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Evaluation of methods for the extraction of nitrite and nitrate in biological fluids employing high-performance anion-exchange liquid chromatography for their determination

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Abstract

Measurements of nitrite (NO_2^-) and nitrate (NO_3^-) in biological fluids are proposed as indices of cellular nitric oxide (NO) production. Determination of NO_2^- and NO_3^- in standard solutions is not difficult, however, determinations which reflect accurately cellular NO synthesis represent a considerable analytical challenge. Problems are often encountered arising from background NO_2^-/NO_3^- contamination in experimental solutions and laboratory hardware, and with methods for sample extraction. We investigated potential procedures for the extraction and determination of NO_2^- and NO_3^- in biological samples. Consequently, a protocol was devised which yielded acceptable results regarding extraction efficiency, assay reproducibility, sample throughput and contaminant minimisation. It entailed rigorous washing of all equipment with water of low NO_2^- and NO_3^- content, sample deproteinisation by centrifugal ultrafiltration through a 3K filter and analysis by high-performance anion-exchange liquid chromatography with UV detection. Retention times for NO_2^- and NO_3^- in standards and plasma were 4.4 and 5.6 min, respectively. Assay linearity for standards ranged between 31 n*M* and 1 m*M*. The limit of detection for NO_2^- and NO_3^- in standards was 3 pmol. Recoveries of NO_2^- and NO_3^- from spiked plasma (1–100 μ*M* KNO_2/KNO_3) and from extracted standards (1–250 μ*M*) were approximately 100%. Intra-assay and inter-assay RSDs for NO_2^- and NO_3^- in spiked and unspiked plasma were 10.6% or less. Assays on washed platelet supernatants demonstrated collagen-induced platelet generation of NO products and analysis of murine and rat cardiac perfusates was achieved. Our procedure may be suitable for routine determination of NO_2^- and NO_3^- in various biological fluids, e.g., plasma.

Keywords: Nitrite; Nitrate

1. Introduction

Nitric oxide (NO) is an extremely labile signalling molecule with a half-life in biological systems of 10–30 s [1]. It is produced by various cell types and has roles in a variety of biological functions. These include in neurotransmission [2], the maintenance of blood vessel tone [3], inflammatory responses [4] and penile erection [5]. Abnormalities relating to the release and/or bioavailability of NO have been

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proposed for various clinical conditions including atherosclerosis [6], hypertension [7], endotoxic shock [8] and impotence [5].

The cellular generation of NO is most often assessed by measuring the nitrite (NO_2^-) and nitrate (NO_3^-) contents of biological fluids [9]. However, whilst the measurement of NO_2^- and NO_3^- in solutions is not difficult, measurements which accurately reflect cellular NO generation, by contrast, present a formidable task analytically. The most commonly encountered problem concerns the environmental contamination of samples with NO_2^- and NO_3^- [9]. Laboratory plasticware and glassware and buffer solutions are frequently sources of contamination and considerable attention needs to be paid to water quality. Problems may also be encountered regarding the procedures for the extraction of samples and assay reproducibility, again often arising from NO_2^- and NO_3^- contamination.

High-performance liquid chromatography (HPLC) is a convenient and rapid technique for the separation of a wide variety of chemical species, including biological molecules, and can be employed for the determination of NO_2^- and NO_3^-. In a recent review focusing on the measurement of NO_2^- and NO_3^- in biological fluids, however, it was stated that with respect to their determination by HPLC that ''not all methods have been fully described and validated'', i.e., vital analytical details are often omitted from the descriptions of methodologies [10].

In this laboratory we have used HPLC for the analysis of various endogenous substances in biological samples [11–13]. In the present study we have addressed the problem of obtaining physiologically relevant measurements of NO_2^- and NO_3^- concentrations in biological fluids, incorporating an evaluation of some of the procedures outlined previously in the literature, although not always in adequate detail. Our aim was to establish an analytical procedure which is convenient, rapid and reliable, and which can be applied to the analysis of NO_2^- and NO_3^- in a variety of biological samples thus permitting as accurate an assessment of cellular NO generation as possible.

