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Peptide bank generated by large-scale preparation of circulating human peptides

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Abstract

Human hemofiltrate (HF) is a source for the purification of circulating regulatory peptides. HF is obtained in large quantities during treatment of patients suffering from chronic renal failure. We have developed a large-scale method for separating peptides from amounts up to 10 000 l HF into 300 fractions in a standardized two-step procedure, employing cation-exchange separation, followed by reversed-phase chromatography. These fractions represent a peptide bank containing bioactive, desalted and lyophilized peptides of blood. Screening for and isolation of regulatory human peptides is simplified by using this peptide bank. © 1997 Elsevier Science B.V.

Keywords: Preparative chromatography; Hemofiltrate; Peptides

1. Introduction

The importance of peptides in the diagnosis and therapy of human diseases has been known for a long time. The use of insulin for the therapy of diabetes mellitus is a good example of the proof of efficacy of a peptide. Although many attempts to treat diabetes mellitus have been made for decades, no alternative has been found to replace the treatment with the extracted, synthetic or recombinant peptides. The same is true for other diseases treated with peptides, e.g., growth hormone for dwarfism, factor VIII for hemophilia A, or erythropoietin for anemia. Up to now, these diseases can only be treated by the use of analogs of the corresponding endogenous human peptides.

Development of new pharmaceuticals based on endogenous peptides begins with the search for such substances in complex biological mixtures and with the identification of their primary structures and functions [1].

To determine the bioactive molecular form of a peptide, isolation from the local synthesizing compartment or that compartment where receptor–ligand interaction takes place, seems the most promising approach. Peptides which act in an endocrine fashion are transported in the blood, entering the interstitial spaces of the effector organs. Peptides that act in a paracrine or autocrine mode in their local compartments will also diffuse in small amounts into the extracellular space and are thus found in the blood plasma, although at very low, systemically mostly inactive concentrations.

Blood plasma is a comprehensive source of regulatory peptides [2]. We used large amounts of human blood ultrafiltrate (hemofiltrate, HF) to isolate circulating peptides with the aim of detecting potentially new targets for drug development as well as lead compounds.

Hemofiltration in patients with chronic renal fail-

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ure is performed to remove uremic toxins, avoiding the loss of large plasma proteins, immunoglobulins and blood cells [3]. The concentration of peptides and proteins in HF ranges between 50 and 70 mg/l, which represents a reduction by a factor of 1000 compared to the entire plasma protein (70 g/l), mostly due to the high content of human serum albumin. However, this protein is still present in the filtrate, but only at a concentration of about 25 mg/l [4]. Due to their molecular masses, regulatory peptides and fragments of plasma proteins in a range M_r<20 000 are filtered almost completely. The rate of turnover of peptide hormones in blood plasma is in the range of a few minutes. Thus, no reduction of hormone concentration is observed in the patient and plasma-like concentrations are obtained in the ultrafiltrate [5]. The specific concentration of a given peptide compared to plasma is increased by a factor of 1000 by hemofiltration. Performance of assay systems and subsequent peptide isolation is therefore possible.

Here, we present our strategy to develop a reproducible large-scale procedure for the isolation of circulating human peptides resulting in the generation of a human circulating peptide bank. This bank contains peptides in quantities which are sufficient for functional analysis and determination of primary structure.

2. Experimental

2.1. Hemofiltrate

Hemofiltrate is obtained from the Nephrologisches Zentrum Niedersachsen, Hannoversch-Münden, Germany, in quantities of 1600 to 2000 l/week. Patients with chronic renal failure are subjected to routine arterio–venous hemofiltration three times per week using ultrafilters with a specified M_r cut-off around 20 000. Filtration is driven by a transmembranous pressure gradient of 60 to 100 mmHg at a blood flow-rate of 250 to 350 ml/min. 20 to 30 l of filtrate are recovered per patient and treatment (1 mmHg= 133.322 Pa).

2.2. Peptide extraction

The sterile filtrate is immediately cooled to 4°C and acidified to pH 3 to prevent bacterial growth and proteolysis. After dilution with deionized water to a conductivity of <8 mS/cm, the batches of 800 to 1000 l HF are conditioned exactly to pH 2.7 using hydrochloric acid (HCl). These batches are applied onto a strong cation-exchanger [Fractogel TSK SP 650(M), Merck, Darmstadt, Germany; 10 cm length×25 cm diameter, Vantage VA 250 column, Amicon, Witten, Germany] using an Autopilot chromatography system (PerSeptive Biosystems, Wiesbaden, Germany). Then, batch-elution is performed with 10 l 0.5 *M* ammonium acetate (2 column volumes). The eluate is stored at −20°C or lyophilized until further use.

