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Quantitative determination of the ligand content in Benzamidine Sepharose 4 Fast Flow media with ion-pair chromatography

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Abstract

A quantitative hydrochloric acid hydrolysis-HPLC method was developed for the analysis of the ligand content of Benzamidine Sepharose™ 4 Fast Flow media. The method requires about 100 mg of dried sample and simple reaction vials can be utilised. Release of the ligand (*p*-aminobenzamidine) from the base matrix (Sepharose 4 Fast Flow) was obtained after hydrolysis for 180 min at 70 °C in concentrated hydrochloric acid. When Benzamidine Sepharose 4 Fast Flow media were treated this way *p*-aminobenzoic acid and *p*-aminobenzamidine were the only products released from the ligand. A chromatographic system based on ion-pair reversed phase separation was used to quantify these ligand products. The mobile phase was made acidic enough to make *p*-aminobenzoic acid and *p*-aminobenzamidine positively charged in order to make ion-pair formation with hexanesulfonic acid possible. The relative standard deviation of the method was below 2% and no systematic errors could be detected when the results were compared to an independent method based on elemental analysis (nitrogen). The new HPLC method was used to analyse ligand densities in the range of 2–20 μmol/ml medium.
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1. Introduction

Advances in biochemistry and biotechnology are increasingly dependent on affinity chromatography as a separation technique for the isolation and characterisation of specific biomolecules. Of all the separation techniques currently used in the purification of macromolecules, affinity chromatography is considered to be the most specific [1]. The ligand plays a very significant role in the success of the purification protocol [2] and the ligand density is one of several important factors that affect the adsorption characteristics of affinity media. For example, the ligand density affects the loading capacity but there is no straightforward relationship between ligand density and the binding capacity [3]. Furthermore, the ligand density needs to be optimised carefully to minimise non-specific interactions. In immunoaffinity chromatography a high ligand density can reduce the accessibility of the immobilised antibody due to steric resistance [4]. The ligand density is an important parameter in other chromatographic techniques as well. For example, it has been found that the protein retention in hydrophobic interaction chromatography (HIC) increases with an increase in ligand density [5]. It has also been shown that adsorption of proteins at HIC occurs at a critical ligand density and that it is a function of the hydrophobicity of the medium [6]. In ion-exchange chromatography it has been observed that the band-broading of proteins was larger at low ligand densities and that the selectivity changes with the ligand density [7]. These examples illustrate that it is of outmost important to monitor the ligand content during the development of new media.

Many different methods have been used for determination of ligand densities on separation media. Previously described methods are based on potentiometric titration [8] and UV

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measurement on a suspended gel solution [9] or hydrolysed medium [10,11], NMR measurement on a partly hydrolysed gel [12], and estimation of the consumption of a specific reagent after a complete reaction with the ligand [13,14]. Chemical release of ligand with concomitant determination of ligand products with a suitable technique is also an approach commonly used for determination of ligand density [15–17].

This article describes a method for the quantification of the affinity ligand (*p*-aminobenzamidine) in Benzamidine Sepharose 4 Fast Flow media. A critical criterion for the method is that it should exhibit a relative precision of less than 3% can be obtained. Furthermore, the method should also be able to verify the identity of benzamidine as the ligand of Benzamidine Sepharose 4 Fast Flow. The *p*-aminobenzamidine group has been used as an affinity ligand for purification of urokinases, serine proteases, plasminogen activators, enterokinase, xanthine dehydrogenase and other similar enzymes that contain a benzamidine binding site [18–21]. Most of the applications are based on the ligand immobilised on beaded chromatographic packing materials such as Sepharose Fast Flow but the ligand has also been coupled to membranes for purification of for example trypsin [22]. It can also be noted that the *p*-aminobenzamidine ligand has been used as an anion-exchanger and it was shown that the ligand exhibit extraordinary strong interactions with a lot of different proteins [23].

2. Materials and methods

2.1. Equipment

For the determination of the dry content of Benzamidine Sepharose 4 Fast Flow media disposable PD-10 columns from Amersham Biosciences (code No. 17-0435-01) and glass filter crucibles (G3) were used.

The HPLC measurements were performed on an HPLC-system (HP 1100 Agilent Technologies) equipped with an RP-18 column (Spheri-5-C 18.5 μm silica particle 80 Å pore, 2.1 mm × 220 mm, Pierce Chemicals) and a guard column (same as above but with the size 2.1 mm × 30 mm, Perkin-Elmer). The mobile phases and the samples were filtered before use by using a sterile filter (0.45 μm).

