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# Influence of the nature of coupling agents on insulin adsorption on supports grafted with sialic acid for high-performance affinity chromatography

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**Abstract**

Porous silica exhibits excellent mechanical properties for use as a stationary phase for high-performance liquid chromatography. However, negative surface charges make it unusable in its native state. For this reason, silica beads are coated with dextran polymers carrying a calculated amount of diethylaminoethyl groups. Both the minimization of non-specific interactions and the hydrophilic character of such supports allow their functionalization with biospecific ligands and finally their use in high-performance affinity chromatography of biological products. The use of these modified supports in high-performance affinity chromatography requires a better understanding of various characteristics of stationary phases. For this purpose, several techniques were utilized, in particular, size-exclusion chromatography and adsorption of radiolabelled albumin. These methods provided complementary information on the structure of these supports. Coated silica-based supports were functionalized with sialic acid by means of different coupling agents. The affinity of these supports for insulin was determined by the establishment of adsorption isotherms and by high-performance affinity chromatography, to evidence the relationships between structural characteristics of the supports and their separation properties. The study of interactions between these supports and insulin allowed us to show the importance of the coupling method on the performances of supports in affinity chromatography. © 1998 Elsevier Science B.V.

*Keywords:* Coupling agents; High-performance affinity chromatography; Adsorption; Insulin; Sialic acid; Albumin

## 1. Introduction

Insulin-dependent diabetes mellitus is a syndrome of disordered metabolism with inappropriate hyperglycemia due either to an absolute deficiency of insulin secretion or a reduction in the biological effectiveness of insulin.

Several experimental approaches have been attempted to reestablish the metabolic equilibrium [1–3]. Nevertheless, the absence of convincing results made insulinotherapy (a daily insulin administration) the only solution to reestablish the glycemic homeostasis. Therefore, it is important to have methods of separation that allow an efficient and rapid purification of insulin.

High-performance affinity chromatography (HPAC) is a technique of separation broadly used for the purification of biological molecules. This method requires, on the one hand, a support that resists high pressures and is chemically modifiable and on the

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other hand, a biospecific ligand to be coupled to the support.

To date, silica has commonly been used to prepare chromatographic supports because this mineral material exhibits excellent mechanical properties; furthermore, silica has a well controlled granulometry and a defined porosity. Nevertheless, several limitations for its utilization in high-performance liquid chromatography (HPLC) exist. Native silica has silanol groups that may strongly interact with proteins, and even denature them [4,5]. Therefore, the silica surface has to be modified in order to obtain a sufficiently neutral support to avoid non-specific interactions with proteins in solution.

Various methods, based on a passivation of the silica surface with polymers can be carried out to obtain these modifications [6], such as a covalent chemical grafting of polymers to the solid support or coating of the silica with a layer of hydrophilic polymer.

Our approach [7] consisted of coating silica beads with dextran polymers carrying a calculated amount of positively charged diethylaminoethyl (DEAE) groups that neutralize negative charges of the support. The dextran layer was then cross-linked in order to reinforce the stability of the final support. These supports were used in high-performance size-exclusion chromatography (HPSEC) [7] and HPAC. Hydroxylic functions at the coated silica surface allowed us to easily graft a number of ligands [8,9].

The existence of N-acetylneuraminic acid (sialic acid) in the insulin receptor suggests that such an acid may develop specific interactions usable in affinity chromatography.

The preparation of an affinity support comprises two steps: activation of the support and ligand coupling. The first step may be defined as the reaction between an inert support and a chemical agent. This produces an intermediate called ''activated support'' that reacts with ligands resulting in their immobilization through a covalent bond.

The presence of specific chemical functions on both the ligand and the support, and the coupling agents properties contributed to the choice of a coupling method.

In the present study, we compared the efficiency of four different coupling agents, i.e., 1,4-butanediol-diglycidyl ether (BDGE), carbonyldiimidazole (CDI), epichlorhydrin (EPC) and divinylsulfone (DVS) on the performances of supports for affinity chromatography of insulin.

