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HIGH-PERFORMANCE SIZE-EXCLUSION LIQUID CHROMATOGRAPHY OF PROTECTED PEPTIDES

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SUMMARY

Methods for the analytical and preparative size-exclusion high-performance liquid chromatography of protected hydrophobic peptides in organic solvents were developed. Columns containing Sephadex LH-20 and various silica gels were used and the selectivity of each sorbent and the behaviour of the peptides on them were examined. The effects of sample volume and concentration and the injection technique were elucidated in preparative-scale work. The applicability of various detection methods for identifying peptides is considered.

INTRODUCTION

A separation method suitable for a wide variety of molecules, especially biopolymers, based on the difference in their molecular masses was proposed by Porath and Flodin over 20 years ago[1]. Over the past 4–5 years the traditional method of gel filtration on strongly swelling soft gels[2–4] has developed into high-performance size-exclusion liquid chromatography (SEHPLC). A number of factors contributed to this development, including the elaboration of techniques for column packing with sorbents of small particle size (5–10 μm), and the application of semi-rigid and rigid sorbents, permitting a large increase in the rate and efficiency of the separation. Size-exclusion chromatography involves very simple procedures and mild conditions, suitable even for such labile compounds as proteins and peptides.

This work was devoted to the development of SEHPLC methods for protected peptides obtained during the synthesis of large peptides and proteins. As such peptides are usually insoluble in water or aqueous–organic solvent systems, it is impossible to use common methods for the analysis and purification of proteins and unprotected peptides such as ion-exchange[5], reversed-phase[6,7]* and partition[9,10] chromatography or counter-current distribution. The limited solubility of long-chain protected peptides in non-polar media and the risk of irreversible sorption restrict the application of adsorption chromatography, although there are some examples of the

* The work of Bakkum *et al.*[8] is an exception; the solubility of protected secretin and its fragments allowed reversed-phase chromatography to be used.

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TABLE I

PARAMETERS OF THE COLUMNS INVESTIGATED

Packing material	*Particle fraction (μm)*	d_p *(μm)*	*Column size (mm)*	*Effeciency, N (theoretical plates)*	*H (μm)*	H/d_p	*F*	*S*	N_c *(theoretical plates)*
Sephadex LH-20	40–56	48	450 × 25	3600	125	2.60	1.00	0.820*	640*
	56–70	63	600 × 8	2500	240	3.81	1.00	0.637**	1058**
	56–70	63	600 × 25	3700	162	2.57	1.00	–	–
Si 60	10–20	15	330 × 25	16,700	19.8	1.32	1.12	–	–
Si 40	6–10	8	200 × 8	6000	33.3	4.16	1.07	0.408	9700
Silica gel L	5–10	7.5	330 × 25	20,000	16.5	2.20	1.10	0.584***	3000***
Silasorb 600	5–8	6.5	250 × 8	27,200	9.2	1.41	1.20	0.585***	2500***
Microspherical silica	10–25	17.5	330 × 25	14,000	23.6	1.35	1.05	–	–
Microspherical silica	5–10	7.5	250 × 7	15,000	16.6	2.21	1.03	0.775***	1100***
Zorbax Sil §	–	5	250 × 4.6	7500	33.3	6.67	2.33	0.384	6400
LiChrosorb RP-8 §	–	10	250 × 22.7	7280	34.3	3.40	1.27	–	–
PSM-60S §	–	5	250 × 4.6	16,000	15.6	3.12	2.13	0.384	6200

* For peptides with molecular masses of about 300 daltons.
** For peptides with molecular masses of about 1000 daltons.
*** For peptides with molecular masses of about 4300 daltons.
§ DuPont columns.

successful use of this method for the purification of hydrophobically protected peptides[11,12].

In many studies on the purification of water-insoluble protected peptides, use was made of gel filtration on commercial, soft, strongly swelling sorbents (Enzacryl K-2[3], Sephadex LH-20[4,13] and LH-60[13] and Bio-Beads SXI[4]). However, under these conditions it proved difficult to separate peptides that differ in molecular mass by less than 2-fold, especially for high-molecular-mass samples.

Therefore, when we started our work there were no effective methods for separating protected peptides that could be applied to a wide range of samples. We have investigated the behaviour of different types of sorbents under SEHPLC conditions, using dimethylformamide as the solvent on account of its availability, solvent properties and chemical inertness. This study is a part of a programme devoted to the synthesis of large peptides and proteins[14]. The peptides involved are the protected segments of neurotoxin II from the Central Asian *Naja naja oxiana* cobra venom, the structures of which are shown in Fig. 1.

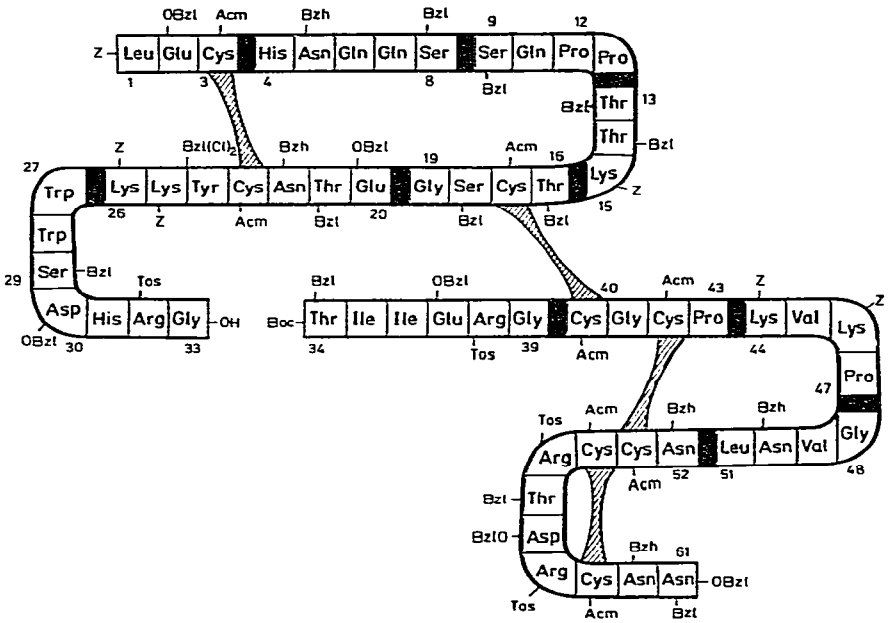

Fig. 1. Molecule fragment scheme for neurotoxin II from the venom of Central Asian cobra *Naja naja oxiana*.

