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Isolation of haemorphin-related peptides from filter membranes collected in connection with haemofiltration of human subjects☆

Greger Nyberg[a], Katarina Sanderson[b], Per Andrén[b,c], Madeleine Thörnwall[b], Margareta Einarsson[b], Bo Danielson[a], Fred Nyberg[b,*]

[a]*Department of Internal Medicine, Uppsala University Hospital, S-751 85 Uppsala, Sweden*
[b]*Department of Pharmacology, University of Uppsala, PO Box 591, S-751 24 Uppsala, Sweden*
[c]*Analytical Chemistry Center, University of Texas Medical School, Houston, TX 77225, USA*

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Abstract

This paper describes the extraction and isolation from dialysis filters of two peptides containing the opioid active sequence haemorphin-7. The filter devices were obtained from uraemic patients subjected to haemofiltration. Following acidic extraction of the filter membranes the peptides were purified by size-exclusion, ion-exchange chromatography and finally by reversed-phase chromatography using different columns and different chromatographic systems. The purification was guided by radioimmunoassay and the structure of the final products was elucidated by N-terminal sequencing and fast-atom bombardment mass spectrometry as well as micro-electrospray mass spectrometry. The isolated peptides were suggested to be identical to fragments 1–41 and 32–41 of the β-chain of human haemoglobin.

1. Introduction

Haemofiltration or haemodialysis has been used in the clinic for renal replacement therapy over a long period of time (see e.g. Refs. [1–4]). The technique has also been applied in the treatment of patients with psychiatric disorders, such as schizophrenia [5]. It is well known that haemofiltration or haemodialysis removes low-molecular-mass plasma constituents ($M_r <$ 20 000) from the circulation [1–4]. These may also include various peptides or peptide fragments. For instance, in a schizophrenic patient, who responded to dialysis and naloxone treatment [6] it was suggested that the dialysis presumable removed most of the opioid peptides. In fact, it was later observed that the plasma levels of receptor active opioids were deceased following haemodialysis (unpublished observation). In the same study it was found that a certain amount of peptides was adsorbed to the filter membrane. This peptide material was extractable by treatment with acetic acid. In the present study we have attempted to recover peptides by acidic extraction from filter membranes obtained

* Corresponding author.

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from uraemic patients undergoing haemofiltration. Following extraction the recovered peptide fraction was subjected to a purification procedure including size-exclusion, ion-exchange chromatography and reversed-phase HPLC. The purified products were further studied by means of mass spectrometry and N-terminal sequencing. The data obtained was compared with the known amino acid sequence of the β-chain of human haemoglobin (Fig. 1).

2. Experimental

2.1. Peptides and chemicals

Synthetic haemorphins were prepared by Dr. G. Lindeberg (Department of Medical Immunology, Uppsala, Sweden). Before use the synthetic peptides were purified by HPLC [7]. Proteins and peptides used for column calibration were purchased from Sigma (St. Louis, MO, USA) and Bachem (Bubendorf, Switzerland) (β-endorphin and Leu-enkephalin-Arg^6). The chromatography material, Sephadex G-50 and SP-Sephadex C-25, was purchased from Pharmacia AB (Uppsala, Sweden). All other chemicals and solvents were of reagent grade from common sources.

Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys-Val-Asn-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg

Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe Glu-Ser-Phe-Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-Lys-Val-Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asn-Leu-Lys-Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-Leu-Leu-Gly-Asn-Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-Gly-Lys-Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His

Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys-Val-Asn-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg

Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe

Fig. 1. The entire amino acid sequence of the β-chain of human haemoglobin. The LVV-haemorphin-7 sequence is boxed. At the lower part of the figure the 1–41 fragment is shown.

2.2. Haemofiltration and collection of filter devices

The conditions for the haemofiltration were as described previously [8]. Filters were collected from uraemic parameters which had been dialysed for 3–24 h. The filters used in this study were of HD-type (Gambro, Lund, Sweden) with filter material of polyamide. Following dialysis the filters were disconnected from the patients and washed with saline. During the transport from the clinic to the laboratory the collected filters were stored in cold boxes at a temperature of ca. +5°C.

