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Size-exclusion chromatography performed in capillaries Studies by liquid chromatography–mass spectrometry

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Abstract

Miniaturization of the chromatographic column led to increased sensitivity and shortened time of analysis. In our work we applied 300 μm I.D. capillaries packed with a novel stationary phase Superdex Peptide for the size-exclusion chromatography, capable of separating molecules within the mass range of 0.1–7 kDa. Here we proved that such capillary columns can operate effectively at high sensitivity. Several peptide mixtures were efficiently chromatographed and analyzed on line with electrospray ionization mass spectrometry as a detection technique. A CNBr peptide map, derived from human globin alpha subunit, was effectively separated using this method. These fragments are difficult to elute from the reversed-phase column at low pH, therefore, such approach can be considered as a complementary to other separation techniques, in particular for analyzing hydrophobic components and complex mixtures.

Keywords: Cyanogen bromide peptide maps; Peptides

1. Introduction

The most commonly used capillary columns are packed with reversed-phase packings, providing higher sensitivity of the measurements as compared to standard LC columns. This is true when the gain in sensitivity between capillary and conventional column is achieved by injecting the same sample amount on both columns. In particular, coupling of liquid chromatography with mass spectrometry (LC–MS) provides a powerful tool for unambiguous identification of molecules of endogenous origin. There is an extensive literature describing such technique [1]. Recently, we have introduced size-exclusion chromatography (SEC) of neuropeptides [2,3], connected on-line to electrospray ionization mass spectrometry (ESI-MS). This method was applied for examining proteolytic processing of neuropeptides in the nervous tissue and was also suitable for separation of the hydrophobic peptide mixtures [2,4]. Moreover, SEC was helpful during

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interpretation of the obtained data where deconvolution process may give misleading conclusions on the true mass assignment [2]. Microcolumn SEC was studied by several groups [5–8] but, so far, such an approach involving the Superdex Peptide and using MS as a specific on-line detector, has not been investigated on a wider scale.

The use of SEC and capillary SEC is of growing interest in the quality control of proteinaceous drug compounds, particularly for controlling the presence and/or to assure the absence of any dimers or other conjugates. Although powerful, SEC is less sensitive than the other chromatographic modes, such as reversed-phase or ion-exchange. This is due to the lack of concentration effect on the separating gel. This urged us to minimize dimensions of the column packed with the Superdex Peptide, a gel-filtration medium suitable for separation of various peptides [9]. The gel is a dextran-based stationary phase, crosslinked with agarose, and is capable of separating molecules between 0.1–7.0 kDa [2,4,9]. This approach was pioneered by Hjertén et al. where the agarose was cross-linked with divinyl sulfone [10].

2. Experimental

2.1. Materials

Peptides were purchased from Bachem (Bubendorf, Switzerland) or synthesized by Dr. Marika Ström (Karolinska Institute, Stockholm, Sweden). Their purity was tested by ESI-MS. Solvents were of the HPLC grade and other chemicals were of the research-grade and came from various commercial sources.

2.2. Preparation of the capillary column

The 30 cm×300 μm I.D. capillary SEC column was packed by LC Packings (Amsterdam, The Netherlands) with a novel stationary phase Superdex Peptide (Amersham Pharmacia Biotech, Uppsala, Sweden). This gel can be purchased from the Amersham Pharmacia Biotech either in a form of the commercially packed columns [9] or as a bulk material. Packing details can be found in the recent literature [1,11].

