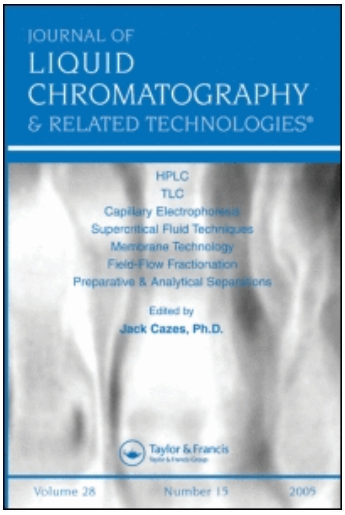

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GEL PERMEATION CHROMATOGRAPHY OF INSULIN

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ABSTRACT

Aquas gel permeation chromatography of insulin under denaturating conditions has been successfully performed on three different chromatographic supports. The separation pattern was identical to that obtained on soft gels (Sephadex, BioGel). The elution time was 10-20 min, recovery 98-100%.

INTRODUCTION

The main field of activity in high pressure liquid chromatography is separation of low molecular weight substances and synthetic polymers. Applications for biological compounds of higher molecular weight (e.g. proteins, nucleic acids) have hitherto been random, primarily due to lack of chromatographic supports.

A good chromatographic support for high-speed gel chromatography of proteins has to be pressure-stable and hydrophilic, thereby eliminating or minimizing the interaction with the proteins. The original material to be used for high pressure

size exclusion chromatography of protein was coated controlled-porosity glasses, but adsorption was a serious problem. A mixture of insulin, glucagon and somatostatin could be resolved on controlled -porosity glass coated with glycerylpropylsilane, but they were eluted in the reverse order expected if the separation should be based on molecular size (1). Recoveries as low as 20% for proteins have been reported for glycophase columns (2).

Recently, silica-based supports with different hydrophilic coatings have been introduced for high pressure gel chromatography of proteins, but only a few applications to biological substances have yet been published (3-7).

In parallel with high pressure size exclusion chromatography, proteins and peptides have been analyzed by reversed phase high pressure liquid chromatography where adsorption problems often are solved by addition of hydrophilic or hydrophobic ion-pairing reagents to the eluants (8). Insulin and insulin-related peptides have been analyzed in several ion-pair reversed phase systems (9-16) and it is possible to distinguish between insulin from different species (bovine-porcine) or between insulin and desamido insulin (9, 12, 13). It has not yet been possible to reach a fractionation capacity for insulin comparable to that of analytical isoelectric focusing or disc electrophoresis.

Gel chromatography of insulin at low pressure may be divided in two classes: either the eluents contain disaggregating substances or not. In the last case the molecular weight of insulin

primarily depends upon concentration, zinc concent and pH (17-19). Under disaggregating conditions, e.g. in 1 M acetic acid crystalline insulin may be separated in the b-component containing the dimer of insulin, proinsulin and intermediary insulin (molecular weight 9.000-12.000) and the c-component containing insulin, deamidated insulin and argininsulin (molecular weight about 6.000) (20).

In the present work the application of aquas gel permeation chromatography to insulin is described. The paper includes retention values and recovery for insulin and insulin-related compounds on different pressure-stable chromatographic supports in aquas buffers containing disaggregating compounds.

MATERIALS AND METHODS

Apparatus

The liquid chromatograph consisted of Waters M 6000 pump U6K injector and 440-UV-detector. Waters data module was used for electronic integration.

Conditions.

All experiments were performed at room temperature. Chemicals were of analytical purity and the water was twice distilled in quartz. The eluants were degassed and Millipore-filtered (0.45 µm) before use.

Bovine and porcine insulin (twice crystallized, 0.5% zinc) were supplied from Nordisk Gentofte. The insulin samples consisted of about 90% pure insulin

plus varying amounts of proinsulin, insulin dimer, intermediary insulin, argininsulin and desamido-insulin. Proteins used as molecular weight markers were supplied from Sigma (bovine serum albumin, ovalbumin, α-chymotrypsinogen, ribonuclease), Merck (cytochrom C), Nutritional Biochemical Corp. (Myoglobin) and Eli Lilly (glucagon). Genapol® SE-150 was supplied from Hoechst.

Separation of insulin and standard proteins was performed on three different silica-based chromatographic supports.

