ELSEVIER

Journal of Chromatography A 817 (1998) 205–214

JOURNAL OF
CHROMATOGRAPHY A

Quantifying biosynthetic human growth hormone in *Escherichia coli* with capillary electrophoresis under hydrophobic conditions

Thomas K. Jørgensen*, Leif H. Bagger, Jesper Christiansen, Gitte H. Johnsen, Jan R. Faarbæk, Linda Jørgensen, Benny S. Welinder

Health Care, Novo Nordisk A/S, DK-2820 Gentofte, Denmark

Abstract

A method has been developed which is able to quantitate the content of precursor biosynthetic human growth hormone (Pre-bhGH) in the cytosol of *E. coli* cells containing the gene for human growth hormone (hGH). The method uses hydrophobic C_{18} coated capillaries with native biosynthetic human growth hormone (bhGH) as an internal standard. This allows for highly robust and precise determinations as well as the evaluation of the presence of deamidated forms in the cytosol samples. Furthermore, by modifying the running buffer with zwitterionic surfactants and an organic modifier, it is possible to detect a related form with a three sulfur atom Cys–Cys bridge (trisulfide Pre-bhGH). Thus, a strong tool for monitoring the effect of fermentation conditions on the biosynthesis of bhGH is obtained. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Escherichia coli*; Cytosol; Growth hormones

1. Introduction

Biosynthetic human growth hormone (bhGH) is produced recombinantly in *Escherichia coli* as an N-terminally extended precursor (Pre-bhGH). The extension is negatively charged which gives the Pre-bhGH an isoelectric point (p*I*) of 4.7, slightly lower than that of hGH, p*I* 4.9 [1]. During the purification process, the N-terminal extension is specifically cleaved to produce the equivalent of native human growth hormone [2].

Several related forms of 22 M_r bhGH are known to occur (Fig. 1). The deamidated forms appear when either Asn_{149} and/or Asn_{152} isomerize via the cyclic imide intermediate to form the more negatively charged Asp or iso-Asp [3]. Another form is a trisulfide bhGH (TS-bhGH) which contains a three-sulfur moiety in one of the two Cys–Cys bridges [4]. This is a structure also seen in the recombinant production of interleukin-6 in *E. coli* [5]. The trisulfide Cys–Cys bridge gives bhGH a higher hydrophobicity than the native form which contains two sulfur atoms at this location (Cys_{182}–Cys_{189}). Deamidated forms as well as TS-Pre-bhGH are detrimental to the purification of bhGH.

In order to monitor the effect of fermentation parameters as early as possible in the production process, it is useful to be able to measure the content of Pre–bhGH, as well as its related forms, as close to the actual fermentation step as possible.

Pre-bhGH is not secreted from the *E. coli* cells, so an appropriate method for lysis is needed in order to expose the Pre-bhGH containing cytosol. The method needed should be efficient in rupturing cell walls, but at the same time gentle enough not to have detrimental effects on Pre-bhGH. Several alternatives

*Corresponding author.

0021-9673/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(98)00424-5

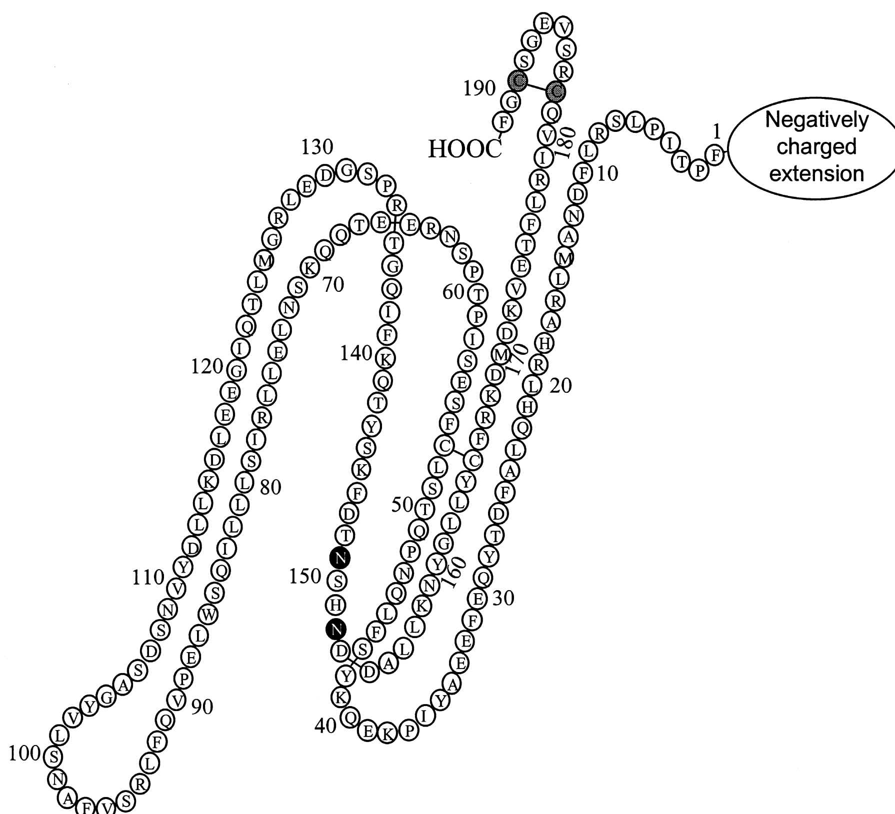

Fig. 1. Biosynthetic human Growth Hormone Precursor (Pre-bhGH): The two cysteines (in grey) at positions 182 and 189 can form a trisulfide bridge. Asn_{149} and Asn_{152} (in black) are known to deamidate to Asp. The negatively charged N-terminal extension is specifically cleaved to produce biosynthetic human growth hormone (bhGH).

exist such as enzymatic lysis, rupture using the combination of shear force and pressure drop in a French Press setup, and sonication. The latter two methods have the advantage that no foreign substances are added which can affect detection of the substance of interest.

