GE Healthcare

Instructions 71-7190-00 AE **Affinity media**

Heparin Sepharose 6 Fast Flow

Heparin is a naturally occurring glycosaminoglycan which serves as an effective affinity binding and ion exchange ligand for a wide range of biomolecules, including coagulation factors and other plasma proteins, lipoproteins, protein synthesis factors, enzymes that act on nucleic acids and steroid receptors. By coupling heparin to Sepharose™ 6 Fast Flow with a chemically optimized linkage, GE Healthcare provides an excellent medium for both laboratory and process scale affinity purification.

Sepharose™

Contents

1. Medium characteristics

Heparin is a glycosaminoglycan consisting of alternating hexuronic (D-glucuronic or L-iduronic) and D-glucosamine residues. The polymer is heavily sulphated, carrying sulphamino (N-sulphate) groups at C-2 of the glucosamine units as well as ester sulphate (O-sulphate) groups in various positions. (Figure 1). The heparin ligand used in Heparin Sepharose 6 Fast Flow is isolated from porcine intestinal mucosa, and has a molecular weight distribution over the range 5 000–30 000.

The base matrix, Sepharose 6 Fast Flow, consists of highly cross-linked 6% agarose beads. It provides the medium with high physical stability and excellent flow characteristics. Heparin is linked to the Sepharose matrix by reductive amination which is a very stable binding, even in alkaline conditions. The stability of this medium is limited only by the heparin itself. The essential characteristics of Heparin Sepharose 6 Fast Flow are summarized in Table 1.

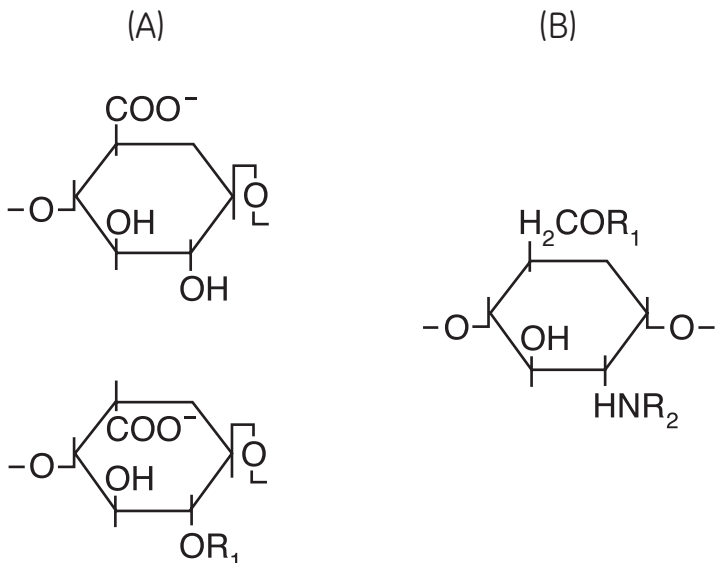


Figure 1. Heparin consists of alternating hexuronic acid (A) and D-glucosamine residues (B). The hexuronic acid can be either D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid. R_1= –H or $–SO_3^-$; R_2= $–SO_3^-$ or $–COCH_3$.

Table 1. Characteristics of Heparin Sepharose 6 Fast Flow.

Ligand density	approx 4 mg heparin/ml drained medium
Average particle size	90 µm (45–165 µm)
Bead structure	6% highly cross-linked spherical agarose,
Flow rate	≥300 cm/h at 100 kPa (XK 50/60 column, bed height 25-cm, eluent distilled water, 20 °C)
Recommended pH	
working and long term	4–12
short term, (cleaning in place)	4–13
Chemical stability (1 week, 40 °C)	0.01 M NaOH
	0.05 M sodium acetate, pH 4.0
	4 M NaCl
	8 M urea
	6 M guanidine hydrochloride
(1 week, 20 °C)	0.1 M NaOH
Autoclavable	121 °C for 20 minutes in distilled water
Delivery and storage conditions	Supplied in 0.05 M sodium acetate containing 20% ethanol

2. Packing columns

Heparin Sepharose 6 Fast Flow is supplied pre-swollen. Decant the 20% ethanol solution and replace it with binding buffer before use.

