ELSEVIER

Journal of Chromatography A, 782 (1997) 199–210

JOURNAL OF CHROMATOGRAPHY A

Analysis of recombinant human growth hormone directly in osmotic shock fluids

Sergio Dalmora[b], João Ezequiel de Oliveira[a], Regina Affonso[a], Elizabeth Gimbo[a], Maria Teresa C.P. Ribela[a], Paolo Bartolini[a,*]

[a]*Department of Application of Nuclear Techniques in Biological Sciences, IPEN-CNEN, São Paulo, Brazil*
[b]*Department of Industrial Pharmacy, Federal University of Santa Maria, Rio Grande do Sul, Brazil*

Received 3 February 1997; received in revised form 28 April 1997; accepted 29 April 1997

Abstract

An isocratic reversed-phase high-performance liquid chromatography (RP-HPLC) method for the determination of human growth hormone (hGH) directly in osmotic shock fluids is described. This methodology allows an initial rapid evaluation of the quality and quantity of hGH being secreted in the bacterial periplasmic space right after, or even during fermentation. Considering that RP-HPLC does not identify size isomers, these were determined via a parallel run of the same osmotic shock fluid on high-performance size-exclusion chromatography, coupled with radioimmunoassay, of the eluted fractions. The methodology provides a complete picture, within 24 h from the beginning of the fermentation process, of the recombinant protein being produced with respect to its activity, identity, yield, and hGH-related contaminants. These latter include sulfoxide and desamido derivatives, dimer and high-molecular-mass forms. © 1997 Elsevier Science B.V.

Keywords: Osmotic shock fluids; Growth hormones; Hormones; Proteins

1. Introduction

Recombinant DNA-derived human growth hormone (rhGH), when expressed in genetically modified bacteria, can be directly stored as insoluble cytoplasmic inclusion bodies [1,2] or secreted in the periplasmic space thanks to the introduction of a suitable leader sequence [3–6] in the constructed expression vector. The recombinant proteins obtained via this last type of construct and extracted from osmotic shock fluids do not have an extra N-terminal methionine, are properly processed and folded, do not need solubilization and renaturation and, consequently, are practically identical to the natural ('authentic') form [3,4,7–9]. Osmotic shock fluids obtained right after, or even during, the bacterial fermentation process are therefore an ideal medium for a reliable, early evaluation of product yield and quality, prior to purification.

Several physico-chemical analytical techniques for the quantitative and qualitative estimation of hGH have been described, such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) frequently combined with immunoblotting [2,4,5,7,10–12], isoelectric focusing [12], capillary electrophoresis [13,14] size-exclusion [15], reversed-phase [16,17] and hydrophobic-interaction [12,18] high-performance liquid chromatography (HPLC). However, with the exception of denaturing SDS-

*Corresponding author. IPEN-CNEN, Travessa R 400, Cidade Universitária, 05508-900 São Paulo, Brazil.

PII S0021-9673(97)00493-7

PAGE and immunoblotting, most of these techniques are applied to purified or semi-purified hGH lots, in order to quantify the unmodified hormone together with the so-called GH-related forms. There have been very few reports of the determination of hGH, or indeed of other recombinant proteins, in fermentation broth matrices. Folena-Wasserman et al. [19] developed an automated RP-HPLC assay based on a linear gradient of acetonitrile containing 0.05% trifluoroacetic acid, to measure the concentration of a recombinant malaria circumsporozoite protein during both fermentation and purification, with the objective of reliably evaluating product yield prior to purification. Hummel et al. [20] purified IGF-I and IGF-II, obtained as fusion proteins from the insoluble fraction of crude bacterial lysate, using a rapid one-step HPLC separation technique based on a C_8 reversed-phase column and a gradient of 2-propanol in formic acid. Strege and Lagu [21] described a method for the isocratic RP-HPLC determination of recombinant methionyl–aspartyl–human growth hormone in *E. coli* fermentation broth via sulfitolysis solubilization employing a mobile phase containing *n*-propanol and SDS under micellar conditions. All these methods are based on labor-intensive sample manipulation and the use of harsh conditions, necessary because the target molecules are present inside the organism as inclusion bodies in an insoluble, denatured state. As far as we are aware, in the case of recombinant proteins secreted in soluble form in bacterial periplasmic space, only one analytical method has been described. Thus, very recently, McNerney et al. [22] described a free-zone capillary electrophoresis (FZCE) method for the direct monitoring and quality control of hGH in *E. coli* cell pellet extracts that can resolve rhGH from the following variants: two-chain forms, hGH lacking the first or first two N-terminal amino acids (desPhehGH and desPheProhGH) and deamidated and dideamidated hGH.

The purpose of the present study is therefore to apply and validate an isocratic RP-HPLC technique for the direct determination of the amount of hGH being secreted in bacterial shock fluids, together with the percentage of undesirable hGH-related forms, like sulfoxide and desamido-derivatives. Concerning the determination of aggregates and dimeric forms of hGH in the same osmotic shock fluids, a study was carried out applying high-performance size-exclusion chromatographic (HPSEC) determination to RP-HPLC eluted samples. Although several sets of experimental conditions were tried, this approach failed to provide satisfactory results, probably because most of the hGH oligomers and aggregates are dissociated by the organic modifier during RP-HPLC [23]. Good results were obtained instead by performing a rapid immunoassay determination on the HPSEC-eluted fractions of the same periplasmic extract, thus providing an early estimate of the amount of dimer and high-molecular-mass (HMM) forms. On the basis of this evaluation it may be possible to adjust and correct the fermentation conditions, avoiding an unnecessary, time-consuming and expensive purification process and, more importantly, permitting investigation of the causes and moment at which alterations occur.

