Purification of Synthetic Peptides by Reversed Phase Chromatography

Application Note

ÄKTA™explorer

- Rapid media screening and method optimisation by use of pre-programmed template methods and scouting schemes.
- Easy scale up from a 3 ml column to a 99 ml preparative RPC column
- Mass spectrometry and second dimension HPLC for purity analysis

Summary

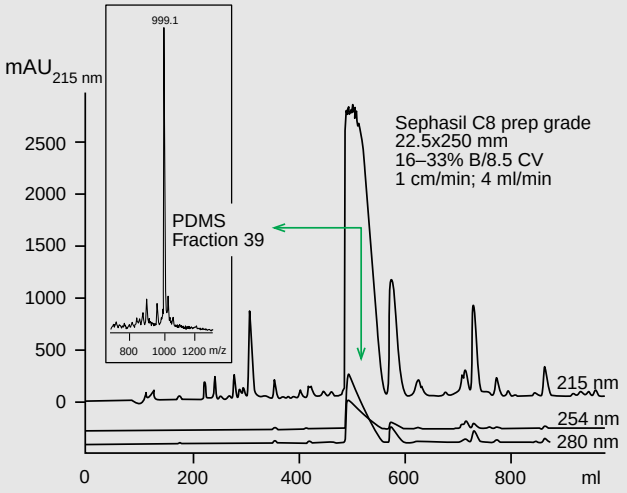
A general strategy for purification of synthetic peptides was used for rapid method optimisation and scale up, using ÄKTAexplorer and UNICORN™.

The strategy is described in detail for the purification of crude myelin basic protein (89-101), synthesised using FMOC technique, and of hemorphin-7, synthesised by the tBoc technique. Media screening and method development were done on 3 ml columns and the optimal purification method was scaled up to purification of 500 mg of material on a 99 ml column. All work was done on the same system.

Media screening was done using RESOURCE® RPC 3 ml, Sephasil® C8 prep grade 4×250 mm and Sephasil C18 prep grade 4×250 mm. Method optimisation was continued on the Sephasil C8 prep grade 4×250 mm column and the final purification was done using the same medium in a 22.5×250 mm column.

The same strategy, media and columns were used for purification of hemorphin-7, synthesised using the tBoc technique. The purified material was analysed by plasma desorption mass spectrometry (PDMS) and micro-HPLC (SMART® System).

Purification of Hemorphin-7
Analysis by PDMS



Synthetic Hemorphin-7 (58 mg) was purified according to the general strategy outlined in the text, using Sephasil C8 prep grade (22.5x250 mm). Solvent A was 0.065% TFA and solvent B was 0.050% TFA in acetonitrile. Fractions were analysed by plasma desorption mass spectrometry (PDMS) as indicated in the figure and by micro-HPLC which revealed a 99.0% purity of the target peptide as judged by peak area integration. Additional peaks in the mass spectrum represent the Na-salt of Hemorphin-7 and peptide fragments generated in the PDMS.

Introduction

The interest in peptides as potential drug candidates is currently very high and the production of chemically synthesised peptides (<20–30 amino acid residues) is now a routine laboratory procedure. The method can prepare quantities of peptides from a few mg up to several g. However, there are numerous ways in which the synthesis can go wrong, so that alternative peptides are produced together with the desired one. Such impurities may originate in a low coupling yield, giving rise to truncated and deleted sequences, or from incomplete removal of protective groups. Other impurities may originate from alteration of sensitive amino acids, e.g. oxidation or alkylation of methionine and tryptophan, ring formation by N-terminal glutamine, dehydration of asparagine or glutamine, and transamidation or deamidation of asparagine and glutamine. In addition, racemisation of the amino acids may occur during coupling. It is obvious that these potential impurities, frequently having quite similar chemical properties, sometimes offer great challenges when purifying the target peptide.

Due to all the potential impurities in peptide drugs the FDA, CDER, CBER provide detailed rules for the characterisation of the synthetic peptide, its synthesis and purification (1). The purification process, the analyses and the interpretation of the results must be described in great detail .

This application note describes a general strategy for purifying synthetic peptides, which takes advantage of a novel chromatograpy software, UNICORN, and ÄKTAexplorer.

18-1111-24

Amersham Biosciences

From a knowledge of the physico/chemical properties of the sample we decide which chromatographic techniques are to be used. Normally reversed phase chromatography (RPC) is considered to be sufficient, but depending on the solubility and amount of material to be purified gel filtration and/or ion exchange chromatography are considered. Next, pre-programmed template methods in UNICORN are used for automatic scouting of a number of chromatographic supports, different mobile phases, gradient shapes and flow rates. In this work the software was set for the method scouting to be performed in a number of analytical separations of a preparation of the peptide VHFFKNIVTPRTP (MB 89-101) and hemorphin-7.

Peptide MB 89-101

Active immunisation of animals with myelin basic protein (MBP) induces experimental autoimmune encephalomyelitis in a variety of animal species. It has been shown that the peptide VHFFKNIVTPRTP (MB 89-101) constitutes an epitope of MBP which, unlike other encephalogenic epitopes of MBP, exerts its action in association with multiple class II MHC molecules (2). MB 89-101 was synthesised and purified to at least 95% purity for studies of its autoimmune properties in animals.

Hemorphin-7

The hemorphins are recently identified peptides with opioid activity, which are enzymatically released from the blood protein hemoglobin (3, 4). Hemorphin-7 (YPWTQRF), has been identified in human plasma and its concentration increases during long distance running. This increase is parallelled by elevated levels of circulating β-endorphin (5). Hemorpin-7 was synthesised and purified to at least 95% purity for use in animal studies.

Choice of purification technique

Since it has five potentially charged amino acid residues, including the N- and C-terminal positions, MB 89-101 could well be a candidate for purification by means of ion exchange chromatography. However, RPC was considered to be the best choice for the purification of the peptides. This choice was particularly influenced by the fact that the different components in a sample obtained from solid phase synthesis are likely to have quite similar amino acid compositions. Since RPC is based on hydrophobic interactions, retention depends on those amino acids that interact with the ligands and is therefore susceptible to the amino acid sequence rather than just the total composition. The selectivity it provides is so high that even peptides with identical compostion but different sequences can be separated (6).

Results

Media screening

Three different media were used:
SOURCE™ RPC 15 µm in RESOURCE RPC 3 ml
Sephasil C8 prep grade 12 µm
Sephasil C18 prep grade 12 µm

A pre-programmed template method in UNICORN was adjusted for media screening, thus enabling automatic control of column switching. The ease with which this is implemented is indicated on top of Figure 1 showing the Run numbers and the scouting scheme.

