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Characterisation of silica-based heparin-affinity adsorbents through column chromatography of plasma fractions containing thrombin[1]

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Abstract

Different heparin–silicas, synthesised in this laboratory via directional end-point attachment of heparin (H) onto various amino-derivatised silicas, have been evaluated in packed-bed and expanded-bed column chromatographic experiments using crude preparations of the therapeutic protein, thrombin (T). Adsorbent capacities, determined through batch adsorption experiments, were verified by employing frontal analysis in packed-bed systems. The performance of these adsorbents was also investigated in terms of thrombin purification factors and recoveries. The potential of the heparin–silicas was further examined in the expanded-bed column chromatographic mode using a scaled-up procedure. With heparin–Fractosil 1000 adsorbents, capacities of around 100 000 U thrombin/ml adsorbent could be achieved. Heparin–Fractosil 1000 adsorbents of intermediate heparin content (around 4 mg heparin/ml sorbent) displayed binding stoichiometries similar to that of the commercial heparin–Sepharose (2.6–2.7 mol T/mol H). Furthermore, binding stoichiometries were largely unaffected by increasing the heparin content on the heparin–Fractosil 1000 adsorbents from 0.8 to 4.6 mg of heparin/ml of sorbent. This result suggests that optimal binding site accessibilities for the thrombin–heparin interaction occurs at lower ligand density values. The binding capacity values determined from frontal analysis were confirmed by the recovery data, thus indicating minimal irreversible adsorption. Specific activities of ca. 2100 U/mg were obtained for thrombin when affinity-purified on these heparin–LiChroprep Si60 or heparin–Fractosil 1000 adsorbents. These values were higher than the maximum achievable purity obtained through alternative, multi-step chromatographic purification procedures reported by other investigators. These results indicated that the packed-bed performances with these silica-based adsorbents were superior to currently available commercial soft gel adsorbents, with the more dense heparin–silicas exhibiting very good potential for use in expanded-bed applications.

Keywords: Affinity adsorbents; Heparin–silicas; Stationary phases, LC; Thrombin; Proteins

1. Introduction

The biospecific affinity interaction of immobilised heparin with the protein coagulation and anti-coagulation factors, including thrombin (T) and anti-thrombin III (ATIII) present in blood plasma, has been a well-known example of heparin-protein specificity for over two decades. In 1973, Gentry and Alexander [1] used an affinity chromatographic method with an immobilised-heparin soft gel adsorbent to purify thrombin. Thrombin preparations of various levels of purity have been obtained from plasma by a single step method [2] or following an ion-exchange step [3,4], by heparin-affinity chromatography. Typically, specific activities of thrombin of

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[1]Part CLVII of the series 'High-performance liquid chromatography of amino acids, peptides and proteins'. For Part CLVI see ref. [24].

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ca. 2000 U/mg have routinely been achieved by such procedures [2,5]. Thrombin preparations of considerably higher specific activities have also been reported, based on multi-step isolation procedures. These earlier investigations included the isolation of thrombin with specific activities of 4500 IU/mg [3] and 3248 U/mg [6]. These higher specific activity values, however, were based on the determination of the total protein content by optical absorbance measurements, using assumed extinction coefficients, and have subsequently been recognised to represent over-estimates of recovery and specific activity.

Traditionally, studies on protein–ligand interactions, including thrombin–heparin interactions, often involve pure protein samples [7]. In the present investigations, a broader characterisation of heparin affinity adsorbents, synthesised through directional end-group attachment of heparin by a reductive amination reaction to silica-based support materials [8,9], was undertaken. This characterisation included assessment of the performance of these new adsorbents in terms of their purification factors as compared to a commercial heparin adsorbent, thereby providing an objective measurement of thrombin selectivities and recoveries as well as of adsorbent performance with more crude feedstocks, similar to those employed during the plasma fractionation by a multi-stage chromatographic process on an industrial scale. A more representative purification situation was also of particular interest since these adsorbents were developed for use in expanded-bed chromatographic systems, where the physical properties of the feedstock, such as its viscosity, play important roles. For these reasons, the use of a crude prothrombin fraction, derived as a complex mixture of proteins from the Cohn fractionation of the plasma cryosupernatant, was chosen for the evaluation of these adsorbents, since thrombin can be readily assayed using chromogenic synthetic peptide substrates [10–12].

2. Experimental

2.1. Chromatographic supports

Heparin Sepharose CL-6B was obtained from Pharmacia, Uppsala, Sweden. The LiChroprep Si60 (25–40 and 40–63 μm), and Fractosil 1000 (40–63 μm) porous silica support materials were obtained from Merck, Darmstadt, Germany.

2.2. Chromatographic equipment

The K16/20, XK16 and HR 5/2 columns, P1 peristaltic pump, Frac-100 fraction collector, FPLC-equipment and the Phast system were obtained from Pharmacia. For larger scale expanded-bed column experiments, constant temperatures were maintained using a 2219 Multitemp II Thermostatic Circulator (LKB, Bromma, Sweden).

2.3. Chemicals

The chromogenic substrate S2238, for thrombin assays, was obtained from Chromogenix, Stockholm, Sweden. The protein assay reagent used in the total protein determinations was a Dye Reagent Concentrate from BioRad Laboratories (Richmond, CA, USA).

2.4. Total protein assay

The assay procedure was based on a modified version of the Bio-Rad microassay. As protein standards, BSA was diluted to 0.01, 0.025, 0.05, 0.125 and 0.25 mg/ml in 10 m*M* Tris–150 m*M* NaCl, pH 7.5 (buffer A). Samples were diluted between 1:1 and 1:100 in the same buffer. The diluted standard or sample (100 μl) was then mixed with distilled water (700 μl) and undiluted Bio-Rad Coomassie reagent (200 μl) was added. As a blank, buffer A (200 μl), distilled water (1.4 ml) and reagent (400 μl) were mixed. All mixtures were then vortex-mixed and 200 μl volumes were added to a 96-well flat bottom microtitre plate (Greiner Labortechnik, Frickenhausen, Germany). The blank mix was placed in column 1 of the plate. The absorbance at 595 nm was measured on a Titertech Multiskan MCC/340 microplate reader (Flow Laboratories, Helsinki, Finland) and protein concentrations in the samples were read from the standard curve using a linear regression program.

2.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed on a Phast System (Pharmacia-LKB, Uppsala, Sweden) using 8–25% gradient gels. Samples applied onto Phast gels were concentrated using a Speedvac Concentrator (Savant Instruments, Hicksville, NY, USA) and were then boiled in sample buffer (500 m*M* Tris–10% SDS, pH 6.8, containing Bromophenol Blue) for 3 min before loading onto the gels using 1 μl loading combs. For reduced samples, 5% (v/v) β-mercaptoethanol was added to the sample buffer. Proteins were visualised by a silver staining method [13]. As molecular mass standards, bovine serum albumin (BSA), ovalbumin (OV), carbonic anhydrase (CA), myoglobin (MYO) and cytochrome C (CYT) were used. A standard curve was generated from the plot of the log M_r of the standard proteins versus their electrophoretic migration distance and the molecular masses of the sample bands were determined using a linear regression analysis program.

2.6. Preparation of thrombin material

Inactive, crude human prothrombin preparations, designated prothrombin PTX-056, -062, -995 and -998 were activated at 37°C with a solution of thromboplastin (15 ml, extracted from placentae) per 100 ml of PTX. A solution of 800 m*M* $CaCl_2$ (3.5 ml per 100 ml PTX) was then added dropwise with continuous mixing. The mixture was allowed to react for 3 h at 25°C, with brief stirring once every hour, was filtered through three layers of dry gauze and was dialysed against 10 m*M* Tris–150 m*M* NaCl, pH 7.5, using 8000 M_r-cutoff, 32 mm dialysis tubing (Union Carbide) at 4°C. The dialysed material, designated batches B056, B062, B995 and B998, was stored at −20°C in 2, 5 or 10 ml aliquots.

2.7. Characterisation of the thrombin materials

The specific amidolytic activities of thrombin (in terms of C_o values as U T/ml) in the starting material batches used in these studies are shown in Table 1 (column 1). The protein composition of the starting materials was also characterised through SDS–PAGE analysis using 10–15% gradient SDS–polyacrylamide gels with the Pharmacia Phast system as described in Section 2.5. The S2238-specific amidolytic activities due to other proteases were found to be negligible in these batches, as determined by activity assays in the presence of hirudin, a thrombin-specific polypeptide protease inhibitor prepared from leeches (*Hirudo medicinalis*).

