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Journal of Chromatography A, 1065 (2005) 327–331
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Application of capillary zone electrophoresis to the analysis and to a stability study of nitrite and nitrate in saliva

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Received 8 October 2004; received in revised form 21 December 2004; accepted 31 December 2004
Available online 20 January 2005

Abstract

The applicability of capillary zone electrophoresis for the determinations of nitrite and nitrate was studied. Using direct UV detection the limit of detection values of the analytes were 0.14 and 0.21 μg/mL, respectively. The developed method was found to be useful to directly determine nitrite, nitrate and thiocyanate in saliva. It was found that adjusting the pH of the sample to 11 and storing the saliva at 4 °C was adequate to make constant the nitrite/nitrate ratio in saliva samples at least 7 days.

Keywords: Nitrite; Nitrate; Saliva

1. Introduction

The knowledge of the quantities of nitrate and nitrite ions has importance in clinical diagnostics and in evaluation of the state of the environment [1,2]. Nitrate and nitrite represent the products of the final pathway of nitric oxide metabolism. The measurement of nitrate and nitrite in blood can be used as an index of nitric oxide production [2]. The role of these ions is contradictory: they are considered as toxic compounds, which may cause infantile methaemoglobinaemia and carcinogenesis, however these compounds are probably necessary for the normal functioning of the body [3,4]. It is estimated that the major nitrite exposure for the human body is due to salivary nitrate reduction by the oral microbiota and the generation from amino acids in the intestines, which then absorbed into the bloodstream.

Numerous methods are described for measurement of nitrate and nitrite. Recently, the simultaneous analysis of the nitrate and nitrite is carried out using ion chromatography (IC) [5,6], which is often used technique for trace analysis of inorganic anions if larger sample volumes available. In last two decades also the capillary electrophoresis (CE) proved its applicability for ion analysis. Numerous reviews [7–9] were summarized in more than 200 papers published about the determination of inorganic anions. The great majority of these works applicable for nitrate and nitrite use indirect UV detection (after adding UV absorbing agent to the buffer electrolyte) and reversal of the electroosmotic flow (EOF) (adding cathionic detergent to the buffer electrolyte). There are some other CE works focusing nitrate/nitrite analysis in samples of different matrix [10–16]. The use of CE in salivary analysis provides advantages compared to IC like smaller operational cost, higher separation efficiency, faster analysis, lower consumptions of electrolytes and samples, and also simplier sample pretreatment.

In this work we wanted to show the possibility to use a simple capillary zone electrophoresis (CZE) method for the determination of nitrite and nitrate in saliva samples that are injected directly into the capillary. The study of the stability of nitrite and nitrate in saliva and the ways of the preservation of these analytes are also planned in order to get true analytical results for the moment of the sampling.

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doi:10.1016/j.chroma.2004.12.085

2. Experimental

2.1. Instrumentation

The capillary electrophoresis instrument was a HP ^{3D}CE model (Agilent, Waldbronn, Germany). The sample solutions were introduced (100 mbar s) at the cathodic end of the capillary. Separations were performed using a fused-silica capillaries of 48 cm (effective length: 40 cm) × 50 μm i.d. (CS-Chromatographie, Langerwehe, Germany). The applied voltage was −25 kV (reversed polarity). The detection was carried out by on-column photometric measurement at 214 nm. The electropherograms were recorded and processed by ChemStation computer program of 7.01 version (Agilent).

2.2. Chemicals and samples

Reagents were mostly of analytical grade. The sodium dihydrogenphosphate and sodium hydroxide for preparing buffer electrolytes were purchased from Reanal (Hungary). The nitrate and nitrite stock solutions (100 mg/L) were prepared from sodium nitrate and sodium nitrite (both from Reanal). The concentration of the stock solution of sodium molybdenate (internal standard) was 1000 mg/L.

The capillaries were preconditioned with the buffer electrolyte for 5 min. In case of analysis of saliva samples, the capillaries were flushed with 0.5 M NaOH (2 min), 0.3 M sodium dodecyl sulfate (SDS) (2 min), destillated water (2 min) and buffer (5 min) between runs to remove all the components which may stick to the capillary walls. Prior to CE analysis all the samples and buffers were filtered through a 0.45 μm syringe filter. The saliva samples were stored in refrigerator at +4 °C.

The saliva samples obtained from healthy persons were centrifugated at 1500 × *g* for 5 min and frozen until analysis. The addition of NaOH to the saliva adjusting its pH to about 11 caused that the solution became more clear and transparent. This high pH prevents the operation of the bacteria which are able to convert nitrate and nitrite.