Recommended columns

Lab-scale columns

- Tricorn™ 5/20 (5 mm i.d.) for bed volumes up to 0.55 ml at bed heights up to 2.8 cm
- Tricorn 5/50 (5 mm i.d.) for bed volumes up to 1.14 ml at bed heights up to 5.8 cm
- Tricorn 10/20 (10 mm i.d.) for bed volumes up to 2.2 ml at bed heights up to 2.8 cm
- Tricorn 10/50 (10 mm i.d.) for bed volumes up to 4.56 ml at bed heights up to 5.8 cm

- Tricorn 10/100 (10 mm i.d.) for bed volumes up to 8.48 ml at bed heights up to 10.8 cm
- XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.
- XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.
- XK 50/20 (50 mm i.d.) for bed volumes up to 275 ml at bed heights up to 15 cm.

Large scale columns

- BPG™ variable bed, glass columns. Inner diameters from 100–450 mm, bed volumes from 2.4–131 litres; bed height max 83 cm.
- BioProcess™ Stainless Steel (BPSS) fixed bed columns. Inner diameters from 400–1400 mm; bed volumes from 12–1500 litres, bed height 10–100 cm.
- INdEX™ variable bed columns. Inner diameters from 70–200 mm; bed volumes up to 24.8 litres; bed heights of max 79 cm.
- CHROMAFLOW™ variable bed columns. Inner diameters from 280–2000 mm.

Packing lab-scale columns

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the column dead spaces by flushing the end-piece and adaptor with packing buffer. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with packing buffer.
3. Resuspend medium stored in its container by shaking (avoid stirring the sedimented medium). Mix the packing buffer with the medium to form 50–70% slurry (sedimented bed volume/slurry volume = 0.5–0.7).
4. Pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.

5. If using a packing reservoir, immediately fill the remainder of the column and reservoir with packing buffer. Mount the adaptor or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adaptor or in the inlet tubing.
6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 6 Fast Flow based media are packed at a constant pressure of approximately 1.5 bar (0.15 MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 500 cm/h (10 cm bed height, 25 °C, low viscosity buffer).
   If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed.

   Note: Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures using the same pump.
7. When the bed has stabilized, close the bottom outlet and stop the pump.
8. If using a packing reservoir, disconnect the reservoir and fit the adaptor to the column.
9. With the adaptor inlet disconnected, push down the adaptor approximately 2 mm into the bed, allowing the packing solution to flush the adaptor inlet.
10. Connect the pump, open the bottom outlet and continue packing. The bed will be further compressed at this point and a space will be formed between the bed surface and the adaptor.
11. Close the bottom outlet. Disconnect the column inlet and lower the adaptor approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

Packing large scale columns

General packing recommendations

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the relevant column instruction manual carefully.

Sepharose 6 Fast Flow based media are easy to pack since their rigidity allows the use of high flow rates, see Figure 2. Four suitable types of packing methods are given:

- CHROMAFLOW packing.
- Pressure packing (for columns with adaptors).
- Suction packing (for large columns with fixed bed heights).
- Hydraulic pressure packing.

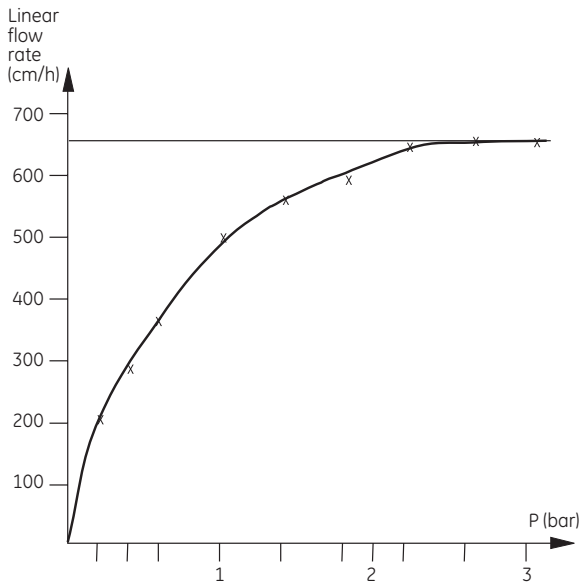


Figure 2. Pressure/flow rate curve for Sepharose 6 Fast Flow medium.

