Journal of Chromatography, 546 (1991) 325–334
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2111

Azide determination in protein samples by ion chromatography

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ABSTRACT

Sodium azide is used as an anti-fungal agent in protein samples in the health related scientific community. Due to its toxic nature, monitoring of the azide level in proteins used in scientific research is necessary. Ion-exchange chromatography has been used to quantitate azide levels in protein samples. Anion-exchange methodology is described which allows for the separation of azide from various common anions found in analytical grade protein sample matrices. The analytical system described utilizes a polymer [poly(styrene–divinylbenzene)] stationary phase which has been surface sulfonated followed by the binding of the aminated latex bead active ion-exchange sites (Dionex, AS4A column). Sodium tetraborate is used as the weak anion-exchange mobile phase. A vendor ion-exchange column comparison is made along with eluent composition and selection studies. Method validation data are presented including: calibration plots for external standardization, limit of detection and method recovery. Various types of proteins are assayed using the described method.

INTRODUCTION

Azide is typically used as an anti-fungal agent in protein samples prepared for scientific research. Due to the toxic nature of azide, its concentration must be accurately monitored in protein samples used in pharmaceutical research. Numerous methods for determining azide in various sample matrices can be found in the literature. Methods include: electrochemical [1], spectrometric [2–5], gas chromatography [6], volumetric [7], liquid chromatography [8] and ion-exchange chromatography [9,10]. Of these techniques, only reversed-phase liquid chromatography and ion-exchange chromatography offer a straight-forward, interference-free approach to quantitating azide in complex matrices such as proteins.

Having a pK_a of 4.8, azide is an excellent candidate for anion exchange with mobile phases having relatively high pH values. The net charge on the azide molecule would be -1 under typical alkaline mobile phase conditions. The primary challenge in developing an ion-exchange method for quantitating azide in proteins would be to completely resolve azide from the similarly sized monovalent common anions (nitrite, bromide and nitrate).

It has been shown that azide can be separated and detected using either single-column or dual-column ion chromatography. By single column, azide has been quan-

0021-9673/91/$03.50

titated on a Wescan anion-exchange column (269-0310) using a nicotinic acid eluent combined with non-suppressed conductivity detection. Azide has also been quantitated using chemically suppressed conductivity detection on a Dionex anion-exchange column with a carbonate–bicarbonate eluent.

The above ion-exchange methods are generally adequate but, they did not completely meet the needs of our laboratory. Using the described carbonate system as a starting point, the authors attempted to quantify low levels of azide in various protein matrices. Incomplete resolution of azide from the common anions (bromide and nitrate) was found. This paper presents an alternative ion-exchange eluent system combined with suppressed conductivity detection which was found to be capable of quantifying azide in various protein samples.

EXPERIMENTAL

Reagents

Sodium tetraborate was obtained from Fluka. Collagen was obtained from Sigma and the bovine serum albumin (BSA) was obtained from both Pierce and Sigma. Sulfuric acid was obtained from Baker. Sodium azide was obtained from Sigma. The sodium salts of the common anions (fluoride, chloride, bromate, bromide, nitrite, nitrate, phosphate and sulfate) were obtained from Fluka. The sodium salts of carbonate and bicarbonate were also obtained from Fluka. Both the metallo enzyme and the growth factor were acquired in-house. All reagents were analytical grade unless otherwise noted.

Stock solutions (100 ppm) of the common anions were prepared from their sodium salts in 18+ MΩ deionized water and were stored in Nalgene volumetric flasks. Working standards of the common anions were serially diluted from the prepared stock using freshly prepared mobile phase. Azide standards were similarly prepared from a 100 ppm azide stock solution and all resulting solutions were stored in Nalgene volumetric flasks. Eluents were prepared from their sodium salts and filtered through a 0.45-μm filter disk. All eluents were thoroughly degassed with helium prior to system start-up. Degassing was found to be especially important with the tetraborate eluents. Shifts in analyte retention times could be seen if eluents were not throughly degassed. This was attributed to carbon dioxide absorption into the tetraborate eluents. Adequate mobile phase degassing with helium eliminated the retention time shifts.

Instrumentation

Instrumentation used was a Dionex 4000i ion chromatograph with gradient pump capabilities and a Dionex conductivity detector. The following different anion-exchange columns were used to perfom a column comparison, Hamilton PRP-X100 [spherical, poly(styrene–divinylbenzene) trimethylammonium exchanger], Waters IC-PAK [poly(methacrylate) resin with a quaternary ammonium exchanger], and a Dionex AS4A [surface-sulfonated poly(styrene–divinylbenzene) with bonded aminated latex beads exchanger]. All azide detection was performed using chemically suppressed conductivity with the Dionex Anion Micromembrane Suppressor.

