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Determination of nitrite and nitrate in human serum

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Abstract

A simple and effective assay for nitrite and nitrate in human serum has been developed using ion chromatography. Initial experiments using isocratic carbonate–bicarbonate elution with conductivity detection on a Dionex QIC system with an AS4A-SC column showed promise but were unsatisfactory because of co-elution problems with nitrite. Carbonate and chloride were investigated as eluents using a gradient system, and direct UV detection at 214 nm was used in place of conductivity detection. Dionex AS4A, AS9A, AS12, Nucleopac PA-100 and Carbopac PA-100 columns were compared for selectivity and resistance to overload. The final method, using a chloride concentration gradient, pH buffering and direct UV detection with a Carbopac PA-100 column, shows good resolution, does not suffer from chloride overload and is simple to use. The method is being used in an investigation of the role of nitric oxide in pre-eclampsia, a hypertensive disorder during pregnancy.

Keywords: Nitrate; Nitrite; Inorganic anions; Nitric oxide

1. Introduction

Nitric oxide (NO) is a vital messenger in many cellular communication and control systems. Because of the short half life of NO in aqueous media, assay of NO is performed on its metabolites, nitrite and nitrate. Classically, these have been determined by the Griess method, in which nitrite is diazotised with sulphanilamide and then reacted with N-1-naphthylethylenediamine to form a coloured product. However, this approach does not work well in the complex matrix of serum, where the reduction step from nitrate to nitrite is difficult to achieve chemically or expensive if enzymatic reduction is used. Redox buffers such as ascorbic acid may be responsible for the reduction problem, but other factors can also affect the assay. Colour and turbidity in the sample will cause interference and the presence of copper in the sample will catalyse the decomposition of the diazonium salt and bring about a reduction in the results [1]. Assay of nitrite alone, without accompanying nitrate, is unsatisfactory because serum samples left at room temperature for more than 4 h may show oxidation of nitrite to nitrate [2].

Ion chromatography has become one of the most powerful tools for the quantitative analysis of anions and cations. The most common procedure for the analysis of anions is an isocratic method with bicarbonate–carbonate buffered eluent, suppressed ion chromatography and conductivity detection, as used by Dionex systems. Ion chromatographic methods for nitrate and nitrite determination have been reported previously using such systems (see Sah [1] for a review). However, the naturally high concentration of chloride present in serum compared to nitrite and nitrate (the concentration of chloride is approx. 10^5 times that of nitrite) swamps out the

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signal from nitrite when using conductivity detection because nitrite elutes shortly after chloride from most anion-exchange columns. This has led previous workers [3] to pretreat samples with silver-loaded resin in order to reduce the chloride concentration. Such an approach is effective but time-consuming, expensive and likely to reduce precision. Silver ions, which may leach from the resin, also ultimately damage the ion-exchange columns.

Direct UV detection at about 214 nm avoids the large positive signal from serum chloride and, if an eluent is chosen that has low absorption at this wavelength, a cleaner trace for double bond-containing ions can be obtained. Ideally, for ion chromatography, the eluent ion should have lower affinity for the column than most of the analyte species and it should have low toxicity. Hydroxyl ions are good eluents, and chloride can also be used.

The use of chloride as an eluent ion for nitrate and nitrite in samples containing high levels of chloride has been reported by Pastore et al. [4]. Haddad et al. [5] reported a working method to quantify low nitrite levels in sea water and Radisavljevic et al. [6] have published an isocratic chloride method for serum samples. The benefit of using chloride as the eluent ion is that the column is coated with this ion prior to injection of the sample and displacement artifacts due to the passage of a large slug of sample-derived chloride through the system are greatly reduced.

Unlike the more commonly used carbonate–bicarbonate system, however, chloride has no inherent pH buffering capacity and so another ion must be added in low concentration to provide buffering. Phosphate has been used as a buffer [6] but obviously this precludes the determination of sample phosphate. Isocratic elution with sufficient resolution to separate nitrite from interferences also resulted in a long run time [6]. Gradient elution is an answer but at low eluent strength it is possible for buffer ions to accumulate on the column; these are displaced at higher eluent strength as the gradient elution progresses, producing an artifactual peak. This work addresses the problem by use of an eluent strong enough to prevent buffer accumulation and a high capacity column with unusual selectivity. A preliminary account of this work has been published elsewhere [7].