2. Materials and methods

2.1. Chemicals and reagents

Water was obtained from a Millipore 'Milli-Q plus' water purification system (Bedford, MA, USA). Acetonitrile and *n*-propanol (HPLC-grade) were from Grupo Química (Rio de Janeiro, Brazil). All other chemicals were analytical reagent grade. Recombinant clinical-grade hGH lots, the secondary standard of rhGH (BRP-3) with a specific activity of 2.5 IU/mg and purified rhGH for radioiodination were prepared in our laboratory [24], while 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). Pituitary human growth hormone reference preparation from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK-hGH-RP-1) and antiserum (NIDDK-anti-hGH-2) were kindly provided by the National Hormone and Pituitary Program (Baltimore, MD, USA). $Na^{125}I$ free of carriers and reductant, was purchased from Nordion Europe (Fleurus, Belgium) with a specific activity of 300–600 mCi/ml (11 100–22 200 MBq/ml) and Sephadex G-100 from Pharmacia Biotech (Uppsala, Sweden). Human blood-

based Immunoassay Quality Controls (Dade Trilevel) were purchased from Baxter Diagnostic (Deerfield, IL, USA).

2.2. Feed batch fermentation of transformed E. coli strains

The equipment consisted of a 20-l Laboratory Bioreactor (MBR, Zurich, Switzerland). The pH, temperature, aeration and foam level were automatically controlled while the agitation and dissolved oxygen tension (DOT) were set up 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 and Carlsen [26]. As carbon source the glucose feed started at the beginning of the process, going on for 5 h with a feeding rate of 1.2 $g/l \cdot h^{-1}$. After 5 h at 30°C, having reached an absorbance (OD) of 6–7 A_{600} units, activation was carried out at 42°C for 6–7 h.

2.3. Osmotic shock

Periplasmic-osmotic shock fluid from hGH-producing or -non-producing transformed *E. coli* strains was obtained by the method of Koshland and Botstein [27]. Briefly, on the microscale, 1.5 ml fermentation broth with 1.0 A_{600} unit was centrifuged at 16 000 *g* for 2 min and the pellet was put on ice. All subsequent steps were carried out at 4°C. Pellets were resuspended in 0.15 ml of ice-cold 10 m*M* Tris–HCl, pH 7.5, containing 20% (w/v) sucrose. Then 5 μl of 0.5 *M* EDTA, pH 8.0, were added and incubation on ice was continued for 10 min. The cells were then microcentrifuged and the pellet rapidly resuspended by vigorous agitation in 0.1 ml of cold 1 m*M* Tris–HCl, pH 7.5, solution. The mixture was incubated for 10 min on ice and then centrifuged again for 5 min. The supernatant was removed and saved as the periplasmic fraction.

2.4. RP-HPLC

An ISCO Model 2350 isocractic HPLC apparatus, coupled to a V4 variable wavelength absorbance detector (ISCO, Lincoln, NE, USA) was used, employing the 'Chem Research 150 Chromatographic Data Management/System Controller' software, also from ISCO. The column was a C_4 Vydac 214 TP 54 (25 cm×4.6 mm I.D., pore diameter of 300 Å and particle diameter of 5 μm) with a guard column (Vydac 214 FSK 54) between the sample injector and the main column and a silica precolumn packed with LiChrosorb Si 60, 7.9–12.4 μm (Merck, Darmstadt, Germany) located between the pump and the injector. All Vydac columns were purchased from The Separation Group (Hesperia, CA, USA). The mobile phase consisted of 71% Tris–HCl buffer (50 m*M*, pH 7.5) and 29% *n*-propanol, as described by Riggin et al. [16], with a flow-rate of 0.5 ml/min, detector wavelength at 220 nm, column temperature maintained at 45°C and a sample volume of 40–70 μl. The guard column lifetime was approximately 100 injections, while the main column lifetime was approximately 200 injections, most of the samples applied (~80%) being osmotic shock fluids.

2.5. HPSEC

The same ISCO system described above was used. Columns were either G2000SW or G3000SW (60 cm×7.5 mm I.D., particle size of 10 μm and pore sizes of 125 or 250 Å, respectively) with a 7.5 cm×7.5 mm I.D. SW guard column. These columns were purchased from TosoHaas (Montgomeryville, PA, USA). The mobile phase was 0.025 *M* ammonium bicarbonate, pH 7.0, with a flow-rate of 1.0 ml/min and detection at 220 nm as described [15]. The sample volume was 10–100 μl. Guard columns could be used for approximately 150 runs, while the main columns were used for approximately 400 runs. Washing the column with 0.5 *M* Na_2SO_4, pH 2.7, extended column lifetime to 50–100 additional runs.

The primary and secondary standards of rhGH were routinely used to check column performance for both RP-HPLC and HPSEC.

2.6. Radioiodination

The ^{125}I-labelling of rhGH was carried out using a modification of the original Chloramine T technique, as described in previous work [24], employing 0.8–1.0 mCi (29.6–37.0 MBq) of radioisotope, 5 μg of

hormone and 0.8 μg of chloramine T. In some experiments, rhGH obtained in this laboratory was purified by HPSEC on a G2000SW column and only the central fraction of the peak used for radioiodination in order to obtain a tracer completely free of dimer and HMM forms.

2.7. Radioimmunoassay

Radioimmunoassays (RIAs) were carried out with simultaneous addition of all reagents: tracer (25 000 cpm), reference preparation or unknown samples and first antibody (1/300 000 final dilution). Incubation was carried out for 2 h at 4°C in 0.01 *M* phosphate buffer, pH 8.6, with 0.1% bovine serum albumin (BSA) using a liquid-phase second-antibody technique, with 1.5-h incubation at room temperature for the separation of the antibody-bound hormone. In each assay, commercial preparations of low-, intermediate- and high-level quality controls were used.

