Journal of Chromatography, 321 (1985) 335–342
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,409

QUANTITATIVE DETERMINATION OF THE LIGAND CONTENT AND THE NEGATIVE AND POSITIVE CHARGES ON BUTYLAMINE-SUBSTITUTED SEPHAROSE 4B

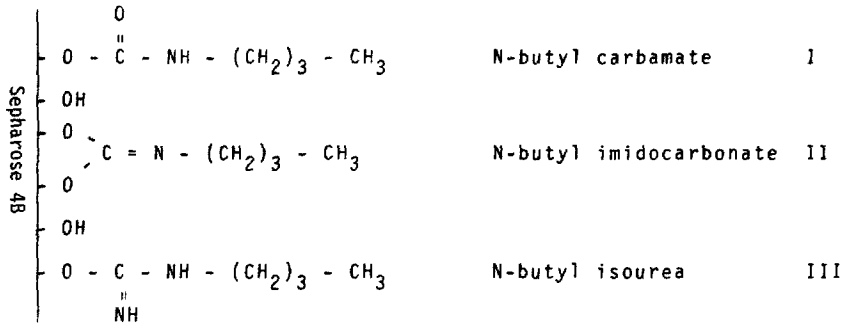
BO-LENNART JOHANSSON* and INGRID DREVIN
Pharmacia Biotechnology, Department of Quality Control, S-751 82 Uppsala 1 (Sweden)
(Received November 15th, 1984)

SUMMARY

Analytical methods based on ion-exchange reactions have been developed for the quantitative determination of the amount of positive and negative charges on butyl-Sepharose 4B at different pH values. A quantitative ^1H NMR procedure is also described for measuring the degree of substitution. These methods constitute the basis for an evaluation of the relative amounts of different coupling structures where butylamine is linked to cyanogen bromide-activated Sepharose 4B.

INTRODUCTION

Agarose gels are widely used as the matrix for the preparation of affinity adsorbents and adsorbents for hydrophobic interaction chromatography[1,2]. The substitution of the polysaccharide matrix is usually performed by coupling free amino groups of a ligand to cyanogen bromide-activated agarose[3,4]. This method introduces three types of ligand–polysaccharide bonds[2,3,5] as exemplified below with butylamine coupled to cyanogen bromide-activated Sepharose 4B.



Structures II and III will carry positively charged nitrogen atoms at acidic pH[6,7]. The gel can consequently act both as an ion exchanger and through hydrophobic interactions[8–10].

0021-9673/85/$03.30

The total amount of the ligand and the relative amounts of the coupling structures I, II and III can be regulated by means of the activation and coupling procedures[1]. Analytical methods are therefore required for monitoring and optimizing the results of the synthetic procedures. Previous methods are based on potentiometric titration[7], radioactively labelled compounds[11,12] and specific reagents[13]. In this study, methods based on ion-exchange reactions were developed for monitoring the amount of cationic and anionic charges on butylamine-substituted Sepharose 4B at different pH. For the determination of the total amount of butylamine coupled to cyanogen bromide-activated Sepharose 4B a quantitative ^1H NMR procedure was developed.

EXPERIMENTAL

Chemicals and apparatus

Nitric acid, silver nitrate, sodium chloride, sodium nitrate and butylamine were purchased from Merck (Darmstadt, F.R.G.) and the reagents were of analytical-reagent grade. ^2H_2O, ^2HCl and dimethylsulphoxide-d_6 (isotopic purity >99.5%) were obtained from Ciba-Geigy (Basle, Switzerland). Butylamine was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to ref. 14. The titrations were performed on Mettler Memo Titrator DL 40 RC, a Varian Techtron AA6 instrument was used in the flame photometric experiments and the NMR spectra were recorded with a Jeol FX 200 instrument at 199.5 MHz. In the pulsed NMR experiments the number of pulses was 100, pulse time 7 μsec, pulse delay 1 sec, acquisition time 2 sec and the delay between pulse and acquisition 50 μsec. The spectral range explored was 4000 Hz.

