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Analysis of recombinant human growth hormone by capillary electrophoresis with bilayer-coated capillaries using UV and MS detection

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Abstract

The characterization of recombinant human growth hormone (rhGH; somatropin) by capillary electrophoresis (CE) with UV-absorbance and mass spectrometric (MS) detection using capillaries noncovalently coated with polybrene (PB) and poly(vinyl sulfonic acid) (PVS) is demonstrated. Compared with bare fused-silica capillaries, PB–PVS coated capillaries yielded more favorable migration-time reproducibilities and higher separation efficiencies. Optimal separation conditions for the bilayer-coated capillaries comprised a background electrolyte (BGE) of 400 mM Tris phosphate (pH 8.5) yielding migration-time R.S.D.s of less than 1.0% and plate numbers above 300,000 for intact rhGH. The protein was also analyzed using the CE method described in the European Pharmacopoeia (Ph. Eur.) monograph. The pharmacopoeial method gave much longer analysis times (22 min versus 8 min), lower resolution and plate numbers, and consecutive shifts in migration time for rhGH, indicating possible interactions between the protein and the inner capillary wall. Due to stable migration times obtained with the coated capillaries, reliable profiling and quantification of rhGH and its byproducts in time was possible. Analysis of thermally degraded rhGH revealed the formation of two main degradation products. CE–mass spectrometry (MS) of this sample, using a PB–PVS coated capillary and a BGE of 75 mM ammonium formate (pH 8.5), suggests that these products are desamido forms of rhGH. Analyses of expired rhGH preparations with CE–UV and CE–MS indicated the presence of both deamidation and oxidation products.

Keywords: Capillary electrophoresis; Mass spectrometry; Recombinant human growth hormone; Biopharmaceutical; Protein stability; Bilayer coating

1. Introduction

Due to the introduction of efficient biotechnological production methodologies, more and more pharmaceutical proteins have become available on our markets. Nowadays, more than 100 pharmaceutical proteins have been approved for marketing and several hundreds are currently in clinical trials [1]. This development increases the demand for suitable methods of analysis that allow not only the separation and quantification of impurities and possible degradation products, but also their identification and, preferably, their physicochemical characterization [2–4]. Regulatory authorities demand a thorough characterization and control of the quality attributes of biopharmaceutical products from the early stages of clinical investigation up to commercial scale production. This presents analytical challenges as biopharmaceuticals may comprise a variety of constituents, including protein isoforms, closely related degradation products and excipients. Furthermore, conformational integrity is also an important issue, and therefore analytical methods are needed that allow detection of proteins in their native, nondenatured state. Yet, another advantage of using nondenaturing conditions is that artifacts caused by denaturation can be avoided.

Capillary electrophoresis (CE) is a powerful separation tool, which in principle is very well suited for the analysis of proteins [5–8]. CE analyses are relatively fast and separation efficiencies can be quite high. The separation mechanism is based on charge-

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to-size ratio of a compound, which makes it complementary to methods such as reversed-phase liquid chromatography (LC) [9]. Moreover, analyses in CE are normally performed using aqueous background electrolytes (BGEs), and, when required, the pH and temperature of separation can be chosen closely to physiological conditions. Separations in CE take place in an open tube in the absence of a stationary phase, which, in contrast to LC, furthermore, decreases the risk of protein denaturation due to interactions. CE can also be coupled to various types of detection systems such as UV-absorbance, laser-induced fluorescence (LIF) and mass spectrometry (MS).

Recombinant human growth hormone (rhGH; somatropin) was one of the first biotechnologically produced proteins in *Escherichia coli* [10]. The protein comprises 191 amino acids and has a p*I* of about 5.1, and its primary use is in the treatment of human growth disorders. Several methods have been used for the analysis of content and purity of rhGH, e.g. reversed-phase [11–15], ion exchange [14,16,17], and size-exclusion LC [12,18,19]. CE also shows great potential for the analysis of rhGH and its degradation products [20–25]. Recently, a CE-based method for detection of charge variants in rhGH was incorporated in the European Pharmacopeia (Ph. Eur.) [15]. However, an inter-laboratory study performed using Ph. Eur. conditions, showed fairly long analysis times and poor migration-time reproducibilities [23]. The shifting migration times might be caused by protein adsorption to the internal wall of the fused-silica capillaries [26–28], which can result in a varying electroosmotic flow (EOF) and band broadening. One way to prevent these problems is to mask the silanols groups on the inner capillary wall using an appropriate coating material. Recently, we have shown that capillaries noncovalently coated with a bilayer of polymers of opposite charge in combination with background electrolytes of high ionic strength yield fast, reproducible and highly efficient separation of proteins [29]. The coating procedure is fast and straightforward, and it can be easily included in a CE method.

