58.5 ± 42.8 μM in diabetics versus 34.5 ± 15.6 μM in non-diabetics; $P < 0.01$) (mean ± S.D.) [50]. Interestingly, in type-2 diabetic patients, the high level of nitrate was positively correlated with serum lipid peroxide and advanced glycation end-products [50]. Thus, reducing oxidative stress may emerge as a strategy to treat diabetic subjects [51].

4.2. Cardiovascular homeostasis

NO is a major endothelium-derived vasodilator, and plays an important role in maintaining vascular tone and regulating blood pressure [52–54]. Therefore, a variety of HPLC methods have been actively developed for conducting cardiovascular research [55–57]. For example, using HPLC analysis with ECD–UV detection, Kelm et al. [32] reported that an increase in serum concentrations of nitrite from 402 ± 59 to 977 ± 82 nM (mean ± S.E.M.) in the antecubital vein of 12 healthy humans were significantly correlated ($r = 0.61$; $P < 0.0001$) with an increase in forearm blood flow from 3.0 ± 0.3 to 10.4 ± 0.9 ml/min per 100 ml tissue (mean ± S.E.M.) in response to the infusion of acetylcholine (an endothelium-dependent vasodilator). Intravenous infusion with L-N^G-monomethylarginine (an endogenous endothelial NO synthase inhibitor) to healthy subjects reduced venous serum nitrite levels and forearm blood flow by 41 and 33%, respectively, whereas intravenous infusion with L-arginine (the substrate for NO synthesis) increased venous serum nitrite levels and forearm blood flow by 322 and 75%, respectively [32]. A recent study by Kelm and co-workers has shown that reduced levels of plasma nitrite are an indicator of endothelial dysfunction and correlate well with an increase in cardiovascular risk [58]. Their data suggest that the endothelial L-arginine/NO pathway is involved in the regulation of blood flow in human forearm circulation. Also, these findings suggest that plasma or serum nitrite is a useful indicator for endothelial NO production.

The function of NO in the coronary circulation has been evaluated using an HPLC–Griess system [53]. Plasma samples were obtained from the coronary sinus and ostium of the left coronary artery of 9 patients with vasospastic angina (VSA) and 9 healthy control subjects. The coronary venous-arterial difference in NO_x (the sum of nitrite and nitrate) was close to zero in control groups, while the value was negative in VSA patients [53]. In addition, the venous-arterial concentration difference was negatively correlated with the basal coronary artery tone ($r = -0.91$), which was increased in VSA patients. These findings suggest that the increased basal coronary tone in the patients with compromised vascular function may be the consequence of reduced NO synthesis by endothelial cells. Thus, the endothelial dysfunction in cardiovascular diseases results, in part, from a decrease in vascular NO biosynthesis. Emerging evidence suggests that nitrite plays a physiological role in signaling, blood flow regulation, and hypoxic NO homeostasis [54].

4.3. Renal function

The kidneys excrete nitrite and nitrate, and thus renal function is an important determinant of plasma levels of nitrite and nitrate [3]. When concentrations of nitrite and nitrate in urine are used as markers of whole-body NO synthesis under various physiological and pathological conditions, renal function and clearance should be considered. A nitrate load study was performed by Himeno et al. [59] to evaluate a relationship between steady-state plasma concentrations of NO_x and in vivo NO_x formation or NO_x clearance. Blood samples from nine healthy subjects were obtained for analyses of nitrite and nitrate using an HPLC–UV method. The authors found that the steady-state plasma levels of NO_x (15.5 ± 1.6 μM, mean ± S.E.M.) were significantly correlated with renal function [59]. Using the same analytical method, Adachi et al. [60] showed that patients with renal failure had higher levels of plasma nitrate compared with those with normal renal function (80.7 ± 44.2 μM, $n = 15$ versus 21.6 ± 4.6 μM, $n = 14$; mean ± S.D., $P < 0.01$). Collectively, these findings suggest that in patients with known renal dysfunction, changes in plasma levels of nitrite and nitrate may be a measure of the extent to which renal clearance is impaired.