2.3. Extraction

At the laboratory the filter devices were perfused with heated (+70°C) acetic acid (1 *M*) and allowed to stand for 30 min before the peptide fraction was eluted by an additional volume (300 ml) of warm 1 *M* acetic acid. Eluates from separate filters were subsequently lyophilized or pooled before further processing.

2.4. Chromatography

Pooled eluates from five separate filters (1.2–1.5 l) were subjected to size-exclusion chromatography on Sephadex G-50. The column (120 × 10 cm) was equilibrated with 1 *M* acetic acid and operated at a flow-rate of 300 ml/h. Fractions of 80 ml were collected and analysed for protein content by measuring the absorbance at 280 nm. The column was calibrated with molecular mass (M_r) standards (bovine serum albumin, M_r 68 000; ribonuclease, M_r 14 000; β-endorfin, M_r 3 500; and Leu-enkephalin-Arg^6, M_r 700). In runs of filter extracts fractions eluting in the molecular mass range 500–5000 were collected for further chromatography on SP-Sephadex C-25. The cation-exchanger was packed in a column (25 × 5 cm), previously equilibrated with

0.018 *M* pyridine–0.1 *M* formic acid (pH 3.1). Prior to application the sample from the size-exclusion step was diluted (1:2) with the above pyridine buffer. After washing with two volumes of 0.1 *M* pyridine–0.1 *M* formic acid (pH 4.5) the column was step-wise eluted at pH 4.5 with increasing concentration of pyridine (0.35 and 1.6 *M*, respectively) using a flow-rate of 200 ml/h. The fractions collected at 0.35 *M* and 1.6 *M* pyridine–0.1 *M* formic acid were pooled and lyophilized. After reconstitution in 60 ml 1 *M* acetic acid this material was subjected to an additional size-exclusion step using a Sephadex G-50 column (90 × 5 cm) with 1 *M* acetic acid as eluent. Fractions of 20 ml were collected at a flow-rate of 60 ml/h and assayed for protein content and immunoreactivity. The active material was lyophilized and taken for further purification by HPLC. In additional experiments the lyophilized extracts eluted from single filter devices were reconstituted in 50 ml 1 *M* acetic acid and directly taken for separation on the second Sephadex G-50 column (90 × 5 cm). The column was operated as before and also in this case the active fractions were pooled and freeze-dried before further separation of HPLC.

2.5. High performance liquid chromatography (HPLC)

The HPLC system (Pharmacia LKB Biotech, Uppsala, Sweden) was in detail described in a preceding paper [9]. For reversed-phase separations at Delta-Pac C_{18} column (300 × 7.8 mm, particle size 15 μm) and an ODS C_{18} column (Spherisorb, 250 × 4.6 mm, particle size 5 μm) were used. Prior to application the lyophilized sample was dissolved in 15% acetonitrile containing 0.04% trifluoroacetic acid (TFA). The columns were developed by linear gradients of acetonitrile in 0.04% TFA and fractions were collected continuously maintaining a constant flow-rate. The UV pattern was recorded at 280 nm. Aliquots of the fractions were evaporated in the Savant Vac concentrator and analysed for hemorphin-7-like immunoreactivity. Micropurification was performed using SMART™ system (Pharmacia Biotech, Uppsala, Sweden) for reversed-phase separation [10,11]. The system was equipped with a μRPC C_2/C_{18} SC 2.1/10 column (100 × 2.1 mm I.D.). The sample was dissolved in 0.04% TFA and applied to the column. The computer-controlled system contains built-in detector cells for UV (U-M II with 214 nm optics) and conductivity measurements. The conductivity scale was set by calibration with eluent A (100%) and B (0%); A = 0.04% TFA; B = 0.034% TFA in 60% acetonitrile. Elution was carried out with a linear gradient of acetonitrile (0–60%) containing TFA (0.04%) at a flow-rate of 240 μl/min. Fractions of 240 μl were collected and analysed by radioimmunoassay. The low-molecular-mass peptide material (M_r 1000–2000) collected in additional experiments was similarly purified to provide additional peptide amounts for analysis by micro-electrospray mass spectrometry.

