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Journal of Chromatography A, 706 (1995) 437–442

JOURNAL OF CHROMATOGRAPHY A

Nitric oxide in biological fluids: analysis of nitrite and nitrate by high-performance ion chromatography

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Abstract

The analysis of nitric oxide-derived nitrite and nitrate ions in biological fluids represents a proven strategy for determining nitric oxide participation in a diverse range of physiological and pathophysiological processes in vivo. In this article we describe a versatile method for the simultaneous measurement of NO_2^- and NO_3^- anions in both plasma and isolated tumour models based on anion-exchange chromatography with spectrophotometric detection (214 nm). This method compares well with the capillary electrophoresis technique, exhibiting an equivalent sensitivity for NO_2^-/NO_3^- anions and short run-times, i.e. not greater than 4 min. Comparisons are also made with two alternative but less satisfactory methods which employ ion-exchange or reversed-phase ion-pair chromatography with conductimetric as well as spectrophotometric detection. Technical problems associated with each method, particularly those arising from nitrate contamination, have been addressed.

1. Introduction

Since the identification of nitric oxide ($NO^\cdot$) as endothelium-derived relaxation factor (EDRF) [1] this free-radical species has been linked to multiple physiological and pathophysiological functions in vivo [2–4]. The signalling properties of EDRF–$NO^\cdot$ which modify vascular smooth muscle tone [5,6] and neurotransmission in peripheral and central nervous systems [7] contrast with the cytotoxic behaviour of $NO^\cdot$ when generated as part of the immune response [8,9].

A number of methods are capable of measuring the continuous release of $NO^\cdot$ [10] but those more commonly used include a chemiluminescence assay based on the reaction of $NO^\cdot$ with ozone [11] or luminol–H_2O_2 [12], spectrophotometric assays based on the oxidation of oxyhaemoglobin [13], electron paramagnetic resonance spectroscopy utilizing cheletropic traps [14] and $NO^\cdot$-selective electrode detectors [15]. However, these methods are not practical for clinical pharmacokinetic studies because of the short half-life (<5 s) of $NO^\cdot$ in vivo. In oxygenated aqueous solution $NO^\cdot$ is rapidly oxidized stoichiometrically to nitrite ions [16] according to Eq. 1:

$$4NO^\cdot + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (1)$$

Endogenous NO_2^- ion production in plasma cannot be assayed because of the nearly complete oxidation of NO_2^- to NO_3^- ions by, for example, oxyhaemoglobin [16] in Eq. 2:

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0021-9673/95/$09.50
SSDI 0021-9673(95)00078-X

$$4HbO_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb^+ + 4NO_3^- + O_2 + 2H_2O \quad (2)$$

The traditional Griess method [17] and the more recent 2,3-diaminonaphthalene method [18] both require back-reduction of plasma NO_3^- ions to NO_2^- prior to analysis [19].

Cancer patients receiving interluekin-2 (IL-2) therapy produce a cytokine-inducible nitric oxide synthase (*i*NOS) which is responsible for increased plasma nitrate production. In patients receiving IL-2 and flavone-8-acetic acid cotherapy, there is a correlation between increased plasma nitrate and remission of advanced malignant melanoma [20]. Clinical trials and studies in tumour models of potential anticancer therapies (which mediate their effects by an increased expression or alternatively inhibition of *i*NOS in tumour-associated macrophages and vasculature [2,21,22]) would benefit from fast quantitation of $NO^{\cdot}$-derived products both systemically and in the tumour microenvironment.

For the determination of many inorganic anions high-performance ion chromatography (HPIC), capillary electrophoresis (HPCE) and liquid chromatography (HPLC) have become the methods of choice and offer a more direct approach to the determination of nitrite and nitrate ions in plasma. A recent HPCE method has been reported with the required sensitivity to measure basal levels of nitrite and nitrate ions in plasma with minimal sample preparation and short run times of no more than 3 min [23]. HPIC analysis of plasma nitrate and particularly nitrite have been hampered by the large amount of chloride ion (typically $[Cl^-] \sim 100$ mmol dm^{-3}) which results in insufficient peak resolution when utilizing conductimetric detection [24]. This problem has in part been circumvented by HPLC utilizing both UV [25] or alternatively electrochemical detection [26] neither of which require prior removal of Cl^- ions.

In this article we describe a versatile method for the rapid (≈4 min), simultaneous measurement of NO_2^-/NO_3^- anions in both plasma and tumour perfusate samples based on anion-exchange chromatography with spectrophotometric detection (214 nm). Comparisons are made with two alternative but less satisfactory methods which employ ion-exchange or reversed-phase ion-pair chromatography with conductimetric as well as spectrophotometric detection. Technical problems associated with each method, particularly those arising from nitrate ion contamination during sample work-up, have been tackled.

