Effect of Mobile Phase Composition on Selectivity in Preparative Hydrophobic Interaction Chromatographic Purification of Glucagon

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A weakly hydrophobic acrylester copolymer, Amberlite XAD-7, has been used in the purification process of glucagon production for elimination of protein impurities in the crude glucagon fractions, obtained from the industrial waste from insulin production.

Varying the mobile phases composition, it was shown that suitable selectivity for the separation of proteins under non-denaturing conditions can be achieved. The purified glucagon, obtained at the proposed chromatographic conditions has a biological activity of above 1 IU/mg.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) has established itself as a method for the separation of proteins. It is based on the interactions between their surface hydrophobic groups (hydrophobic patches) (e.g. Fausnaugh et al., 1984a), under conditions which maintain the original conformation of the molecule (e.g. Fausnaugh et al., 1984; Miller et al., 1984; Heinitz et al., 1988). In HIC the proteins are usually retained by the weakly hydrophobic stationary phase at relatively high salt concentrations and are eluted with a descending salt gradient (e.g. Hjerten et al., 1986; Rassi et al., 1990). Surprisingly few or no publications were found for glucagon purification.

HIC is a chromatography method that has a great deal of flexibility, with numerous operational variables that can be optimized (e.g. Miller and Karger, 1985). HIC on soft gels (as for example those known under the name Phenyl Sepharose) as well as on polymeric adsorbents has been used in the purification of many proteins (e.g. phosphorylase kinase and pohsphorylase phosphatase by Jennissen and Heilmeyer, 1975; ribonuclease A, ovalbumin and lysozyme chimotrypsin by Gooding et al., 1986; human proinsulin by Dimarchi, 1986). The stationary phases, known under the trade name Amberlite and especially Amberlite XAD-7 and Amberlite XAD-8 (Rohm and Haas Company, 1972) are acrylic acid ester copolymers. The aliphatic nature of their matrice with high poor substituents (Rohm and Haas Company, 1981) makes them weakly hydrophobic adsorbents. Their adsorptive properties are due to the combination of macroreticular porosity, controlled pore size distribution and high polar surface area. After some preliminary experiments Amberlite XAD-7 was chosen in this work for studying the effect of mobile phase composition on selectivity in preparative hydrophobic interaction chromatographic purification of crude glucagon.

Glucagon is a polypeptide hormone, secreted by the A-cells of the pancreatic islets of Langerhans. It is a single-chain polypeptide consisting of 29 amino acids. It is known to possess several pharmacological effects, such as a spasmolytic effect on smooth muscle and an inhibitory effect on gastric acid secretion (e.g. Chernish et al., 1972; Miller, 1979). Glucagon is used for the treatment of hypoglycaemia owing to its metabolic effects (e.g. Foa, 1985). The human glucagon DNA sequence encoding has been elucidated (e.g. White et al., 1986; Yoshikawa et al., 1992) and glucaton has been synthesized by a stepwise solid-phase method (e.g. Mojsov and Merrifield, 1981). The amino acid sequences of human, porcine and bovine glucagons are identical (e.g. Lochner et al., 1964), and therefore glucagon is also industrially produced by isolation and purification from the pancreas of cattle or swine (e.g. Maskalick and Anderson, 1986).

Several protein-like impurities and related compounds were found in commercial preparations of glucagon, produced by isolation from the pancreas of both cattle and swine (e.g. Mazzola et al., 1985). For purification of these proteins preparative ion-exchange, gel filtration and hydrophobic chromatographic methods were used. It has been reported that purification of these preparative chromatographic methods requires the application of multi-stage processes of repeat chromatography or additional ion-exchange purification steps (e.g. Cole, 1960; Siltz and Jackson, 1977; Maskalick and Anderson, 1986).

Glucagon is sensitive to proteolytic degradation and the crude product from the extraction of pancreas could be inhomogenous. It includes low molecular weight degradation products, mono- and didesamido glucagon, (des-His)1 glucagon, insulin residues and other pancreatic proteins (e.g. Bromer et al., 1972; Biemond et al., 1979).

Therefore a mild purification process with sufficient separation possibilities has to be used. We consider HIC as a potentially suitable method and in this study its possibilities for glucagon purification were investigated.

