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The role of nitric oxide in cancer Improved methods for measurement of nitrite and nitrate by high-performance ion chromatography

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Abstract

The short lifetime of nitric oxide (NO) in vivo impedes its quantitation directly; however, the determination of nitrite and nitrate ions as the end-products of NO oxidation has proven a more practical approach. High-performance ion chromatographic analysis of nitrite in biological fluids is hampered by the large amount of chloride ion (up to ~100 mmol/l) which results in insufficient peak resolution when utilizing conductimetric detection. Analysis of both anions in small sample volumes is also constrained by the need to minimise sample handling to avoid contamination by environmental nitrate. We report a means to remove Cl^- ions from small sample volumes using Ag^+ resin which facilitates quantitation of either nitrite and nitrate anions in biological samples, using silica or polymer based ion-exchange resins with conductimetric or electrochemical and spectrophotometric detection. Including a reversed-phase guard column before the anion-exchange guard and analytical column also greatly extends column lifetime.

Keywords: Nitrite; Nitrate; Nitric oxide; Inorganic anions

1. Introduction

Endothelium-derived relaxation factor (EDRF) was identified nearly 10 years ago as being the free radical species nitric oxide (NO) [1]. It is involved in many physiological and pathophysiological processes [2–4], and in cancer biology, NO has been shown to modify tumour vascular tone, metastasis, angiogenesis and tumour cell proliferation [5]. The assessment of novel therapeutic approaches which modulate NO levels within tumours therefore relies on the accurate determination of NO within different sample matrices. The short lifetime of NO in vivo (<5 s) impedes its quantitation directly; however, NO is converted quantitatively to nitrite in oxygenated aqueous solutions, and in vivo, nitrite is rapidly oxidized to nitrate [6,7]. Thus, in biological fluids, nitrite and/or nitrate ions are commonly determined as the end-products of NO oxidation, either by spectrophotometric/fluorimetric [8,9] or chromatographic methods [10–16].

Many of our studies in NO biochemistry/physiology require the measurement of nitrite and nitrate in small (<100 μl) samples. We previously described versatile high-performance ion chromatography (HPIC) methods [17,18] for the simultaneous determination of nitrite and nitrate in biological fluids which facilitated this, based on anion-exchange chromatography with spectrophotometric detection (214 nm). However, it was not possible to

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reliably determine the low levels of nitrite present in plasma because of interferences, and we describe here an improved method using a silica based ion-exchange resin with electrochemical and spectrophotometric detection. HPIC analysis of plasma nitrate and particularly nitrite with conductimetric detection has been hampered by the large amount of chloride ion (typically $[Cl^-]$ ~100 mmol/l) which results in insufficient peak resolution from nitrite. We report a means to remove Cl^- ions using Ag^+ resin which facilitates quantitation of nitrite and nitrate anions in small volumes of biological samples.

2. Experimental

2.1. Chemicals

Acetonitrile was from Rathburn (Walkerburn, UK), potassium dihydrogenorthophosphate and orthophosphoric acid were electrochemical grade from Fisons (Loughborough, UK). All other chemicals were from Merck (Poole, UK).

2.2. Chromatography

HPLC was performed on a Waters Millennium system equipped with 616 pumps and 717 autosampler. The silica based column was an Exsil anion-exchanger (Exsil SAX, 125×4.6 mm) with two guard cartridges (Hypersil 5ODS and Exsil SAX, 10×2 mm), all from Hichrom (Reading, UK). The eluent for this system was 26% acetonitrile, 20 m*M* KH_2PO_4, 2 m*M* H_3PO_4, and the flow-rate 2 ml/min. Detection was by absorbance at 214 nm using a Waters 486 detector (Watford, UK), and electrochemically with a Coulochem detector, using a dual porous graphitic electrode, 1st electrode +0.3 V, second (monitoring) electrode, +0.65 V (ESA, St Ives, UK). The polymeric based column was an IonPac anion-exchanger (AS12A, 250×4 mm), with an equivalent guard column (50×4 mm), both from Dionex (Camberley, UK), and a Hypersil guard cartridge as above. The eluent for this system was 0.3 m*M* $NaHCO_3$, 2.7 m*M* Na_2CO_3, and the flow-rate was 1.5 ml/min. Detection was again at 214 nm, or with suppressed conductivity (external water) with a Dionex ED40. A preliminary study was carried out using a Dionex AS4A-SC guard and analytical column, eluent 1.7 m*M* $NaHCO_3$, 1.8 m*M* Na_2CO_3.

2.3. Sample preparation

Samples were prepared as previously described [18]. Briefly, in a flow hood to avoid environmental contamination, a 50 μl sample was aliquoted into a 300 μl limited volume insert (Chromacol, Welwyn, UK), followed by 50 μl acetonitrile and the sample mixed and centrifuged. Injection into the HPLC was made directly from this vial. Where the silver resin (On-Guard-Ag, Dionex,) was used, ~20 mg was weighed into the vial before the sample.