2.8. Thrombin assay

Thrombin was quantified in terms of its proteolytic activity with the chromogenic substrate S2238 [10–12] by measuring the colour formation rate at 405nm. Samples were diluted in assay buffer (20 m*M* Tris–100 m*M* NaCl–0.01% Brij 35, pH 8.0) to obtain a colour formation rate ranging between 0.02 and 0.2 A.U/10 min. Typically, a thrombin standard which contained 578 U/ml was diluted 1:1000. For the determination of thrombin activity, the following microtitre plate assay method was developed to allow small volumes of eluted fractions to be analysed. The reconstituted substrate, S-2238 (H–D-Phe–Pip–Arg–*p*-nitroanaline, 1 m*M* in 0.01% Brij 35) was stored frozen in 500 μl aliquots, and diluted 1:5 in assay buffer immediately before use. In the assay, diluted samples (20 μl) were placed in the wells of a 96-well flat bottom microtitre plate (Greiner), and the diluted substrate (50 μl) was added (at time 0) to the wells in rapid sequence using an Eppendorf multipipette. The absorbance at 405 nm was then measured automatically at 2 min intervals with the aid of a model 3550 Microplate reader (Bio-Rad, Japan).

Sample readings were plotted against time, generating the individual colour formation rates through first order linear regression. The slopes could thus easily be visualised, and the linear range of the colour formation selected. Colour formation rates were converted to thrombin activities relative to a thrombin standard material containing 578 U/ml (±2.1%) which was included in the assays. The standard material (2500 U/bottle, donated by CSL) was calibrated in this laboratory against an accurate thrombin standard containing 83 U of thrombin/ml (donated by CSL). Thrombin activities in the samples were related to the standard material (diluted 1:1000) using the following formula:

Table 1
Frontal analysis data from small-scale packed-bed column chromatography of crude thrombin preraration with heparin–Sepharose and heparin–LiChroprep Si60. In (A) data with commercial heparin–Sepharose (2.64 mg of heparin/ml of adsorbent) while in (B) data for the adsorbent with end-point attached heparin on Sepharose CL-4B HS 5 (1.80 mg heparin/ml adsorbent) are presented. In (C) and (D), frontal analysis data with the heparin–LiChroprep Si60 (HLC 19, 1.66 mg of heparin/ml of adsorbent) and HLC20 (1.73 mg of heparin/ml of adsorbent) are shown

C_0 (U/ml)	v_s (μl adsorbent)	V (ml)	V_0 (ml)	U/ml of adsorbent	Average U/ml of adsorbent
(A) Commercial heparin–Sepharose (2.64 mg of heparin/ml of adsorbent)					
476	245	26.3	4.10	43 130	43 130
952	245	13.7	2.32	44 220	48 240 (±8.5%)[a]
	245	14.5	2.12	48 110	
	193	12.2	1.58	52 390	
(B) HS 5 (1.80 mg of heparin/ml of adsorbent)					
952	196	5.93	1.45	21 760	21 760
(C) HLC 19 (1.66 mg of heparin/ml of adsorbent)					
238	212	10.47	1.34	10 250	10 250
476	196	4.96	1.75	7 800	9 720 (±22%)[a]
	188	6.22	1.49	11 980	
	206	5.04	1.05	9 220	
	206	5.42	1.14	9 890	
952	236	4.29	2.10	8 830	8 740 (±14%)
	206	3.58	1.44	9 890	
	206	3.75	2.13	7 490	
(D) HLC 20 (1.73 mg of heparin/ml of adsorbent)					
238	231	12.14	1.23	11 240	11 240
476	196	4.90	1.10	9 230	9 080 (±1.6%)
	196	4.85	1.17	8 940	
952	216	3.84	1.43	10 620	10 310 (±3.0%)[a]
	216	3.78	1.51	10 010	

The capacities of the adsorbents for thrombin (U/ml of sorbent) at different inlet concentrations (C_0) were determined based on the adsorbent volume (v) and the breakthrough volumes for thrombin under adsorbing (V) and nonadsorbing (V_0) conditions. Average capacities at the different inlet concentrations are also shown as the average U/ml of adsorbent values.
[a] Where multiple experiments were carried out at the same inlet concentration with different bed volumes, the mean value of the U/ml adsorbent for the combined experiments is shown.

$$\text{U/ml} = \frac{RD_s}{R_{std}} \frac{578}{1000} \quad (1)$$

where R is the sample colour formation rate minus the background rate, D_s is the sample dilution and R_{std} is the standard colour formation rate minus the background rate. Least squares regression analysis of the assay data for the 578 U/ml thrombin standard diluted between 1:500 and 1:20 000 indicated that the standard curve was linear through this dilution interval ($r=0.9975$). The lower sensitivity limit using this assay system was determined to be 0.03 U of thrombin/ml.

2.9. Preparation of affinity sorbents

The preparation of the heparin affinity adsorbents involved a two-stage procedure as follows:

2.9.1. Amino-derivatisation of silica

γ-Aminopropyltri-ethoxysilane (1.88 ml) was added to freshly distilled toluene (300 ml). Fractosil 1000 (50 g) or LiChroprep Si60 (50 g), pre-dried under high vacuum for 12 h at 180°C, was then added. The suspension was sonicated for 1 min and then refluxed for 12 h at 150°C. The aminopropyl-derivatised silicas were washed with toluene (200

ml) followed by isopropanol (150 ml) and finally dried at 110°C.

2.9.2. Coupling of heparin onto amino-derivatised silica.

Heparin (in the range of 10–200 and 50–1000 mg/g of silica for LiChroprep Si60 and Fractosil 1000, respectively) was dissolved in 0.2 *M* potassium phosphate buffer, pH 7.0 (5 ml/g of silica). The dry aminopropyl-modified silica and $NaCNBH_3$ were then added (0.1 mg $NaCNBH_3$/mg of heparin). The slurry was degassed for 5 min with careful swirling, and then the mixture was allowed to react at room temperature for 60–70 h with end-over-end mixing using a rotary suspension mixer. The adsorbents were washed on sintered glass funnels with 200 m*M* potassium phosphate buffer, pH 7.0, water and 200 m*M* sodium acetate (40 ml of each per gram of adsorbent), and then were suspended in 200 m*M* sodium acetate (40 ml/g of adsorbent). Unreacted amino groups on the modified silica surfaces were acetylated by sequential addition of acetic anhydride at 2 min intervals over 6 min (0.25 μl/mg sorbent, four times) with vigorous stirring using a suspended impeller. The pH during the acetylation reaction step was kept at between pH 7 and pH 8 by titration with 5 *M* NaOH. The reaction was continued for an additional 4 min after the last aliquot of acetic anhydride had been added. The sorbent was finally washed with 200 m*M* sodium acetate solution, pH 5 (40 ml/g of sorbent) and was stored at +4°C in the same buffer.

2.10. Column chromatographic procedures

For these studies three different experimental configurations were used, as follows:

2.10.1. Small-scale packed-bed column experiments

The heparin sorbents (0.2 ml) were packed into Pharmacia HR 5/5 columns connected to an FPLC system (Pharmacia). All solutions were filtered through a 0.22-μm Millipore filter and degassed prior to use. Duplicate runs were performed at 25°C. The dialysed thrombin-containing feedstocks were diluted to between 1:5 and 1:20 (v/v) in 10 m*M* Tris–150 m*M* NaCl, pH 7.5, (equilibration buffer). After washing the columns with equilibration buffer (4 ml), the diluted crude thrombin solution (ex. batch B056 or the other batches) was applied to the column at a flow-rate of 0.2 ml/min. In experiments where recovery and the degree of purification were studied, the columns were then washed with the equilibration buffer (approx. 40 column volumes) until the A_{280} in the eluent fell to below 0.05 a.u.f.s. The adsorbed material was eluted with 10 m*M* Tris–1.0 *M* NaCl, pH 7.5, (elution buffer). Fractions (1 ml) were collected throughout the runs and analysed for total protein and thrombin content. Columns were regenerated using 10 m*M* Tris–500 m*M* NaCl, pH 8, and 20 m*M* sodium acetate–500 m*M* NaCl, pH 5.0.