4.4. Immunity

Inflammatory and immunological reactions are characterized by a marked increase in NO synthesis via inducible NO synthase [1]. Using an HPLC–UV method of Menyawi et al. [22], Winkler et al. [61] determined serum levels of nitrite plus nitrate in four filariasis patients during microfilaricidal chemotherapy. They noted that all of these patients exhibited the same patterns of change in serum NO_x levels, with a sharp increase to a peak value at 3–4 days after the treatment, followed by a slow decline thereafter to a reduced level that remained higher six months after chemotherapy in comparison with the pre-treatment value [61]. While the relative changes in serum nitrite plus nitrate of the filariasis subjects suggest increased systemic NO production [61], caution should be taken in assessing the actual concentrations of serum nitrite plus nitrate (e.g., basal levels of 1 to 2 μM), which are very low compared with reference values (Table 3). In another study involving HPLC analysis of nitrite and nitrate, Mitaka et al. [62] observed that the plasma NO_x levels in 23 patients with infectious systemic inflammatory response syndrome (sepsis) were increased 2-fold when compared with 10 healthy subjects (51.0 ± 38.5 versus 29.6 ± 8.9 μM; mean ± S.D., $P < 0.01$). Similar analysis with the HPLC–DAN method revealed an induction in nitrite and nitrate production by macrophages treated with a bacterial endotoxin lipopolysaccharide (LPS) [9]. Further, we found that administration of LPS (1 mg/kg body wt) to 60-day-old Sprague–Dawley rats fed a nitrite- and nitrate-free 20%-casein diet [13] increased plasma levels of nitrite (0.73 ± 0.09 μM in control rats versus 3.82 ± 0.41 μM in LPS-treated rats; $P < 0.01$) and of nitrate (14.1 ± 1.2 μM in control rats versus 69.6 ± 5.4 μM in LPS-treated rats; $P < 0.01$) at 24-h post-administration (mean ± S.E.M., $n = 8$). Similarly, LPS administration to rats increased urinary excretion of nitrate by 19-fold [9]. Thus, HPLC methods provide a powerful tool to study immunological reactions in animals and humans.

4.5. Other clinical studies

Preeclampsia is a pregnancy-related multi-organ disorder that is associated with multiple disease states such as hypertension, diabetes, and renal disease. HPLC analyses of plasma/serum nitrite and nitrate have been employed to ascertain a role for NO in the pathogenesis of endothelial dysfunction in pregnant women with preeclampsia [63]. The HPLC–DAN method has been used to determine changes in placental NO synthesis during various stages of ovine fetal development (0.29 to 1.66 nmol/h per g placentomal tissue between Days 30 and 140 of gestation) [64]. These studies will aid in understanding the mechanisms that regulate normal fetal development and underlie the etiology of intrauterine growth retardation, a major problem in both human medicine and animal agriculture [65]. In addition, HPLC methods with UV–ECD or UV detection have been developed to analyze nitrite and nitrate in human cerebrospinal fluid (CSF) [44], which will help define a role for NO in the physiology and pathophysiology of the nervous system. However, the reported CSF concentrations of nitrite (10.4 μM) and nitrate (2.92 μM) in healthy humans [44] were not consistent with those (0.9 μM nitrite and 15.3 μM nitrate) for human subjects, which were measured using capillary electrophoresis with UV detection at 214 nm [66]. Of note, in the above-cited study of Zecca et al. [44], a representative HPLC chromatogram for nitrite and nitrate in a human CSF sample was quantitatively not consistent with that for standards. Further, the HPLC–DAN method has been used to analyze the release of nitrite from the neonatal intestinal epithelium in response to injury and inflammation [67], a significant problem in preterm neonates [68]. Finally, the use of this sensitive and specific HPLC technique in analyzing the release of nitrite and nitrate from adipocytes or adipose tissues [10] will greatly advance research in the field of obesity, an emerging health problem worldwide [69].

