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High-yield purification of biosynthetic human growth hormone secreted in *Escherichia coli* periplasmic space

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Abstract

A six-step, high-yield purification procedure for the preparation of clinical grade recombinant human growth hormone (rhGH) secreted in bacterial periplasmic space is described. Particular emphasis is given to hormone recovery yields and maximum contaminant host cell elimination. The strategy adopted, in addition to using one precipitation and five chromatographic steps in a particularly efficient sequence, was also based on running *E. coli* proteins – immunoradiometric assay profiles right after each chromatographic elution. Thus, an overall rhGH recovery higher than 40%, with a final concentration of *E. coli* proteins below 10 ppm is described for the first time. The accuracy of hGH and total protein quantification, especially in the early steps of the process, and the maximum elimination of hGH-related forms were also studied in detail. For these purposes size-exclusion and reversed-phase HPLC were found to be extremely valuable analytical tools. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: *Escherichia coli*; Proteins; Growth hormones

1. Introduction

In the large scale preparation of recombinant proteins in general and of human somatropin (rhGH) in particular, the purification process represents the major manufacturing cost [1,2]. An optimized design is therefore desired in order to achieve the maximum recovery of the protein of interest, together with elimination of all critical contaminants from the final product. In the case of biosynthetic human growth hormone, secreted in *E. coli* periplasmic space, these include hGH related forms such as polymers, sulfoxides and desamido derivatives, bacterial endotoxins, contaminant host cell proteins (ECP), antibiotic, and bacterial DNA. For injectable somatropin intended for human use, regulatory guidelines require that the presence of these contaminants be maintained within the following limits: 6% for dimers and high-molecular mass forms, 13% for deamidated and methionine sulfoxide derivatives, 5 endotoxin units (EU) per milligram of product, 10 parts per million (ppm, w/w) for ECP, non-detectable levels of tetracycline and *E. coli* DNA [3–14]. Some of these

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limits can be modified by the competent national authorities, but in our opinion they will not be greatly different from these figures.

The purification of recombinant human growth hormone has been studied by several authors [6,15–22], but none of these reported yields of rhGH purification together with ECP elimination schemes based on multistep processes to reduce these contaminants to permissible limits.

Among the authors concerned with either yields or pharmaceutical purity, Fryklund et al. [6] and Flodh [19] reported a reduction of ECP of about 20-fold (from more than 200 ppm to less than 10 ppm) upon addition of an ion-exchange and a precipitation step to their original three-step purification scheme. These authors, however, did not mention the yield of rhGH in their processes. On the other hand, Becker et al. [15] report a yield of 71%, but only hGH electrophoretic purity was shown, nothing being mentioned about host cell-derived contaminants. The fact that only two chromatographic steps were used in their purification process would suggest that this particular product was not ready for human use. Niimi et al. [21], with the aim of further development of the product as a pharmaceutical agent, describe an efficient purification procedure for cytoplasmic methionyl-hGH based on three precipitations and three chromatographic steps. Even in this case, no mention was made of the level of removal of contaminant ECP, merely stating that these were removed step by step and showing only the electrophoretic purity of the final product. Lefort et al. [18], starting from a culture medium of transformed monkey kidney cells, report cumulative yields of rhGH of 28–48% depending on the use of either Octyl- or Phenyl-Sepharose in the first of their three chromatographic steps. Again the purity criteria were limited to classical electrophoretic studies. Other cited authors [16,17,20,22] fail to mention either yields, or ECP elimination.

The present work describes, for the first time, a complete, strictly controlled purification process that is capable of providing pure, pharmaceutical grade human growth hormone, while still maintaining high production yields. Great emphasis has been given to the reduction of ECP to within acceptable limits, this being, in our opinion and that of other authors [6,12,13,19,22], the most difficult task in the purification of recombinant proteins for human injection.