2.10.2. Expanded-bed chromatography using heparin–LiChroprep Si60.

The experiments were performed using 0.3 ml, 3.0 ml or 20 ml of settled adsorbent. In the 0.3 ml-scale column experiments, a three-fold bed expansion of the HLC 20 adsorbent from 1.5 to 4.0 cm bed height was obtained at a flow-rate of 0.22 ml/min (equivalent to a linear flow velocity of 1.12 cm/min) in a Pharmacia HR 5/5 column (I.D. 5 mm). The 3 ml-scale column experiments were performed in a Pharmacia XK 16/20 column (I.D. 16 mm). The adsorbent (HLC 20) was expanded 2.7 times (from 1.5 to 4.0 cm bed height) at a flow-rate of 1.85 ml/min (equivalent to a linear flow velocity of 0.92 cm/min); 3.3 times at 2.75 ml/min (1.4 cm/min linear flow-rate) or was expanded five times at 4.2 ml/min (2.1 cm/min). For the 20 ml-scale column experiments, the HLC 24 adsorbent was expanded 3.4 times (from 3.8 to 12.8 cm bed height) at a flow-rate of 7.3 ml/min (equivalent to a linear velocity of 1.4 cm/min) in a Pharmacia XK 26/60 column (I.D. 26 mm).

All solutions were filtered through a 0.22-μm Millipore filter and were degassed prior to their use. Following equilibration in 10 m*M* Tris–150 m*M* NaCl, pH 7.5 (equilibration buffer), the starting material, e.g thrombin batch B995 (14 ml, 145 ml and 1500 ml with column systems set up at the 0.3 ml, 3.0 ml and 20 ml scales, respectively), previously dialysed and diluted 1:5 in equilibration buffer, was applied. After the adsorption, the columns were washed with equilibration buffer until the A_{280} of the eluent reached a value <0.05 a.u.f.s. and the adsorbed material was then eluted with 10 m*M* Tris–

1.0 *M* NaCl, pH 7.5 (elution buffer). Fractions (1 ml, 10 ml and 20 ml for the 0.3 ml-, 3.0 ml- and 20 ml-scale column systems, respectively) were collected throughout these experiments. The columns were regenerated with 10 m*M* Tris–500 m*M* NaCl, pH 8 and pH 5. For longer periods of storage, the column was washed with 200 m*M* sodium acetate buffer, pH 5.0.

2.10.3. Expanded-bed chromatography using heparin–Fractosil 1000.

Following equilibration, the settled HFS 13 adsorbent (2 ml) was expanded three-fold (from 1.0 to 3.0 cm bed height) in a Pharmacia XK 16/20 column (I.D. 16 mm) at a flow-rate of 1.1 ml/min (equivalent to a linear flow velocity of 0.55 cm/min). The crude thrombin solution (175 ml of batch B998 that had been dialysed and diluted 1:5 in equilibration buffer) was then applied. Whilst still in the expanded mode, the column was washed with equilibration buffer until the A_{280} of the eluent reached a value <0.050 a.u.f.s. The flow was then reversed and bound material was desorbed in packed-bed mode at a flow-rate of 0.5 ml/min with elution buffer. Fractions (2 ml) were collected throughout the experiments. Column regeneration was performed as described in Section 2.10.1. A schematic representation of the experimental set-up is depicted in Fig. 1.

2.11. Evaulation of the adsorption data

Adsorption capacities were derived from frontal analysis measurements as follows:

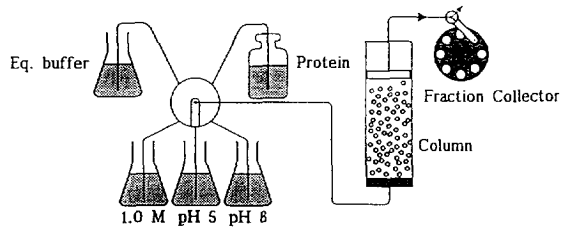

Fig. 1. Experimental arrangement for the expanded-bed experiments for the adsorption of thrombin with the heparin–silicas.

2.11.1. Frontal analysis

Breakthrough curves were generated as described previously [7,14]. Fractions collected during adsorption were analysed for thrombin content, generating a breakthrough profile (Fig. 2 Fig. 3). The total protein breakthrough curves, representing the void and saturation volumes of the system, were determined from the A_{280} measurements. The total protein breakthrough curves determined in this manner for the optical density measurements under

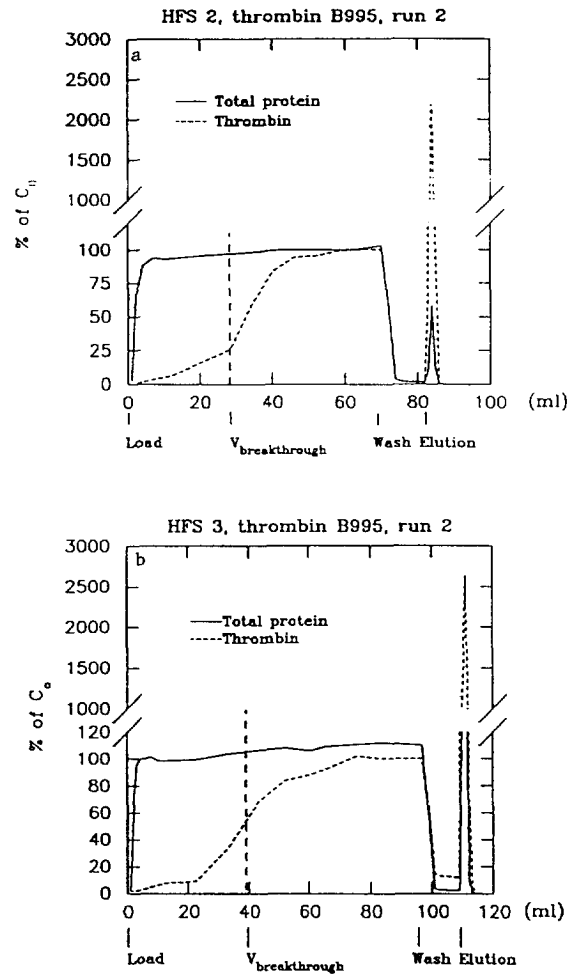

Fig. 2. Typical chromatographic profiles for the frontal analysis and elution of thrombin on heparin–Fractosil 1000. The figures show the breakthrough and elution profiles for the adsorption of thrombin on the heparin–Fractosil 1000 (HFS 2 and HFS 3) adsorbents using thrombin batch B995. Thrombin and total protein profiles are indicated.

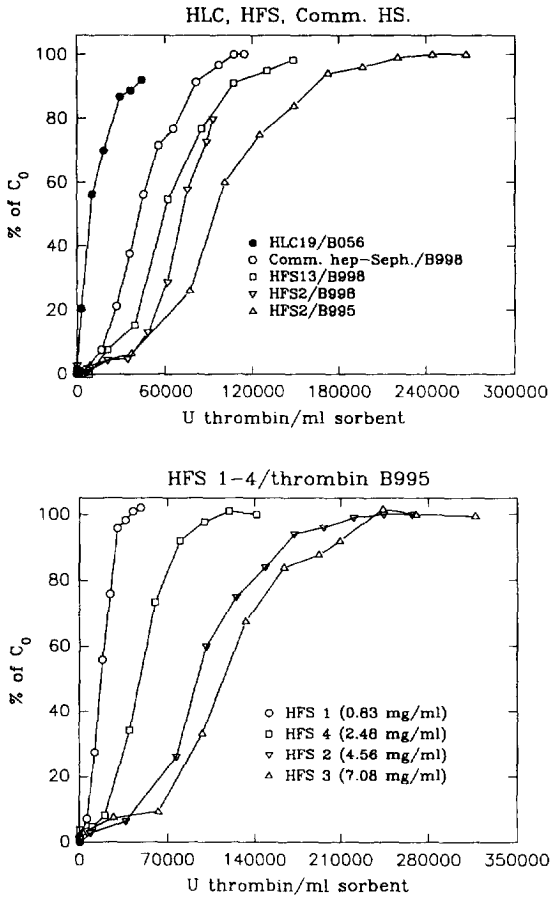

Fig. 3. Frontal analysis–breakthrough curves for thrombin on heparin–LiChroprep Si60, heparin–Fractosil 1000 and commercial heparin–Sepharose. Breakthrough profiles for the adsorption of thrombin on heparin–LiChroprep Si60 (HLC 19), heparin–Fractosil 1000 adsorbents (HFS 1–HFS 4, HFS 13) and commercial heparin–Sepharose using thrombin batches B995 and B998 are shown.

adsorbing conditions were found to be identical to the breakthrough volumes obtained under non-adsorbing conditions. Breakthrough volumes were calculated with the aid of a computer program that had been specially designed for this purpose. In order to allow direct comparisons between breakthrough curves generated in the packed-bed and expanded-bed modes, column volume differences were normalised by specifying the accumulated column output volumes in terms of U of thrombin/ml of adsorbent in the following manner:

$$\mathrm{U/ml_{sorbent}} = \frac{C_o V}{v_s} \quad (2)$$

where: C_o is the initial thrombin concentration (U/ml); V is the accumulated column output and volume (ml); v_s is the settled adsorbent bed volume (ml).

