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Correlation of liquid chromatographic and biological assay for potency assessment of filgrastim and related impurities

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ABSTRACT

In vivo and *in vitro* potency assays have always been a critical tool for confirmation of protein activity. However, due to their complexity and time consuming procedures, it remains a challenge to find an alternative analytical approach that would enable their replacement with no impact on the quality of provided information. The goal of this research was to determine if a correlation between liquid chromatography assays and *in vitro* biological assay could be established for filgrastim (recombinant human granulocyte-colony stimulating factor, rhG-CSF) samples containing various amounts of related impurities. For that purpose, relevant filgrastim related impurities were purified to homogeneity and characterized by liquid chromatography and mass spectrometry. A significant correlation ($R^2 > 0.90$) between the two types of assays was revealed. Potency of oxidized filgrastim was determined to be approximately 25% of filgrastim stated potency (1×10^8 IU/mg of protein). Formyl-methionine filgrastim had potency of 89% of the filgrastim stated potency, while filgrastim dimer had 67% of filgrastim stated potency. A mathematical model for the estimation of biological activity of filgrastim samples from chromatography data was established and a significant correlation between experimental potency values and potency values estimated by the mathematical model was obtained ($R^2 = 0.92$). Based on these results a conclusion was made that reversed phase high performance liquid chromatography could be used as an alternative for the *in vitro* biological assay for potency assessment of filgrastim samples. Such an alternative model would enable substitution of a complex and time consuming biological assay with a robust and precise instrumental method in many practical cases.

1. Introduction

Biopharmaceuticals are medicinal products comprising biotechnology-derived recombinant proteins as active substances [1], which are successfully used in the treatment of many diseases. Growth factors and cytokines are potential therapeutic agents because of their key roles in regulation of production, maturation, and activity of blood, muscle, and bone cells [2].

Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor, cytokine, which has important role in stimulating the proliferation and maturation of granulocytic cell lineages as well as functional activation of neutrophils *in vitro* and *in vivo* [3]. The effects of G-CSF on neutrophils and their progenitor cells are mediated by binding to a specific, cell surface G-CSF receptor [4]. G-CSF is a monovalent ligand that induces receptor dimerization [5].

There are two types of recombinant human G-CSF (rhG-CSF) clinically available: a glycosylated form (lenograstim) which is produced by expression in mammalian cells, and a nonglycosylated form (filgrastim) which is produced in genetically engineered *Escherichia coli*. Molecular cloning, expression of hG-CSF cDNA [6,7] and the purification of the rhG-CSF have been previously described [8–10]. Filgrastim is a 175-amino acid polypeptide chain containing an extra methionine at its N-terminus, with molecular mass of 18.8 kDa. The molecule contains a free cysteine at position 18, two intramolecular disulfide bonds Cys37–Cys43 and Cys65–Cys75 [11], and exhibits a defined four-helical bundle cytokine fold conserved among different species [12].

Abbreviations: G-CSF, granulocyte-colony stimulating factor; rhG-CSF, recombinant human G-CSF; SEC-HPLC, size exclusion high performance liquid chromatography; RP-HPLC, reversed phase high performance liquid chromatography; IC, ion chromatography; f-met, formyl-methionine; WHO, World Health Organization; NIBSC, National Institute for Biological Standards and Control; rDNA, recombinant deoxyribonucleic acid; LC–MS, liquid chromatography–mass spectrometry; PDA, photo diode array; ZQ, Z quadrupole; Q-TOF, quadrupole time-of flight; CV, column volume.

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Filgrastim, the rhG-CSF produced by *E. coli*, has identical biological activity to that of endogenous protein, although it contains an N-terminal methionine residue and is not glycosylated. The biological activity of rhG-CSF can be assessed by *in vivo* assays based on the evaluation of the neutrophil number in mice or leukocyte number in cyclophosphamide or 5-fluorouracil treated mice [13–15] or *in vitro*, using NFS-60 or M-NFS 60 cell lines [16] to determine proliferative activity of the G-CSF molecule.