2.6. Mass spectrometry, amino acid analysis and peptide sequencing

The molecular mass of the purified material was determined by fast-atom bombardment mass spectrometry (FAB-MS) using a Finnigan MAT 90 double-focusing instrument (Bremen, Germany) as described previously [12,13]. Amino acid analysis was carried out on an Alpha Plus amino Acid analyzer (Pharmacia/LKB Biotech) with 24-h hydrolysates (10°C in 6 *M* HCl) of the dried peptide material. N-terminal sequencing of the isolated peptides was performed with an Applied Biosystem 477 A sequencer (Foster City, CA, USA).

2.7. Micro-electrospray mass spectrometry

Electrospray ionization was performed on a Finnigan TSQ 70 triple quadropole mass spectrometer updated with TSQ 700 software (San Jose, CA) that had been fitted with a micro-ES source, a modification of a Vestec electrospray source (Vestec Products, Per Septive Biosystems, Boston MA). Specific source conditions were: needle voltage, 3.0–3.5 kV; spray current, 0.05–0.2 μA; hozzle voltage, 250 V; repeller voltage, 10 V. The mass spectrometer was scanned from

300 to 1000 mass-to-charge (m/z) units in 1 s and all data were acquired in a profile mode. The mass measurements accuracies were ±0.4 amn or less. Samples were dissolved in 50 μl of 50% methanol–0.25% acetic acid and analysed by continuous infusion at a flow-rate of 820 nl/min.

Tandem mass spectrometry (MS–MS) was performed in order to further verify the sequence of the isolated peptide. The first quadrupole of the tandem mass spectrometer was set to transit the parent ion mass with an acceptance window of about ±2 mass units. The parent ion was dissociated by collision with Xe gas (1.0–1.5 mTorr) in the second quadrupole region, and the third quadrupole was scanned over the mass range 300–900 m/z units for the appropriate product ions. Mass measurement accuracy for the product ions was typically ±0.2 m/z units.

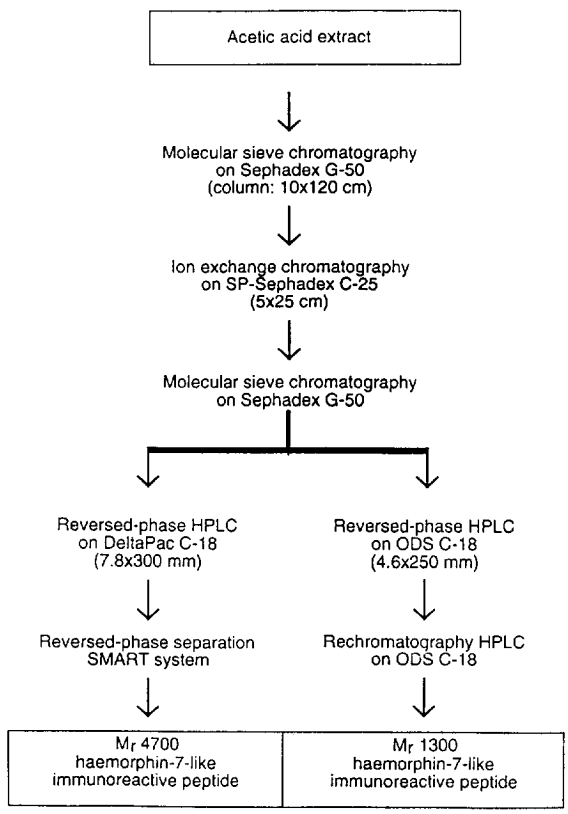

Fig. 2. Purification procedure outlined for the isolation haemorphin-7-like immunoreactive peptides.