In order to analyse the cytosol, a sensitive and selective analysis is needed. Immunochemical methods as well as HPLC can be considered as realistic techniques for analysis of this type of sample. However, both have drawbacks: enzyme-linked immunosorbent assay (ELISA) and Western blotting both lack precision; and HPLC suffers from potential column contamination as well as long analysis times in order to isolate the substance of interest in such complex samples.

Capillary electrophoresis (CE) is an ideal candidate for analyzing such samples. Firstly, the open tube structure of capillaries minimizes the risk of contamination, permitting simple rinsing procedures to give long capillary lifetimes. Secondly, selectivity can be optimized both by a critical choice of the running buffer as well as injection system. Thirdly, if protein adsorption to capillaries and inter-capillary variation can be minimized, a fast and reproducible method can be made. Finally, CE has previously been demonstrated to be a powerful tool for analyzing hGH and its derivatives [6–10].

We present a CE method for quantifying Pre-bhGH in the cytosol of *E. coli* cells taken directly from fermentation tanks. The method can also be used as a basis for an evaluation of the presence of related forms of bhGH. It is demonstrated that the use of C_{18} coated capillaries not only minimizes

protein adsorption, but is found to have low inter-capillary variation, and is highly precise and robust when used with a suitable internal standard.

2. Experimental

2.1. HPLC instrumentation and methods

Separations were carried out on a Waters (Milford, MA, USA) liquid chromatography system with detection at 214 nm. The column used was a C_4 reversed-phase column (dimethylbutyldimethylsilane, 40×4 mm, 5 μm particle size, 300 Å pore size), produced at Novo Nordisk. Elution was carried out by using a gradient of 0.1% (v/v) aqueous trifluoroacetic acid (TFA) to 80% (v/v) acetonitrile in 0.09% (v/v) aqueous TFA. Flow was 1.5 ml/min. Samples of 20 μl of *E. coli* cytosol, containing approximately 50 μg Pre-bhGH were injected.

2.2. CE instrumentation and methods

All CE experiments were carried out on a P/ACE 2100 instrument (Beckman, Palo Alto, CA). The electropherograms were monitored at 200 nm, with a data collection rate of 5 Hz.

Fused-silica capillaries of 50 and 75 μm I.D., 375 μm O.D. were purchased from Composite Metal Services (Worcestershire, UK). C_{18}-coated capillaries with 50 and 75 μm I.D., 375 μm O.D. were purchased from Supelco (Bellefonte, PA, USA) or ISCO (Lincoln, NE, USA). The lengths of the capillaries (to detection window/total length) were as indicated in the text.

A standard running buffer of 150 m*M* Tricine, 7.5% (v/v) methanol, pH 7.55, was used unless otherwise stated in the text. For studies where buffers contained the neutral surfactant Brij-35, C_{18} capillaries were preconditioned for 5 min. with 0.5% (v/v) Brij-35 before analysis, using high-pressure purge (140 kPa).

Samples were low-pressure (3.5 kPa) injected for 5–10 s. The capillaries were maintained at room temperature, during analysis, unless otherwise stated. The field strength during separation is indicated in the text. After each analysis, capillaries were flushed with running buffer for 3 min, using high-pressure purge. Storage: capillaries were first flushed with water for 5 min, then 5 min. of 0.1 *M* NaOH, 5 min of water, and blown dry with air.

2.3. Materials

Purified bhGH, Pre-bhGH, TS-Pre-bhGH as well as *Escherichia coli* fermentation samples containing bhGH were produced at Novo Nordisk.

Tricine, sodium hydroxide, sodium chloride, acetonitrile, 1-propanol and TFA were purchased from E. Merck (Darmstadt, Germany). Methanol (HPLC grade, 99.9% pure) was from Sigma-Aldrich (Steinheim, Germany). The zwitterionic surfactant N-dodecyl-N,N-dimethyl-3-amino-1-propanesulfonate (DAPS) was purchased as Zwittergent 3-12® from Boehringer Mannheim (Mannheim, Germany). The neutral surfactant Brij-35 (30%, w/v) was purchased from Sigma Diagnostics (St. Louis, MO, USA).

2.4. Sample preparation

Samples of purified bhGH (in a dilute phosphate buffer), Pre-bhGH (in a dilute Tris buffer), TS-Pre-bhGH (in a dilute ammonium acetate buffer) were diluted 1:10 with deionized water. *E. coli* cytosol samples were prepared by initially spinning down the cells and removing the fermentation broth. The cells were resuspended in deionised water and lysed by two alternate methods: French Press, where samples were pressed through a narrow bore, causing the cells to rupture due to the shear force as well as pressure drop; sonication of the samples for 100 s in 10 ml test tubes with an Ultrasonic sonicator (Sonics and Materials, Danbury, CT, USA) with an effect of approximately 100 W, while keeping the tubes on ice. The cell debris was removed by centrifugation, and the supernatant was filtered through a 0.22 μm filter. Prior to injection, cytosol samples were diluted 1:10 with deionized water. Total protein content in cytosol samples was determined using a BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA).