3. Results and discussion

3.1. Optimization of the separation

In contrast with the most of the published CE separations of these compounds, we did not applied EOF modifier compound for EOF reversal and light absorbing agent for indirect UV detection. The selection of the simpliest background electrolyte was important not only for economical and easy work, but also for the more efficient determination of the analytes (e.g. in this case, the large amount of UV nonactive chloride in real samples does not interfere the detection of nitrate, different factors can affect to the reproducable adsorption of the EOF modifier on the capillary wall).

As the simplest choice, 25 mM phosphate buffer was used as a running electrolyte. Changing the pH of the buffer the mobility of the nitrite and the rate of the oppositely directed EOF can be influenced (Fig. 1). Since the charge to size ratio of nitrate is independent from the pH, the increase of migration time of nitrate follows the increase of the EOF. At low pH the nitrite has only a slight negative charge, therefore at pH 2.5 it cannot be detected within 25 min, but increasing the pH the mobility of nitrite gradually exceeds that of nitrate. At pH of 4.2, no resolution could be achieved, and at this pH a change of the migration order can be observed. To achieve a proper resolution (higher than 5), short analysis time (smaller than 5 min) and to avoid the possible conversion of nitrite in acidic medium the optimal pH was found to be about 6.8.

The analysis time can be reduced considerably if short-end injection mode (injection from the outlet end of the capillary) is applied when the effective length of the separation is only 8 cm. In this case, the analysis time is only 90 s with a bit smaller resolution of 3.1, while this value is 7.2 in normal injection mode. The short-end injection was generally well applicable for salivary analysis, the peaks of the analytes can be merged (causing difficulty in integration) only in case of non-suitable sample preparation or using capillary with contaminated inner surface (mainly adsorbed component of saliva due to inadequate postconditioning procedure).

3.2. Analytical performance

The nitrate and nitrite have good light absorption over the wavelength range from 190 to 225 nm, while the chloride or sulphate, which are the neighboring peaks and can be present in high concentrations in real samples, absorb only below 200 nm. Therefore, 214 nm was chosen as the detection wavelength for nitrite and nitrate.

In Table 1, the main analytical performance data obtained for the proposed conditions are summarized. The RSD values of the migration times were generally smaller than 1% RSD indicating that the separation system was stable

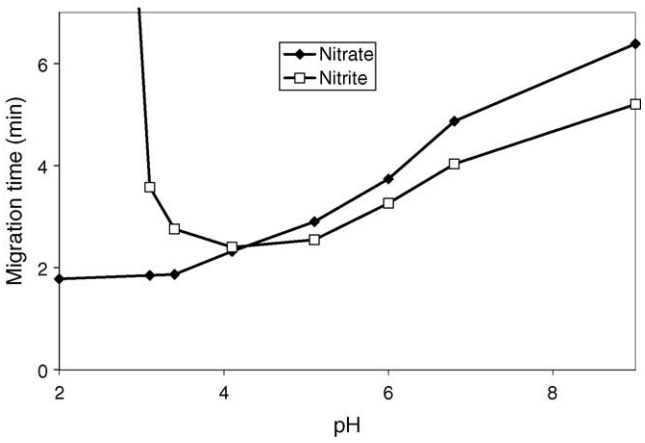

Fig. 1. Dependence of the migration of nitrite and nitrate on the pH of the running electrolyte (electrolyte: 25 mM phosphate).

Table 1
Analytical parameters of nitrite and nitrate

	Migration time (min)	LOD* (μg/mL)	Peak area, RSD (%)**	Migration time, RSD (%)**
Nitrite	4.43	0.14	1.63	0.84
Nitrate	5.14	0.21	1.81	0.89

* 3s (λ = 214 nm).
** c = 5 μg/mL, n = 10.

Table 2
Linearity regression data for nitrite and nitrate determined by CZE

	Regression equation	Correlation coefficient	Range (μg/mL)
Nitrite	$y = 0.5049x + 0.331$	0.9992	1–100
Nitrate	$y = 0.597x + 0.391$	0.999	1–100

during the measurements. The precision of the response was slightly worse (smaller than 2% RSD). The peak areas were found to be linear ($R^2 > 0.999$) in a concentration range of 1–100 μg/mL in each case with a precision better than 2% (Table 2). Very similar linearity data were obtained in undiluted saliva matrix for nitrite and nitrate: 0.9975 and 0.9987, respectively. The analytical validation of the analytical method was checked via standard addition method. In the concentration range 1–100 μg/mL, the differences between the concentration values obtained for standards in water and saliva spiked with the same standards were smaller than 5.5% (e.g. 5.2 μg/mL was obtained for saliva spiked with 5.0 μg/mL).