For ex vivo tumour incubations, tumours were coarsely chopped and placed in 2 ml Eagles minimum essential medium supplemented with 10% foetal calf serum in a 5 cm Petri dish. These were maintained for up to 24 h in an incubator at 37°C gassed with humidified 95% air, 5% carbon dioxide. Cells were grown in a similar manner. Samples of the medium were withdrawn at various times and treated as above.

3. Results

Fig. 1 shows a chromatogram, obtained using the Exsil ion-exchange column, of a human plasma extract, with both absorbance (214 nm) and electrochemical detection. Both detection modes are required since nitrate is not electrochemically active,

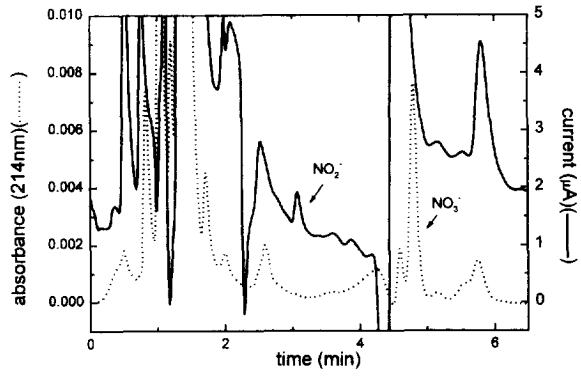

Fig. 1. Chromatogram of an acetonitrile extract of human plasma obtained using the Exsil ion-exchange column with absorbance and electrochemical detection.

while the low concentration (sub-micromolar) of nitrite in plasma and the presence of interfering peaks precludes the use of absorbance detection for reliable determination of this ion [16]. Concentrations of nitrite and nitrate in human plasma were found to be 0.71±0.46 μ*M* and 47.8±15.3 μ*M* (n=5), respectively. The intra-assay R.S.D. was 3.9% for nitrite and 1.2% for nitrate; the corresponding inter-assay figures were 8.1% and 3.9%. Replicate analyses of a sample of normal human plasma gave concentrations of 0.914±0.074 μ*M* (nitrite) and 63.56±2.48 μ*M* (nitrate).

Samples of the rat P22 carcinosarcoma, and the corresponding isolated tumour cells were incubated, and samples of the medium analysed for nitrite and nitrate. The tumour showed an increase in both nitrite and nitrate with time, while the cells showed only an increase in nitrite. Nitrate production by the tumour explants was oxygen-dependent, increasing from 2.49±0.66 nmol/24 h/100 mg at 1% oxygen to 4.12±0.78 nmol/24 h/100 mg at 21% oxygen and 7.08±1.40 nmol/24 h/100 mg at 95% oxygen. In vitro nitrite production amounted to 10.4±1.38 nmol/24 h/10^6 cells initially seeded.

In order to use conductivity detection for both nitrite and nitrate in biological fluids, the high chloride concentration must be decreased, as illustrated in Fig. 2. Panel (a) shows the oxidation of nitrite to nitrate which occurs in blood at 20°C, while panel (b) illustrates that only absorbance detection permits the determination of nitrite in the presence of

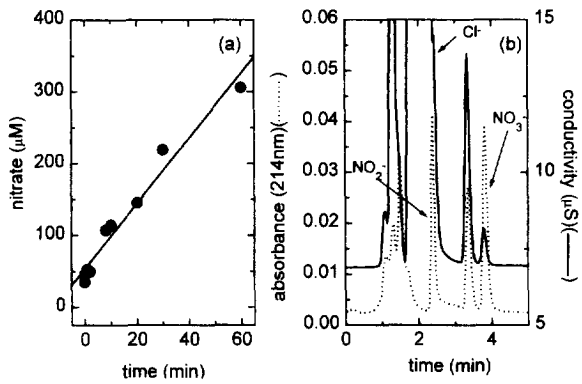

Fig. 2. Oxidation by whole heparinised rat blood of nitrite. (a) Time course of nitrite production; (b) absorbance and conductimetric detection of plasma injected after centrifugation. Chromatography was using a Dionex AS4A-SC column.

