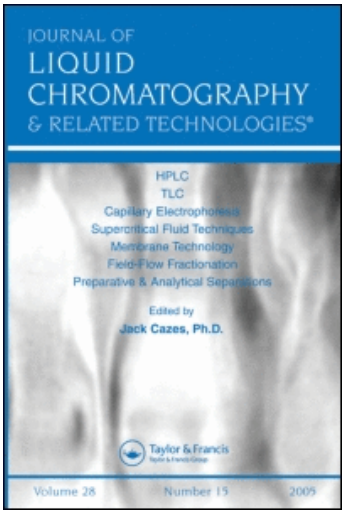

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Liquid Adsorption Chromatography on Preparative Scale of Protected Synthetic Peptides

W. Kullmann[a]
[a] Max-Planck-Institut für biophysikalische Chemie, Göttingen-Nikolausberg, Germany

To cite this Article Kullmann, W.(1979) 'Liquid Adsorption Chromatography on Preparative Scale of Protected Synthetic Peptides', Journal of Liquid Chromatography & Related Technologies, 2: 7, 1017 — 1029
To link to this Article: DOI: 10.1080/01483917908060121
URL: http://dx.doi.org/10.1080/01483917908060121

JOURNAL OF CHROMATOGRAPHY, 2(7), 1017-1029 (1979)

LIQUID ADSORPTION CHROMATOGRAPHY ON PREPARATIVE SCALE OF PROTECTED SYNTHETIC PEPTIDES

W. Kullmann
Max-Planck-Institut für biophysikalische Chemie
Am Faßberg
34 Göttingen-Nikolausberg
Germany

ABSTRACT

This paper reports the purification of synthetic protected peptides on a preparative scale by means of adsorption chromatography on silica gel 60 columns.

The protected peptides described were precursors of a free asymmetrical cystine peptide corresponding to the insulin sequence A^{18-21}-B^{19-26} and were obtained in analytically pure form. Selection of solvent systems for isocratic or stepwise elution depended largely on R_F data obtained from thin-layer chromatograms which were used for monitoring and optimizing synthetic reactions.

INTRODUCTION

The rising demand for synthetic supplies of naturally occuring peptides and their analogues for both therapeutical purposes and biological or conformational studies places an ever growing need on efficient peptide synthesis providing highly purified compounds. To meet the requirement for pure free peptides, it is of utmost importance that the protected intermediates prepared by conventional methods in solution are thoroughly purified. These blocked peptide intermediates normally exhibit a behavior more or less hydrophobic than the contaminants arising during peptide bond formation. Therefore adsorption chromatography, which implies the sole use of organic solvents as mobile phase, offers a good possibility for fractionation of reaction mixtures owing to the different hydrophobicities of their components. This paper describes the purification via adsorption chromatography on silica gel 60 of several partially or fully protected precursors of an asymmetrical cystine peptide comprising the insulin sequence $A^{18-21}\text{-}B^{19-26}$ (1). The procedure was rapid and provided readily crystallizing and analytically pure protected peptides in good yields and thus enabled the preparation of the final free dodecapeptide in homogeneous form.

MATERIALS AND METHODS

Silica gel 60 (60 Å porosity; 0.063 - 0.2 mm particle size (Merck)) served as stationary phase throughout all purification steps using column chromatography.

Thin-layer chromatograms were developed on precoated silica gel 60 plates purchased from Merck. To remove Boc or Bpoc protecting groups the plates were heated to appr. 100° and then sprayed with ninhydrin reagent. Organic compounds other than peptides or amino acids were detected by iodine vapor. TLC screening served as proof for homogeneity of the product and determined the limits of fraction pooling.