EXPERIMENTAL

Chromatography was performed with a Waters Assoc. Model M 6000 A solvent-delivery system, a Model 660 solvent programmer, a U6K universal injector, a Model 440 UV detector (280 nm), a Varian refractive index detector and a Hitachi Perkin-Elmer two-channel recorder.

Dimethylformamide was purified according to the procedure in ref. 16. The

parameters of the columns prepared in this work and DuPont columns are given in Table I. The preparative injection of the sample was carried out with an Eldex B-100-S pump.

Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden), silica gel L and Silasorb 600 from Lachema (Brno, Czechoslovakia) and silica gel Si 60 and Si 40 from Merck (Darmstadt, G.F.R.). Sorbent fractionation was carried out on an Alpine (G.F.R.) separator. The methods for the synthesis of the peptides under investigation are given in ref. 14.

RESULTS AND DISCUSSION

To find the optimal SEHPLC conditions for the analysis and preparative isolation of the protected peptides we had to define the properties of the sorbents, the column characteristics and the efficiency of peptide separation. With this aim, we obtained high-performance columns of high effeciency packed with fractionated soft, semi-rigid and rigid gels. We used Sephadex LH-20 as a soft gel and the rigid gels were represented by various silica gels. In an attempt to define the characteristics of the sorbents, the following considerations were taken into account. The resolution, R_s, of two neighbouring peaks is determined in exclusion chromatography by the expression

$$R_s = \frac{V_2 - V_1}{\omega} \tag{1}$$

Where V_1 and V_2 are the retention volumes of the first and second components (ml) and ω is the width of the chromatographic band (ml), equal to 4 σ (σ is the standard deviation from the Gaussian). To facilitate the comparison of columns of different geometry we used the corrected retention volumes

$$V_i' = \frac{V_i}{V_c} \tag{2}$$

where V_i is the corrected retention volume of the ith component and V_c is the geometrical volume of the column. Subsequently the corrected retention volume of the component, eluting with a void volume (V_0) equal to the column porosity (ε_0), *i.e.*, to the portion of the column volume occupied by the space between the particles (Fig. 2), is determined by the expression

$$V_0' = \frac{V_0}{V_c} = \varepsilon_0 \tag{3}$$

The corrected retention volume of the substance for which all of the sorbent pores (V_t) are accessible can be expressed as

$$V_t' = \frac{V_t}{V_c} = \varepsilon_t \tag{4}$$

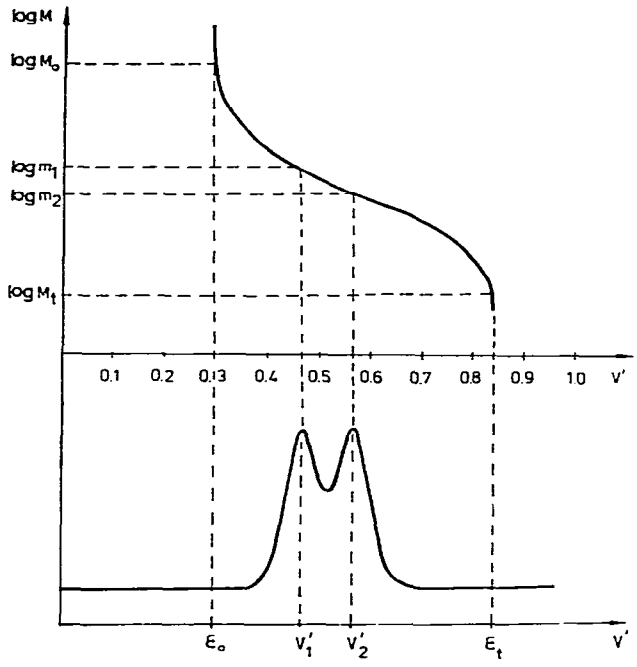

Fig. 2. Dependence of molecular masses of separated compounds on the retention volume: the calibration graph and its parameters. For explanation, see text.

where ε_t is the porosity of the column packing or the portion of the column volume occupied by the sorbent pores and by the space between the particles. The efficiency of the chromatographic column (NTP) under size-exclusion conditions is determined according to the equation

$$N = 16\,(V_t/\omega)^2 \tag{5}$$

or, on the basis of eqn. 4,

$$N = 16\,(\varepsilon_t V_c/\omega)^2 \tag{6}$$

The sorbent selectivity, *i.e.*, the ability to separate two compounds, depends on the dispersion value and the distribution of the pores therein, as the selectivity for the sorbent with a Gaussian distribution of pores (a linear calibration graph) is determined only by the width of the pore dispersion, *i.e.*, by exclusion limits m_0 and m_t, and may be expressed as follows:

$$S = 1/\log\,(m_0/m_t) \tag{7}$$

For sorbents with any sort of pore distribution we have the following more general expression:

$$S = 1/\tan\,\alpha\,(\varepsilon_t - \varepsilon_0) \tag{8}$$

where $\tan \alpha = \mathrm{d} \log m/\mathrm{d}V_i$ is determined from the calibration graph (Fig. 2) and α is the angle of the calibration graph slope at a given point relative to the abscissa.