3. Results and Discussion

3.1. Sample content

To visualize the content of Pre-bhGH in the samples of *E. coli* cytosol, an HPLC separation in a 70 min linear acetonitrile gradient of the cytosol samples was made (Fig. 2). The chromatogram depicts the absorbance of the different cytosol components at 214 nm. Pre-bhGH (approximately 15% of total protein and 20% of total peak area) is seen as a prominent peak, eluting relatively late in the acetonitrile gradient.

3.2. Fused-silica capillaries

The first CE studies with the samples were run under simple free zone conditions in fused-silica capillaries. Dilute Tricine buffers (10 m*M*) were used and NaCl was added to decrease protein adsorption to the fused-silica capillaries. The results show (Fig. 3) that the peak for Pre-bhGH is clearly present, and migrating prominently enough to have potential as a quantitative method. However, with fused-silica capillaries, it was found that there were long equilibration times: peaks did not sharpen completely until after approximately 20 injections, indicating that there was a great deal of sample adsorption to the capillaries. It appeared that multiple injection of sample resulted in a coating of the capillaries which eventually led to a reasonable separation.

3.3. Coated capillaries

Because of the adsorption to the fused-silica capillaries further studies were carried out with coated capillaries. A comparison of different coatings, including hydrophilic, amine, hydrophobic C_8 and C_{18} (results not shown), demonstrated that C_{18} coated capillaries gave the most promising results. In

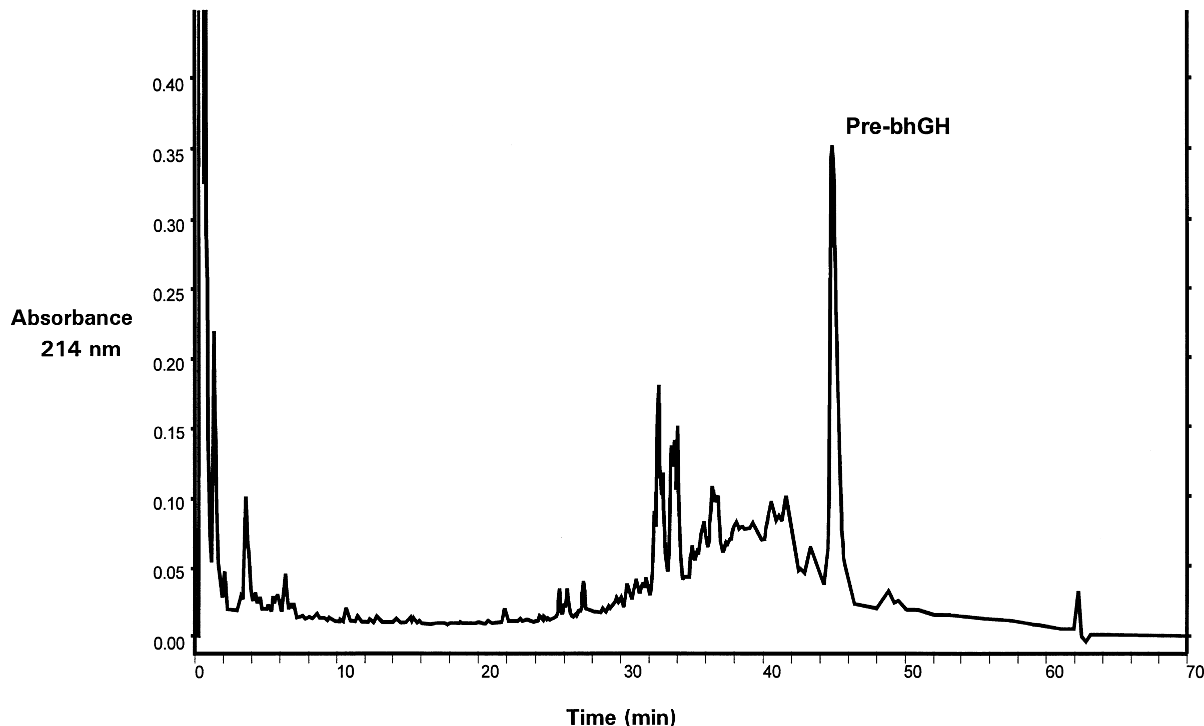

Fig. 2. RP-HPLC of bhGH–*E. coli* cytosol: 0 to 80% (v/v) acetonitrile gradient lasting 70 min. See also experimental.