2.3. Preparation of CNBr fragments from the globin alpha subunit

Human hemoglobin was isolated from peripheral blood obtained from healthy volunteers [12]. Briefly, the cells were washed with physiological saline and subjected to lysis in a water–toluene mixture, followed by an extensive washing of the lysate with water, performed on the 30 kDa cut-off PM-30 membrane (Amicon, Charlotte, NC, USA). To prepare globin alpha subunit, the lysate was precipitated twice by acid acetone and the globins were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using two serially connected Waters Bondapak C_8 Radial Pak cartridges (100×8 mm). The gradient was developed from 0 to 57% of buffer B in 1 min., followed by 57 to 65% B in 20 min. Starting buffer A was 0.1% (v/v) trifluoroacetic acid (TFA) in water and buffer B consisted of 80% (v/v) acetonitrile in water, supplemented with 0.07% TFA. The cyanogen bromide fragments were obtained by 24-h incubation of globin alpha at room temperature in a solution containing 10% (w/v) CNBr in 70% (v/v) TFA, followed by freeze-drying.

2.4. Mass spectrometry

The Finnigan MAT 95S (Finnigan MAT, Bremen, Germany) sector mass spectrometer, equipped with an electrospray source was used in all experiments. A detailed description of the basic operation was given in our previous papers [3,13].

2.5. LC–MS experiments

A capillary column was connected to the four-port Valco injector (Schenkon, Switzerland) equipped with a 0.5-μl internal loop and to the ConstaMetric 4100 MS HPLC pump (Thermo Separation Products, Riviera Beach, FL, USA) via a split, prepared from the zero-dead volume tee (Alltech Associates, Deerfield, IL, USA). Mobile phase consisted of 0.1% (v/v) TFA in water and was purged with helium for 30 min prior to experiments. This solvent proved to be optimal for the hydrophobic peptide separations at high sensitivity [2,4]. The outlet of the column was connected to the transfer line (fused-silica 50 μm I.D.×150 μm O.D.) of the ESI interface via a

grounded zero-dead volume connector (Alltech). The flow-rate through the column was maintained at 2 μl/min and was set experimentally by adjusting the total flow given by the HPLC pump to 180 μl/min. Back-pressure of this system set-up was 60 p.s.i. (1 p.s.i.=6894.76 Pa). To stabilize the signal in the mass spectrometer (due to a lack of the organic solvent in a mobile phase), an additional flow of 10 μl/min of 50% methanol was delivered with the help of Harward 22 infusion pump (Harward Apparatus, South Nattick, MA, USA), connected through an auxiliary port in the ESI flange. Both infusion pump and the syringe containing sheath liquid were additionally grounded. Prior to injections, samples were filtered through 0.45-μm Ultrafree MC disposable filters (Millipore, Bedford, MA, USA). Sample volume, injected to the system was adjusted by shortening injection time, i.e., only part of the loop content was directed into the system. This was performed by turning the valve to the inject position for the calculated period of time, followed by turning the valve back to the load position. This resulted in application of the various sample volumes (250 and 100 nl) on the column. The optimal flow-rate applied on the column was found to be between 1–2 μl/min. This value was estimated empirically to gain the optimal separation time and to provide formation of the stable spray in the instrument. The sample