How well the column is packed will have a major effect on the result of the separation. It is therefore very important to pack and test the column according to the following recommendations.

Begin the packing procedure by determining the optimal packing flow rate. Guidelines are given below for determining the optimal packing flow rates for columns with adaptors and fixed bed heights.

Determining optimal packing flow rate

The optimal packing flow rate is dependent on column size and type, bed height, packing solution and temperature. The optimal packing flow rate must therefore be determined empirically for each individual system.

To determine the optimal packing flow rate, proceed as follows:

1. Calculate the exact amount of medium needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of medium required per litre packed bed is approximately 1.15 litre sedimented medium.
2. Prepare the column exactly as for column packing.
3. Begin running the column at a low flow rate (e.g. 30% of the expected max flow rate) and record the flow rate and back pressure when the bed is packed and the pressure has stabilized.
4. Increase the flow rate in small steps and record the flow rate and pressure at each step after the pressure has stabilized.
5. Continue recording flow and pressure until the maximum flow rate has been reached, i.e. when the flow rate levels off at a plateau indicating bed compression or when the pressure reaches the pressure specification of the column used.
6. Plot pressure against flow rate as indicated in Figure 3. The optimal packing flow rate/pressure is 70–100% of the maximum flow rate/ pressure.

The operational flow rate/pressure should be <70% of the packing flow rate/pressure.

Note: For BPSS columns, first pack the column by suction packing at a low flow rate. Then determine the flow/pressure characteristics as above by pumping buffer downwards through the column.

Packing CHROMAFLOW columns

Procedure

1. Prepare the column for packing as described in the User Manual.

Packing from the top

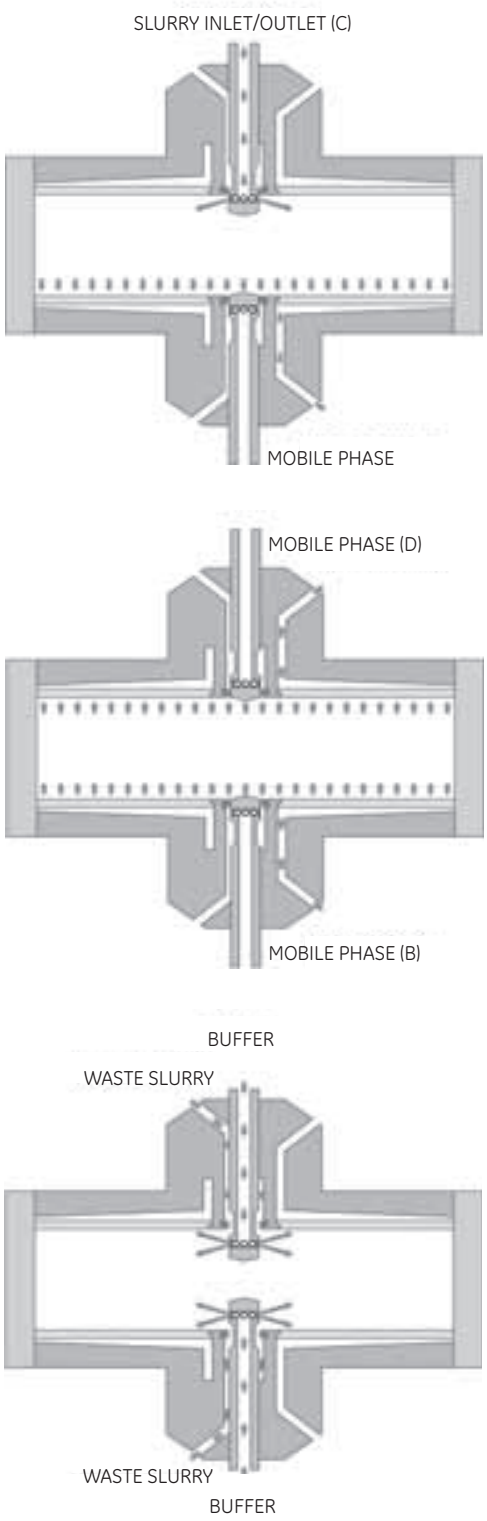
1. Set the top nozzle to the pack position (mid-position).
2. Fully retract the bottom nozzle (run position).