When comparing adsorbents of different heparin contents, the accumulated column output volumes were expressed in terms of U of thrombin/mg of heparin:

$$\mathrm{U/mg_{heparin}} = \frac{C_o V}{v_s h_s} \quad (3)$$

where C_o is the thrombin activity in starting material (U/ml), V is the accumulated column output volume (ml), v_s is the settled volume (ml) and h_s is the heparin content (mg/ml) of the adsorbent.

2.11.2. Determination of adsorption capacities

Adsorbent capacities were determined from the following relationship:

$$C = \frac{C_o(V - V_o)}{v_s} \quad (4)$$

where C is the U of thrombin/ml of adsorbent, C_o is the thrombin activity in the starting material (U/ml), V is the breakthrough volume for thrombin (ml), V_o is the total protein breakthrough volume under either adsorbing or non-adsorbing conditions (ml) and v_s is the settled adsorbent bed volume (ml). Binding capacities in terms of mol thrombin/mol heparin were then calculated as described elsewhere [7,9]. Heparin contents of the adsorbent used in these experiments, specified in terms of settled adsorbent volume, were determined using the dry weight-to-settled volume ratios, as presented elsewhere [8].

2.12. Experimental configuration

The following three experimental configurations were employed in these investigations:

2.12.1. Small-scale packed-bed column experiments with heparin–LiChroprep Si60 and heparin–Sepharose

Two heparin–LiChroprep Si60 sorbents (HLC 19 and HLC 20 of 25–40 and 40–63 μm particle size,

respectively) were compared with commercial heparin–Sepharose and a previously prepared HS 5 heparin–Sepharose adsorbent [8], by frontal analysis on a Pharmacia FPLC system using three different dilutions (1:5, 1:10 and 1:20) of thrombin batch B056 as the crude starting material. The generated data are shown in Table 2.

2.12.2. Small-scale packed-bed column experiments with heparin–Fractosil 1000 and heparin–Sepharose

The chromatographic performance of the different heparin–Fractosil 1000 sorbents was studied using thrombin batches B995 and B998 diluted 1:5 in 10 m*M* Tris–150 m*M* NaCl, pH 7.5, as the starting materials. Thrombin batch B995 was used with the HFS 1–HFS 4 adsorbents, and thrombin batch B998 was used with the HFS 2 and HFS 13 adsorbents and with commercial heparin–Sepharose. The experiment with the HFS 2 adsorbent using thrombin batch B998 was performed for comparison. In order to operate in overload mode for the frontal analysis, much larger volumes of thrombin material were required with the heparin–Fractosil 1000 adsorbents than with the heparin–LiChroprep Si60 adsorbents, due to the greater capacities of the heparin–Fractosil 1000 adsorbents. The capacities determined were also compared with the amount of thrombin recovered on elution.

2.12.3. Expanded-bed chromatographic experiments

Both the low-capacity heparin–LiChroprep Si60 and the high-capacity heparin–Fractosil 1000 sorbents were tested in expanded bed chromatographic experiments. The heparin–LiChroprep Si60 adsorbents of 40–63 μm particle size (HLC 20 and HLC 24) were employed using 0.3 ml and 3 ml settled adsorbent (HLC 20) or 20 ml settled adsorbent (HLC 24). The heparin–Fractosil 1000 (HFS 13) adsorbent was studied at a 2.0-ml scale. Flow-rates were chosen in order to obtain approximately a three-fold bed expansion in each case. In the 3 ml-scale experiments with the HLC 20 adsorbent, the effect of bed expansion was also studied by using three different flow-rates. Due to the relatively low capacity of the HLC 20 sorbent, it was possible to employ frontal analysis procedures at the larger scales.

Table 2
Binding capacity data for packed-bed chromatographic measurements

Adsorbent	Heparin content (mg/ml of adsorbent)	Thrombin batch	Frontal analysis		Elution	
			U T/ml of adsorbent	mol T/mol H	Recovery (%)	Purification factor
Comm HS	2.64	B056	45 680 (±13.6%)	2.72	65±9	10±0.5
HS 5	1.80		21 760	1.90	92±8	8±1.3
HLC 19	1.66		9 570 (±10.8%)	0.92	89±13	6±1.8
HLC 20	1.73		10 210 (±10.2%)	0.93	91±9	12±2.5
HFS 1	0.83	B995	18 000 (±2.6%)	3.41	79±3	12±1.0
HFS 4	2.48		51 610 (±12.8%)	3.26	96±15	13±2.4
HFS 2	4.56		92 040 (±10.7%)	3.17	102±6	14±1.4
HFS 3	7.08		129 900 (±7.5%)	2.88	100±1	13±2.2
HFS 2	4.56		Subsaturating conditions		102	13
HFS 2	4.56	B998	76 390	2.63	86	19
HFS 13	3.89		65 990 (±1.2%)	2.67	90±10	20±3.7
Comm. HS	2.64		45 290 (±11.7%)	2.70	67±3	19±0.3

Capacities of the heparin sorbents for thrombin as determined through frontal analysis are shown expressed in U/ml of sorbent and in molar terms (mol T/mol H). The recovery of thrombin in desorbed material, relative to total applied thrombin is designated "% recovery". The degree of purification (specific activity of desorbed thrombin relative to the specific activity in applied material) is shown in column 7. Variations (S.E.M.) between replicate runs are given in % for frontal analysis capacity data and in absolute numbers for recoveries and purification factors. Comm HS = commercial heparin–Sepharose. HLC = heparin–LiChroprep Si60. HFS = heparin–Fractosil 1000. T = thrombin. H = heparin.

The above procedures therefore offered an opportunity to determine the column capacity of this adsorbent in a scaled-up expanded-bed situation. These measurements were followed by determination of recovery (relative to the capacity determined through frontal analysis) and the degree of purification of the eluted material, through total protein and thrombin assays. Heparin–Fractosil 1000, on the other hand, had a capacity far greater than that of heparin–LiChroprep Si60, and was therefore tested under subsaturating conditions at the 2 ml-scale. With this heparin adsorbent, the performance was studied in terms of recovery (relative to the amount of applied material) and degree of purification of the recovered thrombin.

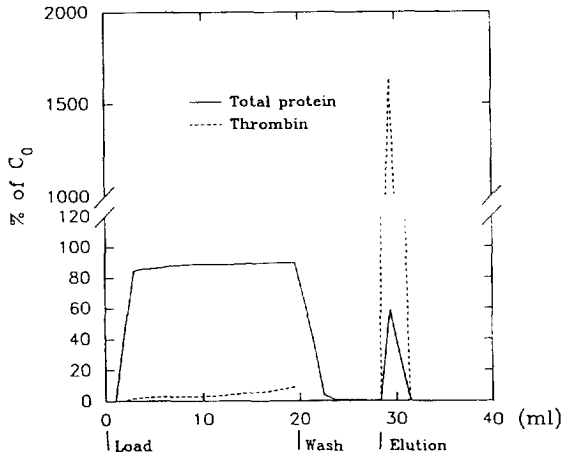

Fig. 4. Frontal analysis–breakthrough curves for thrombin with heparin–Fractosil 1000 under subsaturating conditions. Thrombin batch B995 was applied to a column containing the heparin–Fractosil HFS 2 adsorbent as described in the text. The amount of starting material was chosen in order to operate well within the column adsorption capacity. Breakthrough and elution profiles for thrombin and total protein are indicated.

3. Results and discussion

3.1. Packed-bed chromatographic fractionation of crude thrombin preparations using the heparin–LiChroprep Si60 adsorbents

A lower ligand binding site accessibility for thrombin with heparin–LiChroprepSi60 adsorbents compared to the commercial heparin–Sepharose has been demonstrated previously with batch adsorption experiments [9]. This trend was also apparent in the packed-bed chromatographic experiments carried out as part of the present study (cf. Table 1 and Fig. 3a Fig. 4a). As seen from Table 2, the binding stoichiometries (mol T/mol H), using the thrombin batch B056 with the HLC 19 and HLC 20 adsorbents, were only one third of the stoichiometry values obtained with the commercial heparin–Sepharose. Since the heparin content of the commercial heparin–Sepharose was 36% lower than that of the heparin–LiChroprep Si60 adsorbents, this result demonstrates a ca. nine-fold greater binding site accessibility with the commercial soft gel heparin adsorbent. For a more direct comparison, a laboratory-prepared heparin–Sepharose (HS 5), having similar heparin content as the HLC 19 and HLC 20 adsorbents, was also included in the study. With this heparin–Sepharose adsorbent, the heparin had been immobilised through reductive amination onto epoxy-activated Sepharose CL-4B [15]. The binding stoichiometry value with this soft gel heparin adsorbent was found to be considerably higher than that obtained with the heparin–LiChroprep Si60 adsorbents, indicating that the differences in capacity were not associated with the reductive amination procedure for heparin immobilisation per se, or with the nature of the heparin batch. Binding site accessibilities for LiChroprep Si60-based adsorbents, similar to those obtained for the commercial heparin–Sepharose, could however be achieved when lower heparin contents were introduced onto this silica-based adsorbent. This approach, however, lead to very low capacities in terms of U of thrombin/ml of adsorbent.