3. Results

As shown in Fig. 1, the RP-HPLC chromatogram of an osmotic shock fluid obtained from a bacterial strain transformed with an expression vector in which only the hGH gene had been deleted, exhibited a large amount of *E. coli* proteins, all eluting with low retention time and practically no components with $t_R > 15$ min. Under the same conditions, when the expression vector carries the hGH gene, a clean peak of the hormone appears with a t_R of approximately 31 min. This is confirmed by the hGH RIA profile of the eluted fractions, showing the absence of cross-reactions between hGH and *E. coli* proteins in both chromatograms. These data indicate that RP-HPLC can be used to determine the hGH content directly in bacterial extracts before purification, or even during the fermentation process. Together with the determination of the hGH content it is also possible to estimate the amount of deamidated and sulfoxide derivatives present at a given time, as can be appreciated from the two small immunoreactive peaks preceding the main hGH peak in Fig. 1B, one of which presents the same relative retention time (0.92) as the small altered component present in the International Standard of Somatropin (Fig. 2). The accuracy of hGH determination was confirmed by RP-HPLC analysis of known amounts of hGH in the presence of *E. coli* proteins obtained from osmotic shock fluids of the same strain analysed in Fig. 1A. Recoveries were always above 94%, while the percentage of altered forms did not increase in comparison with the amount (4.3%) present in the original preparation of rhGH used for the test (data not shown). The correlation between added and recovered hGH was highly significant, while practically no bias was introduced: $y_{recov} = 1.005x_{added} - 0.175$ ($r = 0.9997$; $P < 0.001$ for $n = 8$). We should also observe that samples were constructed using either variable amounts (50–90%, v/v) or a fixed amount (50%) of the same shock fluid.

In order to have an immediate indication of the amount of dimer and HMM forms as well, we tried to analyse an aliquot of the hGH peak eluted from RP-HPLC by HPSEC. This study was carried out by using a local standard of rhGH (rhGH-BRP-3), in which the percentage of each of these forms was known. Unfortunately, this determination turned out to be impossible since these oligomers and polymers are apparently partly or totally dissociated to monomeric hGH under RP-HPLC conditions. This is confirmed by the experiment presented in Fig. 3, where an oligomeric variant of hGH, obtained as a side product of the first purification step (size-exclusion chromatography on Sephadex G-100) of bacterially derived hGH, is analysed on HPSEC (Fig. 3A). After running a second sample of the same side product on RP-HPLC, thus confirming that it was mostly hGH, an aliquot of the RP-HPLC-eluted peak was run on HPSEC. As shown in Fig. 3B, at this point practically only the monomeric form of hGH ($t_R = 13.93$ min) is present. In this particular case the oligomeric form had been identified as a trimer on the basis of its relative retention time (0.82 ± 0.01), determined on rhGH-BRP-3, as compared to the retention time of the monomeric form, determined with the International Standard in the same experiment, of 13.83 min.

In the light of these data we decided to employ a rapid RIA scanning of HPSEC-eluted fractions to detect the possible presence of dimer or HMM forms in osmotic shock fluids. In initial tests carried out using the International Standard of Somatropin, shown in Fig. 4, the dimer and HMM forms were

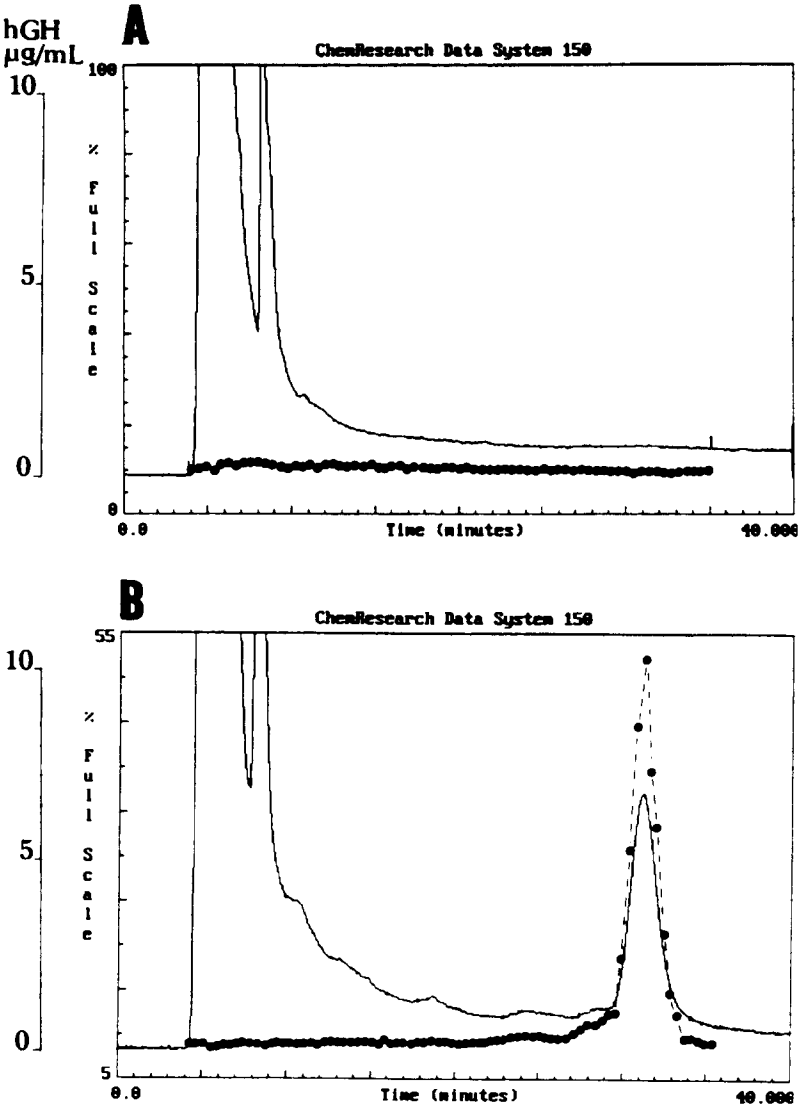

Fig. 1. Isocratic RP-HPLC on a C_4 Vydac column of osmotic shock fluids from two different transformed *E. coli* strains. (A) Strain containing the expression vector in which the hGH gene has been deleted. (B) Same strain, transformed with hGH expression vector. $A_{220\ nm}$ (——); hGH activity (– · –) determined by RIA, using NIDDK-hGH-RP-1 for the standard curve.

