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Chromatographic separation and photoelectrochemical detection of sodium nitroprusside and its degradative and metabolic products

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Abstract: Ion-pair chromatography has been used for the separation of nitroprusside ion and its photochemical hydrolytic and metabolic products. Organic modifier and pH were adjusted for maximum separation of the ions. Methanol was selected as the organic modifier in a pH range 5–8 and tetrabutylammonium perchlorate was used as the ion-pairing reagent.

Ions were detected with a photoelectrochemical detector as described by Krull. A modification of this procedure was used to detect nitroprusside ion in spiked serum samples.

Keywords: *Nitroprusside; degradation ions; thiocyanate; ion-pair chromatography; photoelectrochemical detector.*

Introduction

Sodium nitroprusside (SNP) is the common name for sodium nitrosylpentacyanolferrate(II) dihydrate ($Na_2[Fe(CN)_5NO]2H_2O$), which is an antihypertensive agent. SNP is used as a direct-acting vasodilator in acute cases of hypertension. Its onset of action is almost immediate after the start of infusion [1].

The potential toxicity of SNP is suggested from the molecular structure. The nitroprusside ion decomposes to 5 mol-equivalents of cyanide (CN^-) and is a potential source of cyanide poisoning [3]. Thiocyanate (SCN^-), the final metabolite of CN^-, has a half life of 4 days and can accumulate in the kidneys [2]. Secondary effects of nitroprusside administration cause nausea, vomiting, muscle spasm, and tinnitus [2, 3]. Nitroprusside anion is known to undergo decomposition upon exposure to light [4]. Degradation products of the nitroprusside ion are reported to be [3–5]: ferrocyanide, ferricyanide, nitrite, nitrate and cyanide. Metabolism has been reported to produce thiocyanate in addition to the above mentioned anions. The degradation and metabolic products are all anions with difference of ionic charge, ionic radius and hydrophobicity. These differences can be exploited for chromatographic separation.

This study was concerned with the chromatographic separation of the nitroprusside ion and its photochemical, hydrolytic and metabolic products (i.e. nitrite, nitrate, cyanide, thiocyanate, ferrocyanide and ferricyanide). Due to the ionic nature of the compounds, ion-pair chromatography (IPC) was used for the separation of these anions.

A selective photoelectrochemical detector described by Krull *et al.* [6–14] was employed for the detection of nitroprusside and related anions. This method was also used to assay SNP, its metabolites and degradation products in serum.

Experimental

Instrumentation and equipment

Liquid chromatography. The liquid chromatography system consisted of a Model 6000A solvent-delivery system (Waters Chromatography Division, Millipore Corp., Milford, MA, USA) equipped with a fixed 10-μl loop injector (Model No. 7125, Rheodyne, Cotati, USA). Chromatograms were recorded

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using an integrator (Hewlett Packard, Corvallis, OR, USA).

Analytical columns were Spherisorb ODS-2 (5 μm; 100 or 150 × 4.6 mm, i.d.) (Phase Sep, Hauppauge, NY, USA). Column material was suspended in acetone and packed by the upward-slurry technique [15].

The photoreactor was assembled with Teflon tubing (0.01 inch i.d., 1/16 inch o.d., 10 feet length, Universal Scientific Inc., Atlanta, GA, USA) crocheted in a chain-stitch fashion and wrapped around a quartz immersion well (Ace Glass Inc., Vineland, NJ, USA) into which a Hanovia medium intensity mercury lamp was set. The lamp intensity was 6.75 mW cm^{-1} [2] at 50 cm. The photoreactor was immersed in a constant temperature water bath and circulator (25°) (Forma-Temp Jr, Forma Scientific Inc., Marietta, OH, USA). This photoreactor was connected to the electrochemical detector by a short piece of Teflon tubing (0.01 inch i.d.).

Electrochemical activity was measured using a three electrode electrochemical cell with glassy carbon for the working electrode (Model TL-5A) and a Ag/AgCl reference electrode (Model RE-1, Bioanalytical Systems Inc., West Lafayette, IN, USA), and a potentiostat (Metrohm VA Detector E 611 distributed by Brinkman, Westbury, NY, USA). The photoelectrochemical detector was assembled by connecting the photoreactor in series with the electrochemical detector.