Characterization of a biological product, conducted by appropriate analytical techniques, provides information on efficiency of expression and purification procedures [17]. According to European Pharmacopoeia (Ph.Eur.) monograph for filgrastim concentrated solution, product related impurities are divided into following groups: (a) impurities with molecular masses higher than that of filgrastim; (b) impurities with molecular masses differing from that of filgrastim; (c) impurities with charges differing from that of filgrastim; and (d) other filgrastim related proteins [18].

In the case of filgrastim related impurities formed due to physical or chemical degradation of proteins, some of the most ubiquitous impurities were isolated and *in vivo* or *in vitro* biological activities of these molecular species were determined. Chemical degradation, which includes at least reduction, oxidation and deamidation, was recognized as an important factor of protein inactivation. Loss of biological activity of chemically degraded protein molecules in comparison with biological activity of pure protein was revealed and described in several reports [11,17,19]. rhG-CSF lacking either one or both disulfide bridges has secondary and tertiary structure different from the structure of the native molecule and exhibits lower potency [11]. rhG-CSF dimer, sulfoxides, and deamidates were analyzed by size exclusion and reversed phase liquid chromatography (SEC-HPLC and RP-HPLC), and then injected to neutropenia mice, which showed that upon being modified or degraded to some extent only 15% of the stated potency was retained [17]. Protein dimerization or aggregation represents a challenge during biopharmaceutical production, as such entities may have reduced or no biological activity, and altered immunogenicity [19]. Filgrastim oxidized forms, except Met1-oxidized G-CSF, also show significantly reduced biological activity [4]. *In vitro* biological activity of filgrastim mutants in cysteines involved in disulfide bridges formation was also investigated. All rhG-CSF mutants Cys33 → Ser33, Cys43 → Ser43 and Cys75 → Ser75 exhibited very low activity (1–3%, relative to the native filgrastim). In contrast, rhG-CSF Cys18 → Ser18 exhibited full biological activity. These results suggest that formation of the both disulfide bonds Cys37–Cys43 and Cys65–Cys75 are essential in maintaining a properly folded tertiary structure of the rhG-CSF molecule [11].

Mire-Sluis et al. [19] showed the bioassays as a useful tool for assessment of quality, safety and efficacy of drug product. Although there are a decades of research performed on various pharmaceuticals, there are a few published data on correlation between physicochemical and biological assays. Kenny et al. in their study established the correlation between biological assay and HPLC method for the quantification of ripafentine in human plasma ($r = 0.9804$) [20]. Dalmora et al. [21] attempted to find a chromatographic technique, as a potential alternative for filgrastim *in vivo* potency evaluation. Based on the obtained results a correlation between the SEC-HPLC and the neutropenia mouse bioassay was established (calculated by the Student's *t*-test with the requirement of $P > 0.05$). They proposed SEC-HPLC in combination with the RP-HPLC as a substitute technique for *in vivo* bioassay. Replacement of other biological assays has been attempted in different fields of biochemical analytics, reported in published scientific papers [22,23].

The aim of this work was to isolate and to purify homogeneity filgrastim related impurities (filgrastim dimer, formyl-methionine (f-met) filgrastim and oxidized filgrastim) and characterize those using techniques of liquid chromatography–mass spectrometry. Filgrastim samples containing different amounts of related impurities were analyzed by chromatographic techniques and their *in vitro* bioassay was performed. SEC-HPLC was used for quantification of filgrastim dimer, RP-HPLC for quantification of filgrastim oxidized forms, while ion chromatography (IC) was used for quantification of f-met filgrastim. Based on correlation obtained between chromatographic results and biological assay, in which coefficient of determination (R^2) larger then 0.90 was considered as an indicator of a strong correlation, a simple mathematical model was proposed, with the aim to predict potency of filgrastim samples based on results obtained by RP-HPLC. Such an alternative model would in many cases enable replacement of a complex and time consuming filgrastim *in vitro* biological assay with more robust and precise instrumental method thus improving and facilitating the quality control of biological product, regarding time and cost consumption.

2. Materials and methods

2.1. Samples, solvents and reagents

For experimental work following samples were used: filgrastim bulk solution (2.6 mg/ml), filgrastim prefilled syringes 480 μg/0.5 ml and placebo for filgrastim samples, provided by Hospira Zagreb d.o.o., Croatia. WHO international standard for G-CSF (human, rDNA derived), was purchased from NIBSC (product number 88/502).