2.2. Chemicals

Benzamidine Sepharose 4 Fast Flow media, were obtained from Amersham Biosciences (Uppsala, Sweden). Hydrochloric acid was used at hydrolysis of Benzamidine Sepharose Fast Flow media and 1-hexanesulfonic acid (Fluka, code No. 52862) and phosphoric acid (Fluka) were used for the preparation of the mobile phase at the chromatographic experiments. *p*-Aminobenzamidine dihydrochloride (min. 99%) and *p*-aminobenzoic acid (min. 99%) were purchased from Sigma. All other chemicals and solvents were from Fluka, Aldrich or Sigma unless otherwise noted.

2.3. Sample preparation

2.3.1. Dry content determination

A sample of a Benzamidine Sepharose 4 Fast Flow media was drained in PD-10 columns to determine the gel volume and ca. 10 ml of the sample were transferred quantitatively to a weighed glass filter placed on a filter flask. The medium sample was shrunk with additions of water and successively increased concentrations of acetone and was sucked dry gently with a water aspirator after each addition. Finally acetone was added a few times before the gel was sucked dry.

The glass filter funnel with the gel was dried overnight in an oven at 105 °C and then the glass filter was cooled to room temperature in a dessicator for at least 45 min. Finally, the glass filter was weighed out and the dry content of Benzamidine Sepharose 4 Fast Flow sample was calculated.

2.3.2. Hydrolysis of Benzamidine Sepharose 4 Fast Flow media

100 ± 5 mg of oven dried Benzamidine Sepharose 4 Fast Flow sample were placed in a 100 ml measuring flasks and 5.00 ml of concentrated hydrochloric acid were added. The measuring flask was placed in a water bath adjusted to 70 ± 1 °C. The sample was hydrolysed for 180 ± 1 min and then the measuring flask was withdrawn from the water bath and distilled water (tempered to ca. 20 °C) was added to the mark. This sample solution was filtered (0.45 μm) before HPLC analysis. The described method for hydrolysis of Benzamidine Sepharose 4 Fast Flow media was chosen after optimisation where the sample amount, temperature and hydrolysis time were varied between 50 and 135 mg, 55 and 84 °C, and 35 and 205 min, respectively.

2.4. HPLC determination of ligand products after acidic hydrolysis of Benzamidine Sepharose 4 Fast Flow media

2.4.1. Mobile phases and the separation protocol

Methanol was used as solvent A and as solvent B was 100 ± 0.1 mM hexanesulfonic acid adjusted to pH 2.50 ± 0.02 with phosphoric acid used. Ten microlitres of the sample solution were applied to the column and the flow-rate and the column temperature was set to 0.30 ml/min and 20 ± 0.1 °C, respectively. The separation was obtained by the protocol: 0–4 min at 95% B (isocratic); 4–10 min 95–5% B (linear); 10–12 min 5% B (isocratic); 12–13 min to 5–95% B (linear); 13–20 min 95% B (isocratic). The eluate was monitored at 280 nm.

2.4.2. Calibration and determination of the ligand content

Five standard solutions of *p*-aminobenzamidine dihydrochloride and *p*-aminobenzoic acid were prepared in the

concentration range 0.067–0.54 mM and 0.026–0.21 mM, respectively. The standards were prepared in 5% (v/v) hydrochloric acid solution. The calibration graphs were constructed from the peak areas and the concentration of the substances. The ligand content of Benzamidine Sepharose 4 Fast Flow media was calculated from the concentration of *p*-aminobenzamidine and *p*-aminobenzoic acid in the sample solution. These concentrations were obtained from the constructed calibration graphs.

2.5. Elemental analysis

The total content of nitrogen was determined in Benzamidine Sepharose 4 Fast Flow media and the intermediate product to which *p*-aminobenzamidine is attached (Fig. 1). The ligand density (μmol/ml) of Benzamidine Sepharose 4 Fast Flow media was then determined according to the calculation described in [24]. The dry content of the intermediate product was determined as described above. The intermediate product means that a long spacer arm is attached to the base matrix (Sepharose 4 Fast Flow) through stable ether and amino linkages (Fig. 1). The ligand is coupled to the spacer via an amide bond to the *p*-amino group of *p*-aminobenzamidine. This means that both Benzamidine Sepharose 4 Fast Flow media and the intermediate product contain nitrogen. Elemental nitrogen analysis of the dried samples was performed at Mikro Kemi (Uppsala, Sweden) by using Perkin-Elmer 2400 CHN elemental analyzer.