Here we present an improved CE method for the characterization of rhGH in pharmaceutical preparations. A capillary coated with a bilayer of polybrene (PB) and poly(vinyl sulfonate) (PVS) was used to achieve stable and relatively fast separations. BGE (type and pH) was optimized by studying plate numbers and migration-time reproducibility of rhGH peaks. The applicability of the system was then investigated by the monitoring of the thermal degradation of rhGH and by the analysis of expired preparations of rhGH. In order to gain further information on the identity of degradation products, the samples were also analyzed by CE–MS. Comparisons with the Ph. Eur. method were made.

2. Experimental

2.1. Chemicals and samples

Polybrene (hexadimethrine bromide) and PVS sodium salt were purchased form Sigma–Aldrich (Steinheim, Germany). Tris, ammonium hydroxide (25%), phosphoric acid and hydrogen peroxide (30%) were from Merck (Darmstadt, Germany). Solutions of Tris phosphate were prepared by dissolving Tris to the desired concentration and adjusting the pH with phosphoric acid. The Ph. Eur. BGE was prepared by diluting ammonium hydroxide to 200 mM with Milli-Q water and adjusting the pH of the solution to 6.0 with phosphoric acid. The ammonium formate BGE for CE–MS was made by diluting ammonium hydroxide to 75 mM with Milli-Q water and adjusting the pH to 8.5 with formic acid. These BGEs were weekly prepared and passed through a 0.22 μm hydrophilic filter (Sartorius, Göttingen, Germany) before use. A 1 mg/mL stock solution of terbutaline (Holland Pharmaceutical Supply, Alphen aan de Rijn, The Netherlands) was prepared by dissolving the compound in Milli-Q water.

Somatropin (rhGH) CRS (European Directorate for the Quality of Medicines, Strasbourg) was dissolved in 30 mM Tris phosphate (pH 7.5) to a concentration of 3 mg/mL. A 5 mL volume of this solution was incubated at 40 °C for up to 96 h and aliquots were taken at regular time intervals. To these aliquots, terbutaline was added (from stock solution) to a final concentration of 50 μg/mL and used as internal standard. The liquid preparation Norditropin simplexx (Novo Nordisk, Bagsvaerd, Denmark) (15 mg/1.5 mL) was expired for 12 months. It contained mannitol, histidine, poloxamer 188, and phenol in water. This 1.5 mL solution was diluted before analysis by adding 3 mL of a solution of 50 mM Tris–HCl (pH 7.5). The lyophilized powder Humatrope (Eli Lilly, Houten, The Netherlands), which was expired for 18 months, was reconstituted before analysis with the provided solution. The reconstituted sample solution contained rhGH (1.5 mg/mL), metacresol, glycerin, glycine, mannitol and disodium hydrogen phosphate. Stock solutions of test proteins (3 mg/mL) were weekly prepared in Milli-Q water and stored at 4 °C. All samples contained 0.03% (v/v) of formamide that served as EOF marker.

2.2. CE systems

New bare fused-silica capillaries (50 μm i.d.) from Composite Metal Services (The Chase, Hallow, UK) were rinsed with 20 capillary volumes of water followed by 30 capillary volumes of 1 M sodium hydroxide, and 10 capillary volumes of water. After this treatment, capillaries were used as such or coated with PB–PVS. When using the pharmacopoeia BGE, capillaries were rinsed between analyses with a solution of 100 mM sodium hydroxide for 2 min and then with the BGE for 6 min. When using BGEs of Tris phosphate (pH 7–8.5) or ammonium formate (pH 8.5), capillaries were rinsed in between runs with 4 capillary volumes of BGE. The pressure applied for these rinses was 1380 mbar (20 psi).