## 2. Experimental

### *2.1. Synthesis of chromatographic supports*

The synthesis of a coated silica support functionalized with N-acetylneuraminic acid was carried out as reported previously [10,11].

The preparation of such an affinity support is performed in two steps. First, silica beads are coated with DEAE-substituted dextran in order to mask negative charges at its surface. Second, ligands are immobilized using a coupling agent.

A sample of dextran T70 (68 000 g/mol, Pharmacia, Bois d'Arcy, France) was modified to obtain a polymer substituted with positively charged DEAE groups. The substitution reaction of dextran by 2-chloro-N,N-diethylaminoethane (Janssen, Noisy Le Grand, France) takes place in a very alkaline medium (pH 11), through the formation of an ether bond between the DEAE groups and an hydroxyl function of the glucose unit, probably in position 2.

The substitution rate of dextran with DEAE was determined by elemental analysis of nitrogen. The conditions for dextran modification for an optimal passivation were previously determined to obtain a proportion of dextran units carrying DEAE groups (Dx-DEAE) varying from 4 to 13%.

Silica beads (particle size 15–25 μm, porosity 1000 Å), kindly provided by Biosepra (Villeneuve la Garenne, France), were impregnated with a modified dextran solution (8 g of Dx-DEAE in 100 ml) adjusted to pH 11. Dextran coated silica was cross-linked with BDGE (Sigma, La Verpillière, France). The amount of Dx-DEAE covering the silica beads (SID) was determined by a spectrophotometric assay of the sugar units after acid hydrolysis and by elemental analysis of carbon. Prior to the ligand coupling, the quality of the Dx-DEAE coverage of the silica support was evaluated by testing the elution of standard proteins on the support under HPSEC conditions. A 100 μl amount of these standard proteins was injected on to the column in the presence of a 0.05 *M* phosphate buffer and 0.1 *M* NaCl at a flow-rate of 0.5 ml/min.

The immobilization of NANA extracted from

edible birds nests [11], on SID required the use of a coupling agent to create covalent bonding between the ligand and the support (SID–NANA). In this study, four coupling agents were used: BDGE, CDI, EPC and DVS.

The synthesis of an affinity support was performed in two steps: first, the activation of the support, and secondly, the immobilization of the ligand as described previously [12].

Part of the activated supports was used to perform controls and to compare the efficiency of the different coupling agents. An inactivation was operated on supports carrying coupling agents to eliminate their reactive chemical functions and thus, to avoid the covalent binding of proteins through the column. For this, activated supports were suspended in a solution of 0.1 *M* carbonate buffer at pH 8.7 which contained 1.5 ml of ethanolamine (Janssen). The mixture was slowly stirred for 48 h at room temperature and finally washed with a solution of 0.1 *M* Tris–HCl buffer at pH 8.7.

The amount of the different ligands fixed on the support was determined by a spectrophotometric assay of the coupling solution supernatant using a periodate–resorcinol method [13].

### *2.2. Analysis of accessible surfaces by radiolabelled albumin adsorption*

The protocol of albumin adsorption on the different supports was the following [14]: 8 mg of a support suspension in 100 μl of 0.05 *M* phosphate buffer and with 0.1 *M* NaCl were incubated with 300 μl of radiolabelled human serum albumin ([$^{125}$I]HSA, CisBiointernational, Gif sur Yvette, France) at various concentrations (0–2 mg/ml) for 40 min at room temperature under rotational stirring. The specific activity of albumin was 5 μCi/ml. After five washing steps, the beads were removed and adsorbed albumin was counted using a γ-counter (1470 Wizard/Wallac).

### *2.3. Adsorption isotherms of insulin on different supports*

For the determination of adsorption isotherms, a calculated amount of insulin was incubated with a fixed quantity of support. The amount of adsorbed insulin on the support is a function of its initial concentration. Adsorption isotherms were obtained; the affinity constant of insulin for the support and the maximal adsorption capacity of the active support were determined using the Langmuir model.