Following reagents were used for HPLC and LC–MS: acetonitrile (HPLC grade), trifluoroacetic acid (p.a.), 2-propanol (p.a.), ammonium hydrogen carbonate (p.a.), phosphoric acid (p.a.) all purchased from Merck (Darmstadt, Germany); sodium acetate trihydrate (p.a.) and acetic acid (p.a.) were purchased from Kemika (Zagreb, Croatia); hydrogen peroxide 30% (p.a.), sodium chloride (p.a.), sodium iodide (99%), cesium iodide (99%) were all purchased from Sigma–Aldrich (Steinheim, Germany).

Following reagents were used for biological assay: RPMI-1640 media (with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate), FBS (fetal bovine serum), tripan blue stain 0.4%, phosphate buffered saline (PBS), all purchased from Gibco, Invitrogen, USA. 2-Mercaptoethanol was purchased from Sigma–Aldrich (Steinheim, Germany). CellTiter 96® aqueous one solution reagent (MTS) was purchased from Promega (USA). Cell line M-NFS-60 cells ATCC No. CRL-1838 was obtained from LGC standards GmBH (Germany).

Following reagents were used for preparative chromatography: superdex 75 prep grade purchased from Amersham Bioscienes; SP toyopearl 650 purchased from TOSOH; sodium acetate anhydrous, Tween 80, sodium chloride, urea and acetic acid purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. UV/vis spectroscopy for protein concentration determination

Protein concentration of filgrastim samples was estimated by UV/vis spectroscopy, according to in-house method based on previously described procedure [25,26].

2.2.2. HPLC methods

SEC-HPLC was used for determination of filgrastim dimer, according to the method published in European Pharmacopoiea [18]. HPLC (Agilent 1100 series) was used with diode array detector ($\lambda = 215$ nm). Analyses were performed on Tosoh Bioscience TSKgel G3000SWXL (7.8 mm × 300 mm, 5 μm particle size) analytical column. Column temperature was maintained at 30 °C, and autosampler at 7 °C. Injection volume was 20 μl. Mobile phase was

ammonium hydrogen carbonate buffer (pH 7.0, 0.05 M). Run time was 30 min with isocratic elution. Flow rate was 0.5 ml/min.

RP-HPLC was used for determination of oxidized filgrastim and other filgrastim related proteins, according to the in-house method published in PHARMEUROPA 20.4 [27]. HPLC (Agilent 1100 series) was used with UV detection set on 215 nm (diode array detector). Analyses were performed on Phenomenex Jupiter C_f column (4.6 mm × 250 mm, 5 μm particle size). Column temperature was maintained at 60 °C, and autosampler at 10 °C. Injection volume was 50 μl. Flow rate was 0.6 ml/min. Mobile phase A was 0.1% trifluoroacetic acid diluted in solution of acetonitrile–milli Q water (10:90, v/v). Mobile phase B was 0.1% trifluoroacetic acid diluted in solution of acetonitrile–milli Q water (80:20, v/v). Run time was 60 min with linear gradient elution: 0–35 min (66–73% B); 35–50 min (73–90% B) and 50–60 min (90–66% B).

IC was used for determination of f-met filgrastim and more acidic related impurities according to in-house developed method. HPLC (Agilent 1100 series) was used with fluorescence detector (λ_{ex} = 280 nm, λ_{em} = 280 nm). Analyses were performed on Shodex IEC SP-825 (8.0 mm ID × 75 mm, particle size 8 μm) analytical column, enclosed methacrylic polymer with *n*-propyl-sulfonate functional groups as a stationary phase. Column temperature was maintained at 35 °C, and autosampler at 5 °C. Injection volume was 60 μl. Flow rate was 1.1 ml/min. Run time was 20 min with linear gradient elution: 0–4 min (3.0% B); 4–12 min (3–22.5% B); 12–13 min (22.5–3% B), 13–20 min (3.0% B). Mobile phase solution A was sodium acetate trihydrate (0.01 M, pH 5.6). Mobile phase solution B was sodium chloride (0.5 M)–sodium acetate trihydrate (0.01 M) (50:50, v/v) (pH 5.6).