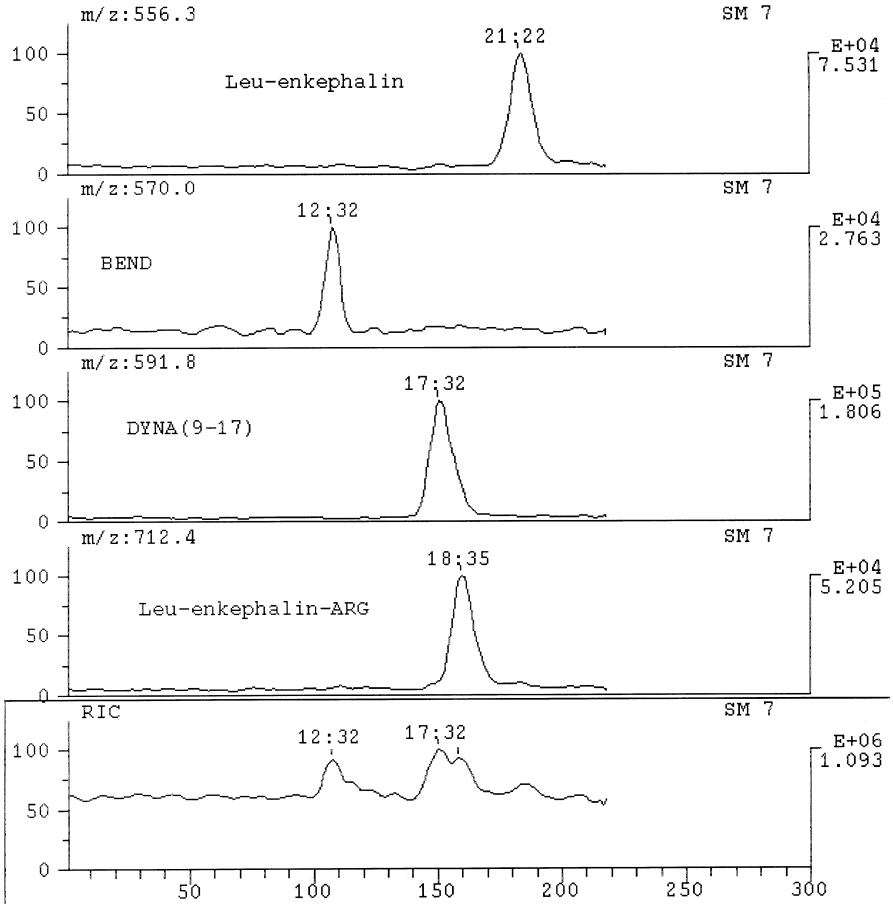

Fig. 1. ESI mass chromatogram of a peptide mixture, separated on the 30 cm×300 μm I.D. Superdex Peptide capillary column. Each component was present at a concentration of 50 pmol except Leu-enkephalin (25 pmol). Sample load: 0.25 μl. The selected traces correspond to the particular peptide (labeled with the appropriate names). For details see Section 2.5.

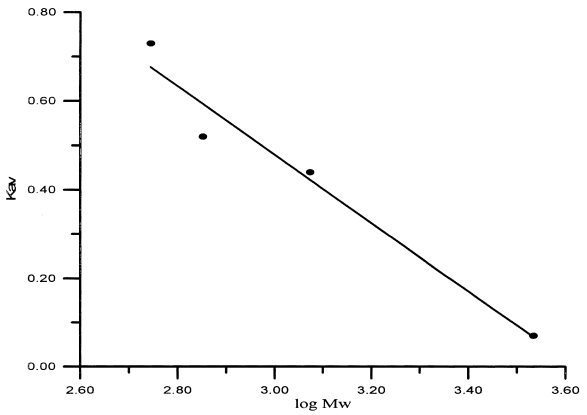

Fig. 2. Selectivity curve for the capillary Superdex Peptide column. The graph was prepared based on the retention times, obtained from Fig. 1. The data are representative for three individual separations.

amount should not exceed 50 pmol per component to avoid peak tailing and to achieve a good resolution during separation of the compounds.

3. Results

To test the separation power of the prepared column, we applied a mixture of synthetic neuropeptides of various sizes. The amount of each component injected into the system was equal to 50 pmol except for Leu-enkephalin (25 pmol). The resulting mass chromatogram is presented in Fig. 1. By using eluting conditions described above, a good separation was obtained within the mass range of 0.5–3.4 kDa. It should be noted that Leu-enkephalin and Leu-enkephalin-ARG[6] were well distinguished,