found to be immunoreactive, even though the larger aggregates present, as expected, a lower activity. Statistical analysis of RIA determinations ($n=6$) on this same standard provided the following data: monomer 98.1%; dimer 1.35%; trimer 0.22%; and aggregate 0.26%; a result that compares well with the average results of the International Collaborative Study [25]: 97.1% monomer; 1.93% dimer; and 0.87% HMM. In order to quantify better the different immunological activities of hGH oligomeric and polymeric forms, this type of determination was repeated on the local standard of rhGH, which contains these altered forms in greater amounts than the International Standard. The trimer was also determined in this study since it is resolved on these long columns (60×0.75 cm G2000SW and G3000SW). The results, presented in Table 1, show a good agreement between RIA and HPSEC de-

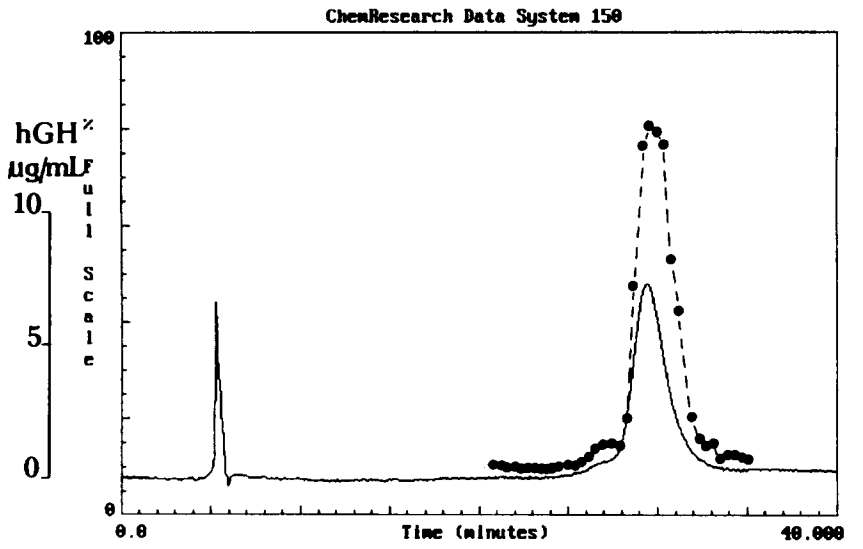

Fig. 2. Isocratic RP-HPLC on a C_4 Vydac column of the First Interrnational Standard for Somatropin (WHO 88/624), 20 µg. $A_{220\ nm}$ (——); hGH activity (– · –) determined by RIA, against NIDDK-hGH-RP-1.

termination (HPLC/RIA close to 1) for the monomer and dimer. In the case of trimer and aggregates, increasingly larger HPLC/RIA ratios were obtained, indicating that these components may present 2–7 times less immunoactivity then the monomer. A cumulative HPLC/RIA=5.1 for HMM forms (trimer+aggregate) was found. Furthermore, as illustrated by this experiment, even if the relative standard deviation (R.S.D.) is quite high for the determination of oligomers and polymers (especially in the case of RIA), there is good agreement between HPLC and RIA total sample quantification, with a bias generally inferior to 5%. This point must be emphasized, especially since two different standards were used: WHO 88/624 for HPLC and NIDDK-RP1 for the RIA.

These results show that it is possible to determine with sufficient reliability the amount of dimer and HMM forms present in different osmotic shock fluids by running a RIA on HPSEC-eluted fractions. In Fig. 5 we show an example of periplasmic extract in which the hGH peak is only partly resolved; without RIA, it would be impossible to estimate hGH correctly or, obviously, to infer the existence of oligomeric and polymeric forms. In Table 2 the results of this type of determination carried out on eight different osmotic shock fluids are reported. Calculations can be simplified by considering the trimer together with the aggregates, thus obtaining, after correction, for the osmotic shocks reported in Table 2, 3–17% HMM together with 2–5% dimeric forms.

We have shown that RP-HPLC can be used to calculate the amount of rhGH and desamido/sulfoxide derivatives present in osmotic shock fluids and that HPSEC, coupled to RIA determination on the eluted fractions, can give an estimate of the amount of HMM forms and dimer. In Fig. 6 and Table 3, an example is given of how the RP-HPLC methodology can be applied to follow as closely as possible the fermentation and activation process. Under our conditions, 6 h seems to be the best activation time, combining the highest hGH secretion with a low amount (6.6%) of altered derivatives. HPSEC–RIA analysis of this same osmotic shock fluid also confirmed the presence of minimal amounts of dimer and HMM forms (about 7% after correction).