2.2.3. Liquid chromatography–mass spectrometry (LC–MS)

LC–MS was performed on Waters HPLC 2795 with 2996 PDA detector. Chromatographic conditions were the same as in the case of RP-HPLC [27]. Analyses were performed on Waters Symmetry 300, butylsilyl-silica gel C4 analytical column (pore size 300 Å, 3.9 mm × 150 mm, 5 μm particle size) with guard column Waters Symmetry 300, butylsilyl-silica gel C4 (pore size 300 Å, 3.9 mm × 20 mm, 5 μm particle size). Two types of MS detectors were used: ZQ 4000 with an electrospray interface, positive ion mode. Scan speed was 1 Da/s. Second detector was Q-TOF Micro mass spectrometer with following parameters: MS scan time 0.50–59.50 min; mass scan 1500–2400 ES+; voltages: capillary 3.00 kV, cone 25 V, extractor 1 V and RF lens 0.1 V; source temperature was 100 °C and desolvation temperature 100 °C.

2.2.4. Filgrastim in vitro biological assay

Filgrastim *in vitro* bioassay was performed using M-NFS-60 cells, according to the described procedure [16,18]. Statistical analyses of the bioassay data were performed by parallel line assay according to European Pharmacopoeia "statistical analyses of results of biological assay and test", using parallel line assay (PLA) Software.

2.2.5. Preparative chromatography methods

2.2.5.1. Isolation of filgrastim dimer. Filgrastim dimer was isolated and purified using laboratory preparative chromatography system GE Healthcare Akta Explorer 100. Preparative gel filtration column XK 16/55 was used, packed with superdex 75 prep grade. Height of stationary phase was 55 cm; volume of stationary phase was 110.5 ml. In all operations 300 mM acetate buffer pH 3.9 with 0.004% Tween 80 (v/v) was used. The flow of mobile phase was 0.6 ml/min. Equilibrating procedure was performed with 2 column volume (CV) of buffer, loading of sample with 1/10 CV of buffer, elution of sample with 1.5 CV of buffer and cleaning/regeneration of column with 1 CV of buffer. UV detection was performed at 280 nm.

2.2.5.2. Isolation of f-met filgrastim. f-met filgrastim was isolated and purified using laboratory preparative chromatography system GE Healthcare Akta Explorer 100. Preparative ion exchange column XK 16/40 was used, packed with SP Toyopearl 650 M. Height of stationary phase was 20 cm; volume of stationary phase was 40.2 ml. In all operational steps, flow of mobile phase was 2 ml/min. Equilibrating procedure was performed with 4 CV of buffer containing 20 mM sodium acetate and 1 M urea pH 5.5, followed with the loading of sample with 19 CV of the same buffer. First step of elution was performed with 2 CV of the same buffer, while second step of elution was performed with 5 CV of 20 mM sodium acetate buffer, pH 5.5. At the end, elution of f-met filgrastim sample was performed with 20 CV of buffer containing 20 mM sodium acetate and 150 mM sodium chloride, pH 5.5. UV detection was performed at 280 nm.

2.3. Procedure for preparation of oxidized filgrastim sample

Oxidized filgrastim was produced by treating 2 ml of 0.2 mg/ml filgrastim with 2 μl 15% H_2O_2 for 24 h at room temperature. Removal of H_2O_2 was performed by ultrafiltration using microcone centricone 10 kDa cut off filter. Samples were centrifuged at 2700 × *g* for 45 min, plus 3 additional cycles of washing with 200 μl of placebo for filgrastim (centrifuging at 2700 × *g* for 30 min), with the aim to remove excess of H_2O_2, and finally turn of filter into clean Eppendorf tube to collect oxidized filgrastim (centrifuging at 1000 × *g* for 5 min). Collected samples were diluted with placebo to the initial volume.