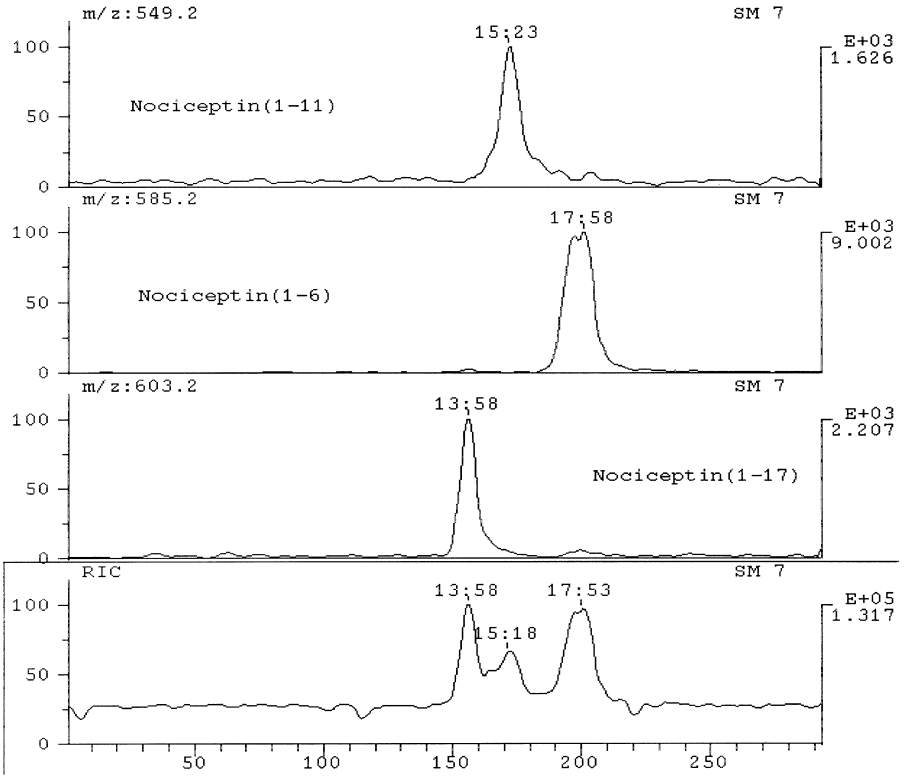

Fig. 3. ESI mass chromatogram of the separation of the neuropeptide nociceptin/orphanin FQ and its major metabolites. Particular fragments are labeled along the chromatogram.

though the difference between these peptides is only one amino acid residue. Sensitivity of the system was estimated at 5 pmol (5 ng, assuming the M_r as 1 kDa) per injected component at a signal-to-noise ratio better than 3:1 on the total ion current (TIC) trace.

The calibration curve for the applied column is shown in Fig. 2. There are minor deviations from the ideal behavior which are probably caused by interactions of small peptides and polar groups with the gel matrix. Such an effect was also observed in our previous report [3] when larger column diameters were applied.

Another example describes separation of the three nociceptin/orphanin (OFQ/N)-derived fragments. This newly discovered heptadecapeptide is involved in pain transmission [14] and its shorter fragments $OFQ/N_{(1-11)}$ and $OFQ/N_{(1-6)}$ possess an opposite sequence of analgesic action to the intact heptadecapeptide [15,16]. The mass chromatogram presented in Fig. 3, shows single traces, corresponding to the particular OFQ/N fragments. An accumulated mass spectrum is shown in Fig. 4. The ion at m/z of 603.7 belongs to the triply-charged intact nociceptin (1–17). The signal at m/z 549.2 is a doubly-protonated ion of nociceptin (1–11). A pseudo-molecular ion of nociceptin (1–6) at m/z 585.2 is also present in the spectrum.

The described technique was also applied for the separation of peptides, derived from the human globin alpha subunit. In this case, a CNBr cleavage was used to yield a peptide map. These fragments often adsorb on the reversed-phase column or elute at low pH as a single, broad peak due to their aggregation. Separation using the Superdex Peptide and 0.1% TFA solution in water (Fig. 5) omits such problems. To verify accuracy of the cleavage procedure, we applied a Swiss-Prot database search using obtained peptide masses. For this purpose, an Internet application of an MS-Fit program was used at http://falcon.ludwig.ucl.ac.uk/ucsfhtml/msfit.htm, and the results of the search are presented in Table 1. The program selected human alpha-hemoglobin with a highest MOWSE score of 3.31.

