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Short communication

Determination of urinary thiocyanate and nitrate using fast ion-interaction chromatography

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Abstract

A simple and rapid chromatographic method for determination of nitrite, nitrate and thiocyanate is reported, and applied to the analysis of non-, medium and heavy smokers' urine samples. Ion-interaction liquid chromatography was carried out on a short 30 mm×4.6 mm C_{18} column (3 μm particle size) with a mobile phase of 10 m*M* tetrabutylammonium phosphate in 20% MeOH. The chromatography was performed at an elevated temperature of 45 °C, at a flow-rate of 1 ml/min. Detection was by direct UV absorption at 230 nm. Sample preparation involved centrifugation and dilution, followed by sample clean-up on a C_{18} solid-phase extraction cartridge. The developed method proved both precise (% RSD <2%) and sensitive (standard detection limits <0.1 mg/l), and yielded total run times of under 10 min when applied to urine analysis of smokers and non-smokers, with thiocyanate eluting in under 5 min.

Keywords: Thiocyanate; Nitrate

1. Introduction

Low concentrations of thiocyanate are usually found in urine, due mainly to the subject's diet. This may be through ingestion of certain vegetables containing glucosinolates (cabbage/turnip, etc.), or foods that actually contain levels of inorganic thiocyanate such as milk and cheese. Higher levels of thiocyanate are found where thiocyanate has been directly administered to treat thyroid conditions, or where sodium nitroprusside (used as a hypotensive agent) is metabolised to contribute to urinary thiocyanate. At high concentrations, thiocyanate can lead to vertigo and unconsciousness, and as such is an important biochemical parameter to consider when studying such conditions.

However, despite the above natural and medical sources of thiocyanate, the majority of studies based on analysis of this anion in urine have actually been performed due to the usefulness of thiocyanate as an important biomarker for inorganic cyanide [1]. Inorganic cyanide (CN^-) can enter the system in a number of ways, including, for example, certain topically applied cosmetics that contain alkyl and arylalkyl-cyanides as fragrance ingredients. However, a much more significant source of cyanide to the body is tobacco smoke. Once absorbed into the body cyanide has a very high affinity for iron in the ferric (trivalent) state, and as such binds to and inhibits the activity of cytochrome oxidase, thereby inhibiting mitochondrial respiration. Renal elimination of low levels of cyanide from the body is

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accomplished by its conversion to thiocyanate by the enzyme rhodanese (EC 2.8.1.1), which is found in the mitochondria of liver and kidney cells [2].

Since tobacco smoke has been shown to be a major contributor to the production of thiocyanate in vivo, levels of urinary thiocyanate can be used to distinguish between smokers and non-smokers and to effectively evaluate and characterise smoking behaviour [1,3–6]. Thiocyanate has been determined in biological samples by a number of analytical methodologies. Spectrophotometric methods have been reported based on derivatisation resulting in the formation of a red complex with iron (III) [7] or on the well-known Konig reaction [8], and, more recently, Zhang et al. [6] determined trace thiocyanate in urine and saliva of smokers and non-smokers using a fluorimetric method, based on the inhibiting effect of thiocyanate on the oxidation of rhodamine 6G by potassium bromate in acidic medium. However, each of these spectrophotometric methods is time consuming and laborious to perform, with some involving the use of harmful reagents. Several electrochemical methods have been developed with varying degrees of complexity, such as the use of pulse-polarography [9] and recently ion-selective electrodes [10], although the complex nature of the matrix in biological samples can prove problematic with such an approach.

The separation techniques applied to thiocyanate determinations in biological samples include gas chromatography [11,12], varying modes of capillary electrophoresis [3,13] and liquid chromatography, with the liquid chromatographic methods generally being based upon some mode of ion chromatography [4,5]. However, the use of ion chromatography with anion-exchange stationary phases for the separation of thiocyanate can result in excessive run times and poor peak shapes due to the highly ''polarisable'' nature of thiocyanate, with mobile-phase additives (MeOH or *p*-cyanophenol), or the use of specialist hydrophilic ion-exchange columns being required to address these problems. In addition to this, expensive anion-exchange columns can easily become fouled when dealing with complex biological samples. An alternative approach that has been investigated is the use of ion-interaction liquid chromatography. For example, Michigami et al. [5] developed an analytical method for thiocyanate in urine on a short 5 cm ODS Capcell Pak column coated with cetyldimethylamine. However, despite the use of a short analytical column, the retention time of thiocyanate with a citrate eluent was still rather long at 16 min, with a total run time of >30 min due to a late eluting peak in the urine sample.