For two compounds with molecular masses m_1 and m_2 the difference in the retained volumes is expressed as follows:

$$V_2 - V_1 = \frac{\log (m_2/m_1)}{\tan \alpha} = \log (m_2/m_1)\, S\, (\varepsilon_t - \varepsilon_0) \tag{9}$$

By inserting eqns. 9 and 6 into eqn. 1, one obtains the following resolution of two peaks:

$$R_s = 0.25 \log (m_2/m_1)\, S \cdot \frac{\varepsilon_t - \varepsilon_0}{\varepsilon_t} \cdot \sqrt{N} \tag{10}$$

For sorbents with linear calibration graphs, eqn. 7 may be taken into account:

$$R_s = 0.25 \cdot \frac{\log (m_2/m_1)}{\log (m_0/m_t)} \cdot \frac{\varepsilon_t - \varepsilon_0}{\varepsilon_t} \cdot \sqrt{N} \tag{11}$$

By transposing eqn. 10 we obtain

$$N = \left[\frac{4R_s}{S} \cdot \frac{\varepsilon_t}{\varepsilon_t - \varepsilon_0} \cdot \frac{1}{\log (m_2/m_1)}\right]^2 \tag{12}$$

Thus one can calculate the necessary efficiency for the separation of two substances with molecular masses m_1 and m_2 according to the calibration graph for a resolution R_s. Eqn. 10 indicates that in order to enhance the resolution of two substances with molecular masses m_1 and m_2 it is necessary to increase the efficiency (N) of the column and to choose a sorbent with maximal selectivity (S), *i.e.*, with the minimal possible pore distribution and with the maximal value of $(\varepsilon_t - \varepsilon_0)/\varepsilon_t$, *i.e.*, with the maximal working range of $V_t - V_0$.

The column efficiency also depends on the packing conditions. To determine the optimal packing conditions for soft gels we studied the dependence of the pressure drop in the column, packed under sedimentation conditions (under the pressure of a liquid column 1 m high), on the flow-rate. As can be seen in Fig. 3, this dependence is represented by a curve with three regions, passing from one to another at definite points. The linear region 0–1 corresponds to the increase in the pressure drop depending on the flow-rate according to Darcy's law. Within this region the packing density does not change and columns packed at flow-rates corresponding to this region have the same efficiency as those packed under sedimentation conditions. Above point 1 the packing becomes denser, bringing about an increase in the pressure drop disproportional to the flow-rate. Within region 1–2 the void volume of the column decreases owing to the elastic deformation of particles, which as a result increases the column efficiency. Maximal column efficiency was achieved at flow-rates corresponding to point 2 (Table I).

Above point 2 the pressure increases considerably owing to non-elastic gel deformation. The elution profile of a substance in the column packed at a flow-rate

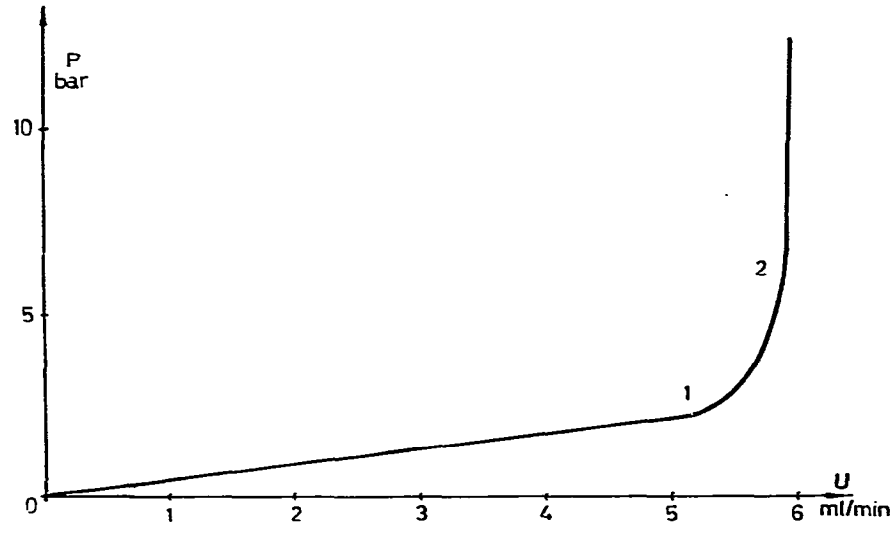

Fig. 3. Dependence of pressure drop (P) on flow-rate (U) for columns packed under suspension conditions (450 × 25 mm). Sorbent: Sephadex LH-20 (40–56 μm). 1, First critical point; 2, second critical point.

above point 2 is asymmetric, apparently owing to the partial inaccessibility of the deformed gel pores.

Similar results have also been obtained with other soft (Sephadex LH-60, Exacryl K-2) and semi-rigid (Spheron P-40, P-300, P-1000) gels[16]. The critical points for each type of gel, particle fraction, column size and eluent differ and are determined experimentally. However, the curve shape is always similar to that shown in Fig. 3.

Using the narrow sorbent particle fractions (40–56 μm), we obtained columns of efficiency 8000 theoretical plates per metre, *i.e.*, with $H = (2–3)\ \bar{d}_p$, where $\bar{d}_p$ is the average particle size (diameter squared) of the sorbent (Table I).