2.4. Procedure for deliberate introduction of impurities into pure filgrastim sample

Pure filgrastim solutions were spiked with solutions of impurities to prepare samples with different volume fractions (%) of filgrastim related impurities. Three groups of filgrastim samples were produced:

(a) group 1: pure filgrastim bulk solution (2.6 mg/ml) was diluted to 0.3 mg/ml with placebo and spiked with purified filgrastim dimer (c = 0.3 mg/ml) to obtain different volume fractions starting from 0% and by increasing volume fraction of impurity by 20% increments, up to 100%;
(b) group 2: pure filgrastim bulk solution (2.6 mg/ml), was diluted to 0.6 mg/ml with placebo and spiked with isolated f-met filgrastim (c = 0.6 mg/ml) to obtain different volume fractions starting from 0% and by increasing volume fraction of impurity by 20% increments, up to 100%;
(c) group 3: pure filgrastim bulk solution (2.6 mg/ml) was diluted to 0.2 mg/ml with placebo and spiked with isolated oxidized filgrastim to obtain different volume fractions starting from 0% and by increasing volume fraction of impurity by 20% increments, up to 100%.

2.5. Preparation of filgrastim samples used for designing mathematical model for filgrastim potency assessment

With the aim to establish a mathematical model for assessment of filgrastim potency, 29 samples with different concentrations of particular filgrastim impurities were prepared. Isolated impurities of filgrastim dimer, f-met filgrastim and oxidized filgrastim were diluted with placebo to the concentration of 0.2 mg/ml and mixed in different volume ratios.

3. Results and discussion

With the aim to establish a correlation between concentration of filgrastim related impurities and the bioassay results, each impurity was purified and characterized.

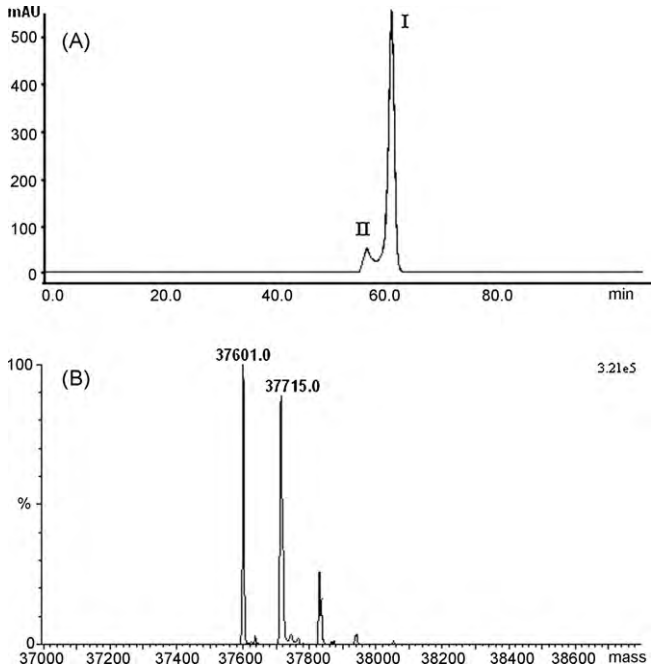

Fig. 1. (A) Chromatogram of filgrastim and filgrastim dimer obtained by preparative size exclusion chromatography. Peak I, filgrastim; peak II, filgrastim dimer. (B) Enlarged mass spectrum of filgrastim dimer fraction after deconvolution, *m/z* range 37,000–39,000 Da. The signal at 37,601 Da corresponds to filgrastim dimer (37,600 ± 1 Da) and the signal at 37,715 Da to the filgrastim dimer–trifluoroacetic acid adduct, originating from the mobile phase.