2. Experimental

2.1. Chemicals

KNO_2, KNO_3, K_2HPO_4, KH_2PO_4, $ZnSO_4$ and chemicals for HEPES and Krebs–Henseleit buffers were purchased from Sigma (Poole, UK). Acetonitrile came from BDH (Poole, UK).

2.2. Solutions and washing of apparatus

All solutions were prepared in deionised, double distilled water low in NO_2^- and NO_3^- content which had been prepared in an all-glass Fistreem Cyclon distillation apparatus (Fisons Scientific Equipment, UK).

Glass and plasticware used in the preparation of solutions, including HPLC eluents, was washed 10 times in the same water.

2.3. Chromatography

The chromatograph comprised a Waters Model 510 pump (Waters, Watford, UK), a Rheodyne Model 7725 injection valve fitted with a 100 μl loop (Rheodyne, Berkeley, CA, USA), a 200 mm×4.6 mm Spherisorb S5 SAX (5 μm particle size) anion-exchange column (Phase Separations, Deeside, UK) and a Waters Model 441 absorbance detector set at 214 nm. The mobile phase was pumped at a rate of 1 ml/min and consisted of 5 m*M* K_2HPO_4 and 25 m*M* KH_2PO_4, pH 3.0.

2.4. Sample extraction

Human plasma obtained from blood taken into heparin (150 units) tubes prepared in the laboratory, supernatants obtained from human washed platelets in a HEPES buffer (10 m*M* HEPES, 140 m*M* NaCl, 2.7 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* glucose, 1 mg/ml bovine serum albumin, pH 7.4) [14] incubated under control and collagen-stimulated conditions, and murine and rat cardiac perfusates (Krebs–Henseleit buffer: 118 m*M* NaCl, 25 m*M* $NaHCO_3$, 4.7 m*M* KCl, 1.22 m*M* $MgSO_4$, 1.21 m*M* KH_2PO_4, 1.84 m*M* $CaCl_2$, 11 m*M* glucose, gassed with O_2–CO_2, 95:5, to pH 7.4) obtained using the Langendorff preparation were analysed for their NO_2^- and NO_3^- contents. Prior to analysis samples (225–1000 μl) were centrifuged for 40 min through Nanosep (500 μl capacity) or Microsep (3.5 ml capacity) 3K microconcentrator filters (Pall Filtron, Northborough, MA, USA) at 11 000 and 3000 *g*, respectively, to remove proteins. Filters had been

pre-washed three times with 500 μl (Nanosep) or 3.5 ml (Microsep) deionised/distilled water with centrifugation and disposal of the washes. Plasma samples were diluted with an equal volume of water before ultrafiltration [15]. The ability of the chromatographic system to resolve NO_2^- and NO_3^- in human urine was also examined. Centrifugal ultrafiltration, as used with the other types of sample is, however, not suitable for the extraction of urine. This is because it fails to remove substances which interfere with the detection of NO_2^- and NO_3^-. Urine (500 μl) was therefore extracted by passing it through octadecyl (C_{18}) silica columns (Supelco, Bellfonte, PA, USA) [16] and extracts were diluted 1:100 with water before analysis. Aliquots of 100 μl of sample extracts and aqueous KNO_2/KNO_3 standards were injected onto the chromatograph. Sample concentrations were corrected for silica column NO_2^-/NO_3^- contamination by subtracting the values obtained with water (500 μl) which had also been subjected to the extraction procedure.

2.5. Calculation of sample NO_2^- and NO_3^- concentrations

Sample NO_2^- and NO_3^- concentrations were calculated by reference to KNO_2/KNO_3 standard mixtures. Chromatogram peak areas in preference to peak heights were used in these calculations as these were found to yield more reproducible results. These were measured manually with peak ''skimming'' being employed where necessary. Where appropriate, sample concentrations were corrected for background contamination with NO_2^- and NO_3^- in water and buffer solutions.