This paper reports on the use of a simple ion-interaction liquid chromatographic method to obtain a rapid separation of nitrate, nitrite and thiocyanate, and its application to the analysis of urine samples. The developed method is based upon the use of a short (3 cm) inexpensive 3 μm ODS analytical column with an eluent containing the ion-interaction reagent, tetrabutylammonium phosphate, and direct UV detection at 230 nm. Run times were <5 min for standards and only 10 min for a real sample (again due to a late-eluting matrix peak). In addition to thiocyanate, the method also resulted in the resolution of nitrate and nitrite from each other and several matrix peaks and allowed the determination of all three anions in real samples taken from non-, moderate and heavy smokers.

2. Experimental

2.1. Equipment

A Dionex DX500 ion chromatograph (Dionex, Sunnyvale, CA, USA), comprising of a GP50 gradient pump, LC25 chromatography oven and an AD20 absorbance detector, was used. The analytical column used was a Phenomenex Hypersil, 3 μm particle size, 30 mm×4.6 mm I.D. column (Macclesfield, Cheshire, UK). The injection loop used was 25 μl. Detection was by direct UV at 230 nm with data acquisition at a rate of 10 Hz and processing of chromatograms performed using a PeakNet 6.0 chromatography workstation (Dionex). Urine samples were centrifuged using a Biofuge 13 centrifuge (Heraeus, Germany).

2.2. Reagents and chromatographic conditions

The water used for mobile phase, standard preparation and for sample pre-treatment was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The tetrabutylammonium hy-

droxide (TBA-OH) used for preparation of the mobile phase was supplied by Aldrich (Milwaukee, WI, USA) as a 50% w/v solution in water, and methanol was obtained from Labscan (Stillorgan, Dublin, Ireland). The optimised mobile phase consisted of 10 m*M* TBA-OH in 20% aqueous MeOH, titrated to an apparent pH of 6 using 85% phosphoric acid (Riedel de Haen, Germany). The mobile phase was degassed and filtered before use using 0.45 μm nylon membrane filters from Gelman Laboratories (MI, USA). The flow-rate used was 1.0 ml/min with column temperature set at 45 °C.

Stock standard solutions of concentration 1000 mg/l were prepared monthly using Milli-Q water, with working standards prepared daily from each respective stock solution. Nitrite, nitrate and thiocyanate standards were prepared from their respective sodium salts (Aldrich).

2.3. Sample pre-treatment

Urine samples were collected from a non-smoker, moderate smokers and a heavy smoker. Samples were centrifuged at 13 000 *g* for 2 min and stored at 4 °C until needed. Before injection onto the column, each sample was diluted 1 in 20 with deionised water. To remove organic interferents, 1 ml of sample was pushed through an SPE C_{18} cartridge (Waters ''Sep-Pak Light'', Waters, Milford, MA, USA), which had been preconditioned with 2 ml MeOH followed by 2 ml of deionised water. The sample was then injected onto the column through a 0.45 μm nylon membrane filter from Gelman Laboratories.

3. Results

3.1. Mobile-phase optimisation

Based on retention data collected in previous work [14], tetrabutylammonium chloride (prepared by titrating a TBA-OH-containing eluent with HCl) was initially chosen as ion-pair reagent to use in this study, with methanol as organic modifier. An initial mobile-phase preparation of 10 m*M* TBA-Cl in 20% MeOH, pH 6.0 (flow-rate, 1.0 ml/min; column temperature, ambient) resulted in baseline separation of nitrate (t_R = 1.4 min) and nitrite (t_R = 1.6 min) with a retention time for thiocyanate of 6.5 min. However, in an attempt to further reduce the retention time for thiocyanate and also add some buffering capacity to the eluent, the chloride counterion was replaced with phosphate, by titrating the TBA-OH with phosphoric acid to pH 6.0. As phosphate has a greater eluting strength than chloride this change resulted in reduced retention times for all three anions, with thiocyanate now eluting at 5.8 min and nitrate and nitrite eluting at 1.1 and 1.3 min, respectively.

As thiocyanate is a less hydrophillic anion than nitrate and nitrite, it can be supposed that its retention was in part due to non-electrostatic attractions to the stationary phase. Therefore, a further reduction in the retention of thiocyanate relative to nitrate and nitrite should result if an increase in column temperature were investigated, as non-electrostatic interactions would be reduced. To test this assumption, the column temperature was raised to 45 °C and used with the above eluent. The resultant optimum standard chromatogram is shown in Fig. 1. As can be seem from the figure, the increase in column temperature reduced the retention time of thiocyanate by approximately 1.5 min, down to 4.4 min, without affecting the retention times or resolution of the nitrite and nitrate peaks. The total run time for the separation of all three analytes in a standard mixture could be reduced to under 2.5 min through the use of a 2 ml/min flow-rate without a