The protocol of insulin adsorption on different supports was the following: 10 mg of a support suspension in 100 μl of 0.05 *M* phosphate buffer, 0.1 *M* NaCl, pH 7.4 were incubated with 300 μl of radiolabelled insulin ([$^{125}$I]insulin), at various concentrations (0–200 n*M*) for 40 min at room temperature under rotational agitation. After five washing steps, the beads were removed and adsorbed insulin was counted with a γ-counter.

### *2.4. High-performance liquid chromatography*

The HPLC apparatus consisted of a pump (L-6210; Merck) monitored by a programmer and equipped with an injection valve (Model 9126, Rheodyne), connected to a UV–visible spectrophotometeric detector (L-4000; Merck), an integrator (D-2520 GPC integrator; Merck) and a fraction collector (Model 203, Gilson).

The insulin used in HPAC, kindly provided by Diosynth (AKZO, Eragny-Sur-Epte, France), was a mixture of porcine insulin with 6% bovine insulin.

A 100 μl volume of insulin was injected on to the column containing the support at a flow-rate of 0.5 ml/min. The fractions corresponding to the elution and desorption peaks were collected and analysed by the Bradford assay to calculate the purification yield.

## 3. Results and discussion

### *3.1. Synthesis of chromatographic supports*

DEAE groups were attached to the saccharidic units of dextran in order to confer a weak anion-exchange capacity to the polymer and to make it usable for silica coating. The dextran substitution rate by DEAE groups was 5%.

The adsorption of Dx-DEAE on silica beads results from interactions between anionic groups on the silica surface and DEAE groups carrying positive charges (Fig. 1). The coverage was further reinforced by cross-linking dextran chains with BDGE, which leads to the formation of ether type bonds with polysaccharide hydroxyl groups. The polymeric

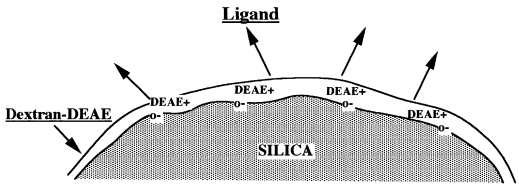


Fig. 1. Schematic representation of coated silica.

Table 1
Coupling yields of sialic acid on coated silica supports

| Supports | Initial NANA (mg/g SID) | Fixed NANA (mg/g SID) | Yield (%) |
|---|---|---|---|
| SID–CDI–NANA | 20 | 8 | 40 |
| SID–BDGE–NANA | 20 | 10 | 50 |
| SID–EPC–NANA | 20 | 5 | 25 |
| SID–DVS–NANA | 20 | 14 | 70 |

coverage on the silica supports was 35 mg of Dx-DEAE per gram of silica.

In order to ascertain that the SID support used for coupling of the ligand will not develop non-specific interactions with standard proteins, the latter were eluted from the SID support under HPSEC conditions (Fig. 2).

The results show an elution of proteins in the order of decreasing molecular mass from the selected support. No interaction occurred between the supports and the different standard polymers under our experimental conditions. The SID support is neutral enough to be coupled to a biospecific ligand.

Coupling of sialic acid with SID support was performed using four different coupling agents: BDGE, CDI, EPC and DVS.

The amount of NANA coupled with SID was determined by a colorimetric assay using a periodate–resorcinol method. Characteristics of the different synthesized supports are presented in Table 1.

The results show that the coupling yields vary. The amount of NANA fixed on the support depends both on the nature and the efficiency of the selected coupling agent. In the present study, the best coupling yield was obtained with DVS and BDGE. This may be due to the higher chemical reactivity of both coupling agents; moreover, the length of their structural chain allows a greater accessibility to the ligand.

### *3.2. Analysis of surfaces by radiolabelled albumin adsorption*

To estimate the surface of the different supports in contact with proteins, we determined the adsorption amount of radiolabelled albumin on the supports. Albumin was chosen as a model protein because it adsorbs on most surfaces in a non-specific way. First, we used non-porous silica beads of known diameter (1.3 μm); for such beads, the surface accessible to proteins was equal to their geometric surface. Their specific surface was 21 $cm^2$ per mg of silica.