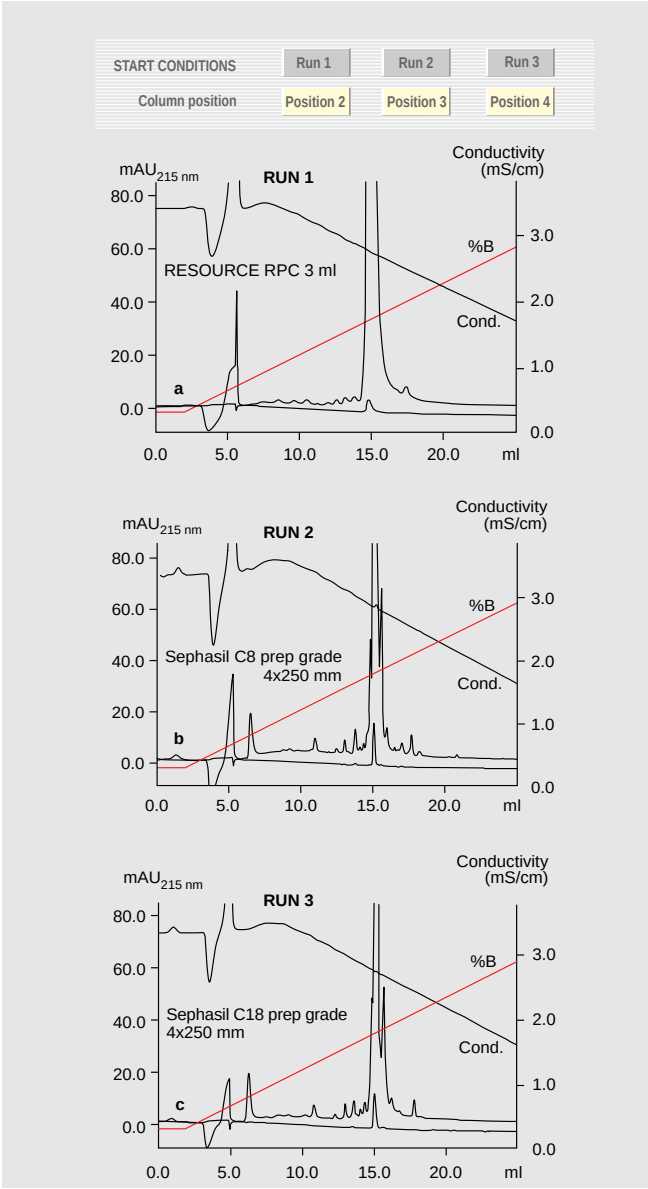


Fig. 1. Media screening was performed using SOURCE™ RPC, Sephasil® C8 prep grade and Sephasil C18 prep grade packed in the columns indicated in Figures 1 a–c. Synthetic MB 89-101 (0.030 mg/ml gel) was automatically loaded on the columns. The scouting scheme used to select media is shown on top of the chromatograms. A simple valve switch was used to select a new column for each run.

The sample was eluted in gradients from 5–100% B in 10 column volumes at a linear flow rate of 4 cm/min (RESOURCE® RPC 3 ml, 1.3 ml/min; Sephasil columns, 0.50 ml/min). Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 215 nm (upper trace), 254 nm (bottom trace) and 280 nm (not indicated).

Aliquots of MB 89-101 (0.030 mg/ml gel) were separated on the columns in gradients from 5–90% B in 10 column volumes (CV) and at a linear flow rate of 4 cm/min. As shown in Figure 1 the highest resolution was achieved with Sephasil C8 prep grade, which was therefore chosen for the subsequent method optimisation.

Gradient and flow rate scouting

A pre-programmed template method in UNICORN was adjusted for automated scouting. Gradient scouting was performed at a constant linear flow of 4 cm/min, while during flow rate scouting the gradient shape was kept constant at a gradient length of 10 CV (see the scouting scheme indicated on top of Figure 2).

From Figure 2 it is seen that gradient reproducibility (as monitored by conductivity), was very good at all flow rates used, giving excellent retention volume reproducibility. The results in Figure 2 show that the most shallow gradient (Run 3) and the lowest flow rate (Run 6) gave the best resolution. A combination of a shallow gradient and a low flow rate was therefore tried for the following method optimisation.