2. Experimental

2.1. Reagents

Stock solutions of nitrite and nitrate were prepared by dissolving the appropriate amount of AnalaR sodium salt (BDH, Poole, UK) in Milli-Q water that had a resistance of not less than 18 MΩ. Trizma buffer (ultra-pure grade, Aldrich, Gillingham, UK) was dissolved in the eluent reservoirs to 5.0 m*M* and the pH was adjusted to pH 7.5 with AnalaR hydrochloric acid. HPLC-grade sodium chloride (BDH) was used to make the eluent. All eluents were thoroughly degassed and were stored under helium.

The location of nitrite and nitrate peaks were determined from known standard solutions in water and were confirmed, where necessary, by spiking serum samples.

2.2. Samples

All control serum samples (prepared from fresh blood) were obtained from the Blood Transfusion Service (Northern General Hospital, Sheffield, UK). Serum samples were diluted four-fold with deionised water and then centrifuged through 3000 M_r cut-off Centricon filters (Amicon, Stonehouse, UK) for 120 min at 7500 *g* prior to analysis. Each filter was washed thoroughly before use with deionised water to remove nitrate left by the manufacturing process. There was no further pre-treatment of serum.

2.3. Griess method

A 200-μl volume of Griess reagent was added to 200 μl of deproteinised serum in a 96-well plate, which was left for 15 min in a dark cupboard and then the absorbance was read at 570 nm on a Labsystems Multiskan MS V1.5 plate reader.

2.4. Isocratic ion chromatography method

A Dionex (Camberley, UK) QIC suppressed ion chromatography system was used with AS4A-SC analytical and guard columns and conductivity detection. The eluent consisted of 1.8 m*M* Na_2CO_3 plus 1.7 m*M* NaHCO3. An LDC/Milton Roy Spec-

tromonitor III (Thermo Analytical Systems, Stone, UK) UV detector set at 214 nm was connected in series to the conductivity cell outlet for comparison of UV and conductivity traces. No other column was used with this system.

2.5. Gradient ion chromatography methods

The chromatographic system contained a Dionex gradient pump and an LDC/Milton Roy Spectromonitor III set at 214 nm. Data were acquired using a VG Minichrom data system, with v1.6 software (VG Data Systems, Altrincham, UK). Samples were injected into the system using a Perkin-Elmer ISS-100 Autosampler (Beaconsfield, UK) with a sample volume of 30 μl. All the stainless steel tubing in the system was replaced with polyether ether ketone (PEEK) tubing and the apparatus was washed after every use with pure water to minimise corrosion due to the use of a high concentration of sodium chloride as the eluent.

Dionex AS4A-SC, AS9A, AS12, Nucleopac-PA 100 and Carbopac-PA 100 columns (all 250×4 mm) were kindly loaned by Dionex (UK). Equivalent guard columns were placed before the analytical columns. The system was fully automated in that the autosampler was the chief controller for starting the Dionex gradient pump and the Minichrom data system at the start of each run, allowing 24 h analysis of samples, if required.

For the AS4A-SC, AS9A, AS12 and Nucleopac PA-100 columns, the chloride gradient programme was as follows: isocratic 1.5 m*M* chloride for 6 min, gradient from 1.5 to 30 m*M* chloride for 9 min, isocratic 30 m*M* chloride for 15 min, isocratic 150 m*M* chloride wash for 1 min and then re-equilibration over 9 min with isocratic 1.5 m*M* chloride. The flow-rate was 1.5 ml min^{-1} throughout and no buffer was present.