4. Discussion

The methodology described here allows a rapid and accurate determination of hGH prior to purification, as well as an estimation of most GH-related

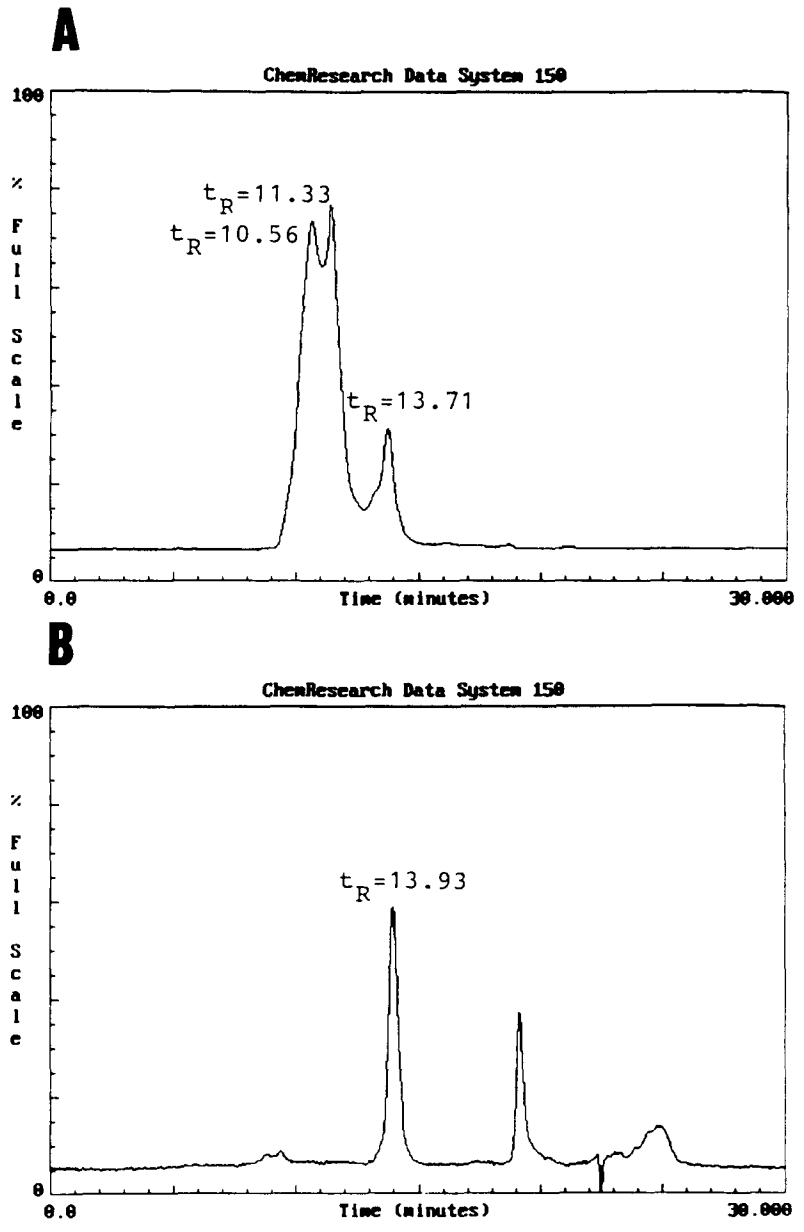

Fig. 3. Isocratic HPSEC on a G3000SW column of a side product from Sephadex G-100, whose elution volume corresponds to the region of hGH oligomers. (A) Direct sample analysis on HPSEC. (B) HPSEC analysis of the hGH peak eluted from RP-HPLC. t_R, retention time (min).

derivatives, secreted in *Escherichia coli* periplasmic space. The isocratic micellar RP-HPLC technique described by Strege and Lagu [21], also based on a C_4 packing, was in fact applied to methionyl–aspartyl–hGH-insoluble inclusion bodies. Besides being more laborious and requiring a long sulfitolysis reaction, unnecessary in our case, their method cannot determine most of the GH-related forms, which are all converted into a single molecular entity by SDS. The same can be said concerning the

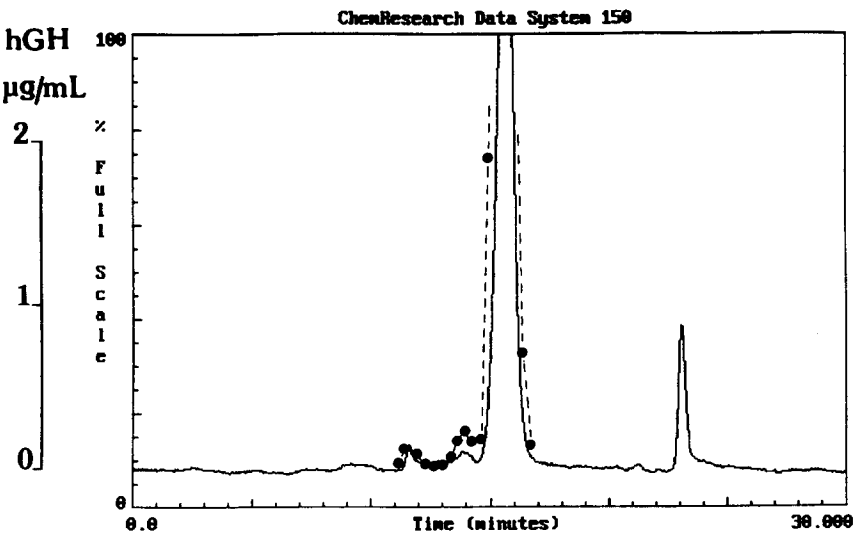

Fig. 4. Isocratic HPSEC on a G2000SW column of the First International Standard for Somatropin (WHO 88/624), 5 µg. $A_{220\ nm}$ (——); hGH activity (– · –) determined by RIA against NIDDK-hGH-RP-1.

determination of other recombinant proteins in fermentation broth [19,20]. The previously mentioned FZCE approach recently described by McNerney et al. [22] for rhGH determination in sonicated *E. coli* extracts, also appears to be a very powerful tool for this type of analysis and, as stated by the authors, it should be applicable to osmotic-shocked cells. This method, however, does not determine sulfoxide, dimer or HMM forms, and has the disadvantage of requiring capillary flushing after each run in order to maintain a reproducible hGH migration time.

