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Set-up of large laboratory-scale chromatographic separations of poly(ethylene glycol) derivatives of the growth hormone-releasing factor 1–29 analogue

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Abstract

In this paper we report the scale-up of the purification of poly(ethylene glycol) (PEG) derivatives of the growth hormone-releasing factor 1–29, from laboratory scale (100 mg of bulk starting material) to larger scale (3 g of bulk), through the use of a cation-exchange TSK-SP-5PW chromatographic column. A one-step purification process capable of purifying large amounts of mono-PEGylated GRF species from the crude reaction mixture was developed. A simple, straightforward stepwise gradient elution separation was developed at laboratory scale and then scaled up with a larger column packed with a chromatographic resin with the same chemistry which maintained the laboratory-scale separation profile. Active material recovery and material purity remained constant through the scale-up from the 13-μm stationary phase to the 25-μm larger column. Overall, the gram GRF equivalent/batch process scale showed to be quite reproducible, and could be considered as a good platform for scale up to production scale.

Keywords: Derivatization, LC; Preparative chromatography; Growth hormone releasing factor; Poly(ethylene glycol); Peptides

1. Introduction

Growth hormone releasing factor (GRF, also called somatorelin) is a peptide secreted by the hypothalamus that, acting on its receptor, promotes the release of growth hormone (GH) from the anterior pituitary [1–6]. The 1–29 residue corresponds to the shortest form of the peptide reported to be fully active [7]. The molecule has a therapeutic value for the treatment of certain growth hormone-related disorders.

The use of GRF to stimulate the release of GH is also a physiological method used to promote long bone growth or protein anabolism.

One problem associated with the use of GRF relates to its short biological half-life (about 12–30 min). Among the numerous chemical and enzymatic degradation pathways [8–10], the peptide is rapidly degraded in the plasma via action of the dipeptidylpeptidase IV (DPP IV) which cleaves the molecule between the Ala^2 and Asp^3 [11]. Long acting and biologically more stable analogues were therefore developed using specific chemical modification of GRF_{1-29} (Fig. 1) in order to prevent or slow down enzymatic degradation [12–15].

Chemical modification with poly(ethylene glycol) (PEG) is a technique widely developed in the field of bioconjugation [16–19]. One of the critical chal-

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H-Tyr[1] Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg **Lys**[12] Val Leu Gly Gln Leu Ser Ala

Arg **Lys**[21] Leu Leu Gln Asp Ile Met Ser Arg-NH_2

Fig. 1. Amino acid sequence of GRF_{1-29}.

lenges for the clinical development of PEGylated conjugates is related to the capability in scaling-up conjugation reactions and purification of the conjugates. Only the preparation and separation of chemically defined conjugates (as well as their characterization) will insure the product consistency required for relating laboratory bench discoveries and development to clinical outcomes.

The liquid-phase modification of GRF_{1-29} on the free amino bearing residues via PEGylation with an activated monomethoxypoly(ethylene glycol) generates a heterogeneous mixture of analogues with different numbers of PEG attached. The synthesis in dimethylsulfoxide (DMSO) leads to the formation of unreacted, monoPEGylated (two positional isomers, respectively, on the Lys^{12} and the Lys^{21} of the peptide) and diPEGylated GRF_{1-29} (both on $Lys^{12}$and Lys^{21}) species.

The monoPEGylated GRF species were identified as functionalized bioconjugates with increased resistance to proteolytic degradation, and adequate receptor-binding affinity.

In this paper we will report the separation at laboratory preparative scale and the identification via HPLC of the PEGmers as well as the unreacted species. We will then show the scale-up of the process to the gram scale (in GRF mass equivalent) and disclose results in terms of material recovery and final bulk purity.

2. Experimental

2.1. Reagents

MethoxyPEG 5000–norleucine–succinimidyl ester ($mPEG_{5000}$–Nle–OSu) was purchased from Shearwater (Huntsville, AL, USA). The GRF_{1-29} peptide, M_r 3358, human, was purchased from Bachem (Torrance, CA, USA). DMSO, triethylamine and ethanolamine, were all purchased from Aldrich. The prepacked TSK-SP-5PW ion-exchange chromatographic columns (sulfopropyl strong cation exchanger n a methacrylate polymer matrix) used for this study were purchased from TosoHaas.