2.3. Cation-exchange subfractionation of peptides from 10 l HF

1.12 g of the lyophilized batch eluate representing 10 l HF are dissolved in 200 ml deionized water to a conductivity of 6.7 mS/cm and adjusted to pH 2.7. Using a BioCad chromatography system (PerSeptive Biosystems), the sample is loaded at a flow-rate of 40 ml/min onto a 15×3 cm strong cation-exchanger [Fractogel TSK SP 650(M)], followed by a washing step using 0.01 *M* HCl. Stepwise elution is performed using seven buffers with increasing pH. Conductivity, pH and A_{280} nm are monitored.

The seven buffers were composed as follows: I: 0.1 *M* citric acid monohydrate, pH 3.6, *C*=2.9 mS/cm; II: 0.1 *M* acetic acid+0.1 *M* sodium acetate, pH 4.5, *C*=4.0 mS/cm; III: 0.1 *M* malic acid, pH 5.0, *C*=6.2 mS/cm; IV: 0.1 *M* succinic acid, pH 5.6, *C*=6.1 mS/cm; V: 0.1 *M* sodium dihydrogenphosphate, pH 6.6, *C*=4.9 mS/cm; VI: 0.1 *M* disodium hydrogenphosphate, pH 7.4, *C*=6.7 mS/cm and VII: 0.1 *M* ammonium carbonate, pH 9.0, *C*=6.7 mS/cm.

The seven pools are collected and subjected to the second fractionation step.

2.4. Reversed-phase fractionation

Each of the seven pools is directly loaded onto a 12.5×1 cm reversed-phase column (Source RPC, 15 μm, Pharmacia, Freiburg, Germany). Following sample application, the column is washed with 2 column volumes of solvent A (0.01 *M* HCl) and eluted in a 25 min linear gradient at 2 ml/min from 100% A to 60% B (80% acetonitrile, 0.01 *M* HCl).

Fractions of 2 ml are collected and subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

2.5. MALDI-MS

1-μL aliquots of the samples from the reversed-phase chromatography, representing a 5 ml hemofiltrate equivalent, are applied on a stainless steel multiple sample tray as admixture to either sinapinic acid or α-cyanohydroxycinnamic acid using the dried drop technique [6]. Measurements are performed in linear mode with a LaserTec RBT MALDI-MS (Perseptive/Vestec, Houston, TX, USA). The instrument is equipped with a 1.2 m flight tube and a 337 nm nitrogen laser. Positive ions are accelerated at 30 kV and up to 64 laser shots are automatically accumulated per sample position. The time-of-flight data are externally calibrated for each sample plate and sample preparation. Data acquisition and analysis is performed using GRAMS software supplied by the manufacturer.

2.6. Preparative separation of peptides from 5000 l HF

Peptides from 5000 l HF are extracted and stored as mentioned in Section 2.2. The extracts are pooled for the first separation step via a 10 l cation-exchange column [Fractogel SP 650(M)] using the Autopilot system. Therefore, the extracts are diluted with deionized water until conductivity reaches less than 8 mS/cm, acidified to pH 2.7 with HCl and loaded onto the column. After sample application, the column is washed with 0.01 *M* HCl until conductivity is below 1 mS/cm. Batch elution of bound peptides is performed using the seven different buffers as in Section 2.3. Following batch elution, all pools (15 to 25 l) are loaded onto a 12.5×10 cm reversed-phase column (Source RPC, 15 μm) at a flow-rate of 200 ml/min and eluted in a 8 l gradient from 100% A (0.01 *M* HCl) to 60% B (80% acetonitrile, 0.01 *M* HCl). Fractions of 200 ml are collected. Aliquots of the fractions for assays are sampled in deep well microtiter tubes in 96-well format using a pipetting robot. The remaining bulks of the fractions are lyophilized and stored at −20°C. Pool III which contains significant concentrations of human serum albumin is ultrafiltered [7] using a membrane with an M_r 20 000 cut-off (cellulose triacetate membrane; Sartorius, Göttingen, Germany) prior to reversed-phase chromatography.