Waters Bondagel E-125 (silica coated with ether groups, 300 x 3.9 mm I.D.)

ToyoSoda G 2000 SW and G 3000 SW (silica coated with hydroxy-groups, 600 x 7.5 mm I.D.)

Waters I-125 protein column (silica coated with hydroxy-groups, 300 x 7.8 mm I.D.)

The exact composition of any of the coating layers is unknown.

The protein samples were dissolved in the respective eluants. The concentration was 10 mg/ml except where anything else is stated.

Columns and actual conditions in each experiment are further explained in the legends to the figures.

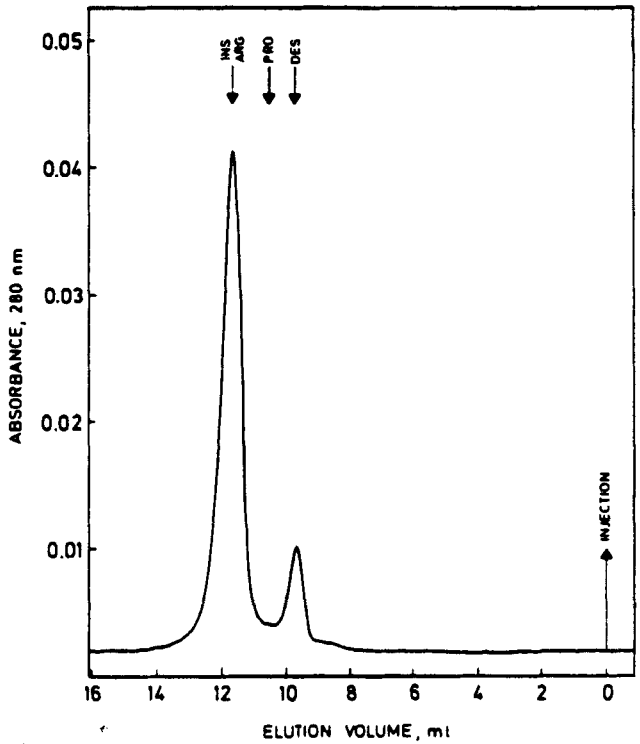

Fig. 1.
Separation of 20 µg crystalline porcine insulin in 5 µl on 2 x Bondagel E-125 eluted with 7 M urea/0.1 M Tris/pH 7.5, 0.5 ml/min. The arrows at the top indicate the elution volumes for some of the components in crystalline insulin (Des= desamidoinsulin, pro=proinsulin, Arg=argininsulin, ins=insulin).

RESULTS

Bondagel E-125.

The first experiments were performed on Bondagel E-125, since it was the only silica gel permeation chromatography column available with some applications for biological substances (21). The

starting point was the well known Sephadex G-50/ 1 M acetic acid separation of re-crystallized insulin in b- and c-components (20). Under these conditions insulin was firmly bound to the E-125 column material.

If urea was added to the eluants (e.g. 7 M urea/ 1 M acetic acid) insulin was eluted as a symmetric peak, but its elution volume was considerably greater than the column volume available to very small molecules. Also at neutral pH adsorption was considerable: in 7 M urea/0.1 M Tris/pH 7.5 crystalline insulin was eluted in two fractions, the first consisting of 12%, the second 88% of the UV-curve area (fig. 1). As can be seen from this figure some components in crystalline insulin (with approximately the same molecular weight) showed different elution volumes, but all much greater than the volume available to small molecules.

Addition of non-ionic detergent to the eluant tends to diminish the retention volumes. The best result was obtained with Genapol® (alkylpolyglycol -ethers), see fig. 2. The separation pattern of twice crystallized porcine insulin is the same as the one obtained on Sephadex G-59 in 1 M acetic acid. The minor component co-elutes with b-component isolated from a preparative separation of the same insulin on Sephadex G-50/1 M acetic acid, the major component has the same retention value as c-fraction from the same Sephadex fractionation.

The recovery based on comparison of UV-absorption of the eluate and the sample was found to be 98% (n=4) and the analysis was completed after 15 min.