Sample preparation

The types of proteins assayed were either soluble or insoluble in the eluent system. BSA, zinc metallo enzyme and a growth factor were all soluble in the eluent. Therefore, sample preparation consisted of dissolving the protein then filtering the sample prior to injecting the sample. Collagen protein was not soluble in the eluent. Azide determination in collagen was accomplished by first performing a solid–liquid extraction of the azide from the parent protein. The extraction step consisted of weighing approximately 250 mg of protein into a centrifuge tube and adding 10 ml of eluent. After sealing the centrifuge tube was placed onto a wrist-action shaker for 15 min. The sample was then filtered and injected.

DISCUSSION

Early efforts to fully resolve azide from bromide and nitrate using the typical anion eluent (carbonate–bicarbonate) on a Dionex ion-exchange column (AS4A) were less than rewarding. In an effort to keep the eluent system simple, an isocratic chromatographic system was sought.

A tetraborate eluent system offers very weak elution strength (greater resolving power) while its background conductivity is also capable of being chemically suppressed under normal suppressor conditions. The weak elution strength of the tetraborate is more likely to separate the early eluting anions such as chloride, nitrite, bromide, azide and nitrate. The suppressor reactions for the tetraborate eluent can be seen in Fig. 1. The tetraborate anion is divalently charged in solution with a relatively high background conductance. Within the suppressor it is converted to the less conductive boric acid.

A comparison of columns from different manufacturers was made in an effort to identify the most appropriate column for the required separation. A standard solution was constructed with approximately 10 ppm of each of the following anions: fluoride, bromate, chloride, nitrite, bromide, azide, nitrate and phosphate. Under typical anion-exchange conditions bromide, azide and nitrate can coelute. The above described test solution was used to compare the following columns: Hamilton PRP-X100, Waters IC-PAK and Dionex AS4A. Column capacities for these columns range from 16–250 μequiv./g [11]. The wide variance of column type and capacity encompassed most of the ion-exchange column technology available.

In an effort to maintain a consistency in azide detection only anion-exchange eluents capable of being chemically suppressed were considered. The previously unsuccessful attempts to resolve bromide and nitrate from azide with dilute carbonate–bicarbonate eluents on the Dionex AS4A column led to the use of the previously

Eluent	Eluting Ion	Suppressor Reaction Product	Elution Strength
$Na_2B_4O_7$	$B_4O_7^{2-}$	H_3BO_3	Very Weak

Suppressor Reaction:

$$B_4O_7^{2-} + 8H^+ \rightarrow H_3BO_3 + 4H_2O$$

Fig. 1. Tetraborate eluent system and chemical suppressor reactions.

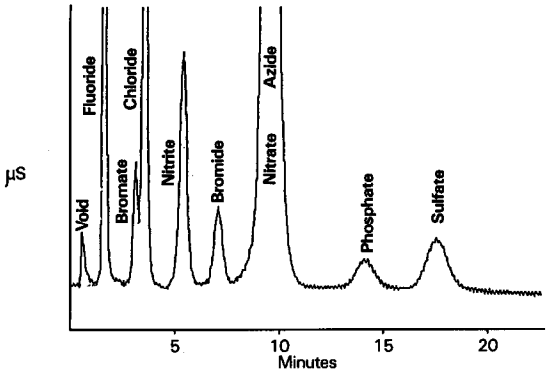

Fig. 2. Ten ppm common anions with azide. Chromatographic conditions: column, Waters IC-PAK A, polymethacrylate resin with quaternary ammonium functional groups; capacity, 30 μequiv./ml; particle size 10 μm; eluent, 0.85 m*M* $NaHCO_3$ and 0.90 m*M* Na_2CO_3; flow, 1.2 ml/min; detection: chemically suppressed conductivity.

described tetraborate eluent with all the above columns. This eluent choice did not improve the separation on either the Waters or Hamilton columns. Therefore, variations of the carbonate–bicarbonate eluent system were developed for the Waters and Hamilton columns. The resulting chromatograms are illustrated in Figs. 2 and 3.