2. Materials and methods

2.1. Materials

The periplasmic fraction used as the starting material for hGH purification was prepared from *E. coli* K12, RRI strain harboring an expression vector, constructed in our laboratory, in which the hGH gene (cDNA) was under control of the λ P_L promoter [23,24]. The First International Standard for Somatropin (recombinant DNA-derived human growth hormone) coded 88/624, with a formally assigned specific activity of 3.0 IU/mg [25], was kindly provided by the National Institute for Biological Standards and Control (South Mimms, UK).

All chromatographic resins were from Pharmacia Biotech (São Paulo, Brazil), culture media from Difco (São Paulo, Brazil), ammonium bicarbonate, analytical grade, was from Riedel-de Haen (São Paulo, Brazil), ammonium sulfate, ammonium acetate, sodium chloride and sodium phosphate, all analytical grade, were from Merck (São Paulo, Brazil).

2.2. Feed batch fermentation of the transformed E. coli strain and osmotic shock

The equipment consisted of a 20 l Laboratory Bioreactor (New MBR, Zurich, Switzerland). The pH (7.2), temperature, aeration and foam level were automatically controlled while the agitation and dissolved oxygen tension (DOT) were set manually, ensuring a DOT of approximately 40% during the whole process. Transformed *E. coli* was grown under selective conditions (12.5 μg/ml of tetracycline) in a complex culture medium which was a two-fold concentrate of the HKSII medium described by Jensen et al. [26]. As carbon source the glucose feed started at the beginning of the process, and was continued for 5 h, with a feeding rate of 1.2 g l^{-1} h^{-1}. After about 5 h at 30°C, having reached an absorbance of about 5 A_{600} units, activation was carried out at 42°C for 6 h. *E. coli* osmotic shock and

direct hGH determination by RP-HPLC on the same periplasmic fluids were carried out as described [27].

2.3. Ammonium sulfate fractionation and first gel filtration chromatography

The total volume of the periplasmic extract, in 1 m*M* Tris–HCl, pH 7.0, was measured and an equal volume of a solution of saturated ammonium sulfate was slowly added under continous stirring at 4°C (50% saturation). The stirring was continued for 1 h after the addition was finished. The precipitate was collected by refrigerated centrifugation for 30 min at 6000 *g* and resuspended in 0.05 *M* ammonium hydrogencarbonate, pH 7.9, centrifuging again under the same conditions. The supernatant thus obtained was chromatographed at 4°C on a Sephacryl S-100 column (100 cm×5 cm I.D.) equilibrated and eluted with the same ammonium bicarbonate buffer, at a flow-rate of 200 ml/h.

2.4. DEAE-Sepharose fast flow (DEAE-FF) anion-exchange and second gel filtration chromatography

The Sephacryl S-100 eluted fractions containing the monomeric form of hGH (distribution coefficient, K_d~0.4) were pooled and applied to a DEAE-FF column (9 cm×2.6 cm I.D.) which had been equilibrated with 0.01 *M* ammonium acetate, pH 8.0, containing 0.05 *M* sodium chloride and washed with at least two bed volumes of this same buffer. The elution was performed with a linear gradient of sodium chloride up to 0.2 *M*, in about 2.5 h, at a flow-rate of 200 ml/h.

The hGH-containing fractions derived from DEAE-Sepharose were re-applied to the same, extensively washed Sephacryl S-100 column, repeating exactly the same steps described above.

2.5. Anion-exchange chromatography on Q-Sepharose fast flow (Q-FF) and hydrophobic interaction chromatography on Phenyl-Sepharose CL4B (Phenyl-CL4B)

The pool of gel-filtered rhGH was then applied to a Q-FF column (11 cm×1.6 cm I.D.) equilibrated in 0.01 *M* ammonium acetate buffer, pH 7.0, with 0.05 *M* sodium chloride. After washing with at least two bed volumes, elution was carried out under conditions of equilibrium with the same buffer, at a flow-rate of 120 ml/h. The pooled hGH-containing fractions were made 0.4 *M* in ammonium acetate, pH 7.0, and applied to a Phenyl-CL4B column (26 cm×2.6 cm I.D.) which had been equilibrated in 0.2 *M* ammonium acetate, pH 7.0. After washing with two bed volumes of 0.2 *M* and 0.05 *M* ammonium acetate buffer, hGH was eluted with 0.3 m*M* sodium phosphate, pH 7.0.