2.2. PEGylation reaction

The PEGylation reaction is carried out at about 7 mg/ml of peptide, with 1 molar equivalent of M_r 5000 PEG–Nle–OSu per mole of peptide, in DMSO, and in the presence of 10 molar equivalent of triethylamine, for about 60 min at room temperature. After PEGylation, the reaction was quenched by addition of 10 molar equivalent of ethanolamine.

2.3. Purification of the monoPEGylated GRF species

The monoPEGylated species of interest were purified using a TSK SP-5PW high-performance strong cation-exchange media (TosoHaas) packed into a 15 cm bed height×2.15 cm I.D. column (13 μm of particle size) for the preparative scale. For the purification at larger scale (gram scale in GRF mass equivalent) we used a 15–25 μm column (20 cm bed height×5.5 cm I.D.) completed with a 15–25 μm precolumn (5 cm bed height×4 cm I.D.). The column was equilibrated with 25 m*M* sodium phosphate, pH 4.5.

After a 10×dilution with the same equilibration solution (25 m*M* NaH_2PO_4), the PEGylation of GRF reaction mixture in 10% DMSO is loaded onto an TSK-SP-5PW column at a maximum concentration of 4.5 mg of peptide/ml of bed volume.

The fractionation of the PEGmers is made by a gradient (stepwise) elution.

The (mono)PEGylated species of interest (one

PEG chains covalently bound to the GRF peptide) was eluted with 0.5 *M* of NaCl in 25 m*M* sodium phosphate, pH 4.5.

The flow-rate throughput of all the steps was 0.1 column volumes per minute. The column was run at room temperature and the elution profile was monitored by 280 nm absorbance.

2.4. RP-HPLC analysis of PEGylation reaction species

The analysis was conducted with a Selectosil RP-C_8 (250×4.6 mm I.D., 300 Å, 5 μm, Phenomenex). A 15-μl volume of the 10-times diluted reaction mixture in DMSO was injected at 25±0.2°C.

Gradient elution was carried out at a flow-rate of 1 ml/min with solvent A [(0.1% (v/v) trifluoroacetic acid (TFA) in water] and solvent B [0.1% (v/v) TFA in acetonitrile].

The following gradient profile was chosen: 20% B for 5 min, 20% B to 35% B over 2 min, 35% B to 55% B over 20 min, 55% B to 95% B over 3 min.

After an additional elution for 3 min with 95% B followed by 10 min with 80% A, the system was ready for the next injection.

3. Results

3.1. Purification of the PEG_{5000}-linked GRF_{1-29} species

The conjugation reaction in DMSO with the $mPEG_{5000}$–Nle–OSu with GRF_{1-29} resulted in a mixture of monoPEGylated GRF (sum of two positional isomers on Lys^{12} and Lys^{21}), diPEGylated species (PEG covalently linked to both Lys^{12} and $Lys^{21)}$, and unreacted species.

The monoPEGylated GRF, which was the molecule selected by the in vitro/in vivo studies, had to be separated from the diPEGylated product that has two PEG chains per molecule of GRF and the unPEGylated GRF species.

Preliminary purification tests performed on the TSK gel SP-5PW column were evaluated by injecting a mixture of PEGylated species into a stainless steel column (15 cm×21.5 mm I.D.) equilibrated with 0.05 *M* of NaH_2PO_4 (pH 4.5) at a flow-rate of 6 ml/min. We observed that the diPEGylated species were eluted within the column flowthrough (Fig. 2). The monopegylated species were eluted by a linear