5. Concluding remarks

The HPLC methodology provides a useful tool to measure nitrite and nitrate, NO oxidation products, in various biological samples, including plasma, serum, urine, fetal fluid, and cerebrospinal fluid. The detection systems include UV/VIS absorbance, electrochemistry, chemiluminescence, and fluorescence. Among the available HPLC techniques, the fluorescence HPLC method involving pre-column derivatization of nitrite with DAN offers the distinct advantages of easy sample preparation, simple derivatization, stable fluorescent derivatives, rapid analysis, high sensitivity, high specificity, lack of interferences, and easy automation for determining nitrite and nitrate in virtually all biological samples. Because nitrite and nitrate can enter the blood circulation from diet, oral medicines, environment, and the intestinal microbial nitrogen metabolism, caution should be taken in determining concentrations of these two anions in biological samples as an indicator of systemic or local NO synthesis. We expect that HPLC methods will continue to play an

important role in studying NO biochemistry, physiology, and pharmacology in the future.

Acknowledgements

Research in our laboratories was supported by National Research Initiative Competitive Grants 2001-35203-11247 and 2003-35206-13694 from the USDA Cooperative State Research, Education, and Extension Service, National Institutes of Health Grant R21HL077401-01, Juvenile Diabetes Research Foundation Grant 5-2004-508, American Heart Association-TX Grant 0655109Y, Texas Agricultural Experiment Station, and a Texas A&M University Faculty Fellowship. We thank guest editor Dr. Dimitrios Tsikas and two anonymous reviewers for constructive comments and suggestions for improving this paper. We also thank Dr. Nancy Turner and Ms. Frances Mutscher for assistance in manuscript preparation.