As a matter of fact, there are no exact theoretical rules for developing an effective preparative separation process. For example, there is no direct correlation between the size of a protein molecule and its hydrophobicity. According to Tanford (Tanford, 1961) the total hydrophobicity of a protein is determined by summing up the hydrophobicities of all residues. Bigelow (1967) calculated the average hydrophobicities from the total hydrophobicities divided by the number of residues. The chromatographic behaviour of proteins in HIC depends further on the hydrophobic surface

properties of both protein molecules and stationary phase (e.g. Gooding *et al.*, 1986). Therefore the development of a hydrophobic interaction chromatographic method for purification of glucagon requires some multiplane experiments. With a preliminary chosen stationary phase, the mobile phase composition remains the only site, and can easily be optimized.

This study is aimed at investigation of the effect of changes in mobile phase composition on selectivity in preparative HIC for purification of glucagon.

EXPERIMENTAL

Materials. Reagents were obtained from the following sources: Natural glucagon (bovine) from Calbiochem Corporation (Lucerne, Switzerland); insulin (porcine) from Novo Industri (Bagsvaerd, Denmark); Amberlite XAD-7 copolymer, obtained from Rohm and Haas Chemical Company (Frans S.A., Paris, France); Coomassie Brilliant Blue from Fluka (Buchs, Schweiz, Switzerland); Analytical standard reagent kits for radioimmunoassay of glucagon from Acad. Sci. Belarous (Minsk, Belarous).

Apparatus. (a) Glass columns (300 mm (30 mm i.d.) and 220 mm (25 mm i.d.) were used. (b) The eluent was monitored, using a UV-2 detector, Dual path monitor (Pharmacia-LKB, Sweden) at 280 nm. The quantitation of PAGE (polyacrylamide gel electrophoresis) were performed on a Glinscan densitometer (Helena Laboratories, Beaumont, TX, USA).

Methods

Polyacrylamide gel electrophoresis (PAGE). The gel electrophoresis method on a 15% polyacrylamide gel (6 mm thick, 85 m long) at pH 8.3 and pH 4.5 was used for determination the glucagon fraction in the glucagon containing material (e.g. Reinsfeld *et al.*, 1962; Ornstein, 1964; Jovin, 1973).

PAGE at pH 8.3
Samples of 100 μg of protein were applied to the gel in a solution of 0.05 M tris (hydroxymethyl) aminomethane (TRIS) and 0.001% bromphenol blue (1 μg/μL). The chamber buffer was a mixture of 0.288 g glycine and 0.032 g TRIS per L of water (pH 8.3). The running time was 4 h at 1.5 mA and 25°C.

PAGE at pH 4.5
Samples of 100 μg of protein were applied to the gel in a solution of 0.01 M β-alanin, 0.001% methyl green (corrected using 10% acetic acid to pH 4.5 (1μg/μL)). The chamber buffer was a mixture of 3.12 g β-alanin and 0.8 mL 10% acetic acid/L of water (pH 4.5). The running time was 6 h at 1.5 mA per tube at 25°C.

In both PAGE experiments (at pH 8.3 and 4.5), peptides were visualized by staining for 1 h in a 12.5% w/v solution of trichloracetic acid, developed with 0.25% Coomassie Blue in 35% methanol for 1 h and destained in 10% acetic acid.

Biological assay of glucagon. The potency of glucagon was estimated by comparing its hyperglycaemic activity with the standard preparation of glucagon. Rabbits were fasted for 20 h and then injected intravenously at doses of 6 and 24
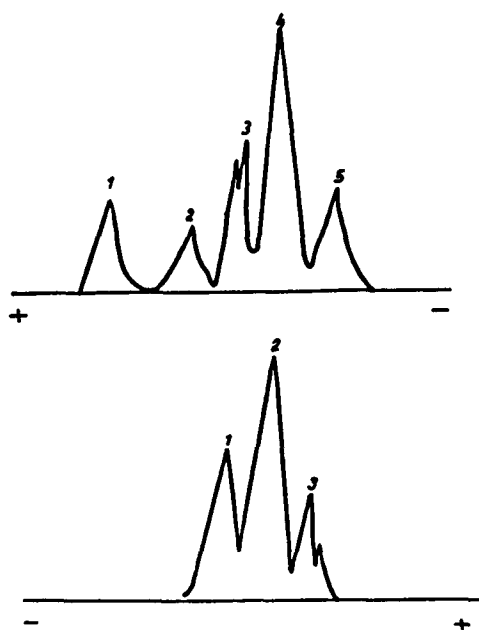