In all chromatographic purifications, the material to be pooled for the next step was chosen on the basis of the ECP immunoradiometric assay (IRMA) profile.

2.6. ECP determination

The detection and quantification of contaminant host cell proteins was carried out by a process-specific, ''sandwich'' format, IRMA, with a sensitivity of the order of 0.03 ng ECP/ml, developed in our laboratory according to the methodology described by Anicetti et al. [28]. Ninety-six well, U-shaped microtiter plates (Dynatech Chantilly, VA, USA) were used as the solid-phase, after having been coated with affinity purified anti-ECP IgG. Incubation times were 24 h at 4°C for standards, samples and controls and, after washing, 24 h at 4°C for ^{125}I-labelled anti-ECPIgG (2×10^5 cpm/well). After the last wash, the wells were cut and counted in a Cobra Auto-Gamma analyser (Packard Instruments, Downers Grove, IL, USA).

2.7. Protein determination

Total protein content was determined by three methods: the classical method of Lowry [29], spectrophotometric reading at 276 nm, utilizing 8.22 as specific absorbance ($E^{1\%}_{276}$) for hGH [25] and a physical method, based on weighing the dialysed and lyophilized samples on a precision balance [30].

2.8. High-performance liquid chromatography (HPLC)

High-performance size-exclusion chromatophaphy (HPSEC) and reversed-phase high-performance liquid chromatography (RP-HPLC), employed to evalu-

ate the quality and quantify rhGH in all different steps of the purification process, were carried out as previously described [27].

3. Results

An accurate quantitative analysis is very difficult to perform in the first two steps (osmotic shock and ammonium sulfate fractionation) of this purification process since, at this stage, only limited amounts of hGH are present, together with a great excess of periplasmic proteins. Five different and complementary analytical techniques were used to quantify proteins and hGH in these two steps: the Lowry method, spectrophotometric reading at 276 nm, dialysis-lyophilization and weighing (DLW), RP-HPLC and HPSEC. The first three techniques were used for total protein quantification and the last two for hGH determination, as shown in Table 1, where the data obtained after the first Sephacryl chromatography are also reported. In the first two steps, good agreement between the method of Lowry and DLW was found, the mean total protein estimate based on these two techniques being 5490±330 (SD) mg for osmotic shock and 1073±199 mg for the ammonium sulfate precipitate. Due to the extreme heterogeneity of the sample, the method based on hGH specific absorbance is obviously not accurate at this stage. However, after the first gel filtration, where we are dealing with a more homogeneous product (~80% hGH), the inter-method reproducibility, considering all techniques, was extremely good: 360±9.2 mg of hGH in a total of 463±13.4 mg of proteins, the coefficient of variation being below 3% in both cases.

Fig. 1 compares typical RP-HPLC and HPSEC profiles of an osmotic shock fluid derived from *E. coli* fermentation broth with those of the international standard. Very good resolution of the human growth hormone peak from all other less hydrophobic components can be observed in the chromatogram shown in Fig. 1A. This was illustrated in previous work by comparing the RP-HPLC chromatograms of osmotic shock fluids from the hGH-producing *E. coli* strain and from the same strain in which the hGH gene had been deleted [27]. Rec-hGH, quantified by this methodology, corresponded to 7.5% of the total periplasmic proteins. HPSEC was used in this step primarily as an identity test and for a semiquantitative evaluation (Fig. 1B).

After ammonium sulfate fractionation, hGH quantification can be done by either RP-HPLC or HPSEC (data not shown). Quantification by the latter technique led, however, to a certain overestimation, since the sample was still quite heterogenous at this stage.

Fig. 2 presents all the chromatographic steps of a typical rhGH purification process. In the same figure, the ECP profile and the associated fraction cut, always determined by IRMA before the next purification step, are also shown.

