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Ion chromatography of azide in pharmaceutical protein samples with high chloride concentration using suppressed conductivity detection

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Abstract

Methods based on reversed-phase liquid chromatography with UV detection of 4-nitrobenzoyl- or 3,5-dinitrobenzoyl azide derivatives lack in accuracy and stability of derivatives to be applied for azide determination in pharmaceutical protein samples with high sodium chloride concentrations. This paper describes a sensitive and selective ion chromatographic method, with simple sample preparation and suppressed conductivity detection, developed for trace determination of azide in protein samples containing sodium chloride in concentrations as high as 11.6 g L^{-1}. Anion exchange stationary phase with quaternary alkyl amine functional groups and gradient elution with sodium hydroxide enabled good resolution of anions with similar retention times: azide, bromide and nitrate, as well as chloride whose retention time was shorter than azide's. Anions with high affinity to stationary phase (phosphate and citrate) were also eluted within acceptable analysis time of 32 min. The stability of sample solutions and the method selectivity, accuracy, precision and sensitivity satisfied the validation criteria of international organizations competent for pharmaceutical industry. The detection and quantitation limit ranges of sodium azide in protein samples were 0.007–0.02 mg L^{-1} and 0.02–0.06 mg L^{-1}, respectively. Both limits increased with the concentration of sodium chloride.

Keywords: Ion chromatography of azide; Suppressed conductivity detection; Pharmaceutical protein samples; Nitrobenzoyl azide derivatives

1. Introduction

Sodium azide is widely used as a preservative in diagnostic reagents, protein samples (for example blood and tissues) and in buffers for long-term storing of immunoaffinity columns. Because of its fungicidal, antibacterial and nematodical properties it is also used in the production of pesticides and herbicides [1–3]. Over the last 20 years automobile industry has dominated sodium azide consumption, where it is used as a propellant for air bags [4,5]. Azide is the precursor of medicines such as antihypertensives, anti-HIV drugs, and some antibiotics [6,7].

On the other hand, sodium azide is a highly toxic compound classified as a first-class poison whose lethal dose for oral ingestion in human (LD_{50}) is less than 50 mg kg^{-1} [8,9]. The mechanism of azide toxicity is akin to cyanide: it obstructs oxidative phosphorylation by inhibiting cytochrome *c* oxidase [10–13].

One of the earliest methods for determining azide was colorimetry based on the detection of red ferric azide complex in the analysis of primer mixtures [14,15]. Azide determination in forensic samples using volumetry was based on iodine reaction with hydrazoic acid and measurement of liberated nitrogen [16]. Another method was spectrophotometry which involved determination of Ce^{4+} reagent excess after azide oxidation [17]. All these methods lacked specificity and selectivity, and could produce false-positive results.

Oxidation with Ce^{4+} was also used for gas chromatographic azide determination with thermal conductivity detection, but detector response was quite insensitive [18]. Azide determination in blood, urine and beverages using gas chromatography–mass spectrometry after its derivatization with pentafluorobenzyl bromide using tetradecyldimethylbenzylammonium chloride as the phase-transfer catalyst, provided low detection and quantitation limits [19,20]. The disadvantages of

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these methods were complex sample preparation and negative interference caused by the presence of other anions in the sample. A biosensor based on catalase inactivation by azide was used for its determination in fruit juices, but in a fairly high concentration range [21].

The majority of methods developed for azide determination are based on reversed-phase liquid chromatography (HPLC) or ion chromatography [22–25]. For HPLC determination on C_{18} stationary phase, azide was derivatized with a reagent containing UV chromophore like 3,5-dinitrobenzoyl chloride [22,23] or nitrobenzoyl chloride [24]. 3,5-Dinitrobenzoyl chloride was also used as a derivatization reagent for azide determination by capillary electrophoresis [26]. These methods were used for azide determination in gaseous, liquid, and solid industrial samples [22], tissues and body liquids [24] and wine [23]. However, the methods involving derivatization have drawbacks such as time-consuming sample preparation, derivative stability shorter than 10 h, and interferences caused by some nucleophiles.

An interesting approach to eliminate some of these problems was the analysis of sodium azide in tap water [25] and plasma samples [27] by ion-interaction reversed-phase liquid chromatography using a C_{18} stationary phase and *n*-octylammonium *ortho*-phosphate as the interaction reagent. In these methods, the only sample preparation step was dilution, and the samples were stable for a longer time.