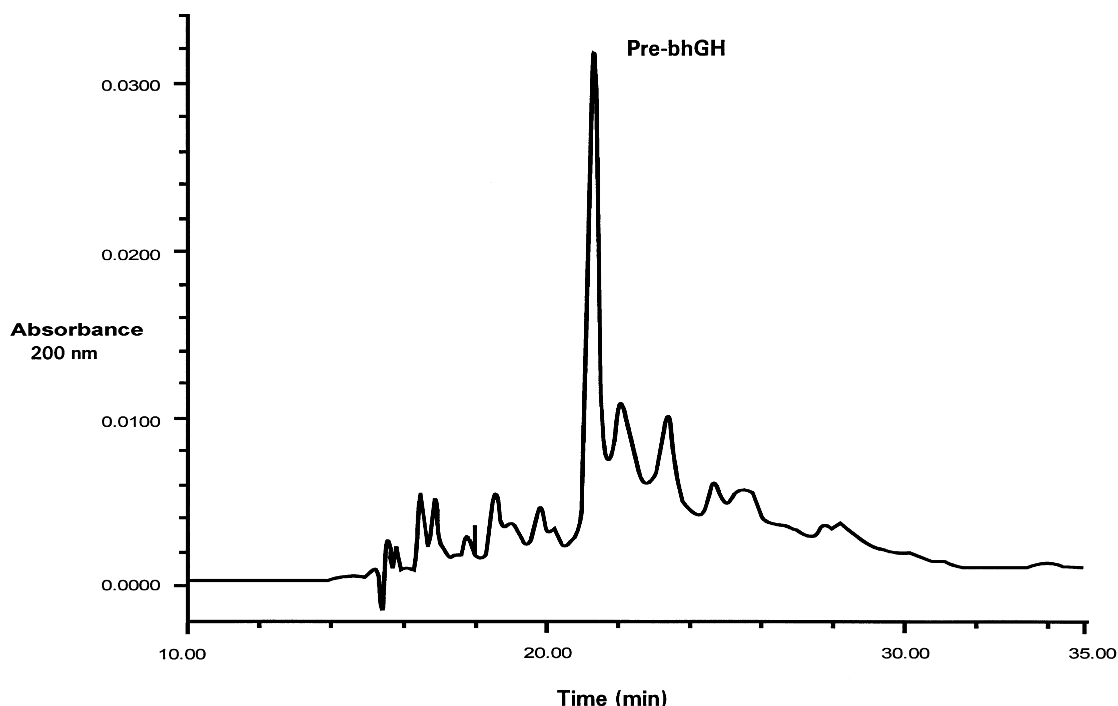

Fig. 3. Free zone electrophoresis of *E. coli* cytosol: A sample of *E. coli* cytosol containing approx. 2.6 mg/ml Pre-bhGH was injected on a running buffer equilibrated [57 cm (effective length 50 cm)×75 μm I.D.] fused-silica capillary. Running buffer: 10 m*M* Tricine, 70 m*M* NaCl, pH 7.4. Volt.: 15 kV. See also Experimental.

Fig. 4 an electropherogram of a sample of *E. coli* cytosol analyzed on this capillary is shown. The Pre-bhGH peak is well separated from the other components of the fermentation sample.

The separation is based on a Tricine system, where the selectivity has been greatly improved by raising the concentration of this zwitterion in the running buffer from 10 to a maximum of 150 m*M*. Furthermore, adding methanol improved the separation. The hydrophobic inner surface of the capillaries seems to remove the problem of sample adsorption and capillaries gave good separations immediately.

To demonstrate that the Pre-bhGH peak was pure, a placebo *E. coli* fermentation, where the hGH gene was omitted, was prepared. A comparison is made of the electropherogram of the placebo *E. coli* fermentation sample with that of a placebo spiked with a purified Pre-bhGH (Fig. 5). It is clear that no peaks in the placebo sample co-migrate with the Pre-bhGH.

3.4. Internal standard

In order to quantify Pre-bhGH it was necessary to choose a suitable internal standard. Due to the risk of sample evaporation as well as poor reproducibility of sample injection size, it was not possible to use independently prepared standard curves. Thus, the requirement for the standard was that it should preferably have similar spectrophotometric absorption characteristics to Pre-bhGH, but have a different electrophoretic mobility than that of Pre-bhGH as well as other components from the cytosol.

The use of bhGH, i.e. the recombinant version of native human growth hormone, proved to be ideal as an internal standard. Due to the negatively charged N-terminal extension of Pre-bhGH, the p*I* is slightly lower (4.7) than that of bhGH (p*I* 4.9). The molar absorption coefficients for the two forms of bhGH are, for practical purposes, the same at 200 nm [11]. After spiking bhGH to an *E. coli* cytosol sample, it is