3. Ensure that the top mobile phase is closed.
4. Open the bottom mobile phase.
5. Open Inlet C and start the packing pump. Adjust the flow to achieve the required packing conditions for the selected medium. Monitor column pressure and the outlet flow rate in order to record column packing parameters. (Remember to stir the medium slurry during packing to prevent it from settling.)
6. Continue pumping until the column is fully packed and the pump stalls due to build-up of medium in its pipelines. Turn off the packing pump.
7. Fully retract the top nozzle to its run position. Close Outlet (C). Open Inlet (B) from the water/buffer tank and open Outlet (D). The pump should now be restarted to rinse the top slurry lines. (If the nozzle is full of liquid when in the packing position, make sure that the waste slurry outlet is open before retracting the nozzle.)
8. To clean-in-place, exchange the buffer tank for wash/buffer tank containing cleaning solution.

Packing from below

To pack from the bottom, carry out the same procedure for the connections and flow path via the bottom nozzle. The column is now ready to equilibrate and test.

Note: It is also possible to use a slightly different packing method where the amount of medium is predetermined. In this case the complete amount of medium is packed into the column causing compression of the bed. When all medium has entered the column the pump is stopped, the top nozzle is retracted, the bottom mobile phase valve closed and the medium is allowed to decompress within the column.



Packing position
The top nozzle is extended part of the way (mid position) into the column. The bottom nozzle is fully retracted. Slurry enters the column via the top nozzle and excess liquid exits via the bottom mobile phase outlet. After packing, the slurry lines are isolated from the mobile phase and can be cleaned independently from the rest of the column.

Running position
The bottom and top nozzles are retracted. Mobile phase enters the column directly into an annulus, immediately behind the bed support. The annulus is cut through at an angle to ensure that linear flow rate is kept constant during distribution of the mobile phase across the bed.

Unpacking position
In this position, both bottom and top nozzles are fully extended into the column, thereby exposing a third passage through which medium leaves the column.

Cleaning solution can be pumped through the nozzles and sprayed into the column. In this way the column is easily and effectively cleaned without exposing the interior or the medium to the environment, or without dismantling the column.

Figure 3. Principle of operation – CHROMAFLOW columns.

Pressure packing (BPG Columns)

BPG Columns are supplied with a movable adaptor. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at a constant flow rate (or back pressure).

1. Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
2. Mix the packing buffer with the medium to form a 50–70% slurry. (sedimented bed volume/slurry volume = 0.5–0.7). Pour the slurry into the column. Insert the adaptor and lower it to the surface of the slurry, making sure no air is trapped under the adaptor. Secure the adaptor in place.
3. Seal the adaptor O-ring and lower the adaptor a little into the slurry, enough to fill the adaptor inlet with packing solution.
4. Connect a pump and a pressure meter and start packing at the predetermined packing flow rate (or pressure). Keep the flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or medium.
5. When the bed has stabilized, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adaptor to 0.5–1.0 cm above the bed surface.
6. Seal the O-ring, start the pump and continue packing. Repeat steps 5 and 6 until there is a maximum of 1 cm between bed surface and adaptor when the bed has stabilized. Mark the bed height on the column tube.
7. Close the bottom valve, stop the pump, disconnect the column inlet and, without loosening the adaptor O-ring, push the adaptor down to approximately 3 mm below the mark on the column tube. The packing solution will flush the adaptor inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Suction packing (BPSS Columns)

BioProcess Stainless Steel Columns are supplied with fixed end pieces. They are packed by suction, i.e. by sucking packing solution through the chromatographic bed at a constant flow rate.