2. Experimental

2.1. Chemicals

Acetonitrile was from Rathburn (Walkerburn, UK) and tetrabutylammonium hydrogensulphate ($TBASO_4$) was from Fisons (Loughborough, UK). All other chemicals were from Merck (Poole, UK). The perfusate solution was Krebs-Henseleit buffer modified to contain 5% albumin.

2.2. Chromatography

Chromatography was performed using three different systems as follows.

System 1: DX100 chromatograph (Dionex, Camberley, UK) fitted with a WISP autosampler (Waters, Watford, UK). The column was an IonPac AS9-SC ion-exchange column, 250 mm × 4 mm (Dionex), and the eluent was 1.7 m*M* $NaHCO_3$, 1.8 m*M* Na_2CO_3, pumped at 1.5 ml/min. Detection was by conductivity (DX100) and absorbance at 214 nm using a 441 detector fitted with a zinc lamp (Waters). Data was acquired and processed using an 840 data system (Waters).

System 2: 820 chromatograph and data system, equipped with 510 pumps and a WISP autosampler. The separation was achieved by an ion-pairing technique using a Hypersil 5ODS reversed-phase column, 125 mm × 4.6 mm (Hichrom, Reading, UK), and the eluent was 4% acetonitrile, 5 m*M* $TBASO_4$, 20 m*M* KH_2PO_4, 20 m*M* H_3PO_4, with a flow-rate of 2 ml/min. Detection was by absorbance at 214 nm using a 486 detector (Waters).

System 3: Millennium chromatograph and data system, equipped with 616 pumps and a WISP autosampler. The column was an IonPac AS9-SC ion-exchange column, 250 mm × 4 mm (Dionex), and the eluent was 5 m*M* K_2HPO_4, 25 m*M* KH_2PO_4, with a flow-rate of 1.5 ml/min. Detection was by absorbance at 214 nm using a 486 detector. All water used to make up eluents and samples was freshly drawn from a Milli-Q system supplied from a Milli-RO unit (Millipore, Watford, UK).

2.3. Sample preparation

Perfusate samples were obtained from an isolated rat tumour or hind limb [27]. Plasma samples were obtained from the rat or mouse. All sample handling was carried out in a laminar flow hood. For use with Systems 1 and 3 (ion exchange), a 50-μl aliquot of sample was pipetted into a 300-μl glass tube (Sci-Vi, Chromacol, Welwyn, UK). Acetonitrile (50 μl) was added, and the tube with an autosampler limited-volume insert spring was put in a 4-ml WISP vial, capped, mixed and centrifuged at 2000 *g* for 2 min. The sample was then ready for injection. For use with System 2 (reversed-phase), samples (50 μl) were pipetted into 1.5-ml polypropylene tubes (Sarstedt, Leicester, UK), 400 μl acetonitrile were added, and the tube was capped, mixed and centrifuged (10 000 *g* 1 min). The supernatant was decanted into another 1.5-ml tube, the pellet washed with a further 400 μl of acetonitrile, the combined extracts were dried under nitrogen and the sample was reconstituted in 200 μl of water. Where ultrafiltration was used for sample preparation, the filters [microcentrifuge tube filters, cellulose triacetate, 12 000 molecular mass cut-off (Whatman, Maidstone, UK), Centricon-10, 10 000 cut-off (Amicon, Stonehouse, UK) or ultra-spin centrifuge filters, 30 000 cut off (Alltech Assoc. Carnforth, UK)] were washed with Milli-Q water, dried, and the sample was applied. After centrifugation [9500 *g*, 5 min (Whatman and Alltech), 5000 *g*, 30 min (Amicon)], the sample was transferred to an autosampler vial for injection.

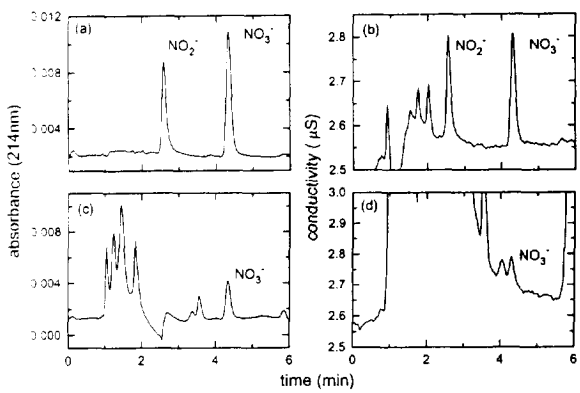

Fig. 1. Chromatograms of an acetonitrile-extracted aqueous standard containing 100 μ*M* nitrite and nitrate (a and b), and control mouse plasma (c and d). (a, c) Absorbance detection; (b, d) conductimetric detection. System 1 (anion-exchange, carbonate–bicarbonate eluent) was used.