For the PB–PVS coating procedure, PB was dissolved in Milli-Q water to a final concentration of 1% (m/v), and a 25% (v/v) solution of PVS was diluted to 1% (v/v) with Milli-Q water. These solutions were weekly made and passed through a 0.22 μm hydrophilic filter before use. Coating was performed by rinsing subsequently with 1.5 capillary volumes of a 1% PB solution at 35 mbar (0.5 psi), 10 capillary volumes of water at 1380 mbar, 1.5 capillary volumes of a 1% PVS solution at 35 mbar, and 10 capillary volumes of water at 1380 mbar. The capillary was then ready for CE analysis with the BGE of choice.

Overnight, capillaries were filled with the 1% PVS solution and tips were immersed in vials with this solution.

CE–UV experiments were performed on a P/ACE MDQ capillary electrophoresis instrument equipped with a DAD detector (Beckman Coulter, Fullerton, CA, USA). Separations performed with the Ph. Eur. BGE involved capillaries with a total length of 70 cm and an effective length of 60 cm. Capillaries were thermostated at 30 °C, the separation voltage was 15 kV and the detection wavelength was 195 nm. Samples were injected hydrodynamically for 7 s at 35 mbar (0.5 psi). Separations performed with BGEs of 300–400 mM Tris phosphate (pH 7.0–8.5) and 75 mM ammonium formate (pH 8.5) were used in combination with bilayer-coated capillaries with a total length of 60 cm and an effective length of 50 cm. The separation voltage was 30 kV, and detection was performed at 220 nm. The current for the BGE of 300 mM Tris phosphate (pH 7.0) typically was 120 μA, whereas for 400 mM Tris phosphate (pH 8.5) a typical current of 100 μA was observed. Samples were injected for 5 s at 35 mbar. Electropherograms were analyzed using 32 Karat Software, Version 4.01 (Beckman Coulter). Plate numbers were calculated from peak widths at half height.

CE–MS experiments were carried out using a PrinCE CE instrument (Prince Technologies, Emmen, The Netherlands) using PB–PVS coated capillaries with a length of 80 cm. The BGE used was 75 mM ammonium formate (pH 8.5) and the separation voltage was 30 kV. To enhance heat dissipation, the CE capillary was placed in a plastic tube (2 mm i.d.) through which a stream of air was continuously led through it. Samples were injected for 12 s at 35 mbar. To minimize adverse effects of the hydrodynamic flow in the CE capillary caused by the nebulizer gas, a reduced pressure in the range of −10 to −60 mbar was applied at the inlet vial during CE analysis; see Ref. [30] for further details.

2.3. MS system

CE was coupled to an Agilent Technologies 1100 Series LC/MSD XCT ion-trap mass spectrometer (Walbronn, Germany) via a coaxial sheath-flow electrospray interface (Agilent). The CE capillary outlet was positioned at 0.1–0.2 mm from the tip of the interface. A sheath liquid of acetonitrile–water–formic acid (75:25:5, v/v/v) was supplied by a syringe pump at a rate of 4 μL/min. The nebulizer-gas pressure was 700 mbar and the flow and temperature of the drying gas were 4 L/min and 200 °C, respectively. The electrospray voltage was 4.5 kV. MS detection was carried out in the positive ion mode and two scans were averaged to one spectrum. The ion charge control (ICC) was set to 100,000. Plate numbers of analyzed peptides were calculated using full width at half height as measured from peaks observed in extracted-ion electropherograms. Spectra were deconvoluted with the DataAnalysis (V. 2.1) software from Agilent Technologies.