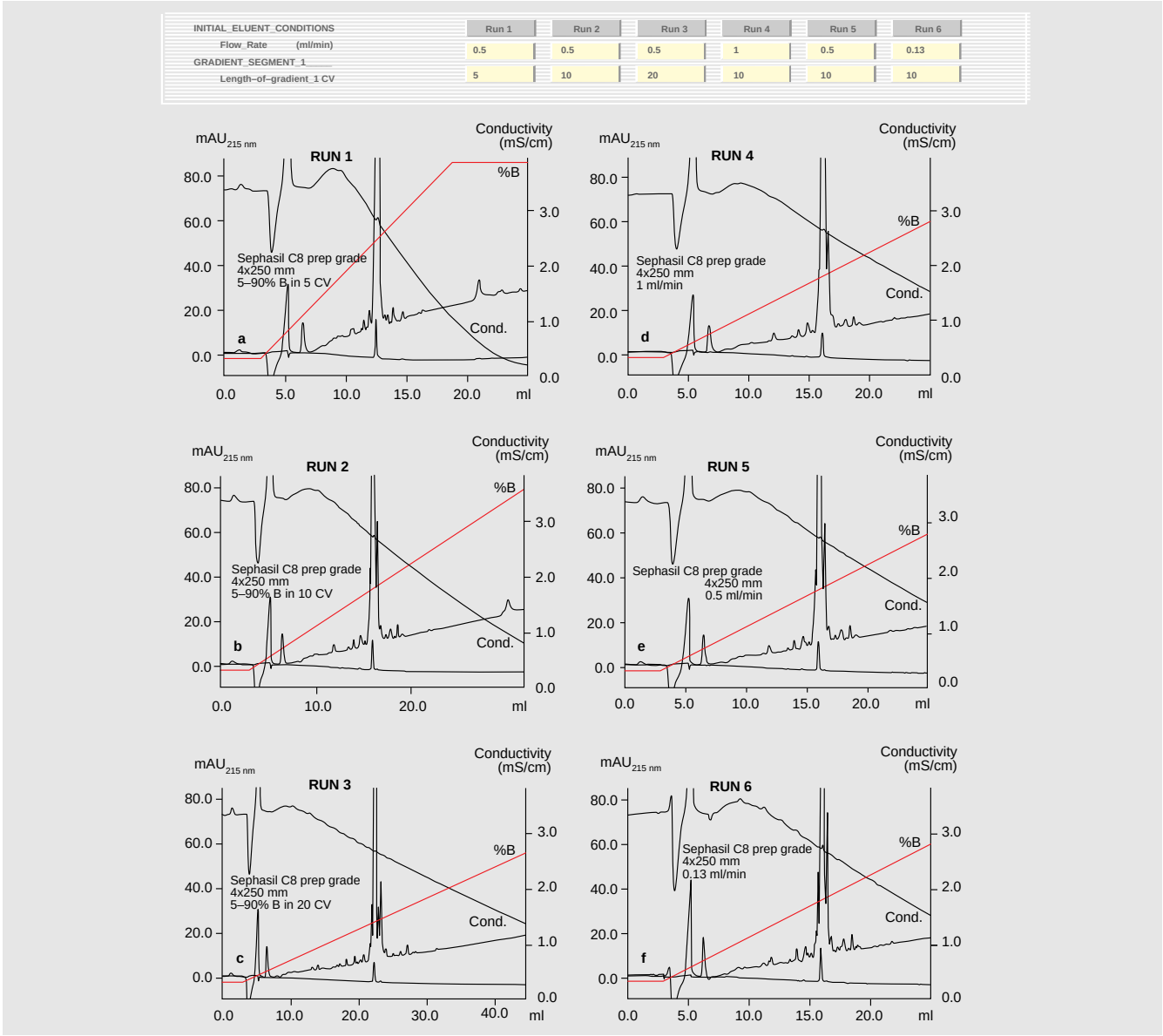
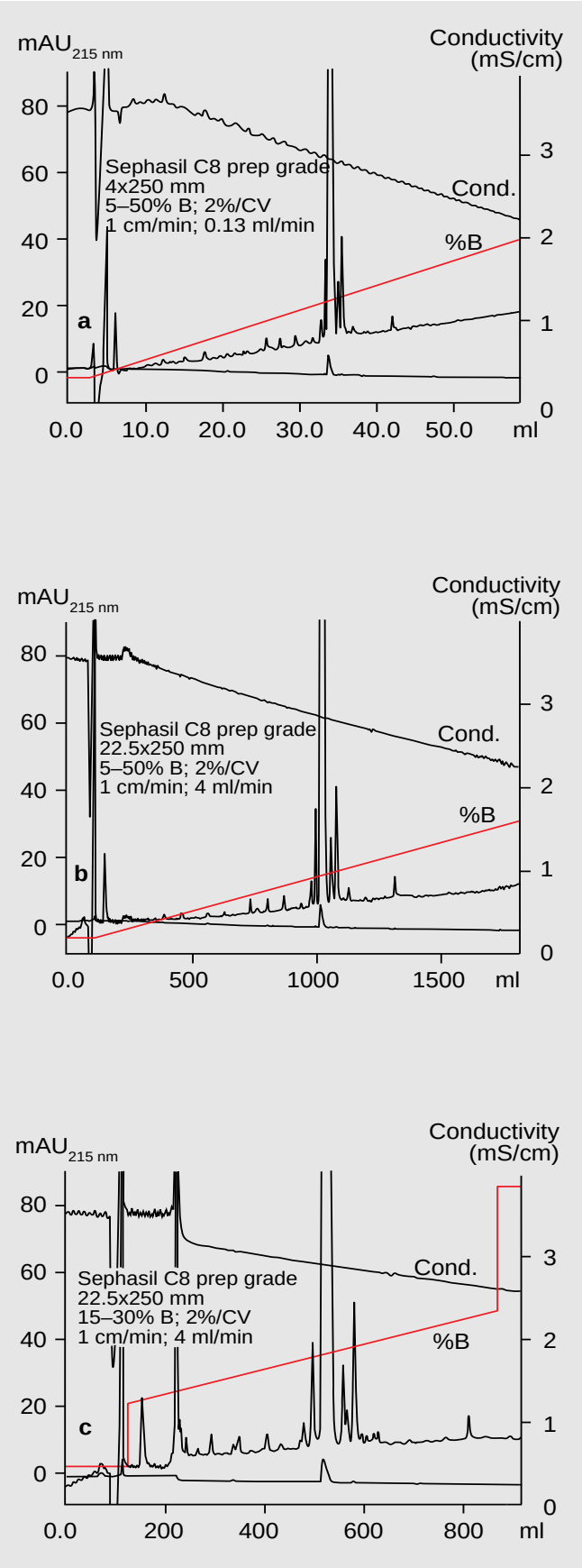


Fig. 2. Gradient and flow rate optimisation were performed on Sephasil C8 prep grade packed in a 4×250 mm column. Synthetic MB 89-101 (0.030 mg/ ml gel) was automatically loaded on the column. The scouting scheme used for the automatic selection of the different runs is shown on top of the chromatograms.

For gradient scouting, the sample was eluted in the gradients indicated in Figures 2a–c at a linear flow rate of 4 cm/min (0.50 ml/min). For flow rate scouting, the sample was eluted in a gradient from 5–100% B in 10 column volumes (CV) at the flow rates indicated in Figures 2d–f. Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 215 nm (upper trace), 254 nm (bottom trace) and 280 nm (not indicated).



Method optimisation and scaling up from a 4 mm i.d to a 22.5 mm i.d column

Scaling up is simple using the template methods in UNICORN. Since the base used for expressing gradient length is column volume instead of time or volume, the same template method can be used for columns of different shapes.

In the subsequent optimisation an even more shallow gradient was run than before. Figures 3a (4×250 mm column) and 3 b (22.5×250 mm column) show an improved resolution compared to the ones obtained from the scouting experiments (Fig. 2). In spite of their different sizes – 3 ml and 99 ml, respectively, the separation on the larger column was quite similar to that achieved using the smaller one.

Using the conductivity trace, displayed in UNICORN as Cond% (not indicated in the figures), it is very easy to find the real concentration of solvent B where the peptide material is eluted. As shown by the conductivity trace, MB 89-101 was found to elute with 17–18% B. That this indicated the true concentration of solvent B where the peptide is eluted, was conformed after binding the peptide to the gel at 5% B followed by elution with step gradients (15%, 17%, 19% B or 16%, 18%, 21% B). Also in these gradients the peptide material eluted with 17% B and 18% B, respectively. Furthermore, it was found that the material bound to the gel at a concentration of solvent B of 15% and that it did not elute even after elution with 20 column volumes at this concentration. In order to optimise the separation in terms of time and solvent consumption, this information was used to implement a combined step and linear gradient for the final method (Fig. 3c), which utilised equilibration at 5% B at a flow rate of 1 cm/min and a gradient from 15% B to 30% B in 7.5 column volumes.

