

Purity Assessment of Recombinant Human Granulocyte Colony-stimulating Factor in Finished drug Product by Capillary Zone Electrophoresis

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Research Article

Purity assessment of recombinant human granulocyte colony-stimulating factor in finished drug product by capillary zone electrophoresis

Current methods for determination of impurities with different charge-to-volume ratio are limited especially in terms of sensitivity and precision. The main goal of this research was to establish a quantitative method for determination of impurities with charges differing from that of recombinant human granulocyte colony-stimulating factor (rhG-CSF, filgrastim) with superior precision and sensitivity compared to existing methods. A CZE method has been developed, optimized, and validated for a purity assessment of filgrastim in liquid pharmaceutical formulations. Optimal separation of filgrastim from the related impurities with different charges was achieved on a 50 μm id fused-silica capillary of a total length of 80.5 cm. A BGE that contains 100 mM phosphoric acid adjusted to pH 7.0 with triethanolamine was used. The applied voltage was 20 kV while the temperature was maintained at 25°C. UV detection was set to 200 nm. Method was validated in terms of selectivity/specificity, linearity, precision, LOD, LOQ, stability, and robustness. Linearity was observed in the concentration range of 6–600 $\mu\text{g}/\text{mL}$ and the LOQ was determined to be 0.3% relative to the concentration of filgrastim of 0.6 mg/mL. Other validation parameters were also found to be acceptable; thus the method was successfully applied for a quantitative purity assessment of filgrastim in a finished drug product.

Keywords:

Capillary zone electrophoresis / Filgrastim / Purity / Recombinant human granulocyte colony-stimulating factor / Validation DOI 10.1002/elps.201300552

1 Introduction

Granulocyte colony-stimulating factor (G-CSF), one of hematopoietic growth factors, belongs to the group of regulatory proteins and peptides known as cytokines that modulate functional activity of individual cells or tissues in the body [1]. It plays an important role in stimulating proliferation and maturation of the granulocyte lineage cells as well as activation of neutrophils in vitro and in vivo [2]. Filgrastim, a recombinant human granulocyte-colony stimulating factor (rhG-CSF), is used primarily to reduce the incidence and duration of neutropenia of different etiology [3]. This nonglycosylated, 175 amino acid polypeptide with molecular mass of 18.8 kDa, has identical sequence as compared to G-CSF isolated from human cells, except for additional methionine at its N-terminus [4]. The absence of glycosylation on the

molecule, and modification of the N-terminus does not have a significant impact on biological activity of filgrastim [5] that is comparable to the activity of endogenous protein.

It is generally a challenge to characterize biological drug due to the fact that many of the contaminants resulting from the active substance are very similar and at the same time have complex structures [6]. Various chromatographic techniques and methods [7–11] provide information about presence of the contaminants in liquid filgrastim formulations, but none of them can be used as standalone to provide satisfactory information on the purity of the product. It has been shown that there is an inverse correlation between the content of related impurities and biological activity of filgrastim in injections using liquid chromatographic methods and mathematical model for the estimation of biological activity [12]. CZE was used to achieve separation of one nonglycosylated and two glycosylated forms of filgrastim, while the estimation of the purity of nonglycosylated filgrastim was achieved using CIEF with characterization by MS [13]. According to the monograph of the European Pharmacopoeia (Ph. Eur.), filgrastim-related impurities for concentrated filgrastim solution are divided into the following groups: (i) impurities with molecular masses higher than that of filgrastim; (ii) impurities with molecular masses differing from that of filgrastim; (iii) impurities with charges differing from that of filgrastim;

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Abbreviations: ICH, International Conference on Harmonization; RH, relative humidity; rhG-CSF, recombinant human granulocyte colony-stimulating factor; TEA, triethanolamine

and (iv) other filgrastim-related proteins [14]. Related compounds in groups (i) and (iv) were evaluated by SEC and RP-HPLC methods, respectively, whereas the related compounds in groups (ii) and (iii) were evaluated using the gel electrophoresis and IEF, respectively.

According to the literature [14], the most reliable determination of filgrastim purity is currently obtained using chromatographic and electrophoretic separation techniques. Moreover, there is an increasing recognition of the complementarities of CE and HPLC in the assessment of purity of drugs due to their differences in the mechanisms of separation that results in different selectivity [15–19]. While the advantages of conventional gel electrophoresis and isoelectric focusing are recognized and used to assess purity of filgrastim in the *Ph. Eur.* monograph for filgrastim concentrated solution [14], the abilities of CE in the same area are inadequately studied [11].