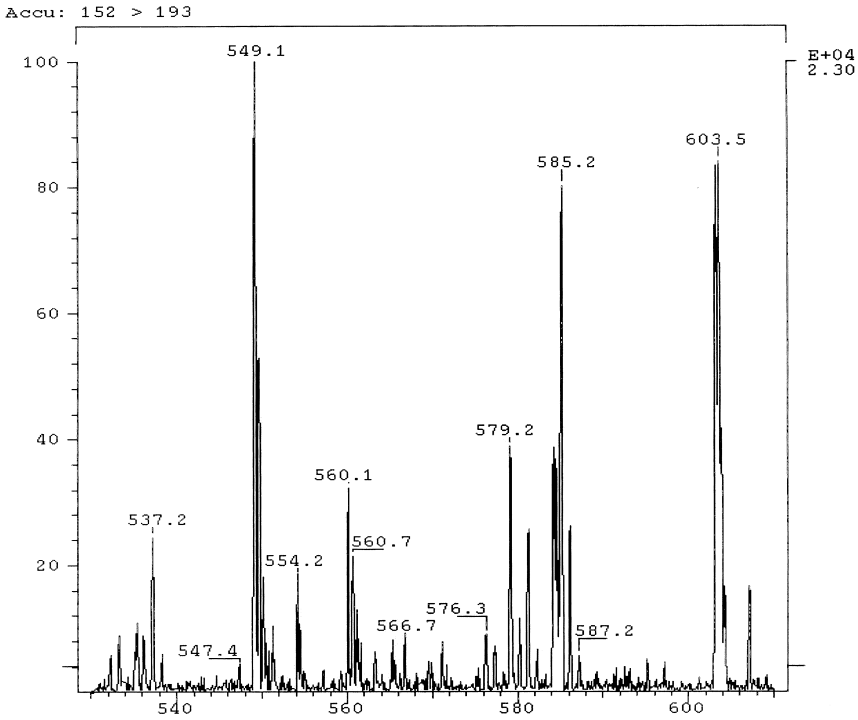

Fig. 4. Accumulated ESI mass spectrum of the separated nociceptin fragments. The ion at m/z 603.7 belongs to the triply-charged intact peptide nociceptin (1–17). The signal at m/z 549.2 is a doubly-protonated ion of nociceptin (1–11). A pseudo-molecular ion of nociceptin (1–6) at m/z 585.2 is also present.