3. Results and discussion

In previous studies, we have shown that peptide hormones are present in HF in plasma-like concentrations and in their bioactive form [2,7–13]. Due to the hemofilters used, plasma proteins are reduced to an extent that total protein content is only 50–70 mg/l compared to 70 g/l in plasma [4]. The filtrate is recovered in quantities of up to 2000 l/week from one nephrological center. Hemofiltrate is a reliable source for our program of isolating and characterizing circulating human peptide hormones. Following the chromatographic purification steps, several peptide hormones have been previously isolated whose specific bioactivity was validated using different bioassay systems, such as relaxation of vascular smooth muscle (atrial natriuretic polypeptide/cardiodilatin-99-126) or stimulation of cyclic guanosine monophosphate (cGMP) production in a colonic tumor cell line [8,9]. To facilitate further purifications, we attempted to establish a reproducible large-scale preparation for batches up to 10 000 l HF.

Certain properties of HF, such as its content of cholesterol, triglycerides, electrolytes and other non-peptidergic compounds, make the careful selection of chromatographic resins essential for successful and reproducible peptide extraction and separation. First, acidification to pH 2.7, dilution to a conductivity of <8 mS/cm and the superior capacity of a strong cation-exchanger proved to be most suitable for peptide extraction. Second, the use of polymeric materials and columns both compatible to cleaning in place procedures employing 0.5 *M* NaOH and 0.5 *M* HCl was shown to be essential for acceptable long-term stability of the media. This proved to be true especially for reversed-phase media, where conventional, silica-based C_{18} and C_4 resins were used with poor results, following repetitive loading with HF. Third, reproducible subfractionation in 300 fractions was necessary to achieve a high ratio of specific activity/total protein for peptide hormones with low systemic concentration. This was especially important for the detection of regulatory peptides that act in a paracrine or autocrine fashion and are only

found in plasma in low concentrations since they diffuse into the vascular bed. The subfractionation also reduces the likelihood of obscuring peptide activities due to the parallel presence of functional antagonists or synergists in the same fraction.

Several two-step procedures combining cation-exchange separation with reversed-phase chromatography were carried out on a small scale and eventually scaled up to a preparation scheme that is able to separate up to 500 g of peptides in 300 fractions.

3.1. Subfractionation on a small scale

For the first separation step, cation-exchange conditions were optimized. Several 10-l HF aliquots from the same crude extract were used to check the reproducibility of the procedure. The peptide concentrate was diluted and acidified to match binding conditions of the peptide extraction performed routinely. Exchange resin identical to that used for peptide extraction (see Section 2.2) was selected to obtain suitable binding and elution. Optimal binding to the column was achieved, as controlled by amino acid analysis of the flow through (data not shown). In a first series of experiments, attempts to perform reproducible gradient elution gave inconsistent results, therefore stepwise elution with the goal of obtaining a total of 5 to 10 fractions was our objective. This takes into account that the fast processing of the resulting fractions to reversed-phase chromatography without freezing–thawing of the peptides is essential to preserve biological activity. In addition, the use of buffers at a pH of 2.7 requires 3 *M* NaCl concentrations to completely elute all bound peptides, resulting in broadened peaks at high salt conditions. At a pH of 5.5, all peptides are readily eluted at 1 *M* NaCl. In this case, equilibration of the resin to pH 5.5 results in an instant breakthrough of acidic peptides. From these findings we concluded that elution has to cover a certain pH range in order to obtain acceptable results for both acidic and basic peptides. Thus, we used seven different buffers, adjusted to pH values close to their buffering optima with comparable ionic strength and low conductivity, covering a pH range from 3.6 to 9.0. Stepwise elutions were performed as depicted in Fig. 1. The seven pools obtained during this stepwise elution are referred to as pH pools I to VII.

This standard procedure proves to be superior, in terms of elution volume, amount of salt required for elution and reproducibility, to a gradient elution using NaCl. Loading studies showed that, using this procedure, up to 35 mg peptide/ml resin were completely bound and eluted with only minor changes in pool composition.