The aim of this work was to establish a quantitative method for determination of impurities with charges differing from that of filgrastim with superior precision and sensitivity compared to isoelectric focusing method described in *Ph. Eur.* based on theoretical advantages of CE over conventional electrophoresis. Our work explores the potential utilization of CZE for the separation of filgrastim-related impurities that might be present in the finished drug. For this reason, a CZE method was developed, and after the selection of optimal separation conditions, the method has been validated according to International Conference on Harmonization (ICH) guidelines [20]. Normalization method has been selected for the quantification of unknown impurities while the validation included determinations of the following parameters: selectivity/specificity, linearity, precision, LOD, LOQ, stability, and robustness.

2 Materials and methods

2.1 Chemicals and reagents

Commercially available finished product Neupogen® 30, Roche, Basel, Switzerland, lot B1047 containing filgrastim (300 µg/0.5 mL) was used throughout this study. Phosphoric acid was purchased from Merck, Darmstadt, Germany, and triethanolamine (TEA) from Fluka, Buchs, Switzerland. Hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide (H₂O₂) were obtained from Kemika, Zagreb, Croatia. Ultrapure water was used in all analyses.

2.2 BGE and sample solution

Optimal BGE consisted of a 100 mM phosphoric acid dissolved in water. pH was adjusted to 7.0 by adding TEA. The buffer was filtered through a 0.22-µm membrane filter (Millipore, Billerica, MA, USA).

Undiluted injection solution of Neupogen® 30 was used for quantitative determination of related impurities with charges differing from that of filgrastim.

2.3 Apparatus

All analyses were performed on an HP 3DCE apparatus (Agilent Technologies, Waldbronn, Germany), equipped with an autosampler, DAD, temperature control of capillary cassette (4–60°C), and a power supply able to deliver up to 30 kV. CE Chem Station software was used for instrument control, data acquisition, and analysis. BGE pH was adjusted using pH meter to within ± 0.02 pH units.

2.4 CE analysis parameters

Fused-silica capillary 50 µm id (Composite Metal, UK) with total length of 80.5 cm (effective length 72 cm), thermostated at 25°C and DAD-detection at 200 nm were selected for separation of impurities under optimal conditions. Prior to each analysis, the capillary was conditioned first with water, 3 min, 900 mbar, and then with BGE solution 10 min, 900 mbar. In order to achieve better reproducibility of migration time and also better efficiency of separation between injections, the working buffer was replaced by a fresh solution before each injection. Samples were injected using hydrodynamic injection for 8 s at 50 mbar; constant voltage of 20 kV was applied during the analysis.

2.5 Forced and accelerated degradation conditions

In order to evaluate selectivity/specificity of the proposed method, forced degradation studies were performed in acidic, alkaline, and oxidative conditions, and also by heating of the sample solution. Acidic hydrolysis was induced by using solution of 0.1 M HCl and alkaline hydrolysis using solution of 0.1 M NaOH. Thermal decomposition was induced by heating the sample solution at 80°C for 30 min. Oxidative conditions were achieved by adding 3% solution of H₂O₂ in the sample solution. For the purpose of accelerated degradation studies, samples were stored under controlled conditions of over a long period of time. One sample was stored for 3 months at 25°C/60% relative humidity (RH) and another for 3 months at 40°C/75% RH.

2.6 Validation of the CE method

CZE method for assessment and determination of potential impurities with charges differing from that of filgrastim in liquid pharmaceutical formulations was validated in terms of: selectivity/specificity, linearity, precision, LOD, LOQ, robustness, stability, all according to the ICH guideline Q2(R1) [20].