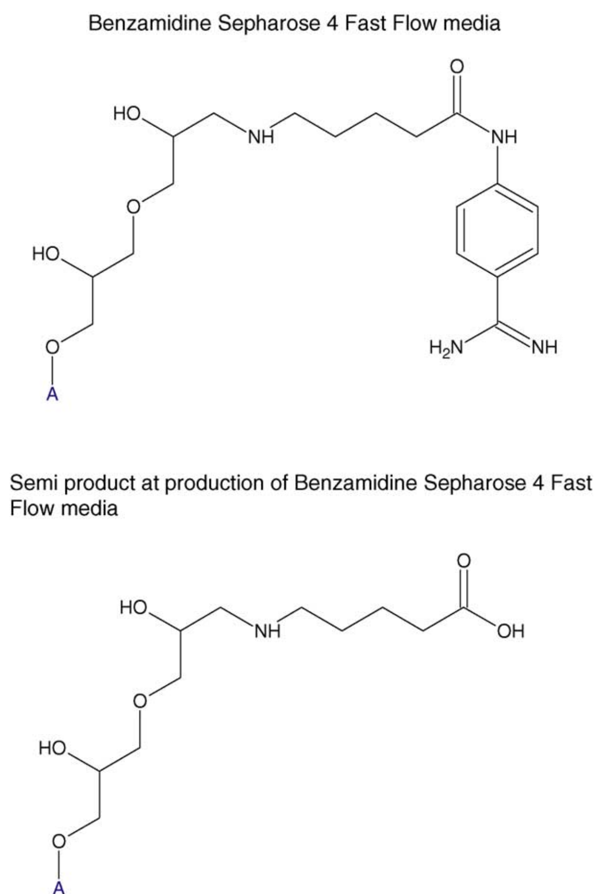

Fig. 1. Structure Benzamidine Sepharose 4 Fast Flow media and the intermediate product (A = Sepharose 4 Fast Flow).

3. Results and discussion

This article describes the acid hydrolysis of Benzamidine Sepharose 4 Fast Flow media and the subsequent HPLC determination of the ligand products released from the medium. The *p*-aminobenzamidine ligand is attached to the Sepharose base matrix via an amide linkage as depicted in Fig. 1. This means that rather harsh conditions (concentrated hydrochloric acid) are required to release the ligand quantitatively from the agarose matrix. Preliminary results from hydrolysis of Benzamidine Sepharose 4 Fast Flow media showed that not only *p*-aminobenzamidine but also *p*-aminobenzoic acid were obtained. These products were identified by HPLC analysis that also was used for quantification of these two compounds.

3.1. HPLC determination of p-aminobenzamidine and p-aminobenzoic acid

A chromatographic system based on ion-pair reversed-phase separation was chosen. The mobile phase was made acidic enough to make *p*-aminobenzoic acid and *p*-aminobenzamidine positively charged to be able to undergo ion-pair formation with hexanesulfonic acid. To evaluate the HPLC method, fractional factorial designed experiments were conducted (Tables 1 and 2). The pH of the mobile phase, the concentration of hexanesulfonic acid, the column temperature and the amount of HCl in the sample were varied. The experimental design means that these main effects are not confounded with two factor interactions but two-factor interactions is aliased with two other two-factor interactions. The largest identified significant effects for determination of *p*-aminobenzamidine appear to be the column temperature, pH of the mobile phase and the concentration of hexanesulfonic acid. These main effects explain 75% of the total variation. It can also be noted that the total spread for the determination of *p*-aminobenzamidine (Table 1) is very low (min. value: 72.79; max. value: 75.99 absorbance units/mg/ml). For the determination of *p*-aminobenzoic acid only two significant factors were observed, namely the pH of the mobile phase and the concentration of hexanesulfonic acid. These two main effects explain 97% of the variability in the observed response and the effect of the pH variation explains about 88%. It can also be noted that the total spread of the results (Table 2) is higher for *p*-aminobenzoic acid compared to *p*-aminobenzamidine (Table 1). To optimise the HPLC performance the three above mentioned important factors were carefully controlled (see Section 2). An analysis of variance was also conducted to test the precision of the HPLC method after optimisation of the hydrolysis procedure of Benzamidine Sepharose 4 Fast Flow media (see below).