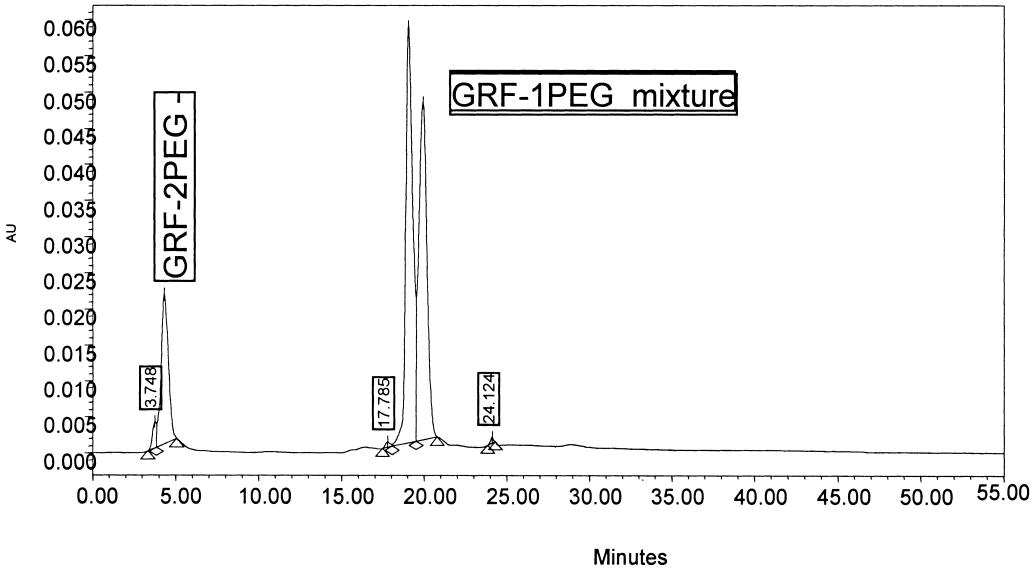

Fig. 2. Ion-exchange preliminary purification of PEGylated GRF species on the laboratory scale.

gradient elution to the same NaH_2PO_4 solution containing 0.5 *M* NaCl.

The retention time of the various PEGmers was found to be inversely proportional to the degree of PEG substitution on the peptide, as has been observed for other proteins [19].

In other experiments (data not shown), we observed that the unreacted GRF_{1-29} species were strongly adsorbed and not washed out even at 0.5 *M* NaCl. Indeed, under the set conditions of elution, e.g. 50 m*M* sodium phosphate, pH 4.5, and gradient in salt with 0.5 *M* NaCl, the GRF is not eluted from the TSK gel column. The adsorbed GRF was eventually washed out from the column with 0.1 *M* NaOH.

In order to improve the ion-exchange purification process, a stepwise gradient elution protocol was designed. After dilution with the equilibration solution the PEGylation of GRF_{1-29} reaction mixture in 10% DMSO was loaded onto a TSK SP-5PW column. The purification of the reaction mixture at the preparative scale on the 21.5 mm I.D. column (150 mg GRF/run), confirmed that cation-exchange chromatography was a suitable method for the purification of monoPEGylated GRF mixture (Fig. 3).

Purification of GRF-$1PEG_{5000}$ material on the ion-exchange TSK SP-5PW preparative column (15 cm×21.5 mm I.D.) also showed good material recovery (average of 90%) once conditions of binding and elution had been optimized (Fig. 4).

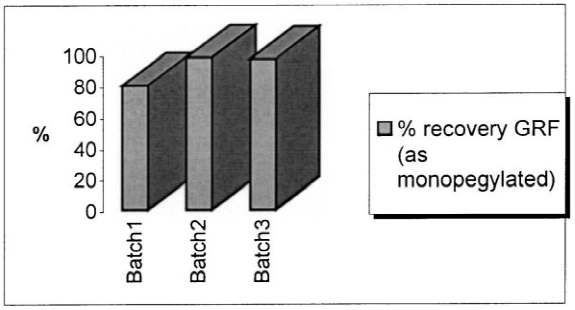

Fig. 4. Recovery of GRF-1PEG-5K material from the ion-exchange TSK SP-5PW column.

3.2. Scale-up of the cation-exchange purification of the monoPEGylated GRF species

The scale-up strategy to reach the gram scale (2.5 g of initial peptide) has been divided into two steps:

Initial scale-up of a factor of 3×, with respect to the laboratory-scale process, was carried out to adjust parameters on the preparative column and make the necessary modifications to the elution conditions (if required) to retain the laboratory scale elution profile.

Second scale-up (10× the initial laboratory scale) was in a successive phase carried out in order to reach the final objective of 1 g of GRF-$1PEG_{5000}$/run.