It is quite obvious that the best separation on the Waters column using a carbonate–bicarbonate eluent still did not resolve azide from nitrate. Likewise on the Hamilton column the best separation achieved still did not result in baseline-to-baseline resolution between bromide, azide and nitrate. The authors realize that this comparison was not an all-inclusive investigation into optimum mobile phase conditions with these two columns. But as seen in Fig. 4, complete resolution of bromide,

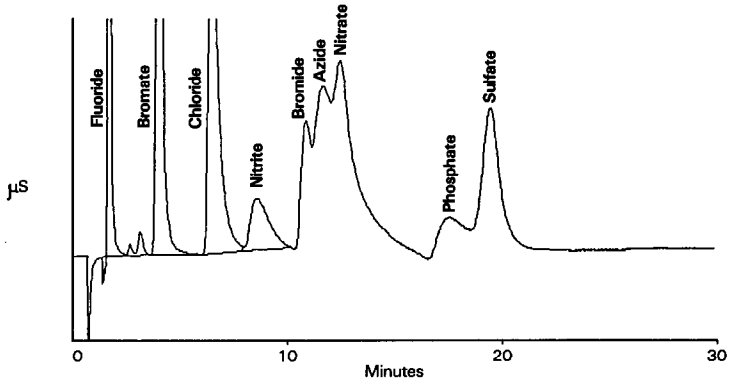

Fig. 3. Ten ppm common anions with azide. Chromatographic conditions: column, Hamilton PRP-X100, poly(styrene–divinylbenzene) resin with surface quarterized (trimethylamine) functional groups; capacity, 200 μequiv./g; particle size 10 μm; eluent, 2.55 m*M* $NaHCO_3$ and 2.70 m*M* Na_2CO_3; flow, 2.0 ml/min; detection: chemically suppressed conductivity.

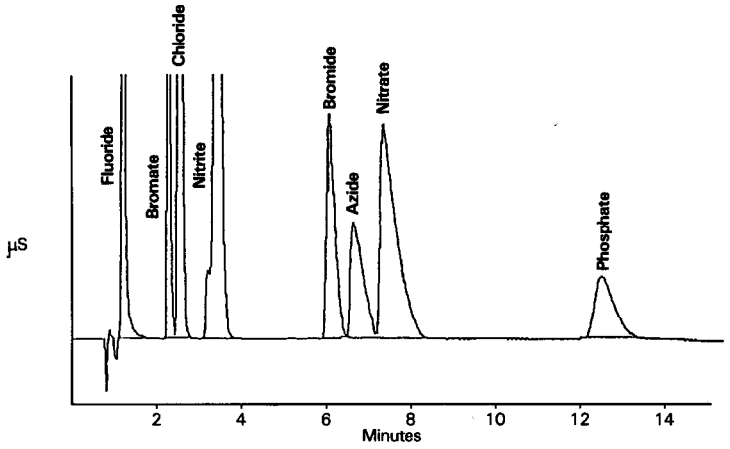

Fig. 4. Ten ppm common anions with azide. Chromatographic conditions: column, Dionex, poly(styrene–divinylbenzene) resin surface sulfonated with aminated latex bead functional groups, AS4A/AG4A; capacity, relatively high loadin capacity; particle size 10 μm; eluent, 16 mM $Na_2B_4O_7$; flow, 2.0 ml/min; detection: chemically suppressed conductivity.

azide and nitrate was obtained on the Dionex column using a tetraborate eluent system. At this point the decision was made to further define the azide chromatography on the Dionex AS4A column using a tetraborate eluent system.

Although sulfate is retained strongly under the tetraborate eluent system it was never a problem throughout these protein investigations. One should consider the effect of sulfate levels in each individual sample. The late eluetion of sulfate or other divalent anions could pose a problem for multi-sample automated analyses. This problem can be solved by establishing the required elution volume for sulfate and including a sufficient delay in the automation schedule. This would allow sulfate to elute between sample injections and eliminate sample carry-over related interferences. Fig. 5 is a typical 10 ppm (in solution) azide standard using the AS4A (Dionex)

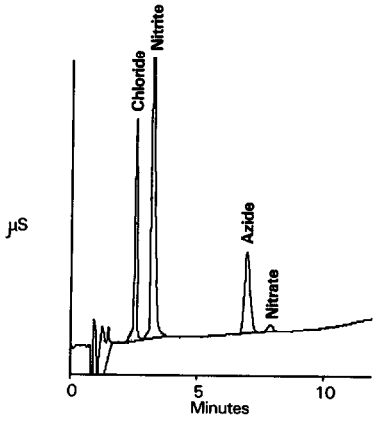

Fig. 5. Ten ppm (in solution) azide standard. Chromatographic conditions: column, Dionex, AS4A/AG4A; eluent, 17 mM $Na_2B_4O_7$; flow, 2.0 ml/min; detection, chemically suppressed conductivity.