Flow injection analysis. The flow injection analysis system used in this study was identical to the chromatographic system described above except the injector was connected directly to the Teflon tubing in the photoreactor by omitting the chromatographic column.

Reagents and chemicals

All reagents used in this study were reagent grade and obtained from commercial sources. Sodium nitroprusside (SNP) contained 2.0 H_2O per mole when assayed (Sigma Chemical Co., St. Louis, MO, USA). Sodium nitrite, potassium thiocyanate, potassium ferricyanide, citric acid and tetrabutylammonium perchlorate (TBA perchlorate) were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA); potassium nitrate from Merck and Co., Inc. (Rahway, NJ, USA) and potassium cyanide and potassium ferrocyanide from Fisher Scientific Co. (Fair Lawn, NJ, USA). Sodium tetraborate was obtained from Matheson Coleman and Bell (Norwood, OH, USA). Electrolytes, lithium perchlorate ($LiClO_4$) and sodium perchlorate ($NaClO_4$) were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA; EM Science, Cherry Hill, NJ, USA). Water used in this study was double distilled and freshly prepared. Organic solvents used in this study were methanol and acetonitrile (HPLC grade, J.T. Baker Chemical Co.).

Stock solutions

Stock solutions in water (1.0 mM) were prepared for ferrocyanide and ferricyanide. Stock solutions of 10.0 mM were prepared for nitrite, nitrate, thiocyanate and cyanide in water. An aqueous solution of 1.0 mM sodium nitroprusside (29.8 mg/100 ml) was prepared fresh daily and wrapped in aluminium foil to prevent light-induced degradation.

SeraChem Normal Clinical Chemistry Control Serum (Human), unassayed was reconstituted with 10.0 ml water (Fisher Scientific, Orangeburg, NY, USA).

Procedures

Hydrodynamic voltammograms (HDV). Determination of the hydrodynamic voltammograms was carried out by flow injection analysis. The potentiostat setting was varied in 0.05 V increments in the range 0.70–1.00 V. The flow rate was set at 1 ml min^{-1}.

The mobile phase used was 0.007 M lithium perchlorate adjusted to pH 6.5 with dilute perchloric acid and 0.01 M tetrabutylammonium perchlorate in water–methanol (70:30).

Decimal dilutions of the anionic stock solutions were made with mobile phase until a response was received that did not deflect off scale. Replicate measurements, 3–5, of the electrochemical response were made for each ion at each respective potential with the photoreactor on and off.

Ion-pairing chromatography. The system consists of the detector system as described above and an ODS-2 reversed-phase column, either 10 or 15 cm in length. The potential on the working electrode was set at 0.90 V versus Ag/AgCl and the potentiostat was set at 50 nA full scale. The flow rate was set at 1 ml min^{-1}.

Tetrabutylammonium perchlorate was used as the ion-pairing agent. The buffer used was

lithium perchlorate/perchloric acid. The variable parameters of the eluent were pH, the concentration of the ion-pairing agent, the composition of the organic solvent modifier and the ratio of organic solvent modifier to water. The concentration of electrolyte was held constant at 0.007 M.

Quantitative assay of nitroprusside

Minimal detectable quantity. The reversed-phase IPC system was employed in determining the minimal detectable quantity (MDQ) of nitroprusside. The electrolyte used was 0.007 M lithium perchlorate. The ion-pairing agent was 0.01 M tetrabutylammonium perchlorate in methanol–water (30:70) adjusted to pH 6.5 with dilute perchloric acid. The photoelectrochemical detector was employed with the potential at the working electrode set at 0.90 V versus Ag/AgCl. The flow rate was maintained at 1 ml min^{-1}. The potentiostat attenuation was decreased to 5 nA full scale where the noise level was approximately 1.5% of the full scale deflection.

Calibration. A standard curve for SNP was constructed in the range 12–96 ng SNP using 10-μl injections of aqueous aliquots of the SNP stock solution. The mobile phase was 0.01 M tetrabutylammonium perchlorate, 0.007 M $LiClO_4$ in methanol–water (35:65, v/v) with the apparent pH adjusted to pH 6.5 with dilute perchloric acid.