In the first ion chromatographic method developed for azide determination in water-soluble protein samples, the quaternary amine was used as the stationary phase and tetraborate solution as the mobile phase [28]. Azide was detected by measuring mobile phase conductivity. With tetraborate and carbonate mobile phases it was not possible to apply gradient elution, which restricted the number of anions that could be separated with acceptable retention times and resolutions. Therefore, methods for azide determination in blood and beverages were modified by lowering the sample pH and azide extraction as gaseous hydrazoic acid [29,30]. This modification enhanced the method selectivity but the sample preparation became more complex and the method less accurate and precise.

The use of hydroxide mobile phase for isocratic elution and suppressed conductivity detection provided efficient separation of azide ions from chloride, sulphate and citrate ions in diluted human plasma [31].

Besides conductometric detection, nitrogen chemiluminescence [32] and UV detection [33] were also applied in ion chromatography of azide.

The aim of this study was to develop a chromatographic method for azide determination in pharmaceutical protein samples with high sodium chloride concentration. Special requirements were simple sample preparation, small sample consumption, the lowest possible quantitation limit, and stability of standard calibration and sample solutions for longer than 12 h. In our study, azide was a component of conservation buffer for immunoaffinity column used in the production of a pharmaceutically active protein for its separation from reaction mixture. Since produced protein should become a pharmaceutical product, it was necessary to prove that it had not been contaminated with azide during production. In addition to the protein and azide the samples also contained chloride, bromide, nitrate, citrate, and phosphate. Chloride was in much higher concentration than other anions. The method was to meet the validation criteria for the application in the pharmaceutical industry. None of the methods reported in literature completely meets these requirements.

This paper describes a new ion chromatographic method developed for tracing the determination of azide in pharmaceutical protein samples. Acceptable selectivity and sensitivity was achieved by gradient elution with hydroxide mobile phase and suppressed conductivity detection. In addition, the experiments were performed to investigate the influence of high chloride concentrations on the determination of azide as a 4-nitrobenzoyl or 3,5-dinitrobenzoyl derivative by reversed-phase HPLC with UV detection.

2. Experimental

2.1. Chemicals and reagents

Sodium hydroxide, sodium chloride, concentrated hydrochloric acid, sodium hydrogenphosphate, phosphoric acid, potassium nitrate and sodium sulphate, all of analytical grade purity, were products of Kemika (Zagreb, Croatia). Sodium azide, ultra pure, and bromthymol blue indicator were purchased from Sigma (Steinheim, Germany) and potassium bromide of analytical grade, 3,5-dinitrobenzoyl chloride and acetonitrile of HPLC grade from Merck (Darmstadt, Germany). 4-Nitrobenzoyl chloride was purchased from Aldrich (Steinheim, Germany). Water was purified with the Milli-Q purification system Millipore (Molsheim, France). All solutions were prepared in purified Milli-Q water.

2.2. Chromatographic system

A modular liquid chromatographic system LC-10 (Shimadzu, Kyoto, Japan) consisted of in-line degasser DGU-14A, two binary pumps LC-10ADvp, autosampler SIL-10A, column oven CTO-10Avp, UV detector SPD-10AVvp, conductivity detector CDD-6A and control unit SCL-10Avp. Electrochemical suppression was achieved by using an anion self-regenerating suppressor ASRS-Ultra-II, 4 mm (Dionex, Sunnyvale, USA). Chromatographic signals were integrated using Class VP-5 software (Shimadzu, Kyoto, Japan).

2.3. HPLC conditions

A 100 mm × 4.6 mm i.d. ChromSep HPLC SS column with C_{18} functional groups, particle size 3 μm (Varian, Palo Alto, USA), was used to separate azide 4-nitrobenzoyl or 3,5-dinitrobenzoyl derivatives from other sample ingredients. Column temperature was 28 °C, injection volume 50 μL, and mobile phase flow rate 1 mL min^{-1}. The mobile phase consisted of phosphate buffer and acetonitrile in equal proportions. Phosphate buffer was prepared by dissolving of 1.42 g of sodium hydrogenphosphate in 1000 mL of ultra pure water. The buffer pH was adjusted to 6.8 ± 0.1 with 6% phosphoric acid. Elution

was isocratic and time of analysis was 6 min. Detection was performed by monitoring the absorbance at 254 nm.