The affinity constant of albumin for silica, $K_{aff}$, amounts to $1.75 \cdot 10^5$ $M^{-1}$; the $B_{max}$ value of bound

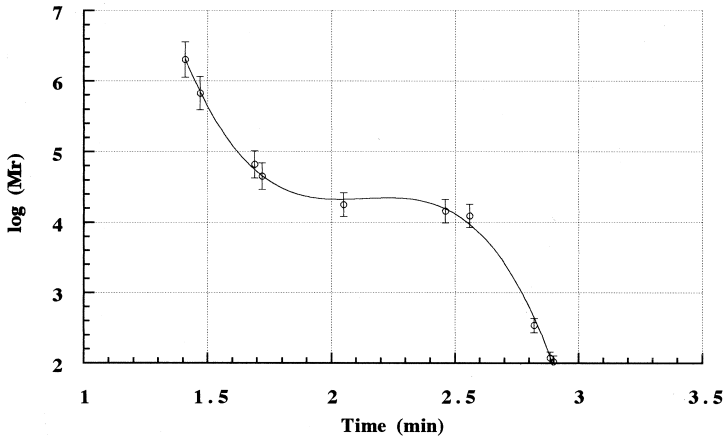


Fig. 2. Standard curves of proteins on coated silica support.

[$^{125}$I]HSA concentration at saturation is around 0.11 $\mu g/cm^2$ and represents the formation of an albumin monolayer at the silica surface.

In this way, [$^{125}$I]HSA adsorption was performed onto different supports (porous silica, SID and SID-NANA).

Adsorption isotherms presented in Fig. 3 show that the amount of adsorbed [$^{125}$I]HSA increased with the initial [$^{125}$I]HSA concentration until it reached a plateau corresponding to the maximal adsorption capacity of the support.

Adsorption isotherms were analysed according to the Langmuir model; the affinity constant is $10^5\ M^{-1}$ whatever the support; this value reveals a non-specific adsorption of albumin on different supports.

The $B_{max}$ values allow the determination of the surfaces accessible to proteins if it is considered that adsorbed albumin forms a monolayer whatever the support (Table 2). The surface accessible to albumin developed by each support was calculated as follows:

$$SAA = \frac{B_{max}\ (\mu g/mg)}{0.11\ (\mu g/cm^2)}\ (cm^2/mg)$$

where, SAA = surface accessible to albumin; $B_{max}$ = maximal adsorption capacity; and 0.11 $\mu g/cm^2$ = $B_{max}$ of albumin on non-porous silica beads.

In the case of porous silica, the surface value amounts to 73 $cm^2$ per mg of support which indicates that non-specific interactions due to silanol groups on silica surface are important. The coating of silica with Dx-DEAE minimized non-specific interactions between albumin and the support by hindering silanol functions and resulted in a decrease in the accessible surface (2.35 $cm^2/mg$).

Table 2
Parameters of radiolabelled albumin adsorption on different supports

| Supports | $K_{aff}$ ($M^{-1}$) | $B_{max}$ ($M$) | SAA $cm^2/mg$ |
|---|---|---|---|
| Silica | $1.72 \cdot 10^5$ | $2.52 \cdot 10^{-6}$ | 73 |
| SID | $4.08 \cdot 10^5$ | $7.95 \cdot 10^{-8}$ | 2.35 |
| SID–BDGE–NANA | $6.09 \cdot 10^5$ | $8.50 \cdot 10^{-8}$ | 2.51 |
| SID–EPC–NANA | $5.36 \cdot 10^5$ | $4.70 \cdot 10^{-8}$ | 1.38 |
| SID–DVS–NANA | $2.03 \cdot 10^5$ | $1.3 \cdot 10^{-7}$ | 3.84 |
| SID–CDI–NANA | $5.38 \cdot 10^5$ | $1.28 \cdot 10^{-7}$ | 3.79 |

SAA = Surface accessible to albumin.