3.2. Packed-bed chromatographic fractionation of crude thrombin preparations using heparin–Fractosil 1000 adsorbents

3.2.1. Application of saturating conditions with the heparin–Fractosil 1000 adsorbents

With a larger accessible surface area at the same ligand densities, higher binding stoichiometries (mol T/mol H) were anticipated with the heparin–Frac-

tosil 1000 adsorbents than with the heparin–Li-Chroprep Si60 adsorbents. As evident from Fig. 3b Fig. 4b and the results listed in Table 2, the binding stoichiometries for the HFS 2 and HFS 13 adsorbents were 2.63 (±10%) and 2.67 (±1.2%) mol T/mol H, respectively, using thrombin batch B998. These values were similar to the binding stoichiometry of 2.70 (±11.7%) obtained with commercial heparin–Sepharose. As well as having similar ligand accessibilities, these three silica-based heparin adsorbents also displayed similar efficiencies for the purification of thrombin; namely 18.4-, 19.9- and 18.4-fold purification factors, respectively. Since the syntheses of these HFS 2 and HFS 13 adsorbents were based on an aqueous and an anhydrous silylation procedure for amination, respectively [8], the similar results with these two adsorbents furthermore demonstrated that, for these purposes, the choice of activation and immobilisation chemistry was not critical to adsorbent performance.

Binding stoichiometries obtained with the commercial heparin–Sepharose were practically identical regardless of whether thrombin batch B056 or B998 was used (2.72 mol T/mol H (±13.6%) and 2.70 mol T/mol H (±11.7%), respectively). The HFS 2 and HFS 13 data could therefore be directly compared with the data obtained with the heparin–Li-Chroprep Si60 adsorbents. Thus, the binding stoichiometries (and therefore the binding site accessibilities) obtained with the HFS 2 and HFS 13 adsorbents were approximately three times higher than found with the HLC 19 and HLC 20 adsorbents. This increased accessibility was obtained despite the considerably higher heparin content, in terms of the amount of heparin (mg)/ml of adsorbent on the heparin–Fractosil 1000 adsorbents, which could potentially lead to a reduced binding site accessibility through steric crowding effects.

As evident from the data shown in Table 2, the binding stoichiometries (similar in value to that found for the commercial heparin–Sepharose) only decreased marginally with a gradually increased ligand density from 0.83 mg of heparin/ml to 7.08 mg of heparin/ml of adsorbent. In particular, the access of thrombin to the ligand binding-sites was effectively unrestricted with these adsorbents, in the range of heparin contents of between 4–5 mg of heparin/ml of adsorbent. Although the lower stoichiometry observed with the HFS 3 adsorbent (2.88 mol T/mol H) indicated that the accessibility to some thrombin-binding sites on the immobilised heparin molecules had become restricted at a heparin content of 7.1 mg of heparin/ml of adsorbent, the overall decrease in binding stoichiometry (mol T/mol H) was only 15% compared to a ca. seven-fold increase in heparin content (from 0.83 to 7.1 mg of heparin/ml of adsorbent).

These results suggest that a heparin content of around 4 mg of heparin/ml of adsorbent on heparin–Fractosil 1000 (representing on average 0.05 μmol of heparin/m^2) would be an optimal value with regards to the capacity and utilisation of the binding sites present on the immobilised sulphated polysaccharide ligands. However, with lower ligand densities, a shift in the position of the breakthrough curve can be anticipated, as evident from the results shown in Fig. 3, with thrombin batches B056, B995 and B998, respectively.

A significant difference in the binding stoichiometry with the heparin–Fractosil 1000 adsorbents was observed when different batches of thrombin were used. For example, the binding stoichiometry of the HFS 2 adsorbent was 3.17 (±10%) mol T/mol H with batch B995, but only 2.63 (±10%) mol T/mol H with batch B998 (Table 2). These two thrombin batches had specific activities of 153 (±16%) and 95.2 (±20%) U/mg respectively. The binding stoichiometries were thus proportional to the specific activities of the starting materials. The capacity variations could therefore be explained either by a larger amount of other protein species competing with thrombin for the binding sites on the immobilised heparin, or be due to the presence of non-functional thrombin lacking amidolytic activity (and therefore not being detected in the enzyme assay). In practical terms, the overall capacities (in terms of U/ml of adsorbent) with some heparin–Fractosil 1000 adsorbents significantly surpassed that of the commercial heparin–Sepharose. As an example, the heparin–Fractosil 1000 with the highest heparin content (HFS3) had a thrombin capacity of ca. 130 000 U/ml of adsorbent. This value was approximately three times higher than that obtained with the commercial heparin–Sepharose.

3.2.2. Subsaturating conditions with a heparin–Fractosil 1000 adsorbent

In order to verify the capacity determinations made with frontal analysis, column experiments were performed with the HFS 2 adsorbent under subsaturating conditions, using thrombin batch B995. The capacity of the HFS 2 adsorbent was determined in preliminary studies to be ca. 92 000 U/ml of adsorbent from frontal analysis measurements using a 1:5 dilution of batch B995 (Table 2). The breakthrough profile indicated that the column was beginning to saturate after approximately 20 ml of the feedstock had been loaded onto the bed (see Fig. 3a). A 1:5 dilution of thrombin batch B995 (19.5 ml) was therefore applied onto the packed-bed column with the heparin–Fractosil HFS 2 adsorbent (0.225 ml). This applied amount was chosen in order to operate within the range of the column capacity, and thus minimise breakthrough losses. A chromatographic profile is shown in Fig. 4 and the recovery data are given in Table 2.

As can be seen from Fig. 4, the thrombin concentration at the column outlet had only reached approximately 9% of the C_0 value at the end of the loading step. The column was thus operating well within its total binding capacity. In total, 14 810±1350 units of thrombin were recovered. Since 14 600±1400 units had been applied to the packed bed, this value is indicative of (nearly) complete recovery of the applied material. Losses in the dropthrough can thus be considered insignificant, even though the amount of thrombin applied was theoretically very close to the maximum capacity value of this adsorbent. In total, 65 820 U of thrombin/ml of adsorbent were adsorbed in this experiment, representing a 71.5% saturation of the column. Furthermore, the specific activity of the eluted thrombin material was similar to that obtained when operating in the overload mode, namely 1629–1876 U/mg.

3.3. Expanded-bed column chromatographic fractionation of crude thrombin preparations using heparin–Lichroprep Si60 adsorbents

Capacity determinations, based on frontal analysis data for the adsorption of thrombin onto the expanded heparin–LiChroprep Si60 adsorbents, are shown in Table 3, together with thrombin recoveries based on the elution data. Average values for replicate runs expressed in U/ml of adsorbent and binding stoichiometries (mol T/mol H) are summarised in Table 4. As evident from these tables, the thrombin capacities of the adsorbents found by frontal analysis were similar to the thrombin recovery values found in the desorbed fractions. Thus,

Table 3
Frontal analysis data for heparin–LiChroprep Si60 in expanded-bed mode

Scale (ml)	Bed expansion	C_0 (U/ml)	V (ml)	V_0 (ml)	Capacity (U/ml of adsorbent)	Recovery (U/ml of adsorbent)
0.3	2.60	1056	5.41	2.21	11 260	11 320
	2.61	685	5.70	2.15	8 110	9 190
	(two runs)	756	6.55	2.15	11 090	9 300
3.0	2.67	621	71.6	18.1	11 070	11 820
	(two runs)	723	77.6	18.8	14 170	10 030
	3.33	753	58.8	17.2	10 440	11 600
	(two runs)	842	51.3	16.8	9 680	11 560
	5.00	777	72.0	28.8	11 190	9 800
	(two runs)	723	44.0	24.2	4 770	9 680
20	3.41	1034	783	85.2	36 080	35 260

Capacities of the HLC 20 and HLC 24 adsorbents for thrombin on three scales were determined by frontal analysis. The capacities of the adsorbents for thrombin (U/ml of sorbent) at different inlet concentrations (C_0) were determined based on the adsorbent volume and the breakthrough volumes for thrombin under adsorbing (V) and non-adsorbing (V_0) conditions. The recovery of thrombin in the desorbed fractions, relative to the adsorbent volume is shown in the last column. HLC 20 (1.70 mg of heparin/ml of adsorbent) was used in the 0.3 and 3.0 ml scales; in the 20 ml scale HLC 24 (2.50 mg of heparin/ml of adsorbent) was used.