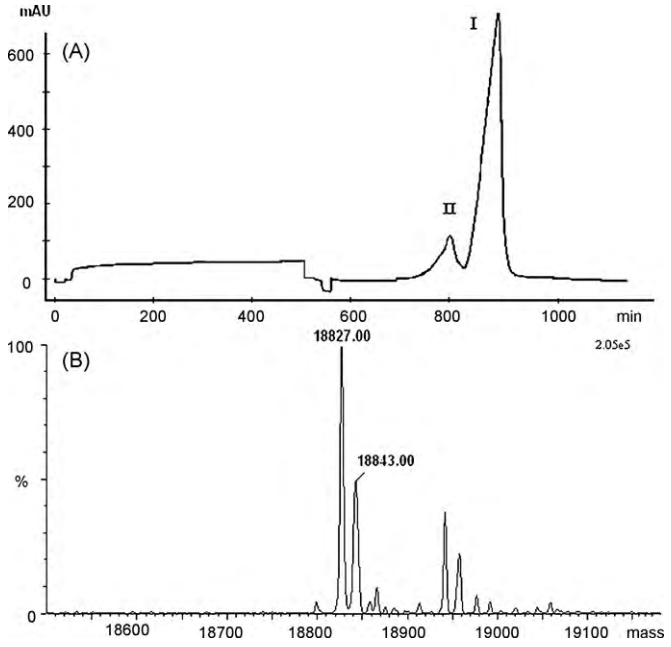

Fig. 2. (A) Chromatogram of filgrastim and f-met filgrastim obtained by preparative cation exchange chromatography. Peak I, filgrastim; peak II, f-met filgrastim. (B) Deconvoluted mass spectrum of f-met filgrastim peak reveals f-met filgrastim mass (18,828 ± 1 Da). Peak of oxidized f-met filgrastim mass (18,844 ± 1 Da) was also detected.

3.1. Isolation and characterization of filgrastim related impurities

3.1.1. Filgrastim dimer

Filgrastim dimer was isolated using preparative gel chromatography (Fig. 1A). After chromatographic isolation of filgrastim dimer, the fraction was subjected to SEC for purity determination. Purity of filgrastim dimer was determined to be 95%. Molecular mass of the intact dimer molecule was determined to be 37,601 Da (Fig. 1B). Theoretical average mass of the protonated filgrastim macromolecule is 18,800 Da, which is in line with the mass obtained for filgrastim dimer.

3.1.2. f-met filgrastim

Preparative ion exchange chromatography (IC) was used during filgrastim purification process. f-met filgrastim elutes before filgrastim, therefore, this chromatographic step was used for substantial or complete depletion of f-met [28]. As shown in Fig. 2A, f-met filgrastim was purified using preparative laboratory procedure. The purity of isolated f-met filgrastim determined by ion chromatography (IC) was 92%, while molecular mass of intact molecule determined by LC–MS was 18,827 Da, which is in line with its theoretical mass (Fig. 2B).

3.1.3. Oxidized filgrastim

Reversed phase high performance liquid chromatography is the most suitable technique for separation of filgrastim oxidized forms. Methionines at position Met1, Met122, Met127 and Met138 are sites most prone to oxidation [4].

Sample was prepared as described in Section 2.3 and analyzed by RP-HPLC. Purity of oxidized filgrastim was determined to be 97% (Fig. 3).

Identification of oxidized filgrastim was performed by LC/MS. Peaks II and III (Fig. 3) were identified using Q-TOF Micro mass spectrometer. For peak III (filgrastim oxidized form I), obtained mass spectrum is given in Fig. 4A, and for peak II (filgrastim oxidized form II), in Fig. 4B. Both filgrastim oxidized forms mainly contain monooxidized filgrastim (mass 18,816.00), while di- and tri-oxidized forms were observed at lower extent. The presence of multiply oxidized forms (di- and tri-) can be attributed to the excessive oxidation under the conditions used. Mass increments of 16 ± 1, 32 ± 1 and 48 ± 1 Da correspond to mono-, di- and tri-oxidized variants, respectively, relative to the intact protein (18,800 ± 1 Da).

3.2. Determination of correlation between content of individual filgrastim related impurity and potency of the sample

Three groups of samples were prepared, according to the procedure described in Section 2.4, and tested by SEC-HPLC (group 1), IC (group 2) and RP-HPLC (group 3). Each sample from each group was prepared and analyzed in triplicate. Chromatograms of analyzed groups of samples were quantitatively assessed by normalization of peak areas to provide a quantitative analysis of the analyzed mixture. Average content for each impurity was calculated and expressed as area %. In parallel, *in*

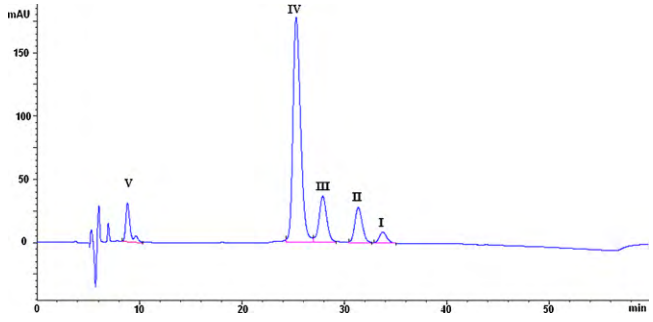

Fig. 3. RP-HPLC chromatogram of oxidized filgrastim sample. Peak I, filgrastim; peak II, filgrastim oxidized form II; peak III, filgrastim oxidized form I; peak IV, filgrastim oxidized form III, product of excessive H_2O_2 oxidation; peak V, filgrastim related proteins.