Determination of the amount of cationic charge at different pH

About 3 ml of butylamine-substituted Sepharose 4B was packed in a column of 9 mm I.D. The gel volume and gel weight were observed after the gel had been washed with 15 ml of water and settled at an operating pressure of 10 cmH_2O. The gel was transferred to a glass filter-funnel and subsequently saturated with chloride using 2 × 20 ml of 0.100 *M* buffer solution (see below) for 10 min each time. The chloride-saturated gel was further washed with 4 × 20 ml of 1.00 m*M* buffer solution (the same buffer as in the saturation process) for 5 min each time. Removal of as much as possible of the washing solution was accomplished by centrifugation of the glass filter-funnel at 318 *g* for 20 min.

The chloride ion capacity of the gel was then determined by potentiometric titration. Titration of the gel was performed in 50 ml 0.10 *M* sodium nitrate, acidified to pH 3 with HNO_3, using 10.0 m*M* silver nitrate as the titrant. The positively charged buffers used in the determination of the amount of cationic charge on butyl-Sepharose 4B at different pH are shown in Table I. Buffer solutions and chloride ion concentrations were 0.100 *M* for the saturation step and 1.00 m*M* for the washing step. At pH 3 the gel was washed with 1.00 m*M* hydrochloric acid after prior saturation with 0.50 *M* hydrochloric acid.

Determination of the amount of anionic charge at different pH

With the exception of the choice of buffers and the counter ion, the same

TABLE I

BUFFERS USED FOR VARIOUS pH RANGES IN THE DETERMINATION OF CATIONIC CHARGE ON BUTYLAMINE-SUBSTITUTED SEPHAROSE 4B

Buffer	*pH range*	*pK_a (25°C)*
N-Methylpiperazine	4.5–5.0	4.8
Piperazine	5.5–6.0	5.7
Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane	6.5–7.0	6.5
Triethanolamine	7.5–8.5	7.8
Ethanolamine	9.0–10.0	9.5
1,3-Diaminopropane	10.0–10.5	10.5

general procedure was followed as in the determination of cationic charge. Acetate, phosphate and carbonate buffers were used. The counter ion was sodium. The results at pH 11.0 were achieved with 0.100 *M* sodium hydroxide as the saturation buffer and the gel was washed with 1.00 m*M* sodium hydroxide.

The sodium ion capacity was determined by flame emission spectroscopy. After the sodium-saturated gel had been centrifuged the Na^+ ions were eluted with 4 × 5 ml of 1.00 m*M* hydrochloric acid for 5 min each time. The eluate was diluted to 25 ml with 1.00 m*M* hydrochloric acid and analysed. Standard solutions were prepared from sodium chloride and 1.00 m*M* hydrochloric acid in the range 1–10 ppm.

Determination of the degree of substitution by NMR spectroscopy

The sampling of butyl-Sepharose 4B was carried out as above. This sample was further dehydrated by successive washes with acetone and finally dried to constant weight at 100°C. A 10-mg amount of the dried gel was hydrolysed with 100 µl of 6.5 *M* ^2HCl at 70°C for 15 sec. This mixture was cooled in an ice-bath for 1 min, then 1.00 ml of DMSO-d_6 was added. The NMR spectrum of this solution was received and the degree of substitution was evaluated with standard solutions of butylamine. These standards were prepared in exactly the same way as the sample and with the same bottle of DMSO-d_6 as used for the gel. The peaks from protons H_A, H_B and H_C were integrated and isotopic impurities in DMSO-d_6 served as the internal standard (Fig. 1). A calibration graph was prepared for the range between 0.1 and 0.4 mg of butylamine.

RESULTS AND DISCUSSION

Sources of errors in the determination of charged sites on butyl-Sepharose 4B

The blank value. The washing buffer solutions contain counter ions in order to prevent elution of ions from the charged sites on the gel. Some buffer will be retained in the gel beads after washing and consequently give rise to a positive error in the determination of charged sites. This blank value was minimized by centrifugation and determined by using unsubstituted Sepharose 4B as a blank gel. Sepharose contains a small number of sulphate and carboxyl groups[15]. In order to avoid ion exchange with Sepharose 4B, Cl^- ions are consequently used in the determination of