3 Results and discussion

3.1 Optimization of electrophoretic conditions

During the optimization we focused on the adjustment of parameters affecting primarily resolution/selectivity and sensitivity in order to obtain maximum selectivity with sufficient sensitivity. Method development has started with the selection of BGE. In order to obtain adequate separation conditions some electrolyte solutions containing various combinations of phosphoric acid, Tris, TEA, trimethylammonium propane sulfonate, SDS, and lithium chloride were investigated and the best results were achieved with a combination of phosphoric acid and triethanolamine. The effects of pH were tested over a range from 3.0 to 10.0. In the pH range around the *pI* of filgrastim, separation was not possible, and the best resolution was achieved in the pH range from 7.0 to 8.0. Impurity profiles varied considerably with small changes in pH, and pH 7.0 has proven to be optimal based on the obtained separation. Resolution between the main peak of filgrastim and the most abundant of all impurities — impurity A served as a useful indication of separation power of the method. It was found that resolution between filgrastim and impurity A decreases as pH of the BGE changes from 7.0 in both directions. Influence of small changes of pH in range from 7.0 to 8.0 is shown in Fig. 1. The temperature at which the separation was performed was varied from 16 to 40°C; 25°C was chosen as optimal because of favorable relationship between peak widths and differences in migration times thus providing the optimal separation conditions. Influence of ionic strength was tested by increasing the concentration of phosphoric acid ranging from 20 mM to 140 mM. Generally, the increase in ionic strength had a positive effect on selectivity, but this increase was limited because of a simultaneous increase of Joule heating in the capillaries and prolonged duration of the analysis, which together had a negative impact on the reproducibility of analysis and on the sensitivity. Therefore, a solution of 100 mM of phosphoric acid was selected as optimal. The impact of voltage was tested in the range from 5 to 30 kV and a voltage of 20 kV (0–20 kV in 1 min) was chosen as optimal due to the favorable balance of separation efficiency, analysis time, and current in the capillary. Capillaries with internal diameters of 75 and 50 μm have been tested, and although the larger internal diameter capillary had the advantage of better sensitivity, a smaller internal diameter capillary was chosen due to a considerably better selectivity and separation efficiency. Capillary length was varied in the range from 21.5 to 111.5 cm eff. and the length of 72 cm eff. was selected on the same principles as the ionic strength and/or voltage, because at this length the optimum ratio of selectivity, sensitivity, and reproducibility is obtained. Wavelength was examined between 190 and 300 nm, and the best signal-to-noise ratio was obtained at 200 nm. Special attention was paid to reproducibility testing, and for this purpose conditioning of the capillary was carried out in detail. Immediately before each injection, the capillary was flushed with water for 3 min, followed by flushing by BGE

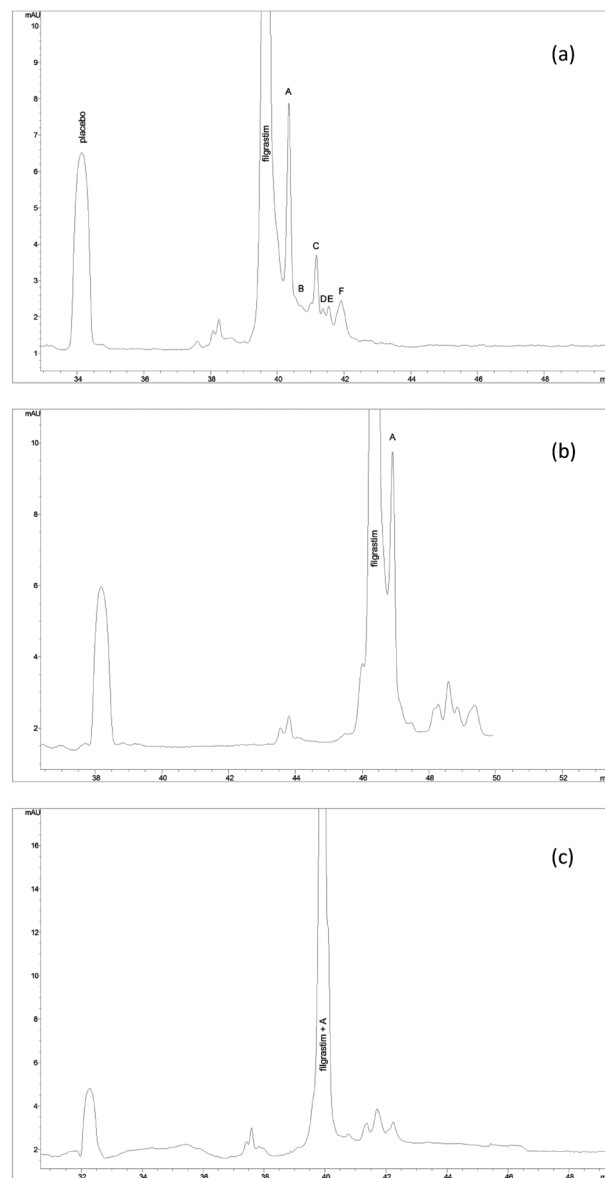
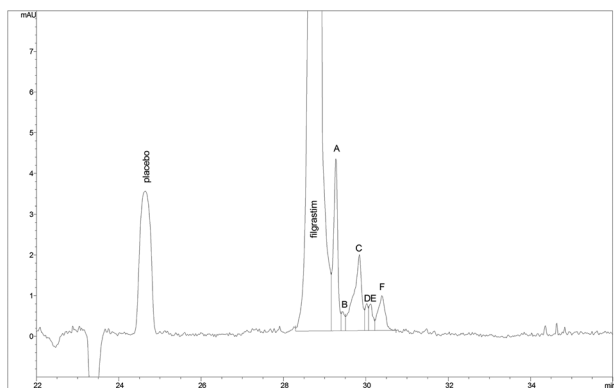


