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Short communication

Analysis of recombinant human growth hormone and its related impurities by capillary electrophoresis

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Abstract

A convenient free solution capillary electrophoretic method is described for the simultaneous quantitation of human growth hormone (hGH) and its related impurities in pharmaceutical preparations. The separation of the cleaved form, the monodeamidated form and two new compounds which may arise from post-translational modifications in *Escherichia coli*–a succinylated hGH with a blocked amino terminus and a modified hGH having a His to Gln replacement at sequence position 18–is described. The proposed capillary electrophoretic method shows good specificity, linearity and precision. A limit of detection of 0.03% was calculated for the impurities.

1. Introduction

Human growth hormone (hGH) is one of the first generation pharmaceutical products made by rDNA technology. Recognition of most of the degradation impurities comes from the study of the native hGH. Deamidated forms, oligomeric aggregates, cleaved and oxidized forms are the major related proteins found in the native [1–3] as well as in the recombinant hGH [4,5].

The assessment of the absence of such impurities by a set of analytical methods is now well established [6]. However, these methods are often not capable to detect traces of new impurities which may arise from the recombinant product during processing. We have found two new isoforms of the recombinant growth hormone expressed in *Escherichia coli*. The first one is a succinylated hGH with a blocked amino terminus. In the pituitary, on the other hand, only an acetylated form of hGH has been described [7]. The second isoform is growth hormone with a His to Gln replacement at position 18. This variant may originate from an amino acid substitution during translation. Such a variant was also found in the human granulocyte colony stimulating factor produced in *E. coli* [8]. The development of a new analytical method was necessary to demonstrate that these impurities were eliminated during the purification of the hormone.

High-performance capillary electrophoresis (HPCE) had been proved highly efficient for the analysis of complex mixtures, especially in aqueous media, and in recent years some attempts have been made to promote its use in the quality control of pharmaceutical proteins [9,10]. This paper describes the validation of a free solution capillary electrophoretic (FSCE) method for the assessment of the purity of biosynthetic hGH.

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Some reports have described the separation by FSCE of the two deamidated forms originating from the two deamidation sites at Asn 149 and Asn 152 [11,12]; this separation can be performed in a few minutes due to the net charge differences between the proteins. In the present paper, the conditions of the separation were obtained after optimization of the free solution (manuscript in preparation). The validation criteria used were very similar to those applicable to the validation of an HPLC method.

2. Experimental

2.1. Reagents

All common reagents were of analytical grade. Diammonium hydrogenphosphate was purchased from Riedel-de-Haën (Hannover, Germany) and phosphoric acid from Prolabo (Paris, France). Water, HPLC grade, was obtained from Rathburn (Walkerburn, UK). Acetonitrile was from J.T. Baker (Deventer, Netherlands) and trifluoroacetic acid from SDS (Peypin, France). Recombinant human growth hormone (re-hGH) and related impurities were obtained from Sanofi (Labège, France).

An artificial mixture was made with re-hGH in 50 m*M* ammonium carbonate (pH 8.3) spiked with small amounts of impurities. The total protein content of this artificial mixture, determined with reversed-phase chromatographic reference method, was 12.8 mg/ml. All FCZE solutions were diluted with water.

2.2. High-performance capillary electrophoretic apparatus and conditions

All analyses were carried out on a Model 270 A (Applied Biosystems, San Jose, CA, USA) analytical capillary electrophoretic system used in the normal polarity mode (anode on the injection side). The separations were monitored on-column by measuring the UV absorption at 195 nm. The capillary was thermostated at 30°C. The electrophoregrams were acquired and stored on a Model C-R4A data collection system (Shimadzu, Kyoto, Japan).

Untreated fused-silica capillaries (50 μm I.D.) were obtained from Applied Biosystems and cut to a total length of 92 cm with the detection window placed at 70 cm.

The running buffer was a solution of 0.1 *M* diammonium hydrogenphosphate, adjusted to pH 6.0 with phosphoric acid. The solution was filtrated through a disposable filter from Nalge (Rochester, USA) prior to use.

Each new capillary was conditioned by rinsing with 1 *M* sodium hydroxide for 20 min, then by washing with water for 10 min and filling with the running buffer during 20 min.