Table 1
Results of deliberate introduction of impurities into pure filgrastim sample.

Sample	Group 1		Group 2		Group 3	
	Filgrastim dimer (%)	Potency (%)	f-met filgrastim (%)	Potency (%)	Oxidized filgrastim	Potency (%)
1	0.2	117.9	2.7	122.6	1.5	126.1
2	14.9	101.2	18.4	120.1	20.5	101.7
3	33.1	88.8	36.5	112.0	38.0	91.4
4	51.7	86.0	54.1	100.8	51.8	69.7
5	72.3	72.3	73.5	103.2	73.8	51.4
6	95.1	66.9	92.0	89.7	99.7	25.5
R^2	0.94		0.93		0.99	

vitro potencies of these samples were determined. For all three groups of samples, obtained coefficient of determination R^2 was above 0.9 thus confirming significant correlation between the content of impurity and potency. Results are summarized in Table 1.

Results also showed that 95% pure sample of filgrastim dimer had a significantly lower potency then pure filgrastim (0.67×10^8 IU/mg or 66.9% in comparison with 1×10^8 IU/mg or 100%, a stated potency of pure filgrastim). Potency of 92% pure f-met filgrastim sample was 89.7% of the stated filgrastim potency. Obtained value for f-met filgrastim potency is comparable with the results obtained in the study where three variants of acidic isoforms of rhG-CSF were purified, isolated by a series of cationic exchange chromatographic separations and tested by *in vitro* bioassay. They exhibited full *in vitro* biological activity (isoform I: 1.09×10^8 IU/mg or 109%; isoform II: 0.75×10^8 IU/mg or 75%; isoform III: 0.89×10^8 IU/mg, or 89%) [28]. Finally, potency of 97% pure oxidized filgrastim sample was 25% of the stated filgrastim potency. Obtained results revealed that oxidation of filgrastim caused a significant decrease of its potency, far more pronounced than the loss of potency caused by filgrastim dimerization or formylation. Obtained value is also comparable with the result obtained for rhG-CSF sulfoxide forms, which were analyzed by liquid chromatography (RP-HPLC), and then subjected to neutropenia mouse bioassay, giving biological activity of about 15% [17]. Similar result was obtained by studying selective oxidation of G-CSF (methionine oxidation) by H_2O_2 and *t*-butyl hydroperoxide. Different oxidized forms of G-CSF were produced and isolated, and all of them showed only residual *in vitro* biological activity (less than 20%), except of oxidized filgrastim at position Met1, which retained approximately 80–85% activity [4].

Table 2
Calculated coefficients of quadratic polynomial function.

Coefficient	Value
a_0	−70,8246.0
b_1	14,555.6
b_2	17,919.2
b_3	15,044.2
b_4	−16,961.0
c_{11}	−74.7
c_{22}	−108.4
c_{33}	−79.6
c_{44}	238.9
c_{12}	−183.1
c_{13}	−154.3
c_{14}	165.7
c_{23}	−188.0
c_{24}	132.5
c_{34}	161.0

3.3. Mathematical model for filgrastim biological activity estimation

According to the currently available literature data, mathematical model for *in vitro* potency estimation of filgrastim was not proposed until now. Group of authors [20] analyzed pharmaceutical samples of filgrastim ($n = 11$) by chromatographic methods and compared results with the *in vivo* biological assay (neutropenia mouse bioassay), showing average difference between the estimated potency of 2.04% lower for the RP-HPLC, and 4.03% lower for the SEC-HPLC, with significant correlation ($P > 0.05$). Based on obtained results, they proposed a SEC-HPLC, in combination with RP-HPLC, as alternative methods for assessment of filgrastim potency.