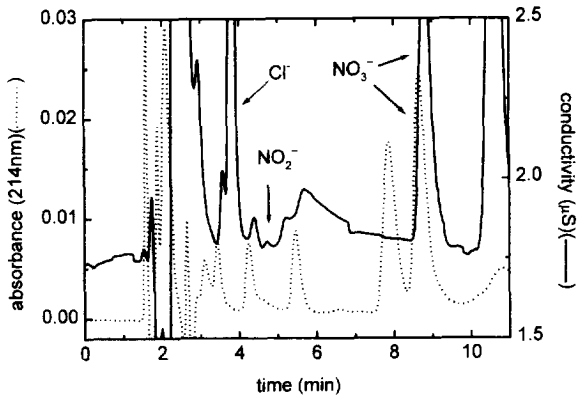

Fig. 3. Chromatogram of an acetonitrile extract of human plasma treated with silver resin, obtained using the Dionex AS12 column with conductimetric and absorbance detection.

physiological levels of chloride ion. In order to remove the chloride, samples were extracted in the presence of silver resin. Fig. 3 was obtained using the AS12 system, and shows the use of conductivity in parallel with absorbance detection for a human plasma extract after this treatment. The chloride peak is sufficiently reduced to permit the detection of nitrite, as well as nitrate, by conductivity, while an interference precludes the determination of nitrite by absorbance detection using this system. Note that the conductivity scale in this figure is an order of magnitude more sensitive than in the previous figure. Figs. 4 and 5 show the same samples chromatographed on the silica based Exsil column with

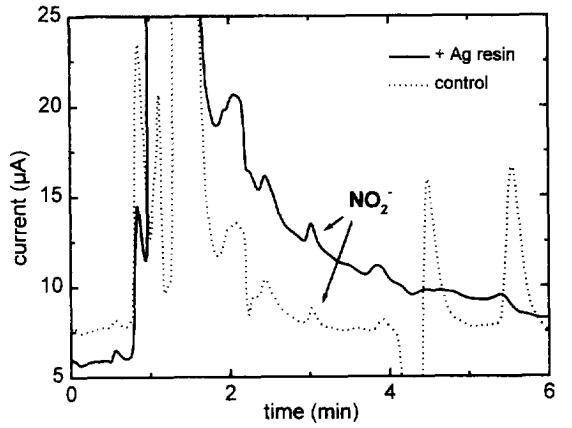

Fig. 4. Chromatogram of an acetonitrile extract of human plasma treated with silver resin, obtained using the Exsil ion-exchange column, with electrochemical detection.

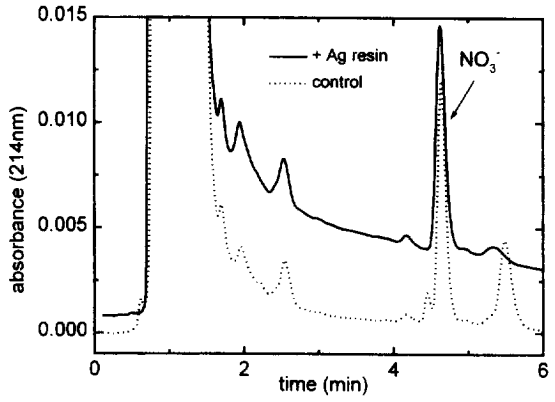

Fig. 5. Chromatogram of an acetonitrile extract of human plasma treated with silver resin, obtained using the Exsil ion-exchange column, with absorbance detection.

electrochemical and absorbance detection, respectively.

4. Discussion

The determination of nitrite and nitrate in small volumes of biological fluids has proved a challenge for a number of reasons. The potential for contamination by environmental nitrate is a particular problem with small samples. Our early experiences with determination of plasma nitrate prior to the use of flow hoods for sample preparation, and with re-use of washed sample vials, gave large variations in concentrations, often up to 3–4 fold, regardless of the chromatographic system employed. Our one step sample preparation technique, [18], has proved extremely useful in a variety of analyses. However, with the column used in that study (Dionex AS9-SC), we could not satisfactorily determine nitrite at the low concentrations present in plasma, and we also found that column lifetime was rather short. At that time it was not thought that significant concentrations of nitrite occurred in human plasma, because of the oxidation by haem iron, but there were reports that nitrite was detectable in man [13–15,19,20]. In addition, it became necessary to determine both species in other in vitro systems where oxidation did not occur.

We therefore assessed the use of silica based ion-exchange resins, and found that using electrochemical detection, we could reliably determine plasma nitrite and nitrate, although this required the use of two detectors (Fig. 1). This system has been used successfully for analysis of a large number of both plasma samples and tumour explants and tumour cell in vitro incubations. One factor which we have found to be of great importance in maintaining column lifetime is the use of two guard columns, the first a reversed-phase and the second an anion-exchange column. With careful washing of the column, and regular replacement of guard cartridges, this has allowed the analysis of ~2000 samples before changing the column. A disadvantage of the use of this column is that when a new column is first used, the retention times move quite markedly earlier after the first few injections, sometimes necessitating some adjustment to the eluent. Occasionally small adjustments are required to the eluent dependent on the sample matrix being analysed.