3. Results and discussion

3.1. System optimization

Initially, we used the CE method for rhGH that was recently included in the Ph. Eur. [15]. A solution of rhGH CRS (3 mg/mL) was analyzed using a BGE of 100 mM ammonium phosphate (pH 6.0) in combination with a bare fused-silica capillary. CE analysis reveals one main peak (intact rhGH) with a plate number of ca. 40,000. According to the Ph. Eur. monograph, the CRS solution should also contain impurities: the so-called cleaved, Gln-18 and deamidated forms of rhGH. Indeed, some minor peaks that probably corresponded to these compounds were observed in the electropherogram (inset in Fig. 1A). It should be mentioned that other studies using the same separation conditions reported various migration times of rhGH (in the range of 50–75 min) [23,31]. In our hands, repeated analyses of the rhGH CRS yielded basically the same profile but migration times successively increased as more runs were performed (Fig. 1A). The migration-time R.S.D. ($n = 5$) for the main compound and the impurities was 3.1%. These shifts in migration times were most probably caused by protein adsorption onto the capillary inner wall, which affects the magnitude of the EOF. A poor repro-

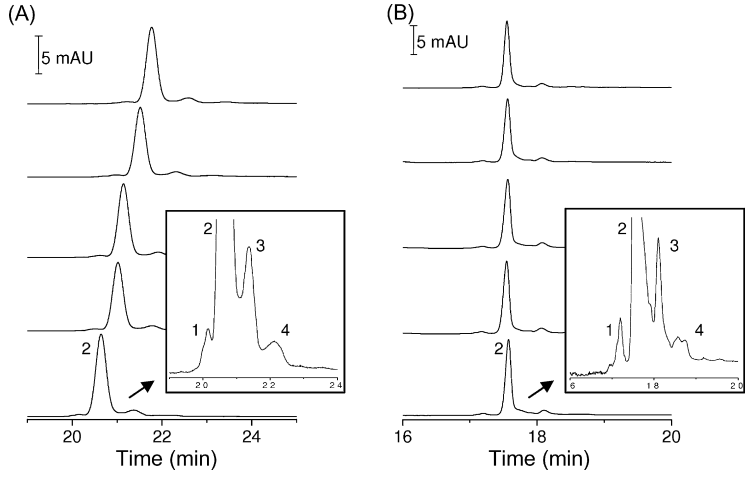

Fig. 1. Repeated CE–UV analysis of a solution of rhGH CRS (3 mg/mL) using Ph. Eur. conditions (see Section 2) in combination with (A) a bare fused-silica capillary, and (B) a PB–PVS coated capillary. Inserts show expanded views of selected time windows of bottom traces. Provisional peak assignment: 1, cleaved form; 2, intact rhGH; 3, Gln-18 rhGH; 4, deamidated rhGH.

ducibility in migration times has also been reported by others when using the Ph. Eur. method for the analysis of rhGH [23].

In a previous study, we have demonstrated that capillaries coated with a bilayer of PB and PVS are very suitable for the reproducible CE analysis of proteins at medium pH yielding a constant EOF [29]. Therefore, we used a PB–PVS coated capillary for the analysis of rhGH using the same BGE as in the Ph. Eur. method. Analysis time now was somewhat shorter, but peak width decreased considerably yielding a plate number of ca. 180,000 for the main compound. This gain in separation efficiency led to an improved resolution of rhGH and its related impurities. Moreover, the PB–PVS coated capillary presented much more stable and repeatable CE system with an R.S.D. of 0.68% ($n=5$) for the migration times of rhGH and the observed impurities (Fig. 1B). These experiments demonstrated that bilayer-coated capillaries had good potential for the CE-based characterization of rhGH samples.