2.3.1. Azide standard solutions

Azide stock solution for testing the linearity of detector response was prepared by dissolving 77.15 mg of sodium azide in 100 mL of ultra pure water, $\gamma(N_3^-) = 500\ mg\ L^{-1}$. The stock solution was further diluted to prepare aqueous standard solutions of azide ion mass concentrations ranging from 10 mg L^{-1} to 100 mg L^{-1}.

Azide stock solution for testing the method accuracy and the stability of azide nitrobenzoyl derivatives was prepared by dissolving 77.15 mg of sodium azide in 100 mL of sodium chloride solution, $\gamma(NaCl) = 11.6\ g\ L^{-1}$. Standard solutions of azide ion mass concentrations of 10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, and 100 mg L^{-1} were prepared by further dilution of stock solution with the solution of sodium chloride.

2.3.2. Derivatization procedures

Derivatization of azide with 3,5-dinitrobenzoyl chloride was performed according to the procedure described by Swarin and Waldo [22]. For azide derivatization with 4-nitrobenzoyl chloride, 0.2 mL of acetonitrile was added to a mixture of 0.5 mL of azide solution, 5 μL of hydrochloric acid diluted at ratio 1:10, and 5 μL of 100 mg L^{-1} acetonitrilic 4-nitrobenzoyl chloride solution. Reaction mixture was manually shaken, left at room temperature for 10 min and then stored at 4 °C until analysis.

2.4. Ion chromatographic conditions

Azide ion was separated from other anions using a 250 mm × 4 mm i.d. IonPac AS11 column with quaternary alkyl amine functional groups attached to 13 μm particles. Analytical column was coupled with a 50 mm × 4 mm i.d. IonPac AG11 guard column (Dionex, Sunnyvale, USA). Column temperature was 32 °C, injection volume 50 μL and the mobile phase flow rate 1 mL min^{-1}. Ultra pure water and 15 mmol L^{-1} sodium hydroxide solution were used for gradient elution. The water–sodium hydroxide mobile phase gradient began with an initial 1.2 mmol L^{-1} sodium hydroxide concentration held for 14 min, then the sodium hydroxide concentration was increased to 15 mmol L^{-1} over 1 min and held for 7 min, and then decreased to the initial value of 1.2 mmol L^{-1} over 1 min.

Fresh sodium hydroxide solution should be prepared on a daily basis because carbon dioxide is absorbed from the ambient air, which makes retention times irreproducible and causes baseline drift. After passing the analytical column, mobile phase conductivity was suppressed in self-regenerating electrochemical suppressor using 100 mA current. Anions were detected by monitoring the mobile phase conductivity with detector gain of 0.1 μS cm^{-1}.

2.4.1. Azide standard solutions

A sodium azide stock solution of 100.0 mg L^{-1} was prepared in ultra pure water and further diluted as experiments required. Standard solutions of sodium azide concentrations ranging from 0.02 mg L^{-1} to 0.80 mg L^{-1} were used to calibrate and test method linearity. Solutions for testing method selectivity, precision, quantitation limit, accuracy, and other validation parameters were prepared by spiking the analysed samples with an adequate volume of a 4.00 mg L^{-1} sodium azide solution.

2.5. Samples

Mass concentration of sodium azide was determined in:

(1) Eluates from the immunoaffinity column collected while the column was flushed from conservation buffer. When it was not used, the immunoaffinity column was filled with conservation buffer A (Table 1) containing sodium azide. Prior to use the column was rinsed with buffer B (Table 1) that contained all components as buffer A, except sodium azide.
(2) Protein fractions eluted from the immunoaffinity column. After rinsing and equilibration, the reaction mixture (fermentation broth) consisting of cell fragments that produced the active protein, supplements, enzymes necessary for cell fragmentation, and active protein was loaded on the immunoaffinity column. The active protein was retained on the column while other ingredients were flushed from the column with buffers B and C (Table 1). Thereafter, the active protein was eluted from the column with buffer D (Table 1).
(3) Final protein product after all purification steps that followed immunoaffinity chromatography: microfiltration, nanofiltration, desalinization, ultrafiltration, anion-exchange chromatography, and stabilization. In the final product, the active protein was dissolved in buffer E (Table 1).