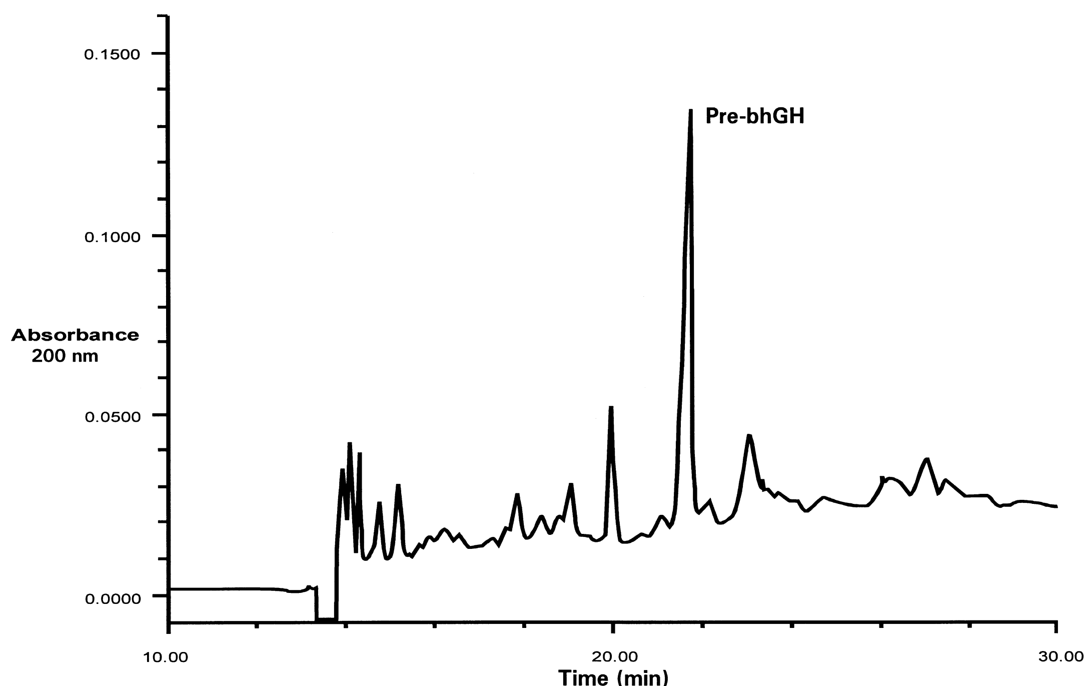

Fig. 4. Separation of bhGH–*E. coli* cytosol on a C_{18} coated hydrophobic capillary: A sample of *E. coli* cytosol containing approx. 2.6 mg/ml Pre-bhGH was injected on a running buffer equilibrated [67 cm (effective length 60 cm)×75 μm I.D.] C_{18} coated hydrophobic capillary. Running buffer: 150 m*M* Tricine, 7.5% (v/v) methanol, pH 7.55. voltage: 28 kV. See also Experimental.

seen (Fig. 6) that bhGH migrates not only well away from the peak of Pre-bhGH but also in a region without other cytosol components.

3.5. Method performance

In order to further verify that sample adsorption to the hydrophobic surface was not detrimental in this system, capillaries were coated with the neutral surfactant Brij-35 as suggested by Towns and Regnier [12] as well as several capillary vendors. Our method was compared to an otherwise identical separation system, except that the capillary was equilibrated with 0.5% (v/v) Brij-35 for 5 min and subsequently run with 0.01% (v/v) Brij-35 added to the running buffer (Fig. 7). Clearly, the baseline has been improved, but the migration times are longer. However, robustness was greatly reduced; with neutral surfactants, capillaries became unusable after approximately 50 runs, even after re- equilibration with the high concentrations of Brij-35. Without neutral surfactant as a coating modifier, capillaries are usable up to 300 or 400 times—clearly much more robust, and demonstrating that adsorption is not detrimental.

Good correlation over a broad range was found between our expected and measured content of Pre-bhGH in our samples (results not shown). This was demonstrated in experiments where either the concentration of internal standard varied in samples where purified Pre-bhGH was spiked into placebo *E. coli* fermentations. In both cases it was verified that there are no peaks which interfere with either that of Pre-bhGH or the internal standard.

A quantitation limit of 115 μg/ml Pre-bhGH was found. This is a value which is considerably higher than quantitation limits that exist for immunochemical methods. For our purpose, however, and for many other proteins that are recombinantly expressed in *E. coli*, it is more than adequate. This is all the more relevant seen in the light of the simple sample preparation and UV detection.

The precision and robustness of the method was studied with especially one source of variation in

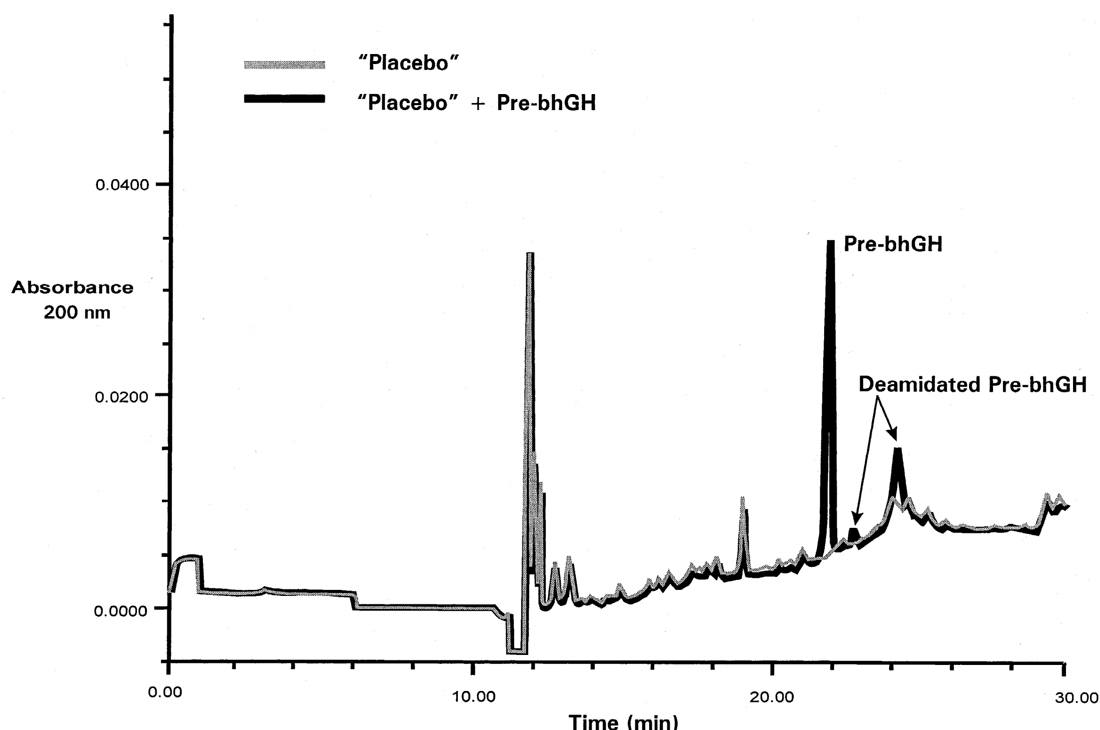

Fig. 5. 'Placebo' *E. coli* cytosol and 'Placebo' spiked with Pre-bhGH (approx. 2.6 mg/ml) injected on a running buffer equilibrated [67 cm (effective length 60 cm)×75 μm I.D.] C_{18} coated hydrophobic capillary. Running buffer: 150 m*M* Tricine, 7.5% (v/v) methanol, pH 7.3. Voltage: 28 kV. See also Experimental.