3. Results

As seen in Figs. 1–3 separation of NO_2^- and NO_3^- was achieved in extracts prepared from human plasma, human washed platelet supernatants and murine cardiac perfusates by centrifugal ultrafiltration. In plasma extracts NO_2^- peaks eluted as a shoulder on the solvent front. These peaks, however, could be accurately and reproducibly measured manually employing peak skimming. The retention times for NO_2^- and NO_3^- in standard solutions were (mean±SD) 4.4±0.2 and 5.6±0.2 min, respectively

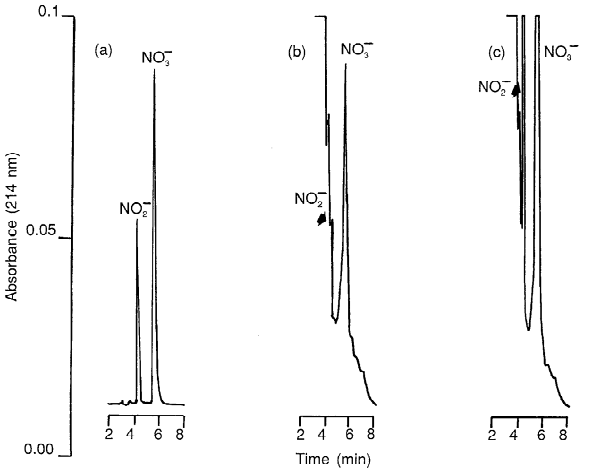

Fig. 1. Chromatograms of an aqueous standard containing 25 μ*M* nitrite and nitrate (a), human plasma (b) and human plasma spiked with 50 μ*M* nitrite and nitrate (c).

(*n*=12). In plasma extracts retention times were 4.4±0.1 and 5.6±0.1 min, respectively (*n*=10). Chromatogram peak identities for sample extracts were confirmed in spiking experiments in which known quantities of NO_2^- and NO_3^- standards were added.

With non-extracted KNO_2/KNO_3 standards NO_2^- and NO_3^- determinations were linear over the range of concentrations examined (i.e., 31 n*M* to 1 m*M*) after, in the case of standards at the lower end of the scale (31 n*M*–10 μ*M*), subtraction of the back-

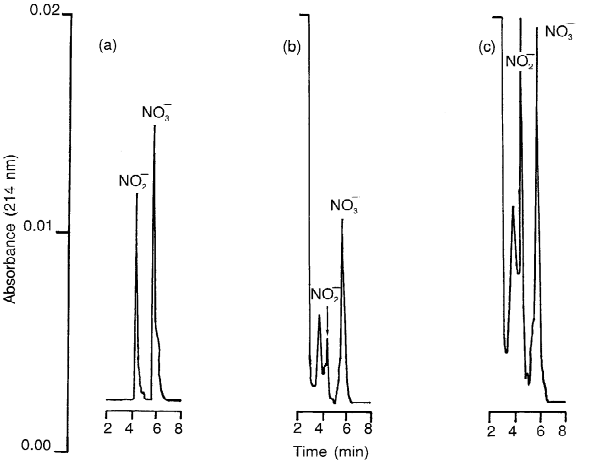

Fig. 2. Chromatograms of an aqueous standard containing 5 μ*M* nitrite and nitrate (a) and washed human platelet supernatants obtained under control (b) and collagen-stimulated conditions (c).

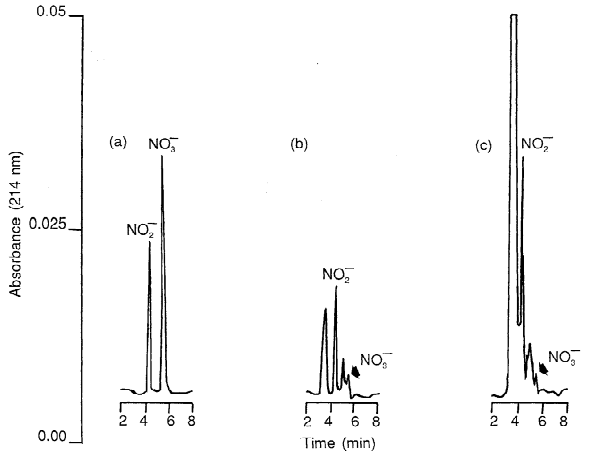

Fig. 3. Chromatograms of an aqueous standard containing 10 μ*M* nitrite and nitrate (a) and murine cardiac perfusates obtained under control (perfused) conditions (b) and on reperfusion following a period of ischaemia (c).