A good purification can already be observed on the first Sephacryl S-100 (I) column, where the hormone is resolved from at least two other components of higher molecular mass (Fig. 2A). Distribution coefficient (K_d) analysis showed a K_d for peak III that, according to previously pooled data [31], corresponds to the monomeric form of hGH.

Table 1
Protein and hGH quantification in the first three steps of the purification process

Step	Protein determination			hGH determination	
	Lowry (mg)	A_{276} (mg)	DLW[b] (mg)	RP-HPLC (mg)	HPSEC (mg)
Osmotic shock	5724	4330[a]	5257	412	–
$(NH_4)_2SO_4$ Precipitation	1213	1766	932	385	465
Sephacryl I	448	469	473	367	354

[a] Sample dialysed and centrifuged; determination in mg based on the specific absorbance proper of pure hGH ($A^{1\%}_{276}=8.22$).
[b] Dialysis, lyophilization and weighing.

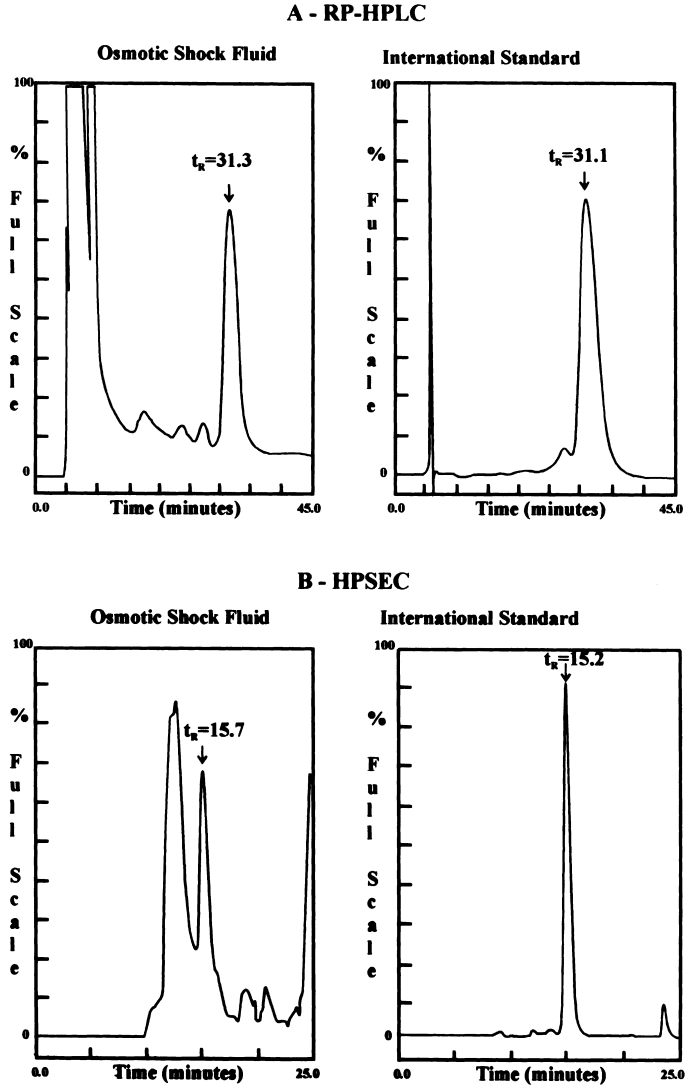

Fig. 1. HPLC analysis of an osmotic shock fluid obtained from an hGH-producing *E. coli* strain and of the First International Standard of rec hGH, coded 88/624. (A) Isocratic RP-HPLC: 50 μl of osmotic shock fluid and 20 μl (20 μg) of standard rhGH were applied to a C_4 Vydac 214 TP 54 column (25 cm×4.6 mm I.D., pore diameter of 300 Å and particle diameter of 5 μm), connected to a Vydac 214 FSK 54 guard column. The mobile phase consisted of 71% Tris–HCl buffer (50 m*M* pH 7.5) and 29% *n*-propanol, with a flow-rate of 0.5 ml/min and column temperature maintained at 45°C. (B) Isocratic HPSEC: 36 μl of osmotic shock fluid and 10 μl (10 μg) of standard rhGH were applied to a G2000 SW column (60 cm×7.5 mm I.D., particle size of 10 μm and pore size of 125 Å) connected to a 7.5 cm×7.5 mm I.D. SW guard column. The mobile phase was 0.025 *M* ammonium hydrogencarbonate, pH 7.0, with a flow-rate of 1.0 ml/min. t_R, Retention time.