Further gain in speed and separation efficiency could be achieved by using a separation voltage of 30 kV in combination with Tris phosphate buffers of pH 7.0 and 8.5 with relatively high concentrations. In our previous study [29] we have shown that BGEs of 300–400 mM Tris phosphate can provide high performance protein separations. As Tris ions contribute only modestly to the conductivity when compared to sodium ions, the current could still be kept within acceptable levels (100–120 μA) by using Tris phosphate instead of sodium phosphate. Analysis times obtained for rhGH CRS were about 7.6 and 9.0 min for the 400 mM Tris phosphate (pH 8.5) and 300 mM Tris phosphate (pH 7.0), respectively. Higher BGE concentrations could not be used due to excessive Joule heating. Migration-time reproducibilities for the main compound and the observed minor impurities were quite favorable. For the BGE of pH 8.5 the migration-time R.S.D.s were 0.97% ($n=5$), whereas the R.S.D.s at pH 7.0 were 0.92% ($n=5$). Peaks obtained with these BGEs were narrower than those obtained with the Ph. Eur. BGE. For the BGE at pH 8.5 and 7.0, plate number for intact rhGH were ca. 350,000 and 250,000, respectively. As 400 mM Tris phosphate (pH 8.5) yielded faster analysis and higher plate numbers, it was used in further experiments. It should be mentioned that hydrolysis of rhGH is facilitated at alkaline pH [32,33], however, in the short time frame of the CE analysis (less than 9 min) the actual degradation appeared to be negligible. This is confirmed by the fact that high plate numbers as well as symmetric peaks were still obtained at pH 8.5. Especially the formation of deamidation products (see below) during CE analysis would have led to peak deformation.

3.2. Thermal degradation of rhGH CRS

Stability testing should provide information on how the quality of drug substances or drug products varies with time under the influence of a variety of environmental factors [34]. Forced degradation studies are carried out in time by exposing drugs to various factors such as oxidative agents (e.g. hydrogen peroxide), intense light (photolysis) and heat (thermal degradation). During stability studies, it is of vital importance that stable separation profiles can be obtained allowing reliable comparisons and providing the ability to detect and characterize small changes in sample composition. In order to test the suitability of the bilayer-coated CE system, an aqueous solution of rhGH CRS was exposed to heat (40 °C) and aliquots were taken at regular time intervals and analyzed using a BGE of 400 mM Tris phosphate (pH 8.5) as it provided optimum performance (see Section 3.1). Next to the minor impurities present in rhGH CRS, the CE–UV system revealed a gradual formation of degradation products, which appear at a fixed position in each trace. First the originally minor peak at 7.9 min considerably increased, and prolonged thermal exposure yielded a second degradation product at 8.2 min (Fig. 2A). Because of the stable migration times, it was possible to reliably annotate the peaks among the successive electropherograms, which allowed the quantification of intact rhGH and degradation products (Fig. 2B) by measuring migration-time-corrected peak areas. This can be of great use for the determination of the degradation rate of biopharmaceuticals.

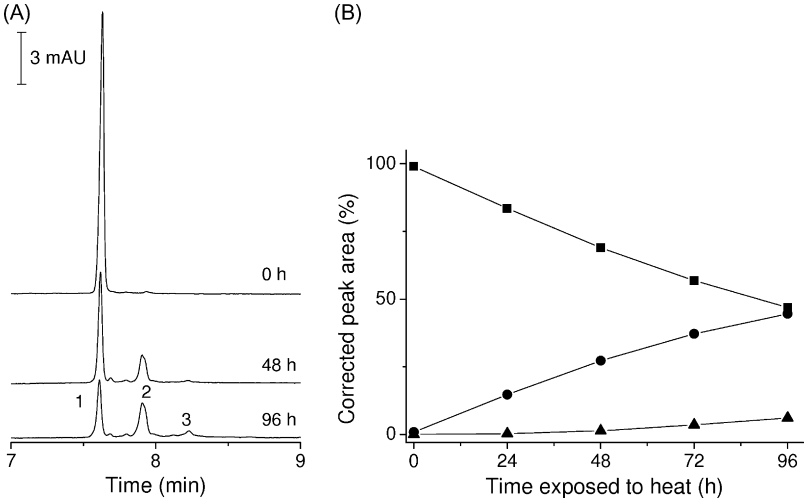

Fig. 2. (A) CE–UV analysis of aliquots taken at the indicated times of an rhGH CRS (3 mg/mL) sample solution exposed to 40 °C. A PB–PVS coated capillary was used with a BGE of 400 mM Tris phosphate (pH 8.5). Peaks: 1, intact rhGH; 2, degradation product; 3, degradation product. The internal standard (terbutaline) migrated at 4.6 min (peak not shown). (B) Migration-time-corrected peak areas of rhGH (■) and the two main degradation products (● and ▲).