ground concentrations in distilled/deionised water. Regression analysis, achieved using the least squares method, yielded regression coefficients for NO_2^- and NO_3^- of 0.92±0.004 and 1.45±0.04, respectively (n=4). Correlation coefficients (r^2) for NO_2^- and NO_3^- were 0.995±0.005 and 0.998±0.001, respectively (n=4). The intercepts for the lines were at or near zero. The limit of detection for both ions with these standards was 3 pmol, for a 100 μl injection onto the chromatograph. At the detector sensitivities routinely employed for the determination of NO_2^-/NO_3^- in sample extracts contamination of the deionised, double distilled water with NO_2^- and NO_3^- was not evident. When however the detector was used at its maximum setting (i.e., 0.005 absorbance units) background concentrations for NO_2^- and NO_3^- in water were estimated to be 37.2±4 and 60.4±4.2 n*M*, respectively (n=4).

With a series of extracted standards NO_2^- and NO_3^- determinations were, as for non-extracted standards, linear over the concentration range studied (31 n*M*–250 μ*M*) with the intercepts for the lines passing through zero. Regression coefficients calculated for NO_2^- and NO_3^- were 1.1±0.02 and 1.63±0.02, respectively, and r^2 values were 0.998±0.001 and 0.999±0.0001, respectively (n=4). Extraction recoveries for NO_2^- ranged between 90.7±2.1% for 31 n*M* KNO_2/KNO_3 and 97.1±2.4% for the 250 μ*M* standard, whilst for NO_3^- the respective recoveries were 20.9±6.3 and 97.3±1.4% (Table 1 presents complete data). Plasma was spiked with 1–100 μ*M* KNO_2/KNO_3 and extracted. Recoveries and relative standard deviations (RSDs) for NO_2^- and NO_3^- were then calculated following subtraction of the plasma background concentrations (Table 2). Recoveries of NO_2^- and NO_3^- from plasma spiked with 10, 25 and 100 μ*M* KNO_2/KNO_2 were approximately 100% for all samples. Intra-assay RSDs for NO_2^- were 6.5% or less, whilst inter-assay RSDs did not exceed 5.4%. RSDs, both intra-assay and inter-assay, for NO_3^- were 3.3% or less. Recoveries for NO_2^- and NO_3^- from plasma which had been spiked with lower concentrations of KNO_2/KNO_3 exhibited greater variability, whilst RSDs generally exceeded those observed with higher KNO_2/KNO_3 concentrations (Table 2). Nevertheless, the values obtained fell within acceptable limits. For an unspiked plasma sample containing 0.98 μ*M* NO_2^- and 47.5 μ*M* NO_3^- intra-assay RSDs for NO_2^- and NO_3^- were 8.2 and 3.8%, and inter-assay RSDs were 8.5 and 5.6%, respectively.

Table 1
Recoveries of NO_2^- and NO_2^- in extracted KNO_2/KNO_3 standards (mean±SD, n=4)

Concentration	Recovery (%)	
	NO_2^-	NO_3^-
31 n*M*	90.7±2.1	20.9±6.3
62 n*M*	94.5±10	65.4±4.3
125 n*M*	94.7±4	87.8±5.7
250 n*M*	92.1±1.9	80.8±1.5
1 μ*M*	93.7±2.9	92.4±4.5
5 μ*M*	91.8±5.8	87.7±6.2
10 μ*M*	102±8.9	95.3±1.8
25 μ*M*	99±9.6	100.4±9.2
100 μ*M*	100±2.4	98.5±1.6
250 μ*M*	97.1±2.4	97.3±1.4

Mean plasma NO_2^- and NO_3^- concentrations for a group of normal subjects were 3.5±1.6 and 46.8±18.6 μ*M*, respectively (n=12). NO_2^- and NO_3^- concentrations in plasma samples which had been diluted 1:3 and 1:1 yielded similar values. Hence, in plasma diluted 1:3 NO_2^- and NO_3^- concentrations were, respectively, 4.3±1.3 and 55.4±13 μ*M*, and in plasma diluted 1:1, 4.2±1.7 and 54.2±14.6 μ*M* (n=5).