For the Carbopac PA-100 column, the short gradient programme used (no resolution of organic acids) was: gradient from 120 to 300 m*M* chloride over 10 min, isocratic 1000 m*M* chloride wash for 4 min and then re-equilibration over 6 min with 120 m*M* chloride. 5 m*M* Tris buffer was present throughout and the pH value of all eluents was adjusted to 7.5. The flow-rate was 1.0 ml min^{-1} throughout.

The longer programme with the Carbopac PA-100 column for separation of organic acids was: isocratic 30 m*M* chloride for 1 min, gradient from 30 to 300 m*M* chloride over 15 min, a 4-min was with 1000 m*M* chloride and then re-equilibration over 10 min with isocratic 30 m*M* chloride. The buffer and the flow-rate were as for the previous programme.

3. Results and discussion

Fig. 1 shows the traces obtained with conductivity and UV detection from an isocratic elution with carbonate eluent and a Dionex AS4A-SC column.

The lower chromatogram shows a typical serum sample with conductivity detection. The largest peak is from chloride and clearly obscures the signal from nitrite, which elutes just after chloride. The result of pretreatment with silver-loaded resin, as advocated by Lippsmeyer et al. [3], is shown in the upper two traces of Fig. 1. Once prepared, the resin is added to serum that has been centrifuged through a membrane filter. The resulting precipitate is filtered off and then the sample is injected onto the column. The resulting conductivity chromatogram (Fig. 1, middle trace) shows that this procedure greatly reduces the chloride content, but does not always reduce it sufficiently to allow quantification of nitrite. The use of

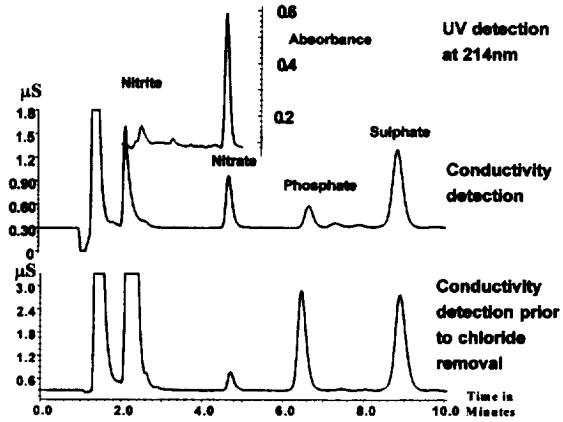

Fig. 1. Chromatograms of ultrafiltered human serum obtained using an AS4A-SC column with isocratic carbonate elution. The amount of chloride was decreased by pretreatment with silver resin (middle and upper traces).

direct UV detection at 214 nm (Fig. 1, upper trace) renders the chloride invisible and allows quantification of the nitrite peak. However, there are many other UV-absorbing components in serum (particularly organic acids), which, under these conditions, elute very close to the nitrite peak. Reducing the eluent strength to improve resolution in the early part of the chromatogram results in an unacceptably long retention time for nitrate. Additionally, the use of resin pretreatment in this method creates additional manipulations which are possible sources of error.

A modified elution protocol is needed to improve the resolution of the early-eluting species (including nitrite) while retaining a reasonable elution time for nitrate and for late-eluting species. A concentration gradient of carbonate–bicarbonate can be used to improve the resolution of early-eluting species, however, removal of chloride is still necessary, even if UV detection is used, because the amount of chloride present causes a large negative peak in the UV trace [8,9]. This is probably due to displacement of UV-absorbing eluent ions by the chloride slug as it passes down the column. The effect is to render nitrite determination impossible.

The use of chloride as an eluent ion has been reported previously as a means of overcoming chloride-induced artifacts without resorting to its removal from each sample [4–6]. Haddad et al. [5] determined trace amounts of nitrite in seawater in a method designed to solve essentially the same problem as here, namely a high concentration ratio of chloride to nitrite. Use of this procedure, an isocratic method with 15 m*M* chloride as the eluent (data not shown), did remove the negative peak due to chloride in the UV trace, but organic acids co-eluting with the nitrite peak caused interference.