References

[1] G. Wu, S.M. Morris Jr., Biochem. J. 336 (1998) 1.
[2] D. Tsikas, Free Radic. Res. 39 (2005) 797.
[3] G. Ellis, I. Adatia, M. Yazdanpanah, S.K. Makela, Clin. Biochem. 31 (1998) 195.
[4] P.M. Rhodes, A.M. Leone, P.L. Francis, A.D. Struthers, S. Moncada, Biochem. Biophys. Res. Commun. 209 (1995) 590.
[5] D. Tsikas, F.-M. Gutzki, D.O. Stichtenoth, Eur. J. Clin. Pharmacol. 62 (2006) 51.
[6] L. Castillo, L. Beaumier, A.M. Ajami, V.R. Young, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 11460.
[7] D. Tsikas, Methods Mol. Biol. 279 (2004) 81.
[8] V. Di Matteo, E. Esposito, J. Chromatogr. A 789 (1997) 213.
[9] H. Li, C.J. Meininger, G. Wu, J. Chromatogr. B 746 (2000) 199.
[10] W. Fu, T.E. Haynes, R. Kohli, J. Hu, W. Shi, T.E. Spencer, R.J. Carroll, C.J. Meininger, G. Wu, J. Nutr. 135 (2005) 714.
[11] X.X. Gu, T.Z. Zhou, D.Y. Qi, Talanta 43 (1996) 169.
[12] N.P. Sen, P.A. Baddoo, S.W. Seaman, J. Chromatogr. A 673 (1994) 77.
[13] G. Wu, N.E. Flynn, S.P. Flynn, C.A. Jolly, P.K. Davis, J. Nutr. 129 (1999) 1347.
[14] H. Preik-Steinhoff, M. Kelm, J. Chromatogr. B 685 (1996) 348.
[15] W.E. Utermahlen Jr., D.W. Mellini, H.J. Issaq, J. Liquid Chromatrogr. 15 (1992) 3315.
[16] C. Verdon, B. Burton, R. Prior, Anal. Biochem. 224 (1995) 502.
[17] T.P. Misko, R.J. Schilling, D. Salvemini, W.M. Moore, M.G. Currie, Anal. Biochem. 214 (1993) 11.
[18] G. Wu, J.T. Brosnan, Biochem. J. 281 (1992) 45.
[19] M.R. Stratford, Methods Enzymol. 301 (1999) 259.
[20] C.C.T. Smith, L. Stanyer, D.J. Betteridge, J. Chromatogr. B 779 (2002) 201.
[21] S.A. Everett, M.F. Dennis, G.M. Tozer, V.E. Prise, P. Wardman, M.R.L. Stratford, J. Chromatogr. A 706 (1995) 437.
[22] I.E. Menyawi, S. Looareesuwan, S. Knapp, F. Thalhammer, B. Stoiser, H. Burgmann, J. Chromatogr. B 706 (1998) 347.
[23] C. Stein, H.-G. Classen, G. Schwedt, Clin. Chim. Acta 175 (1988) 167.
[24] Z. Radisavljevic, M. George, D.J. Dries, R.L. Gamelli, J. Liquid Chromatogr. Rel. Technol. 19 (1996) 1061.
[25] D. Tsikas, S. Rossa, J. Sandmann, J.C. Frolich, J. Chromatogr. B 724 (1999) 199.
[26] M.N. Muscara, G. de Nucci, J. Chromatogr. B 686 (1996) 157.
[27] K. Yamada, T. Nabeshima, J. Neurochem. 68 (1997) 1234.
[28] M.R.L. Stratford, M.F. Dennis, R. Cochrane, C.S. Parkins, S.A. Everett, J. Chromatogr. A 770 (1997) 151.
[29] L. Calo, S. Cantaro, D. Paleari, D. Vianello, F. Zerbo, L. Bonfante, S. Favaro, A. Antonello, A. D'Angelo, Biomed. Chromatogr. 12 (1998) 97.
[30] B.C. Lippsmeyer, M.L. Tracy, G. Moller, J. Assoc. Off. Anal. Chem. 73 (1990) 457.
[31] M.I.H. Helaleh, T. Korenaga, J. Chromatogr. B 744 (2000) 433.
[32] M. Kelm, H. Preik-Steinhoff, M. Preik, B.E. Strauer, Cardiovasc. Res. 41 (1999) 765.
[33] V. Rizzo, L. Montalbetti, A.L. Rozza, W. Bolzani, C. Porta, G. Balduzzi, E. Scoglio, R. Moratti, J. Chromatogr. A 798 (1998) 103.
[34] V. Jedlickova, Z. Paluch, S. Alusik, J. Chromatogr. B 780 (2002) 193.
[35] R. Cox, Anal. Chem. 52 (1980) 332.
[36] D. Tsikas, I. Fuchs, F.-M. Gutzki, J.C. Frolich, J. Chromatogr. B 715 (1998) 441.
[37] R. Marley, M. Feelisch, S. Holt, K. Moore, Free Radic. Res. 32 (2000) 1.
[38] F. Yang, E. Troncy, M. Francoeur, B. Vinet, P. Vinay, G. Czaika, G. Blaise, Clin. Chem. 43 (1997) 657.
[39] C.J. Meininger, G. Wu, Methods Enzymol. 352 (2002) 280.