No significant variation of the surface was observed after ligand grafting whatever the coupling agent. We can assume that the small amount of ligand fixed on the different supports did not modify the polymeric layer at the supports surface; therefore, the maximal adsorption capacity remained the same.

Moreover, the ligand grafting did not generate non-specific interactions that could increase albumin adsorption. Eventually, the presence of ligand on the support did not influence albumin adsorption.

### 3.3. Insulin adsorption isotherm on supports grafted with NANA

Adsorption isotherms for insulin on different supports were established in order to define the potential selectivity of the ligand and to estimate the

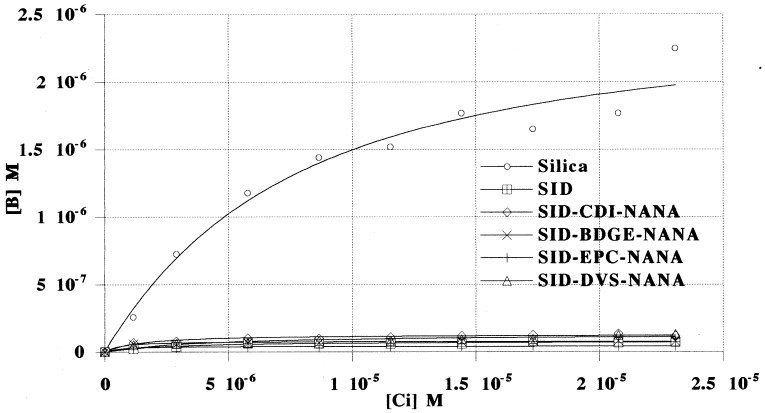


Fig. 3. Adsorption isotherms of radiolabelled HSA on different supports.

strength of the ligand–insulin interactions by measurement of the affinity constants.

Adsorption isotherms were obtained by incubating a variable amount of insulin with a fixed amount of support. They are presented in Fig. 4.

From these adsorption isotherms, the affinity constants of insulin for the different supports ($K_{aff}$) and the maximal adsorption capacities ($B_{max}$) of the latter were determined according to the Langmuir model (Table 3). In this model, it is assumed that insulin interacts with a single active site so that the amount of adsorbed insulin at saturation represents the number of accessible sites for insulin on each support.

Affinity constants and maximal adsorption capacities amount respectively, to about $10^8$ $M^{-1}$ and $10^{-7}$ $M$ whatever the support. The $K_{aff}$ values indicate the existence of strong interactions between insulin and the different supports grafted with NANA.

Furthermore, the nature of the coupling agent did not influence the affinity of insulin for immobilized sialic acid. The grafting of sialic acid on the support by using four coupling agents was probably achieved in a similar way, and the chemical functions generated from coupling agents did not influence ligand–insulin interaction.

Table 3
Parameters of [$^{125}$I]insulin adsorption on supports functionalized with sialic acid

| Supports | $K_{aff}$ ($l/M$) | $B_{max}$ ($M/g$) |
|---|---|---|
| SID–BDGE–NANA | $3.0 \cdot 10^8$ | $2.7 \cdot 10^{-7}$ |
| SID–CDI–NANA | $1.9 \cdot 10^8$ | $2.5 \cdot 10^{-7}$ |
| SID–DVS–NANA | $7.0 \cdot 10^8$ | $3.8 \cdot 10^{-7}$ |
| SID–EPC–NANA | $1.8 \cdot 10^8$ | $2.3 \cdot 10^{-7}$ |

### *3.4. Elution of insulin on different functionalized supports*

We proceeded to the elution of an insulin mixture (porcine + 6% bovine) on the different supports grafted with sialic acid by using four coupling agents: CDI, BDGE, EPC and DVS.

The different elution profiles are reported in Fig. 5. The protein concentrations in the different fractions were determined by a Bradford assay (Table 4). The adsorption yields were calculated as the ratio between the amount of adsorbed insulin and the quantity of insulin injected onto the column.

The yields of adsorbed insulin on different supports are high and are comprised between 60 and 90%. They vary with the amount of ligand fixed on each support.