To obtain columns with rigid sorbents we used the suspension packing method. The columns packed in this manner are usually characterized by highly asymmetric peaks. Under preparative conditions the peak asymmetry should be at a minimum for complete isolation of the compounds. We obtained columns with an asymmetry factor, F, of 1.1 (the calculation of the asymmetry factor is described in ref. 17). The height equivalent to a theoretical plate (H) for these columns is (1.5–2.5) $\bar{d}_p$ (Table I). An example of the separation of an artificial peptide mixture is given in Fig. 4 and an example of the analysis and preparative isolation of a 28-membered peptide on a silica gel L column in Fig. 5.

According to eqn. 10, in order to obtain a better resolution of two peaks, one needs a high-performance column, a highly selective sorbent and a wider operating area. In order to compare the various sorbents, it seems reasonable to introduce the sorbent characteristic efficiency (N_c), including both of the factors mentioned and corresponding to the efficiency necessary for resolution with $R_s = 1$ of two compounds differing 2-fold in their molecular masses:

$$N_c = 180\left[\frac{\varepsilon_t}{(\varepsilon_t - \varepsilon_0)\ S}\right]^2 \tag{13}$$

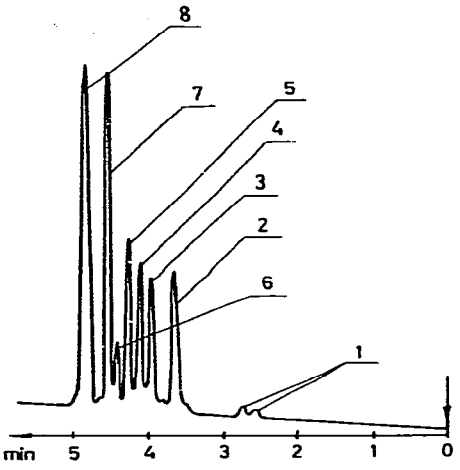

Fig. 4. Chromatogram of the artificial mixture of protected peptides: 1, high-molecular-mass impurities; 2, Boc-34–61-OBzl (molecular mass 5417); 3, Boc-48–61-OBzl (3125); 4, Aoc-55–61-OBzl (1962); 5, Aoc-58–61-OBzl (1265); 6, Boc-60–61-OBzl (768); 7. Boc-Asn(Bzh)-OBzl (488); 8, dimethyl sulphoxide (78). Column, Silasorb 600 (250 × 8 mm I.D.). Flow-rate, 2 ml/min. Refractive index detector.

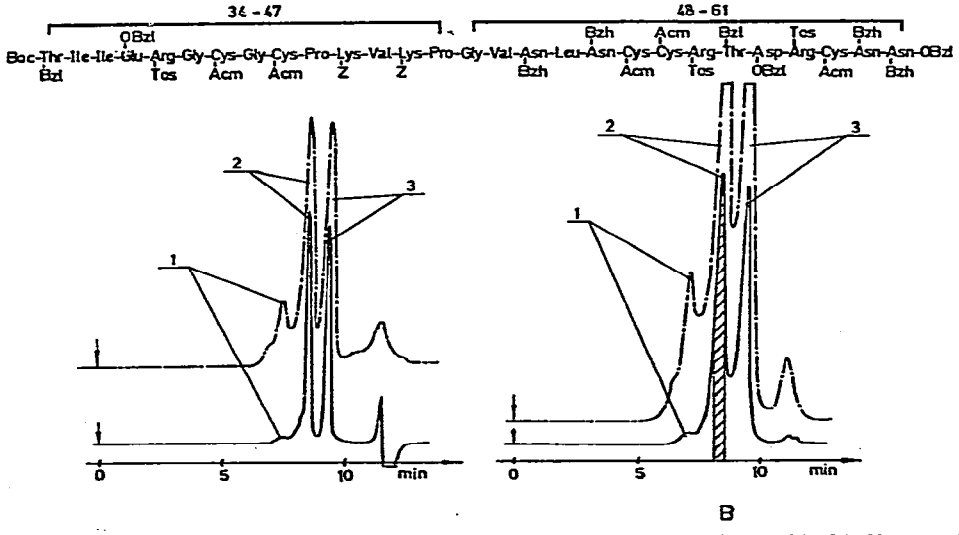

Fig. 5. (A) Analytical and (B) preparative chromatography of synthetic peptide 34–61 on a silica gel L column (330 × 25 mm I.D.). Flow-rate, 10 ml/min. Cross-hatched region corresponds to the isolated fraction. 1, High-molecular-mass impurities; 2, Boc-34–61-OBzl; 3, initial fragments. Broken lines, UV detector; solid lines, refractometer. Loading under preparative conditions, 50 mg per 2 ml. The fragment 34–61 sequence is shown at the top. The initial fragments are designated by brackets.

Soft Sephadex gels have a relatively narrow pore distribution; in addition, they can be easily packed into a column at $\varepsilon_0 = 0.25$, which is an excellent indicator of the gel packing properties.

Of the gels investigated earlier[16] and in this work, Sephadex LH-20 is the most suitable packing for the separation and analysis of synthetic protected peptides with

molecular masses of less than 2000 daltons. The mechanical properties of Sephadex LH-20 allow one to obtain high-performance columns with which high flow-rates can be achieved. This sorbent has an S-shaped calibration graph (Fig. 7) and maximal selectivity ($S = 0.820$) at about 300 daltons; at about 1000 daltons the selectivity is lower ($S = 0.637$), so this sorbent is more suitable for the separation of low-molecular-mass peptides. An example of the separation of an artificial mixture of peptides on the Sephadex LH-20 column is given in Fig. 6.