mind, namely inter-capillary reproducibility. We had previously experienced a great deal of variation in the quality of fused-silica capillaries and were worried that this would also be the case for C_{18} coated capillaries. This did not seem to be the case with our method.

Five different capillary batches were tested—-one batch of 75 μm capillary from ISCO and four different batches of Supelco capillary with internal diameters of either 50 or 75 μm. The values obtained for content of Pre-bhGH in an *E. coli* cystosol sample were very consistent (Table 1). In fact, compared to the results found when the same sample was analysed with three capillaries cut out of the same spool, there was no significant difference.

The results demonstrate that the method is robust: Firstly, this is due to an apparently small difference between capillaries; secondly, the analysis itself is robust, shown by the fact that the precision of the actual quantitation of Pre-bhGH was not affected by changes in the internal diameter of the capillaries from 50 to 75 μm — even though this obviously affected migration times. Due to the stabilizing effect of the C_{18} capillaries, protein adsorption is diminished and the electroosmotic flow is decreased; consequently, both of these parameters are, to a lesser extent than in fused-silica capillaries, a cause of variation.

4. Detection of related bhGH forms

As can be seen with the spiked placebo sample (Fig. 5), several peaks occur just after the Pre-bhGH peak. These are Pre-bhGH forms with deamidated Asn_{149} and Asn_{152}. The spiked Pre-bhGH sample contains several of these forms, and the electropherogram demonstrates that this method can also be used to make a qualitative evaluation of the

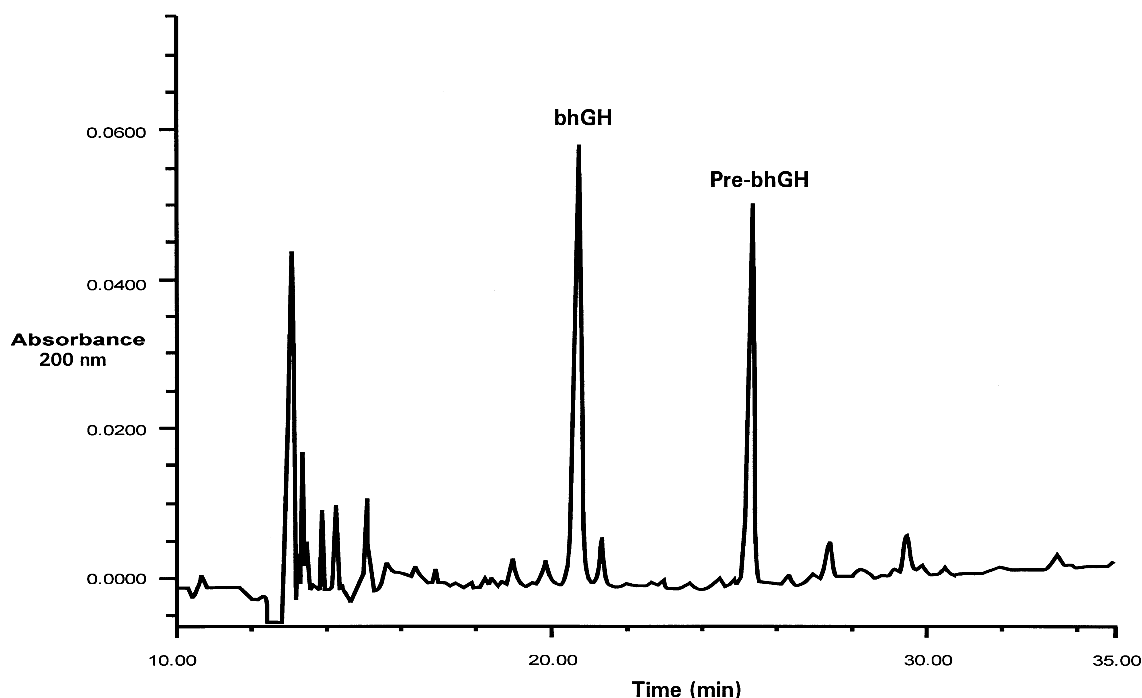

Fig. 6. Quantitating Pre-bhGH in *E. coli* fermentation samples: *E. coli* cytosol sample (approx. 2.6 mg/ml Pre-bhGTH) spiked 1:1 with bhGH on a running buffer equilibrated [67 cm (effective length 60 cm)×75 μm I.D.] C_{18} coated hydrophobic capillary. Running buffer: 150 m*M* Tricine, 7.5% (v/v) methanol, pH 7.55. voltage: 28 kV. See also Experimental.

presence of these forms in fermentation samples. An actual quantitation of these deamidated forms is, however, not possible.