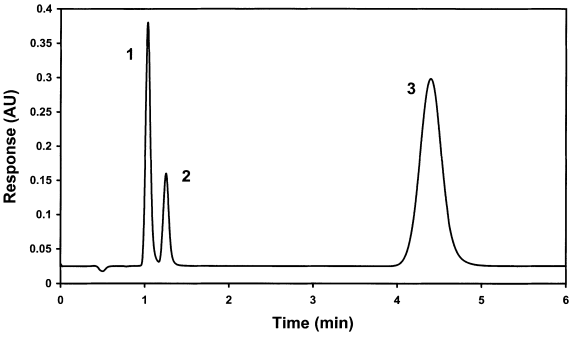

Fig. 1. Optimised chromatographic conditions. Column, 30 mm × 4.6 mm C_{18} 3 μm column. Flow-rate, 1.0 ml/min; loop size, 25 μl; column temperature, 45 °C; detection, direct UV at 230 nm. Peaks: 1 = 2.5 mg/l nitrite, 2 = 2.5 mg/l nitrate, 3 = 5 mg/l thiocyanate.

detrimental affect upon resolution or peak shape, although for complex biological matrices the use of the lower flow-rate was more suitable due to the large number of matrix peaks likely to be present.

3.2. Detection

To optimise detection sensitivity a mixture of nitrate/nitrite (2.5 mg/l) and thiocyanate (5 mg/l) was injected at wavelengths ranging from 190 to 235 nm. Signal-to-noise ratios for each peak were plotted versus wavelength. The baseline noise was at a minimum above 215 nm, and the signal-to-noise ratio for thiocyanate was at a maximum at 230 nm. This also corresponded to the optimal signal-to-noise value for nitrite. The optimum response for nitrate was obtained at 220 nm, although as nitrate is present at higher levels than thiocyanate in urine, 230 nm was used as the detection wavelength.

3.3. Precision, linearity and detection limits

Method precision was determined by the repeat injection ($n=6$) of a standard mixture at a concentration of 5 mg/l nitrite, nitrate and thiocyanate. The resultant % RSD values for peak area were 0.87% for nitrite, 1.44% for nitrate and 1.10% for thiocyanate. Method linearity for standard solutions was determined by peak area over the range 0.5 to 10 mg/l ($n=5$) using mixed standards, with each standard injected in triplicate. Correlation coefficients obtained were $R^2=0.9993$ for nitrite, $R^2>0.999$ for nitrate and $R^2>0.999$ for thiocyanate, illustrating the method showed excellent linearity over the concentration range of interest. Using a 25 μl injection loop, limits of detection in standards were found to be approximately 0.02 mg/l for nitrite, 0.04 mg/l for nitrate and 0.02 mg/l for thiocyanate (calculated using a signal equivalent to three times the standard deviation of the baseline noise). Improved concentration detection limits were possible with the use of larger injection volumes. Loop sizes of 25, 50 and 75 μl were studied by injecting the same standard mix as above. With standard solutions increasing loop size up to 75 μl had no deleterious effect on peak shape or efficiency. However, a 25 μl loop was chosen for use with real samples to reduce potential fouling of the short column due to the complex nature of the sample.

The linearity of the method in real samples was determined using the standard addition of nitrite, nitrate and thiocyanate to the diluted (1 in 40) urine sample of a medium smoker. Standard addition curves for both nitrite and nitrate ($n=6$) were constructed over the range 0–10 mg/l (representing 0–400 mg/l nitrite and nitrate in the undiluted urine sample). Again, acceptable correlation coefficients were obtained, $R^2=0.9979$ for nitrite and $R^2=0.9982$ for nitrate. For thiocyanate a standard addition curve over the range 0–5 mg/l was constructed ($n=6$) (representing 0–200 mg/l thiocyanate in the undiluted urine sample). For this curve a correlation coefficient of $R^2=0.9956$ was obtained. From the standard addition work carried out a quantifiable working range of 10–400 mg/l nitrate and 2–200 mg/l thiocyanate in undiluted samples was determined. This was deemed sufficient to cover the concentrations of the two anions likely to be present in the majority of samples.

3.4. Analysis of real samples

Urine samples were collected from a non-smoker (male), moderate smokers (male and female, 10 to 20 cigarettes per day) and a heavy smoker (male, 30 to 40 cigarettes per day) in order to demonstrate the effectiveness of this method as a means of evaluating smoking behaviour. Samples were analysed against a standard curve for each of the three analytes respectively (standard five point curves could be constructed in under an hour with each standard injected in duplicate). The run time for samples was extended to 10 min due to a late-eluting sample matrix peak at ~9 min. Nitrate and thiocyanate peaks were evident in all four samples, with the retention time for thiocyanate slightly reduced due to probable ''self-elution'' resulting from the complex sample matrix. As is recommended when analysing samples of a complex nature, standard addition was again used to further identify and quantify the analyte peaks. It was found that analyte sensitivity was not affected by the analytes being present in the diluted urine matrix. Fig. 2 shows the chromatogram obtained from the analysis of a urine sample taken from a heavy smoker. Overlaid is the same sample spiked