This analysis was then confirmed by Sephacryl II chromatography, where a perfectly resolved, symmetric peak is observed (Fig. 2C). When peak III from Sephacryl I was analysed on HPSEC (Fig. 3), it appeared indeed to be mostly the monomeric form (87%), while only 8% was dimer, and about 4% aggregate. The other two peaks eluted from the same column, representing 57% of the total eluted pro-

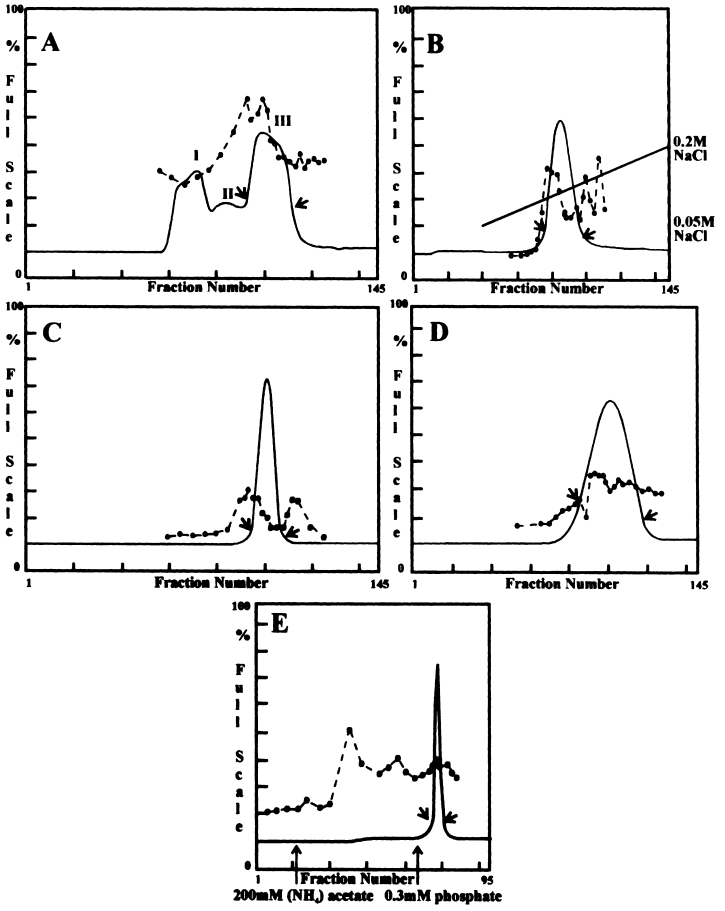

Fig. 2. Complete chromatogram sequence of a typical purification process of rhGH. (A) Sephacryl I (100 cm×5 cm I.D.); (B) DEAE-FF (9 cm×2.6 cm I.D.); (C) Sephacryl II (100 cm×5 cm I.D.); (D) Q-FF (11 cm×1.6 cm I.D.); (E) Phenyl-CL4B (26 cm×2.6 cm I.D.). —— A_{280}, ·– ––– · ECP-IRMA determination (cpm).

teins, revealed the presence of extremely small amounts of hGH (<7%) when analysed on HPSEC and RP-HPLC.

Table 2 collects the main parameters related to each purification step. Specific activities have been calculated considering all rhGH (fundamental + related forms) to be a percentage of the total protein content determined by the method of Lowry. The high yield obtained in the overall process and in each purification step is quite remarkable: not considering the peak cuts for maximum ECP elimination, the final yield in hGH was ~60%, each purification step showing an average recovery of more than 90%.