Table 1
Composition and pH value of the buffers used for conservation (buffer A) and equilibration (buffer B) of immunoaffinity column, for separation of active protein from fermentation broth (buffers C and D), and for dissolving the final protein product (buffer E)

Buffer	γ (g L^{-1})						pH
	NaH_2PO_4	Sodium citrate	NaCl	Tween® 20[a]	NaN_3	Tris–HCl[b]	
A	2.4	–	11.6	0.2	1.0		7.2
B	2.4	–	11.6	0.2	–		7.2
C	2.4	–	47.2	0.2	–		7.2
D	–	0.0214	11.6	0.2	–		2.2
E		0.0214	8.8	0.2	–	1.6	7.2

[a] Polyoxyethylene(20)sorbitan monolaurate.
[b] 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

Table 2
Dilution factors, linearity range and detection and quantitation limits for azide determination by ion chromatography in samples of different sodium chloride mass concentrations

γ(NaCl) ($g\,L^{-1}$)	Dilution factor	$\gamma(NaN_3)$ ($mg\,L^{-1}$)		
		Linearity range	Detection limit	Quantitation limit
<3.9	1	0.02–0.80	0.007	0.02
3.9–7.8	2	0.04–1.60	0.014	0.04
7.8–11.6	3	0.06–2.40	0.020	0.06

Eluates from the immunoaffinity column described under (1) could contain up to $1.0\,mg\,L^{-1}$ of sodium azide. Possible ingredients of all samples were bromide and nitrate: bromide was an impurity contained in sodium chloride in mass fraction of 0.005%, and nitrate was the product of protein degradation. Protein samples described under (2) and (3) contained from $0.15\,g\,L^{-1}$ to $10\,g\,L^{-1}$ of active protein. All samples were filtered through cellulose filters with 0.2 μm pores (IC Acrodisc, Gelman Laboratory, USA) and diluted with ultra pure water, depending on sodium chloride concentration as shown in Table 2.

3. Results and discussion

3.1. HPLC analysis of azide nitrobenzoyl derivatives

Based on the published data [22–24] the HPLC analysis of azide in (protein) samples with high chloride mass concentration was preceded by azide derivatization with either 4-nitrobenzoyl chloride or 3,5-dinitrobenzoyl chloride. The linearity of UV detector response for 4-nitrobenzoyl azide ($y = 1.49 \times 10^5 x + 1.36 \times 10^6$; $N = 11$; slope and intercept standard deviations 5.47×10^3 and 2.84×10^5; standard error 5.70×10^5) and 3,5-dinitrobenzoyl azide ($y = 2.26 \times 10^5 x + 5.09 \times 10^5$; $N = 12$; slope and intercept standard deviations 6.32×10^3 and 2.07×10^5, standard error 3.61×10^5) was tested at azide ion mass concentrations in standard solutions ranging from $10\,mg\,L^{-1}$ to $100\,mg\,L^{-1}$ and from $10\,mg\,L^{-1}$ to $50\,mg\,L^{-1}$, respectively (Table 3).

The accuracy of azide determination after conversion to 4-nitrobenzoyl- or 3,5-dinitrobenzoyl azide was tested by derivatizing standards containing 10–100 $mg\,L^{-1}$ of azide ion and $11.6\,g\,L^{-1}$ of sodium chloride, which was the highest expected sodium chloride mass concentration in samples to be analysed. Other possible sample components were omitted on purpose in order to isolate the influence of chloride ion on azide determination. The detector response for azide derivative in these samples was compared to the response in standards not containing chloride. The chloride ion high concentration significantly influenced the accuracy of azide determination by lowering the efficiency of azide derivatization by both derivatization agents. The average accuracy of azide determination after derivatization with 4-nitrobenzoyl chloride and 3,5-dinitrobenzoyl chloride was (65 ± 8)% and (59 ± 5)%, respectively (Table 3).

The stability of azide nitrobenzoyl derivatives in solutions of high chloride concentrations was tested by subsequent analyses of derivatized standards containing 5–50 $mg\,L^{-1}$ of azide and $11.6\,g\,L^{-1}$ of sodium chloride. The samples were stored in autosampler at room temperature for 6 h. The highest chromatographic peak area of 3,5-dinitrobenzoyl azide was recorded 30 min after sample derivatization. However, only 360 min after the sample preparation the peak area decreased to the 83% of its highest value. The highest chromatographic peak area of 4-nitrobenzoyl azide was recorded 20 min after sample derivatization, and it decreased only 1.5% in the next 2 h.