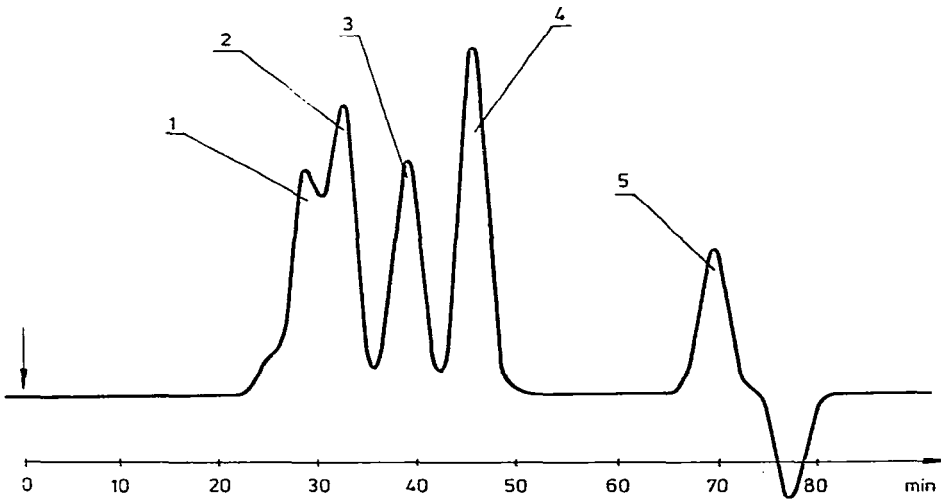

Fig. 6. Chromatogram of artifical mixture of protected peptides and naphthalene. 1, Aoc-55–61-OBzl (molecular mass 1963); 2, Aoc-58–61-OBzl (1265); 3, Boc-60–61-OBzl (768); 4, Boc-Asn(Bzh)-OBzl (488); 5, naphthalene (130). Column, Sephadex LH-20 (600 × 8 mm I.D.). Flow-rate, 0.4 ml/min. Refractive index detector.

For separation and analysis of peptides with molecular masses higher than 2000 daltons we used silica gels of different types.

The application of rigid sorbents in exclusion cnromatography has opened up new opportunities for performing separations and analyses of peptides. Although the parameter value is 0.4–0.5 for columns packed with rigid adsorbents, they have a number of advantages in comparison with soft and semi-rigid gels. For example, the accessibility of silica gels with small particle sizes allows one to obtain columns of extremely high efficiency (about 100,000 theoretical plates per metre). In addition, the mechanical properties also allow high flow-rates to be used. One can select silica gels functioning in various ranges of molecular masses and, by modifying the silica gel surface, one can obtain an adsorbent suitable for the separation of a wide variety of compounds, including labile biopolymers, in preparative amounts.

We investigated the selectivity of different silica gels in order to find an optimal sorbent for size-exclusion chromatography of the protected peptides in organic solvents.

As can be seen in Fig. 7, Zorbax Sil and PSM-60S have virtually linear calibration graphs, *i.e.*, these sorbents have the same selectivity over the whole operating range, but the selectivity is low ($S = 0.384$). The characteristic efficiency for these

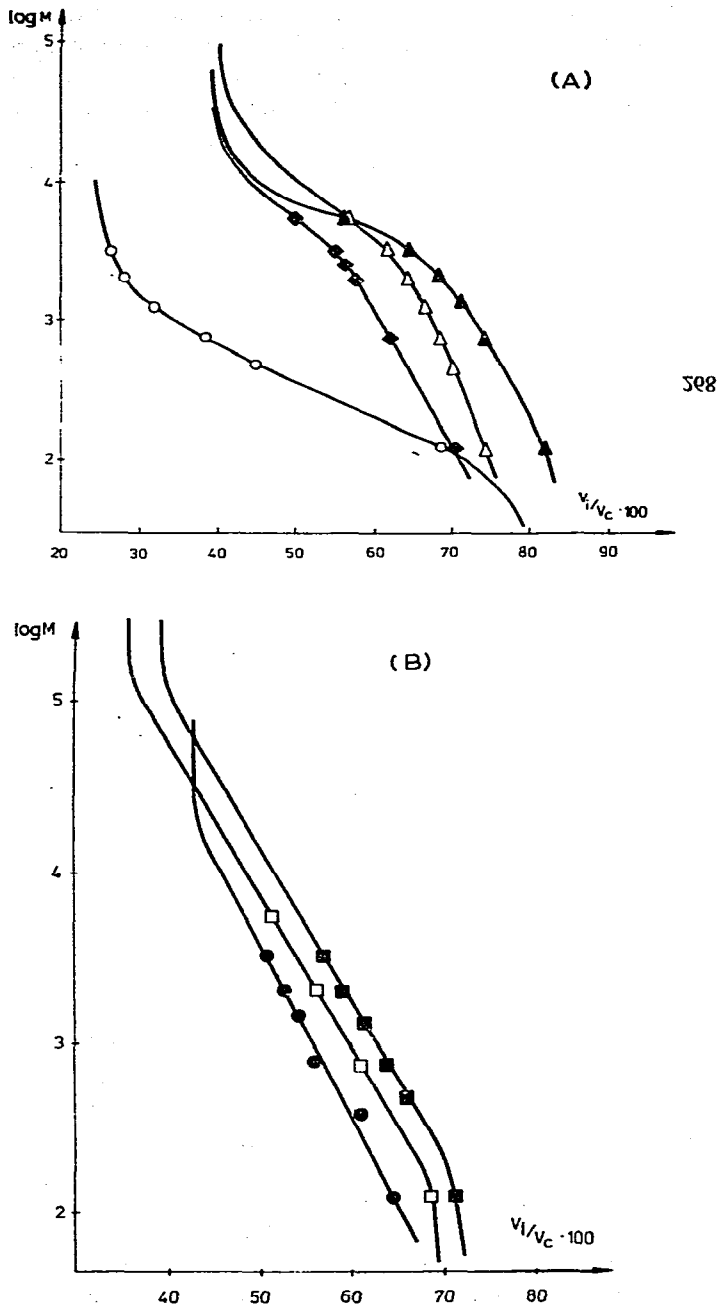

Fig. 7. Calibration graphs for synthetic protected peptides in dimethylformamide. (A) ○, Sephadex LH-20; ◆, silica gel L; △, Silasorb 600; ▲, spherical silica gel. (B) ●, Si 40; □, PSM-60S; ■, Zorbax Sil. Synthetic peptides as in Fig. 4, plus a Boc-52–61-OBzl fragment (molecular mass 2575) and naphthalene used for calibration. The void volume was determined using high-molecular-mass impurities.