2.8. Radioimmunoassay (RIA)

Radioimmunoassay for hemorphin-7 was based on the charcoal adsorption technique and conducted as described elsewhere [7]. The antiserum was raised in rabbits and the iodinated (^{125}I) synthetic hepta-peptide was used as tracer. Crossreaction between antibodies and LVV-hemorphin-7 was 100% and LVV-hemorphin-6 and shorter fragments less than 1%. The detection limit of the RIA was 4 fmol/tube.

3. Results

The fractionation scheme for the hemorphin-7-like immunoreactivity is shown in Fig. 2. In the size-exclusion step the major active material was eluted in area corresponding to peptides of molecular mass in the ranges M_r 1000–5000. This

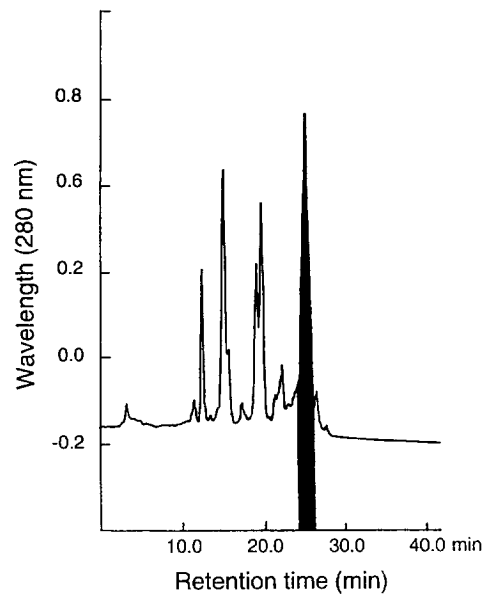

Fig. 3. Reversed-phase HPLC of the 3000–5000 peptide fraction from the second size-exclusion step. The lyophilized fraction was redissolved in 3 ml of 15% acetonitrile in 0.04% TFA and 1.0 ml was applied. The column (Delta Pac, 300 × 7.8 mm) was eluted with a 40-min linear gradient of acetonitrile (15–60%) containing 0.04% TFA. Fractions of 2.5 ml were collected at a flow-rate of 2.5 ml/min. The shaded area indicates the immunoreactive peak.

peptide fraction was further purified and concentrated on the cation exchanger (SP-Sephadex C-25) before a second size-exclusion step on the smaller Sephadex G-50 column. By this step the immunoreactive material was separated in two major fractions eluting in the areas corresponding to peptides of M_r 3000–5000 and M_r 1000–2000, respectively.

The active material collected from the second Sephadex G-50 size-exclusion in the range M_r 3000–5000 peptides was further separated by reversed-phase HPLC using a Delta-Pac column (Fig. 3). As shown in Fig. 3, by this chromatographic step the UV-absorbing material was resolved in four major components, one of which was found to crossreact in the radioimmunoassay. This active component was subsequently taken to micropurification using the SMART™ system, where additional inactive components were removed (Fig. 4). The peak associated with haemorphin-7-like immunoreactivity (80–100 μg/filter) was collected for sequence analysis, amino acid analysis and molecular mass determination by mass spectrometry. N-terminal sequencing revealed that at least the first ten residues were identical with the β-chain of human haemoglobin (only the amino acids from the first 10 cycles were analysed). By FAB-MS the molecular mass of the isolated peptide was determined. The spectrum obtained in a positive-ion mode gave a molecular ion at m/z 4569.9, which was found to correspond to the 1–41 fragment of the human haemoglobin β-chain after calibration of the system. The amino acid composition of the hydrolyzed compound was also compatible with the isolated peptide being identical to the β-chain fragment 1–41.