Table 3
Acceptance criteria for method application in pharmaceutical industry and the results of method validation for azide determination in pharmaceutical protein samples by ion chromatography; the results of azide determination by HPLC as azide nitrobenzoyl derivatives are presented for comparison

Validation parameter	Acceptance criteria	Validation results		
		Ion chromatography	HPLC	
			4-Nitrobenzoyl azide	3,5-Dinitrobenzoyl azide
Selectivity	$R_S \geq 1.0$	$R_S \geq 1.0$	$R_S \geq 1.0$	$R_S \geq 1.0$
Linearity	$r \geq 0.98$ $R.S.D._{RF} \leq 20.0\%$	$r = 0.999$ $R.S.D._{RF} = 15.3\%$	$r = 0.994$ $R.S.D._{RF} = 14.7\%$	$r = 0.996$ $R.S.D._{RF} = 6.9\%$
Precision	R.S.D. ≤ 10.0%	$R.S.D._{measurement} = 1.8\%$ $R.S.D._{preparation} = 5.7\%$		
Recovery (%)	80–120	86–106	53–71	55–63
Detection limit	S/N ≥ 3	$\gamma(NaN_3)_{DL} = 0.007$–$0.02\,mg\,L^{-1}$		
Quantitation limit	S/N ≥ 10 R.S.D. ≤ 10.0%	$\gamma(NaN_3)_{QL} = 0.02$–$0.06\,mg\,L^{-1}$ R.S.D. = 1.4%		
Stability of calibration solutions	>12 h at room temperature, R.S.D. ≤ 10.0%	294.5 h R.S.D. = 3.0%		
Sample stability		45.5 h R.S.D. = 2.5%	<6 h	<6 h

RF: response factor (in HPLC RF = $\gamma(N_3^-)$/azide ion peak area; in IC RF = $\gamma(NaN_3)$/azide ion peak area); QL: quantitation limit; DL: detection limit.

Twenty-four-hour testing of 4-nitrobenzoyl azide stability in samples stored at 8 °C showed an average decrease of 22% of the initial chromatographic peak area, i.e. of the azide derivative mass concentration.

Considering the accuracy of HPLC analysis and the stability of azide nitrobenzoyl derivatives, this analytical approach was not appropriate for azide determination in pharmaceutical protein samples with high chloride concentrations. The results of the experiments with 4-nitrobenzoyl chloride and 3,5-dinitrobenzoyl chloride as derivatizing agents were the same: the derivatives were stable for too short a time for routine analysis of the usually long sequences of samples in pharmaceutical laboratories, and the accuracy of azide determination was too low for the application in pharmaceutical industry.

3.2. Ion chromatographic determination of azide

The main problem to be solved by optimising ion chromatographic conditions for trace analysis of azide in protein samples with high chloride concentrations is to achieve good separation and well-resolved chromatographic peaks of these two anions contained in samples in extremely different concentrations. Due to the similarity of chemical and physical properties between sodium chloride and sodium azide, all attempts to reduce the concentration or to eliminate chloride from the sample before analysis also affected the concentration of azide. For example, it is not possible to purify the samples by passing them through filters impregnated with silver salt because azide, which is a pseudohalide, as well as chloride, produces insoluble salt with silver ion.

Azide was efficiently separated from other anions contained in the protein samples on an ion chromatographic column with quaternary alkilamine functional groups enabling the elution of anions with sodium hydroxide concentration gradient. Fig. 1 shows a chromatogram of the mixture of anions dissolved in ultra pure water and the changes in mobile phase sodium hydroxide concentration over time. Chloride, as an ion with low affinity to stationary phase, was eluted at the initial sodium hydroxide concentration (1.2 mmol L^{-1}), and its retention time was eight minutes shorter than that of azide. Increase in sodium hydroxide concentration enabled an efficient, down-to-the-baseline separation of azide, bromide, and nitrate peaks, as well as the elution of anions with higher affinity to stationary phase within an acceptable time of analysis. Strongly retained phosphate and citrate were eluted from the column between the 21st minute and 23rd minute. However, after nitrate was eluted from the column, the baseline was disturbed due to the change in mobile phase composition resulting in a large signal overlapping the phosphate and citrate peaks. The change in the mobile phase composition affected the conductivity detector response four minutes after increasing the sodium hydroxide concentration. After the 23rd minute, hydroxide concentration was reduced to the initial value to equilibrate the analytical column for new analysis. The baseline signal fell to 0 at about the 30th minute. The baseline drift could be minimised with the use of an eluent generator and carbonate removal device produced by Dionex, which recently became a standard ion chromatographic approach with hydroxide eluents.