1. Fit a packing device on top of the column tube.
2. Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2–3 cm of liquid in the column.
3. Mix the packing buffer with the medium to form a 50% slurry (sedimented bed volume/slurry = 0.5). Pour the slurry into the column. Stir gently to make sure it is homogeneous.
4. Connect the column outlet valve to the suction side of a pump and start packing the bed by suction through the bed at the predetermined flow rate. Keep the flow rate constant during packing.
5. When the bed has stabilized, the top of the bed should be just below the junction between the column and the packing device.

   If, when stabilized, the level of the bed is incorrect, add or remove slurry. Always stir the slurry thoroughly before packing.
6. Just before the last of the solution has entered the packed bed (before the surface starts to dry), close the valve at the column outlet, stop the pump, quickly remove the packing device and replace it with the lid.

   Note: This final operation should be completed as quickly as possible because the bed will expand when the flow stops.
7. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

Hydraulic packing (INdEX Columns)

INdEX Columns are supplied with a hydraulic function which allows an extremely simple, rapid and reproducible packing procedure. The medium is packed at the same time as the adaptor is lowered into position at the correct pressure.

The adaptor is pushed down by a constant hydraulic pressure, forcing water or packing buffer through the slurry and compressing it so that a packed bed is gradually built up. The hydraulic pressure can be generated using a pump and a pressure relief valve.

When the adaptor reaches the surface of the settled medium, it continues downwards under hydraulic pressure compressing the medium. The extent to which the medium is compressed depends upon the pressure from the adaptor and the elasticity of the medium. The quantity of medium required when packing Sepharose 6 Fast Flow based media by hydraulic pressure is approximately 1.2 litre sedimented medium per litre packed bed.

1. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
2. Pour the 75% slurry into the column. Fill the column with packing solution up to the top of the glass tube and mix the slurry. Allow the medium to sediment to just below the bevel of the glass tube (G), see Figure 4.
3. Put the adaptor in a resting position against the bevel of the glass tube. Avoid trapping air bubbles under the adaptor by slightly tilting the adaptor while mounting.
4. Lower the lid and secure it in place.
5. Connect a pump to the inlet of the hydraulic chamber (A), with a manometer and a pressure reliefe valve in-line between the pump and the hydraulic chamber. The manometer should be placed after the valve in the direction of the flow.
6. Open the hydraulic inlet (A), and the hydraulic outlet (C). Start the pump and flush the hydraulic chamber (E) free of air and any residual medium.
7. Close (C) and open the elution inlet/outlet (B) to allow trapped air in the adaptor bed support to escape.
8. Close (B) and open the elution inlet/outlet (D) to start the packing, applying a predefined constant hydraulic packing pressure. When packing Sepharose 6 Fast Flow based media in an INdEX column to a bed

height of 15 cm, the recommended hydraulic packing pressure is 1.5 bar for INdEX 100 and 0.8 bar for INdEX 200.

9. When the adaptor has reached the surface of the settled bed, continue to run the pump until the adaptor has been lowered 5 mm into the packed bed.
10. Close (A) and (D) and stop the pump.
11. Run the column with upward flow for a few minutes to remove residual air trapped in the adaptor. The column is now ready for use.
12. To unpack the column, connect the outlet from the pump to (B) and open (C) while keeping (D) closed. This will cause the adaptor to rise from the bed surface.