The pH pools were further fractionated using reversed-phase chromatography. Fractionation into about 40 fractions per pH pool, as shown in Fig. 2, resulted in a total of 300 salt-free fractions. These

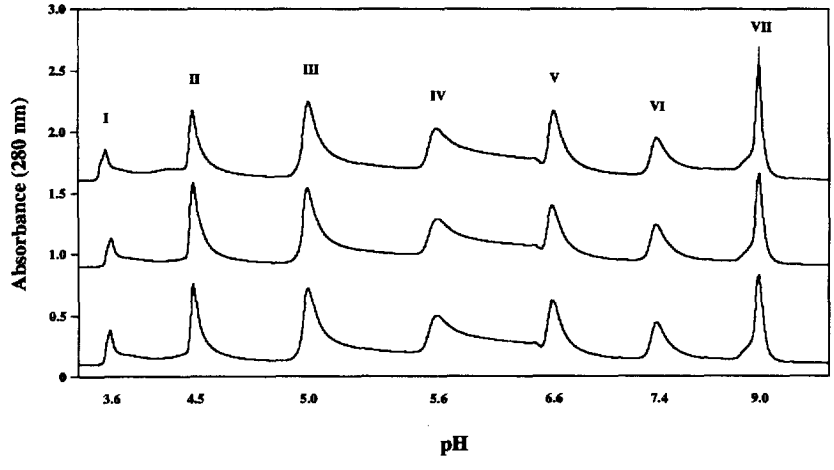

Fig. 1. Cation-exchange separation of HF peptides. Peaks I to VII represent peptides from 10 l HF which are reproducibly separated in seven pH pools using buffers of increasing pH. For details see Section 2.3.

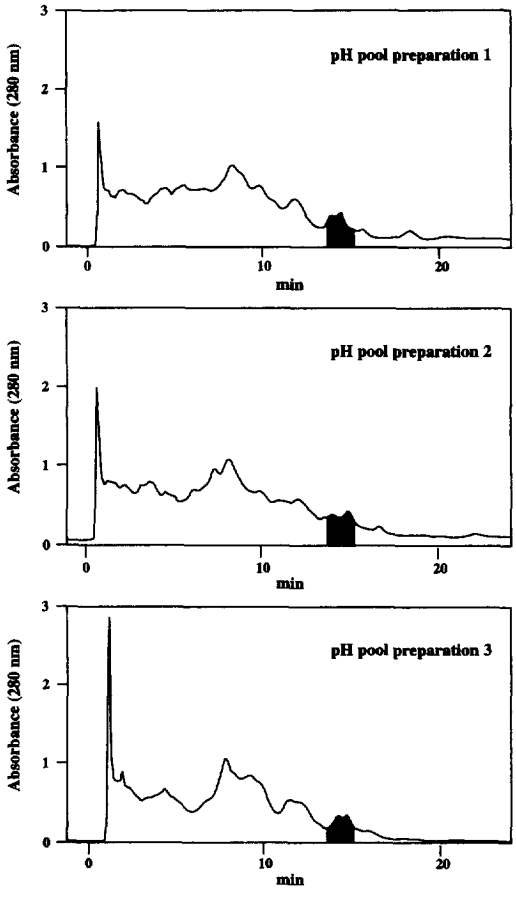

Fig. 2. Reversed-phase chromatography. As shown here for pool I, a similar elution profile is seen in three different small-scale pH pool preparations. Fractions were analysed by MALDI-MS, the mass spectra of the shaded fraction 19 are given in Fig. 3.

fractions were lyophilized. They dissolved readily in aqueous solutions.

To compare the composition of single fractions prepared in an identical manner, MALDI-MS was performed. As shown in Fig. 3, the samples from three different preparations that underwent pH pool elution and reversed-phase chromatography showed a comparable peptide pattern supporting the chromatographic profile seen in Figs. 1 and 2. Not only was the identity of peptides in these fractions very similar, but the relative quantity was also comparable. At this purification stage, the detection of more than 60 different peptides occurring at higher concentrations in one single fraction underlines the complexity of the peptide spectrum in human plasma and the necessity to perform a certain degree of subfractionation to detect peptides in the picomolar range. From the MALDI-MS results, we conclude that the preparative chromatographic media in combination with our separation protocol are selective enough to upscale the preparation scheme to much larger amounts of HF peptides.

3.2. Upscaling

In a second series of experiments the processing of up to 500 g HF peptides per run was performed. In general, peptides from 1 000 l HF (50 to 70 g) were completely bound by 2 l of Fractogel TSK SP 650(M). Pooled extracts from up to 10 000 l HF were adjusted to pH 2.7 with a conductivity of <8 mS/cm and loaded onto an Amicon Vantage column with a variable column volume of 2 to 25 l. The column is operated in hydraulic axial compression mode and routinely cleaned in place under reversed flow using 0.5 *M* NaOH. In combination with the Autopilot chromatography system, flow-rates of up to 3 l/min were used for sample application. Elution was performed at 0.5 to 1 l/min and volumes of the pH pools obtained range between 15 and 25 l for a 5000 l preparation, as shown in Fig. 4. For practical reasons, the flow-rates used were varied according to the sample volume to allow fast processing. The transfer of the small-scale pH pool elution to large scale has shown to yield stable, reproducible results.