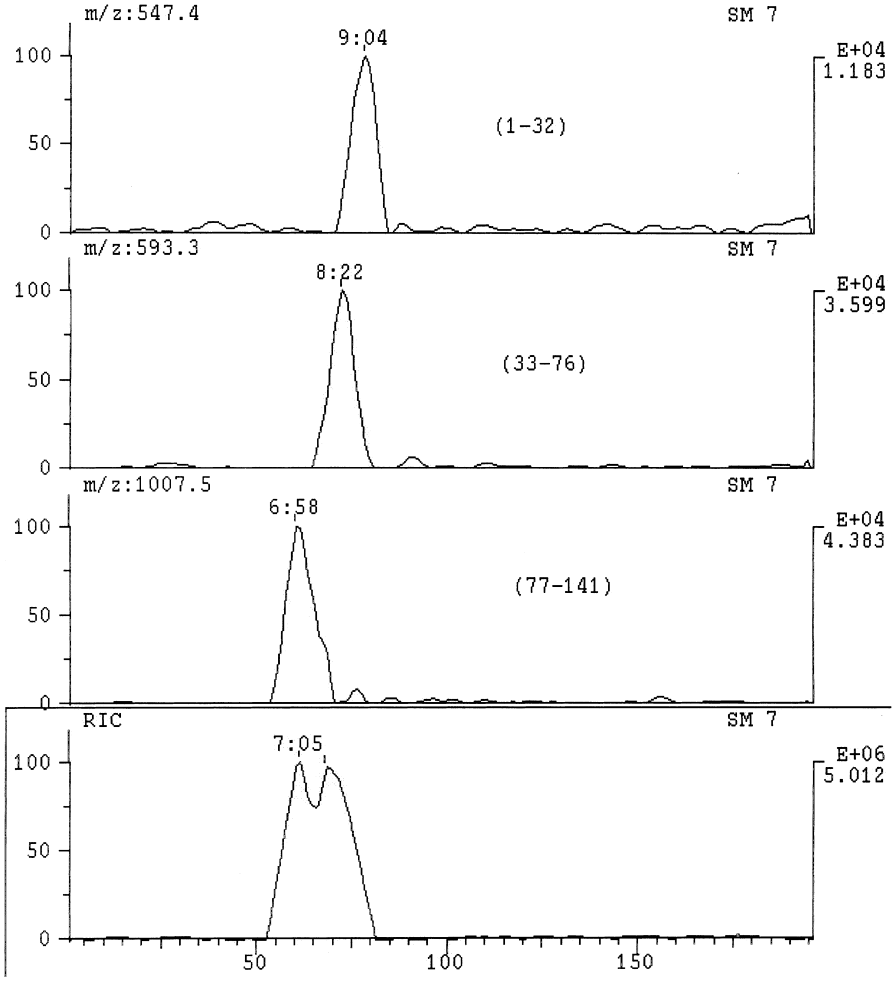

Fig. 5. Separation of the alpha−hemoglobin fragments, released by CNBr. Ion at m/z of 547.4 belongs to the fragment (1–31) of alpha−hemoglobin. Signal at m/z 593.3 is an 8-times protonated peptide (33–76) and the peak at m/z of 1007.5 is an $[M+7H]^{+7}$ fragment (77–41). Cysteines were not modified in this experiment, but methionines were converted to homoserine lactone. Separation was performed in 0.1% TFA in water. For details see Section 2.

Table 1
Database search for protein sequences based on the peptide masses[a]

m/z submitted	$[M+H]^+$ matched	δ	Position
7045	7043.8	1.2	77–141
4737	4737.4	−0.4	33–76
3278	3278.7	−0.7	1–32

[a] The peptide map was prepared using CNBr cleavage (for details see Section 2.3). Cysteines were not modified. Methionines have been modified to homoserine lactone. Human globin alpha was selected by the MS-Fit software with the MOWSE score of 3.31.

4. Discussion

We applied a novel approach in separation and identification of peptides using capillary size-exclusion technique linked to ESI-MS. The stationary phase Superdex Peptide used in the experiments was tested by us previously as a powerful tool for the rapid analysis of complex mixtures [2,3,13]. Column diameter, however, did not provide sensitivity which was high enough to be comparable with capillary or even narrow-bore reversed-phase columns. There-

fore, we tested the possibility of using size-exclusion columns having diameters less than 0.30 mm. Elution patterns obtained from such columns are similar to those shown for the commercial column (300×3.1 mm) connected to the SMART micropurification system [3], but separation can be performed with 100–1000-fold better sensitivity. The commercial column required application of 0.5–5 nmol of material which corresponds to approximately 0.5–5 μg of a peptide having an average molecular mass of 1 kDa, whereas separations run on the capillaries required as little as 5 pmol of the substance. It is known, that the Superdex Peptide possesses not only properties of a size-exclusion gel but, under certain conditions, other interactions may play a role during separation of molecules [2–4]. Therefore, careful selection of the mobile phase content may significantly improve analysis. Nevertheless, under conditions applied, it was possible to separate peptides differing only by one amino acid residue. Mobile phase consisted of 0.1% TFA because this solution was found to be the most efficient for the separations at a desired sensitivity level. Addition of acetonitrile (above 20%, v/v) to the solution caused reduction of the detection limit. However, methanol was added to the electrospray interface to stabilize the signal due to the better ionization efficiency in the presence of organic solvents. It has also been found that formic acid, which is commonly used in the electrospray mass spectrometry, is not suitable at the concentrations of up to 1%, for peptide separations on the Superdex Peptide columns. In this case, components were retained on the column. Therefore, only TFA or acetic acid could be applied for such purpose. The major drawback of these acids is that they increase current in the electrospray source which is potentially dangerous using sector instruments, where the ion source is connected to the high voltage [2].