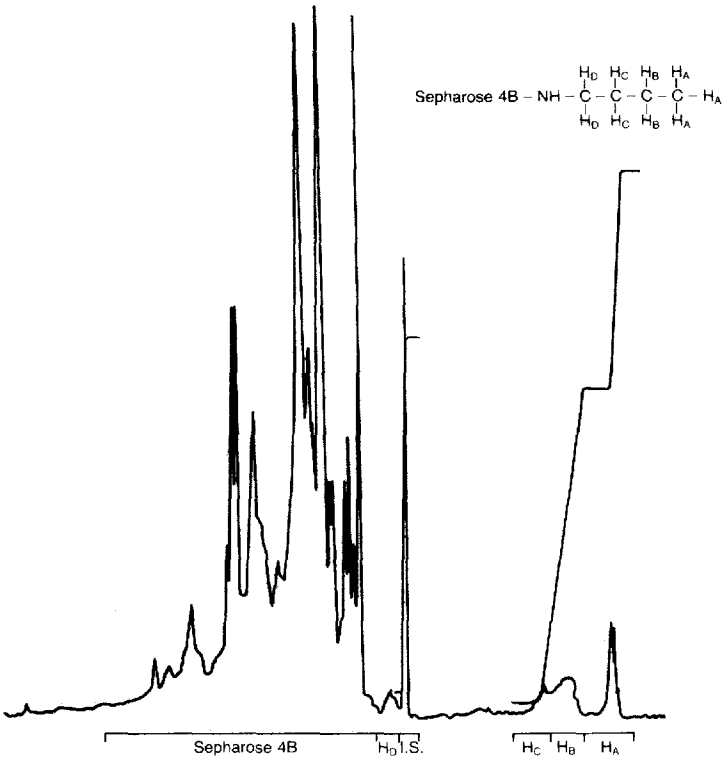


Fig. 1. NMR spectrum of partially hydrolysed butylamine-substituted Sepharose 4B in DMSO-d_6. A small amount of DMSO containing H was used as an internal standard (I.S.).

the blank value. The variation of the blank value with the centrifugation velocity is shown in Table II. Centrifugation at 318 *g* for 20 min was chosen as a standard method giving a background of 0.201 μmole/g swollen gel, which must be subtracted from the determined values. This blank value was also obtained when the amount of cationic charge on butyl-Sepharose 4B was determined at pH $\geqslant$ 10 (Fig. 2). The absence of protonated/positive groups at these high pH values is in accordance with

TABLE II

VARIATION OF THE AMOUNT OF CHLORIDE IONS RETAINED IN SEPHAROSE 4B WITH THE VELOCITY OF CENTRIFUGATION

The gel was saturated with 0.50 *M* HCl and washed with 1.00 m*M* HCl. The centrifugation time was 20 min in all instances.

Centrifugation velocity		*Amount of Cl^- in the gel (μmole/g swollen gel) (mean* ± standard deviation)*
rpm	*g*	
1900	155	0.391 ± 0.016
3200	261	0.226 ± 0.004
3900	318	0.201 ± 0.006

* Determined from three experiments.

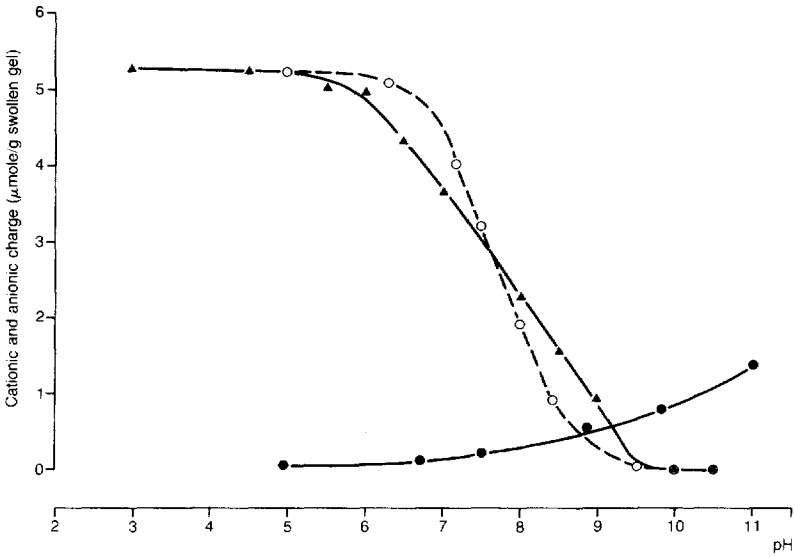


Fig. 2. Variation of the amount of cationic (▲) and anionic (●) charges on butylamine-substituted Sepharose 4B with pH. The dotted line shows the theoretical variation of a positive ionizable group having a pK_a of 7.8.

the proposed structures I, II and III for butyl-Sepharose 4B. The reliability of the blank value is further confirmed by the results from the pH dependence of the amount of anionic charge on butyl-Sepharose 4B. As shown in Fig. 2, the blank value is approached when the pH decreases.