Preparative reversed-phase chromatography of pH pools from 1000 up to 5000 l HF equivalents resulted in complete binding and reliable separation except for pH pool III which at 2000-l aliquots showed peak broadening and increasing amounts of unbound peptides in the flow through. This was due to the fact that this pH pool III contains most of the filtered albumin (albumin content of HF is around 25 mg/l). pH pool III was therefore subjected to ultrafiltration using a membrane with an M_r cut-off at 20 000, removing 80% of total protein from the pool. Following this ultrafiltration, this pool was processed as the other pools. As shown in Fig. 5 for a 5000 l HF-preparation, up to October 1996 the peptides from 30 000 l HF were separated into 300 fractions using cation-exchanger and reversed-phase gradient elution with acetonitrile. Every 6–8 weeks, peptides from as much as 10 000 l HF were treated in the

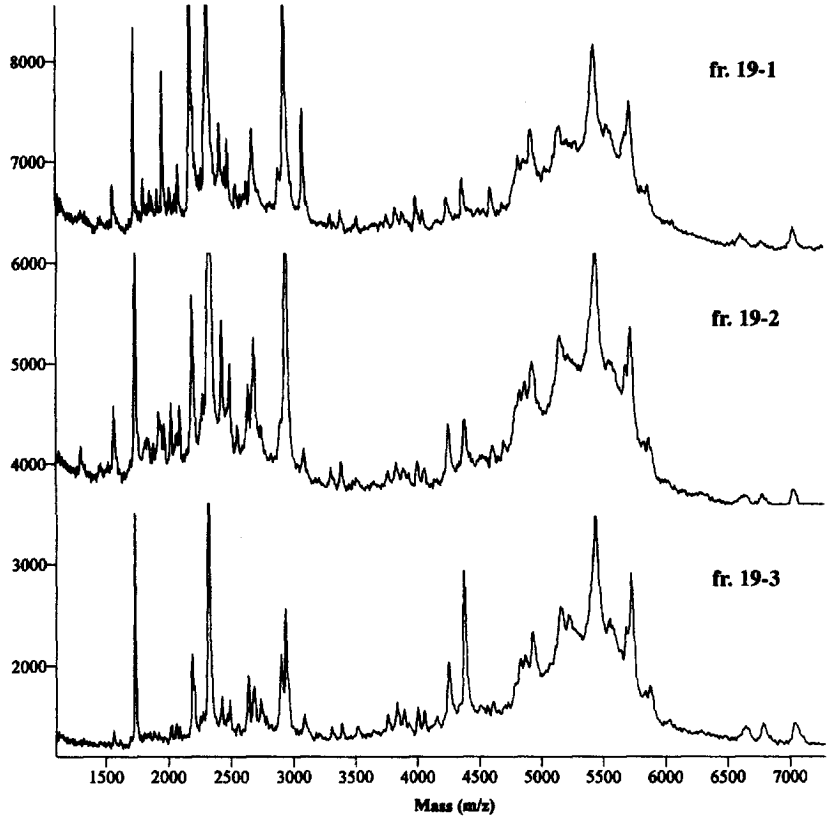

Fig. 3. MALDI-MS analysis. The mass spectra of fraction 19 from different reversed-phase chromatographies show the complex composition of the samples. A comparable pattern is observed in all three traces.

same way. For every preparation, aliquots of 0.1, 1 and 5 l HF were drawn in deep well microtiter racks in 96-well format using an pipetting robot. Specific activity of single compounds was increased by a factor of >50 000 compared to plasma.

The fractions generated using the combined procedure of pH elution via cation-exchanger and reversed-phase chromatography form a comprehensive peptide bank of human circulating peptides. This peptide bank can be used for different applications. Most importantly, screening for biological activities in a variety of bio-assay systems was performed. So far, several peptide hormones such as the insulin-like growth factors (IGF)-I and -II, guanylin, angiotensin I and others have been isolated using specific assay systems (data not shown). These peptides were found to occur in one to three RP fractions of one or two adjacent pH pools, demonstrating the good resolution achieved during the standardized procedure.