As an internal standard molybdenate was used. In Fig. 2, the electropherogram of a mixture containing nitrite, nitrate, thiocyanate and molybdenate is shown (in saliva samples high amount of thiocyanate is present).

3.3. Analysis of saliva samples, study of changing the ratio of nitrite and nitrate

The optimized method using short-end injection was employed to analyze human saliva samples. The samples were measured directly without pretreatment (there was only a spiking with NaOH) or dilution. Using direct injection of biofluids postconditioning (washing with NaOH, SDS and buffer electrolyte) of the separation capillary is necessary. The effectiveness of the postconditioning can be indicated by the changes in migration times or shape of the peak of the same component for consecutive runs. The nitrate, nitrite and thiocyanate could be determined, neither dilution of the sample nor addition of different agents to the buffer electrolyte was necessary but the amounts of nitrite and nitrate were rapidly changed in time, while the amount of the thiocyanate remained constant. The decrease of the amount of the nitrate was faster, because the different bacteria of the oral cavity reduce the nitrate to nitrite. Ninety minutes after sampling saliva all amount of nitrate and nitrite were disappeared from the sample, while the amount of the thiocyanate was quite constant. The changes of the amount of the three components in saliva (stored in room temperature) were shown in Fig. 3.

Generally the task of the analyst is the determination of the components present in the sample just in the time of sampling. Since the concentration of the studied analyte in saliva

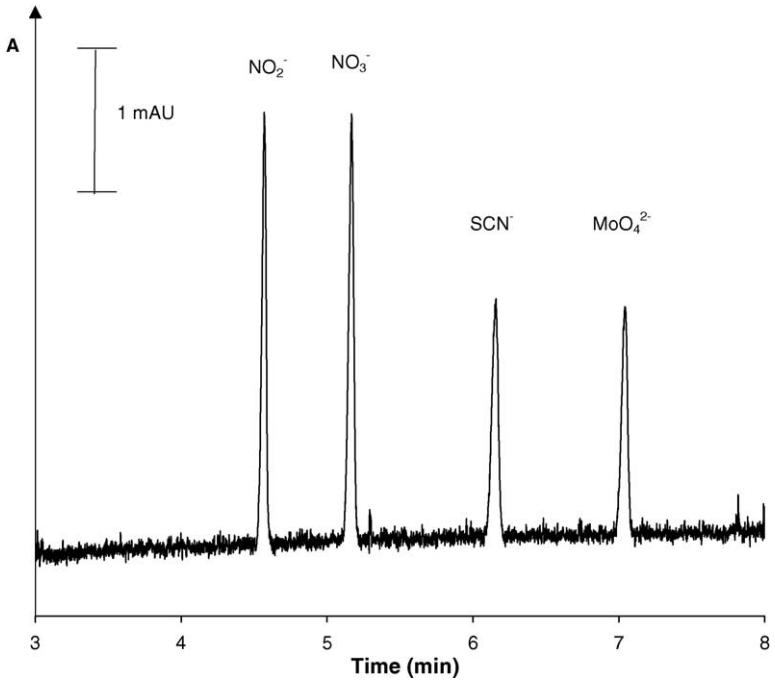

Fig. 2. Electropherogram of a mixture containing nitrite, nitrate, thiocyanate and molybdenate [electrolyte: 25 mM phosphate, pH: 6.8, nitrite: 10 μg/mL, nitrate: 10 μg/mL, thiocyanate: 10 μg/mL, molybdenate (internal standard): 20 μg/mL].

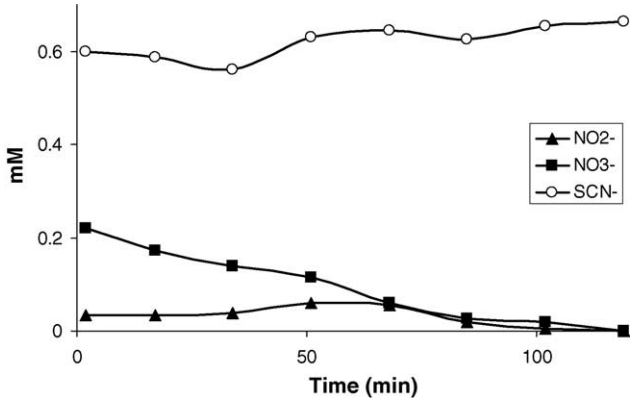

Fig. 3. The changes of the amount of the nitrite, nitrate and thiocyanate content of saliva stored at 25 °C.