3. Results

Figs. 1–3 show chromatograms of standards and plasma extracts using the three systems. In each case the separation of nitrate is acceptable, but quantification of nitrite is compromised in the first two systems by a large baseline disturbance when absorbance detection is used, or by the chloride peak with conductimetric detection. Only using the phosphate-based eluent was the nitrite sufficiently separated from the baseline dip. This is illustrated in Fig. 4 which shows chromatograms of perfusate buffer before and

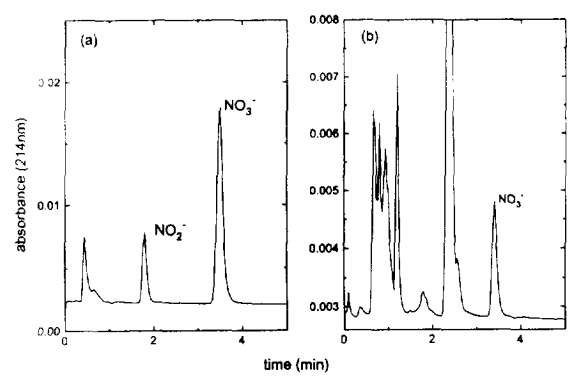

Fig. 2. Chromatograms of an acetonitrile-extracted aqueous standard containing 100 μ*M* nitrite and nitrate (a) and control mouse plasma (b) with absorbance detection at 214 nm. System 2 (reversed-phase chromatography) was used.

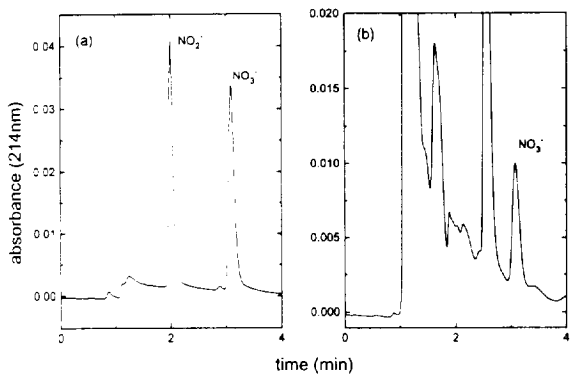

Fig. 3. Chromatograms of an acetonitrile-extracted aqueous standard containing 100 μM nitrite and nitrate (a) and control mouse plasma (b) with absorbance detection at 214 nm. System 3 (anion-exchange, phosphate eluent) was used.

after spiking with 5 μM nitrite and nitrate. Replicate extractions were also made of a perfusate sample using the three systems, and these are shown in Table 1. Because System 3 showed the lowest standard deviation, and permitted the measurement of nitrite, this system was selected for more detailed study.

The linearity of this method was assessed using extracted aqueous standards; calibration curves for both nitrite and nitrate were found to be linear over the range investigated (0.2–100 μM) (correlation coefficients, $r > 0.9993$ and >0.9997, respectively). The detection limit for nitrite was ~0.1 μM, governed by the shape of the baseline in this region. It was not possible to set a lower

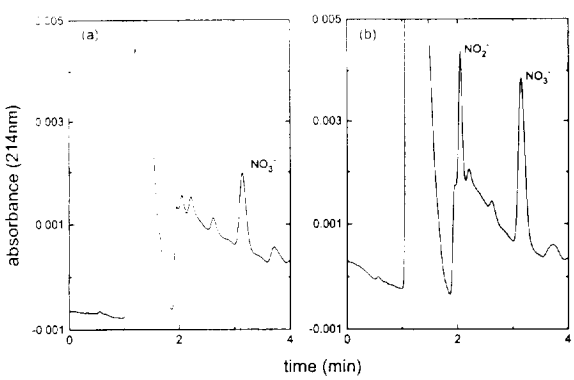

Fig. 4. Chromatograms of an acetonitrile-extracted perfusate buffer. (a) Control; (b) spiked with 5 μM nitrite and nitrate. System 3 (anion-exchange, phosphate eluent) was used.

Table 1
Repeat extractions of a perfusate using Systems 1, 2 and 3

	Nitrate concentration (μM)		
	System 1	System 2	System 3
	5.0	29.4	10.4
	10.5	32.6	8.6
	9.6	10.4	10.9
	6.5	11.1	11.4
		11.6	11.3
Mean ± S.D.	7.9 ± 2.6	19.0 ± 11.0	10.5 ± 1.1

detection limit for nitrate since all samples, including the Milli-Q water, contained detectable levels ≥0.5 μM. Recoveries of nitrite and nitrate from spiked plasma were 93.6 ± 2.9% and 94.6 ± 4.6% and from perfusates were 101.4 ± 3.1% and 99.4 ± 3.9%. Endogenous peak areas were subtracted prior to calculating these values. Intra- and inter-assay precision and accuracy and reproducibility were determined (Tables 2 and 3).