The present RP-HPLC determination of hGH in osmotic shock fluid can be carried out in about 30 min, at any moment during or after the fermentation process, also providing an estimate of the amount of desamido and sulfoxide derivatives. This is extremely important for setting up good fermentation and purification conditions, since a preparation with a low secretion yield will be very difficult to purify, especially from host cell-derived proteins. On the other hand, high amounts of desamido/sulfoxide derivatives are also highly undesirable, since these components cannot easily be separated from the unaltered form of the hormone due to the similarity of their physico-chemical properties. We have shown in this respect that, by carefully controlling the

Table 1
Analysis by HPSEC–RIA of the secondary standard of rhGH

Assay no.	Monomer		Dimer		Trimer		Aggregates		Total	
	RIA (µg)	HPLC (µg)	RIA (µg)	HPLC (µg)	RIA (µg)	HPLC (µg)	RIA (µg)	HPLC (µg)	RIA (µg)	HPLC (µg)
1	23.0	21.0	1.30	1.75	0.06	0.26	0.051	0.35	24.4	23.4
2	23.3	22.3	3.08	2.80	0.20	0.50	0.092	0.59	26.7	26.2
3	23.8	22.8	2.02	1.88	0.11	0.23	0.050	0.46	26.0	25.4
4	23.3	22.5	2.85	2.45	0.11	0.32	0.051	0.34	26.3	25.6
5	24.2	22.0	1.63	2.04	0.10	0.20	0.047	0.39	26.0	24.6
Average	23.5	22.12	2.18	2.18	0.12	0.30	0.058	0.43	25.9	25.0
±R.S.D. (%)	2.0	3.1	35.2	20.0	42.7	39.7	32.7	24.0	3.4	4.3
% of Total	90.7	88.5	8.4	8.7	0.46	1.2	0.23	1.72	100	100
HPLC/RIA	0.94		1.0		2.5		7.4			

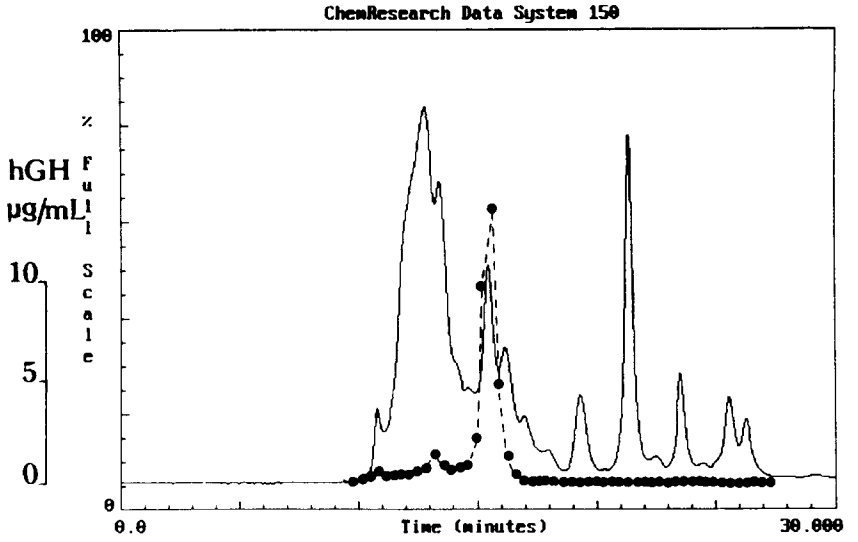

Fig. 5. Isocratic HPSEC on a G2000SW column of an osmotic shock fluid obtained from an hGH-secreting *E. coli* strain. $A_{220\ nm}$ (——); hGH activity (– · –) determined by RIA against NIDDK-hGH-RP-1.

activation time, these derivatives can be reduced to less than 7% (see Table 3). This is relevant if we consider that the latest monograph of the European Pharmacopeia on Somatropin established a limit of acceptability for these forms of 10% for bulk material and of 13% for the injectable product [28]. In addition, this methodology applied to osmotic shock fluids may also allow the simultaneous determination of *E. coli* proteins present in the extract by analysing the same chromatogram at a lower amplification (see Fig. 1) in order to quantify the sum of peaks eluting at $t_R < 15$ min, which are perfectly resolved from hGH.

We were not able to detect accurately dimer and HMM forms by sequential RP-HPLC–HPSEC confirming previous observations of partial or total dissociation of oligomers and polymers under these RP-HPLC conditions [15,29]. This approach can, however, be utilized preparatively to obtain rapidly small amounts of relatively pure product, useful for different applications such as antigen or tracer preparation. The immunochromatographic detection of these forms, combining HPSEC with RIA, was successful and the whole analysis can be carried out in about 4 h. Thus, if necessary, the entire set of results, including the two chromatographic analyses and RIA, can be obtained on the same day that fermentation starts. Dimer and HMM forms can be

Table 2
HPSEC–RIA analysis of osmotic shock fluids

Shock no.	HMM (%)	Dimer (%)	Monomer (%)	Fermentation yield (µg hGH/ml/A_{600})
F-065	2.40	2.5	95.1	1.73
F-066	2.10	3.1	94.8	3.11
F-077	0.63	2.3	97.1	1.64
F-079	3.90	3.1	93.0	0.64
F-082	3.10	4.6	92.3	1.55
F-088	0.68	3.2	96.1	0.77
F-089	0.82	3.0	96.2	0.69
F-100	1.50	2.3	96.2	1.13