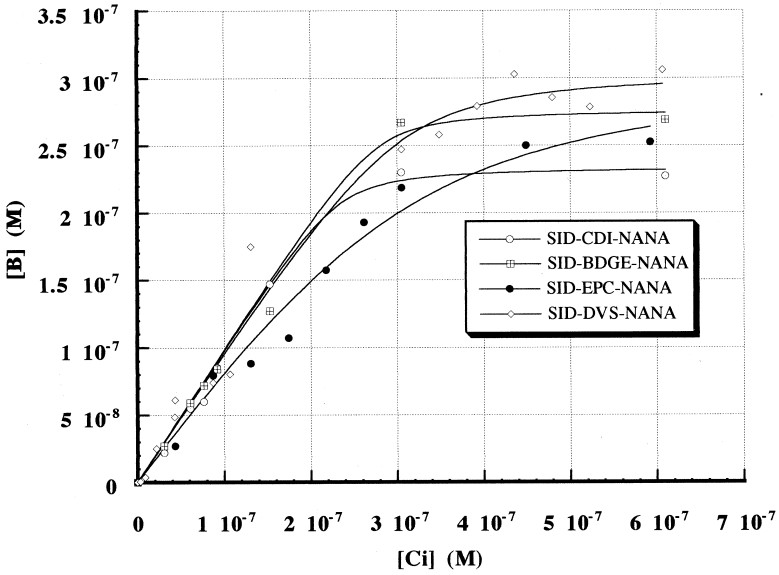


Fig. 4. Adsorption isotherms of radiolabelled insulin on different supports.