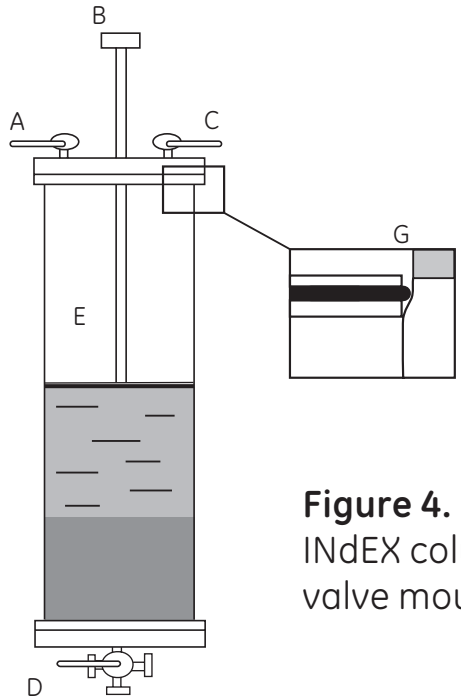


Figure 4. Schematic representation of INdEX column with a 4-port (2-way) valve mounted at the bottom outlet.

3. Evaluation of packing

To check the quality of the packing and to monitor this during the working life of the column, column efficiency should be tested directly after packing, prior to re-use, and when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate, HETP, and the asymmetry

factor, As. These values are easily determined by applying a sample such as 1% (v/v) acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent.

The calculated plate number will vary depending on the test conditions and it should therefore be used as a reference value only. It is also important that conditions and equipment are kept constant so that results are comparable. Changes in solute, solvent, eluent, sample volume, flow rate, liquid pathway, temperature, etc., will influence the results.

For optimal results, the sample volume should be at max. 2.5% of the column volume and the flow velocity between 15 and 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as part of the acceptance criteria for column use.

Method for measuring HETP and A_s

To avoid dilution of the sample, apply it as close to the column inlet as possible.

Conditions

Sample volume:	2.5% of the bed volume
Sample conc.:	1.0% (v/v) acetone
Flow velocity:	15 cm/h
UV:	280 nm, 1 cm, 0.1 AU

Calculate HETP and As from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

$$HETP = L/N$$

$$N = 5.54(V_e/W_h)2$$

where

L = Bed height (cm)
N = number of theoretical plates
V_e = Peak elution distance
W_h = Peak width at half peak height

V_e and W_h are in the same units.

To facilitate comparison of column performance the concept of reduced plate height is often used.

The reduced plate height is calculated:

$$HETP/d$$

where d is the diameter of the bead. As a guideline, a value of <3 is normally acceptable.

The peak should be symmetrical, and the asymmetry factor as close as pos-sible to 1 (values between 0.8–1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor calculation:

$$A_s = b/a$$

where
a = 1st half peak width at 10% of peak height
b = 2nd half peak width at 10% of peak height.

Figure 5 shows a UV trace for acetone in a typical test chromatogram in which the HETP and A_s values are

Column: BPG 300
Medium: Sepharose 6 Fast Flow
Bed height: 57.5 cm
Bed volume: 40.6 litres
Eluent: Distilled water
Sample: 1.05 litres (1% (v/v) acetone)
Flow velocity: 19 cm/h
V_e =18.7
W_h =0.9
HEPT=0.024 cm
a: 0.90
b: 0.85
A_s: 0.94