The solvents used were reagent-grade and eluant systems were composed on the basis of thin-layer chromatographic data as to provide satisfactory separation in short time. Silica gel was suspended in the respective solvent, degassed, and filled into preequilibrated columns. The columns were subsequently equilibrated with the starting eluant using 4-6 column volumes. The samples were poorly soluble in the starting eluants and were therefore applied in dimethylformamide and eluted by a single solvent or solvent mixture during the entire run (isocratic elution) or by stepwise elution changing the solvents during the fractionation. The flow rates used varied between 0.4 and 0.6 ml per minute. Eluted compounds were detected by a UV absorbance monitor at 280 nm and collected by an LKB Ultrorac 7000 fraction collector. Fresh silica gel was used for each new separation problem.

The isolated protected peptides were characterized by standard methods, i.e. melting point, optical rotation, and their homogeneity was confirmed by elementary and amino acid analyses.

RESULTS AND DISCUSSION

The examples presented below are to demonstrate that under appropriate conditions peptides with free or blocked NH_2-terminus and free or protected COOH-terminus could be obtained in analytically pure form as confirmed by standard criteria. Acid labile protecting groups including the 2-(4-biphenyl)-2-propyloxycarbonyl (Bpoc) and triphenylmethyl (Trt) groups remained unaffected under the condition of the chromatographic procedures.

Preparation of Bpoc-Asn-Tyr(2,6-Cl_2-Bzl)-Cys(Trt)-Asn(OBzl)

Removal of the N^{α}-t-butyloxycarbonyl(Boc) group from the tripeptide Boc-Tyr(2,6-Cl_2-Bzl)-Cys(Trt)-Asn-(OBzl) using boron trifluoride etherate (2) provided the deacylated compound which was contaminated with a by-product as revealed by thin-layer chromatography. This contaminant could not be removed by extraction techniques because its solubility properties were very similar to those of the desired product. Therefore 1,6 g of the mixture, dissolved in dimethylformamide, was applied to a silica gel 60 column (75x2.4 cm) equilibrated with chloroform-methanol (60:1). Elution with the same solvent system followed by chloroform-methanol (20:1) yielded the desired tripeptide well separated from the by-product which was eluted after addition of chloroform-methanol (5:1) (Fig. 1). The contaminant showed the same amino acid composition as the main product but it contained a free sulfhydryl group as shown by staining with sodium nitroprusside and could therefore be identified as the detritylated

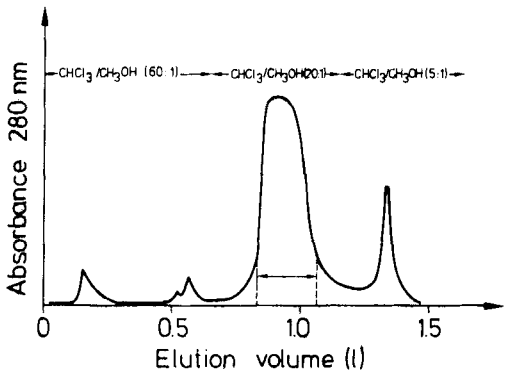

FIGURE 1. Chromatography of H-Tyr(2,6-Cl_2-Bzl)-Cys(Trt)-Asn (OBzl) on a silica gel column (75x2.4 cm). The sample, 1.6 g, was applied in 2 ml of DMF and eluted with the solvent mixtures indicated above the peaks at a flow rate of 0.5 ml/min. Fractions were collected at 10-min intervals. The limits of fraction pooling are given by dashed lines.

analogue of the deacylated tripeptide. The latter was homogeneous by TLC and the amino acid analysis was in close agreement with theoretical values (Asp, 1.00(1); Cys(O_3H), 0.91(1); Tyr, 1.01 (1)).