Table 4
Average capacities derived from frontal analysis experiments with expanded-bed adsorption of thrombin on heparin–LiChroprep Si60 at different scales

Scale (ml)	Bed expansion	Frontal		Elution	
		U/ml of sorbent	mol T/mol H	U/ml of sorbent	mol T/mol H
0.3	2.61	9 600±15.5%	0.87	9 250±0.6%	0.84
3.0	2.67	12 620±12.2%	1.1	10 930±8.2%	0.99
	3.33	10 060±3.8%	0.92	11 580±0.2%	1.0
	5.00	7 980±4.0%	0.72	9 740±0.6%	0.89
20	3.41	36 080	2.2	35 260	2.2

Average binding capacity values (U/ml of sorbent) are compared with recoveries in eluted material.
The values have also been converted to molar values (mol T/mol H) to demonstrate stoichiometries in the interactions.

the average capacity of HLC 20 (a heparin–LiChroprep Si60 adsorbent containing 1.70 mg of heparin/ml of adsorbent) for thrombin in the 0.3 and 3.0 ml column volume scale was 10 060 U/ml of adsorbent, as compared to an average recovery of 10 370 U/ml of adsorbent. These values represent binding stoichiometries of 0.91 (±23%) and 0.94 (±11%) mol T/mol H, respectively. Furthermore, the binding capacities were similar to values obtained with the HLC 20 adsorbent in packed-bed experiments: 102 102 (±10%) U/ml (see Table 2, column 4).

3.3.1. Packed- and expanded-bed mode operation with the heparin–LiChroprep Si60 adsorbents

The experimental evaluation of the HLC 20 adsorbent was carried out in both the packed- and expanded-bed modes in Pharmacia HR 5/5 columns using thrombin batch B056. As evident from the breakthrough curves in Fig. 5, representing the packed-bed and expanded-bed separations, the adsorbent performed similarly in these two modes. The binding capacity determinations from the frontal analyses were thus 10 210 and 11 260 U/ml of adsorbent in the packed-bed and expanded-bed mode, respectively (Tables 2,3). These values can be considered as equivalent when the experimental errors in the system are taken into account.

3.3.2. Scale effects with the heparin–LiChroprep Si60 adsorbents

Breakthrough curves generated using the HLC 20 adsorbent at the 0.3 ml-, 3.0 ml- and 20 ml-scale are shown in Fig. 6. As evident from Fig. 6, a difference in the binding capacity existed between the 20 ml-

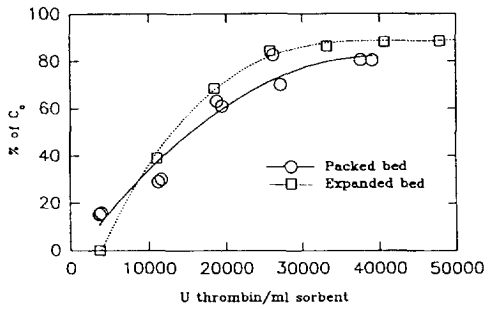

Fig. 5. Comparison between the packed-bed and expanded-bed breakthrough curves for the adsorption of thrombin on the HLC 20 adsorbent. Frontal analysis using thrombin batch B056 diluted 1:5 was performed in packed-bed and expanded-bed mode, as described in the text. The accumulated volumes in the breakthrough curves have been expressed in terms of U of thrombin/ml of adsorbent.

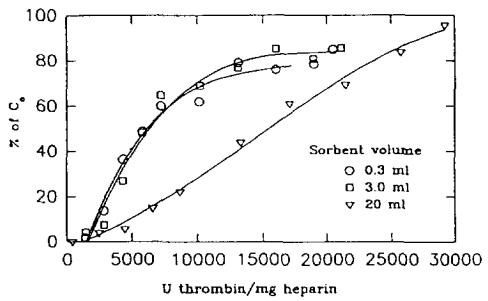

Fig. 6. Influence of column scale-up on the shape of the breakthrough curve with heparin–LiChroprep Si60 in expanded beds. Frontal analysis using thrombin batch B056 diluted 1:5 was performed in expanded-bed mode on 0.3, 3.0 and 20 ml scales, as described in the text. Accumulated volumes in the breakthrough curves have been expressed in terms of U of thrombin/mg of heparin.

scale and the two smaller scales. This difference is further evident from the results tabulated in Tables 3,4, with an approximately three-fold increase in adsorption capacity when at the 20 ml-scale. This frontal analysis capacity determination was confirmed by the recovery data (Table 4). These results thus confirm a significant scaling effect from the 3- to the 20-ml column system. No such change in capacity was observed when the same adsorbents were used in an analogous manner at the 0.3- and the 3.0-ml column scale. The adsorption capacity with the 20 ml column scale indicated a ligand binding-site accessibility that was similar to that observed with heparin–Sepharose and heparin–Fractosil 1000 in packed-bed experiments (Table 2). One possible explanation for this capacity increase is that the flow patterns with these larger column dimensions are more advantageous than in the smaller columns.

3.3.3. Effect of bed expansion with the heparin–LiChroprep Si60 adsorbents

The performance of the HLC 20 adsorbent in the 3.0 ml scale was studied at three different levels of bed expansions, by applying different flow-rates. Breakthrough curves at these bed expansions are shown in Fig. 7. Accumulated column output volumes are expressed in terms of U of thrombin/ml of adsorbent, as described in Section 2. Although the breakthrough profiles were similar at the different levels of bed expansion, a tendency towards less sharp breakthroughs with increased bed expansion was evident. The adsorption capacities, however, shifted towards lower values with greater bed expansion as seen in Table 4.

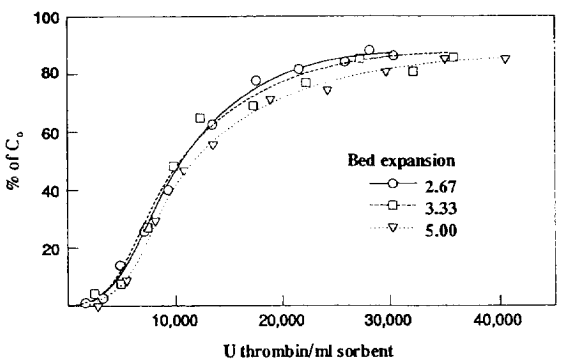

Fig. 7. Effect of bed expansion on the shape of the breakthrough curves with expanded-bed heparin–LiChroprep Si60 using 3.0 ml of adsorbent. Frontal analysis using thrombin batch B056 diluted 1:5 was performed in expanded-bed mode on the 3.0 ml scale, as described in the text. Three different flow-rates, resulting in 2.67, 3.33 and 5.0-fold bed-expansions, were used.

3.4. Expanded-bed column chromatographic fractionation of crude thrombin preparations using heparin–Fractosil 1000 adsorbent

In a production situation, columns are rarely operated in the complete overload mode, since the starting material is usually valuable. In order to minimise breakthrough losses, the adsorption is therefore terminated before the maximum column capacity is reached. The high-capacity heparin–Fractosil 1000 adsorbent (HFS 13) was tested under such subsaturating conditions in a 2 ml-scale expanded-bed system. Experiments were performed at the level of a yield of 50 000 units of thrombin per run. The capacity of the HFS 13 adsorbent for thrombin from batch B998 had been earlier determined to be 66 000 U/ml of adsorbent from packed-bed adsorption studies. In order to operate well below the maximum capacity of this adsorbent, thrombin batch B998, containing 1638 U of thrombin/ml (35 ml, diluted 1:5), was therefore applied per run on 2 ml of adsorbent (total capacity 130 000 units). Under these conditions, approximately 18% saturation was reached during the adsorption step. The recovery data indicated that, at this saturation level, the recovery of the bound thrombin from the crude sample was 91±7%, with an average specific activity of the product of 1840±16% and an average purification factor of twenty-fold. The results thus indicated that conditions for maximum yield (minimal breakthrough losses) could be accurately chosen, based on the capacity determinations for the heparin–Fractosil 1000 adsorbent.