Concerning the strategy adopted of eliminating ECP as much as possible by sacrificing some product after each step (Table 3 and Fig. 2) (not considering the first chromatographic step, in which the ECP IRMA assay is extremaly inaccurate), with DEAE-Sepharose we could eliminate 39% of the interfering ECP. Cutting the edges of the Sephacryl II peak it was possible, with very limited loss of hGH, to eliminate 45% of ECP that would otherwise be present in the pool. The same procedure did not appear to be very efficient on Q-FF since only 14% of ECP could be eliminated in this way. However, this step presented a good purification factor and, without Q-FF, we never succeeded in obtaining an acceptable product (ECP<10 ppm) (data not shown). Hydrophobic interaction chromatography on Phenyl-Sepharose CL4B seems to be the most efficient

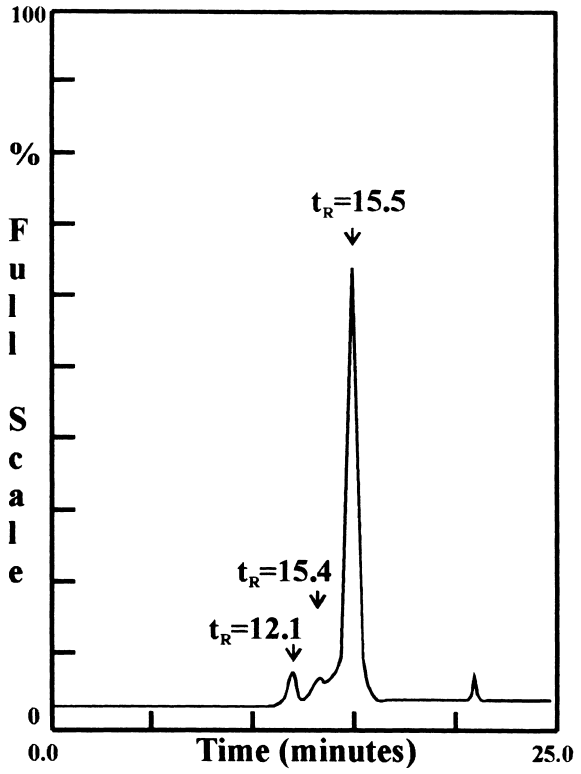

Fig. 3. Isocratic HPSEC on a G 2000 SW column of peak III obtained from Sephacryl I (5 μl;10 μg).

technique in this respect. However, the task of this last column is greatly facilitated by the good performance of all previous steps.

Regarding hGH-related forms (polymers, sulfoxide and desamido derivatives), after running HPSEC and RP-HPLC analysis, Fig. 4 shows the quality of the final lyophylized product and Table 4 the progress of the elimination of these contaminants. In this Table the hGH-related forms are reported as a percentage of total hGH (fundamental + related forms) determined by RP-HPLC or HPSEC. Sulfoxide and desamido derivatives are evidently also produced during the purification process, reflecting the effect of oxygen, temperature, light and high pH [32], and are by far the most difficult to eliminate (Fig. 5). No purification step was very efficient in eliminating these derivatives, though Q-FF seemed to be the most effective. To confirm this, a parallel study was run comparing the three chromatographic systems that, at least in principle, are capable of performing this purification. In all cases, an impure product with 15% altered forms was used and the amount of impurities remaining in the same fixed central fraction of the peak was determined, together with the amount of hGH (Table 5). The data confirmed that Q-FF is indeed the most efficient chromatographic step for desamido and sulfoxide elimination, indicating also that hydrophobic interaction chromatography has indeed little effect on them. Clearly, preventing their formation is the best way to obtain a product completely free of these forms.