sorbents is $N_c = 6500$ (Fig. 8). Silica gel Si 40 also has a linear calibration graph, but its operating range is apparently smaller than those of other silica gels (see Table I and Fig. 7B) owing to the larger volume of the micropores. For the same reason the characteristic efficiency reaches $N_c = 10{,}000$.

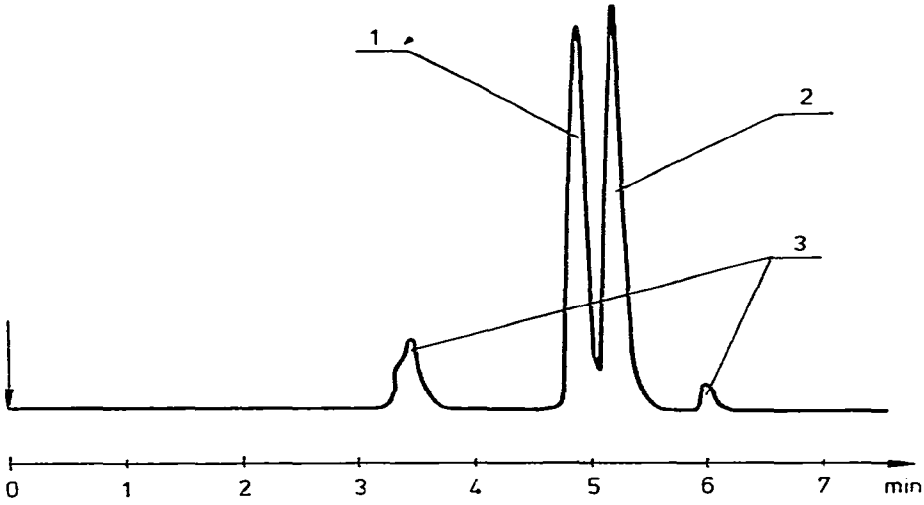

Fig. 8. Chromatogram of a mixture of peptides differing 2-fold in molecular mass. Column, Zorbax Sil (250 × 4.6 mm I.D.). Flow-rate, 0.5 ml/min. Detector, UV (280 nm). 1, Boc-52–61-OBzl (molecular mass 2575); 2, Aoc-58–61-OBzl (1265); 3, impurities.

The best selectivity was found for the silica gel obtained by Unger's method[17]. The calibration graph of this silica gel is non-linear, *i.e.*, its selectivity is mass-dependent. For peptides with molecular masses around 4000 daltons $S = 0.775$.

It should be noted that the nature of the silica gel surface also plays an important role in the successful separation of the protected peptides. For instance, only fully protected peptides were eluted from columns with unmodified surfaces according to the pure size-exclusion mechanism (Fig. 5). Peptides containing free amino groups were sorbed on a gel matrix and eluted later than peptides with the same molecular masses (Fig. 9). A complex elution profile was observed for histidine-containing peptides. Peptides with an unprotected imidazole group of the histidine were partially sorbed on the matrix of unmodified silica gel (Fig. 10). Treatment of the sorbent with trimethylchlorosilane or the use of silica gel RP-8 suppressed the adsorption of the histidine-containing peptides almost completely.

Their ability to form aggregates resulted in a complicated and unpredictable elution profile of the peptides in dimethylformamide on a column packed with LiChrosorb RP-8 (Fig. 11). The isolated fractions corresponding to several peaks reproduced the same complicated elution profile on re-chromatography. It should be noted that no aggregates were observed in the chromatography of histidine-containing peptides on Sephadex LH-20 and LH-60, because during the interaction of the compound with the carbohydrate groups of the Sephadex gels the association is suppressed, whereas the hydrophobic surface of the modified trimethylsilyl or octyldimethylsilyl groups of silica gels is unable to destroy the aggregates formed in solution.

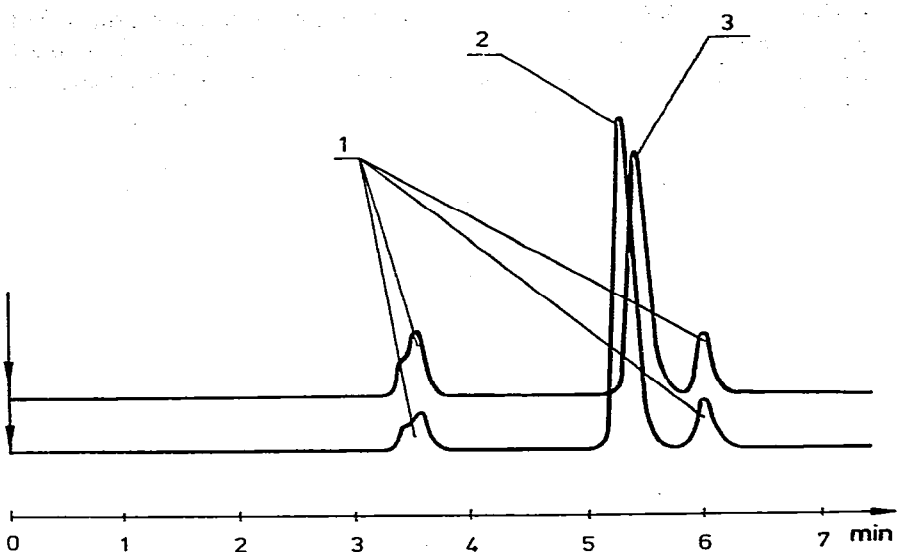

Fig. 9. Elution profiles of fragment 52–61 in a Zorbax Sil column (250 × 4.6 mm I.D.). Flow-rate, 0.5 ml/min. Detector, UV (280 nm). 1, Impurities; 2, Boc-52–61-OBzl; 3, H-52–61-OBzl.