Reversed-phase separation of the material eluting in the low-molecular-mass region (M_r 1000–2000) is shown in Fig. 5. As shown in the figure, one UV-absorbing component was found to crossreact in the radioimmunoassay. This component was rechromatographed on the same system using a different gradient (Fig. 6). By this step the active material (about 100 μg/filter) was still found to be associated with a single UV-peak

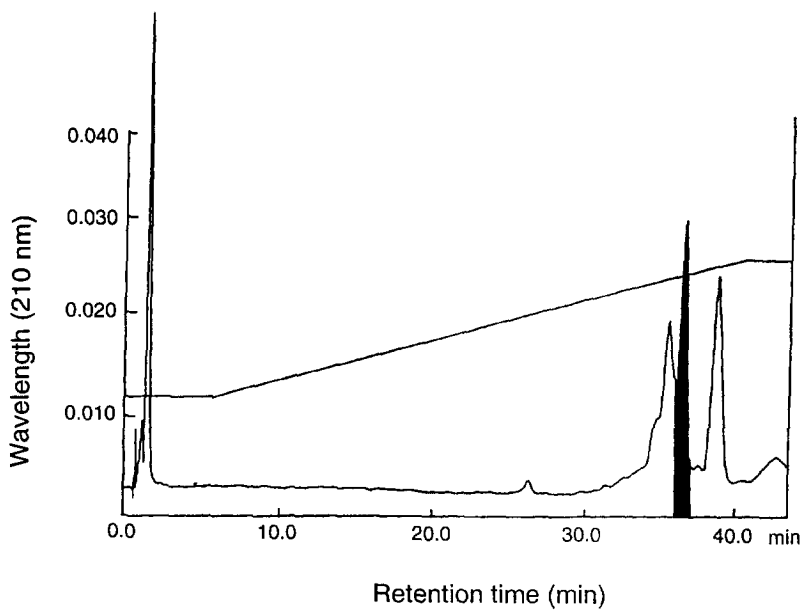

Fig. 4. Reversed-phase separation using the SMART™ system of the active component recovered in the experiment shown in Fig. 3. The column, μRPC-C_2/C_{18}SC 2.1/10 (100 × 2.1 mm I.D., particle size 3 μm), was eluted with a linear gradient of acetonitrile (20–60%) containing 0.04% TFA. Fractions of 100 μl were collected at a flow-rate of 100 μl/min and analysed by radioimmunoassay. The shaded area indicates the immunoreactive peak. Prior to injection the sample (100 μl) was centrifuged at 10 000 *g* for 1 min.

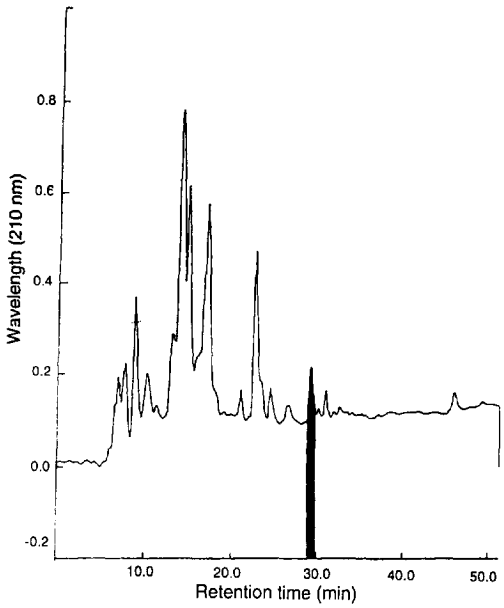

Fig. 5. Reversed-phase separation of peptide containing fractions M_r 1000–2000 from the second size-exclusion step on Sephadex G-50. The sample (100 μl) was injected onto a column (ODS C_{18}, 25 × 4.6 mm) equilibrated with 20% acetonitrile containing 0.04% TFA. Elution was carried out with a 40-min linear gradient of acetonitrile (20–60%) maintaining a flow-rate of 0.5 ml/min. Fractions of 0.5 ml were collected and evaporated for further analysis. The shaded area indicates the immunoreactive peak.