Fig. 3. Continued method optimisation and scaling up experiments were done on Sephasil C8 prep grade in 4×250 mm (3 ml) and 22.5×250 mm (99 ml) columns. Synthetic MB 89-101 (0.030 mg/ml gel) was loaded to the 3 ml and 99 ml columns and eluted as indicated in each figure.

Figures 3a and 3b show the results from eluting the sample from two different columns using similar gradients and flow rates.

Figures 3b and 3c show the results from using the same column and gradient slopes. The overall time and solvent consumption was reduced as shown in Figure 3c by using a gradient running from 15–30% B in 7.5 column volumes (CV) after the sample had been bound to the gel at 5% of solvent B.

Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 215 nm (upper trace), 254 nm (bottom trace) and 280 nm (not indicated).

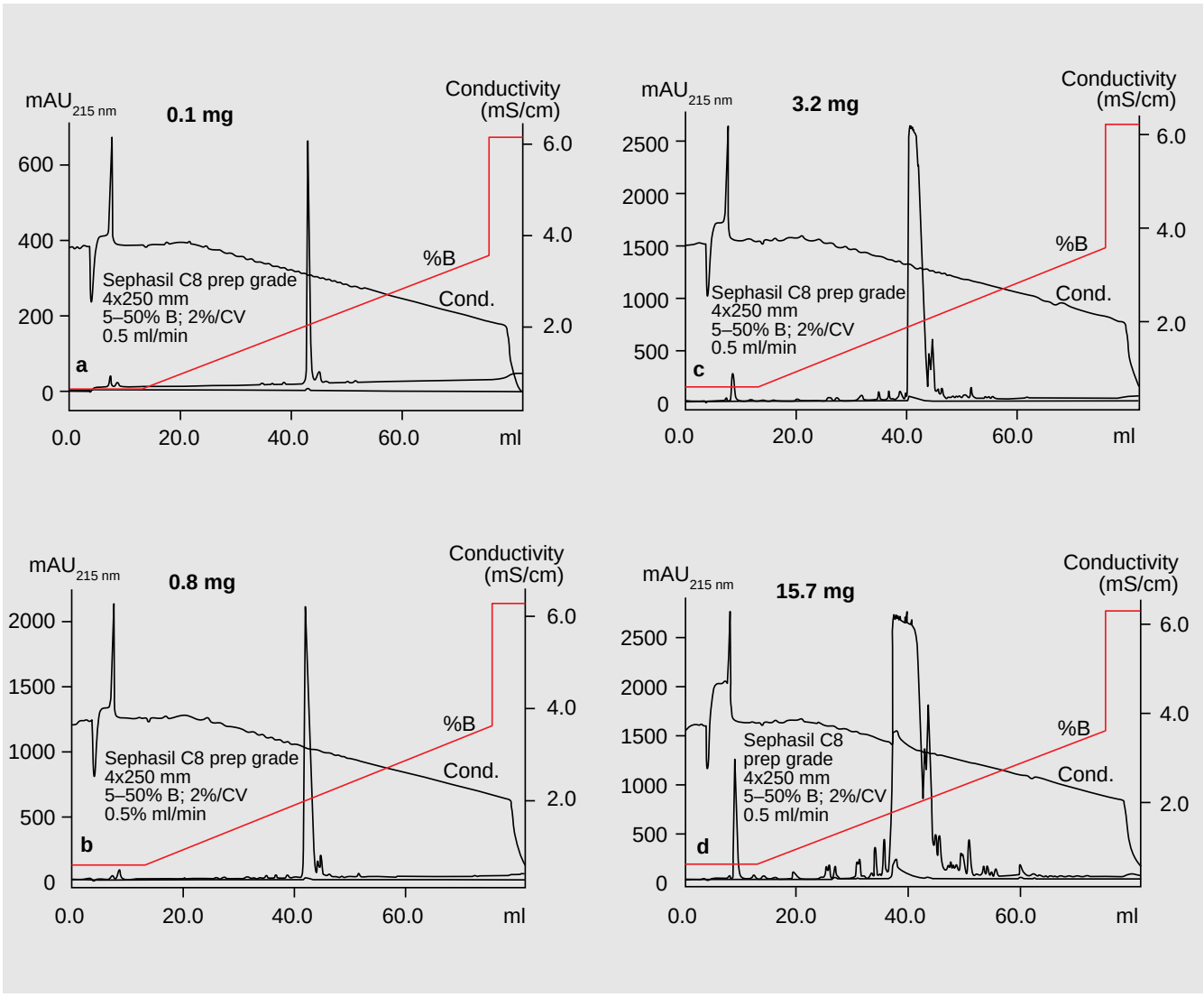


Fig. 4. The loading capacity of Sephasil C8 prep grade was tested in four experiments. Synthetic MB 89-101 was loaded on a 4×250 mm column and eluted as indicated in Figures a–d.

Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 215 nm (upper trace), 254 nm (bottom trace) and 280 nm (not indicated).

The loading capacity of Sephasil C8 prep grade

The loading capacity of Sephasil C8 prep grade for this particular sample, was tested in a series of experiments. From the results shown in Figure 4 it was concluded that the maximum load on the gel was 5 mg of peptide material per ml of gel. The conclusion was based on the observation that the contaminant visible as a separate peak at the backslope of the main peak, still at the highest load, eluted adequately resolved from the main peak. Other contaminants were still sepatated either in front of the main peak or well after it. It is also seen that, probably as a result of self displacement of the peptide material, the front of the main peak eluted earlier at the higher loads, while the peak at the back slope eluted with a nearly constant retention volume at all situations. Thus, at the highest load the front material started to elute at about 16.5 % of solvent B. Since this was very close to the starting concentration of the linear gradient of the optimised method, it was considered that higher loads would not be compatible with effective separation of the sample components.

Purification of 500 mg of MB 89-101

The method optimised as described above was used to purify 500 mg of synthetic MB 89-101. The peptide material was dissolved in 20 ml of 0.065% TFA and filtered through a 0.20 µm filter (see Experimental) and the sample was loaded to the column via Superloop. The material eluted in the main peak was collected in 2 ml fractions (Figure 5).