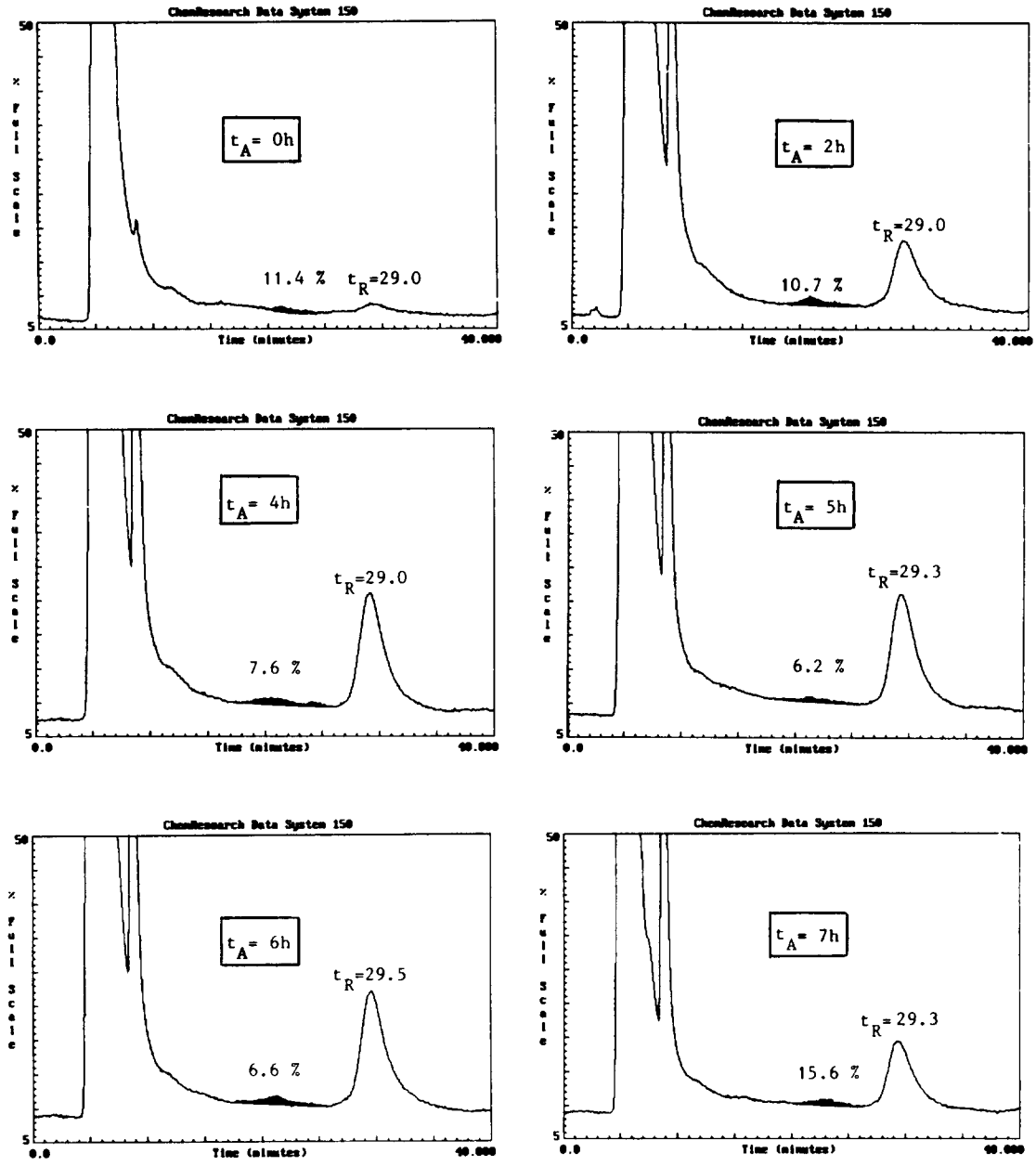

Fig. 6. Isocratic RP-HPLC of different osmotic shock fluids obtained during fermentation, at different activation times. t_R, retention time (min); t_A, activation time (h). Darkened areas indicate the amount of desamido/sulfoxide derivatives.

more readily eliminated than desamido/sulfoxide derivatives, especially considering the usual presence of a gel filtration step in any purification process. However, these polymers can form very easily, especially during lyophilization and, since their limit of acceptability is quite low (4% for bulk material and 6% for the injectable product), it is very important to control their presence and formation

Table 3
Fermentation analysis: choice of ideal activation time

Activation time (h)	RP-HPLC			HPSEC–RIA		
	t_R (min)	Yield (μg hGH/ml/A_{600})	Desamido/sulfoxide (%)	t_R (min)	Dimer (%)	HMM (%)
0	29.00	0.06	11.4	14.38	6.1	1.9
2	29.00	0.47	10.7	14.27	9.0	2.6
4	29.00	0.76	7.6	14.00	4.9	2.3
5	29.30	0.72	6.2	13.93	5.3	1.4
6	29.50	0.84	6.6	14.77	3.0	0.8
7	29.30	0.45	15.6	14.50	3.6	1.1

from the outset. Surprisingly enough, we have found that the amount of these forms is relatively low in bacterial shock fluids, suggesting that, under our conditions, bacteria do not seem to secrete critical amounts of aggregates of rhGH resulting from the incorrect processing of disufide bonds, at least in soluble form [22]. In our case, we believe that such polymers probably arise during the initial manipulations.

Finally, it is interesting to observe that the dimeric form of rhGH consistently presented the same immunological activity as the monomer, confirming to a certain extent the data obtained by Becker et al. [30] in a polyclonal RIA system. This is quite different from the reported bioactivity of GH dimer (human or bovine) which, according to literature data, ranges from 0 to 60% of the full activity of the monomer [15,30–33]. On the other hand, aggregates of rhGH (we did not find any data for the trimer) always presented a very low immunoactivity in the present work, a result that is consistent with literature data [32,34]. As far as we are aware, this is the first time that the immunological activity of this HMM form of hGH has been determined.