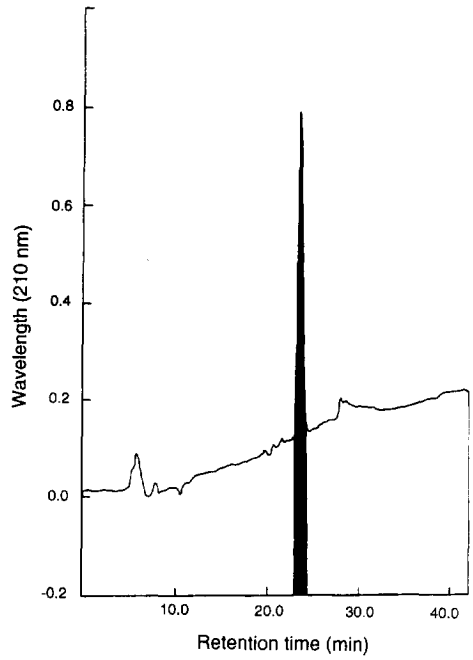

Fig. 6. Reversed-phase rechromatography of the active component collected from the experiment shown in Fig. 5. The conditions were identical to those of the previous run (Fig. 5) with the exception that a gradient of 20–50% acetonitrile was used in this experiment. The shaded area indicates the immunoreactive peak.

and no significant impurities could be detected. Micro-electrospray mass spectrometry gave a peak that corresponded to the haemoglobin β-chain fragment 32–41 or LVV-haemorphin-7 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe). Thus, a recorded m/z 655.4 corresponds to the doubly-charged ion $[M + 2H]^{2+}$ of LVV-haemorphin-7 (M_r 1308.5). To provide sequence verification of this peptide tandem MS (MS–MS) was also performed. By this technique several product ions that could only originate from LVV-haemorphin-7 were observed thus confirming the identity of the isolated peptide and the haemoglobin β-chain fragment 32–41.

4. Discussion

It is evident from this study that during haemofiltration of uraemic patients a considerable amount of various peptides are adsorbed to the filter membrane. A certain amount of these peptides seems to represent hydrolytic fragments from haemoglobin. The present data suggests that at least two of these fragments derive from the β-chain of human haemoglobin and contain the opioid active sequence haemorphin-7 (i.e. residues 35–41 of the β-chain). Based on the results obtained by N-terminal sequencing and FAB-MS the large peptide presumable is identical to fragment 1–41 of the β-chain. The smaller peptide fragment seems undistinguishable from fragment 32–41 of the β-chain (LVV-haemorphin-7). Further support for the larger structure being identical to the 1–41 fragment of human haemoglobin β-chain comes from its crossreactivity with antibodies used in the radioimmuno assay.

It thus seems that during the course of haemofiltration red blood cells are lysed and haemoglobin is exposed to proteolytic activity capable of releasing the actual fragments from its β-chain. The fragment LVV-haemorphin-7 could be further converted to haemorphin-7. Both these fragments seem to be stable towards enzymatic degradation [14] and both are known to posses opioid activity [15,16]. Whether all released peptides are adsorbed to the filter membrane or a certain fraction is left in the circulation of the dialysed patient remains to be determined. At high concentrations the hemorphins might have profound effects on the patients. For instance, the ability of haemorphins to inhibit angiotensin converting enzyme (ACE) [14] may affect the blood pressure in patients undergoing dialysis. Interestingly, a hypotension-inducing effects of haemodialysis or haemofiltration is a well-known phenomenon (see e.g. Ref. [16]). Other effects of the haemorphins may be similar to those of other peptides acting on μ-opioid receptors [15].

The present study also shows the usefulness of various HPLC procedures for peptide purification. The introduction of SMART™ system (Fig. 4) gave an improved resolution and allowed us to purify the large β-chain fragment at a comparatively high yield. The total amount of peptides extractable from the filter membrane recovered by the purification procedure also seems to be high. From a single filter device it was thus possible to recover up to 100 μg of each peptide. It also seems clear from this study that a number of other so far unidentified peptides or protein fragments are present in the material extracted from the filter device. Their final isolation and structure elucidation will be a challenge for future work.

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