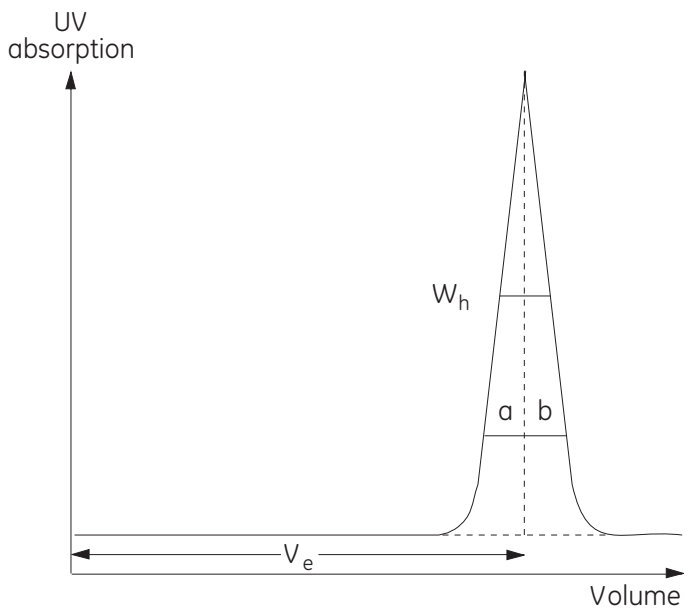


Figure 5. UV trace for acetone in a typical test chromatogram showing the HETP and A_s value calculations.

4. Operation

Binding

Immobilized heparin has two main modes of interaction with proteins. Heparin Sepharose 6 Fast Flow can be used as an affinity chromatography media; e.g. for purification of coagulation factors or nucleic binding proteins. Different substances may differ in their affinity for Heparin Sepharose 6 Fast Flow. The binding capacity of a particular protein will depend upon parameters such as buffer composition, pH, flow rate and temperature. Heparin Sepharose 6 Fast Flow might also function as a cation exchanger due to the negatively charged sulphate and carboxylate groups on the immobilized heparin ligand.

A commonly used binding buffer for the purification of plasma proteins is 10–20 mM sodium citrate buffer, pH 7.4. Since the heparin ligand acts as an affinity ligand in these cases, it may be advisable to include low concentration of NaCl in order to eliminate unspecific ionic interactions.

In other applications 10 mN sodium phospate, pH 7.0 or 20 mM Tris-HCl, pH 8.0 are often recommended as binding buffers.

Elution

Elution is commonly performed by increasing the ionic strength of the buffer. Elution using a continuous linear gradient or step gradient with NaCl, KCl or $(NH_4)SO_4$ up to 1.5–2 M is most frequently used.

5. Maintenance

For best performance from Heparin Sepharose 6 Fast Flow over a long working life, follow the procedures described below.

Cleaning-In-Place

Cleaning-in-place (CIP) is a cleaning procedure that removes contaminants that may remain in the packed column after regeneration. Regular CIP also prevents the build-up of these contaminants in the media bed and helps to maintain the capacity, flow properties and general performance of Heparin Sepharose 6 Fast Flow.

Heparin Sepharose 6 Fast Flow withstands exposure to 0.1 M NaOH for long periods with no significant loss of binding capacity for antithrombin III. When contamination is severe, 0.5 M NaOH can be used, however, a decrease in functionality will be seen over time (Figure 6). Other reagents in which the medium is stable include 8 M urea and 6 M guanidine hydrochloride.

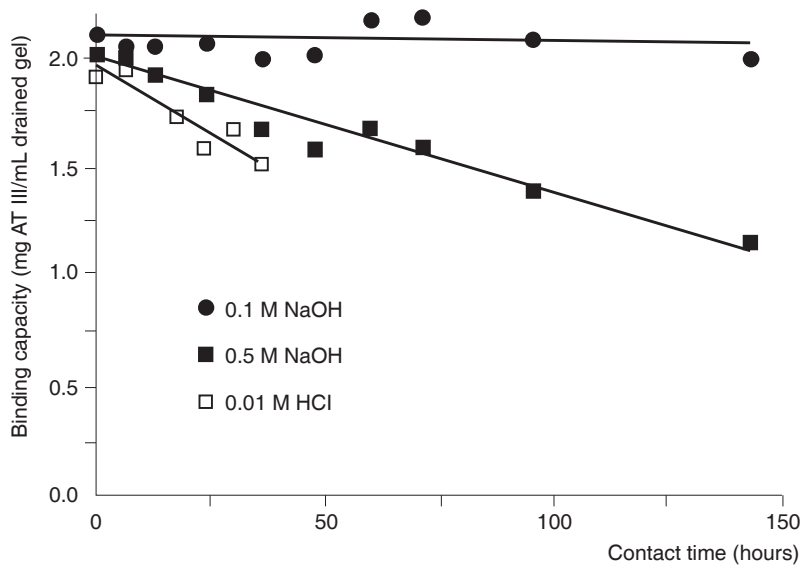


Figure 6. Functional stability of Heparin Sepharose Fast Flow was tested in three different CIP studies. CIP with 0.5 M NaOH can be used effectively over shorter periods.