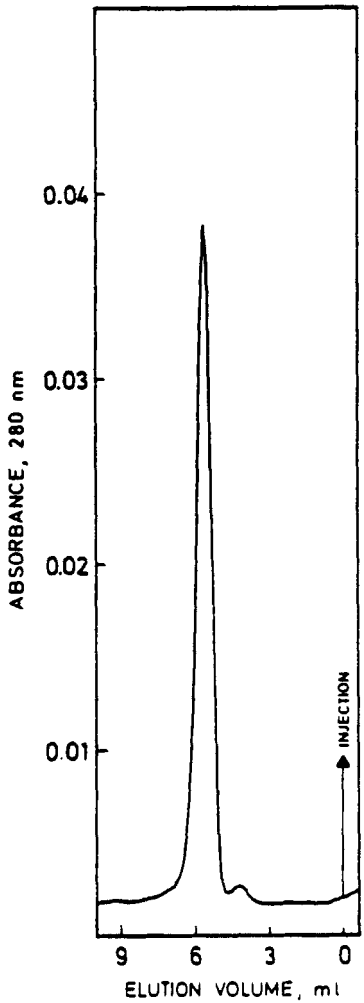

Fig. 2.
Separation of twice crystallized porcine insulin on 2 x Bondagel E-125 eluted with 7 M urea/0.1% H_3PO_4 (v/v)5% Genapol® SE-150, 0.5 ml/min. 30 µg protein in 10 µl was injected. Based on electronic integration, the content of c-fraction (the major fraction) was found to be 97.38, S.D.=0.05%, n=8.

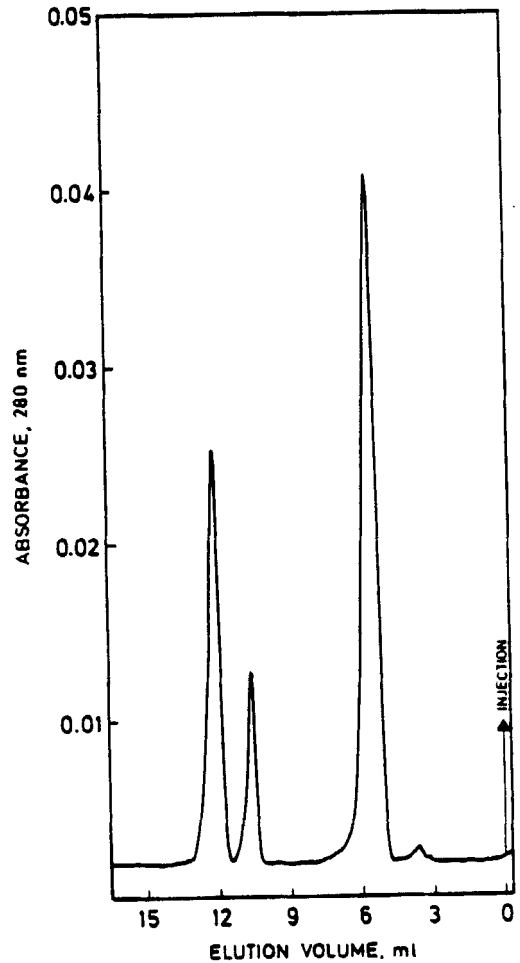

Fig. 3.
Separation of 35 µg protamin-insulin in 3.5 µl, conditions as in fig. 2.

Fig. 3 shows a chromatogram of protamin-insulin. The two base-line separated peaks after the insulin sample are the preservatives phenol and m-cresol.

Fig. 4 shows the elution volume for a few proteins-peptides and acetone in relation to their molec-