Table 2
Relative standard deviations (RSDs) and recoveries for NO_2^- and NO_3^- from plasma spiked with 1–100 μ*M* KNO_2/KNO_3 (mean±SD, *n*=3–6)

Concentration (μ*M*)	NO_2^-			NO_3^-		
	Intra-assay RSD (%)	Inter-assay RSD (%)	Recovery (%)	Intra-assay RSD (%)	Inter-assay RSD (%)	Recovery (%)
0*	8.2	8.5	–	3.8	5.6	–
1	9.0	8.9	107.9±10.1	8.3	10.6	101.3±11.9
2.5	7.3	9.6	108.8±16.4	7.4	10.5	101.9±7.6
5	9.1	9.8	93.1±8.4	2.9	2.7	100.6±2.9
10	6.5	5.4	102.1±6.6	2.2	3.0	98.9±2.2
25	5.9	–	94.4±5.6	3.3	–	106.9±3.5
100	4.2	5.9	104.6±4.4	1.2	2.6	96.6±1.2

*Unspiked plasma, i.e., plasma to which exogenous NO_2^-/NO_3^- has not been added.

Assays performed on supernatants derived from human washed platelets which had been pre-incubated for 2 min at 37 °C in a Born aggregometer followed by the addition of saline (control) or the strong platelet agonist collagen (25 μg/ml) and a further 5 min incubation demonstrated that platelet activation is associated with substantial platelet efflux of NO products (Fig. 2).

Murine and rat cardiac perfusate samples obtained in classical Langendorff experiments yielded data consistent with flow-mediated endothelial generation of NO (chromatograms for murine extracts presented in Fig. 3). An unidentified peak ran between NO_2^- and NO_3^- on chromatograms for perfusate samples but did not interfere with either peak. Perfusate samples were found to be stable with respect to NO_2^- and NO_3^- measurements for 6 months with storage at −85 °C.

Analysis of human urine yielded unacceptable results. A peak corresponding to NO_2^- was subsequently found to be due to contamination associated with the C_{18} silica extraction columns (Fig. 4). Thus, when 500 μl of water was taken through the extraction procedure a contaminating NO_2^- concentration equivalent to 0.14±0.01 m*M* (*n*=3) was obtained. Peaks eluting at the position appropriate for NO_3^- were broad and contained shoulders, indicating that additional, contaminating substances were co-eluting with NO_3^-. Contamination of water (500 μl) which had been passed through C_{18} extraction columns with NO_3^- was equivalent to 0.16±0.002 m*M* (*n*=3).

A number of methods of sample preparation prior to analysis were examined before alighting on the method which yielded optimal results and is now used routinely, as described in Section 2.4. Various ultrafiltration devices for sample deproteinisation were tested at detector sensitivities normally used but were found, with the exception of the Nanosep and Microsep 3K filters, to perform unsatisfactorily.