Each day, before starting the analysis, the capillary was first purged with 1 *M* sodium hydroxide for 10 min, followed by water for 10 min. Afterwards it was filled with the running buffer for 20 min. No rinsing sequences were performed between two runs.

Samples were introduced hydrodynamically (vacuum: 12.7 cmHg) with the following sequence: sample injection (3 s), separation buffer injection (1 s) and then analysis with an applied voltage of 20 kV.

2.3. Quantitation conditions

All peak areas were normalized with corresponding migration times [13].

According to the high/low sample loading described by Altria [14], each solution to be analyzed was diluted to concentrations of 0.6 mg/ml (low sample loading) and 4.5 mg/ml (high sample loading) prior to injection. The low sample loading was selected to give a main peak which was within the linear range of the detector.

The area of the main peak in the off-scale separation of the high sample loading was estimated by multiplying the main peak area obtained in the on-scale separation of the low sample loading by the factor of the increase in sample loading.

This calculated main peak area was used in the calculation of impurity level as % area/area in the high concentration sample.

The contents of hGH in the solutions to be analyzed were calculated on the nominal electrophoregram (0.6 mg/ml) with reference to an external working standard and corrected for the dilution.

2.4. Reversed-phase chromatography reference method

A 100-μg amount of protein sample was applied to a C_{18}, 300 Å, 250 × 4.6 mm I.D. column (Symchrom, Lafayette, IN, USA) and eluted with a mixture of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.08% trifluoroacetic acid in acetonitrile) using a linear 25–70% gradient of solvent B over 35 min at a flow-rate of 1 ml/min. Elution was monitored at 220 nm. The accuracy of the protein content expressed in mg ml^{-1} was 5%.

3. Results and discussion

3.1. Specificity

Fig. 1 shows a typical electrophoregram of an artificial mixture of human growth hormone and its related products. The separation of the deamidated forms and the Gln 18-hGH is remarkable as these compounds differ by only 0.05 p*I* unit and because there is no calculated charge difference between Gln 18-hGH and hGH (Genetic Computer group, software: Wisconsin package version 8). For these two proteins the separation could probably be correlated to their tertiary structure and the ability of the mutational residue Gln 18 to interact with the eluting solvent and (or) the capillary wall.

The crystal structure of the hGH [15] and the ability of Zn^{2+} ions to promote the formation of an hGH dimer in which His 18, His 21 and Glu 174 participate in coordinating the Zn^{2+} suggest that the residue in position 18 is effectively pointing out of the structure and therefore could affect the migration.

Moreover, we obtained two peaks for the deamidated forms, as expected from the deamidation mechanism of the protein. If there are interactions between the impurities and the capillary wall–no mobile phase additives were used–they do not affect the migration order according to the isoelectric points measured by isoelectrofocusing on an immobiline gel (Table 1).

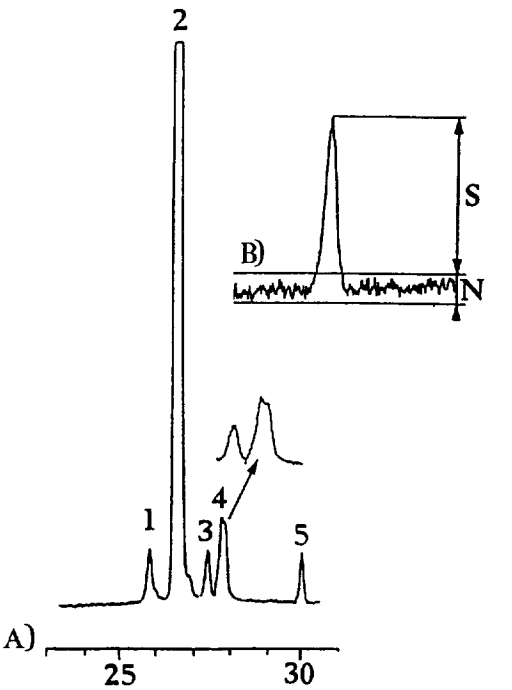

Fig. 1. (A) Typical electrophoregram of an artificial mixture of hGH and its related products. Peaks: 1 = cleaved hGH; 2 = hGH; 3 = Gln 18-hGH; 4 = deamidated-hGH; 5 = N-succinylated hGH. (B) Determination of the detection limit for the N-succinylated hGH obtained with a concentration of 5.2 μg/ml (N = 70 mm gave LOD = 1.45 μg/ml; S = 13 mm gave LOQ = 4.8 μg/ml).