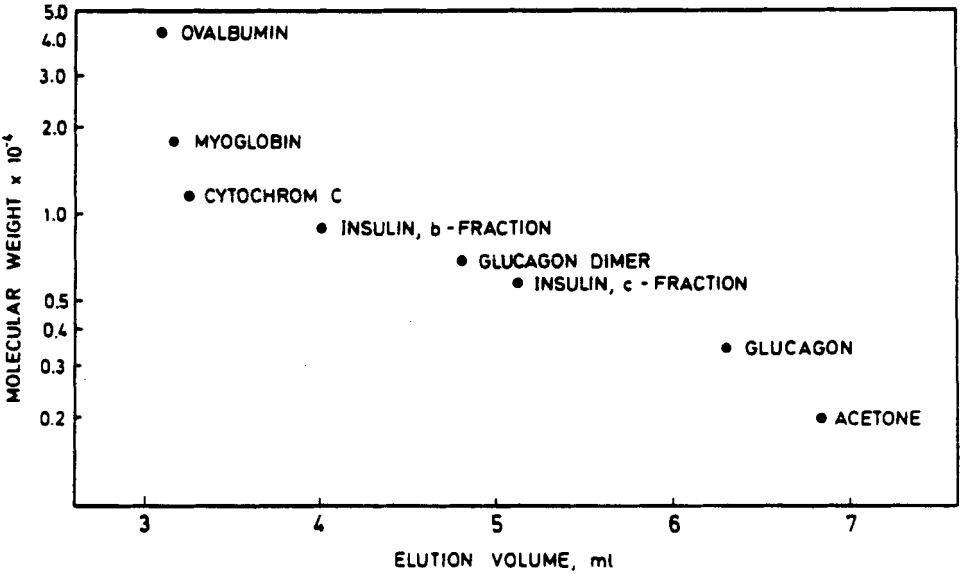

Fig. 4.
Elution volume for some proteins and acetone in relation to molecular weight. Conditions as in fig. 2.

ular weight. The separation range is MW 3000-12000 and the available separation volume (for 2 columns) is 3-6.5 ml. By comparing to the elution volume for phenol and m-cresol in fig. 3 it is clearly seen that these aromates are heavily retarded, as they are on Sephadex and BioGel.

TovaSoda G 2000 SW.

The separation of recrystallized insulin in b- and c-component on TSK gel G 2000 SW could be performed in 7 M urea/0.1% phosphoric acid (v/v) with the same separation pattern as found on Bondagel. It was further examined whether this separation could be

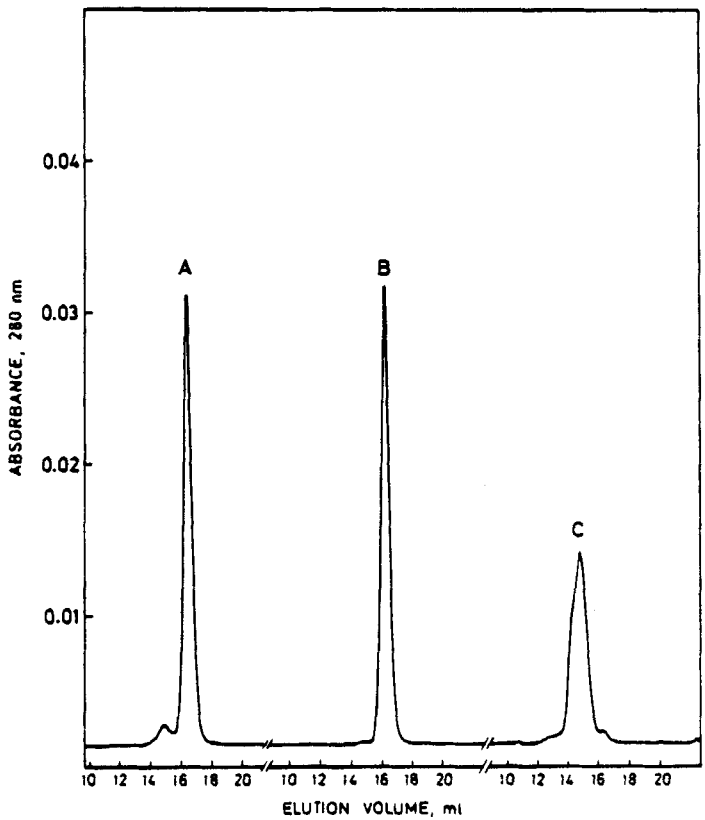

Fig. 5.
Separation of 50 µg twice crystallized bovine insulin (A), 50 µg bovine c-fraction (B) and 50 µg bovine b-fraction (C) on TSK-gel G 2000 SW eluted with 7 M urea/0.1 M tris/0.1 M NaCl, pH 7.30. The column (600 x 7.5 mm I.D.) was eluted with 1.0 ml/min.

performed at a more neutral pH. Fig. 5 shows the separation of crystalline bovine insulin and the Sephadex fractioned b- and c-components in 7 M urea/0.1 M Tris/0.1 M NaCl, pH 7.30. The heterogeneity of the b-component is clearly seen. The minor content of b-component seen in the isolated c-com-