The in vitro and ex vivo rat P22 rat tumour data highlight the importance of determining both nitrite and nitrate, since the tumour explants produced both anions, while the cells only produced nitrite. This presumably reflects haemoglobin from residual blood in the tumour samples which oxidises the nitrite produced to nitrate [6]. Oxygen is also required for nitric oxide biosynthesis [21], which may explain the oxygen dependence of the production of nitrate.

Direct determination of nitrite in plasma samples using conductivity detection is not possible because of the large chloride peak (~100 m*M*) (Fig. 2b), and the requirement to work with small volumes precluded the use of the silver resin cartridges directly. However, adding the resin directly to the sample vial avoids introducing an additional step to the procedure, minimising the potential for sample contamination. This does permit the detection of nitrite using conductivity detection with the AS12 column (Fig. 3), which has the advantage of allowing quantitation of both ions using one detector. However, this system does not appear as robust as using the silica based ion-exchange column with electrochemical and absorbance detection, with peaks on either side of the nitrite which in other samples interfere with its determination. The silver treatment also appears to enhance the chromatography of the nitrate on the Exsil column (Figs. 4 and 5).

We have successfully used the extraction method

described here for the analysis of small volumes of a large number of both plasma and other samples of biological origin for nitrite and nitrate using electrochemical and absorbance detection. The low concentration of nitrite observed in plasma agrees well with that published by some workers [13,14,19,20], although others have noted higher levels [11,15]. It is very quick, both in terms of sample preparation and chromatography. The extra step of removing silver ions at the same time as sample extraction may prove of value where only conductimetric detection is available, and may also prove useful in the system described here by removing other interfering peaks.

Acknowledgments

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References

[1] R.M.J. Palmer, A.G. Ferrige and S. Moncada, Nature, 327 (1987) 524.
[2] S. Moncada, M.A. Marletta, J.B. Hibbs, Jr. and E.A. Higgs (Editors), The Biology of Nitric Oxide, Portland Press, London, Vol. 2. 1992.
[3] J.F. Kerwin, Jr. and M. Heller, Medicinal Res. Rev., 14 (1994) 23.
[4] A.R. Butler and D.L.H. Williams, Chem. Soc. Rev., (1993) 233.
[5] S.M. Sagar, G. Singh, D.I. Hodson and A.C. Whitton, Cancer Treat. Rev., 21 (1995) 159.
[6] L.J. Ignarro, J.M. Fukuto, J.M. Griscavage, N.E. Rogers and R.E. Byrns, Proc. Natl. Acad. Sci. USA, 90 (1993) 8103.
[7] P.C. Ford, D.A. Wink and D.M. Stanbury, FEBS Lett., 326 (1993) 1.
[8] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannenbaum, Anal. Biochem., 126 (1982) 131.
[9] T.P. Misko, R.J. Schilling, D. Salvemini, W.M. Moore and M.G. Currie, Anal. Biochem., 214 (1993) 11.
[10] E. Bianchi, R. Bruschi, R. Draisci and L. Lucentini, Z. Lebensm. Unters. Forsch., 200 (1995) 256.
[11] D. Crowther, J.M. Monaghan, K. Cook and D. Gara, Anal. Comm., 33 (1996) 51.
[12] P. de Jong and M. Burggraaf, Clin. Chim. Acta, 132 (1983) 63.
[13] A.M. Leone, P.L. Francis, P. Rhodes and S. Moncada, Biochem. Biophys. Res. Commun., 200 (1994) 951.
[14] B.C. Lippsmeyer, M.L. Tracy and G. Moeller, J. Assoc. Off. Anal. Chem., 73 (1990) 457.
[15] Y. Michigami, Y. Yamamoto and K. Ueda, Analyst, 114 (1989) 1201.
[16] J. Osterloh and D. Goldfield, J. Liq. Chromatogr., 7 (1984) 753.
[17] M.R.L. Stratford, M.F. Dennis, G.M. Tozer, V.E. Prise and S.E. Everett, in J. Stamler, S. Gross, S. Moncada and E.A. Higgs (Editors), The Biology of Nitric Oxide, Vol. 5, Portland Press, London, 1996, p. 175.
[18] S.A. Everett, M.F. Dennis, G.M. Tozer, V.E. Prise, P. Wardman and M.R.L. Stratford, J. Chromatogr. A, 706 (1995) 437.
[19] M. Kelm, M. Feelisch, R. Grube, W. Motz and B.E. Strauer, in S. Moncada, M.A. Marletta, J.B. Hibbs, Jr. and E.A. Higgs (Editors), The Biology of Nitric Oxide, Vol. 1, Portland Press, London, 1992, p. 319.
[20] A. Wennalm, B. Lanna and A.S. Peterson, Anal. Biochem., 187 (1990) 359.
[21] K.A. White and M.A. Marletta, Biochemistry, 31 (1992) 6627.