Figure 1. Electropherogram of impurity profile of Neupogen 30 at: (A) pH 7.0. Working conditions: 125 mM phosphoric acid buffer, 20 kV, 25°C, 400 mbar*s injection, 81.5 cm eff./50 μm fused-silica capillary. R (filgrastim, impurity A) = 2.6; (B) pH 7.5. Working conditions: 125 mM phosphoric acid buffer, 20 kV, 25°C, 400 mbar*s injection, 81.5 cm eff./50 μm fused-silica capillary. R (filgrastim, impurity A) = 1.4; (C) pH 8.0. Working conditions: 100 mM phosphoric acid buffer, 20 kV, 25°C, 400 mbar*s injection, 81.5 cm eff./50 μm fused-silica capillary. Filgrastim and impurity A are not separated.

electrolyte for 10 min (all at 900 mbar). As the prerequisite for the reliable separation, a 30 min conditioning preceding the injection has been introduced. Also, an important detail that ensures reproducibility of the analysis is the replacement of the BGE electrolyte with a fresh one between injections. In addition, there was an attempt of injecting the water plug in front of the sample, so-called “sample stacking,” but without any improvement. A summary of optimization of the

Table 1. Optimization of the CZE conditions for purity analysis of filgrastim in liquid pharmaceutical formulations

| Parameter | Investigated range | Optimal value |
|------------------------------------|--------------------|---------------|
| Phosphoric acid concentration (mM) | 20–140 | 100 |
| Temperature (°C) | 16–40 | 25 |
| Injection (mbar*s) | 50–600 | 400 |
| Wavelength (nm) | 191–300 | 200 |
| pH | 3.0–10.0 | 7.0 |
| Applied voltage (kV) | 5–30 | 20 |
| Capillary length, total (cm) | 30–120 | 80.5 |

**Figure 2.** Representative electropherogram of impurity profile of Neupogen 30. Peaks labeled A–F present unknown impurities related to filgrastim. Integration events: Slope sensitivity 0.6; peak with 0.01; area reject 1; height reject 0.4; shoulders OFF (for integration of impurities B, D, and E shoulders ON was used).

electrophoretic parameters is given in Table 1, and representative electropherogram of the impurity profile of Neupogen[®] 30 obtained after running optimized CZE method is shown in Fig. 2.

3.2 Method validation

3.2.1 Definition of the method

The method was intended for quantification of impurities with different charge-to-volume ratio from that of filgrastim in the finished drug Neupogen[®] 30 by normalization procedure (area% – area of the given peak is expressed as a percentage of the sum of the areas of all peaks).

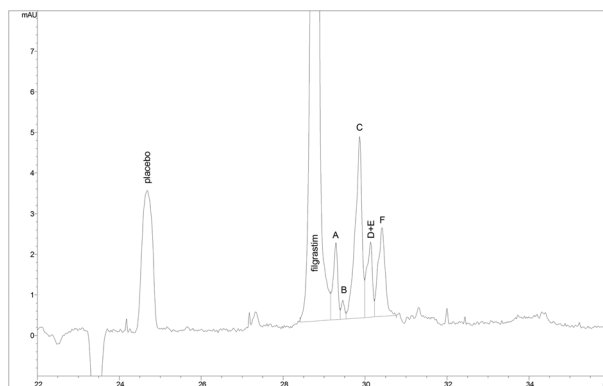
3.2.2 Selectivity/specificity

Selectivity/specificity was evaluated through forced and accelerated degradation studies. The applied conditions are described in Section 2.5.