Table 1
Isoelectric points of hGH and its related impurities

Protein	Isoelectric point
Cleaved hGH	5.25
hGH	5.2
Gln18-hGH	5.1
Deamidated-hGH	5.05
Succinylated-hGH	4.9

3.2. Repeatability

The repeatability of the peak areas was calculated from the results of seven determinations which were carried out by the same analyst under the same conditions at the nominal concentration. The relative standard deviations (R.S.D.) obtained are shown in Table 2. Acceptable levels of precision for the injection were obtained for the main hGH peak: 1.9%.

3.3. Linearity and detection limit

The linear range for hGH was demonstrated from 0 to 1.17 mg/ml. Three replicate injections for each concentration were performed. The correlation between peak area and the concentration of hGH is excellent ($r^2 = 0.9984$) and the regression line obtained passes through the origin (confidence interval of the y-axis at the origin: −953.3 to 494.1 in area units).

In Fig. 1 a signal-to-noise ratio of greater than 3 is shown for the succinylated form, given as an example, at a concentration of 5.2 μg/ml. Finally, a limit of detection of 1.45 μg/ml and a limit of quantitation of 4.8 μg/ml were calculated for this impurity. The detection and quantitation limits were of the same order as for the other impurities (detection limit 0.25% for a 0.6 mg/ml solution of hGH). To improve this detection limit to 0.03% we have used a more concentrated solution (4.5 mg/ml) in this study (high sample loading).

3.4. Quantitation

The precision of the determination of the related impurities and of the hGH protein itself was calculated for the artificial mixture from the results of six determinations carried out over 4 days. All the results obtained (Table 3) show good levels of performance in terms of precision. They illustrate the gain in performance when employing high/low sample loading as described by Altria [14].

It is interesting to compare the hGH content determined for the artificial mixture and corrected for the dilution (11.8 mg/ml) with the one obtained with the gradient reversed-phase HPLC reference method (12.8 ± 0.6 mg/ml). Indeed, if we correct the FCZE value for the content of impurities, which are not separated in the HPLC method, we calculate a value of 12.5 mg/ml, which is in good agreement with the value determined with reversed-phase chromatography.

Table 2
Repeatability of the peak areas

Areas	hGH	Cleaved hGH	Gln 18-hGH	Deam.-hGH	Succ.-hGH
	45835.8	1276.8	992.4	2362.0	645.0
	45006.2	1417.0	1122.1	2530.0	720.9
	45130.5	1278.9	1026.3	2321.9	620.3
	47423.7	1394.6	1163.4	2775.7	735.2
	45982.3	1190.7	929.7	2255.5	632.9
	46898.3	1452.7	1201.1	2330.1	805.1
	46450.4	1315.9	1004.2	2398.3	765.4
Average	46103.9	1332.1	1062.7	2424.8	703.7
Standard deviation	888.1	92.9	100.1	176.6	71.5
R.S.D. (%)	1.9	7.0	9.4	7.3	10.2

Table 3
Quantitative determinations

Day	hGH (mg/ml)	Cleaved hGH (%)	Gln 18-hGH (%)	Deam.-hGH (%)	Succ.-hGH (%)
1	0.57	1.44	0.99	2.36	0.67
2	0.57	1.52	0.99	2.50	0.63
	0.59	1.73	1.09	2.83	0.67
3	0.59	1.63	0.99	2.59	0.64
	0.60	1.80	1.04	2.62	0.67
4	0.58	1.68	1.01	2.49	0.61
Average	0.58	1.63	1.01	2.56	0.65
Standard deviation	0.01	0.13	0.04	0.16	0.03
R.S.D. (%)	2.1	8.2	4.0	6.2	4.0

4. Conclusions

The validation of the proposed capillary electrophoretic method showed good performance in terms of specificity, linearity and precision.

The results obtained showed that capillary electrophoresis is a convenient, alternative method to HPLC. The ruggedness of this method has now to be demonstrated by an interlaboratory collaborative study.

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