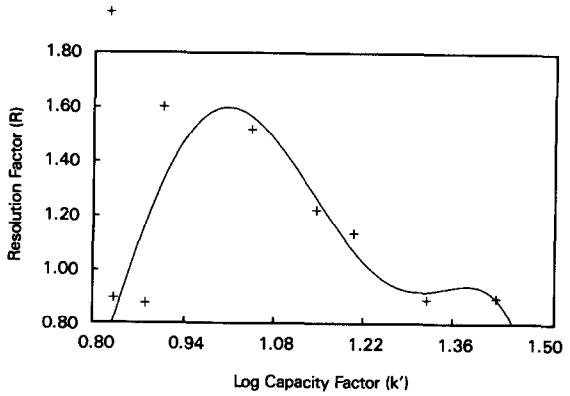

Fig. 6. Bromide and azide resolution map. Tetraborate eluent, AS4A anion column (Dionex). Flow: 2 ml/min, chemically suppressed conductivity detection.

column with a tetraborate eluent. With a flow-rate of 2 ml/min and a tetraborate concentration range of 12–18 m*M* one can expect retention times of 8–12 min for the azide peak.

A study of capacity factor, k', for azide *versus* resolution factors (azide/bromide and azide/nitrate) was made using the tetraborate eluent system on the AS4A (Dionex) column. The goal of the study was to determine the most optimum eluent concentration for the resolution of azide from the primary potential interferences bromide and nitrate. Figs. 6 and 7 are the resulting resolution maps of the log of the capacity factor *versus* resolution factors for bromide and nitrate respectively. In this study k' for azide was increased beyond the optimum range ($1 \leqslant k' \leqslant 10$) [12] to illustrate that typically little is gained in resolution beyond a k' value of 10. This is quite evident in the nitrate/azide plot (Fig. 7).

The maximum resolution obtained for bromide and azide occurs at an azide log k' between 0.95 and 0.98 ($8.9 \leqslant k' \leqslant 9.5$) which corresponds to an eluent concentration of 14–16 m*M* tetraborate. As illustrated in Fig. 6, the resolution map for bromide

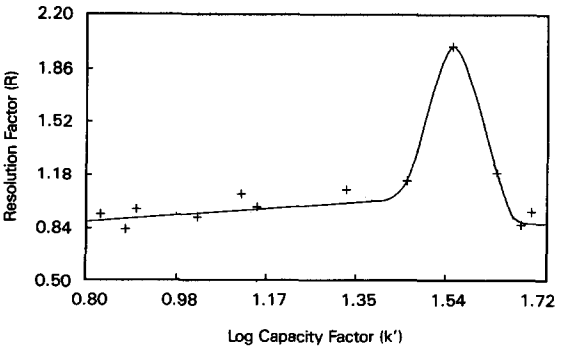

Fig. 7. Azide and nitrate resolution map. Tetraborate eluent, AS4A anion column (Dionex). Flow: 2 ml/min; chemically suppressed conductivity detection.

and azide is quite complex. It was found that if one alters the mobile phase within the above given concentration range then resolving bromide from azide in a given protein sample matrix is possible.

As illustrated in Fig. 7, between an azide k' of 6–20 there is little change in the resolution of azide and nitrate. Considering the fact that the majority of protein matrices will contain some level of nitrate this situation is helpful to the chromatographer. With a larger suitable k' range available to resolve azide from nitrate, method development is not as critical with respect to eluent concentration requirements. Therefore, one has the extended ability to address the bromide–azide interference issue without losing resolution of nitrate and azide. From this resolution map for azide and nitrate it can be concluded that a mobile phase concentration range of 10–18 mM tetraborate will provide adequate separation of azide from nitrate on the AS4A (Dionex) column.

Peak height response for azide using conductivity detection was found to be linear over the typical working range (0–15 ppm azide in solution). A comparison of calibration slopes at varying k' values for azide was made. While optimizing the chromatography for azide with a k' of 10–15, increased sensitivity for azide at the lower k' values was found. The detection limit for azide using the AS4A column and the tetraborate eluent system is estimated to be 30 ppb (in solution). Depending on the sample concentration this detection limit provides quite adequate assurance that this method can be used for trace level azide determination. The concentration range investigated for azide response linearity encompasses the levels that were expected in the protein samples studied.