Table 1
Experimental worksheet for HPLC determination of *p*-aminobenzamidine

Experimental factors				Response peak area ((mAU s)/(mg ml))
pH of the mobile phase	Concentration of hexanesulfonic acid (mM)	Column temperature (°C)	Amount of HCl in the sample solution % (v/v)	
2.4	75	17	3	73.04
2.4	75	17	3	73.78
2.4	75	23	7	73.77
2.4	75	23	7	74.52
2.4	125	23	3	72.75
2.4	125	23	3	73.26
2.4	125	17	7	72.79
2.4	125	17	7	73.03
2.6	75	23	3	74.98
2.6	75	23	3	75.99
2.6	75	17	7	74.14
2.6	75	17	7	73.91
2.6	125	17	3	72.89
2.6	125	17	3	73.27
2.6	125	23	7	74.60
2.6	125	23	7	74.39
2.5	100	20	5	73.14
2.5	100	20	5	73.48
2.5	100	20	5	73.62
2.5	100	20	5	73.35

Hydrolysed (according to the procedure described in Section 2) Benzamidine Sepharose 4 Fast Flow prototypes were used as sample.

3.2. *Hydrolysis of Benzamidine Sepharose 4 Fast Flow media*

About 10 ml of settled media was sampled. The sample was dried (see Section 2) before hydrolysis. Dried Benzamidine Sepharose 4 Fast Flow medium was treated with hydrochloric acid at various times and temperatures in order to optimise the release of the ligand from the medium. In all experiments concentrated hydrochloric acid was used. To evaluate the ligand density the total amount of the two identified hydrolysis products (*p*-aminobenzamidine and *p*-aminobenzoic acid) was quantified from calibration graphs (Fig. 2). A central composite design was used for the optimisation of hydrolysis time and temperature (Table 3). A contour plot of the results is depicted in Fig. 3. It is shown that a hydrolysis temperature above ca. 65 °C is necessary to obtain a quantitative release of the ligand (Ligand density >12.5 μmol/ml). At this temperature a hydrolysis time of about 190 min must be used. However, the hydrolysis time can be decreased if a higher temperature is applied (Fig. 3). Chromatograms obtained from two different samples, prepared under different hydrolysis conditions, are shown in Fig. 4. The peak at about 5 min is attributed to UV-absorbing agarose products since it appears also if non-substituted Sepharose 4 Fast Flow is hydrolysed. It can also be noted that this peak decreases with hydrolysis time. At low hydrolysis time a peak can be observed at about 14 min. This peak probably arises from molecules containing both agarose and ligand. This conclusion is based on the fact that the UV–vis spectrum of the peak is similar to the spectrum of *p*-aminobenzamidine.

Table 2
Experimental worksheet for HPLC determination of *p*-aminobenzoic acid

Experimental factors				Response peak area ((mAU s)/(mg ml))
pH of the mobile phase	Concentration of hexanesulfonic acid (mM)	Column temperature (°C)	Amount of HCl in the sample solution % (v/v)	
2.4	75	17	3	95.75
2.4	75	23	7	98.12
2.4	125	23	3	109.25
2.4	125	17	7	105.90
2.6	75	23	3	128.07
2.6	75	17	7	126.65
2.6	125	17	3	135.23
2.6	125	23	7	134.25
2.5	100	20	5	112.69
2.5	100	20	5	112.63
2.5	100	20	5	112.56

Hydrolysed (according to the procedure described in Section 2) Benzamidine Sepharose 4 Fast Flow prototype was used as sample.

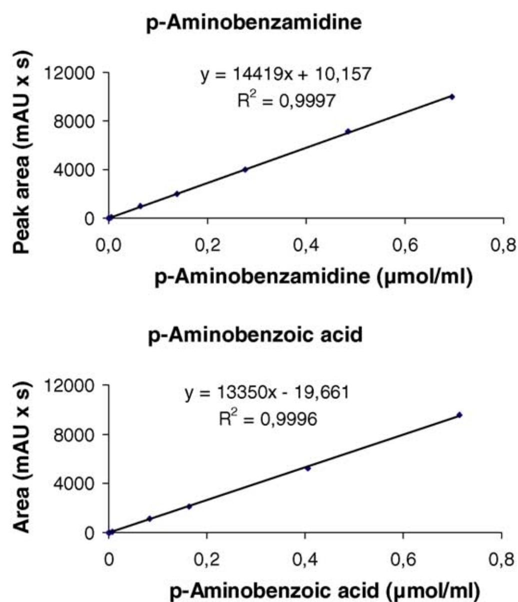

Fig. 2. Calibration graphs for *p*-aminobenzamidine and *p*-aminobenzoic acid.