Under the acidic conditions of forced degradation, there was a slight increase in the content of impurities over a time at the expense of reduction of filgrastim content (Table 2).

Table 2. Impurity analysis of samples treated with 0.1 M HCl

| Sample treatment with 0.1 M HCl | Filgrastim (%) | A (%) | B (%) | C (%) | D + E (%) | F (%) |
|---------------------------------|----------------|-------|-------|-------|-----------|-------|
| Without | 95.0 | 2.0 | 0.2 | 1.7 | 0.5 | 0.6 |
| Immediately after | 91.1 | 2.1 | 0.2 | 2.7 | 1.0 | 2.9 |
| After 15 h | 86.9 | 2.1 | 0.4 | 3.8 | 1.6 | 5.0 |
| After 35 h | 83.4 | 2.3 | 0.5 | 4.9 | 2.2 | 6.7 |

**Figure 3.** Electropherogram of impurity profile of sample stored for 3 months at 40°C/75% relative humidity. Peaks labeled A–F present unknown impurities related to filgrastim. Integration events: Slope sensitivity 0.6; peak with 0.01; area reject 1; height reject 0.4; shoulders OFF.**Table 3.** Impurity analysis of samples stored for 3 months in controlled conditions

| Storage conditions | Filgrastim (%) | A (%) | B (%) | C (%) | D + E (%) | F (%) |
|--------------------|----------------|-------|-------|-------|-----------|-------|
| Prior to storage | 95.0 | 2.0 | 0.2 | 1.7 | 0.5 | 0.6 |
| 25°C/60% RH | 84.2 | 6.3 | 0.5 | 5.0 | 1.7 | 2.3 |
| 40°C/75% RH | 79.3 | 2.8 | 0.7 | 9.0 | 3.2 | 5.0 |

RH, relative humidity.

Under alkaline and oxidative degradation conditions, filgrastim has been almost completely degraded and there was no possibility of getting any meaningful data analysis concerning these samples. Under the influence of high temperature, there was a significant decrease in the content of filgrastim in the treated sample solution (due to a possible coagulation and/or precipitation), but there was no increase in the amount of impurities or the appearance of new ones. Storing of the samples over a long period of time under controlled conditions mentioned above, resulted in a slight decrease in the content of filgrastim at the expense of an increased amount of existing impurities (Fig. 3). Expectedly, this change was more pronounced in the sample exposed to higher temperature and relative humidity (40°C/75% RH) (Table 3). Possible interference of the excipients was studied by the injection of placebo (sample containing in-house mixture of all excipients). No interference was observed. The results of the selectivity tests showed that the method is sensitive to changes in the impurity profile and that these changes can be quantitatively

Table 4. Overall precision of the method

| Day/analyst | | Filgrastim | A | B | C | D + E | F |
|-------------|-------------------------|------------|-------|-------|------|-------|------|
| 1 | Mean ^{a)} (%) | 95.0 | 2.0 | 0.2 | 1.7 | 0.5 | 0.6 |
| | RSD ^{b)} (%) | 0.15 | 4.55 | 22.26 | 5.63 | 10.38 | 6.80 |
| 2 | Mean ^{a)} (%) | 93.7 | 2.5 | 0.5 | 1.9 | 0.7 | 0.7 |
| | RSD ^{b)} (%) | 0.23 | 3.19 | 14.00 | 4.81 | 6.15 | 1.47 |
| | Mean of day 1 and 2 (%) | 94.4 | 2.3 | 0.4 | 1.8 | 0.6 | 0.7 |
| | RSD of day 1 and 2 (%) | 0.97 | 15.65 | 65.27 | 8.29 | 23.73 | 6.75 |

a) Mean of six measurements.

b) RSD, relative standard deviation.

monitored. As evident from the changes in the impurity profile and their quantification, the method is suitable for the separation and quantification of potential contaminants formed by moderate decomposition of filgrastim and therefore, it is suitable for quality control of filgrastim liquid pharmaceutical formulations.