Acknowledgments

This work was supported by FAPESP, São Paulo (Project N. 92/0052-2), CNPq-RHAE, Brasília and the International Atomic Energy Agency (IAEA), Vienna, Austria (Technical Cooperation Project BRA 6/012).

References

[1] D.V. Goeddel, H.L. Heyneker, T. Hozumi, R. Arntzen, K. Itakura, D.G. Yansure, M.J. Ross, G. Miozzari, R. Crea, P.H. Seeburg, Nature 281 (1979) 544–548.
[2] M. Ikehara, E. Ohtsuka, T. Tokunaga, Y. Taniyame, S. Iwai, K. Kitano, S. Miyamoto, T. Ohgi, Y. Sakuragawa, K. Fujiyama, T. Ikari, M. Kobayashi, T. Miyake, S. Shibahara, A. Ono, A. Sakurai, T. Oishi, O. Chisaka, K. Matsubara, Proc. Natl. Acad. Sci. USA 81 (1984) 5956–5960.
[3] G.L. Gray, J.S. Baldridge, K.S. Mc Keown, H.L. Heyneker, C.N. Chang, Gene 39 (1985) 247–254.
[4] H.M. Hsiung, N.G. Mayne, G.W. Becker, Bio/Technology 4 (1986) 991–995.
[5] C.N. Chang, M. Rey, B. Bochner, H. Heyneker, G. Gray, Gene 55 (1987) 189–196.
[6] P. Bartolini, L.E. Morganti, Y. Murata, M.T.C.P. Ribela, I. Schwarz, M.H. Bellini, C.R.J. Soares, in IAEA Staff (Eds.), Developments in Radioimmunoassay and Related Procedures, IAEA, Vienna, 1992, pp. 197–204.
[7] G.W. Becker, H.M. Hsiung, FEBS Lett. 204 (1986) 145–150.
[8] P. Riggs, Current protocols in molecular biology, in: F.N. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidan, J.A. Smith, K. Struhl (Eds.), Greene Associates, Wiley-Interscience, New York, 1990, pp. 16.6.1–16.6.14.
[9] C. Lauritzen, E. Tuchsen, P.E. Hansen, O. Skovgaard, Protein Expression Purif. 2 (1991) 372–378.
[10] S. Lefort, P. Ferrara, J. Chromatogr. 361 (1986) 209–216.
[11] D.R. Bangham, R.E. Gaines Das, D. Schulster, Mol. Cell. Endocrinol. 42 (1985) 269–282.
[12] P. Gellerfors, G. Eketorp, K. Fhölenhag, B. Pavlu, S. Johansson, L. Fryklund, J. Pharm. Biomed. Anal. 7 (1989) 173–183.
[13] C. Arcelloni, I. Fermo, G. Banfi, A.E. Pontiroli, R. Paroni, Anal. Biochem. 212 (1993) 160–167.
[14] P. Dupin, F. Galinou, A. Bayol, J. Chromatogr. A 707 (1995) 396–400.
[15] R.M. Riggin, C.J. Shaar, G.K. Dorulla, D.S. Lefeber, D.J. Miner, J. Chromatogr. 435 (1988) 307–318.
[16] R.M. Riggin, G.K. Dorulla, D.J. Miner, Anal. Biochem. 167 (1987) 199–209.

[17] G. Teshima, E. Canova-Davis, J. Chromatogr. 625 (1992) 207–215.
[18] S. Wu, W.S. Hancock, B. Pavlu, P. Gellerfors, J. Chromatogr. 500 (1990) 595–606.
[19] G. Folena-Wasserman, R. Inacker, J. Rosenbloom, J. Chromatogr. 411 (1987) 345–354.
[20] M. Hummel, H. Herbst, H. Stein, Eur. J. Biochem. 180 (1989) 555–561.
[21] M.A. Strege, A.L. Lagu, J. Chromatogr. A 705 (1995) 155–161.
[22] T.M. McNerney, S.K. Watson, J.H. Sim, R.L. Bridenbaugh, J. Chromatogr. A 744 (1996) 223–229.
[23] R.M. Riggin, personal communication.
[24] M.T.C.P. Ribela, Y. Murata, L. Morganti, D. Toniolo, P. Bartolini, J. Immunol. Methods 159 (1993) 269–274.
[25] A.F. Bristow, R. Gaines Das, S.L. Jeffcoate, D. Schulster, Growth Regul. 5 (1995) 133–141.
[26] E.B. Jensen, S. Carlsen, Biotechnol. Bioeng. 36 (1990) 1–11.
[27] D. Koshland, D. Botstein, Cell 20 (1990) 749–760.
[28] European Pharmacopeia Convention, European Pharmacopeia, 3rd ed., Council of Europe, Strasbourg, 1997, pp. 1518–1526.
[29] J.P. Chang, R.C. Tucker, B.F. Ghrist, M.R. Coleman, J. Chromatogr. A 675 (1994) 113–122.
[30] G.W. Becker, R.R. Bowsher, W.C. Mackellar, M.L. Poor, P.M. Tackitt, R.M. Riggin, Biotechnol. Appl. Biochem. 9 (1987) 478–487.
[31] T. Hayakawa, S. Niimi, E. Uchida, Pharmeuropa 3 (1991) 33–40.
[32] V. Borromeo, A. Berrini, C. Secchi, G.F. Brambilla, A. Cantafora, J. Chromatogr. B 669 (1995) 366–371.
[33] J.P. Chang, T.H. Ferguson, P.A. Record, D.A. Dickson, D.E. Kiehl, A.S. Kennington, J. Chromatogr. A 736 (1996) 97–104.
[34] M. Wallis, J. Ivanyi, K. Surowy, Mol. Cell. Endocrinol. 28 (1982) 363–372.