4. Discussion

A purification process, concerned primarily with the recovery of the protein of interest and the elimination of contaminant host cell proteins, is described for rhGH secreted in bacterial periplasmic

Table 2
Purification of rhGH from periplasmic osmotic shock fluid

Step	Total protein (Lowry) (mg)	hGH content (HPSEC) (mg)	Specific activity (mg hGH/mg protein)	Yield (%)	Selected pools (%)
Shock	5724	412[a]	0.072	100	100
Precipitation	1213	385[a]	0.318	93.5	93.5
Sephacryl I	448	354	0.790	93.4	85.9
DEAE-FF	328	285	0.869	78.2	69.2
Sephacryl II	298	273	0.916	77.7	66.3
Q-FF	239	229	0.958	69.2	55.6
Phenyl-CL4B	173	177	1.022	60.5	43.0

[a] RP-HPLC determination.

Table 3
Contaminant *E. coli* proteins (ECP) in the selected pools of a typical rhGH purification

Type of column	Selected pool				Eliminated fraction		
	hGH (mg)	ECP (mg)	ECP (ppm)	Purification (- fold)	hGH (mg)	ECP (mg)	ECP (ppm)
Sephacryl I	354	44.9	126911		82	17.8	217574
DEAE-FF	285	12.4	43664	2.9	52	7.7	148856
Sephacryl II	273	1.99	7299	6.0	28	1.6	56564
Q-FF	229	0.11	480	15.0	27	0.018	681
Phenyl-CL4B	177	0.00013	0.71	676	—	—	—

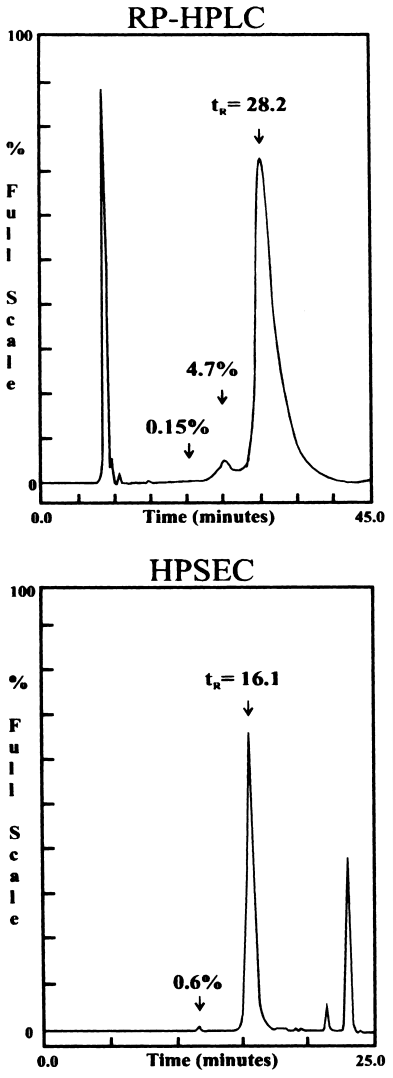

Fig. 4. Isocratic RP-HPLC (C_4 Vydac 214 TP 54) and HPSEC (G2000 SW) analysis of lyophilized rhGH for injection. The percentages of altered products are reported. Retention times for the International Standard of rhGH in RP-HPLC and HPSEC, were 27.4 and 16.2 min respectively.

space. Besides the high purity level, the process provided a final yield of more than 40% after six purification steps. Not only is this value among the highest reported for rec hGH purification from either bacteria or mammalian cells [15,18,21], but, as far as we know, no report has ever described a strategy for ECP elimination. Niimi et al. [21] described a six-step purification process for cytoplasmic methionyl-hGH also directed toward ECP elimination; their recovery of hGH was, however, only 19%. The present results were obtained by careful optimization of each step with respect to hGH recovery and ECP elimination and by adopting the practical strategy of alternating gel filtration and ion-exchange chromatography, with an efficient hydrophobic interaction chromatography as the last step [33].