For spiked serum samples the IPC system was modified. A guard column packed with Perisorb RP-18, 30–40 μm pellicular particles (Uptight Guard Column Kit, No. 1602, Upchurch Scientific Inc., Oak Harbor, WA, USA) was connected in series with the 15 cm ODS-2 column. The nitroprusside ion was detected with the photoelectrochemical detector.

A standard curve of nitroprusside was prepared from spiked serum samples. Aliquot volumes in the range 10–70 μl of 1.0 mM sodium nitroprusside were diluted to 500 μl with Fisher reconstituted human serum and then diluted with 1.0 ml acetonitrile, mixed on a Vortex mixer for 1 min and centrifuged for 15 min. One ml mobile phase was added to the supernatant and centrifuged for an additional 10 min. Triplicate 10-μl injections (containing 10 to 70 ng) were made.

Results and Discussion

The photoelectrochemical detector

The applicability of Krull's photoelectrochemical detector to nitroprusside and its degradative and metabolic products was determined. Experiments with the photoreactor on and off established that nitroprusside and nitrate were detectable with the photoelectrochemical detector on but are not detectable with the UV light off. In addition, the photoelectrochemical detector increased the response of thiocyanate, cyanide and ferricyanide but reduced that of ferrocyanide compared with responses obtained by the amperometric detector alone. An appropriate potential to permit optimum detection of all the anions was determined from the results of the hydrodynamic voltammograms.

Data collected from the HDV of the seven anions are summarized in Table 1. Nitroprusside ion produced a signal with the photoelectrochemical detector, but not with the amperometric detector. Nitroprusside ion has been reported to decompose upon exposure to UV light presumably due to the Fe—NO bond undergoing cleavage to form aquapentacyanoferrate(II) [4, 5]. The nitrosyl group is free to react with water and produce the oxidizable nitrite which is most likely the actual species detected. The following equation presents the proposed photolytic reaction:

$$[Fe(CN)_5NO]^{2-} + 2H_2O \rightarrow [Fe(CN)_5H_2O]^{3-} + NO_2^- + 2H^+. \quad (1)$$

The current response obtained for nitroprusside ion increased with increasing potential with the limiting current obtained at approximately 1.0 V versus Ag/AgCl. Response and therefore, sensitivity, could possibly be increased by reducing the flow rate or increasing exposure time by lengthening the PTFE reaction coil. A 0.005 M solution of nitroprusside was used to yield a response with a signal to noise ratio (S/N) in the range 2–5.

Reduction of nitrate by the photoreactor permitted detection of this analyte as well [16]. The nitrate voltammogram showed a steep increase in the range 0.85–1.0 V versus Ag/AgCl with no indication of reaching a limiting current. The voltammogram for nitrite indicated that the detection of nitrite remained unchanged, with the photoreactor on or off. The limiting current was found to be in the range 0.85–0.95 V versus Ag/AgCl.

Table 1
Hydrodynamic voltammogram data for sodium nitroprusside and its degradation and metabolic ions

	Cyanide		Thiocyanate		Nitrite		Nitrate		Ferrocyanide		Ferricyanide		SNP	
Light*	Off	On	Off	On	Off	On	Off	On	Off	On	Off	On	Off	On
0.70†	0.00‡	0.00	0.00	0.50	0.00	0.00	—§	0.22	0.00	0.00	0.00	4.72	—§	5.81
0.75	0.00	5.70	0.00	0.46	0.00	0.00	—	0.47	0.00	0.00	0.00	3.39	—	4.81
0.80	0.00	17.20	0.18	1.21	0.00	3.61	—	1.73	0.00	0.00	0.00	8.07	—	8.72
0.85	1.80	17.60	0.21	1.90	18.50	19.16	—	1.99	67.57	24.27	2.41	6.95	—	9.06
0.90	3.30	19.20	0.16	2.96	36.35	17.05	—	3.82	78.07	22.95	2.06	7.15	—	21.79
0.95	1.70	20.00	0.29	2.35	46.39	24.15	—	6.01	69.71	21.81	1.92	7.30	—	18.11
1.00	0.30	22.50	0.09	—	76.77	79.33	—	8.45	65.95	17.05	1.68	5.52	—	30.31

*Hanovia mercury lamp (medium intensity).
†Working electrode voltage.
‡Current (μAmps μmol^{-1}).
§No response with UV light off.