Figure 1. (A) PAGE of glucagon containing material from porcine pancreas at pH 8.3: 1, insulin; 2, proinsulin; 3, desamido glucagon; 4, glucagon; 5, polypeptide of porcine pancreas. (B) PAGE of glucagon containing material from porcine pancreas at pH 4.5: 1, insulin together with proinsulin; 2, glucagon; 3, desamido glucagon; polypeptide of porcine pancreas not eluted.

m/U of glucagon. The concentration of glucose in the serum was determined by a colorimetric glucose assay by using the enzyme system of glucoseoxidase and peroxidase (British Pharmacopeia 88).

Radioimmunoassay of glucagon. A radioimmunoassay of glucagon was adapted from the method of Heding (Heding, 1971) with modifications.

The usual incubation mixture (0.3 mL) for the immunoassay of samples contains: 4 pg ^{125}I glucagon, Trasylol (300 U/mL), 0.05 mL nonradioactive glucagon at various concentrations, human serum albumin (1 mg/mL) and an appropriate dilution of antiserum in a total volume. For dilution was used 0.04 M sodium phosphate, pH 7.4, containing sodium chloride (6 mg/mL).

The incubation was done at 4°C for 2 days. For precipitation was added 0.6 mL ethanol. The mammalian crystalline glucagon standard was employed in all assays.

Preparative chromatography

The starting glucagon-containing material was a waste product from the manufacture of insulin. It was a water solution of crude glucagon, obtained directly after ion-exchange isolation of insulin from porcine pancreas. It contained about 20 mg/mL total solids, 3 mg/mL proteins and 0.5 mg/mL glucagon.

The assay of glucagon was performed by radioimmunoassay (RIA). The PAGE carried out at both pH 4.3 and 8.3 showed high heterogeneity (see Fig. 1). Polyacrylamide electrophoresis using sodium dodecyl sulphate was also used, and although no better results were obtained they confirm the observed heterogeneity.

The purification process was started with a diluted glucagon solution to give an extinction of 2.6–2.8 at 280 nm. The salt concentration indispensable to induce an

interaction between the weakly hydrophobic matrix and exposed hydrophobic moieties of some native polypeptides was determined experimentally to be 0.4 M sodium chloride. The glucagon solubility was also taken into account. It is known that glucagon is sparingly soluble (about 0.05 mg/mL) in aqueous solutions with a pH of between pH 3.5 and pH 8.5. Preparative significant concentrations exceeding 10 mg/mL can be obtained only in the pH ranges below 3 and above 9 (Gratzer, 1967).

In view of this two solutions with a pH adjusted to 3.2–3.6 and 8.5–9.5 were prepared. It was experimentally determined that, at the lower pH region, the desorption step showed better selectivity.

The column was first equilibrated with 0.4 M sodium chloride at a flow-rate of 4 mL/min. A volume of 2 L of glucagon solution was then applied to the Amberlite XAD-7 support: 300 mm (30 mm i.d.) with a flow-rate of 6 mL/min. After the loading step, the column was washed with 300 mL of the same sodium chloride solution. At the accepted loading condition, the desorption was investigated by changing the mobile phase composition in isocratic and gradient elution. The mobile phases for the purification were composed of 0.1 M sodium chloride, brought to pH 9.8–10 using a 10% solution of ammonium hydroxide (Eluent A). A gradient elution with an increasing quantity of ethanol (Eluent B) was checked. Eluent A and Eluent B were mixed at various ratios (v/v) to search for optimum

separation. The desorption flow-rate was 4 mL/min. The following variations were tested in desorption steps:

1. Gradient elution. 1.1 Elapsed time 0 min. Gradient: 0–80% eluent B in eluent A for 12.5 h.

1.2 100 min (400 mL) pure water. Gradient: 0–80% eluent B in eluent A for 10.5 h.

Step-wise elution.