3.2.3 Linearity

Linearity was determined in the range of 1–100%, which corresponds to filgrastim concentrations of 6–600 µg/mL. The amount of impurities with different charges is limited to 10% of each by European Pharmacopoeia [14]. Nevertheless, a calibration up to 100% was made due to the fact that the normalization procedure (area%) was used to calculate the amount of the impurities. A calibration curve was constructed based on filgrastim peak area measurements at six concentration levels (1, 5, 20, 40, 60, and 100%). Solutions were prepared by diluting the filgrastim finished drug injection solution of the declared concentration of 600 µg/mL. Dilutions were made with working buffer, and each solution was injected twice. A linear relationship was determined by linear regression using least squares method, which resulted in the regression curve $y = (3.709 \pm 0.034)x - (14.633 \pm 10.478)$ with a determination coefficient $R^2 = 0.9991$, where x is concentration (µg/mL) and y corrected peak area of filgrastim (mAU*s) in the measuring solutions. Relative standard error of the slope can be taken as a parameter that describes the precision of regression, as well as the general criteria for the acceptability of the linearity of analytical method. This parameter should be comparable to the RSD obtained in the test of method precision. The result obtained for the RSD of the slope was 0.9% and it was comparable with the RSD of 0.2% obtained in the precision test (or 1.0%, which was the overall precision, also including the results for the within-laboratory reproducibility).

3.2.4 Precision

Precision of the method was determined in terms of repeatability and within-laboratory precision. Repeatability was determined by six consecutive injections of the finished product sample solution (nominal concentration of 600 µg/mL of filgrastim). Repeatability was checked in the same way for the

diluted sample containing filgrastim at the concentration of 6 µg/mL (1% of the nominal concentration). The value of RSD for major peak of filgrastim in undiluted sample was 0.15%, while RSDs for the six detected impurities arbitrarily designated from A to F were: 4.55, 22.26, 5.63, 10.38, and 6.80%, respectively (Due to the lack of resolution among them, impurities D and E were calculated as one impurity D+E where their areas were summed up and RSD% was calculated for the precision of impurity D+E.). The RSD of the peak area of filgrastim for six consecutive injections in the diluted sample of the concentration near LOQ 6 µg/mL (1%) was 3.12%. Within-laboratory reproducibility was determined in a way that the sample was analyzed by different analyst on different days with preparation of a new working buffer and by utilization of a new capillary. Undiluted sample was injected six times consecutively. The value of relative standard deviations for the major peak of filgrastim in undiluted sample was 0.23%, while RSDs for the six detected impurities amounted to: 3.19, 14.00, 4.81, 9.10, 6.16, and 1.47%. Overall results for the within-laboratory reproducibility are given in Table 4.

Obtained results indicate a good reproducibility of the method, suggesting that the estimation of the amount of filgrastim-related impurities in the finished product can be made with sufficient precision. Likewise, the values of RSDs for impurities realistically reflect the limitation of achieved resolution among impurities, which are not baseline separated, as well as the limited sensitivity of the method. Amounts of impurities A, C, and F can be estimated without any major difficulties and with sufficient precision. The amount of impurity B is below the LOQ, while the assessment of impurities D and E is aggravated due to the insufficient resolution and the quantities that are close to the limit of quantification, but still with sufficient precision.

3.2.5 Limits of quantification and detection

LOQ and LOD were determined experimentally on the basis of signal to noise ratio according to the guidelines given in the ICH Q2(R1) document. Signal to noise ratio of 10:1 was used as the limit of quantification; due to problems with the dilution of the sample [21] the ratio was determined by computing on the basis of characteristics of the signals in 1%

solution (6 $\mu\text{g}/\text{mL}$). Calculated using this approach, LOQ was 2.0 $\mu\text{g}/\text{mL}$, i.e. 0.34% compared to the declared concentration of filgrastim in a finished drug. Good reproducibility that was achieved at a concentration level of 1% (RSD% = 3.12%) is also expected at the LOQ. LOD was calculated in the same way; the signal to noise ratio of 3:1 was taken as the LOD, and it was 0.6 $\mu\text{g}/\text{mL}$, i.e. 0.10% of the declared concentration of filgrastim in a finished drug. Thus, the determined values of LOQ and LOD refer to filgrastim, i.e. the major peak. Looking at Fig. 1 it is clear that impurity B as well as D and E are not really resolved. For these impurities the values of LOQ and LOD will definitely be higher. This can also be seen at the high RSD values found in precision tests for these compounds as they cannot be reliably determined.