The indeterminate error. In order to establish the indeterminate uncertainty of the method for analysis of cationic charge, the amount of positively charged groups

TABLE III

DETERMINATION OF THE AMOUNT OF CATIONIC CHARGE ON BUTYLAMINE-SUBSTITUTED SEPHAROSE 4B AT pH 3

The gel was saturated with 0.50 *M* HCl and further washed with 1.00 m*M* HCl.

Weight of swollen gel (g)	*Amount of cationic charge (μmole/g swollen gel)*
2.37	5.30
2.19	5.43
2.15	5.46
233	5.17
233	5.27
2.11	5.39
2.21	5.24
2.33	5.26
1.92	5.20
Average:	5.30 ± 0.12 (t = 99%)
Standard deviation:	0.102
Relative standard deviation:	1.92%

was determined a number of times at pH 3 (Table III). All other results from the measurements of cationic and anonic charges are mean values from two replicate analyses and the spread from the mean value is always less than 5%.

Method parameters. The validity of the method for the determination of the cationic charge was further elucidated by showing that neither variation of the saturation buffer concentration between 0.1 and 0.5 *M* nor variation of the saturation time between 1 and 20 min resulted in additional binding of chloride ions. Moreover, more than four washings did not influence the results. The sample quality/stability of butyl-Sepharose 4B was regularly checked during this investigation. It was found that the cationic charge at pH 3 did not change with time.

Effect of pH on the amount of cationic charge

In the actual pH range studied butylamine-substituted Sepharose 4B contains two structures II and III, which may be expected to carry positively charged nitrogen. The apparent pK_a values for the N-substituted imidocarbonate group (II) and the N-substituted isourea group (III) are 5 and 10, respectively[6,7]. Accordingly, the maximum cationic charge capacity should appear below pH 5, where all basic groups on butyl-Sepharose 4B are ionized. The model of butyl-Sepharose 4B further predicts that the cationic charge should be zero at pH > 10. The results in Fig. 2 follow these predictions. However, the variation of the amount of cationic charge at intermediate pH values cannot be directly explained from the two structures II and III. The S-shaped relationship merely indicates that butylamine is bonded to cyanogen bromide-activated Sepharose 4B with one structure having an apparent pK_a value of 7.8 (pH at the half maximum capacity). The theoretical variation of the cationic charge in that case was calculated and is depicted in Fig. 2, showing that the relationship found cannot be explained by a single pK_a value/coupling structure. A probable interpretation of the pH dependence is therefore that the same coupling structure (II or III) can have different pK_a values. This would mean that the ionization process cannot occur independently of the surrounding gel structure. Variation in the ionization processes of II and III can therefore be related to inhomogeneity in the gel structure. Titration curves affected by the gel structure have been observed previously[16].

Effect of pH on the amount of anionic charge

As shown in Fig. 2, the amount of anionic charge on butyl-Sepharose 4B is not higher than 0.2 μmole/g swollen gel at pH < 7. Further, the net charge of the gel is zero at about pH 9.2 and becomes negative at higher pH.

The amount of anionic charge on the underivatized gel (Sepharose 4B), determined using the described method, is 1.5 μmole/g swollen gel in the pH range 5–8. This fact indicates that the procedure for the preparation of butyl-Sepharose 4B from Sepharose 4B decreases the anionic charge by nearly 90% in this pH range. Hence butyl-Sepharose 4B contains mainly weak acidic groups that can be ionized to negatively charged groups (Fig. 2).

Total concentration of butylamine immobilized on butyl-Sepharose 4B

Acidic hydrolysis of butyl-Sepharose 4B as described above splits the agarose gel into shorter polygalactans which are soluble in dimethyl sulphoxide. The NMR

spectrum of such a solution is shown in Fig. 1. By integration of the resonance lines of protons H_A, H_B and H_C (Fig. 1) in the butyl group, the degree of substitution was calculated from the calibration graph for butylamine. A correlation between the amount of butylamine in milligrams (M) and the ratio (R) of the sample resonance peaks (protons H_A, H_B and H_C) and the internal standard peak areas was found to be

$$R = 5.162\,M - 0.0395 \quad (1)$$

The correlation coefficient of the calibration graph, calculated by linear regression, is 0.9998 in the investigated range. This relationship is subject to some variation because of the batch-to-batch variation of the quality of DMSO-d_6.