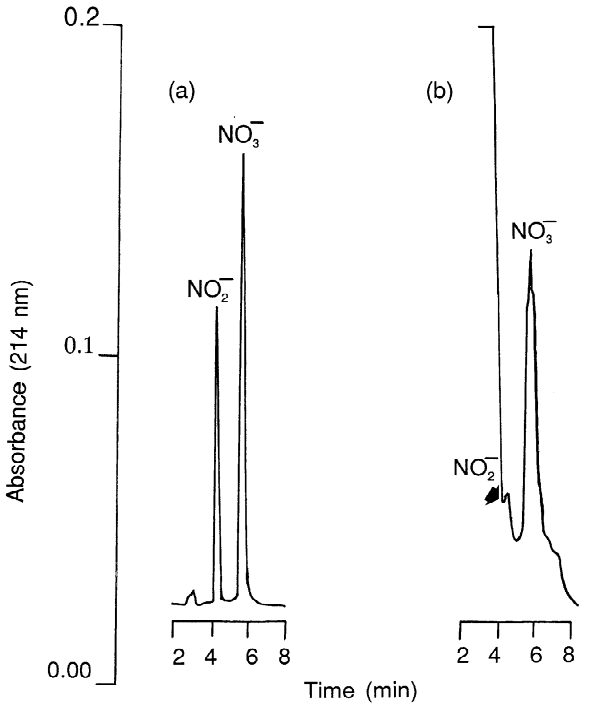

Fig. 4. Chromatograms of an aqueous standard containing 50 μ*M* nitrite and nitrate (a) and human urine (b).

Problems encountered related to poor sample deproteinisation and the introduction into sample extracts of contaminants which interfered with the chromatography. The introduction of contaminating NO_2^- and NO_3^- themselves into extracts was also observed. Devices manufactured by Millipore-Amicon, Pall Filtron and Whatman were investigated and data regarding the contamination of water ultrafiltrates with NO_2^- and NO_3^- are presented (Table 3). In our hands a previously described acetonitrile extraction procedure was found to yield poor separations and a large solvent front which interfered with the chromatography [17]. A sample deproteinisation procedure utilising $ZnSO_4$ [18] was also found to be inadequate for our purposes.

Employing the eluent described by Wennmalm et al. [15], i.e., 1 m*M* phosphate buffer, pH 9.0, separation of NO_2^- and NO_3^- was not achieved. By contrast, the eluent system specified by Everett et al. [17], i.e., 5 m*M* K_2HPO_4, 25 m*M* KH_2PO_4, after pH mapping to establish the optimal working pH (i.e., 3.0) for NO_2^- and NO_3^- separation, yielded good results.

Commercially available glass heparin tubes (143 units) filled with 10 ml distilled deionised water were found to be contaminated with NO_2^- (0.34±0.05 μ*M*, *n*=3) and NO_3^- (2.1±0.14 μ*M*, *n*=3). Heparin tubes (150 units) prepared in the laboratory incorporating washed 15-ml plastic centrifuge tubes (Greiner Labortechnik, Frickenhausen, Germany), however, yielded more acceptable results, i.e., NO_2^- concentrations were 0.3±0.07 μ*M* and NO_3^- was undetectable (*n*=3). Plastic centrifuge tubes containing no anticoagulant which had been washed as outlined in Section 2.2 exhibited no contamination. For washed platelet experiments blood samples were collected into tubes containing acid citrate–dextrose (0.8%, w/v, citric acid; 2.2%, w/v, trisodium citrate; 2.4%, w/v, glucose; anticoagulant:blood ratio 1:9). Any contamination associated with acid citrate–dextrose blood collection tubes did not result in any problems as following platelet washing a clear NO signal was evident on platelet stimulation.

4. Discussion

After an exhaustive investigation we believe that we have identified procedures which may have application in the routine extraction and determination of NO_2^- and NO_3^- in biological/clinical samples, and offer opportunities for the acquisition of data which reflects cellular NO generation. The method entails centrifugal ultrafiltration of samples through pre-washed Nanosep or Microsep 3K filters and anion-exchange HPLC with UV detection utilising a phosphate eluent system [17]. It has the potential benefits of being simple and rapid, and is of sufficient sensitivity for some biological applications, in particular the determination of NO products (especially NO_3^-) in plasma. Previously it has been stressed that sample manipulations should be kept to a minimum so as to reduce sample NO_2^-/NO_3^- contamination, a requirement which our method satisfies [17]. Contrasting with our procedure, assays employing the Griess reagent are more time-consum-

Table 3
NO_2^- and NO_3^- contamination associated with various ultrafiltration devices (mean±SD, *n*=3)