Calculation of the electrophoretic mobilities of the compounds 1–3 (Fig. 2A) showed that the difference in electrophoretic mobility between compounds 1 and 2 matches the difference in mobility between compounds 2 and 3. As the electrophoretic mobility is proportional to the net charge of the protein, the mobility differences could be explained by discrete increments of charge. This would suggest that with respect to compound 1 (intact rhGH), compound 2 has gained one negative charge, and compound 3 has gained two negative charges. It is quite likely that deamidation of rhGH occurs when it is exposed to elevated temperatures [32,33,35]. During deamidation, an asparagine moiety is converted to aspartic acid adding a negative charge to the protein. The change in protein mass due to deamidation (i.e. 1 Da only) will practically not affect the protein mobility. Therefore, the compounds 2 and 3 might well be monodesamido and didesamido forms of rhGH, respectively.

CE–MS is a powerful tool for the characterization of (therapeutic) proteins since it allows one to effectively separate a complex mixture and gain mass information of analytes. In order to confirm the identity of degradation products, the 96-h stressed sample was analyzed by CE–MS using a PB–PVS coated capillary with a BGE of 75 mM ammonium formate (pH 8.5). The use of bilayer-coated capillaries in combination with volatile BGEs of pH 7–8.5 for CE–MS of proteins has shown to yield efficient and reproducible separations [36]. Fig. 3A shows the electropherogram obtained, revealing a separation profile that is comparable to the one obtained with CE–UV. The minor impurities present in the rhGH CRS (see Section 3.1) were not detected, most probably because their levels were below the detection limits for proteins of our CE–MS system. The deconvolution of the mass spectra obtained for the detected peaks (Fig. 3B–D) shows that the decomposition products have virtually the same mass as rhGH (22124 Da). This observation supports the suggestion that peaks 2 and 3 are desamido forms of rhGH. As stated above, mono and dideamidation adds only 1 or 2 Da, respectively, to the total protein mass. Detection of such a mass difference would require the use of a mass analyzer with significantly higher mass resolution than the used ion-trap instrument such as, e.g. a time-of-flight (TOF) instrument. It should be noted that these results also underline the importance of an efficient separation step prior to MS analysis.

3.3. Expired formulations of rhGH

Biopharmaceuticals are proteins that can easily suffer chemical or physical changes during storage, which might change or reduce their biological activity. Therefore, it is important to have analytical methodologies that are able to reliably detect, characterize and quantify degradation products in formulations. A liquid preparation of rhGH (Norditropin), which was expired 12 months beyond the approved shelf life of 24 months, was analyzed with CE–UV using a PB–PVS coated capillary and a BGE of 400 mM Tris phosphate (pH 8.5). Fig. 4A depicts the separation obtained for this sample, showing two degradation products. Migration times of these compounds matches those obtained for the desamido forms of rhGH mentioned above. This suggests that this preparation has undergone significant deamidation during long term storage. The sample was also analyzed with CE–MS using a bilayer-coated capillary and a BGE of 75 mM ammonium formate (pH 8.5) (Fig. 4B). Unfortunately, the sensitivity was not sufficient to reveal the second degradation product (cf. Fig. 4A). Mass spectra for the obtained peaks (Fig. 4C and D) were deconvoluted, showing a major mass of 22124 Da for both peaks, suggesting peak 2 indeed to be desamido rhGH. However, the deconvoluted spectra also revealed the presence of minor constituents with a mass of 22140 which co-migrated with both peaks. Such mass difference indicates that rhGH and desamido rhGH have been oxidized, which could be caused by the oxidation of a methionine residue, thus forming sulfoxide products. The presence of oxidized products in rhGH formulation has also been observed by others [37]. As oxidation induces a very small relative change in protein mass (and no change in

