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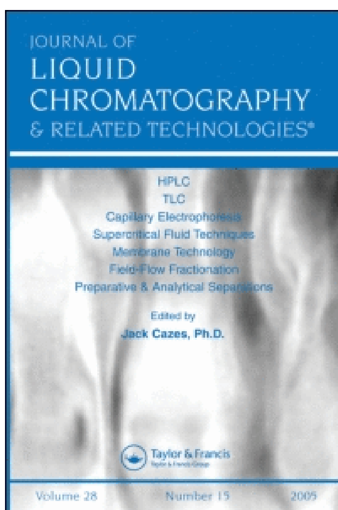

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Determination of Nitrate and Nitrite Ions in Human Plasma by Ion Exchange-High Performance Liquid Chromatography

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DETERMINATION OF NITRATE AND NITRITE IONS IN HUMAN PLASMA BY ION EXCHANGE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Acetonitrile precipitation of plasma samples followed by injection of supernatant onto a reverse phase precolumn coupled to an anion exchange column allowed ultraviolet detection (214 nm) of eluting nitrate and nitrite ions. Sensitivity in plasma is about 0.01 mM for both ions and linearity is excellent from 0.02 to 1.0 mM. Nitrite accuracy assessed by diazotization coupling was good. Reproducibility studies demonstrated within-run coefficients of variation of < 4%. Interferences were few. Random endogenous serum nitrate concentrations (0.03-0.12 mM) were determined. Serum nitrite and nitrate concentrations were measured in a patient following an overdosage of isobutyl nitrite. The method is applicable for nitrite/nitrate studies in plasma at these concentrations.

INTRODUCTION

Many methods for measuring nitrite and nitrate are available (1). Few are applicable to measuring these analytes in biologic material (2). Modified Griess reactions with and without reduction of nitrate to nitrite are commonly employed (3,4).

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Variability is introduced for nitrate quantitation by the reduction process through interferences and blanking procedures (5). However, the diazotization coupling reaction for NO_2^- alone performs well. Nitration assays followed by gas chromatography may suffer from poor recoveries or use of dangerous chemicals (2,6). Preparatory cleanup with anion exchange columns may be incorporated into any of the methods. This requires larger samples for preconcentration and may introduce some variation while reducing background and increasing sensitivity (7). Anion exchange pretreatment has been used successfully prior to chemiluminescence determination on urine samples for NO_2^- and NO_3^- (8). Neither nitrate nor nitrite were measured directly and although quite sensitive for nitrite, the procedure is long and special apparatus is required.

Measurement of nitrate and nitrite by high pressure chromatographic anion exchange or ion pair technique offers a more direct approach (9-13) at least in water, waste waters, and brine. These techniques have not been applied to serum samples possibly due to interferences from many other anions (approximately 150 mM total plasma anions). In rare environmental situations sample preconcentration is required for determinations below 0.02 mM (1 ppm) by these techniques. However, low ppm sensitivity is adequate for nitrate in human serum as will be demonstrated. Suppression of chemical and electronic backgrounds is normally necessary in other methods, but is not required of the liquid

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chromatographic method presented herein because of the unique combination of sample preparation, precolumn-column pairing, mobile phase, and detection techniques used. This method was developed for use in studying nitrite-nitrate interconversion in human blood.

EXPERIMENTAL

High pressure liquid chromatographic conditions

A Beckman (Berkeley, CA, USA) Model 110A high pressure pump and Model 210 injector equipped with 20 or 100 μl sample loops for injection and elution of a Whatman (Clifton, NJ, USA) pre-column (2.1 mm I.D. x 60 mm) packed with 35 μ CO:PELL ODS (C-18 pellicular) coupled to a polystyrenedivinylbenzene-based strong anion exchange column (4.1 mm I.D. x 250 mm) from Wescan Instruments (Santa Clara, CA, USA) were used. Detection was by ultra-violet absorption at 214 nm using a Beckman Model 160 detector at 0.1-0.5 AUFS per 10 mV output. Final mobile phase composition was 50 mM NaH_2PO_4, 3mM NaCl, and 4 mM acetic acid in water (final pH = 3.95). Reverse osmosis/deionized water was used and all mobile phases were filtered with Ultipor NR nylon-66, 0.22 μ inert filters (Woburn, MA, USA) under 5 mm Hg vacuum. Flow rate was 4.0 ml/min.