3.2.6 Robustness

The robustness of this method was determined during its development, using variations of certain parameters. Since the resolution among impurities is the major criteria according to which the method was developing, evaluation of the robustness is based on the impact of changes on the separation. Thus it was determined that small changes in the concentration of BGE (± 2 mM), capillary temperature ($\pm 2^\circ\text{C}$), operating voltage (± 2 kV), wavelength of detection (± 2 nm) do not significantly affect the impurity profile and the electropherogram appearance in general. Unlike these parameters, the pH of the working buffer must be accurately set to 7.00 ± 0.02 , because the change of just ± 0.1 unit has a negative effect on the separation. Likewise, injection time must be accurately set to 8 s (with the hydrodynamic pressure of injection set to 50 mbar). If the time is extended by 1 s, an adverse effect on the resolution occurs, and if it is reduced by 1 s, the sensitivity is reduced.

3.2.7 Stability

The stability of filgrastim in the sample solution was tested in the following way: a microvial has been stored in autosampler and it was protected from light at room temperature. Injections from the same microvial were made at different time intervals during a period of 51 h. The slope of the obtained line was -0.0103 while the intercept on the y -axis was 95.1% with RSD of 0.28%. These results clearly indicate that the sample is stable and that it can be reliably determined during a period of at least 51 h.

3.2.8 System suitability

A system suitability test was introduced in order to make analysis useful for other laboratories. From validation studies, it can be seen that the reproducibility of peak areas of filgrastim and the resolution between main peak of filgrastim and the most abundant of all impurities — impurity A are critical parameters for effective determination of impurities

and filgrastim amount. Therefore, a quantitative estimation of filgrastim and related impurities should be carried out by five consecutive injections of the sample solution. The system suitability test showed RSD value of 0.2% for the peak area of filgrastim ($< 1.0\%$ is acceptable) and the lowest value for resolution between filgrastim and impurity A was 1.7 (> 1.5 is acceptable). The tested parameters were within the acceptable range, indicating that the method is suitable for the analysis intended.

3.3 Comparison of the purity of filgrastim in biosimilar formulations assessed with other techniques

The results regarding impurity levels obtained during this research could be compared with literature reported impurity profiling during degradation studies with biosimilar filgrastim formulations [9]. Purity of filgrastim was assessed under accelerated degradation conditions (40°C for 12 weeks) with various techniques such as RP-HPLC, IEC, SDS-PAGE, and IEF. After 12 weeks at 40°C , increased levels of filgrastim impurities were detected with all of these techniques. This is in line with the increased impurity levels observed in samples stored under the stress conditions during this research. RP-HPLC and IC methods revealed a decrease in filgrastim content and could measure it quantitatively. The decrease of filgrastim content found in samples stored under the stress conditions during this research was also consistent with reported levels in these studies.

A direct comparison could be made between the proposed CZE method and pharmacopoeial IEF method [14] utilized in reported degradation studies due to the fact that both methods are able to determine impurities with charges differing from that of filgrastim. It is not clear if the proposed CZE method could also separate proteins with different masses that would then make possible to assess additional set of analytes by this method. This possibility could not be sufficiently justified based on theoretical principles of CZE separation only. Perhaps, some additional experimental work in different CE operational modes, especially in capillary gel electrophoresis could provide some useful information in relation to this issue. Although it was possible to detect an increase in impurities with IEF method, this was done by estimation of band intensities compared to the intensity of the reference solution band at the concentration level of 10% of the sample solution. Thus, LOQ of IEF method was 10%. At the same time, it was not possible to determine a decrease in filgrastim content with IEF method. On the other hand, with proposed CZE method it was possible to measure quantitatively both changes in filgrastim content and increase in impurities with different charges at the same time (Table 3). This comparison indicates that the chosen approach of development and validation of the proposed CZE method resulted with significant improvement in purity assessment of filgrastim in liquid formulations giving quantitative insight

in the stability of filgrastim in stressed conditions with precise quantification of impurities with different charges.

4 Concluding Remarks

A CZE method for purity assessment of filgrastim in liquid pharmaceutical formulations has been developed and validated. Based on the obtained validation results, it can be concluded that proposed method is selective/specific, precise, linear, robust, and sensitive enough for detection and quantification of impurities with charges differing from that of filgrastim. The method is characterized by calculated LOQ of 0.3% in comparison to filgrastim concentration of 0.6 mg/mL that is present in the finished product. This value refers to the main peak of filgrastim while it is expected to be higher for some of the less efficiently resolved impurities. Due to its wide range of linearity, good precision, and acceptable selectivity this method enables detection and quantification up to six unknown filgrastim-related compounds and even the determination of the filgrastim content in a single run. The proposed method is almost two orders of magnitude more sensitive compared to the IEF method published in Ph. Eur. [14] and at the same time it is expected that it is at least as precise as the Ph. Eur. IEF method. Therefore, it can be used for filgrastim impurities determination during different stages of product and process development and also for the quality control of the finished product.