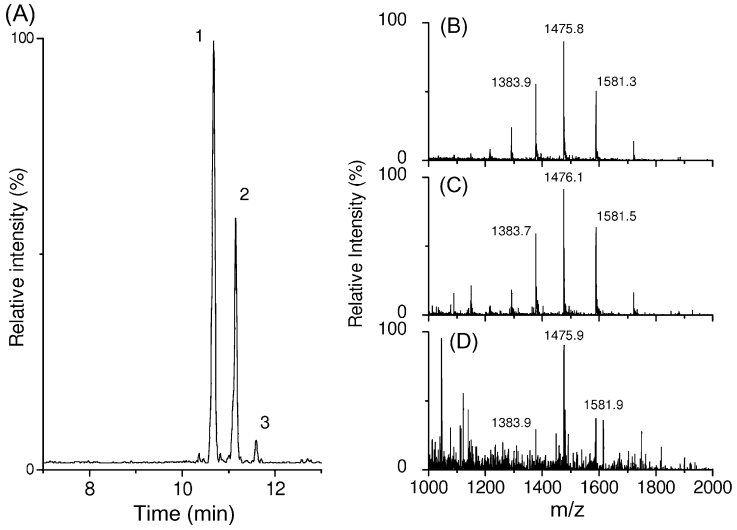

Fig. 3. CE–MS of an rhGH CRS sample exposed to heat (40 °C) for 96 h. A PB–PVS coated capillary was used with a BGE of 75 mM ammonium formate (pH 8.5). (A) Sum of extracted-ion electropherograms obtained at *m/z* 1475.9 and 1581.5; (B–D) averaged mass spectra of peak 1 (intact rhGH), 2 (degradation product), and 3 (degradation product), respectively.

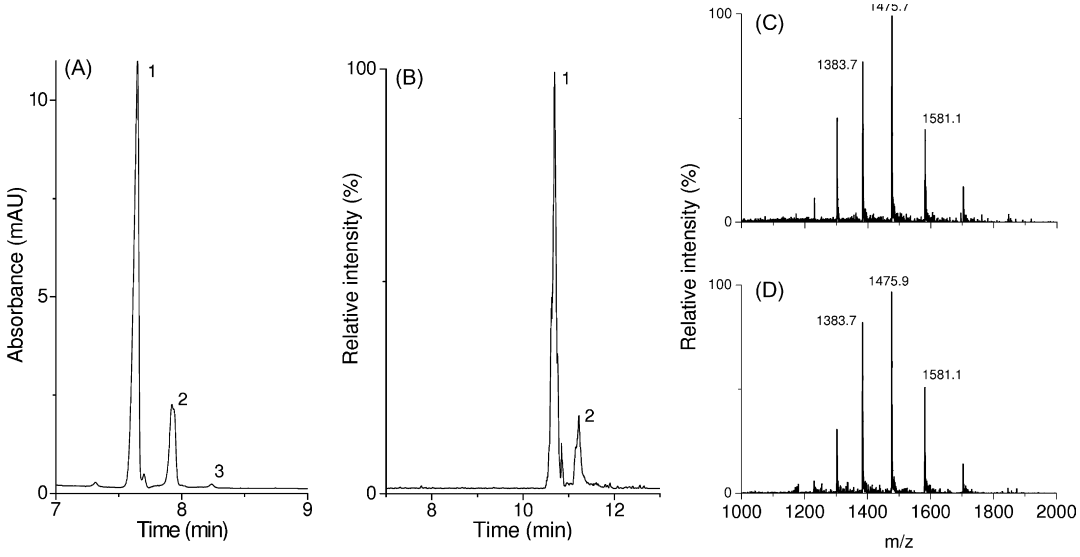

Fig. 4. Analysis of a 12-month expired liquid formulation of rhGH (Norditropin simplexx) by: (A) CE–UV and (B) CE–MS (sum of extracted-ion traces at *m/z* 1383.7 and 1475.8). (C and D) represent the averaged mass spectra of peaks 1 and 2, respectively. A PB–PVS coated capillary was used with a BGE of: (A) 400 mM Tris phosphate (pH 8.5) or (B) 75 mM ammonium formate (pH 8.5). Peaks: 1, intact rhGH; 2, degradation product; 3, degradation product.

the protein charge), its effect on the electrophoretic mobility is negligible, and thus no CE separation with the non-oxidized form can be expected.