Good reproducibility between runs was also observed, with the purification factors for the thrombin activity being slightly higher in these repeated runs than the values earlier achieved with the packed-bed experiments. Due to the high capacity of this type of affinity adsorbent, relatively small expanded beds should thus be sufficient for the recovery of thrombin and for a wide range of other purification applications.

3.5. Recoveries and purification factors

Since the expanded-bed heparin–LiChroprep Si60 experiments were performed in the overload mode, recoveries were calculated as the ratio of desorbed material to the amount bound (determined through frontal analysis measurements). These values, shown in Table 5, indicate almost complete recoveries when the experimental errors in the system are taken into account. The recoveries with heparin–Fractosil 1000 adsorbents were slightly smaller. This difference was, however, linked to the nature of the calculations, since the heparin–Fractosil 1000 experiments were performed under subsaturating conditions, and recoveries in those cases were calculated relative to the amount of thrombin applied.

As seen from a comparison of Tables 2 and 5, the purification factors in the expanded-bed system closely reflect values achieved in packed-bed experiments using the different thrombin batches. Thus, thrombin was purified 9.25-fold (±1.7) from batch B056 using the HLC 20 adsorbent in expanded-bed mode, compared to 10-fold (±0.5) using the commercial heparin–Sepharose in packed-bed mode. Under conditions of bed saturation or deliberate overload, thrombin was purified between 13.7- and 15.4-fold from batch B995 using the HLC 20 and HLC 24 adsorbents in expanded-bed mode, compared to between 11.4- and 14.1-fold using the HFS 1–HFS 4 adsorbents in the packed-bed mode, whereas thrombin was purified 17-fold (±6.3) from batch B998 using the HFS 13 adsorbent in expanded-bed mode and was purified 19.9-fold (±3.7) in packed-bed experiments using the same adsorbent. The degree of purification was thus dependent on the specific activity of thrombin in the starting material, rather than on the nature of the adsorbent used in both the packed-bed and the expanded-bed systems.

Comparison of the specific activities of thrombin purified with packed-bed or expanded-bed chromatographic systems using these new immobilised heparin silica-based adsorbents revealed that the purities achieved with the specific heparin affinity chromatographic systems prepared in this laboratory corresponded extremely favourably, and in many cases considerably exceeded, the purities achieved in multi-step isolations reported by other investigators with heparin–agarose adsorbents [2,5,16,17].

3.6. SDS–PAGE analyses

Samples of the purified fractions from the various experiments were concentrated five-fold and analysed, together with starting material, on 10–15% gradient SDS–polyacrylamide gels as described in Section 2.5. Shown in Fig. 8 is a representative SDS–PAGE result for the non-reduced samples of the eluted peak fractions from the expanded-bed purification of thrombin with the heparin–Fractosil 1000 (HFS 13, run 3, lane 2) with the corresponding starting material in lane 3. The extent of purification of thrombin achieved with an expanded heparin–

Table 5
Recovery and purification factors in the expanded-bed mode of thrombin with heparin–LiChroprep Si60 and heparin–Fractosil 1000 adsorbents

Sorbent	Thrombin batch	Scale (ml)	Bed expansion	Recovery (%)	Purification (peak fraction)
HLC 20	B056	0.3	2.60	100	9.3±1.7
HLC 20	B995		2.61	99±15	15.4±3.4
		3.0	2.67	89±18	13.4±3.6
		3.0	3.33	115±4	15.4±8.2
		3.0	5.00	145±58	14.7±3.1
HLC 24		20	3.41	98	13.7±1.8
HFS 13	B998	2.0	3.0	90.2±7.1	17.0±6.3

Recoveries are expressed as a percentage of the capacity determinations for heparin–LiChroprep Si60 (HLC) sorbents and as a percentage of the applied thrombin amounts for heparin–Fractosil 1000 (HFS). The degree of purification (specific activities of desorbed thrombin in peak fractions relative to the specific activity in applied material) is shown in the last column. Error ranges represent the variations between runs.

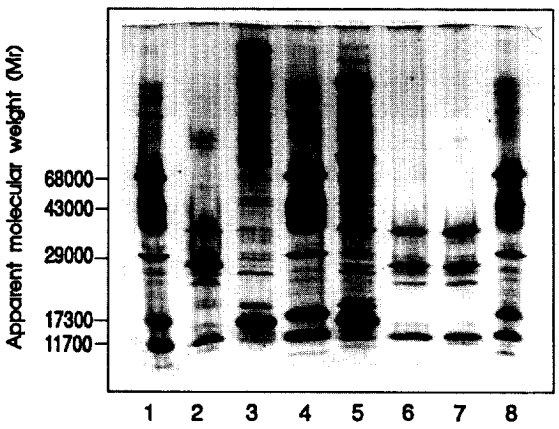

Fig. 8. SDS–PAGE results for the thrombin material purified with the heparin–Fractosil 1000 and heparin–LiChroprep Si60 adsorbents following expanded-bed runs. Samples (0.2 mg) of the crude thrombin feedstock (batches B995 and B998) and the thrombin purified using the expanded-bed mode with the HFS 13 and HLC 20 adsorbents were analysed by SDS–PAGE with protein bands visualised using a silver-staining procedure. A mixture of bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 29 000), myoglobin (M_r 17 300) and cytochrome *c* (M_r 11 700) was used as the molecular mass standards. Key: (1) M_r standards; (2) HFS 13–B998: run 3, eluted pool fraction; (3) HFS 13–B998: run 3, S/M; (4) M_r standards; (5) HLC 20–B995: S/M; (6) HLC 20–B995: eluted pool fraction; (7) HLC 20–B995: run 2, eluted fraction 4; (8): M_r standards.

LiChroprep Si60 (HLC 20, bed expansion 5.0, run 2) is shown with purified material in lanes 6 and 7 and starting material in lane 5. In particular, the intensely staining contaminating band at ca. M_r 16 000 present in the starting material is almost completely absent in the purified samples, regardless of which thrombin batch or heparin affinity adsorbent was used. A wide range of other high molecular mass material was also completely removed. An intensely staining band at M_r 37 000 in the purified material corresponds to thrombin. There are however other minor bands of lower molecular mass present, most notably bands at M_r 25 800 and 12 800. The abundance of these bands parallels the specific activities in the purified material. As evident from Fig. 8, the M_r 25 800 band is more prominent relative to the thrombin band in lane 2 than in lane 7. Lane 2 contains thrombin which was affinity purified from batch B998 on the HFS 13 heparin–Fractosil adsorbent, whilst lane 7 contains thrombin purified from batch B995 on the HLC 20 heparin–LiChroprep adsorbent. These purified materials had the specific activities 1590 and 2560 U/mg, respectively.

Similar SDS–PAGE results were also obtained for non-reduced samples of purified thrombin obtained with the packed-bed column experiments using heparin–Fractosil 1000, with no major variations in band composition in replicated experiments. Also evident in these SDS–PAGE patterns, which were independent of the adsorbent or thrombin batch used, was a band with a M_r of 34 500 that was close to the thrombin band and may be tentatively identified as non-functional γ-thrombin [18,19]. Similar results were also obtained when a sample of thrombin was affinity-purified on expanded heparin–LiChroprep Si60 (HLC 20, bed-expansion 5.0, run 1). For example, under reducing and non-reducing conditions, the SDS–PAGE results of the eluted peak confirmed that the M_r 37 000 band was replaced by a M_r 31 000 band on reduction of the recovered fraction with β-mercaptoethanol. This change in apparent molecular mass corresponds well with the molecular mass of the β-chain of thrombin, M_r 32 500, showing that the two chains migrate independently under these conditions.

These lower-molecular-mass bands in the more highly purified samples may represent breakdown products of thrombin. Thrombin is known to be autolytic [18,20]. As evident from the SDS–PAGE experimental results, the extent of self-digestion becomes more prominent as the purity of the sample increases. Boissel et al. [18] identified β-thrombin as an autolytic derivative of α-thrombin, resulting from two cleavages at Arg^{62} and Arg^{73} in the β-chain, and the β- to γ-thrombin conversion caused by the additional loss of a fragment of the B-chain extending from residues Ile^{124} to Lys^{154}. Thrombin contains 21 arginine residues, some of which are highly surface accessible. These sites include Arg^{106} and Arg^{216}, with cleavage at these amino acid residues generating a mixture of fragments, with molecular masses of 13 300, 23 700, 13 800, 9900 and 27 000, respectively. These fragments could possibly be represented as the lower molecular mass bands seen in the SDS–PAGE analyses. Since heparin is a multifunctional ligand, it is however also possible that these bands could represent other proteins that co-elute with thrombin under the experimental conditions used. Further investigations to identify the

molecular nature of these components will be described elsewhere [21].