A chloride gradient was then used to resolve the co-elution problem, using a range of anion-exchange columns with a gradient from 1.5 to 30 m*M* chloride followed by a 150 m*M* chloride wash before re-equilibration. An AS4A-SC column (capacity ca. 20 μequiv. per column) produced reasonable results (not shown) but became blocked after several runs with serum and required extensive cleaning. A higher capacity column that was designed for gradient elution, the AS9A (capacity 30–35 μequiv. per column), gave improved results but still did not clearly separate the nitrite peak from interfering species (Fig. 2).

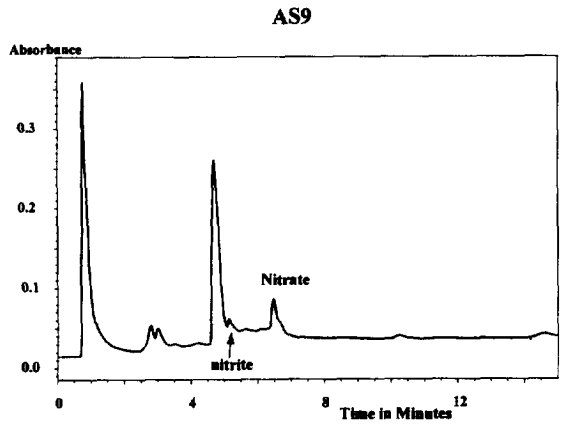

Fig. 2. Chromatogram of ultrafiltered human serum obtained using an AS9A column with low concentration chloride gradient elution and UV detection.

An AS-12 column (Fig. 3) showed better separation of the nitrite peak from interferences in the serum samples but at the expense of overall resolution.

Because the co-elution problem seemed to be with organic acids, where elution behaviour might also be affected by hydrophobic interactions with the stationary phase, column packings designed for the separation of organics were also investigated. Fig. 4 shows the chromatogram obtained with a Dionex

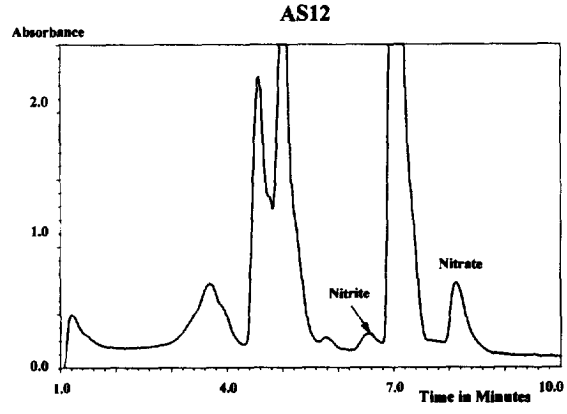

Fig. 3. Chromatogram of ultrafiltered human serum obtained using an AS12 column with low concentration chloride gradient elution and UV detection.

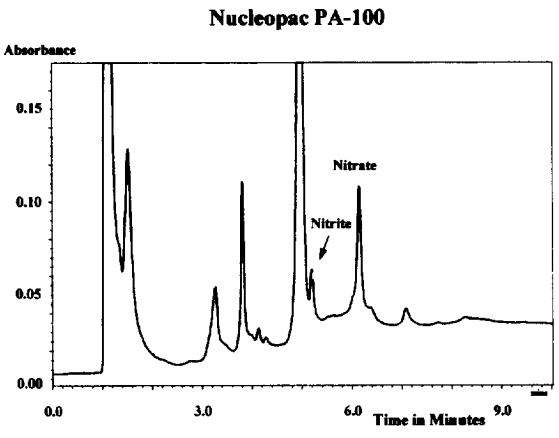

Fig. 4. Chromatogram of ultrafiltered human serum obtained using a Nucleopac PA-100 column with low concentration chloride gradient elution and UV detection.

Nucleopac PA-100 column (capacity 25 μequiv. per column), where reasonable resolution is evident but where nitrite still elutes on the tail of a larger unidentified peak.