Pre-bhGH could also be separated from the more hydrophobic trisulfide form of Pre-bhGH (TS-Pre-bhGH) in *E. coli* cytosol samples. Experiments were carried out which were based on systems which could separate native Insulin-like Growth Factor I from an improperly folded form, where disulfide bonds were mismatched [13]. Hydrophobic selectivity had been introduced by using organic solvents to modulate the interaction of proteins with surfactants in the running buffer. A zwitterionic surfactant DAPS was added to the original Tricine running buffer, and 1-propanol replaced methanol as the organic modifier. By optimizing the Tricine concentration as well as capillary temperature, a separation of TS-Pre-bhGH and Pre-bhGH was obtained in a sample of *E. coli* cytosol (Fig. 8). By adding a dilute solution of cysteine to the sample, the TS-Pre-bhGH peak reverts to a non-trisulfide structure, confirming that the peak is in fact TS-Pre-bhGH.

5. Conclusion

It is demonstrated that hydrophobic C_{18} coated capillaries give robust and reproducible results when quantitating Pre-bhGH in complex samples such as those from *E. coli* cytosols. No detrimental protein adsorption or any other kind of contamination that could affect reproducibility is observed. This, in turn, results in long capillary life and good consistency. The method may (by itself or with a modification of the running buffer to add hydrophobic selectivity) also be used to evaluate the presence of certain related variants of bhGH. Therefore, the system represents a powerful tool for monitoring the effect of fermentation parameters on bhGH synthesis.

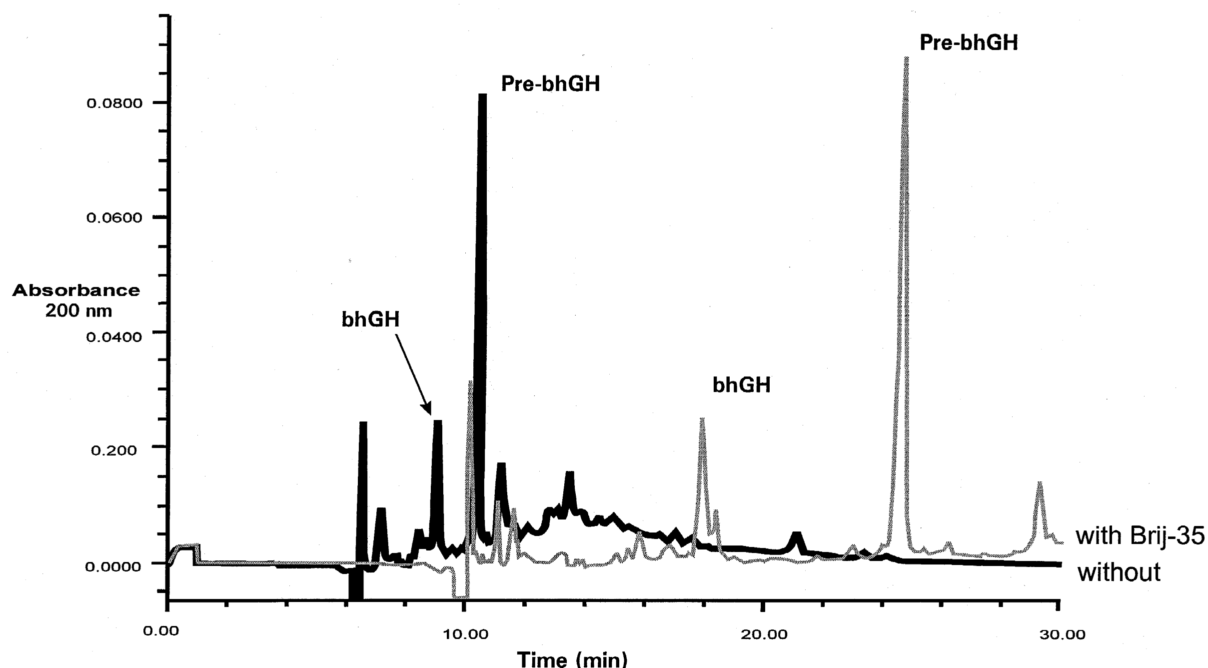

Fig. 7. Comparison of results with and without a neutral surfactant as a running buffer additive: *E. coli* cytosol sample (approx. 2.6 mg/ml Pre-bhGH), spiked 1:1 with bhGH, injected on a 0.5% (v/v) Brij-35 washed, and running buffer equilibrated [47 cm (effective length 40 cm)×75 μm I.D.] C_{18} coated hydrophobic capillary. Running buffer: 150 m*M* Tricine, 0.01% (v/v) Brij-35, 7.5% (v/v) methanol, pH 7.3. voltage: 20 kV. Brij-35 was omitted from the equilibration and running buffers in the ordinary system (see also Experimental).

Table 1
Precision/robustness[a]

Results from five different capillary batches (two suppliers)[b]	
N	140
Mean content of Pre-bhGH (mg/ml)	2.60
S.D.	0.044
R.S.D. (%)	1.69
Results from three capillaries of the same batch[c]	
N	60
Mean content of Pre-bhGH (mg/ml)	2.63
S.D.	0.032
R.S.D. (%)	1.23

[a] Study of the effect of inter-capillary variation on the measured content of Pre-bhGH in *E. coli* cytosol samples
[b] Supplier 1: ISCO: 1 batch of C_{18} coated capillary, 75 μm I.D.; Supplier 2: Supelco: 4 different batches of C_{18} coated capillary, 50 and 75 μm I.D.
[c] Supplier: Supelco: C_{18} coated capillary, 75 μm I.D.