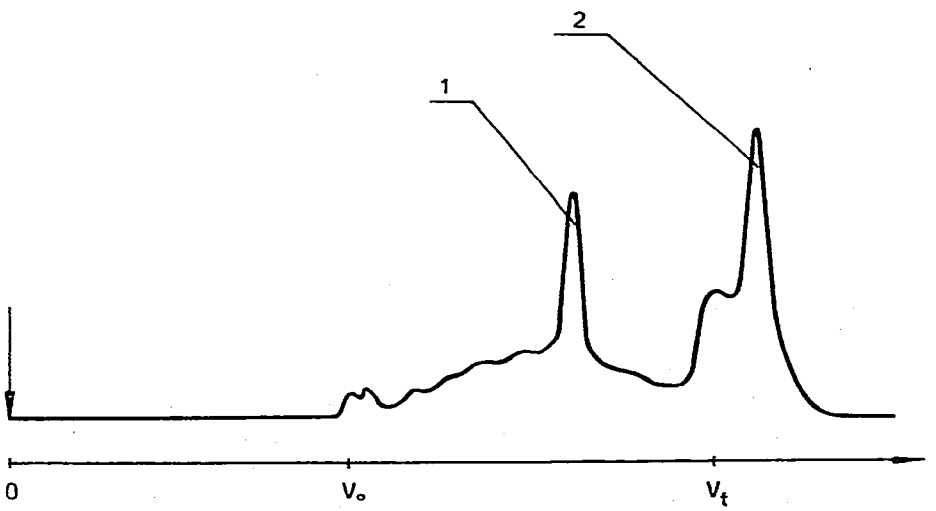

Fig. 10. Elution profile of peptide 1–19 on a 330 × 25 mm I.D. column packed with silica gel L. Flow-rate, 10 ml/min. 1, Z-1–19(Tos)-OH (His-protected Tos); 2, Z-1–19-OH (His unprotected).

Special attention should also be focused on the technique used for injection of the substance into the column under preparative chromatographic conditions and on the detection method when working with protected peptides. The injection of the sample into the column is one of the most important factors influencing the resolving power of the column. The total diffusion of the chromatographic zone is determined by the expression[19]

$$\sigma_{tot}^2 = \sigma_{col}^2 + \sigma_{ex}^2 \qquad (14)$$

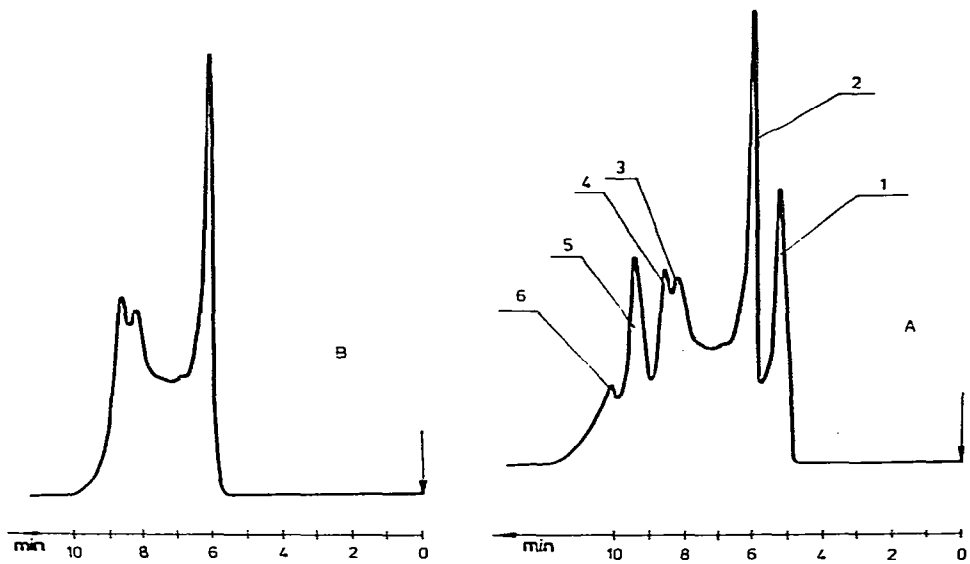

Fig. 11. Elution profile of peptide Z-1–33-OH on a 250 × 22.7 mm I.D. column. Flow-rate, 8 ml/min. Sorbent, LiChrosorb RP-8. Detector, UV (280 nm). (A) Chromatogram of the initial compound; 1–6 represent the isolated peaks. (B) Re-chromatography of peak 2. Peaks 3 and 4 have identical profiles.

where σ_{tot} is the total dispersion of the peak, σ_{col} is the dispersion of the peak in the column and σ_{ex} is the dispersion of inter-column effects, being a function of three components:

$$\sigma_{ex}^2 = \sigma_{inj}^2 + \sigma_d^2 + \sigma_{cap}^2 \tag{15}$$

As the diffusion contribution in the detectors (σ_d^2) and capillaries (σ_{cap}^2) can easily be minimized under preparative chromatographic conditions, one may assume that

$$\sigma_{ex}^2 \approx \sigma_{inj}^2 \tag{16}$$

With ideal injection of the sample into the column, the dispersion is[19]

$$\sigma_{inj}^2 = \frac{V^2}{12} \tag{17}$$

where V is the volume of sample injected.