2.1. Mixtures of eluent B with eluent A

Volume	Mixture	Step
300 mL	pure water	1st
300 mL	1:9	2nd
300 mL	2:8	3rd
200 mL	3:7	4th
200 mL	4:6	5th

2.2. Method as 1. with the following changes in the steps

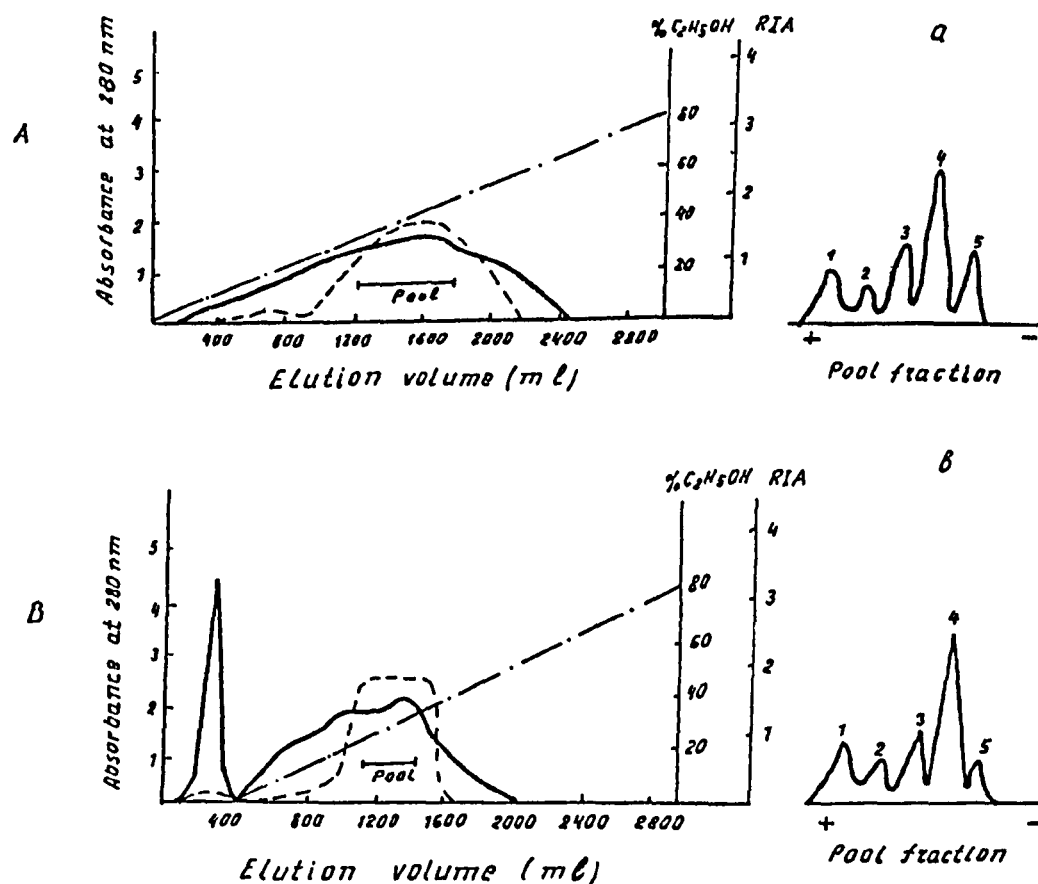| Volume	Mixture	Step
300 mL	pure water	1st
400 mL	1.5:8.5	2nd
300 mL	3:7	3rd
200 mL	4:6	4th