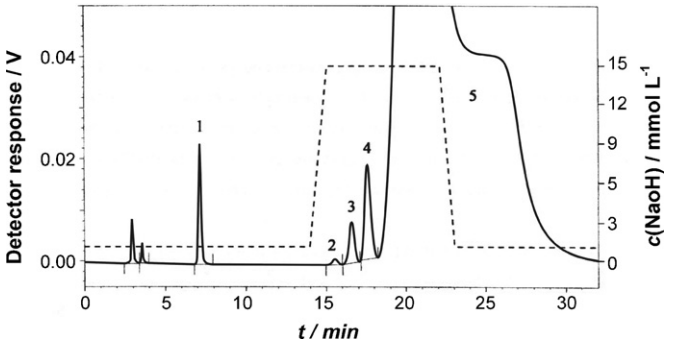

Fig. 1. Chromatogram of anions dissolved in water: (1) chloride, $\gamma(Cl^-) = 0.5\,mg\,L^{-1}$; (2) azide, $\gamma(NaN_3) = 0.08\,mg\,L^{-1}$; (3) bromide, $\gamma(KBr) = 0.5\,mg\,L^{-1}$; (4) nitrate, $\gamma(KNO_3) = 1.0\,mg\,L^{-1}$; (5) baseline disturbance, caused by gradient elution and carbon dioxide absorption from air, overlapping the peaks of strongly retained phosphate and citrate. Dotted line represents sodium hydroxide concentration in time. Chromatographic conditions are described in Section 2.4.

The linearity of the conductivity detector response was tested by analysing sodium azide standard solutions at five mass concentrations ranging from 0.02 mg L^{-1} to 0.80 mg L^{-1}. Each solution was prepared in triplicate and injected twice. The relationship between the azide ion peak area and sodium azide mass concentration in standard solutions was linear, with the correlation coefficient 0.999 ($y = 1.99 \times 10^{-1}x + 5.69 \times 10^{-4}$; slope and intercept standard deviations 1.66×10^{-3} and 6.76×10^{-4}, respectively; standard error 2.58×10^{-3}; number of data points 30). Relative standard deviation of the response factor, calculated by dividing sodium azide mass concentration with azide ion peak area, was 15.3%.

Good separation of azide from other anions in protein samples is illustrated by chromatograms of three protein samples compared in Fig. 2. Two samples containing 11.6 g L^{-1} of sodium chloride were eluted from the immunoaffinity column and spiked with 0.133 mg L^{-1} (Fig. 2A) and 0.060 mg L^{-1} (Fig. 2B) of sodium azide. The latter concentration corresponded to sodium azide quantitation limit in protein samples of chloride mass concentration higher than 7.8 g L^{-1}. Sodium chloride mass concentration in this sample was more than 190,000 times higher than the mass concentration of azide, but their peaks were still well resolved. The third sample was the final protein product containing 8.8 g L^{-1} of sodium chloride and no traces of azide ion (Fig. 2C).

Trace analysis of azide in protein samples required optimization of the sample injection volume and of detector gain to achieve detection and quantitation limits as low as possible. After a series of experiments, the injection volume was set to 50 μL, which ensured reasonable sample consumption and good peak symmetry. With detector gain of 0.1 μS cm^{-1}, the quantitation limit, based on a signal-to-noise ratio of 10, depended on chloride mass concentration in protein sample, and ranged from 0.02 mg L^{-1} to 0.06 mg L^{-1} of sodium azide (Table 2). Namely, if chloride mass concentration was too high, its large chromatographic peak overlaid the peak of azide. To avoid peak overlapping, protein samples have to be adequately