Change of column type to a Dionex Carbopac PA-100 (a high capacity column, of 90 μequiv. per column) and use of a higher concentration of chloride as the eluent yielded the best results (Fig. 5). This method showed good separation of nitrite from adjacent peaks, good overall resolution and good resistance to overload. An analysis time of 10 min plus another 10 min for washing and re-equilibration

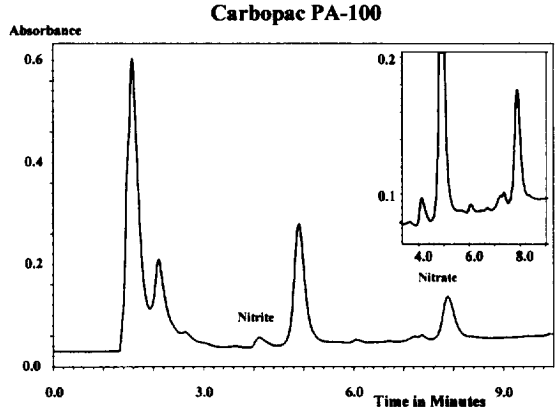

Fig. 5. Chromatogram of ultrafiltered human serum obtained using a Carbopac PA-100 column with high concentration chloride gradient elution (short run) and UV detection.

of the column were adequate for nitrate and nitrite quantification in each serum sample.

With this 20 min cycle time, organic acids in the sample elute early and are not fully resolved. A modified elution programme, starting at a lower eluent strength and with a 1-min isocratic step before a longer gradient time, was found to allow separation of some of the organic acids present in serum (Fig. 6), with a full cycle time of 30 min. Organic acids indicated (identification probable, by retention time comparison only) include lactate, pyruvate, formate, acetate, malonate, hippurate, fumarate, citrate and maleate. Further work is needed to investigate the efficiency of separation of the serum organic acid complement, which can prove difficult to separate fully, but the method appears promising.

All of the columns used are of the pellicular type, where small latex particles bearing quaternary ammonium ion-exchange groups are electrostatically bound to larger modified styrene–divinylbenzene beads. The nature of the organic side chains of the quaternary ammonium groups forms a secondary selectivity mechanism in ion chromatography. Here, the AS-12 and Carbopac PA-100 columns have essentially hydrophobic groups while the AS9A and

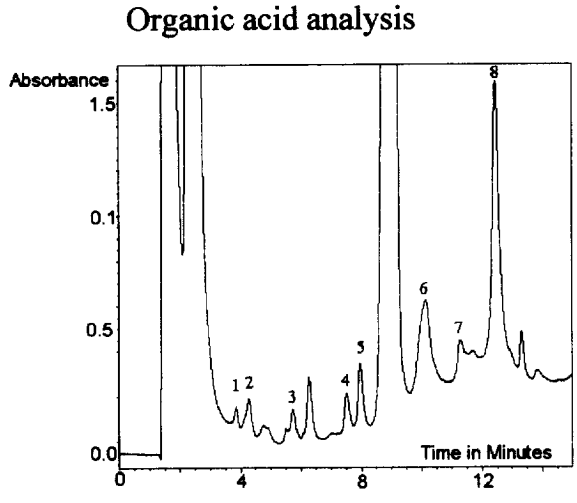

Fig. 6. Chromatogram of ultrafiltered human serum obtained using a Carbopac PA-100 column with wide concentration range chloride gradient elution (long run) and UV detection. 1=Lactate, 2=acetate, 3=pyruvate, 4=malonate, 5=nitrite, 6=fumarate, 7=citrate and 8=nitrate.