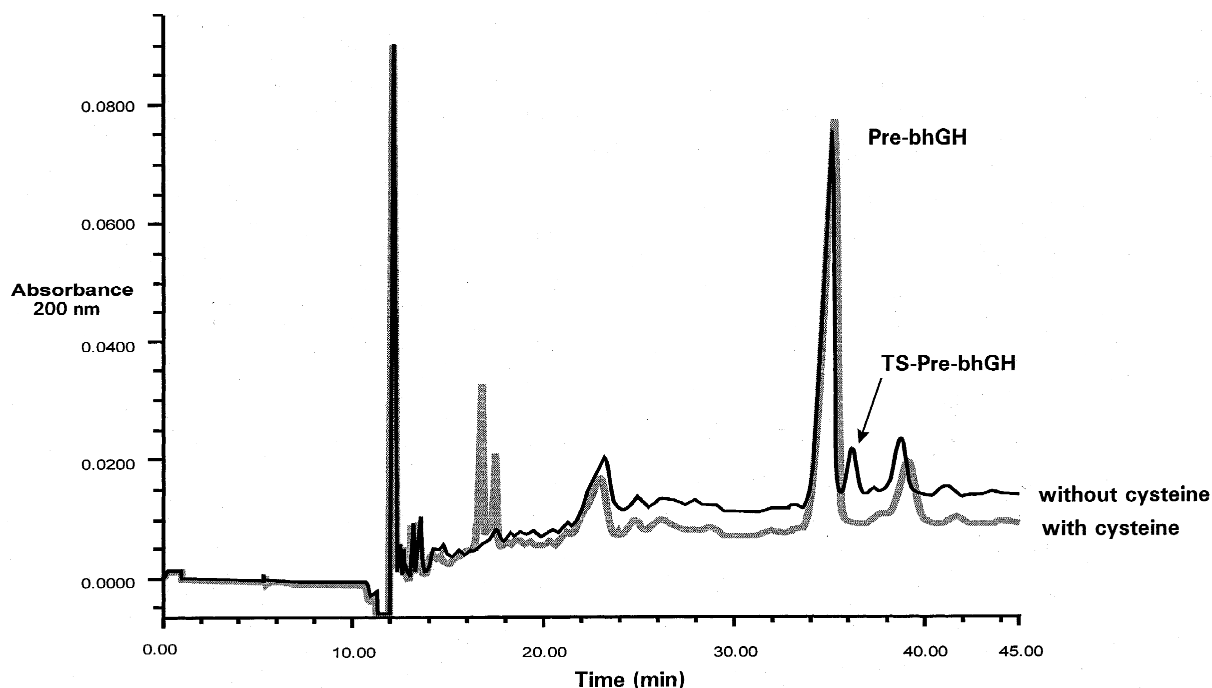

Fig. 8. Measuring TS-Pre-bhGH in *E. coli* cytosol by adding zwitterionic surfactants and organic modifier to the running buffer: *E. coli* cytosol sample containing Pre-bhGH/TS-Pre-bhGH (approx. 2.5 mg/ml) was injected on a running buffer equilibrated [67 cm (effective length 60 cm)×75 μm I.D.] C_{18} coated hydrophobic capillary. Running buffer: 58 m*M* Tricine, 15% (v/v) propanol, 12 m*M* N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (DAPS), pH 7.55. Voltage: 20 kV. Temperature: 35°C. See also Experimental.

Acknowledgements

The authors wish to acknowledge the technical assistance of Birgitte S. Brendstrup.

References

[1] T. Christensen, Novo Nordisk A/S, Personal communication.
[2] J.T. Jørgensen, T. Christensen, M. Jørgensen, G. Jakobsen, Indian J. Pediatr. 58(Suppl) (1991) 23–32.
[3] D.W. Aswad (Editor), Deamidation and Isoaspartate Formation in Peptides and Proteins (CRC Series in Analytical Biotechnology), CRC Press, Boca Raton, FL, 1995.
[4] A.M. Jespersen, T. Christensen, N.K. Klausen, P.F. Nielsen, H.H. Sørensen, Eur. J. Biochem. 219 (1994) 365–373.
[5] J. Breton, N. Avanzi, B. Valalsasina, S. Sgarella, A.L. Fiura, U. Breme, G. Orsini, E. Wenisch, P.G. Righetti, J. Chromatogr. A 709 (1995) 135–146.
[6] P. Dupin, F. Galinou, A. Bayol, J. Chromatogr. A 707 (1995) 396–400.
[7] T.M. McNerney, S.K. Watson, J.H. Sim, R.L. Bridenbaugh, J. Chromatogr. A 744 (1996) 223–229.
[8] R.G. Nielsen, G.S. Sittampalam, E. Rickard, Anal. Biochem. 177 (1989) 20–26.
[9] K. Shimura, B.L. Karger, Anal. Chem. 66(1) (1994) 9–15.
[10] A. Vinther, H. Søeberg, H.H. Sørensen, A.M. Jespersen, Talanta 38(12) (1991) 1369–1379.
[11] G.H. Beaven, E.R. Holiday, Adv. Protein Chem. 7 (1952) 364–367.
[12] J.K. Towns, F.E. Regnier, Anal. Chem. 63 (1991) 126–1132.
[13] W. Nashabeh, K.F. Greve, D. Kirby, F. Foret, B.L. Karger, Anal. Chem. 66 (1994) 2148–2154.