The TosoHaas column used for the 100 mg batch

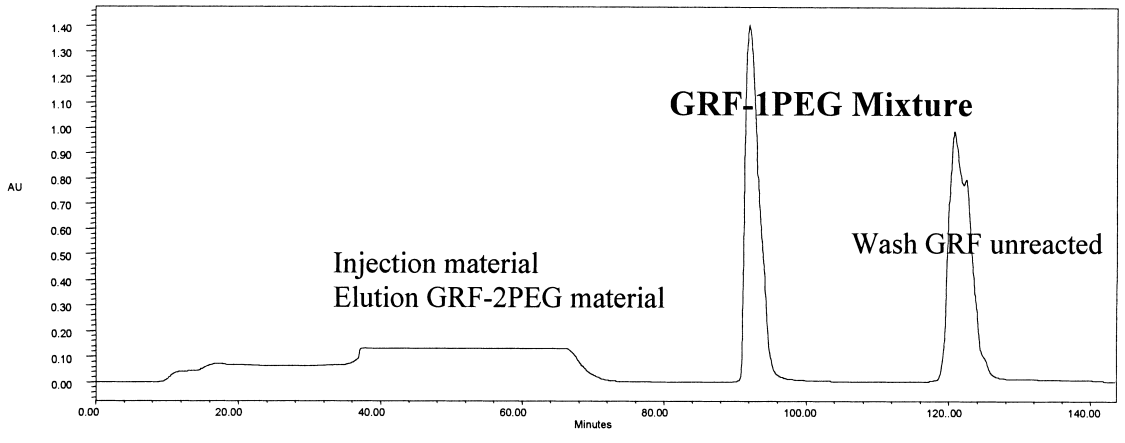

Fig. 3. Ion-exchange purification of GRF-$1PEG_{5000}$ material at the preparative scale.

scale purification of the monoPEGylated GRF species was substituted by a larger column, packed with a chromatographic resin with the same chemistry but with a larger particle size (15–25 μm instead of 13 μm).

Table 1 summarizes the results obtained at a larger-batch scale and compares them to the results obtained at the 100-mg scale.

Sample loading on the larger size column was proportional to the loading on the preparative column. In order to have equivalent elution profiles between the preparative and process column, variables such as linear flow-rate were maintained constant.

Purification at the 300-mg scale of GRF-1PEG$_{5000}$ (3× scale-up factor) showed good material recovery for both of the chromatographic steps, and a final bulk material conforms to the study specifications (purity≥95%, ratio between the two single monoPEGylated GRF species within specifications). No significant modification to the laboratory scale process was necessary.

Final material analyses reported in the Table 1 (purity and ratio between the two positional isomers) were made onto a reversed-phase chromatographic column which is not described in this article. The analytical method we developed allowed a complete separation of the two monopegylated species.

The final analyses were performed on the lyophilized bulk that had been subjected to an additional desalting step on a column packed with Sephadex G-25 fine media.

Analyses made during the development phase onto the GRF-1PEG$_{5000}$ bulk in solution after ion-exchange purification showed that the optimized ion-exchange chromatographic purification procedure was giving an identical level of purity for the intermediate material.

Passage to the gram scale showed equivalents results, in line with the expected values. Although the preparation time of GRF-1PEG$_{5000}$ at this scale had been increased by a factor 2, no loss of batch quality was observed by RP-HPLC analysis. The purity level was maintained (>96%).

3.3. Reproducibility of the GRF-1PEG$_{5000}$ production process at the gram scale

The maximum scale of 1 g GRF equivalent/batch process proved to be quite reproducible (Table 2), not only in terms of yields and product recovery after the two purification steps, but also in terms of purity and ratio between the two monoPEGylated species (RSD of 1.16%).

The material GRF-1PEG$_{5000}$ obtained showed a consistent purity profile with an average purity of 97.4%. An important parameter for the future production and from a regulatory viewpoint, was the consistency of the ratio between the two monoPEGylated species (RSD of 1.2%), with PEG on Lys^{12} and on Lys^{21} which constitutes the final GRF-1PEG$_{5000}$ product.

Fig. 5 shows a typical chromatographic profile of the purification at the gram scale. Selectivity is nearly the same, and elution time roughly equivalent.

Table 1
Recovery and final purity of GRF-1PEG$_{5000}$ material during the scale up from laboratory scale to large process scale

GRF bulk (mg)	GRF peptide (mg)	GRF1PEG$_{5000}$ input on ion-exchange column (mg)[a]	Recovery after ion-exchange purification (%)[a]	Recovery after desalting (%)[a]	GRF-1PEG$_{5000}$ output (mg)[a]	Purity (%)[b]	Process yield (%)[b]	Lys21/(Lys21+Lys12) (%)[b]
252	209	83	99	88.0	73	96.0	35	52
750	625	256	96	96.0	235	96.0	38	52
750	624	256	99	95.5	242	96.6	39	53
2386	2040	902	95	95.0	811	96.4	40	53

[a] Samples are quantified by C_8 RP-HPLC analysis.
[b] Samples are analyzed by RP-HPLC which procedure is not described in this article.