Table 1
Measured and previously published normal range values for nitrite and nitrate in human serum (or plasma, where indicated). Deviations shown represent the full population variation observed, not experimental variations

Author	Mean nitrite concentration (μM)	Mean nitrate concentration (μM)	Total NO_x (μM)	n
Michigami et al. [10]	4.4±2.8	61.1±36.8	65.5±39.6	34
Jong and Burggraaf [12]	–	36.0±19.0[a]	–	20
Lyall et al. [13]	–	–	28.8±1.07[a]	
Curtis et al. [14]	9.5±0.6[a]	85±8.0[a]	94.5±8.6[a]	59
Radisavljevic et al. [6] (plasma)	3.1±0.4	10.3±0.3	13.4±0.4	22
Leone et al. [15] (plasma)	0.45±0.2	41±15	41±15	5
Meulemans and Delsenne [16] (plasma)	not detectable (<0.5)	92±19	92±19	10
Monaghan (this work)	4.2±3.9	39.9±22.0	44.1±22	200

[a]result quoted for females

Nucleopac PA-100 columns have hydrophilic groups. This difference in selectivity may account for the apparent change in elution order of the nitrite peak and the large unidentified peak seen in Figs. 2–5.

Using the Carbopac PA-100 column with the chloride gradient programmes, calibration curves from aqueous nitrate and nitrite standards were linear to 30 μM. The limits of detection for both anions were approximately 0.25 μM (S/N=2:1) with the UV detector used. Improvement of these limits of detection should be possible with a UV detector with a better S/N ratio. Comparison with the Griess method for nitrite showed a linear correlation over the working range of the method.

Using the quick Carbopac PA-100 method, 200 individual serum samples (in duplicate) from the local Blood Transfusion Service were analysed. Male and female samples were separated because there are conflicting reports in the literature concerning sex linked differences in serum nitrate and nitrite concentrations [10,11]. Results for the 200 serum samples are shown in Table 1, along with previously reported values. It is clear that there is a wide individual-to-individual variation in levels and that there may be systematic (technique dependent) or inter-population variations as well. Sample storage conditions may play a large part in these variations. Oxidation of nitrite to nitrate will occur in whole blood or in partially haemolysed samples.

4. Conclusions

The aim of this work was to produce a simple, versatile and robust ion chromatographic method that had distinct advantages over previously published methods for analysis of nitrite and nitrate in serum. A gradient system has been used with a selected anion-exchange column to enhance resolution of nitrite and, as a bonus, to enable separation of a number of organic acids. The use of a chloride eluent has removed the need for silver resin pretreatment of samples and UV detection removes the need for a conductivity suppressor. The method developed combines these features to produce a reliable and simple procedure for studies of nitric oxide in mammalian systems. It should also be suitable for any study that requires the analysis of UV-absorbing anions in complex matrices, such as foodstuffs, waters (including brine, sea water and waste waters) and environmental samples.

The advantages of the method are:

1. Specificity: The method shows good resolution for the target analytes in nearly all samples investigated.
2. Reproducibility: Buffering of the eluent allows reliable quantification because retention times remain stable and repeats can be assured. Full loop injections gives reproducible sample volumes.
3. Minimal sample pretreatment, yielding faster and more reproducible assays. A "one-step" centrifugal ultrafiltration is all that is required.
4. Cheapness: The reagents required for the enzymatic reduction of nitrate to nitrite are expensive and many reaction steps are required. Once the ion chromatography equipment has been obtained, the system is relatively cheap to run.

The wash step is advantageous for prolonging the life of the columns, periodic replacement of which is the principal running cost.

5. Automation: The automated system is advantageous in clinical studies where many samples can be analysed in batch mode without operator attendance.
6. Direct detection of target analytes: Unlike the Griess method, no chemistry is performed on the analytes and thus there are no problems with inhibition of essential reactions.

The method described has clear benefits over previously described methods and, in one year of use, has shown only two problems.

Firstly, co-elution of nitrite with other UV-absorbers (even though normally removed by this method) is not always completely eliminated. So far, two serum samples of over 300 analysed have shown an unidentified interfering peak that partly co-eluted with nitrite and made quantification difficult. Inspection of chromatograms is always recommended and the Griess method should be employed for the nitrite component, either on the original sample or as a post-column derivatisation, if such a problem is noticed.

Secondly, serum contains a diverse array of anionic substances and molecules with high affinity for the column packing. The result is eventual column blockage, which requires comprehensive cleaning with organic solvents to remove. The life of the analytical column is, however, greatly extended by the wash step in the gradient program and by the use of a guard column.

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