Figure 2. Conditions and elution profile of the crude glucagon on Amberlite XAD-7-gradient elution. Protein sample: 2.0 L glucagon containing material (E=2.6 at 280 nm) from porcine pancreas. Other conditions as given in the Experimental section.
(A) Peptide elution profile under conditions of case 1.1; —— chromatogram, obtained by UV monitoring at 280 nm; ------ concentration of glucagon (mg/mL), monitored by radioimmunoassay (RIA); -.-.-.-.-. gradient of eluent B in eluent A.
(a) PAGE of pool fraction (pH 8.3), see Fig. 1A for exaplantion of the figures; (b) peptides elution profile under conditions of case 1.2; (b) PAGE of pool fraction (pH 8.3), see Fig. 1A for explanation of the figures.
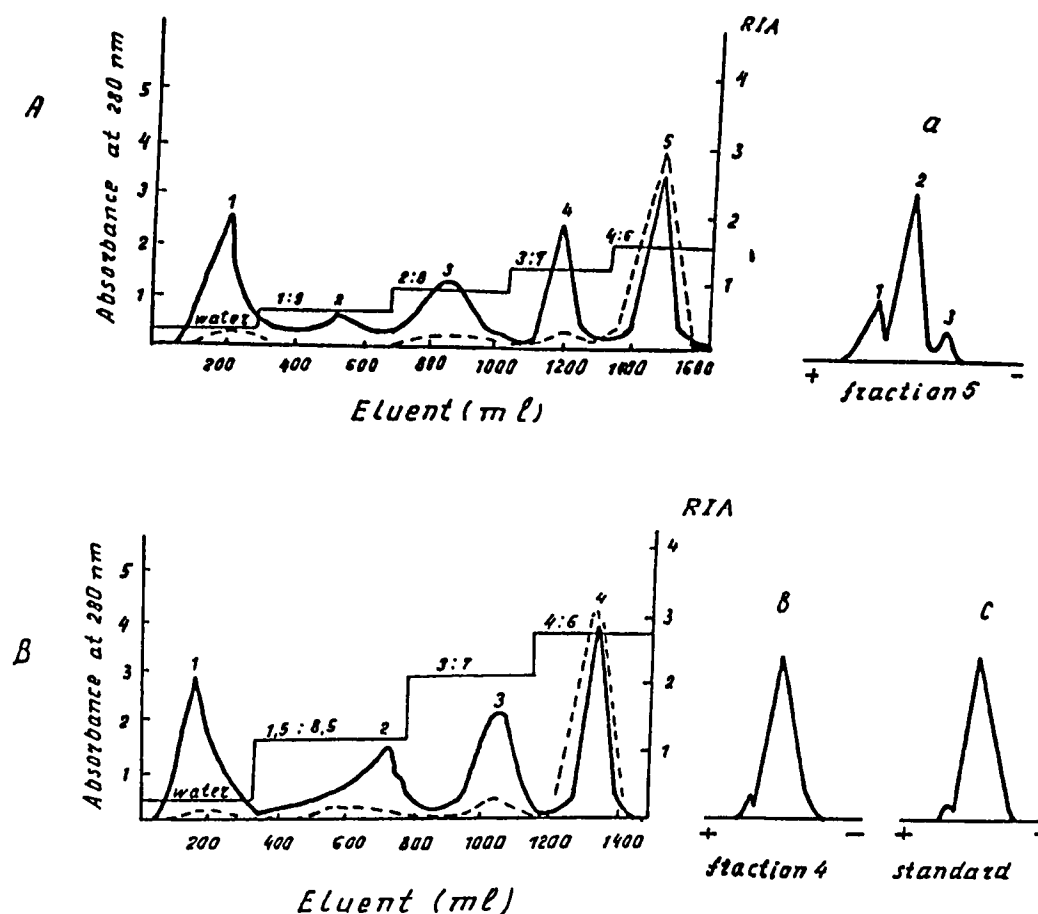
Figure 3. Conditions and elution profile of the crude glucagon on Amberlite XAD-7-step wise elution. Protein sample: 2.0 L glucagon containing material from procine pancreas. (A) Peptides elution profile under conditions of case 2.1; ——— chromatogram obtained by UV monitoring at 280 nm; ------- concentration glucagon (mg/mL) by RIA; 1, polypeptides of porcine pancreas; 2, mixed fraction, containing low molecular weight peptides, polypeptides of porcine pancreas; 3, mixed fraction, containing glucagon like peptides, determined by radioimmunoassay; low molecular weight peptides; 4, insulin, determined by PAGE (pH 8.3), together with glucagon like peptides; 5, glucagon. (B) Peptides elution profile under conditions of case 2.2. 1, polypeptides of porcine pancreas; 2, mixed fraction, containing glucagon like peptides, determined by radioimmunoassay; low molecular weight peptides, polypeptides of porcine pancreas; 3, insulin, determined by PAGE (pH 8.3) together with glucagon like peptides; 4, glucagon. (a) PAGE of fraction A5 (pH 8.3). 1, Desamido glucagon; 2, glucagon; 3, polypeptide of porcine pancreas. (b) PAGE of fraction B4 (pH 8.3). (c) PAGE of standard glucagon (pH 8.3).
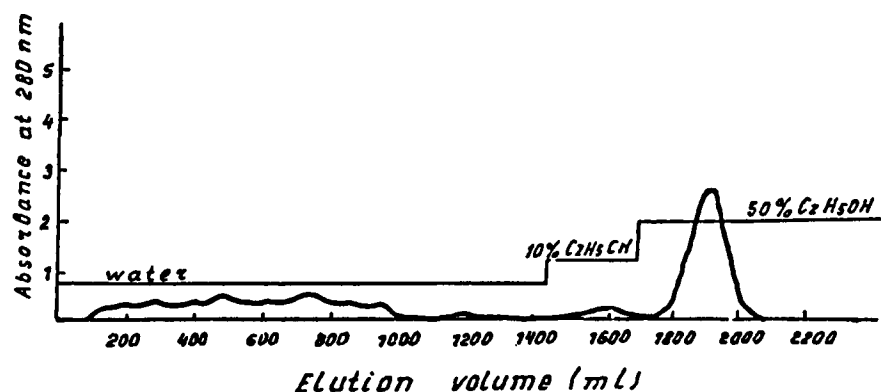