The proteins used in this study consisted of BSA, zinc metallo enzyme, collagen and a growth factor. From a sample preparation viewpoint all but the collagen protein were soluble in the tetraborate mobile phase system. Azide determination in collegen was accomplished by first performing a solid–liquid extraction of the azide from the parent protein. Figs. 8 through 11 are the resulting chromatograms of the various proteins which were assayed for azide content.

It was assumed that the soluble proteins (BSA, metallo enzyme and growth

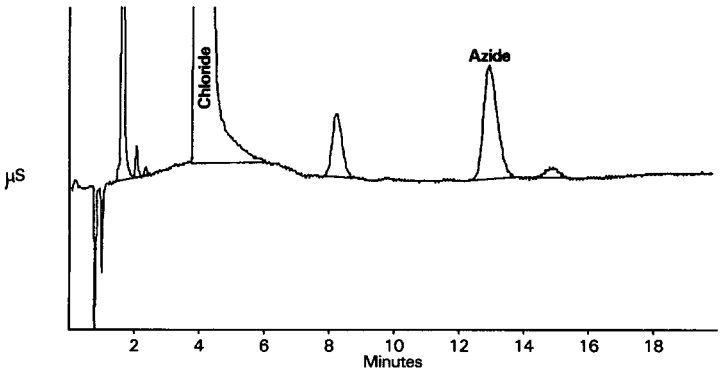

Fig. 8. BSA from Sigma (1000 ppm); 51 ppm chloride and 3.2 ppm azide. Chromatographic conditions: column, Dionex, AS4A/AG4A; eluent, 15 mM $Na_2B_4O_7$; flow, 2.0 ml/min; detection, chemically suppressed conductivity.

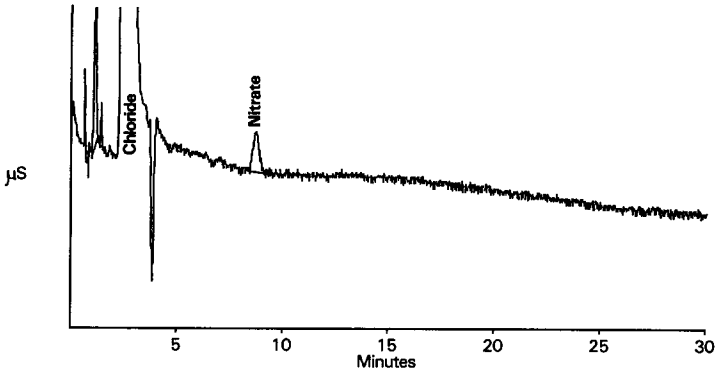

Fig. 9. Zinc metallo enzyme (0.5 ml in 5 ml of eluent). Chromatographic conditions: column, Dionex, AS4A/AG4A; eluent, 15 m*M* $Na_2B_4O_7$; flow, 2.0 ml/min; detection, chemically suppressed conductivity.

factor) were either removed by the sample pretreatment filter (0.45 μm) or were hung-up on the analytical guard column (AG4A, Dionex) or were washed completely through the entire system. If the proteins were absorbed onto either the guard or analytical columns during an assay one would expect column pressures to increase dramatically throughout the study. There was no significant change in the column backpressure from the start through to the end of the study. Approximately 50 protein injections were made through the course of the study. In agreement with the manufacture's literature, final column clean-up with a strong (0.1 *M*) sodium hydroxide column wash is advised. Although, if the tetraborate eluent system is to be used on the column at a later date, consideration for an extended initial equilibration time must be allowed.

BSA samples obtained from two different manufacturers (Sigma and Pierce) were assayed for azide. Fig. 8 is a representative chromatogram for the Sigma sample.

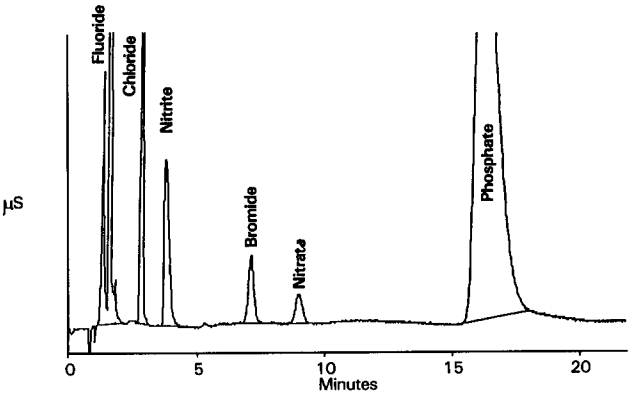

Fig. 10. Collagen (24 mg/ml, insoluble, Bovine Achilles Tendon, Sigma). Chromatographic conditions: column, Dionex, AS4A/AG4A; eluent, 16 m*M* $Na_2B_4O_7$; flow, 2.0 ml/min; detection, chemically suppressed conductivity.