Filter	Wash volume	μ*M*	
		NO_2^-	NO_3^-
Nanosep 3K	500 μl	Not detectable	Not detectable
Nanosep 10K	500 μl	0.4±0.1	0.6±0.1
Microsep 3K	3.5 ml	Not detectable	Not detectable
Whatman VectaSpin Micro 5K	400 μl	0.6±0.1	0.6±0.4
Whatman VectaSpin Micro 12K	400 μl	0.3±0.1	0.8±0.1
Ultrafree 30K	500 μl	0.2±0.05	13.6±3.2
Centricon 3K	2 ml	0.7±0.1	0.4±0.1
Centricon 10K	2 ml	0.7±0.1	0.7±0.1

Assays were performed on the third wash. Wash volumes in most cases are as specified by the manufacturer.

ing and complicated, and require multiple sample manipulations which increase the likelihood of introducing errors [18]. Additionally, when cadmium/copper is used to catalyse the conversion of NO_3^- to NO_2^- (a prerequisite for this method as it only measures NO_2^-) it involves handling a toxic material (i.e., cadmium) [16]. Despite our best efforts to minimise sample contamination it must be admitted that on occasion spurious results were obtained. It has been suggested that air-borne, nitrate-laden dust particles may be a source of non-specific contamination [17]. We found however that when problems arose regarding contamination, repeat extraction of suspect samples often remedied the situation.

A detailed examination of various commercially available ultrafiltration devices resulted in the identification of two (i.e., Nanosep and Microsep 3K filters) that performed satisfactorily with respect to extract purity and NO_2^-/NO_3^- contamination. Previously, such devices have been rejected as being unsuitable for the extraction of NO_2^- and NO_3^- in biological samples [17]. However, previous studies have not involved the use of Nanosep and Microsep 3K filters. It is our opinion that the identification of ultrafiltration devices which are efficient and permit rapid and easy clean-up of samples, whilst avoiding problems relating to the introduction into sample extracts of contaminants and interfering compounds contained in reagents used in alternative extraction procedures, will assist others in the field of NO research. We found that extraction procedures involving acetonitrile and $ZnSO_4$ led to interference with and poor separation of NO_2^- and NO_3^- on chromatography.

The chromatographic system which we adopted after investigating alternatives readily resolved NO_2^- and NO_3^-, the peaks being separated by more than a minute, as observed by Everett et al. [17]. Anion-exchange HPLC was found to be eminently suitable and has been advocated by several laboratories for the separation of NO_2^- and NO_3^- in biological samples [17,19]. As we observed, however, careful manipulation of eluent pH is necessary to achieve good separation. We failed to separate NO_2^- and NO_3^- using the eluent system described by Wennmalm et al. [15]. This however may merely reflect the fact that different types of analytical column were used by these workers and ourselves. Certainly we confirmed in our study that as has been reported by Everett et al. [17] a phosphate-based eluent is required for the determination of NO_2^- when using anion-exchange HPLC.

The extraction of KNO_2/KNO_3 standard mixtures employing our procedure revealed that the recoveries for NO_3^- but not for NO_2^- at the lower concentrations examined (i.e., <1 μM) were reduced. We would suggest that this may be due to binding of NO_3^- to the plastic from which the ultrafiltration units are constructed. Despite this apparent limitation, problems should not arise with regard to the analysis of NO_3^- in biological fluids, particularly blood, as NO_3^- concentrations in these samples are usually at the micromolar level.

Using our procedure the extraction of plasma NO_2^- and NO_3^- was found to yield highly reproducible results. Admittedly, NO_2^- eluted as a shoulder on the solvent front, however, accurate measures of NO_2^- peaks were possible when employing peak skimming. Plasma NO_2^- and NO_3^- concentrations were comparable to previously published values [9], with mean NO_3^- levels exceeding those for NO_2^- 13-fold. In previous studies the extents to which plasma samples were diluted for extraction have varied from 1:1 [15] to 1:5 [16], plasma:water. We found that whether plasma samples were diluted 1:1 or 1:3 it made little difference regarding the NO_2^- and NO_3^- values obtained.