changes in time, different content of nitrite and nitrate can be detected depending on the duration from the sampling. Therefore, the transformation of the analytes due to the bacteria should be stopped just at the sampling. This aim can be achieved by reduction of the temperature or increasing the pH of the sample above 11. The stability of nitrite/nitrate a saliva sample was studied in different temperatures: 25, 4 and −20 °C. It was found that both the decrease of the temperature to 4 °C and adjust of the pH of the sample to 11 were adequate to make constant the nitrite/nitrate ratio even up to 7 days (Fig. 4). Similar stability of nitrite/nitrate ratio was found in samples that were frozen at −20 °C. The ratio of the studied analytes in saliva samples, which were frozen at −20 °C can be preserved at least 2 months.

The saliva samples obtained from the different places inside the oral cavity will contain nitrite and nitrate in largely different ratio. In Fig. 5, the electropherograms of saliva samples collected from the resting mixed whole saliva, from the submandibular and sublingual glands and from the parotid gland are shown. In whole saliva samples the salivary nitrate is converted to nitrite by bacteria on the posterior surface of the tongue (*Veillonella* spp., *Staphylococcus aureus* and *Staphylococcus epidermidis*, etc. [17]. However, in case of

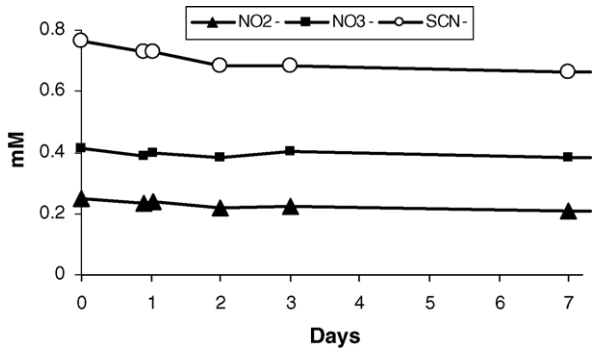

Fig. 4. Monitoring the changes of the amount of the nitrite, nitrate and thiocyanate content of saliva spiked with NaOH and stored at 4 °C up to 7 days. (Saliva sample was spiked with 10–10 μg/mL nitrite, nitrate and thiocyanate and 20 μg/mL molybdenate).

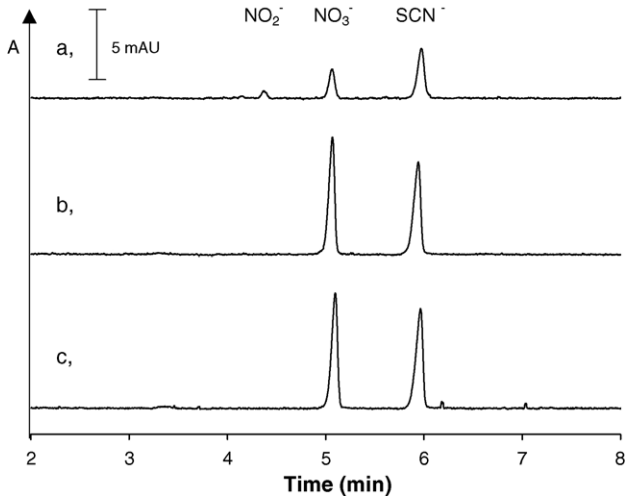

Fig. 5. Electropherograms of saliva samples collected from the resting mixed whole saliva (a), from the submandibular and sublingual glands (b) and from the parotid gland (c) (samples were spiked with NaOH and cooled after the sampling).

saliva samples, which are collected directly from the saliva ducts (Stenson's duct and Wharton's duct), nitrate (but not nitrite) can be present, while whole saliva contains nitrite, too. These results agree with the works of others [17].

4. Conclusion and outlook

Capillary electrophoresis enables direct, qualitative and quantitative determination of nitrate and nitrite in saliva samples. Direct UV detection at 214 nm was suitable for measurement of nitrate and nitrite in a concentration range of 1–100 μg/mL. The simplicity, rapidity, relative cheapness of the analytical procedure give outstanding advantages for CE compared to other techniques. It was found that decreasing the temperature to 4 °C and adjusting the pH of the sample to 11 were adequate to make constant the nitrite/nitrate ratio in saliva samples even up to 7 days.

Acknowledgement

Our work was supported by the Hungarian Scientific Research Fund (F040468).

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