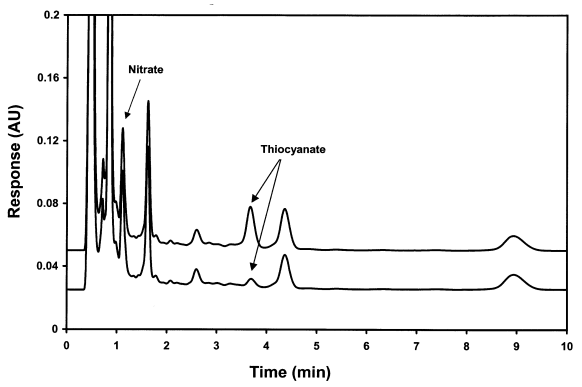

Fig. 2. Analysis of urine for thiocyanate. Lower trace: sample (heavy smoker) diluted 1:20. Upper trace: sample (heavy smoker) diluted 1:20 and spiked with thiocyanate. Chromatographic conditions as in Fig. 1.

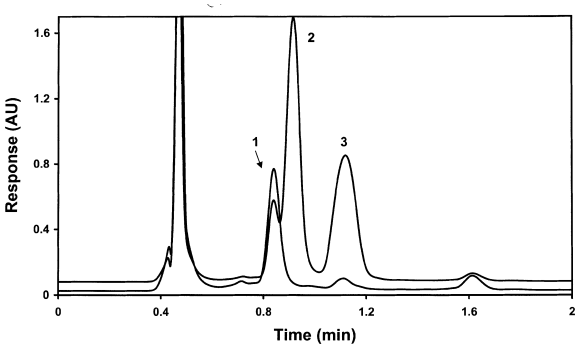

Fig. 3. Analysis of urine for nitrate and nitrite. Lower trace: sample (heavy smoker) diluted 1:20. Upper trace: sample (heavy smoker) diluted 1:20 and spiked with nitrate and nitrite. Peaks: 1=unidentified sample matrix peak, 2=nitrite, 3=nitrate. Chromatographic conditions as in Fig. 1.

with thiocyanate. As can be seen from the two chromatograms, thiocyanate is clearly well resolved from all major matrix peaks, the majority of which elute in under 2 min. Although the exact profile of individual urine samples will vary, there was no interference from matrix peaks for any of the samples studied, indicating the method is generally applicable for this particular sample type.

The ability of the method to simultaneously detect nitrite and nitrate in the above samples is illustrated in Fig. 3. The figure shows overlaid urine sample chromatograms, one of which has been spiked with both nitrite (peak 2) and nitrate (peak 3). The reduction in size of peak 1 is simply due to the dilution of the spiked sample. As can be seen, nitrate is well resolved from nitrite (none detected in each of the real samples), which partially co-elutes with a sample matrix peak (peak 1). It should be noted that the co-elution of nitrite with a sample matrix peak would make the quantitative determination of low levels of nitrite difficult, although the presence of nitrite in the sample would be clearly seen. Levels of nitrate found in the samples were 141 mg/l, 152 mg/l (male) 259 mg/l (female) and 224 mg/l in the non-smoker, moderate smokers and heavy smoker samples, respectively. The fact that nitrate levels are lower in the heavy smoker than the moderate smoker reflects the fact that significant alternative sources of urinary nitrate exist and further illustrates the potential of the use of urinary thiocyanate as a rough indicator of smoking behaviour. Table 1 details the results obtained for thiocyanate determinations. The results obtained in this brief study are compared to urinary thiocyanate levels determined in alternative studies and also compares analysis times. The results

Table 1
Typical urinary thiocyanate levels (mg/l) as determined by different analytical methodologies

Ref.	Analytical method	SCN^- found			Total run time (min)
		Non-smoker	Medium smokers	Heavy smoker	
[5]	Ion-interaction chromatography	2.91	12.20	–	30
[3]	Capillary electrophoresis	4.65	12.78	–	10
This work	Ion-interaction chromatography	6.48	11.62 (female) 17.10 (male)	30.21	10

obtained in this study compare well with average values found previously.

4. Conclusions

A very simple, rapid and selective method for urinary nitrite, nitrate and thiocyanate determinations has been shown. The method uses only a short inexpensive analytical column, resulting in shorter runs times, allowing rapid method development, system calibration and sample analyses. Applied to urine samples taken from smokers and non-smokers, the method showed how the chromatography can be used to qualitatively indicate the smoking behaviour of the donor.

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