4. Conclusions

The performance of a range of heparin–silica adsorbents, prepared by end-group attachment of heparin to aminopropyl-derivatised silicas, has been evaluated using human plasma fractions containing thrombin. These affinity adsorbent were compared with a commercial soft gel heparin adsorbent, heparin–Sepharose, in packed-bed column chromatography experiments. The rigid silica-based heparin adsorbents were further evaluated in expanded-bed column applications. The data from the packed-bed and expanded-bed column experiments can be directly compared to batch equilibrium adsorption data described elsewhere [9]. Adsorption capacities at high thrombin concentrations, determined through batch equilibrium adsorption studies, were verified by the present experiments based on frontal analysis methods. These current experiments further yielded information regarding the recovery and purification efficiencies. Recoveries with these heparin–silicas were close to 100%, indicating that very little irreversible adsorption of thrombin occurred with these adsorbents.

The results using subsaturating amounts of thrombin-containing feedstock with the large pore HFS 2 adsorbent showed that accurate predictions of column performance could be made based on the binding capacities derived from batch adsorption and frontal analysis experiments. These results from the packed-bed chromatographic applications with the rigid silica-based heparin affinity adsorbents confirm that the characteristics of high capacity, ligand accessibility and specificity can be introduced into amino-derivatised silicas through reductive amination and directed end-point attachment methods via the aldehyde group of heparin.

Based on the data from packed-bed experiments, the rigid adsorbents were then examined in expanded-bed column chromatography systems. Chromatographic experiments in expanded-bed mode were performed on three different scales with the heparin–LiChroprep Si60 adsorbent, and on a single scale with the heparin–Fractosil 1000 adsorbent. In experiments with heparin–LiChroprep Si60 adsorbents, expanded-bed frontal analysis and elution data were found to be similar to results obtained in packed-bed experiments. Expanded-bed experiments with the high-capacity heparin–Fractosil 1000 adsorbent were performed under subsaturating conditions, thus simulating larger scale production situations. On this scale, the results on the yield and purification could be anticipated on the basis of the earlier packed-bed column experiments performed on a ten-fold smaller scale. Enzyme activity measurements and SDS–PAGE analysis confirmed the high degree of purification of thrombin that could be obtained with this single heparin-affinity step.

The stoichiometries (mol of thrombin/mol of heparin), determined through batch and column experiments and summarised in Table 6, indicate that the values determined by batch adsorption measure-

Table 6
Interaction stoichiometries (mole thrombin/mole heparin) based on the comparison between the batch/column data

Sorbent	Batch data (high concentration range)	Column data	
		Packed bed	Expanded bed
Commercial heparin-Sepharose	1.81±12% (B998)	2.72±14% (B056) 2.70±12% (B998)	Not applicable
HLC 19	1.55±11% (B995)	0.92±11% (B056)	Not determined
HLC 20	1.51±4% (B995)	0.94±10% (B056)	0.94±8% (B995)[x]
HFS 13	2.37±5% (B998)	2.67±1% (B998)	Not determined

Variations (S.E.M.) in the ratio of mole T/mole heparin between chromatographic replicate runs and between replicates in batch-adsorption experiments are shown.
× = average of 0.3 and 3.0 ml scale.

ments were generally slightly lower than those obtained with the packed-bed systems using the HFS 13 (heparin–Fractosil 1000) adsorbent, a trend that was even more pronounced with the commercial heparin–Sepharose. The opposite trend was found for the heparin–LiChroprep Si60 adsorbents, with a lower capacity being obtained with the packed-bed determinations. These trends coincide with the differences in the relative porosity of the adsorbents; Sepharose and Fractosil 1000 are both macroporous materials (i.e. pore size $\geq$1000Å), whilst LiChroprep Si60 is a meso-porous material (pore-size 60Å). Pore-diffusion effects will thus contribute to these capacity/stoichiometry differences and apparent K_d effects.

The influence of mass-transfer resistance effects, such as pore diffusion, on the adsorption kinetics with dye affinity and ion-exchange adsorbent has been investigated previously in this laboratory [22–24] and similar effects were noted. For example, these earlier studies have shown that higher binding capacities could be achieved in batch equilibrium adsorption ("bath") experiments for the binding of lysozyme and human serum albumin (HSA) to dye affinity ligands immobilised to silica than those found with frontal analysis experiments. The opposite trend (a higher capacity value determined by frontal analysis) was however found with the adsorption of HSA to Trisacryl GF 2000 dye affinity adsorbent at low ionic strength. It can thus be concluded from the present study, as well as from these earlier studies, that equilibrium is expected on the basis of stochastic arguments to be reached more rapidly with the non-porous and mesoporous adsorbents than with the macroporous adsorbents, where restricted diffusion of the solute molecules within pores can occur. Secondary surface rearrangements, or reorientation of the adsorbed proteins, may also be a factor resulting in prolonged equilibrium times with porous as well as non-porous adsorbents [23], or with adsorbents in which the pores are lost through ligand immobilisation. Data obtained in the present investigations with the mesoporous HLC 19 and HLC 20 adsorbents and thrombin-containing feedstocks thus are largely consistent with our findings [22] on the interaction between proteins and other types of small pore and non-porous affinity adsorbents.

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References

[1] P.W. Gentry and B. Alexander, Biochem. Biophys. Res. Commun., 50 (1973) 500.
[2] B. Nordenman and I. Björk, Thromb. Res., 11 (1977) 799.
[3] M. Miller-Andersson, P.J. Gaffney and M.J. Seghatchian, Thromb. Res., 20 (1980) 109.
[4] M.J. Seghatchian, M. Miller-Andersson and P. Gaffney, Thromb. Haemostas., 38 (1977) 218.
[5] F.L. Zhou, D. Muller and J. Jozefonvicz, J. Chromatogr., 510 (1990) 71.
[6] I. Danishefsky, F. Tzeng, M. Ahrens and S. Klein, Thromb. Res., 8 (1976) 131.
[7] F.B. Anspach, A. Johnston, H.-J. Wirth, K.K. Unger and M.T.W. Hearn, J. Chromatogr., 476 (1989) 205.
[8] M. Björklund and M.T.W. Hearn, J. Chromatogr. A, 728 (1996) 149.
[9] M.Q. Mao and M.T.W. Hearn, Biotechnol. Bioeng. Prog., (1996), in press.
[10] U. Abildgaard, M. Lie and O.R. Ødegård, Thromb. Res., 11 (1977) 549.
[11] G. Axelsson, K. Korsan-Bengtsen and J. Waldenström, Thromb. Haemost., 36 (1976) 517.
[12] Kabi Diagnostica AB. S2238. Determination of antithrombin–heparin cofactor in plasma (laboratory instruction). Kabi Diagnostica, Stockholm, Sweden.
[13] H. Blum, H. Beier and H.J. Gross, Electrophoresis, 8 (1987) 93.
[14] K.-I. Kasai and Y. Oda, J. Chromatogr., 376 (1986) 33.
[15] H. Sasaki, A. Hayashi, H. Kitagaki-Ogawa, I. Matsumoto and N. Seno, J. Chromatogr., 400 (1987) 123.
[16] A.R. Thompson and E.W. Davie, Biochim. Biophys. Acta, 250 (1971) 210.
[17] G. Schmer, Hoppe-Seyler's Z. Physiol. Chem., 353 (1972) 810.
[18] J.-P. Boissel, B. Le Bonniec, M.-J. Rabiet, D. Labie and J. Elion, J. Biol. Chem., 259 (1984) 5691.
[19] F.C. Church, C.W. Pratt, C.M. Noyes, T. Kalayanamit, G.B. Sherrill, R.B. Tobin and J.B. Meade, J. Biol. Chem., 264 (1989) 18419.

[20] J. Hofsteenge, P.J. Braun and S.R. Stone, Biochemistry, 27 (1988) 2144.
[21] M. Björklund, G. Dasari and M.T.W. Hearn, (1996), in preparation
[22] F.B. Anspach, A. Johnston, H.-J. Wirth, K.K. Unger and M.T.W. Hearn, J. Chromatogr., 499 (1990) 103.
[23] H.-J. Wirth, K.K. Unger and M.T.W. Hearn, J. Chromatogr., 550 (1990) 383.
[24] F.W. Fang, M.I. Aguilar and M.T.W. Hearn, J. Chromatogr. A, 729 (1996) 67.