Sample and standards preparation

Serum or plasma preparation was by acetonitrile precipitation of proteins. Chromatographic grade acetonitrile (400 μl) and

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serum (200 µl) were vortexed in 1.5 ml Sarstedt (Princeton, NJ, USA) capped centrifuge tubes and then centrifuged at 15,000 r.p.m. on a Brinkmann (Westbury, NY, USA) Model 5412 centrifuge for 2 min. Exactly 20 or 50 µl of the supernatant was injected. A sample injection loop improves reproducibility. Peak heights of sample nitrite and nitrate peaks were compared with standard curve peak heights for quantitation.

Other preparatory cleanup procedures were attempted including dilution, methanol precipitation, ultrafiltration, and bonded anion exchange resins. Background interferences were problems with all but the exchange resin techniques and acetonitrile precipitation. Exchange resins provided clean baselines and the opportunity for approximately a twofold preconcentration of the sample. However, it was determined that a separate column type was required for each nitrate or nitrite and variation in recovery was difficult to control. Analytichem (Harbor City, CA, USA) Bond-Elute primary amine-bonded column could be used for nitrite and Bond-Elute quarternary amine column could be used for nitrate. Elution of nitrate required strong molarity salt solutions that interfered with subsequent ion chromatography. Nitrate was not retained on the weaker anion exchange column. Reproducibility and recovery varied on these columns probably because of the many competing anions already present in serum. Acetonitrile precipitation was easy and was expected to introduce little variation and have adequate recovery.

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Standards were made in banked plasma, serum, and water using sodium nitrate and sodium nitrite at 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 1.0, 2.0, and 3.0 mM. Nitrate and nitrite are stable for several weeks in dilute aqueous solutions that are kept cold and dark. Nitrite plasma standards lose 5% of initial concentrations by 3 hr at room temperature. Fresh plasma standards should be made daily. Linearity, reproducibility, background specificity, nitrite accuracy by diazo-coupling assay (14) and interference studies were performed. Interferences were tested using analytic grade chemicals dissolved in water and mixed with acetonitrile as described earlier. Random assayed serum samples were frozen (-15°C); i.e., clinical specimens and one sample from a patient who had ingested isobutyl nitrite.

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RESULTS

Table 1 shows the various experimental mobile phases and nitrate and nitrite retention times. For all the listed mobile phases, capacitances and resolutions were adequate. Peak efficiency was poor (tailing) with phosphate buffers alone. Addition of sodium chloride improves peak efficiency alone or in combination with phosphate buffers, but sodium chloride has high background absorbance. Therefore, sodium chloride and phosphate buffer was optimized to give the least background absorbance but adequate peak efficiency. Addition of acetic acid effected elution of interfering anions (probably organic) after the nitrate

TABLE 1

Effect of various mobile phase compositions on retention time of nitrite and nitrate ions

Mobile phase composition (mM)			Retention time (min)*		
NaH_2PO_4	NaCl	CH_3COO^-	NO_2^-	NO_3^-	Comment
50	0	0	6.5	10.0	Tailing peaks
60	8	0	6.5	9.8	Endogenous interference at NO_2^-
100	8	0	4.8	7.8	Endogenous interference at NO_2^-
110	20	0	3.5	4.8	Endogenous interference at NO_2^-
120	3	0	4.0	5.5	Endogenous interference at NO_2^-
50	3	0	5.8	8.5	Endogenous interference at NO_2^-
35	3	0	6.8	10.5	Minor interference at NO_2^-
35	3	4	5.0	7.5	No interference
65	3	2	4.0	6.0	No interference
60	3	3	4.0	6.0	No interference

* Flow rate = 3.0 ml/min.

and nitrite peaks. Sample chromatograms of nitrate and nitrite in plasma are shown in Figure 1. Retention times of nitrite and nitrate ion were 3.2 and 4.6 min (4.0 ml/min), respectively. Because of the acetonitrile precipitation, the majority of proteins are not introduced onto the column. The reverse phase (C-18)