[40] N. Gharavi, A.O.S. El-Kadi, J. Pharm. Pharmaceut. Sci. 6 (2003) 302.
[41] J. Woitzik, N. Abromeit, F. Schaefer, Anal. Biochem. 289 (2001) 10.
[42] D. Tsikas, Anal. Chem. 72 (2000) 4064.
[43] D. Tsikas, Clin. Chem. 50 (2004) 1259.
[44] L. Zecca, M. Rosati, R. Renella, M. Galimberti, A. Ambrosini, R.G. Fariello, J. Neural Transm. 105 (1998) 627.
[45] H.O. Steinberg, A.D. Baron, Diabetologia 45 (2002) 623.
[46] C.J. Meininger, S. Cai, J.L. Parker, K.M. Channon, K.A. Kelly, E.J. Becker, M.K. Wood, L.A. Wade, G. Wu, FASEB J. 18 (2004) 1900.
[47] W. Shi, C.J. Meininger, T.E. Haynes, K. Hatakeyama, G. Wu, Cell Biochem. Biophys. 41 (2004) 415.
[48] R. Kohli, C.J. Meininger, T.E. Haynes, W. Yan, J.T. Self, G. Wu, J. Nutr. 134 (2004) 600.
[49] G. Wu, Diabetes 44 (1995) 360.
[50] K. Maejima, S. Nakano, M. Himeno, S. Tsuda, H. Makiishi, T. Ito, A. Nakagawa, T. Kigoshi, T. Ishibashi, M. Nishio, K. Uchida, J. Diab. Complications 15 (2001) 135.
[51] G. Wu, Y.Z. Fang, S. Yang, J.R. Lupton, N.D. Turner, J. Nutr. 134 (2004) 489.
[52] G. Wu, C.J. Meininger, J. Nutr. 130 (2000) 2626.
[53] T. Hori, T. Matsubara, T. Ishibashi, M. Yamazoe, T. Ida, K. Higuchi, M. Takemoto, S. Ochiai, Y. Tamura, Y. Aizawa, M. Nishio, Jpn. Heart J. 41 (2000) 583.
[54] M.T. Gladwin, A.N. Schechter, D.B. Kim-Shapiro, R.P. Patel, N. Hogg, S. Shiva, R.O. Cannon, M. Kelm, D.A. Wink, M.G. Espey, E.H. Oldfield, R.M. Pluta, B.A. Freeman, J.R. Lancaster, M. Feelisch, J.O. Lundberg, Nat. Chem. Biol. 1 (2005) 308.
[55] J.M. Monaghan, K. Cook, D. Gara, D. Crowther, J. Chromatogr. A 770 (1997) 143.
[56] P. Kleinbongard, A. Dejam, T. Lauer, T. Rassaf, A. Schindler, O. Picker, T. Scheeren, A. Godecke, J. Schrader, R. Schulz, G. Heusch, G.A. Schaub, N.S. Bryan, M. Feelisch, M. Kelm, Free Radic. Biol. Med. 35 (2003) 790.
[57] A. Wennmalm, G. Benthin, A. Edlund, L. Jungersten, N. Kieler-Jensen, S. Lundin, U.N. Westfelt, A.-S. Petersson, F. Waagstein, Circ. Res. 73 (1993) 1121.
[58] P. Kleinbongard, A. Dejam, T. Lauer, T. Jax, S. Kerber, P. Gharini, J. Balzer, R.B. Zotz, R.E. Scharf, R. Willers, A.N. Schechter, M. Feelisch, M. Kelm, Free Radic. Biol. Med. 40 (2006) 295.
[59] M. Himeno, T. Ishibashi, S. Nakano, K. Furuya, J. Yoshida, T. Kigoshi, K. Uchida, M. Nishio, Clin. Exp. Pharmacol. Physiol. 31 (2004) 591.
[60] J. Adachi, S. Morita, H. Yasuda, A. Miwa, Y. Ueno, M. Asano, Y. Tatsuno, Clin. Chim. Acta 269 (1998) 137.
[61] S. Winkler, I.E. Menyawi, K.F. Linnau, W. Graninger, Am. J. Trop. Med. Hyg. 59 (1998) 523.
[62] C. Mitaka, Y. Hirata, K. Yokoyama, H. Wakimoto, M. Hirokawa, T. Nosaka, T. Imai, Shock 19 (2003) 305.
[63] F.M.E. Diejomaoh, A.E. Omu, N. Al-Busiri, S. Taher, S. Al-Othman, T. Fatinikun, S. Fernandes, Arch. Gynecol. Obster. 269 (2004) 237.
[64] H. Kwon, G. Wu, C.J. Meininger, F.W. Bazer, T.E. Spencer, Biol. Reprod. 70 (2004) 679.

[65] G. Wu, F.W. Bazer, T.A. Cudd, C.J. Meininger, T.E. Spencer, J. Nutr. 134 (2004) 2169.
[66] G. Zunic, S. Spasic, Z. Jelic-Ivanovic, J. Chromatogr. B 727 (1999) 73.
[67] J.L. Gookin, J.M. Rhoads, R.A. Argenzio, Am. J. Physiol. 283 (2002) G157.
[68] G. Wu, L.A. Jaeger, F.W. Bazer, J.M. Rhoads, J. Nutr. Biochem. 15 (2004) 442.
[69] W.S. Jobgen, S.K. Fried, W.J. Fu, C.J. Meininger, G. Wu, J. Nutr. Biochem. 17 (2006) 571.