Another important feature of the size-exclusion column was the capability of separating hydrophobic peptides such as Alzheimer amyloid fragments [2], biotinylated peptides [4] or the peptide map obtained after CNBr digestion of the globin alpha chain. Chromatography of such components, cause experimental difficulties during elution from the reversed-phase columns at acidic pH [13,14] and tend to adsorb on the stationary phase. The known protocols [8,17,18] describe application of isopropanol as an eluting solvent but this solvent produces high back-pressure in the chromatographic system and separations strongly depend on the column choice. Therefore, SEC might be one of the solutions to the described problems.

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References

[1] K. Tomer, M. Moseley, L. Deterding, C. Parker, Mass Spectrom. Rev. 13 (1994) 431.
[2] J. Silberring, Methods in Molecular Biology, Humana Press, New Jersey, 1997, pp. 129–140.
[3] I. Nylander, K. Tan-No, A. Winter, J. Silberring, Life Sci. 57 (1995) 123.
[4] R. Bhikhabhai, J. Sandin, I. Nylander, J. Silberring, presented at The Protein Society, 10th Symposium, 3–7 August 1996, San Jose, CA, Poster No. 595-T
[5] A. Hirose, D. Ishii, J. Chromatogr. 411 (1987) 221.
[6] C.L. Flurer, C. Borra, F. Andreolini, M. Novotny, J. Chromatogr. 448 (1988) 73.
[7] H.J. Cortes, B.M. Bell, C.D. Pfeiffer, D. Graham, J. Microcol. Sep. 1 (1989) 278.
[8] R.T. Kennedy, J.W. Jorgenson, J. Microcol. Sep. 2 (1990) 120.
[9] Amersham Pharmacia Biotech Data File No. 18-1106-06.
[10] S. Hjertén, B.-L. Wu, J.-L. Liao, J. Chromatogr. 396 (1987) 101.
[11] S. Hsieh, J.W. Jorgenson, Anal. Chem. 68 (1996) 1212.
[12] C. Vasseur, Y. Blouquit, J. Kister, D. Prome, J.S. Kavanaugh, P.H. Rogers, C. Guillemin, A. Arnone, F. Galacteros, C. Poyart, J. Rosa, H. Wajcman, J. Biol. Chem. 267 (1992) 12682.
[13] J. Silberring, F. Nyberg, J. Chromatogr. A 777 (1997) 41.
[14] R.K. Reinsheid, H.-P. Nothacker, A. Bourson, A. Ardati, R. Henningsen, J. Bunzow, D. Grandy, H. Langen, F. Monsma, O. Civelli, Science 270 (1995) 792.
[15] G.C. Rossi, L. Leventhal, E. Bolan, G. Pasternak, J. Pharmacol. Exp. Ther. 282 (1997) 858.
[16] P. Suder, J. Kotlińska, M.T. Smoluch, M. Sällbeg, J. Silberring, Peptides, (1998) in press
[17] W.C. Mahoney, M.A. Hermodson, J. Biol. Chem. 255 (1980) 11199.
[18] J. Näslund, A. Schierhorn, U. Hellman, L. Lannfelt, A. Roses, L. Tjärnberg, J. Silberring, S. Gandy, B. Winblad, P. Greengard, Ch. Nordstedt, L. Terenius, Proc. Natl. Acad. Sci. USA 91 (1994) 8378.