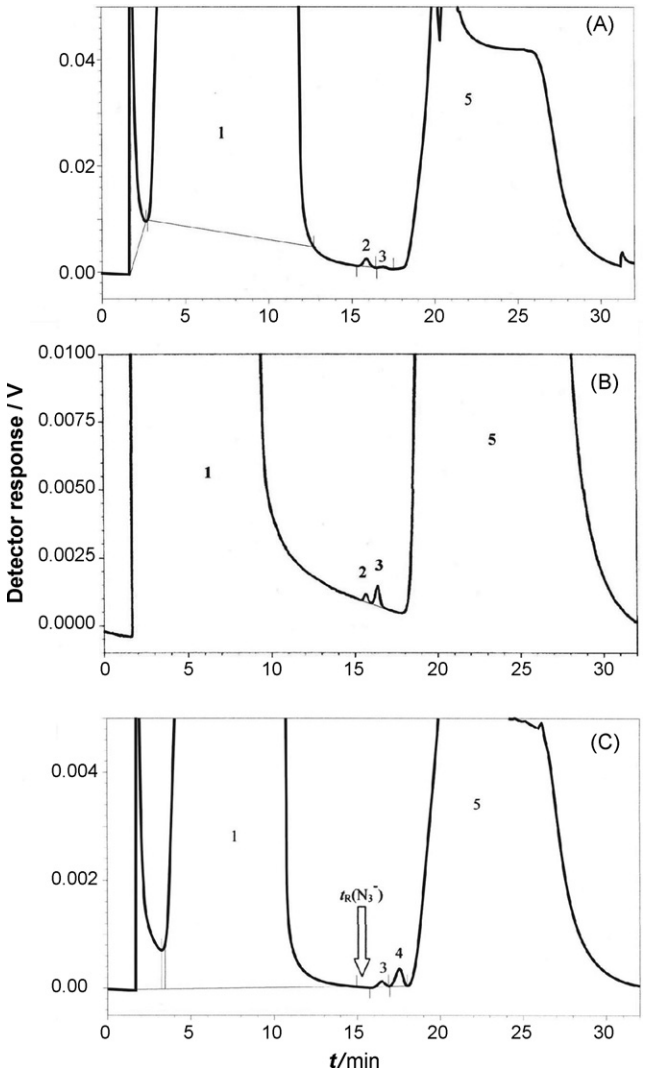

Fig. 2. Chromatograms of (A) protein sample eluted from immunoaffinity column containing sodium chloride in concentration of 11.6 g L^{-1} and spiked with sodium azide in concentration $\gamma(NaN_3) = 0.133$ mg L^{-1}; (B) protein sample eluted from immunoaffinity column and spiked with sodium azide in concentration equal to concentration limit, $\gamma(NaN_3) = 0.06$ mg L^{-1}, shown in enlarged scale; (C) final protein product with sodium chloride concentration of 8.8 g L^{-1}. Peaks: (1) chloride, (2) azide, (3) bromide, (4) nitrate and (5) baseline disturbance, caused by gradient elution and carbon dioxide absorption from air, overlapping the peaks of strongly retained phosphate and citrate. Chromatographic conditions are described in Section 2.4.

diluted with ultra pure water (Table 2). The measurement precision (relative standard deviation) at azide concentrations of 0.06 mg L^{-1} (quantitation limit) and 0.133 mg L^{-1} was 1.4% and 1.8%, respectively. The sample preparation repeatability (relative standard deviation of results obtained by the analysis of six sample preparations) at the concentration of 0.133 mg L^{-1} was 5.7%.

The mass concentration of sodium azide equal to its detection limit in protein samples was estimated from quantitation limits and the signal-to-noise ratio higher than 3 (Table 2). Sodium azide detection limit, ranging from 0.007 mg L^{-1} to 0.02 mg L^{-1}, was lower than the limits reported in literature for azide determination in protein samples soluble in water [26], blood samples [28,29], and in samples of human plasma [31].

The accuracy of azide ion chromatographic determination in protein samples using suppressed conductivity detection was estimated by recovery experiments. Nine protein samples containing 11.6 g L^{-1} of sodium chloride were spiked with sodium azide at three mass concentrations in the range of 0.08–0.80 mg L^{-1}. The average recovery of azide, calculated against the calibration standards, was (95 ± 8)%. This recovery did not depend on azide mass concentration and values above and below 100% were equally distributed, which suggests that there was no systematic error in measurement. The recovery of azide from a collagen sample at azide mass fraction of 10 μg g^{-1} was 97%, and from bovine serum albumin at mass fraction of 100 μg g^{-1} was 92% [28]. The recovery of azide from human plasma at mass concentration of 1 mg L^{-1} was 117% [31]. Azide recoveries from beverages increased with mass concentration [30]. At sodium azide concentration of 0.1 mg L^{-1} the recovery was 54%, while at concentration of 10.0 mg L^{-1} azide recovery increased to 93.3%.

The robustness of the developed method was evaluated by varying the chromatographic column temperature, flow rate and mobile phase sodium hydroxide concentration for ±5% of optimum values defined in Section 2.4. The monitored parameters were azide retention time and the resolution of bromide and azide chromatographic peaks. The comparison between the resolutions of chloride and azide peaks was not suitable because their difference in width is too great. The efficiency of bromide and azide separation was only influenced by the increase in sodium hydroxide concentration. At higher concentrations, the resolution of azide and bromide peak was lower than 1.4.