Analysis by mass spectrometry (Figure 6) revealed the correct molecular mass, Mr 1557 in the fractions in the back slope of the main peak (fractions 30–34), while the material eluted in the front (fractions 22–26) also contained contaminants as shown by additional mass peaks. The contaminants may originate from condensed material (Mr 1539.6) lacking water and a truncated, acetylated peptide (Ac-FFKNIVTPRTP-OH, calculated Mr=1361.6) in fraction 22. Peaks in the mass spectra with masses 45 Da lower than the expected ones correspond to fragmentation of the target peptides in the mass spectrometer resulting in a loss of a carboxyl group.

The presence in fraction 22 of a peptide different from the desired one, although with a similar mass, was detected in the SMART System analysis. Analysis of fraction 22 showed that the material had a significantly lower retention time (Rt 29.77 min) (Fig. 7a) than the material eluted in the main peak (Rt 30.16 min, Fig. 7c), and may represent a variant form in which threonine has been subjected to a N-O shift. A mixture of fractions 22 and 30 separated in two different peaks (Fig 7d).

As judged from peak area integration of the chromatograms from the SMART System analysis the main peak (fractions 28–34) contained the desired material at 97.3% purity.

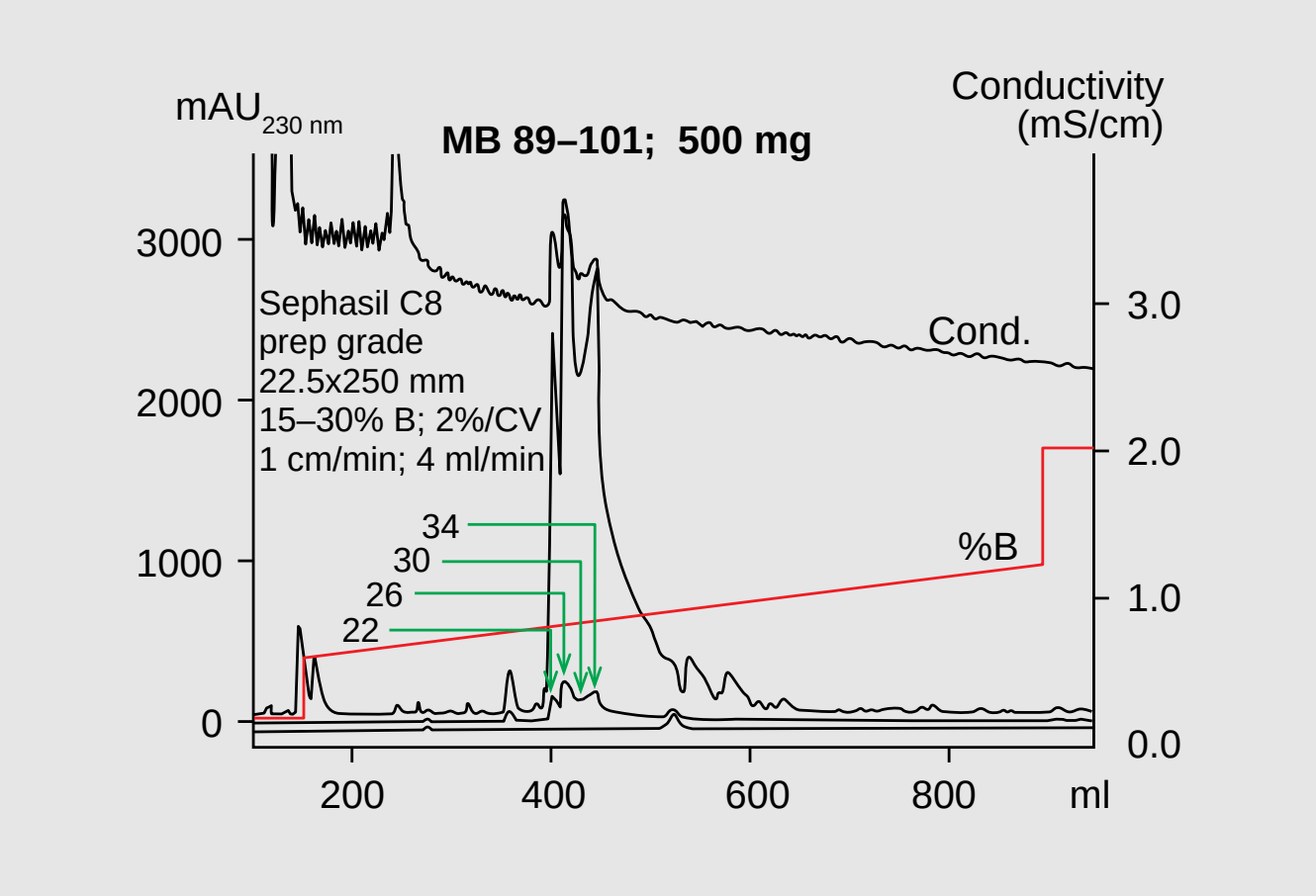


Fig. 5. Synthetic MB 89-101 (500 mg) was purified using Sephasil C8 prep grade (22.5×250 mm). The sample was dissolved in 20 ml of solvent A, loaded on the column via Superloop and eluted as indicated in the figure. Fractions were collected between 360 ml and 530 ml and fractions 22–34 (positions indicated by arrows) were analysed by plasma desorption mass spectrometry (PDMS) and micro-HPLC (SMART System).

Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 230 nm (upper trace), 254 nm (middle trace) and 280 nm (bottom trace).