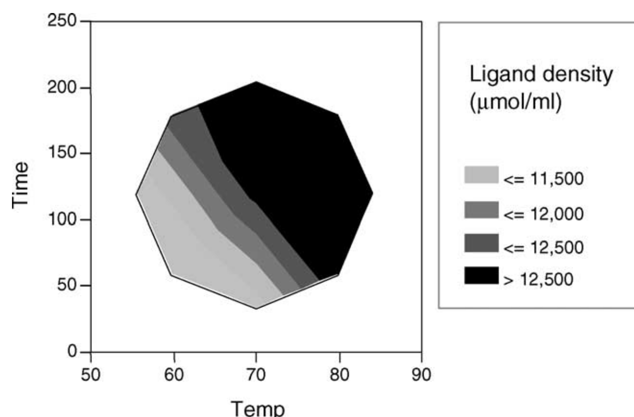

Fig. 3. Contour plot for the optimisation of the time and temperature at hydrolysis of Benzamidine Sepharose 4 Fast Flow prototype (see Table 3 for details).

Table 3
Central composite design worksheet for optimisation of hydrolysis of a Benzamidine Sepharose 4 Fast Flow prototype

Hydrolysis time (min)	Temperature at hydrolysis (°C)	Ligand density[a] (μmol/ml)
60	80	12.97
120	70	12.68
120	56	10.57
120	84	13.18
120	70	12.63
205	70	13.04
120	70	12.63
120	70	12.73
120	70	12.64
60	60	10.06
35	70	10.76
180	60	12.26
180	80	13.29

[a] The ligand density was determined by quantification of the hydrolysis products *p*-aminobenzamidine and *p*-aminobenzoic acid.

Also the fact that the peak disappears after long hydrolysis times indicate that all ligands are eventually released from the agarose matrix. The *p*-aminobenzoic acid peak increases slightly with hydrolysis time but is much smaller compared to the *p*-aminobenzamidine peak (Fig. 4). From the results obtained we decided to hydrolyse Benzamidine Sepharose media with concentrated hydrochloric acid at 70 ± 1 °C for 180 ± 1 min.

3.3. Studies of possible errors in the determination of the ligand density of Benzamidine Sepharose 4 Fast Flow media

To test the possible effect of sample size on the result 50, 75, 100 and 120 mg of Benzamidine Sepharose 4 Fast Flow medium were hydrolysed with 5.00 ml of hydrochloric acid at 70 °C for 180 min. No trends in the results were obtained and the relative spread, based on the numerical difference between the highest and lowest result, was 0.9%.

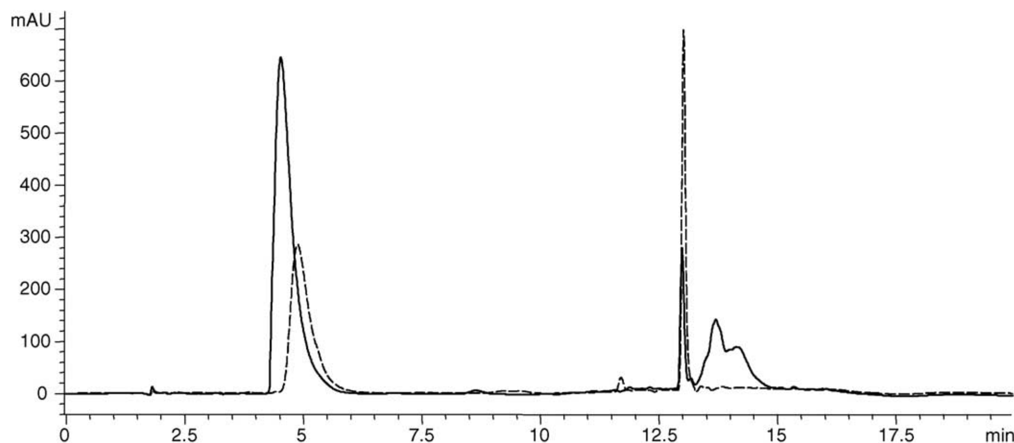

Fig. 4. Separation of hydrolysis products of a Benzamidine Sepharose 4 Fast Flow prototype after hydrolysis with hydrochloric acid at 70 °C in 15 (solid line) and 120 min (dashed line). The peaks at 11.7 and 13.0 min are attributed to *p*-aminobenzoic acid and *p*-aminobenzamidine, respectively.