The deacylated tripeptide H-Tyr(2,6-Cl_2-Bzl)-Cys(Trt)-Asn-(OBzl) served as amino component in the following condensation step using Bpoc-Asn-OH as carboxyl component, 1-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) as condensing agent. The resulting tetrapeptide Bpoc-Asn-Tyr(2,6-Cl_2-Bzl)-Cys (Trt)-Asn(OBzl) was shown by TLC to contain several by-products the R_F value of one of which was very similar to that of the main product. The reaction mixture (1,53 g in DMF) was applied to a

silica gel 60 column (75x2.4 cm). Elution was started with chloroform to remove more lipophilic contaminations which were probably N-acyl urea derivatives. Upon addition of chloroform-methanol (35:1) the by-product mentioned above was eluted, followed closely by the desired tetrapeptide (Fig. 2). The by-product and the protected tetrapeptide had identical amino acid composition. After staining a chromatogram with the ninhydrin spray the by-product gave a bluish-green spot indicative of a nitrile compound. Probably, the side chain of the N-terminal asparagine had been partially dehydrated during the activation step and, after addition of the amino component, a nitrile analogue of the protected tetrapeptide was formed. The latter was recovered in good yield and crystallized after evaporation of the solvents upon addition of me-

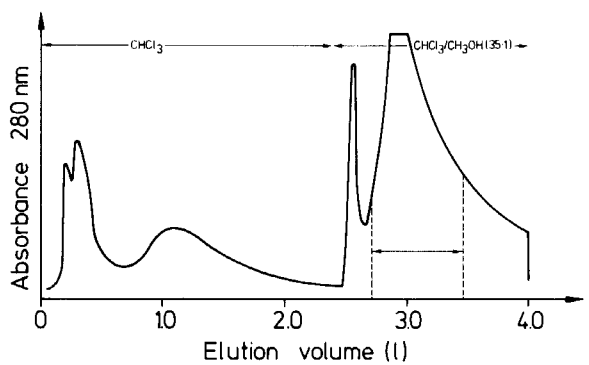

FIGURE 2. Adsorption chromatography of Bpoc-Asn-Tyr(2,6-Cl_2-Bzl)-Cys(Trt)-Asn(OBzl). The sample (1.5 g) was dissolved in 1.5 ml of DMF and applied to a silica gel column (75x2.4 cm) equilibrated with chloroform. Elution proceeded at a flow rate of 0.6 ml/min. Fractions were collected at 10-min intervals.

thanol. Yield: 1.06 g; m.p. 180-181°. Elementary analysis: Found: C, 66.56; H, 5.39; N, 6,81; S, 2.37; $C_{69}H_{66}N_6O_{10}S\ Cl_2$ (1242.2) requires: C, 66.71; H, 5.36; N, 6.77; S, 2.58. Amino acid analysis of a performic acid-oxidized hydrolysate gave: Asp, 2.00 (2); Cys(O_3H), 0.88 (1); Tyr, 1.00 (1).

Preparation of Bpoc-Asn-Tyr(2,6-Cl_2-Bzl)-Cys-Asn(OBzl)
/
Boc-Cys-Gly-OH

Prior to selective formation of the asymmetrical protected disulfide peptide (A^{18-21}-B^{19-20}) the tetrapeptide was treated with ethoxycarbonylsulfenyl chloride (3) replacing the S-trityl group by an ethoxycarbonylsulfenyl (Sce) moiety. As shown by TLC the resulting product mixture contained a small amount of the original tetrapeptide together with an iodine-positive contamination which was probably tritylmethylether usually formed during removal of S-trityl groups in the presence of methanol. A DMF solution of the reaction mixture (950 mg) was applied to a silica gel column (47x2.3 cm) equilibrated and eluted with chloroform-methanol (20:1) (Fig. 3). The by-products were eluted first followed by the desired protected tetrapeptide Bpoc-Asn-Tyr(2,6-Cl_2-Bzl)-Cys(Sce)-Asn(OBzl) which was obtained as an analytically pure compound. Yield: 625 mg; m.p. 175-178°. Elementary analysis: Found: C, 55.40; H, 5.05; N, 8.32; S, 4.92; $C_{53}H_{56}N_6O_{12}S_2Cl_2$ (1104.0) requires: C, 57.66; H, 5.11; N, 7.61; S, 5.80.