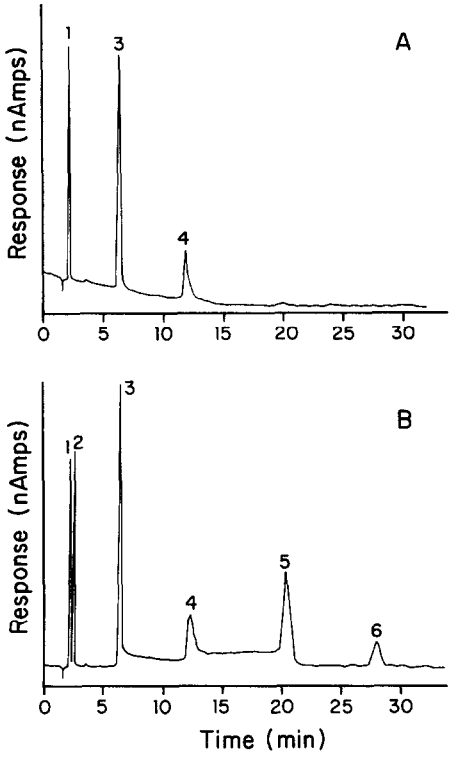

Figure 1
Chromatographic separation of 0.01 mM nitrite (1), 0.1 mM nitrate (2), 1.0 mM thiocyanate (3), 0.1 mM ferrocyanide (4), 0.1 mM ferricyanide (5) and 0.01 mM nitroprusside (6), on 15 cm ODS-2 column; (A) photoreactor off; (B) photoreactor on mobile phase, 0.01 M TBA perchlorate, 0.007 M $LiClO_4$, methanol–water (30:70, v/v), pH 6.5 adjusted with $HClO_4$; flow rate, 1.0 ml min^{-1}; detector, 0.90 V Ag/AgCl, 50 nAfs.

The photoelectrochemical detector increased the current response of ferricyanide and thiocyanate with limiting currents at 0.80 and 0.90 V versus Ag/AgCl, respectively, compared with the responses obtained by the amperometric detector. The response for ferrocyanide, however, was reduced with the photoreactor on. This may be due to the photo-degradation of ferrocyanide to ferrihydroxide [17]. The current response of both ferrocyanide and ferricyanide decreased towards the higher potentials as a result of fouling of the working electrode. The voltammogram of cyanide showed enhanced detection with the photoreactor on. However, the degree of ionization of HCN is 0.2% at the pH of the buffer (6.5) which is almost three units less than the pK_a of HCN (9.2).

HDV measurements were concluded at 1.0 V versus Ag/AgCl as background noise from the mobile phase increased. The current responses remained either constant or decreased below 0.75 V versus Ag/AgCl. A potential of 0.90 V was selected for this study because it allowed greater response with the photoreactor on, minimal oxidation of mobile phase components and minimal fouling of the electrode.

Ion-pairing chromatography

Separation by IPC is dependent on pH, concentration of ion-pairing agent, and the selection and concentration of organic solvent modifier. Change in the pH from 5 to 8 had little effect on the capacity factors as all detectable analytes are fully ionized in this pH range. Therefore, the median pH of 6.5 was maintained in future eluents.

Detection of cyanide anion at pH <8 was not possible. The use of methanol and acetonitrile as organic solvent modifiers was investigated in various concentrations. Increasing the concentration of methanol produced a decrease in the capacity factor for the three polyvalent ions, ferricyanide, ferrocyanide and nitroprusside. The monovalent ions, thiocyanate, nitrite and nitrate produced a very small decrease in capacity factors on increasing the concentration of methanol. Methanol increased the distribution of the tetrabutylammonium ion-pairs into the mobile phase. If a more aqueous eluent were used, these ion-pairs would have been retained longer due to their adsorption onto the reversed-phase column. With very low concentrations of methanol (1–10%) there was an increase in capacity factors for the polyvalent ions with increased concentration of methanol. This response is atypical for ion-pairing chromatography and may reflect increased solvation of the lipophilic stationary phase at low methanol concentrations.