Recommended cleaning-in-place procedures are summarized in Table 2. Always wash the column thoroughly with equilibration buffer after cleaning-in-place.

Table 2. CIP protocol.

For removal of time	Wash with	Column volumes	Contact
Ionically bound proteins	2 M NaCl	0.5	10–15 min
Precipitated or denatured proteins	0.1 M NaOH	4	1–2 h
	or 6 M guanidine-HCl	approx. 2	30 min–1 h
	or 8 M urea	approx. 2	30 min–1 h
Hydrophobically bound proteins	0.1–0.5% non-ionic detergent	4	1–2 h

A specific CIP protocol should be designed for each process according to the type of contaminants present. The frequency of CIP depends of the nature and the condition of the starting material, but one CIP cycle is generally recommended every 5 separation cycles.

Sanitization

For inactivation of microbial contaminants, equilibrate the column with buffer consisting of 0.1 M NaOH and 20% ethanol and allow to stand for 1 h.

Note: Alternatively, equilibrate with 70% ethanol and allow to stand for 12 h if working in an explosion-proof environment.

Wash the column thoroughly with running buffer after sanitization.

Sterilization

Autoclaving is the only recommended sterilization treatment. Equilibrate the medium with 0.5 M NaCl, pH 7. Dismantle the column and autoclave the medium at 120 °C for 30 minutes. Sterilize the column parts according to the instructions in the column manual. Re-assemble the column, then pack and test it as recommended.

Storage

Store Heparin Sepharose 6 Fast Flow at +4 to 30°C in 0.05 M sodium acetate containing 20% ethanol as preservative.

6. Ordering information

Product	Pack size	Code No.
Heparin Sepharose 6 Fast Flow	50 ml	17-0998-01
Heparin Sepharose 6 Fast Flow	250 ml	17-0998-25
Heparin Sepharose 6 Fast Flow	1 L	17-0998-03
Heparin Sepharose 6 Fast Flow	5 L	17-0998-04

Related products

Lab scale columns:		
Tricorn 5/20 column (5 mm i.d.)	1	18-1163-08
Tricorn 5/50 column (5 mm i.d.)	1	18-1163-09
Tricorn 10/20 column (10 mm i.d.)	1	18-1163-13
Tricorn 10/50 column (10 mm i.d.)	1	18-1163-14
Tricorn 10/100 column (10 mm i.d.)	1	18-1163-15
XK 16/20 (16 mm i.d.)	1	18-8773-01
XK 26/20 (26 mm i.d.)	1	18-1000-72
XK 50/20 (50 mm i.d.)	1	18-1000-71
Prepacked columns:		
HiTrap™ Heparin HP, 1 ml	5x1 ml	17-0406-01
HiTrap Heparin HP, 5 ml	1x5 ml	17-0407-01
HiPrep™ 16/10 Heparin FF	1 (20 ml)	17-5189-01
Large scale columns:		
Data File CHROMAFLOW columns		18-1138-92
Data File BPG columns		18-1115-23
Data File BPG 450 columns		18-1060-59
Data File INdEX columns		18-1115-61
Data File Bio Process Stainless Steel columns		18-1121-08

Reference literature

For general advice on lab scale use check the Affinity Chromatography Handbook from GE Healthcare, Code no. 18-1022-29.

For general advice on optimization, scaling up and other aspects relating to process chromatography we recommend:

Handbook of Process Chromatography: A Guide to optimization, scale-up and validation. Academic Press, pp 188-214 (1997). Sofer G. and Hagel, L.

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