The addition to plasma samples of KNO_2/KNO_3 at concentrations which ranged between 1 and 100 μM yielded data acceptable with respect to RSDs and NO_2^- and NO_3^- recovery. As might have been expected, however, assay accuracy was reduced for plasma samples treated with KNO_2/KNO_3 at the lower end of the concentration range (i.e., 1μM and 2.5 μM). These inaccuracies may have resulted from errors associated with the addition of low concentrations of KNO_2/KNO_3 to plasma. Alternatively, the binding of KNO_2/KNO_3 by plasma proteins or, as may have been the case for lower concentrations of extracted standards, the extraction filters, may have played a role. Nevertheless, the observations made with plasma samples spiked with low concentrations of KNO_2/KNO_3 would indicate that the acquisition of meaningful data at these levels of concentration should be possible. The fact also remains that when untreated plasma was repeatedly assayed, RSDs for both NO_2^- and NO_3^- were well within acceptable limits.

Assays performed on cardiac perfusates and platelet supernatants demonstrated clearly that the application of physiological stimuli to tissues results in the generation of NO products. In the case of collagen-stimulated generation of NO by washed platelets the data obtained have been discussed in detail previously [20]. The results of Langendorff experiments in which the generation of NO by the heart was studied have also been published [21]. The appearance on chromatograms of additional unidentified, non-NO related peaks or peaks which increased in size after physiological stimulation is, perhaps, not surprising. Many unidentified substances, some of which will be UV-absorbing, may be released when tissues are stimulated. What is important is that none of these peaks interfered with the resolution of NO_2^- and NO_3^-.

Contrasting with cardiac and platelet samples and plasma, the analysis of human urine proved unsuccessful. The C_{18} silica columns used for the extraction of urine were found to introduce contaminants (NO_2^-) into extracts and failed to remove substances which interfered with the resolution of NO_3^-, as evidenced by the appearance of shoulders on NO_3^- peaks.

In addressing the question of the contamination of samples and sample extracts with NO_2^- and NO_3^- and other interfering substances, particular attention was focused on water quality and the washing of plastics and glassware used for the preparation of solutions. The problem of contamination of sample collection tubes was also considered. Commercially available blood collection tubes containing anticoagulants, e.g., EDTA and heparin, have been reported to contain significant amounts of nitrite and nitrate [10]. We found that commercial glass heparin tubes were, indeed, contaminated with NO_2^- and to a greater extent NO_3^-. By contrast, heparin tubes prepared in the laboratory although exhibiting some contamination with NO_2^-, were not contaminated with NO_3^-. Rigorously washed centrifuge tubes were found to be free of NO_2^- and NO_3^- contamination. For routine measurement of circulating (serum) NO products therefore we would suggest that plastic centrifuge tubes containing no anticoagulent should be used.

Regarding the assays we performed on human samples, particularly plasma, it should be noted that account had not been taken of subject dietary intake or infection. Both factors influence circulating NO_2^- / NO_3^- concentrations [9]. It is possible, therefore, that some of the values recorded in this study were higher than would have been the case if conditions had been more carefully controlled [9].

To conclude, it is our opinion that the procedure described in this paper is suitable for the routine determination of NO_2^- and, particularly, NO_3^- in biological fluids. If however an assessment of NO production at the low nanomolar level is required the anion-exchange HPLC procedure of Preik-Steinhoff and Kelm [19] may be more appropriate. This procedure offers greater sensitivity, particularly with respect to the determination of NO_2^-, by utilising electrochemical and UV detectors in combination, although the measurement of low NO_2^- concentrations may not be relevant regarding plasma [17]. However, because this arrangement is more complicated than normally found in biological laboratories, one could perhaps envisage problems arising. The principal strength of our procedure, we believe, is its simplicity. For a laboratory versed in standard HPLC techniques setting up the method should be straightforward. It cannot be stressed enough however that in order to obtain biologically meaningful data stringent measures must be applied to reduce sample contamination. Careful manipulation of chromatographic conditions (i.e., eluent pH) must also be undertaken to maximise NO_2^- and NO_3^- separation and reduce interference by other eluting substances. Once, however, the methods outlined have become routine any problems encountered initially in setting up the procedure should be abrogated.

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