Table 2
Yield and purity of GRF-1PEG$_{5000}$ production at the gram scale

Batch production	GRF peptide (mg)	GRF-1PEG$_{5000}$ output (mg)	Purity (%)	Process yield (%)	Lys^{21}/(Lys^{21}+Lys^{12}) (%)
1	2040	803	96.4	39	53.0
2	2505	982	96.2	39	55.0
3	2507	976	97.6	38	54.3
4	2505	1060	96.0	42	55.0
5	2512	1065	98.0	42	54.4
6	2552	1040	98.4	40	54.6
7	2510	1042	97.5	41	54.5
8	2510	1057	98.1	42	54.2
9	2508	1169	98.5	46	54.9
10	2508	1130	97.6	45	55.4
Mean			97.4	41.4	54.5
SD			0.92	2.6	0.65
RSD			0.94	6.26	1.2

After purification on the ion-exchange column, total bulk material volume had been decreased by a factor 10.

3.4. RP-HPLC analysis of the PEGylated reaction mixture and the purified monoPEGylated GRF species

The RP-C_8 HPLC analysis allowed us to characterize the crude reaction mixture by separating the various species obtained during the synthesis step (Fig. 6).

The retention time increases with the degree of PEGylation of GRF, somehow correlating with an increase of hydrophobicity of the molecule when the PEG chains are known as amphiphilic polymeric entities [20]. Fig. 7 shows the elution profile of the purified monoPEGylated GRF species. The order of

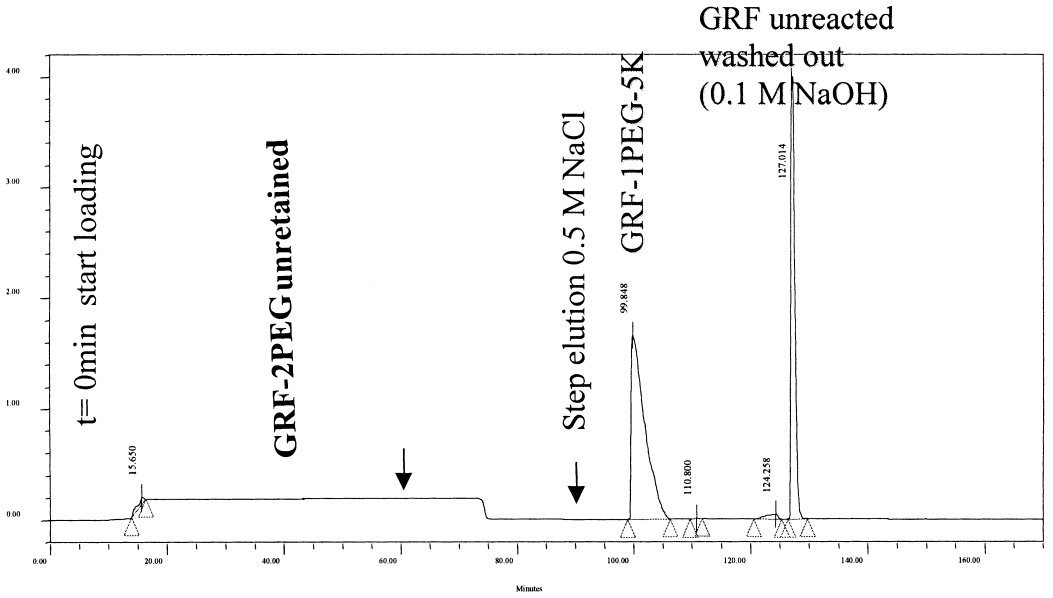

Fig. 5. Ion-exchange purification of GRF-1PEG-5000 material at the gram scale.