Table 4
Worksheet and results of ANOVA

Day	Sample	Ligand density[a] (μmol/ml)
1	1	13.08
1	1	13.11
1	2	13.33
1	2	13.44
2	1	13.62
2	1	13.67
2	2	13.58
2	2	13.61
3	1	13.28
3	1	13.33
3	2	13.21
3	2	13.23
4	1	13.14
4	1	13.14
4	2	14.02
4	2	13.95
R-square	0.983	
Mean of response	13.421	
Variance component of day ($S^2_{\text{between-day}}$)	0.0000415	
Variance component of sample (nested with day; $S^2_{\text{between-sample}}$)	0.0914	
Variance component of residual (S^2_{residual})	0.00150	

Two samples from Benzamidine Sepharose 4 Fast Flow prototype were prepared and analysed twice each day for four different days.
[a] The ligand density was determined by quantification of the hydrolysis products *p*-aminobenzamidine and *p*-aminobenzoic acid.

To estimate the precision of the method an analysis of variance was performed (Table 4). The analysis was performed at four different days on two samples (hydrolysed Benzamidine Sepharose 4 Fast Flow media) prepared freshly each day. Both samples were analysed by the HPLC method twice each day. In Table 4 the statistical evaluation of the results is presented. Between-day variance is not significant but between-sample variations differ significantly (F-ratio = 203) from zero. The variance estimates can be used to calculate the standard deviation (S_M) for the method (based on hydrolysis of two samples and two HPLC analyses on each sample) according to

$$S_M = \sqrt{S^2_{\text{between-day}} + \frac{S^2_{\text{between-sample}}}{2} + \frac{S^2_{\text{residual}}}{2}}$$

In this case the standard deviation S_M is 0.216 μmol/ml medium and the relative standard deviation 1.6%.

To test if constant errors exist that are due to interfering contaminants from reagents or base matrix (Sepharose 4 Fast Flow) blank analysis were performed. Analysis of Sepharose 4 Fast Flow gave no interfering peaks in the chromatogram. The accuracy of the method was also tested with an independent method based on elemental analysis of nitrogen before and after coupling of the ligand (see Section 2). The results from a number of different prototypes of Benzamidine Sepharose 4 Fast Flow with different ligand densities are shown in Table 5. From the regression line it is clear that the calculated slope and intercept do not differ significantly from the ideal values of 1 and 0, respectively. Consequently, there is no evidence of systematic differences between the two methods.

Table 5
Comparison of two methods for determination of ligand density of different prototypes of Benzamidine Sepharose 4 Fast Flow

HPLC method	Nitrogen analysis
Ligand density (μmol/ml)	
2.1	2.1
3.8	4.2
5.9	6.2
6.4	6.4
8.6	9.3
8.8	8.7
9.5	10.3
11.0	11.0
12.2	13.5
Statistical evaluation of the regression line of the result from the two methods	
Regression line	Nitrogen = 1.069 HPLC − 0.148
Correlation coefficient	0.993
Standard deviation of intercept (S_a)	0.388
Standard deviation of slope (S_b)	0.0473
Confidence limits for intercept (t = 2.36)	−0.148 ± 0.916
Confidence limits for the slope (t = 2.36)	1.069 ± 0.112

4. Conclusions

A quantitative method has been described for the analysis of the amount of *p*-aminobenzamidine covalently attached via an amide bond to Sepharose 4 Fast Flow. The method is based on acid hydrolysis of Benzamidine Sepharose 4 Fast Flow media and subsequent HPLC analysis of two products obtained from the ligand. No systematic errors in the determination of the ligand content could be observed as the results of the HPLC method compared to a method based on analysis of nitrogen gave similar results. The relative precision of the method is below 2% and the method has been developed for ligand densities between 2 and 20 μmol/ml.

The HPLC method is presently used in the authors' laboratory for quality control of the ligand content in the products Benzamidine Sepharose 4 Fast Flow (high sub) and Benzamidine Sepharose 4 Fast Flow (low sub).

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