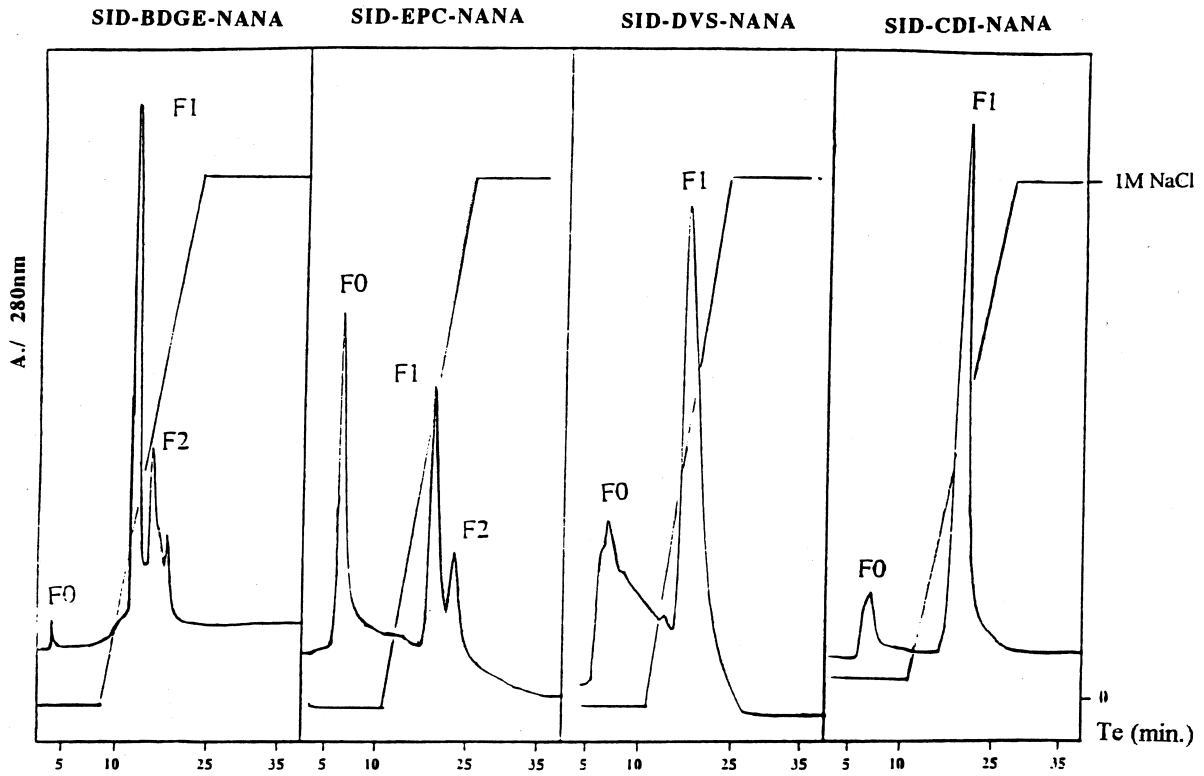


Fig. 5. Elution of 100 μl of porcine insulin (6% bovine) on SID–NANA. Ta: bidistilled water with 5% methanol, pH 8.0. Tb: 0.05 *M* phosphate buffer, 1 *M* NaCl, pH 8.0. Ta: Adsorption buffer; Tb: desorption buffer.

Chromatograms show the presence of two types of profiles including either one or two desorption peaks. Fraction analysis by the Bradford assay shows that the ratio of both peak areas is consistent with the initial insulin mixture. Bovine insulin (6%) develops a higher affinity for sialic acid than porcine insulin since it is eluted the last.

A quantitative comparison between the different supports was difficult because the amount of immobilized ligand on each support was different. Nevertheless, SID–BDGE–NANA and SID–EPC–NANA supports appeared significantly more selective as can be deduced from the presence of two desorption peaks corresponding to the elution of each type of insulin.

Among the characteristics of the prepared station-

Table 4
Protein concentration in the different functions

| Supports | Injected quantity (μg) | F0 (μg) | F1 (μg) | F2 (μg) | $\frac{(F1+F2)}{Q}$ (%) |
|---|---|---|---|---|---|
| SID–BDGE–NANA | 93 | – | 86 | 5 | 97 |
| SID–EPC–NANA | 100 | 34 | 58 | 4 | 62 |
| SID–DVS–NANA | 100 | 12 | 83 | – | 83 |
| SID–CDI–NANA | 80 | 15 | 64 | – | 80 |

F0: Non retained fraction; F1 and F2: retained fractions.

ary phases, the coating quality is certainly the most important parameter because it can strongly influence the coupling capacity of the supports. Indeed, the polysaccharidic chains coating silica exhibit a dynamic conformation and can be considered as a succession of ''loops'', ''trains'' and ''tails''. These structures may influence the steric accessibility of the active site, depending on whether the ligand is fixed on a ''loop'' or on a ''train''. Nevertheless, we assume that the accessibility to the ligand does not influence insulin fixation on the support because it can enter the pores of the support to access to the ligand.

This difference has two main causes: on the one hand, the fixation mechanism of the ligand depends on the coupling agent used; this can lead to conformational changes of the ligand and therefore may change both selectivity and activity of the support; on the other hand the coupling agent itself can modify the steric accessibility of the active site. For instance, BDGE creates a spacer arm between the support and the ligand, thus, improving the steric accessibility of the ligand.

### *3.5. Control of the affinity of the supports for insulin*

To compare and demonstrate the specificity of the functional supports, we performed the elution of a mixture of porcine insulin with 6% bovine insulin on both SID support and the four supports bearing inactivated coupling agents.

Insulin elution on a SID support shows that the whole insulin is present in the non-retained fraction. We deduce that the silica-based coated support is inert and does not develop any notable interaction with insulin in solution under our experimental conditions.

The elution of insulin on the supports carrying coupling agents (Fig. 6) shows its retention on these supports. The insulin concentration in the different fractions was determined by the Bradford assay (Table 5).