Figure 4. Desalting chromatography step by Amberlite XAD-7. Protein sample: 150 mL glucagon fraction (E=3 at 280 nm). Steps: First step, 1400 mL pure water; second step 250 mL 10% ethanol in water, which pH is adjusted to 9.7–10.2 with 10% ammonium hydroxide); third step 50% ethanol in water, which pH is adjusted to 9.7–10.2 with 10% ammonium hydroxide.

Table 1. Biological assay of samples glucagon

Samples	IU/mg, calculated with reference to the dried substance
1. Case 1.1, pool fraction lyophilized powder (after desalting)	0
2. Case 1.2, pool fraction lyophilized powder (after desalting)	0
4. Case 2.1, pool fraction lyophilized powder (after desalting)	0.68
5. Case 2.2, pool fraction lyophilized powder (after desalting)	0.92
6. Case 2.2, pool fraction crystalline powder (after desalting)	1.08
7. Standard used	1.1

RESULTS AND DISCUSSION

Figure 2A shows the elution profile of case 1.1. The eluent was monitored using a UV detector at 280 nm (solid line). Radioimmunoassay (RIA) results also are presented in Fig. 2A (dotted line). The pool fraction was analysed by PAGE at pH 8.3 (Fig. 2a). Unfortunately no practical results were obtained.

Better result was obtained in case 1.2. The chromatogram is given in Fig. 2B. As seen, a water washing as first step elutes a considerable quantity of non-active (RIA) impurities. The analysis of pool fraction by PAGE once more shows, however, unacceptably high heterogeneity (Fig. 2b). The only improvement was a slightly lower content of impurity eluted in peak 5.

Evidently, gradient elution seems not to be promising for preparative purification of glucagon.

The elution steps in case 2.1 and case 2.2 are presented in Fig. 3 A,B (solid line). Radioimmunoassay results for glucagon are also presented in Fig. 3 A,B (dotted line).

The glucagon fractions, obtained in case 2.1 and case 2.2 were analysed by PAGE (Fig. 3 a,b). A PAGE of the standard is given for comparison (Fig. 3 c).

As seen, the main fraction is glucagon in case 2.1 and this was eluted at a composition of eluents B:A=4:6 (Fig. 3A). The separation between the target compound glucagon (peak 5) and the neighbouring impurities as well as between the impurities themselves was acceptably good. If the latter is not necessary, the elution time can be shortened, preserving the high purity of the target compound. Optimization by changes in the step duration led to case 2.2. The purity of the glucagon fraction is presented by PAGE on Fig. 3 b and is similar to the purity of the standard (Fig. 3 c).

The desalting step was tested with the purified glucagon fraction, obtained in case 2.2 (peak 4 from Fig. 3 B). It was carried out again on a Amberlite XAD-7 column 220 mm (25 mm i.d.). The elution profile is presented in Fig. 4. Again a step-wise desalting process is preferable.

The desalted glucagon was further crystallized. The pure product was characterized by biological assay (see Table 1). and showed hyperglycaemic activity of 1.08 IU/mg.

CONCLUSION

The proposed step-wise hydrophobic interaction chromatography seems to be a powerful preparative technique for the preparation of glucagon. The mobile phase compositions used and the step durations for purification of glucagon by HIC on Amberlite XAD-7 showed base-line separation between the impurity peaks and the target peak. This approach could be used for tuning the mobile phase composition and chromatographying conditions for the best purifying of glucagon on Amberlite XAD-7, batches of which have slightly different properties.

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