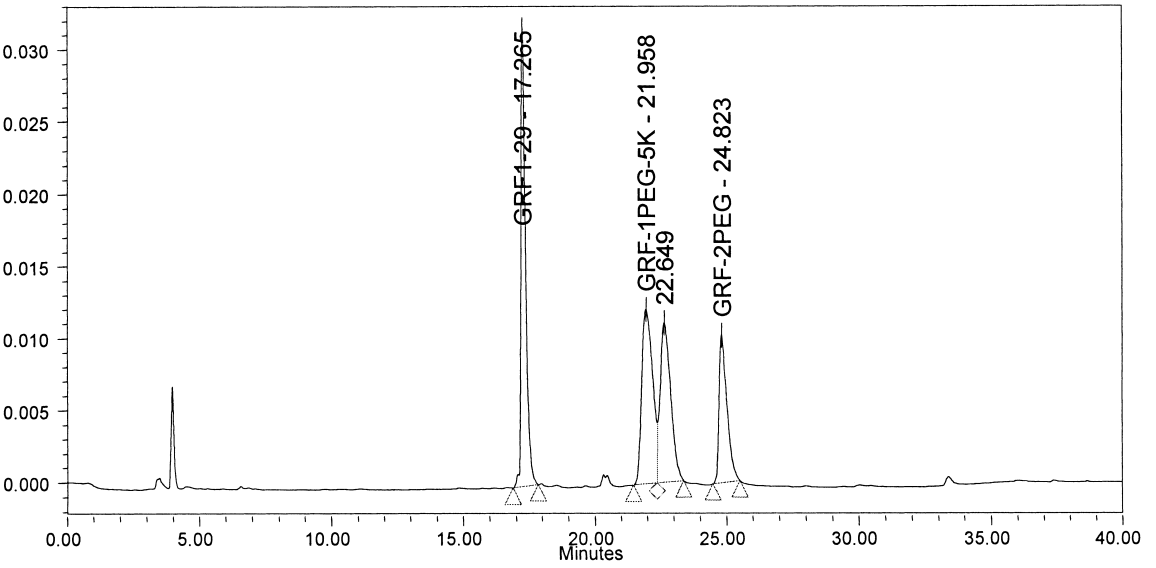

Fig. 6. RP-HPLC C_8 analytical chromatogram of the crude reaction mixture in DMSO before purification on the ion-exchange purification column.

elution has been found to be Lys^{21} first and then Lys^{12}.

The C_8 Selectosil also proved to be a useful method for assaying GRF concentration.

4. Discussion

A high-performance strong cation-exchanger (sulfopropyl), with potentially an increased resolution compared to conventional gel, allowed us to efficiently purified the GRF-1PEG$_{5000}$ material from the diPEGylated species and the unreacted GRF.

We showed the scale-up of the ion-exchange chromatographic purification on a TSK-SP 5PW column of monoPEGylated derivatives of the growth factor releasing hormone GRF_{1-29} from the laboratory scale (100 mg) to the large scale (gram).

A simple and efficient stepwise gradient elution procedure was set-up for the separation of the monoPEGylated species of GRF present in the PEGylation reaction mixture.

The peptides PEGylated at the lysines groups of

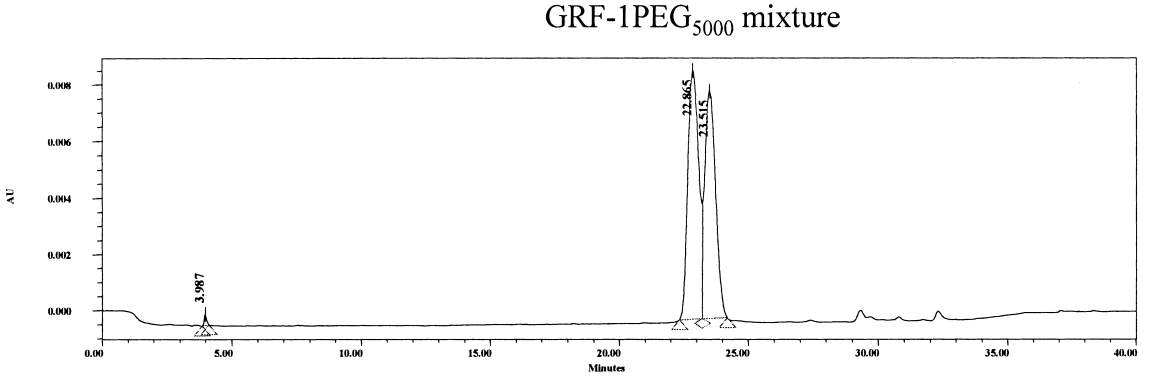

Fig. 7. RP-HPLC C_8 analytical chromatogram after purification on the ion-exchange purification column.

the GRF_{1-29} were probably separated in function of the net charge density shift due to the neutralization of charge on the amino nitrogen, although, as already reported by other authors [19], a charge shielding effect of PEG could also take place.

The procedure was scaled-up to a scale that showed its potential for a scale-up to a pilot plant production suitable for a therapeutic development of the product.

The 55-mm I.D. column with 25-μm particles allowed a identical selectivity and similar recoveries than the preparative purification on the 21.5 mm I.D. column.