Hence it follows that the resolving power of the column can be increased if σ_{ex}, *i.e.*, the diffused sample volume, is minimized.

We investigated two ways of injecting the sample into the column: loop injection using a U6K universal injector and direct pump injection (Fig. 12) (other injection techniques appeared to be less efficient for work under preparative conditions). The outlet of the injectors was connected directly to the detector, and the concentration profile of the eluting sample was observed. With the loop injection method a

large (1.5-fold) increase in the sample volume was observed at the injector outlet (Fig. 12A). The contribution of the injection volume (σ^2_{inj}) to the total peak dispersion in this instance is within the range $V^2/4$ to $V^2/5$, depending on the rate of injection of the sample. Virtually no additional broadening was observed (*i.e.*, $\sigma^2_{inj} = V^2/12$) with the injection pump connected directly to the detector (Fig. 12B). The pump can inject a volume of sample $\sqrt{3}$ times greater than with the loop injection method, which adds to the utility of the former technique.

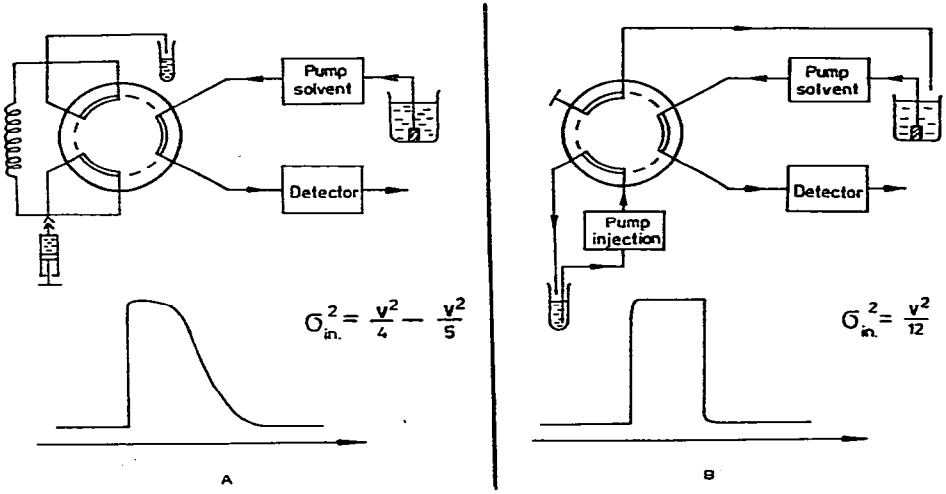

Fig. 12. Concentration profile of injected sample and diagram of pump connection, sample injection and detector sites for loop (A) and pump (B).

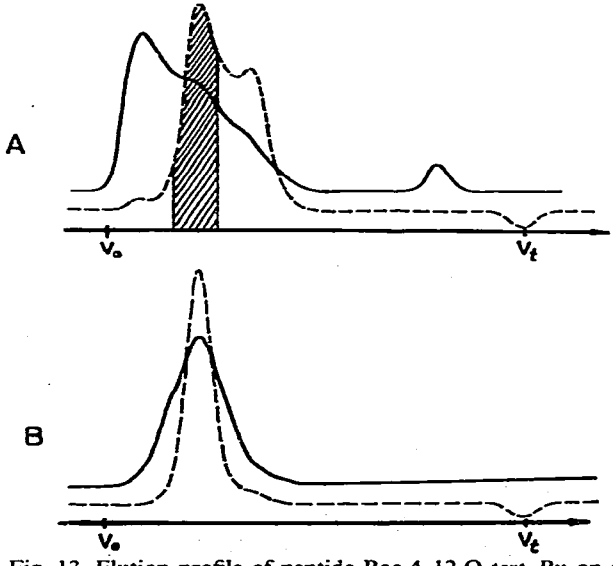

Fig. 13. Elution profile of peptide Boc-4–12-O-*tert.*-Bu on a Sephadex LH-20 column (1200 × 16 mm I.D.). Flow-rate, 2 ml/min. Solid lines, UV detector (280 nm); broken lines, refractive index detector. (A) Chromatogram of a crude reaction product. The cross-hatched region corresponds to the isolated fraction. (B) Re-chromatography of the isolated fraction.

The influence of the sample concentration on the diffusion of the chromatographic zone was more pronounced for higher molecular masses. Probably the concentration overload in this instance is due to the increased viscosity of the sample and therefore it manifests itself mainly at the column head. The load capacity could be increased by injecting the sample in an eluent diluted with a low-viscosity solvent, such as diethyl ether or acetonitrile. This helped to increase the load 1.5-fold and thus maintain the resolution as indicated in Fig. 6.

Most investigators use a UV detector in studies on peptides. However, this does not provide adequate information on the purity and composition of the peptide fractions obtained after chromatography. In certain instances additional information can be obtained with the use of a refractometric detector. Especially convenient is the application of a two-detector control monitor, which provides information on the composition of the material being chromatographed and considerably facilitates the task of identifying and isolating the required product (Fig. 13).

From the above considerations, it follows that with the aid of SEHPLC, the analysis and separation of protected peptides in organic solvents can be performed with soft, semi-rigid and rigid gels. For peptides with molecular masses less than 2000 daltons, fractionated Sephadex LH-20 proved to be the most suitable sorbent. Peptides with a higher molecular mass may be analysed and separated on columns packed with silica gel. High-performance columns packed with highly selective sorbents allow the separation of peptides with molecular masses that differ less than 1.1-fold. For example, 5–10 min are sufficient to remove an impurity from a 30-membered peptide differing in chain length by 2–3 amino acid residues.

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