Analysis by mass spectrometry

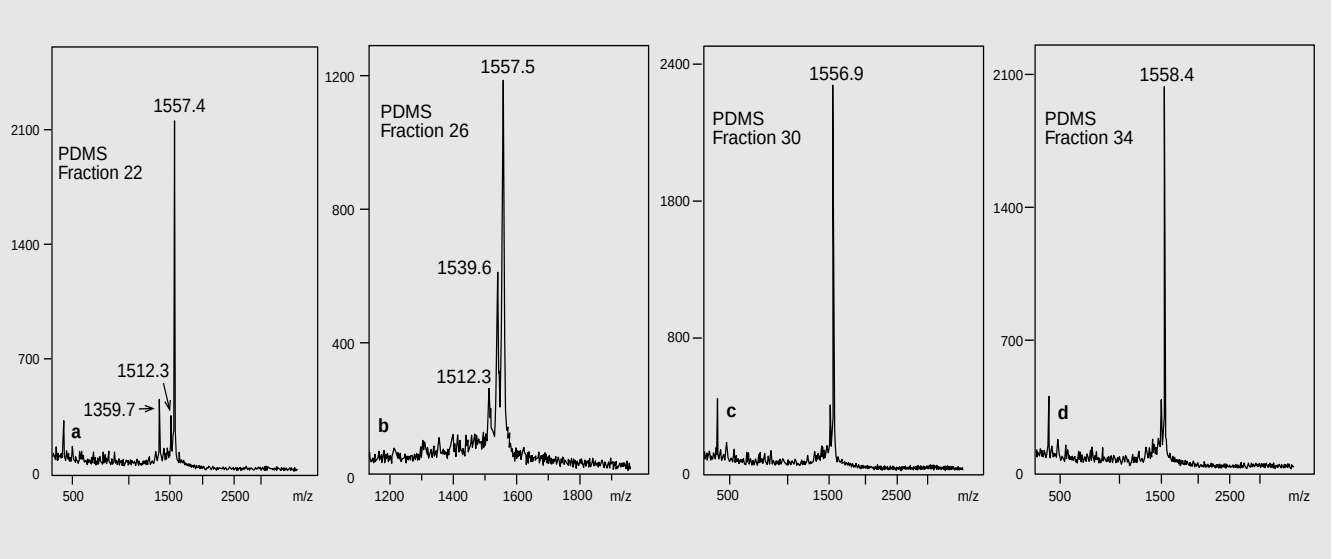


Fig. 6 a–d. Spectra from the plasma desorption mass spectrometry (PDMS) analysis of fractions 22, 26, 30 and 34 from the purification of 500 mg of synthetic MB 89-101. M/z values are indicated on top of the peaks (calculated mass = 1557 Da).

Analysis by micro-HPLC

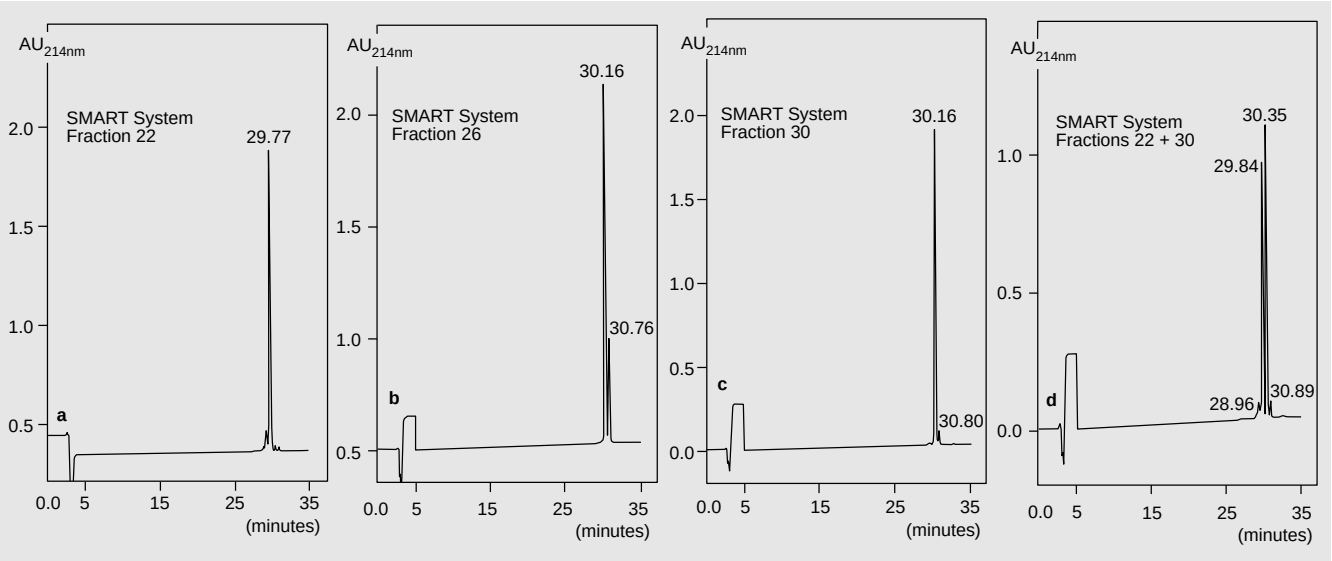


Fig. 7 a–d. Chromatograms from the micro-HPLC (SMART® System) analysis of fractions 22, 26, 30 and a mix of fractions 22+30, collected during the purification of 500 mg of synthetic MB 89-101. The separations were done using a µRPC SC 2.1/10 column. The gradient was from 0–40% B in 40 min at a flow rate of 100 µl/min.

Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 214 nm. Retention times are indicated on top of the peaks.

Purification of 58 mg of hemorphin-7

A method for the preparative purification of hemorphin-7 was rapidly and conveniently developed according to the strategy outlined above.

Having three aromatic amino acid residues, the peptide is particularly suitable for tracing using triple wavelength monitoring. Monitor UV-900 was therefore set at 215 nm to detect peptide bonds and at 254 nm and 280 nm enabling peptide identification by calculation of the absorbance (A) ratio of A_{254} nm/ A_{280} nm.

In media screening experiments there was a very little difference in selectivity between Sephasil C8 prep grade and Sephasil C18 prep grade. The former was chosen for the purification.