The quality of the product and the performance of the process were unaffected by the scale-up procedure.

5. Nomenclature

CH_3CN or ACN	Acetonitrile
D or kD	Dalton or kilodaltons
GRF_{1-29}	Growth hormone releasing factor 1–29
$GRF\text{-}1PEG_{5000}$ or GRF species	GRF1PEG or monoPEGylated GRF covalently linked to polyethylene glycol 5000 D
IEX	Ion-exchange
IEX–HPLC	Ion-exchange–high-performance liquid chromatography
Lys^{12}	Lysine in position 12 of the peptide GRF_{1-29}
Lys^{21}	Lysine in position 21 of the peptide GRF_{1-29}
NaCl	Sodium chloride
$NaH_2PO_4 \cdot 1H_2O$	Sodium dihydrogen phosphate, monohydrated
NaOH	Sodium hydroxide
PEG	Poly(ethylene glycol)
mPEG–Nle–OSu	Monomethoxypolyethylene glycol 5000 norleucine succinimidyl ester
RP-HPLC	Reversed-phase high-performance liquid chromatography
Sephadex G-25	Dextran cross-linked with epichlorohydrin
SP-5PW	Sulfopropyl strong cation-exchange on a methacrylate polymeric matrix
TEA	Triethylamine
TFA	Trifluoroacetic acid

References

[1] R.M. Campbell, Y. Lee, J. Rivier, E.P. Heimer, A.M. Felix, T.F. Mowles, Peptides 12 (1991) 569.
[2] D.A. Kloosterman, T.A. Scahill, R.M. Hillman, D.L. Cleary, T.M. Kubiak, Pept. Res. 4 (n°2) (1991) 72.
[3] L. Lefrancois, P. Gaudreau, Neuroendocrinology 59 (1994) 363.
[4] R.M. Campbell, J. Bongers, A.M. Felix, Biopol. (Peptide Sci.) 37 (1995) 67.
[5] L.A. Cervini, C.J. Donaldson, S.C. Koerber, W.W. Vale, J.E. Rivier, J. Med. Chem. 41 (1998) 717.
[6] L.M. Bilezikjian, H. Seifert, W. Vale, Endocrinology 118 (5) (1986) 2045.
[7] P. Gaudreau, L. Boulanger, T. Abribat, J. Med. Chem. 35 (10) (1992) 1864.
[8] A.R. Friedman, A.K. Ichhpurani, D.M. Brown, R.M. Hillamn, L.F. Krabill, R.A. Martin, H.A. Zurcher-Neely, D.M. Guido, Int. J. Pept. Prot. Res. 37 (1991) 14.
[9] J. Bongers, E.P. Heimer, T. Lambros, Y.E. Pan, R.M. Campbell, A.M. Felix, Int. J. Pept. Prot. Res. 39 (1992) 364.
[10] L.A. Frohman, T.R. Downs, E.P. Heimer, A.M. Felix, J. Clin. Invest. 83 (1989) 1533.
[11] J. Bongers, T. Lambros, M. Ahmad, E.P. Heimer, Biochim. Biophys. Acta 1122 (1992) 347.
[12] Y. Lu, A.M. Felix, React. Polymers 22 (1994) 221.
[13] Y. Lu, A.M. Felix, Pept. Prot. Res. 43 (1994) 127.
[14] A.M. Felix, Y. Lu, R.M. Campbell, Int. J. Pept. Prot. Res. 46 (1995) 253.
[15] R.M. Campbell, E.P. Heimer, M. Ahmad, H.G. Eisenbeis, T.J. Lambros, Y. Lee, R.W. Miller, P.R. Stricker, A.M. Felix, J. Pept. Res. 49 (1997) 527.
[16] P. Caliceti, F.M. Veronese, S.T.P. Pharma. Sci. 9 (1999) 107.
[17] K.C. Lee, S.C. Moon, M.O. Park, J.T. Lee, D.H. Na, S.D. Yoo, H.S. Lee, P.P. DeLuca, Pharm. Res. 16 (1999) 813.
[18] T. Sakane, W.M. Partridge, Pharm. Res. 14 (1997) 1085.
[19] J.E. Seely, C.W. Richey, J. Chromatogr. A 908 (2001) 235.
[20] J. Snider, C. Neville, L. Yuan, J. Bullock, J. Chromatogr. 599 (1992) 141.