To check the reproducibility of the NMR method, three separate determinations of butyl-Sepharose were performed. The results gave a mean value of 9.69 μmole/g swollen gel with a relative standard deviation of 4.0%. The reliability of the method was further confirmed by showing that unsubstituted Sepharose 4B does not interfere in the evaluation of peak areas.

Relative concentration of the coupling structures I, II and III

The amount of I can be calculated if it is assumed that the positively charged groups on butyl-Sepharose 4B emanate only from the coupling structures II and III. Subtracting the amount of cationic charge at pH 3 (5.30 μmole/g swollen gel), where II and III are positively charged, from the total concentration of butylamine coupled to the gel (9.69 μmole/g swollen gel) gives the amount of I as 4.39 μmole/g swollen gel. However, it is not possible to evaluate the individual concentrations of the coupling structures II and III, as the pH dependence of the cationic charge does not differentiate between the two structures (Fig. 2). The total concentration of II and III is 5.30 μmole/g swollen gel (see above) and the way in which this amount is distributed between the charged and uncharged forms of II and III at different pH can easily be calculated from Fig. 2. For example, at pH < 5 and pH > 10 the total concentration of the uncharged forms of II and III is 0.00 and 5.30 μmole/g swollen gel, respectively, whereas at pH 7.0 and 8.0 it is 1.65 and 3.00 μmol/g swollen gel, respectively.

CONCLUSIONS

It has been demonstrated that butylamine–agarose structures (I, II and III) formed when butylamine is coupled to cyanogen bromide-activated Sepharose 4B can be characterized with respect to the total amount of attached ligand. Further, the amount of I, the total amount of II and III (C_{II+III}) and the fraction of C_{II+III} that is positively charged at various pH values can also be determined.

The amount of negative charges on butyl-Sepharose 4B has shown to be low (*ca.* 0.1 μmole/g swollen gel) at acidic pH. However, the amount increases at basic pH, showing that butyl-Sepharose 4B contains mainly weakly acidic groups that can be ionized to negatively charged groups. Consequently, when determining the amount of III by acid–base titration these weak acids must be taken into account.

REFERENCES

1 I. Kohn and M. Wilchek, in T. C. J. Gribnau, J. Visser and R. I. F. Nivard (Editors), *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam, 1982, pp. 235–244.
2 C. R. Lowe, *An Introduction to Affinity Chromatography*, Elsevier Biomedical Press, New York, 1979, pp. 346–358.
3 R. Axen, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
4 W. B. Jakoby and M. Wilchek, *Methods Enzymol.*, 34 (1974) 1.
5 R. Axen, P. A. Myrin and J. C. Janson, *Biopolymers*, 9 (1970) 401.
6 B. Svensson, *FEBS Lett.*, 29 (1973) 167.
7 R. Jost, T. Miron and M. Wilchek, *Biochim. Biophys. Acta*, 362 (1974) 75.
8 B. H. J. Hofstee, *Biochem. Biophys. Res. Commun.*, 50 (1973) 751.
9 B. H. J. Hofstee, *Biochem. Biophys. Res. Commun.*, 53 (1973) 1137.
10 H. P. Jennissen, *Biochemistry*, 15 (1976) 5683.
11 R. L. Schnaar, T. F. Sparks and S. Roseman, *Anal. Biochem.*, 79 (1977) 513.
12 H. P. Jennissen and L. M. G. Heilmeyer, *Biochemistry*, 14 (1975) 754.
13 M. M. Werber, *Anal. Biochem.*, 76 (1976) 177.
14 W. B. Jakoby and M. Wilchek, *Methods Enzymol.*, 34B (1974) 130.
15 *Gel Filtration Theory and Practice*, Technical Bulletin, Pharmacia Fine Chemicals, Uppsala, 1979.
16 T. K. Korpela, *J. Chromatogr.*, 242 (1982) 33.