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5 References

- [1] Barreda, D. R., Hanington, P. C., Belosevic M., *Dev. Comp. Immunol.* 2004, 28, 509–554.
- [2] Metcalf, D., *Science* 1985, 229, 16–22.
- [3] Siena, S., Secondino S., Giannetta, L., Carminati, O., Pedrazzoli, P., *Crit. Rev. Oncol. Hematol.* 2003, 48S, S39–S47.
- [4] Lu, H. S., Clogston, C. L., Narhi, L. O., Merewether, L. A., Pearl, W. R., Boone, T. C., *J. Biol. Chem.* 1992, 267, 8770–8777.
- [5] Tanaka, H., Tanaka, Y., Shinagawa, K., Yamagishi, Y., Ohtaki, K., Asano, K., *Cytokine* 1997, 9, 360–369.
- [6] Barnes, H. J., Ragnarsson, G., Alván, G., *Int. J. Risk. Saf. Med.* 2009, 21, 13–22.
- [7] Codevilla, C. F., Brum Jr., L., De Oliveira, P. R., Dolman, C., Rafferty, B., Dalmora, S. L., *J. Liq. Chrom. Relat. Technol.* 2004, 27, 2689–2698.
- [8] Dalmora, S. L., Krug Masiero, S. M., De Oliveira, P. R., Da Silva Sangoi, M., Brum Jr. L., *J. Liq. Chrom. Relat. Technol.* 2006, 29, 1753–1767.
- [9] Skrlin, A., Radic, I., Vuletic, M., Schwinke, D., Runac, D., Kusalic, T., Paskvan, I., Krsic, M., Bratos, M., Marinc, S., *Biologicals* 2010, 38, 557–566.
- [10] Reubsaet, J. L. E., Beijnen, J. H., Bult, A., Hop, E., Scholten, S. D., Teeuwesen, J., Underberg, W. J. M., *J. Pharm. Biomed. Anal.* 1998, 17, 283–289.
- [11] Reubsaet, J. L. E., Beijnen, J. H., van Bennekom, W. P., Bult, A., Hoekstra, A. J., Hop, E., van Os, P. J. H. J., Teeuwesen, J., Underberg, W. J. M., *J. Pharm. Biomed. Anal.* 1999, 19, 837–845.
- [12] Skrlin, A., Krnic, E. K., Gosak, D., Prester, B., Mrsa, V., Vuletic, M., Runac, D., *J. Pharm. Biomed. Anal.* 2010, 53, 262–268.
- [13] Zhou, G. H., Luo, G. A., Sun, G. Q., Cao, Y. C., Zhang, X. D., Zhang, X., *J. Pharm. Biomed. Anal.* 2004, 35, 425–432.
- [14] European Pharmacopoeia 7.6., Filgrastim concentrated solution 07/2010:2206 corrected 7.6., EDQM, Council of Europe, 2012.
- [15] Engelhardt, H., Beck, W., Schmitt, T., *Capillary Electrophoresis Methods and Potentials*, Friedr. Vieweg & Sohn Verlagsgesellschaft mbH, Wiesbaden 1996.
- [16] Huikko, K., Kostianen, R., *J. Chromatogr. A* 2000, 893, 411–420.
- [17] Fernandez, H., Ruperez, F. J., Barbas, C., *J. Pharm. Biomed. Anal.* 2003, 31, 499–506.
- [18] Bertinatto Rodriguez, J. A., Desimone, M. F., Iglesias, S. L., Giorgieri, S. A., Diaz, L. E., *J. Pharm. Biomed. Anal.* 2007, 44, 305–308.
- [19] Sungthong, B., Jač, P., Scriba, G. K. E., *J. Pharm. Biomed. Anal.* 2008, 46, 959–965.
- [20] International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH), November 2005. Validation of analytical procedures: text and methodology Q2(R1).
- [21] Sweetman, S. C. (Ed), *Martindale: The Complete Drug Reference*, 37th ed. Pharmaceutical Press, London 2011.