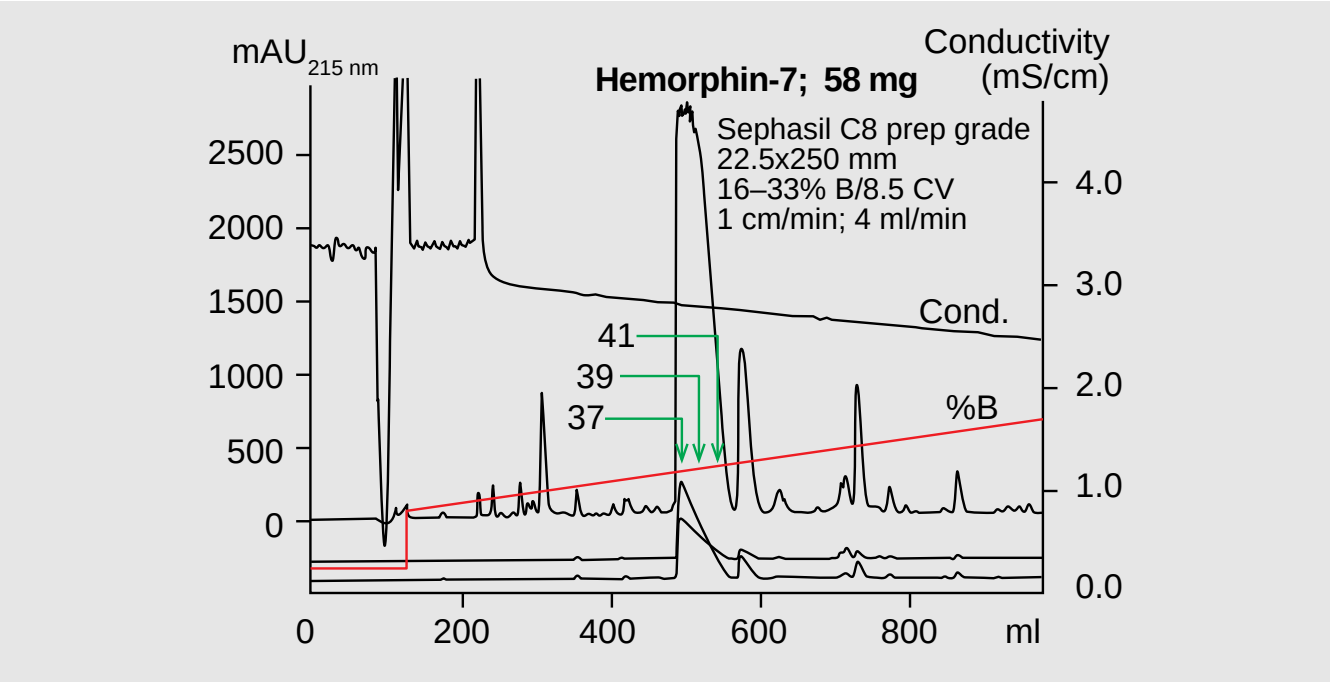


Fig. 8. Synthetic Hemorphin-7 (58 mg) was purified using Sephasil C8 prep grade (22.5×250 mm). The sample was dissolved in 10 ml of solvent A, loaded on the column via Superloop and eluted as indicated in the figure. Fractions eluting between 490 and 610 ml were collected and fractions 37–41 (positions indicated by arrows) were analysed by plasma desorption mass spectrometry (PDMS) and micro-HPLC (SMART System).

Solvent A was 0.065% TFA in water and solvent B was 0.050 % TFA in acetonitrile. Detection was done at 215 nm (upper trace), 254 nm (bottom trace) and 280 nm (not indicated in the figures).

Analysis by mass spectrometry

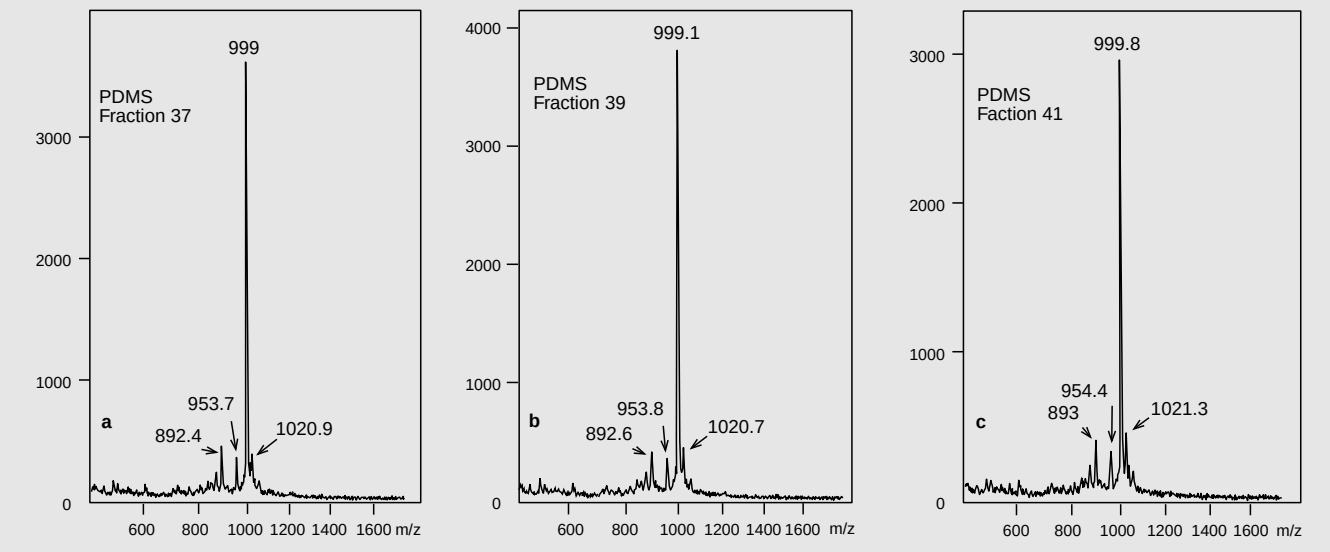


Fig. 9. Spectra from the plasma desorption mass spectrometry (PDMS) analysis of fractions 37, 39 and 41 from the purification of 58 mg of synthetic Hemorphin-7. M/z values are indicated on top of the peaks (calculated mass = 997.1 Da).

The optimised method used for the purification involved column equilibration at 5% B at a flow rate of 4 ml/min, and a gradient from 16% B to 33% B in 8.5 column volumes (CV).

The peptide material, 58 mg, was dissolved in 10 ml of 0.065% TFA and, after filtration through a 0.22 µm filter, loaded to the 22.5×250 mm column via a 10 ml Superloop.

The material eluted in the main peak (fractions 37, 39, 41) (Figure 8) was analysed by PDMS and micro-HPLC using SMART System. This material revealed the correct mass (calculated mass 997.1 Da) and one dominating peak in the SMART System analysis, indicating a purity of the material of 98.1–99.0%, as judged by peak area integration.

The additional peaks in the mass spectra, represent the Na-salt of hemorphin-7 (Mr 1021), and fragments generated in the mass spectrometer, one lacking a carboxyl group (Mr 954) and one unidentified (Mr 893). The latter has a mass corresponding to that of a hemorphin-7 molecule lacking the side chain of tyrosine, although it may be a different entity. It has however, no likely origin in the chemistry from the peptide synthesis.

Experimental

Materials

All media and columns for the synthetic peptide purification were from Amersham Biosciences.

Peptide MB 89-101

The peptide was synthesised (7) using the FMOC-technique. The synthesis utilised capping with acetic anhydride to eliminate peptide variants with deletions. The peptide was cleaved off the resin with trifluoroacetic acid (TFA) and scavengers, filtered from the resin and precipitated and washed with diethylether. For the scouting experiments aliquots of the peptide were dissolved in 0.065% TFA to a concentration of 150 µg/ml. Prior to separations, the sample was centrifuged at 10,000 rpm using a Eppendorff Centrifuge 5414 to eliminate particulate material.

Prior to preparative separations the dissolved peptide material was filtered through 0.20 µm filter (Sartorius Minisart N 0.20 my) (8).