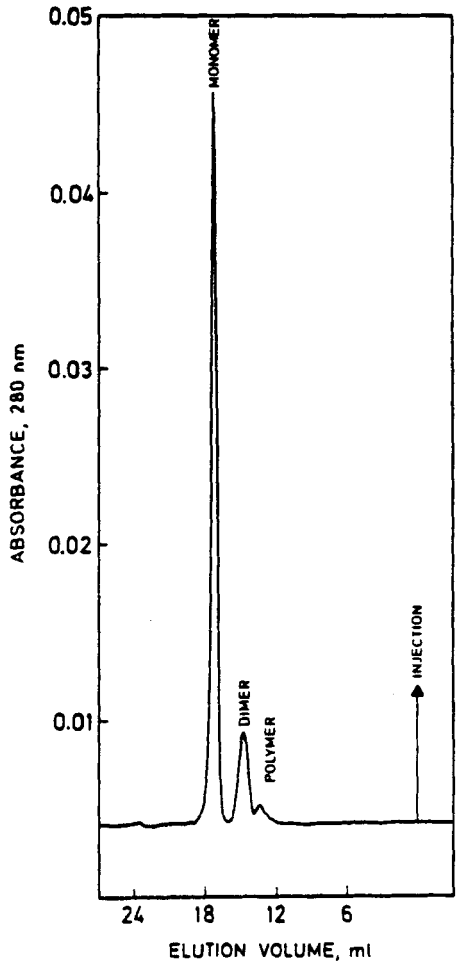

Fig. 6.
Separation of commercial bovine serum albumin in monomer, dimer and higher polymers on G 3000 SW eluted with 0.15 M phosphate buffer/0.1 M NaCl/pH 7.30, flow rate 1.0 ml/min. Identification of mono and dimer was based on co-chromatography of the actual components isolated by Sephadex G 100 chromatography of the same sample. The content of monomer, based on electronic integration, was found to be 86.2%, S.D.=0.4%, n=10.

ponent derives from a small amount of dimerized insulin produced by freeze drying of the c-component from 1 M acetic acid. The separation is completed in less than 20 min. and the recovery, based on UV-absorption as described above, is 100%.

Proper separation of insulin in b- and c-component could not be performed without addition of urea to the eluant. Other proteins could be fractionated in inorganic buffers containing a small amount of neutral salt, fig. 6 shows the separation of a commercial sample of bovine serum albumin on G 3000 SW.

I-125 protein column.

Finally, the separation capacity on protein column I-125 was examined. The separation in 7 M urea/0.1% phosphoric acid (v/v), fig. 7, is essentially the same as found on G 2000 SW and Bondagel. The separation is terminated after 11 min. and the recovery is 99%.

The elution volumes for some proteins on the I-125 column are shown in fig. 8.

DISCUSSION

In the last few years attempts to separate peptides and proteins on high pressure liquid chromatographic supports have been almost exclusively based on reverse phase chromatography. This method, with or without addition of ion-pairing reagents, seems very well suited for the separation of peptides, whereas applications for proteins still are very limited.

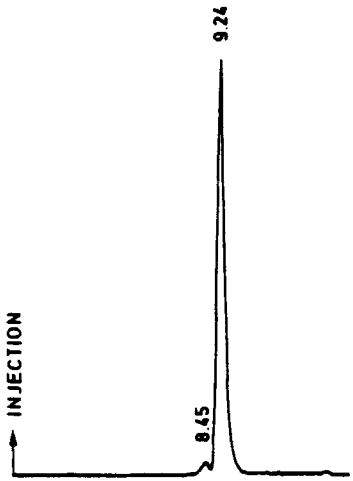

Fig. 7.
The separation of 50 µg twice crystallized porcine insulin in 5 µl on a Waters I-125 column (300 x 7.8 mm I.D.) eluted with 7 M urea/0.1% H_3PO_4 (v/v), 1.0 ml/min. The numbers printed vertically are retention times in min. and 1/100 min., the ordinate is the absorption at 280 nm in arbitrary units.