The utilization of two HPLC methodologies, one of which is capable of determining hGH in crude extracts [27], has allowed us to follow, efficiently and with great accuracy, all partial recoveries. This methodology also facilitated the detection and elimination of hGH-related impurities such as polymeric, deamidated and sulfoxide derivatives. Emphasis has

Table 4
hGH-related forms determined by HPSEC and RP-HPLC after each step of the rhGH purification process

Step	hGH-related forms	
	HPSEC (%)	RP-HPLC (%)
Osmotic shock	52.4	11.1
Precipitation	30.2	8.3
Sephacryl I	11.9	5.2
DEAE-FF	7.7	4.9
Sephacryl II	1.6	10.4
Q-FF	0.58	6.9
Phenyl-CL4B	0.56	4.9

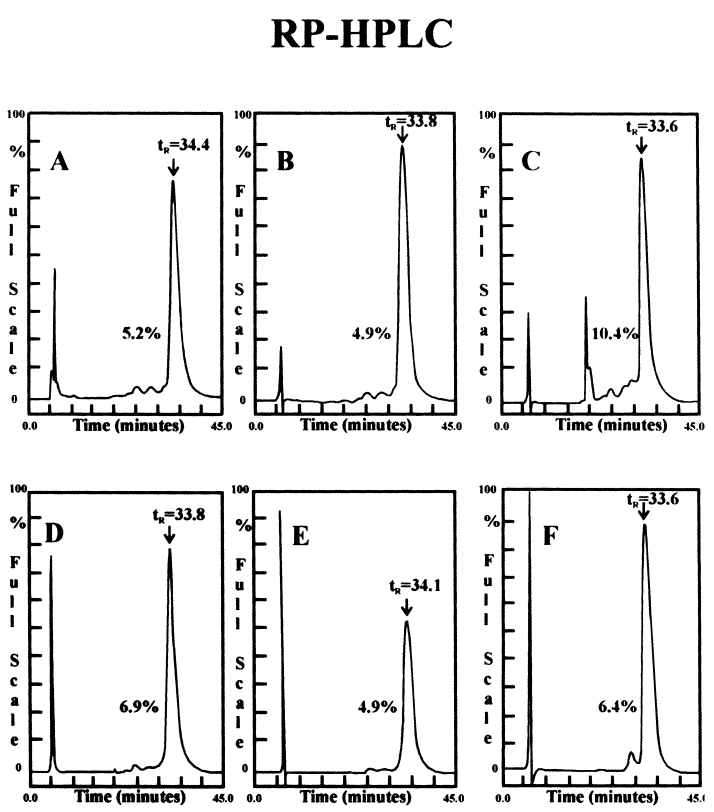

Fig. 5. Isocratic RP-HPLC on a C_4 Vydac 214 TP 54 of the products of each step of a typical purification process of rhGH. (A) Sephacryl I; (B) DEAE-FF; (C) Sephacryl II; (D) Q-FF; (E) Phenyl-CL4B; (F) First International Standard of rhGH. The percentages of altered products are reported.

also been given to accurate hGH and protein quantification in the early steps of the process, a stage in which these determinations are particularly critical and inaccurate. We emphasize that it is practically impossible to obtain reliable data on total rhGH recovery or provide real purification factors when total protein and hGH content in the starting material are not accurately known. This concept has been stressed by other authors, especially regarding immunoassay, a technique that can be highly misleading when used to accurately quantitate a protein in a complex matrix [34]. We have found, for example, that determination of the protein of interest in osmotic shock fluids, by radioimmunoassay, frequently leads to great dilution effects and consequent overestimation.

Finally, where possible, we employed fast flow resins and chromatographies to shorten the duration

Table 5
Elimination of sulfoxide and desamido derivatives by different chromatographic procedures

Column	Altered forms[a] (%)	Total hGH recovery (%)	Central fraction recovery (%)
DEAE-FF	8.9	69	35
Q-FF	8.5	75	56
Phenyl-CL4B	12.7	81	34

[a] Before purification, the product contained 15% altered forms.

of the whole process, that, excluding fermentation and lyophilization, never exceeded five days. Besides the economic advantage of using conventional, relatively unsophisticated methodologies, our experience confirms, as emphasized in a recent review on this topic [35], that yields of high quality recombinant proteins are also very much related to the speed of the purification process.

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