Hemorphin-7

The peptide was synthesised (9) using the tBoc-technique, cleaved off the resin with hydrogen fluoride and scavengers and stored with the formyl protection of the tryptophan. The formyl protection was removed with piperidine prior to purification. For the scouting experiments aliquots of the peptide were dissolved in 0.065% TFA to a concentration of 150 µg/ml.

Chemicals

TFA (No 808260) was purchased from Merck-Schuchardt, Germany.
Acetonitrile, HPLC grade (No C2502) was from Labscan Ltd, Dublin, Ireland.

The water used was filtered through a Milli-Q Reagent Water System (Millipore) (10).

Chromatography

ÄKTAexplorer was used, equipped with Pump P-901, Monitor UV-900, Monitor pH/C-900. Fraction Collector Frac-900. The plumbing was made using the low dispersion tubing kit (0.5 mm i.d. PEEK capillaries), which was used also with the 22.5 mm i.d. colum. This was considered to be benefitial due to the low flow rate used during the separation (1 cm/min; 4 ml/min) but should not be used at flow rates above 30 ml/min. The flow path excluded the reversed flow valve and the pH-monitor flow cell. The mixer volume used was 2 ml for the 4×250 ml columns and 5 ml for the 22.5 ml one. Other experimental details are described in the figures.

Triple wavelength monitoring

Monitor UV-900 was set at triple wavelength monitoring at 215 nm, 254 nm and 280 nm during the scouting experiments. The 254 nm trace, from its content of two Phe residues, was used to monitor the target peptide of the MB 89-101 material and the absorbance ratio from the 254 nm (Tyr, Trp, Phe) and 280 nm (Tyr, Trp) traces, was useful for following the target peptide from the hemorphin-7 material.

At the preparative separation of MB 89-101 (500 mg) the wavelength 230 nm instead of 215 nm was used.

Analysis

Collected fractions were analysed (7) by plasma desorption mass spectrometry (PDMS, BIOION, Uppsala, Sweden) (mass accuracy ± 0.1 %) and by micro-HPLC on a SMART® System equipped with a µRPC SC 2.1/10 column providing a selectivity significantly different from that of Sephasil C8 (11, 12). The samples were eluted with a gradient running from 0–40% B in 40 min at a flow rate of 100 µl/min. Solvent A was 0.1% TFA and solvent B was 0.1% TFA in acetonitrile. UV monitoring was at 214 nm, 254 nm and 280 nm.

References

1. Guidance for Industry for the submission of chemistry, manufacturing, and controls information for synthetic peptide substances. PDA, Inc. Publication Service (301) 986-0293. Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). U.S. Government Printing Office: 199 4-616-023-1302/02759. November 1994.

2. Jones, R.E., Bourdette, D., Offner, H. and Vandenbark, A.A. The synthetic 87–99 peptide of myelin basic protein is encephalitogenic in Buffalo rats. *J. Neuroimmunol., 37:203–212; 1992.*

3. Brantl, V., Gramsch, C., Lottspeich, F., Mertz, R., Jaeger, K.-H. and Herz, A. Novel opioid peptides derived from hemoglobin: Hemorphins. *Eur. J. Pharmacol., 125:309–310; 1986.*

4. Glämsta, E.-L., Marklund, A., Hellman, U., Wernstedt, C., Terenius, L. and Nyberg, F. Isolation and characterization of a hemoglobin-derived opioid peptide from the human pituitary gland. *Regul. Pept., 34:169–179; 1991.*

5. Glämsta, E.-L., Mørkrid, L., Lantz, I. and Nyberg, F. Concomitant increase in blood plasma levels of immunoreactive hemorphin-7 and β-endorphin following long distance running. *Regul. Pept., 49:9–18; 1993.*

6. Aguilar, M. I., Mougos, S., Boublik, J., Rivier, J., Hearn, M.T.W. High-performance liquid chromatography of amino acids, peptides and proteins. CXXVIII. Effect of D-amino acid substitutions on the reversed-phase high-performance liquid chromatography retention behaviour of neuropeptide Y [18–36] analogues. *J. Chromatogr., 646:53–65; 1993.*

7. Synthesis of MB 89-101 and analysis of the collected fractions from the purification of MB 89-101 and hemorphin-7 were done by Dr. Åke Engström at the Department of Medical and Physiological Chemistry, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden.

8. Minisart is a registered trade mark owned by Sartorius

9. Synthesis of hemorphin-7 was done by Dr. Gunnar Lindeberg at the Department of Medical and Physiological Chemistry, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden.

10. Milli-Q is a registered trade mark owned by Millipore Corporation.

11. Renlund, S., Erlandsson, I., Hellman, U., Silberring, J.,Wernstedt, C., Lindström, L., Nyberg, F. Micropurification and amino acid sequence of β-casomorphin-8 in milk from a woman with postpartum psychosis. P*eptides, 14:1125–1132, 1993.*

12. Hellman, U. Isolations of peptides for microsequencing by "in-gel" proteolytic digestion. In: R. M. Kamp, T. Choli-Papadopoulou, B. Wittmann-Liebold (Eds.). Advanced methods on protein structure analysis. Springer Verlag (Heidelberg); 1996.

Ordering information

Designation	Code No.
ÄKTAexplorer	18-1112-41
Pump P-901	18-1114-00
Monitor UV-900	18-1108-35
Monitor pH/C-900	18-1107-76
Fraction Collector Frac-900	18-1104-99
RESOURCE RPC 1 ml	17-1181-01
RESOURCE RPC 3 ml	17-1182-01
Sephasil C8 prep grade 4 × 250 mm	CDP
Sephasil C8 prep grade 22.5 × 250 mm	CDP
Sephasil C18 prep grade 4 × 250 mm	CDP
Sephasil C18 prep grade 22.5 × 250 mm	CDP

Related product literature

Product	Code No.
Data File Monitor UV-900	18-1111-17
Data File Pump P-901	18-1111-18
Data File Monitor pH/C-900	18-1111-19
Data File UNICORN control system	18-1111-20
Data File ÄKTAexplorer	18-1111-21
Data File ÄKTAexplorer ancillary equipment	18-1111-22
Application Note ÄKTAexplorer Rapid development of a purification process for a recombinant antigen-binding fragment expressed in *E. coli*	18-1111-23
Application Note ÄKTAexplorer Automated pilot scale purification of synthetic phosphorothioate oligonucleotides	18-1111-25
Data File Sephasil C8, Sephasil C18	18-1060-40
Data File SOURCE 15RPC, RESOURCE RPC	18-1104-37

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