It would therefore be of interest to examine the present possibilities for performing high pressure liquid chromatographic separations of polypeptides and proteins based on their molecular size. According to the experiments reported here 2 separation "classes" can be described: one needs the addition of detergent, urea and acid to elute insulin properly (µ-Bondagel) and another can elute insulin under acid as well as neutral conditions with urea as the only denaturating agent (TSK gel, I-125 column).

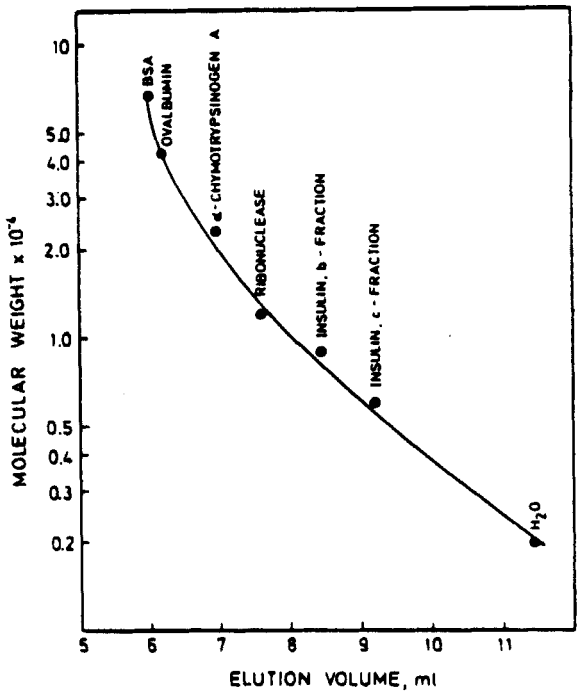

Fig. 8.
Elution volume for some proteins and water on a I-125 protein column. Conditions as described for fig. 7.

It is obvious that the last class of column materials constitute a great step towards the "ideal" column material, which should allow very rapid separation and be free of adsorption to proteins and nucleic acids. The most serious drawbacks are the following:

1. Some adsorption between the column material and the proteins still exists, probably due to unreacted silanol groups in the silica back-bone. It is therefore generally necessary to incorporate a small amount of a neutral salt in the eluants.

2. When TSK-G 2000 SW columns are eluted with commercially used buffers (Tris, phosphate, ammonium hydrogencarbonate) at neutral pH with 0.1-0.2 M NaCl in the buffers, adsorption of some low molecular weight compounds to the matrix suspends the linear relationship between molecular weight and elution volume. Such deviations were found for insulin, insulin A-chain, insulin B-chain, glucagon and aprotinin (22). From the literature aprotinin is known to bind to G 3000 SW column-packings (23). In 0.1% sodiumdodecylsulphate containing 0.02-0.5 M sodiumphosphate, insulin began to adsorp to the G 3000 SW and G 4000 SW column-packings at 0.1 M sodiumphosphate and at 0.2 M severely tailing peaks were obtained (24). It therefore seems as if the adsorption problems increase with the decreasing molecular weight of the sample. A few experiments with the I-125 column indicated a similar problem.

It should be noticed that incorporation of urea in the eluants decreases the effective separation range for proteins. The fractionation range for Bondagel E-125 measured with a polyethyleneglycol in water is 2000-50000 (25). Eluted with urea/Genapol/phosphoric acid as described here this column shows very poor resolution for proteins with a molecular weight higher than 12000. This tendency, but to a much lesser degree, has been described for Bondagel columns in Tris/7 M urea as neutral pH (3). Also G 2000 SW and I-125 columns show a reduced fractionation range in urea containing buffers, the primary sign seems to be a shift in the upper part of the standard curve from linear to bend (see fig.

6), thereby reducing the efficiency. The same effect is seen in other denaturating eluants, e.g. 0.5% sodiumdodecylsulphate and 6 M guanidinium hydrochloride (24).

In spite of these drawbacks it should be pointed out that the present level in aquas gel permeation chromatography allows molecular weight estimation for proteins in a variety of solvents and molecular weight ranges and with a consumption of time around 1-2% of that used for low-pressure gels. The separation capacity for the columns investigated in this report lies in the range 0.05-1 mg, but since preparative columns allowing size fractionation of up to 100 mg protein are commercially available, this technique will probably very soon be developed to the point as low-pressure gel chromatography.

ACKNOWLEDGEMENTS

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