The amounts of insulin retained on supports activated by CDI and DVS are important and show the contribution of both coupling agents in the support–insulin interaction. The adsorption of insulin on supports functionalized with sialic acid and

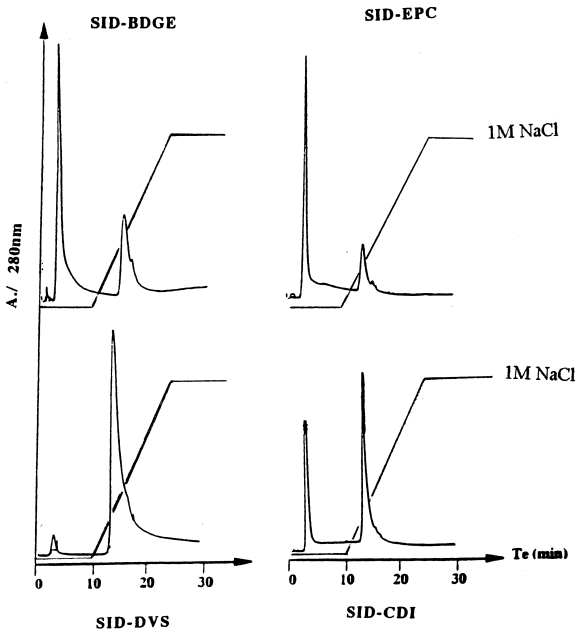


Fig. 6. Elution of 100 μl of porcine insulin (6% bovine) on supports bearing coupling agents. Ta: Bidistilled water with 5% methanol, pH 8.0. Tb: 0.05 *M* phosphate buffer, 1 *M* NaCl, pH 8.0.

activated by CDI and DVS is essentially a non-specific mechanism under our experimental conditions. So, it appears necessary to find more selective elution conditions to use these supports in affinity chromatography.

The weak fixation yields of insulin on supports activated by BDGE and EPC may indicate that the latter are weakly involved in the interaction with insulin. These supports exhibit a selectivity towards insulin and allow the fixation of both types of insulin (porcine and bovine) with a distinct affinity and great selectivity. They are high-performance affinity phases.

## 4. Conclusions

In the present study, we showed the influence of surface characteristics of supports on their performances in HPAC.

The passivation of silica with Dx-DEAE minimizes non-specific interactions due to silanol groups between proteins and silica. The grafting of sialic

Table 5
Yields of insulin adsorption on supports grafted with coupling agents

| Supports | Injected quantity (μg) | F0 (μg) | F1 (μg) | Yield (%) |
|---|---|---|---|---|
| SID–BDGE | 100 | 87.2 | 11.4 | 11 |
| SID–EPC | 100 | 92.3 | 7.4 | 7 |
| SID–DVS | 100 | 4.8 | 94.7 | 95 |
| SID–CDI | 100 | 32.3 | 67.4 | 67 |

acid with different coupling agents does not influence the surface accessible to albumin and shows that the presence of this ligand does not create non-specific interactions between the protein and the affinity supports.

Affinity constants of insulin for different supports are high and indicate the existence of specific interactions between functionalized supports and the protein.

The results show the sensitivity of chromatographic methods and, in particular, of affinity chromatography. The study of the affinity of insulin for supports grafted with sialic acid shows no difference between these supports under static conditions.

With affinity chromatography, we noticed a different behaviour of each support towards insulin. Two types of supports emerged: supports that allow or do not allow the separation of the two forms of insulin. This difference may be explained by the fixation mechanism of the ligand and to its steric accessibility to the active site.

The elution of insulin on the supports bearing coupling agents shows the contribution of such activation agents in the interaction mechanism between insulin and chromatographic supports. The importance of these interactions varies with the nature of the coupling agent used. In the present study, the best coupling yield was obtained with DVS and BDGE which adsorbed a negligible amount of insulin. This quantity of insulin was eluted within one peak.

The grafting of sialic acid on supports allows a better adsorption of insulin (between 60 and 90%) and a selective separation of both forms of insulin. Furthermore, the amounts of insulin retained on supports activated by CDI and DVS are important and show that sialic acid grafting may hinder the accessibility of coupling agents and therefore may minimize their interactions with insulin.

Finally, supports that allow one desorption peak of insulin (SID–CDI–NANA and SID–DVS–NANA) could be used to study the influence of different physico-chemical parameters in order to find selective conditions for insulin retention. Such supports develop ''thiophilic-like'' interactions [12]. Supports that permit to obtain two desorption peaks were used to separate the different forms of insulin [15].

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