An alternative method of sample preparation was assessed using ultrafiltration to remove protein from the sample. However, all three filter types were heavily contaminated with nitrate which proved difficult to remove; the data are shown in Table 4.

4. Discussion

We have shown that nitrite and nitrate can be readily separated using several chromatographic and detection techniques as has already been demonstrated by a number of workers [24–26,28,29]. However, only one group [23] has addressed the question of sample contamination which we have found to be a major problem for nitrate determination. The contamination introduced by ultrafiltration, even after washing, did not encourage us to continue with the use of filters, although they have apparently been used successfully [23]; this may be a reflection of the larger sample volumes used.

We found that sample manipulations needed

Table 2
Intra- and inter-assay precision and accuracy of the HPIC procedure for nitrite and nitrate in perfusate buffer (System 3)

Component	Intra-assay		Inter-assay	
	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
Nitrite	1.58	99.35	1.27	100.20
Nitrate	5.21	100.80	3.35	100.80

Table 3
Mean and standard deviation ($n = 5$) of replicate analyses of mouse plasma (System 3)

Component	Mean concentration (μM)	S.D. (μM)
Nitrite	1.93[a]	0.24
Nitrate	15.60	0.72

[a] Peak co-elutes with nitrite.

to be kept to a minimum as illustrated by the standard deviation in Table 1. The reversed-phase method (System 2) would not tolerate injections of high concentrations of acetonitrile, and therefore the samples were dried down prior to injection. This involved two additional transfers, which resulted in large errors in the calculated concentrations. We attributed this contamination to nitrate-laden dust particles, and the situation was improved by carrying out the sample manipulations in a laminar flow hood where the air is filtered. By doing this, standard errors could be reduced, but were still much higher than using the single-step process. This was due in part to the fact that even new tubes were contaminated with 1–2 μM nitrate, occasionally much higher. Late-eluting peaks in plasma samples necessitated the use of a solvent gradient greatly extending the analysis time. This and the need to reduce sample manipulations to a minimum led us to discard the reversed-phase method (System 2). Also, in order to prevent a negative peak where nitrate eluted, it was necessary to prepare the eluent using freshly dispensed water; otherwise low concentrations of nitrate were not detected.

Measurement of low concentrations of nitrite are probably not relevant in plasma, but could be of importance in haemoglobin-free systems such as the perfused tumour. Both System 1 and

Table 4
Nitrate introduced by ultrafiltration treatments

Treatment	Nitrate concentration (μM)		
	Amicon	Whatman	Alltech
100 μl water[a]	19.4	–	–
50 μl water	–	10.4 ± 2.3	–
2 × 500 μl wash	–	1.9, 2.6[b]	–
50 μl water			
500 μl	–	4.1	17.9
2 × 500 μl wash	–	1.6, 2.9[b]	1.6, 2.3[b]
500 μl water			
2 × 500 μl wash	–	26.3 ± 1.8[c]	34.9 ± 2.3[c]
50 μl buffer + 450 μl water			

[a] Water contains up to 2 μM nitrate.
[b] Two observations.
[c] Perfusate buffer alone contains ~5 μM nitrate when analysed using the standard extraction method.

System 2 preclude the measurement of nitrite in either matrix. In System 2 there is a large interfering peak, while using System 1, the high concentration (~100 m*M*) of chloride gives a very large peak using conductimetric detection and is probably responsible for the dip using absorbance detection. Also, with conductimetric detection, there was an interference which would adversely affect the quantification of the nitrate (Fig. 1d). Although we did not assess the use of silver-based cation-exchange resins to remove chloride, we felt that the additional sample pretreatment steps involved were likely to exacerbate the contamination problem. Also, it is difficult to use such an approach with volumes of <0.5 ml, and sample amounts from biological systems are frequently rather limited. Absorbance detection for plasma nitrate appeared satisfactory, but we occasionally saw a distorted peak shape which suggested the presence of an interfering peak (not shown). Use of the phosphate-based eluent (System 3) has a number of advantages: it allows the determination of nitrite, while manipulation of the pH seemed to give more flexibility in altering the relative retention of potentially interfering peaks. However, it is not compatible with suppressed conductimetric detection.

Acknowledgements

This work is supported by The Cancer Research Campaign. Sponsorship from The Dionex Corporation is gratefully acknowledged. We thank Mr. Ralph Cochrane of Dionex (UK) for his many helpful discussions.

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