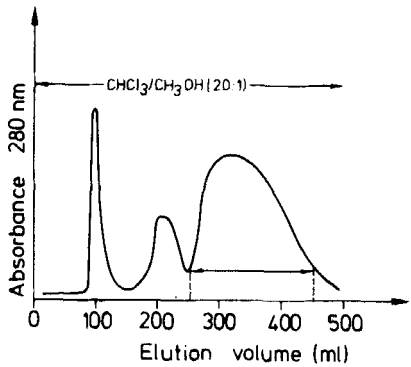

FIGURE 3. Chromatography of Bpoc-Asn-Tyr(2.6-Cl_2-Bzl)-Cys(Sce)-Asn(OBzl). The sample (0.95 g), dissolved in 1.5 ml of DMF, was loaded on a silica gel column (47x2.3 cm) equilibrated and eluted with chloroform-methanol (20:1) at 0.4 ml/min. Fractions were collected every 8 min.

The protected asymmetrical cystine peptide Bpoc-Asn-Tyr (2,6-Cl_2-Bzl)-Cys-Asn(OBzl) Boc-Cys-Gly-OH was prepared by reacting Bpoc-Asn-Tyr(2,6-Cl_2-Bzl)-Cys(Sce)-Asn(OBzl) with the dipeptide Boc-Cys-Gly-OH. TLC of the reaction mixture (510 mg) revealed the presence of at least three contaminants which could be removed by adsorption chromatography on a silica gel 60 column (47x2.3 cm) equilibrated and eluted with chloroform-methanol (50:1) (Fig. 4). One of the by-products emerged from the column between 250 and 350 ml; it was tentatively identified as a symmetrical cystine peptide possessing the same amino acid composition as the starting tetrapeptide. A second contaminant, Boc-Cys-Gly-OH, was washed from the column between 725 and 825 ml. The protected asymmetrical hexapeptide eluted between 525 and 700 ml.

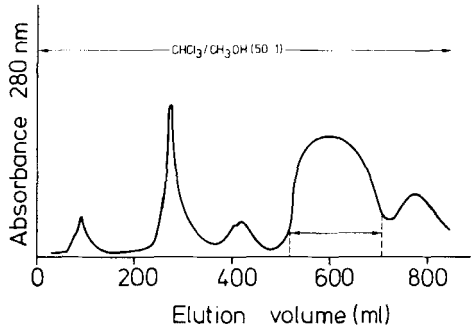

FIGURE 4. Chromatography of Bpoc-Asn-Tyr(2.6-Cl_2-Bzl)-Cys-Asn-(OBzl) Boc-Cys-Gly-OH on a silica gel column (47x2.3 cm). The crude sample (0.51 g) was applied in 1.5 ml of DMF and eluted with chloroform-methanol (50:1) at 0.5 ml/min. Fractions were collected at 5-min intervals.

Solvent evaporation gave an oil which crystallized upon addition of methanol. Yield: 350 mg; m.p. 165-167°. Elementary analysis: Found: C, 56.66; H, 5.62; N, 9.16; S, 5.04; $C_{60}H_{68}N_8O_{15}S_2Cl_2$ (1276.2) requires: C, 56.47; H, 5.37; N, 8.87; S, 5.02. Amino acid analysis of a performic acid-oxidized hydrolysate gave: Asp, 1.98 (2); Gly, 1.00 (1); Cys(O_3H), 1.89 (2); Tyr, 1.04 (1).

Preparation of the free asymmetric dodecapeptide A^{18-21}-B^{19-26}

The protected hexapeptide was coupled to the hexapeptide B^{21-26} resin to give the protected dodecapeptide resin. The peptide was cleaved from the resin and deprotected using liquid HF and the free dodecapeptide (A^{18-21}-B^{19-26} of bovine insulin) was submitted to gel filtration on a Sephadex G-25 column followed by partition chromatography on a silica gel 60 column