Over the last few years, we have developed the concept of peptide trapping for a systematic analysis of circulating peptides [13]. Using the elution position in high resolution microbore reversed-phase HPLC and their precise molecular masses as determined by MALDI or electrospray-MS, several thousand peptides were unambiguously identified in the peptide bank. Purification of selected peptides was carried out according to their molecular masses during rechromatography. Using this approach, many bioactive fragments of plasma proteins [10,11] and novel peptides have also been discovered. For example, a new member of the chemokine family, the circulating chemokine HCC-1 [12], and the first

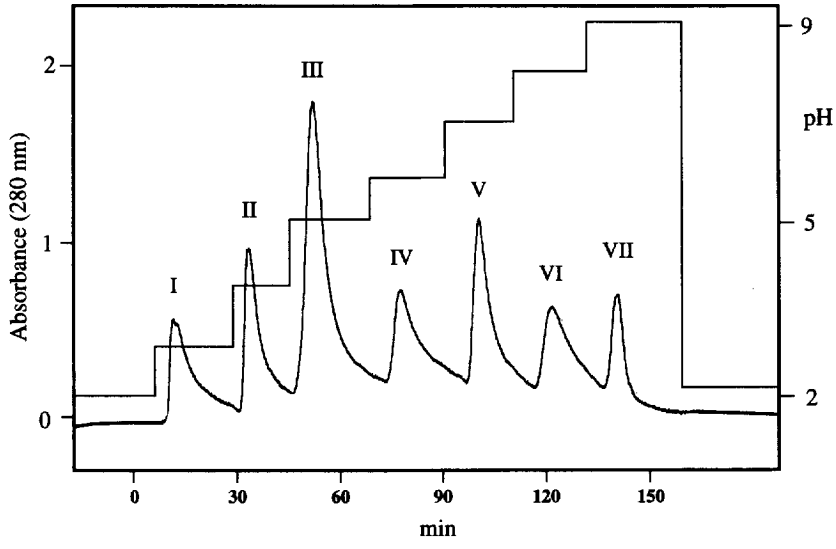

Fig. 4. Preparative cation-exchange chromatography. Peaks I to VII represent the peptides of 5000 l HF eluted stepwise with buffers of increasing pH, as explained in Section 2.3. Upscaling factor compared to Fig. 1: 500.

member of the β-defensin family, human β-defensin I (hBD-1) [7] were characterized and subsequently isolated in milligram quantities.

4. Conclusions

Screening for circulating human peptides and their isolation is possible using blood ultrafiltrate, the hemofiltrate. The reduced protein content and high availability of HF allows large-scale preparation of thousands of liters with conventional column chromatography. The two-step procedure of cation-exchange separation into pH pools followed by reversed-phase chromatography generates a peptide bank of 300 fractions covering a comprehensive peptide spectrum of plasma. In general, peptide hormones in femto- to nanomolar concentrations can

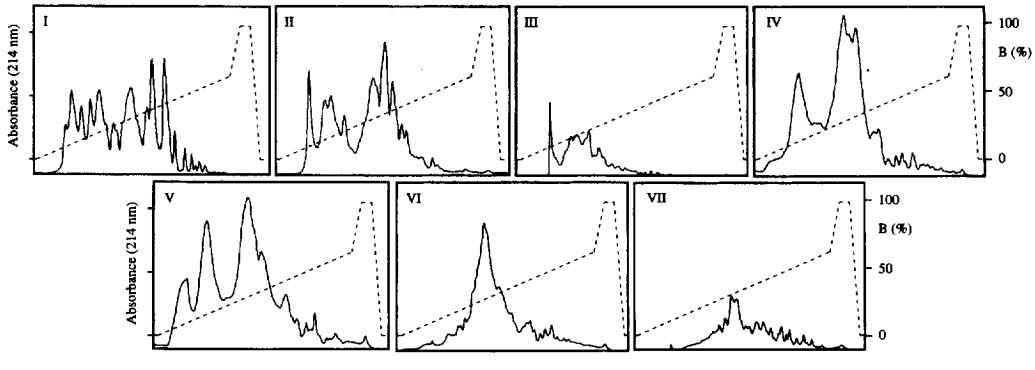

Fig. 5. Preparative reversed-phase chromatography. Numbers I to VII show the chromatograms and gradients of the individual pH pools obtained from 5000 l HF. 46 fractions of 200 ml are collected per pH pool and subsequently lyophilized. These fractions form the peptide bank from human blood. Upscaling factor compared to Fig. 2: 500.

be isolated and subjected to biochemical and functional analysis. By using our large scale peptide bank from human plasma, identification of many endogenously circulating peptides is possible. Thus, this peptide bank offers a novel approach for the detection of new targets for drug development.

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