As the method was intended for the analysis of long sequences of samples, it was necessary to test the stability of calibration and sample solutions. Both tested solutions were stored in sealed vials at room temperature. Calibration solutions were stable for 295 h. Over that period calibration solutions were analysed 12 times, and the relative standard deviation of the azide peak area was only 3.0%. In the last measurement, the azide peak area decreased to 95.6% of its initial value. The stability of azide in protein samples was tested over 45 h: its peak area decreased only 1.9% and the peak area relative standard deviation was 2.5%.

One of the steps in the preparation of protein samples for ion chromatographic analysis was filtration through filters with 0.22 μm pores. The potential impact of filtration on sodium azide concentration in protein samples was assessed by analysing six protein samples spiked with sodium azide. Three of these samples were filtered while the other three were centrifuged prior to injection. The results of azide analysis in the two groups of samples, compared by uncorrelated *t*-test, were not significantly different, which suggests that sample filtration does not affect sodium azide concentration in the sample.

The method was applied to determine azide in fractions eluted from the immunoaffinity column used to separate active protein from the reaction mixture, while it was washed out from conservation buffer. Sodium azide was one of the conservation buffer constituents. It was necessary to prove that azide was com-

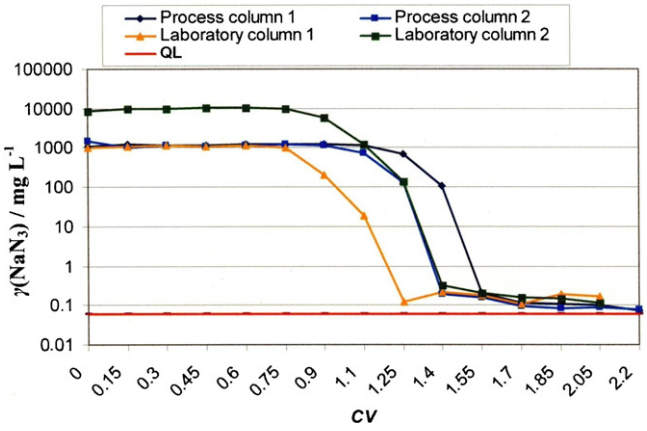

Fig. 3. Dependence of sodium azide mass concentration in eluate from immunoaffinity column on volume of buffer used for washing out the conservation buffer (CV: column volume). Volume of laboratory column was 6 mL, while volume of process column was 300 mL.

pletely washed out from the column to avoid any contamination of the active protein. In our experiment two 6 mL laboratory and two 300 mL process immunoaffinity columns were filled with conservation buffer containing 1000 mg L^{-1} of sodium azide. Columns were washed out with buffer for conditioning and equilibration, and sodium azide concentration was measured in collected fractions. After rinsing the column with a volume of conditioning buffer corresponding to about 2.2 column volumes, sodium chloride concentration in the collected fractions decreased to values below the quantitation limit (Fig. 3). The immunoaffinity column used in the protein production process has to be washed out with almost a hundred column volumes of conditioning buffer for a complete elimination of azide.

Ion chromatographic analysis of purified protein solution eluted from the immunoaffinity column and of the final protein product after all purification steps (Fig. 2C) confirmed that protein samples were not contaminated with azide; it was not detected in any of these samples.

4. Conclusions

Ion chromatography with suppressed conductivity detection and gradient sodium hydroxide elution proved suitable for an efficient separation of azide and chloride contained in the sample in extremely different concentrations, as well as of bromide, nitrate, citrate and phosphate within an acceptable time of analysis. High selectivity of ion chromatographic analysis contributed significantly to the sensitivity of azide determination with detection and quantitation limits at the level of microgram per litre. The most important factor influencing azide detection and quantitation limits was sodium chloride mass concentration in analysed samples. However, the peaks of these two anions were well resolved, even in chromatograms of samples containing sodium azide at the limit of quantitation, i.e. in concentration more than 190,000 times lower than the maximum concentration of sodium chloride. Simple sample preparation contributed to high precision and accuracy of the method, even at azide trace concentrations. As shown in Table 3, long-term sample stability and method selectivity, linearity, accuracy, precision, and sensitivity meet the criteria for its application in pharmaceutical industry. For the moment, the method has proved itself as suitable for trace analysis of azide in raw and final pharmaceutical protein products, as well as in eluates of immunoaffinity columns or ultrafiltration filters which were conserved with sodium azide.

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