Using acetonitrile as the organic solvent modifier resulted in a reduced retention of all the analytes. The capacity factors of all ions were <5. Also, tetrabutylammonium perchlorate, the ion-pairing agent, is poorly soluble at low concentrations of acetonitrile. Increasing the concentration of acetonitrile resulted in elution of the analytes at the solvent front with little resolution. Methanol therefore was selected as the organic solvent modifier.

Capacity factors of nitroprusside, ferricyanide and ferrocyanide were reduced with increasing concentrations of tetrabutylammonium perchlorate. However, the retention of thiocyanate, nitrite and nitrate ions remained constant under these conditions.

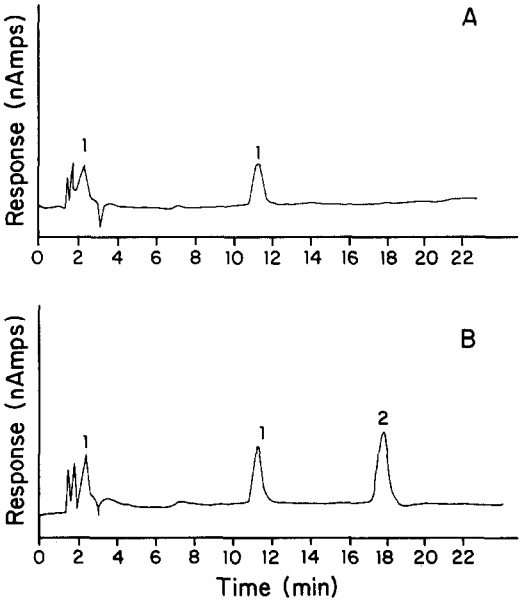

Figure 2
Chromatographic analysis of nitroprusside from blank (A) and spiked (B) human serum samples. Chromatographic conditions are: 15 cm ODS-2 column; mobile phase, 0.01 M TBA perchlorate, 0.007 M $LiClO_4$, methanol–water (35:65), pH 6.5 adjusted with $HClO_4$; flow rate, 1.0 ml min^{-1}; detector, 0.90 V Ag/AgCl, 50 nAfs; (1) serum constituents; (2) nitroprusside.

Ion-pair chromatography of a solution of nitrite, nitrate, thiocyanate, ferrocyanide, ferricyanide and nitroprusside ions with the photoelectrochemical detection system was performed using 0.01 M tetrabutylammonium perchlorate, 0.007 M $LiClO_4$ in methanol–water (70:30) at pH 6.5. Resolution of all six anions was achieved (Fig. 2a,b). Ferrocyanide and ferricyanide were well resolved. Cyanide could not be detected due to the more acidic eluent required by the ODS-2 columns. The analysis of aqueous solutions of nitroprusside ion was linear in the range 12–96 ng on column (r^2 = 0.984).

Quantitative analysis of nitroprusside

Analysis of nitroprusside from spiked serum samples was successful, suggesting applicability to therapeutic drug monitoring (Fig. 2).

The chromatographic method used in the quantitative analysis of nitroprusside from serum required the removal of protein; 1 ml acetonitrile and 1 ml mobile phase were used in the precipitation of protein and dilution of nitroprusside. Protein precipitation with 6% perchloric acid or 0.5 M trichloracetic acid left clear supernatants, however, minimal response was obtained for nitroprusside.

Table 2
Recovery and accuracy data for nitroprusside (n = 3)

Added (ng)	Found (ng)	RSD (%)	% Error*
12	11.77 ± 0.26†	2.21	1.92
24	22.06 ± 4.53	20.53	8.08
36	36.51 ± 4.76	13.04	1.42
48	47.63 ± 6.49	13.63	0.77
60	65.22 ± 11.38	17.45	8.77
72	74.35 ± 0.93	1.25	3.26
84	84.91 ± 1.29	1.52	1.08

*ABS [(weight added − weight found) * 100/weight added].
†Standard deviation.

Linearity of response was achieved from spiked serum samples in the range 10–70 ng (r^2 = 0.983). Recovery and percent error were calculated (Table 2). Percent error was in the range 1–9%.

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