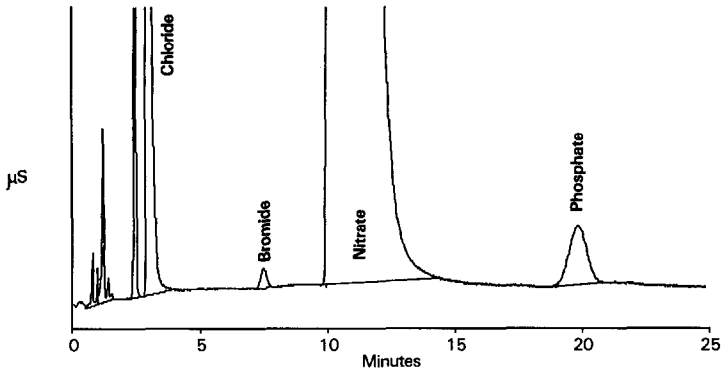

Fig. 11. Growth Factor (4 mg/ml, in solution). Chromatographic conditions: column, Dionex, AS4A/AG4A; eluent, 16 m*M* $Na_2B_4O_7$; flow, 2.0 ml/min; detection, chemically suppressed conductivity.

At a sample concentration of 1 mg/ml, the Sigma BSA was found to contain 0.3% azide while the Pierce BSA at a sample concentration of 0.02 mg/ml contained 16% azide. This level of azide in the Pierce BSA seems to be high and the authors do not have an explanation at this time.

A metallo enzyme (zinc, non-azide binding) was also assayed for azide. The resulting chromatogram of the solubilized protein can be seen in Fig. 9. Obtained from an "in-house" source, the original enzyme samples contained azide and this method was used to prove that azide was no longer present in a new sample lot. As an azide monitoring system, this separation works quite well.

Fig. 10 is the resulting chromatogram for a collagen sample extracted with mobile phase. No azide was detected in this sample and had it been present it would have eluted between the bromide and nitrate peaks. Due to the insolubility of collagen in the mobile phase, sample preparation consisted of a solid–liquid extraction. The extraction as performed at a sample concentration of approximately 25 mg/ml collagen in mobile phase. A wrist-action shaker was used for the azide extraction. Standard addition recoveries were performed with this sample and the results are in Table I.

The final protein assayed for azide was another "in-house" obtained growth factor. As illustrated in Fig. 11, no azide was detected in this sample. Confirmation of

TABLE I

METHOD RECOVERY SOLUBLE *VERSUS* INSOLUBLE PROTEIN SAMPLES

Protein sample	Sample preparation	Standard addition level (ppm in sample)	Recovery (%) (peak area)
Collagen	Solid–liquid extraction: sample is insoluble in the mobile phase	10	97
Albumin bovine serum	Sample is soluble in the mobile phase	100	92

this result was made by spiking azide directly into the sample prior to injection. Azide if present elutes just before the high levels of nitrate found in this sample. Limited available sample minimized further work with this particular protein. It has been proven that this system is capable of quantitating low levels of azide in this type of growth factor.

Method recoveries for azide were determined with two different proteins. The first protein being BSA which is soluble in the mobile phase and the second protein being collagen which is insoluble in the mobile phase. By determining azide recovery with these proteins the sample preparation of insoluble *versus* soluble proteins can be evaluated. The results are summarized in Table I.

Quite good recoveries for azide were obtained from both proteins. The authors found that using a wrist-action shaker provided the best wetting of the insoluble collagen with the tetraborate mobile phase. This, as expected, improved the recoveries of azide to greater than 90%.

CONCLUSIONS

The method described is selective and sensitive for the determination of azide in various types of proteins. In general, both soluble and insoluble proteins assayed for azide resulted in excellent analyte recoveries. The simplicity of the technique enables it to be used as a diagnostic tool in many pharmaceutical quality assurance laboratories.

The authors did not investigate any "azide binding" type proteins (hemoglobin, iron containing) and future work is planned to address this issue. The goal would be to break the bond between the active site (Fe^{3+}) and azide ion, allowing for the quantitation of the free azide ion.

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