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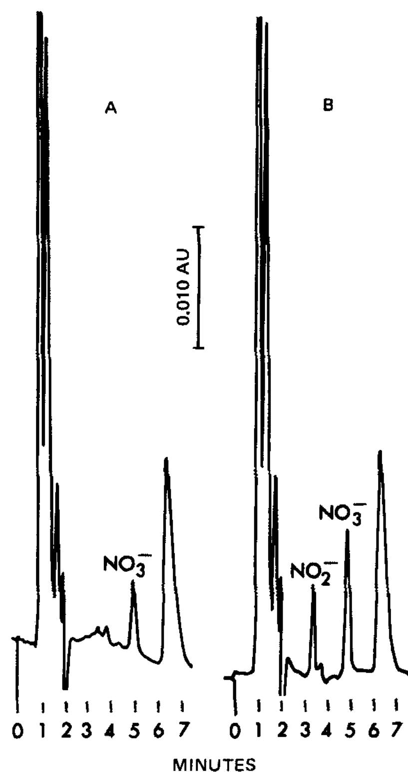

Figure 1. Chromatographic tracings: A, blank plasma with 0.05 mM endogenous nitrate; B, a plasma standard with 0.10 mM nitrite and nitrate added.

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precolumn is effective in further removing organic substances. Five millimolar concentrations of fluoride, chloride, phosphates, and sulfates are transparent at 214 nm UV. Bromide (3.2 min) and iodide (9.2 min) absorb at millimolar concentrations, but usually are not present in sufficient quantities. Chloride in serum (100 mM) precipitates produces a peak at 1.5 min.

Previous ion exchange techniques have used conductivity detectors and elution with an organic anion such as phthalate (11-13). High phthalate absorbance prohibits measurement of several other anions with ultraviolet absorbance detection. With conductivity detectors high concentrations of serum anions (chloride 100 mM, bicarbonate 20 mM) can overwhelm the detector relative to the lower concentrations of nitrite and nitrate. In a preliminary attempt on a similar anion-ion exchange high pressure liquid chromatographic system with a conductivity detector, neither supernatants of acetonitrile-precipitated serum nor high molarity salt eluates from preparatory anion exchange columns could be analyzed because of this problem. Therefore, in the described system, the wavelength of ultraviolet detection, phosphate rather than phthalate buffer, sample preparation and the reverse phase precolumn improved the assay specificity and allowed measurement of nitrate and nitrite in serum.

Linearity studies demonstrated regression to zero in aqueous standards and demonstrated a positive bias of 0.04 mM for nitrate and 0.004 mM for nitrite in plasma. The nitrate bias is due to endogenous nitrate in plasma. The nitrite bias is due to unknown interferences that prohibited sensitivity to < 0.01 mM in serum. Sensitivity for nitrite and nitrate in water was 0.005 mM. Sensitivity for nitrate in plasma was 0.01 mM above the endogenous nitrate value. Linearity was good over the fiftyfold range tested with aqueous and plasma regression coefficients of 0.9995

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and 0.9996, respectively, for nitrite and 0.9996 and 0.9993, respectively, for nitrate. When plasma samples were compared with aqueous samples at the same concentrations, the recovery was 97% for nitrate and 82.8% for nitrite. Because sample manipulation is minimal, nitrite losses must be due to protein binding or chemical reaction in plasma. Even though biases can be measured and subtracted using aqueous standards, plasma standards must be used to account for recovery because the method cannot be conveniently standardized internally with another anion. Internal standardization is difficult because other suitable anions cannot be detected, interfere, or compete with the anion exchange process. Accuracy of nitrite determinations by high pressure liquid chromatography was compared with the diazotization-coupling reaction method. Actual plasma samples were used when nitrite concentrations were changing with time (2.73 mM to 0.2 mM) and both analyses on each sample were performed within 10 min of each other. The correlation coefficient, slope, and intercept between two methods were 0.9983, 0.821, and -0.003 (y = high pressure liquid chromatography method), respectively. Although the correlation was quite good between the two methods with no constant bias (intercept), the proportionate bias (slope) of 0.821 indicates lesser recovery by the high pressure liquid chromatography method and is consistent with the recovery study mentioned earlier. Within-run reproducibility (n = 5) was excellent at concentrations of 0.10 mM for both nitrate and

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nitrite showing within-run coefficients of variation of 2.6% and 3.3%, respectively. This is probably a result of minimal preparatory sample manipulation.

Five random normal sera were tested for nitrate and nitrite. Nitrate concentrations ranged from 0.03-0.12 mM (mean = 0.06) and nitrite concentrations were not detectable. In a case of ingested isobutyl nitrite overdose, serum taken several hours after the overdose showed a nitrite concentration of 0.14 mM and nitrate of 1.08 mM. The assay presented is simple, reproducible, linear, and accurate. Single serum samples can be assayed in 8 min. Such a procedure is useful for rapid analysis of nitrite and nitrate during their transformation in human plasma.

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