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Technical note

High-performance liquid chromatographic analysis of nitrite and nitrate in human plasma as *S*-nitroso-*N*-acetylcysteine with ultraviolet absorbance detection

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Abstract

A rapid HPLC method with UV absorbance detection at 333 nm for the measurement of nitrite and nitrate in ultrafiltrate samples of human plasma is described. The method is based on hydrochloric acid-catalyzed conversion of nitrite by *N*-acetyl-L-cysteine to *S*-nitroso-*N*-acetyl-L-cysteine and isocratic elution using 10 m*M* NaH_2PO_4 in acetonitrile–water, pH 2.0 (15:85, v/v). The limit of detection of the method is 50 n*M* nitrite. The method was validated by gas chromatography–mass spectrometry.

Keywords: Nitrate; Nitrite; *S*-Nitroso-*N*-acetylcysteine

1. Introduction

In fasted humans about 90% of circulating nitrite has been shown to endogenously originate from the L-arginine:nitric oxide (NO) pathway [1]. The major metabolite of L-arginine-derived NO has been shown to be nitrate [1]. Various analytical methods have been developed for the quantitation of nitrite and nitrate in biological fluids (reviewed in Ref. [2]). In the present work, we report a new and rapid HPLC method for the measurement of nitrite and nitrate in human plasma. This method is based on the acid-catalyzed conversion of nitrite by *N*-acetylcysteine (NAC) to *S*-nitroso-*N*-acetylcysteine (SNAC). The method was validated by a previously described gas chromatographic–mass spectrometric (GC–MS) method [3].

2. Experimental

2.1. Materials and chemicals

NAC was obtained from Sigma (Munich, Germany). Sodium nitrite, *o*-phosphoric acid, hydrochloric acid and acetonitrile of gradient grade were obtained from Merck (Darmstadt, Germany). Centrisart I® ultrafiltration cartridges (pore size 4 μm, cut-off 20 kDa) from Sartorius (Göttingen, Germany) were used to obtain ultrafiltrate by centrifugation (1800 *g*, 2°C, 15 min) of 2-ml aliquots of plasma.

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2.2. Derivatization procedure and HPLC analysis

Aliquots (200-μl) of nitrite-containing samples were treated with a freshly prepared solution of NAC in twice-distilled water (50 m*M*; 40 μl) and with HCl (4 *M*; 10 μl). Five minutes after incubation at room temperature, 200-μl aliquots of these solutions were injected into the HPLC system. In the derivatization mixture the concentration of SNAC was found to remain unchanged for at least 2 h at room temperature and for at least 24 h when stored at 4°C. Nitrate was reduced to nitrite as described elsewhere [3]. The recovery was 78±12% (mean±SD, $n=10$) as measured by GC–MS [3].

HPLC analyses were performed on an LKB delivery system model 2150 coupled with a variable UV–Vis LKB detector model 2151 (Bromma, Sweden). The chromatographic column (250×4.6 mm I.D.) was packed with ODS Hypersil, 5 μm particle size (Shandon, UK). The mobile phase was 10 m*M* of NaH_2PO_4 in acetonitrile–water, 15:85, v/v, the pH of which was adjusted to 2.0 by addition of *o*-phosphoric acid. Isocratic runs were carried out at a flow-rate of 1.0 ml/min. SNAC was monitored at 333 nm. The retention time of SNAC was measured as 5.3±0.12 min (mean±SD, $n=60$).

3. Results and discussion

UV–Vis spectra of nitrite and SNAC in HCl solutions revealed molar absorptivity coefficients of $a=769\ M^{-1}\ cm^{-1}$ at $l_{max}=333$ nm for SNAC, $a=11\ M^{-1}\ cm^{-1}$ at $l=333$ nm and $a=33\ M^{-1}\ cm^{-1}$ at $l_{max}=358$ nm for nitrite. The mean enhancement factor was calculated as 39.4 from the ratio of the slopes of the straight lines which were obtained from regression analysis between the peak areas (y) from HPLC analyses of nitrite after conversion to SNAC ($y=-13951+53201x$, $r^2=0.999$) and prior to *S*-nitrosylation ($y=2221+1332x$, $r^2=0.997$) and the amount of nitrite (x) (0 to 150 nmol).

Standard curves of nitrite (0–50 μ*M*) and nitrate (0–100 μ*M*) in plasma samples were linear in these ranges ($r^2=0.975$ and 0.995, respectively). The following regression equations were obtained by linear regression analysis between the data obtained from analysis by HPLC (y) and those by GC–MS (x): $y=0.156+1.156x$ ($r^2=0.995$) for nitrite and $y=-1.43+0.985x$ ($r^2=0.938$) for nitrate. Intra-assay RSD were 6.3% for nitrite and 2.5% for nitrate from analysis of 10 μ*M* nitrite and 50 μ*M* nitrate added to plasma. The limit of detection of the method for aqueous nitrite was 50±15 n*M* ($S/N=3:1$) at a RSD

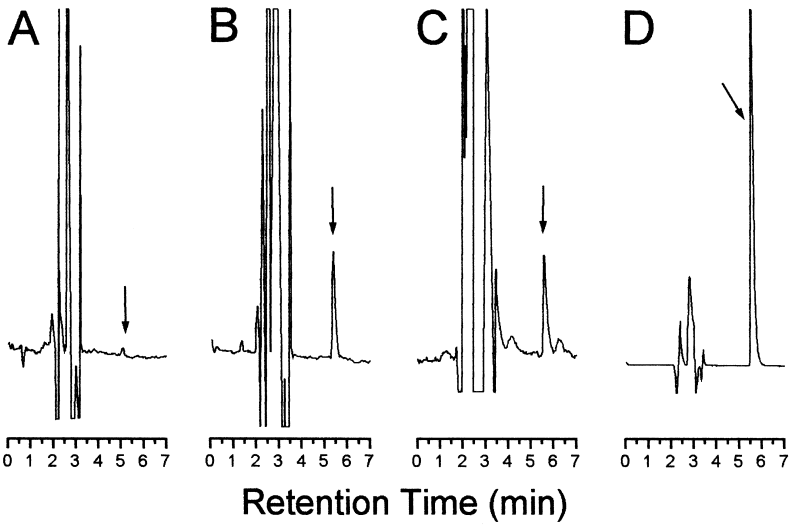

Fig. 1. Chromatograms of nitrite and nitrate as *S*-nitroso-*N*-acetyl-L-cysteine (indicated by arrows at a retention time of 5.5 min). Analysis of nitrite (A) in unspiked distilled water and (B) in distilled water spiked with 1.0 μ*M* nitrite. Analysis of nitrite (C) and nitrate (D) in a unspiked plasma sample of a healthy volunteer. Note the different integrator attenuation values in A, B, C (attenuation 2^2) and D (attenuation 2^6).

value of 17%. In healthy volunteers plasma nitrite and nitrate levels were determined as (mean±SD) 0.55±0.16 μ*M* (*n*=26) and 27.4±3.3 μ*M* (*n*=8), respectively. Representative chromatograms from analyses of nitrite and nitrate as SNAC in aqueous solution and in ultrafiltrates of plasma are shown in Fig. 1. The HPLC method described in this paper is useful for routine analysis of nitrite and nitrate in human plasma.

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