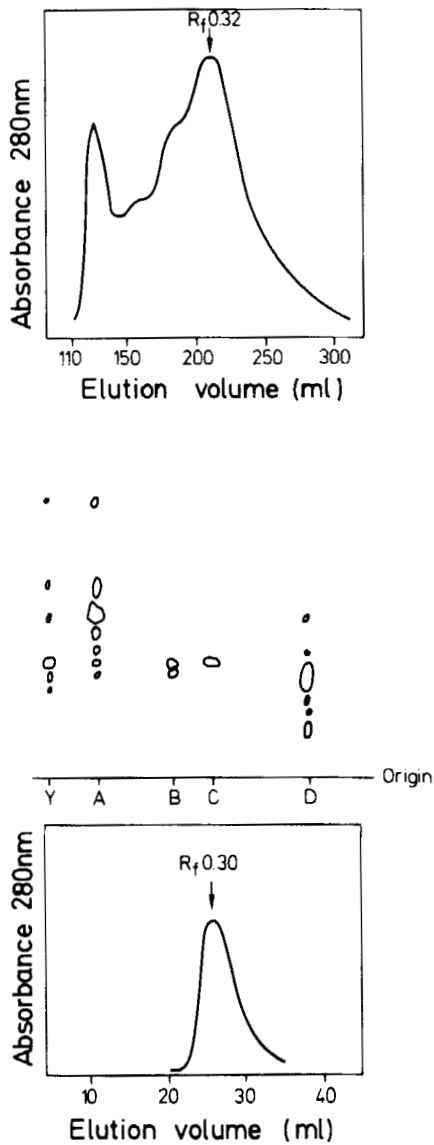

FIGURE 5. (Top) Partition chromatography of 14 mg of dodecapeptide (obtained after rechromatography on Sephadex G-25) on silica gel 60 in the organic layer of the solvent system 1-butanol-acetic acid-water (4:1:5); column, 42x2 cm. The volume per tube was 1.83 ml. Fractions 65-76 (A), 95-109 (B), 110-125 (C),

(45x2.0 cm) equilibrated sequentially with the aqueous and the organic phase of the solvent system 1-butanol-acetic acid-water (4:1:5) (Fig. 5). Elution with the organic phase yielded the pure target peptide which was shown to be homogeneous on TLC and electrophoresis. The amino acid composition was in close agreement with the theoretical values. Amino acid analysis of the pure dodecapeptide: Asp, 2.07 (2); Cys(O_3H), 2.03 (2); Glu, 1.04 (1); Gly, 2.00 (2); Tyr, 1.87 (2); Phe, 2.08 (2); Arg, 1.06 (1).

It was demonstrated that satisfactory fractionation of reaction mixtures can be achieved by adsorption chromatography providing analytically pure protected peptide precursors on a preparative scale in relatively short time as compared to alter-

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Figure 5 (continued)

and 126-150 (D) were collected. The R_F was determined as follows:

$$R_F = \frac{\text{hold-up volume}}{\text{elution volume}}$$

The volume at which the aqueous phase was displaced from the column by the organic phase was the hold-up volume.
(Middle) T.l.c. of the peptide fractions (A-D) isolated above and of the starting material (Y) on silica gel in the solvent system 1-butanol-pyridine-acetic acid-water (20:10:3:10). Peptide spots were detected by Pauly spray. (Bottom) Partition chromatography on silica gel 60 of 0.8 mg of the peptide isolated from fractions 110-125 (C). Column, 19x1cm; the volume per tube was 0.65 ml. The solvent system used was the same as above.

native purification methods such as repetitive crystallization or counter current distribution which are more time-consuming and give lower yields. The last example was selected to underline the advantages of rigorously purified protected intermediates which enabled the preparation of a homogeneous free dodecapeptide readily available by partition chromatography.

The elution volumes of both the desired peptides and the by-products were found to be in good correlation with the R_F values obtained from thin-layer chromatograms on silica gel 60 plates. This correlation is given by the equation $1/R_F-1=K'$ (K', the capacity factor equals the column volume exceeding the void volume). It was generally found that the elution of the products was somewhat faster than expected from R_F values.

In summary, adsorption chromatography on silica gel columns was shown to be a convenient procedure for time-saving and efficient recovery of protected peptides from complex reaction mixtures on a preparative scale.

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