The system using bilayer coatings and a BGE of 400 mM Tris phosphate (pH 8.5) was also used for the analysis of another expired rhGH preparation (Humatrope) which is provided in powder form and has to be reconstituted before analysis. CE–UV of this sample revealed two main degradation products appearing after the main peak (not shown). The first and main degradation product matched the migration time of the monodesamido form of rhGH. The migration time of the second degradation product, however, did not correspond to the time found earlier for the didesamido form of rhGH. In an attempt to identify these degradation products, CE–MS of this sample was performed using a bilayer-coated capillary and a BGE of 75 mM ammonium formate (pH 8.5). Fig. 5A presents the base peak electropherogram obtained, showing two degradation products at 11.1 and 11.5 min. Deconvolution of the mass spectra of peaks 1–3 (Fig. 5B–D) gave masses of 22,124, 22,140 and 22,252 Da, respectively. These masses suggest that peak 1 corresponds to intact rhGH, peak 2 to rhGH with one extra oxygen atom, and peak 3 to a yet unknown compound, respectively. The fact that the degradation products were separated from rhGH indicates that they have gained extra negative charge, most probably through deamidation. Identification of the second degradation product (peak 3) would require further experiments such as pep-

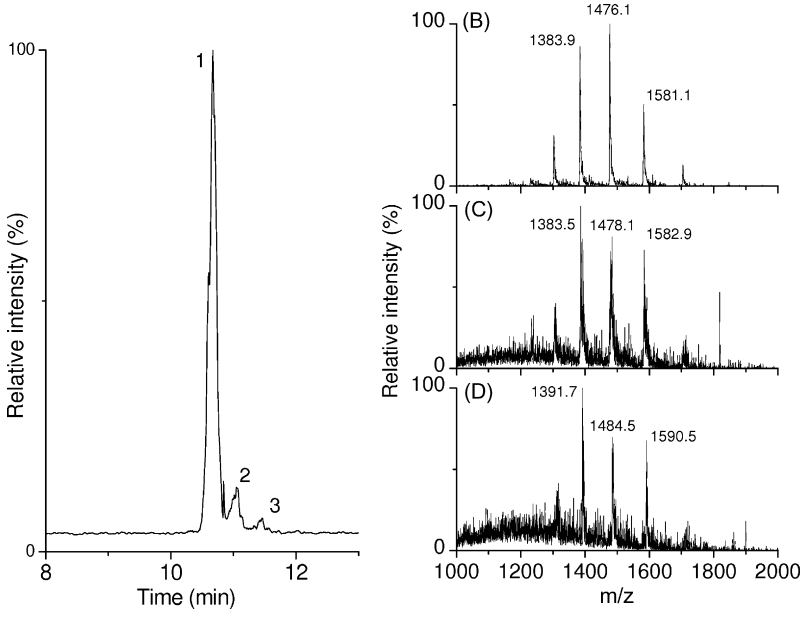

Fig. 5. CE–MS of an 18-month expired formulation of rhGH (Humatrope) stored in powder form and reconstituted before analysis. A PB–PVS coated capillary was used with a BGE of 75 mM ammonium formate (pH 8.5). (A) Base peak electropherogram; (B–D) averaged mass spectra of peaks 1–3, respectively.

tide mapping and/or the use of a TOF mass spectrometer to obtain the exact mass of the compound.

4. Conclusions

PB–PVS coated capillaries were used to characterize thermally stressed and aged samples of the biopharmaceutical rhGH by CE. Bilayer-coated capillaries are shown to yield faster and more efficient and reproducible separations than bare fuse-silica capillaries as used in the Ph. Eur. method for rhGH. This is most probably due to a minimized interaction between the proteins and the capillary wall when a coating is applied. Migration-time reproducibilities are highly favorable with the PB–PVS coated capillaries which exhibit a significant and constant EOF in the studied pH range (6.0–8.5). The stable migration times are of great value for consistent peak annotation and allow for reliable quantification of degradation products in stressed and expired samples. The bilayer coating is compatible with MS detection and CE–MS could be used to obtain further information on the identity of degradation products in rhGH samples. Overall, the developed CE system shows good potential for the analysis and characterization of biopharmaceuticals.

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