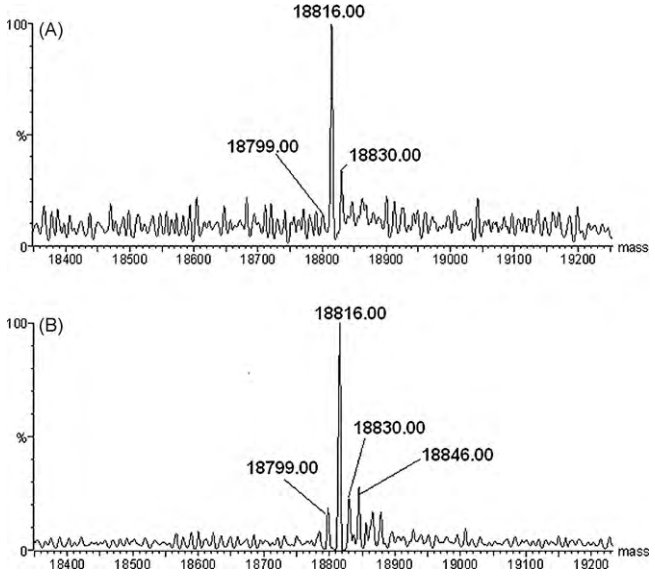

Fig. 4. (A) Deconvoluted mass spectrum of filgrastim oxidized form I. Mono- and di-oxidized filgrastim with mass increments 17 and 31 Da, respectively, relative to filgrastim (18,799 Da). (B) Deconvoluted mass spectrum of filgrastim oxidized form II. Mono-, di- and tri-oxidized filgrastim forms with mass increments 17, 31, and 46 Da, respectively, relative to filgrastim (18,799 Da).

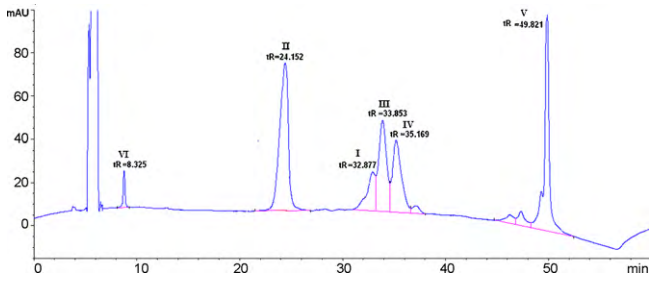

Fig. 5. Representative RP-HPLC chromatogram of one of the 29 samples used for mathematical model development. Peak I, filgrastim; peak II, filgrastim oxidized form, product of excessive H_2O_2 oxidation; peaks III and IV, peaks due to f-met filgrastim and oxidized f-met filgrastim; peak V, filgrastim dimer; peak VI, filgrastim related proteins.

Table 3
Prepared samples for determination of suitable mathematical function. Results for X_1, X_2, X_3 and X_4 are expressed as area %.

Sample	X_1 (filgrastim dimer)	X_2 (f-met filgrastim)	X_3 (oxidized filgrastim)	X_4 (filgrastim)	Potency (%) calculated (Y_c)	Potency (%) experimentally (Y)
1	0.0	41.6	49.5	8.9	77	74
2	21.0	36.6	35.8	6.6	72	82
3	40.8	27.4	27.3	4.5	67	70
4	60.7	18.1	18.1	3.1	63	68
5	80.7	8.5	8.8	2.0	60	61
6	98.9	0.0	0.0	1.1	55	58
7	52.2	0.0	47.2	0.6	46	43
8	43.3	17.3	35.7	3.7	58	55
9	30.6	33.8	28.8	6.8	68	63
10	17.5	52.3	21.8	8.4	90	85
11	8.3	70.8	11.3	9.6	116	120
12	25.9	51.5	16.0	6.6	92	83
13	47.0	45.3	2.2	5.5	96	103
14	40.0	34.9	19.6	5.5	75	87
15	30.6	24.1	39.9	5.4	62	70
16	19.6	14.7	61.3	4.4	58	62
17	6.7	5.7	85.4	2.2	50	42
18	0.0	0.0	100.0	0.0	25	29
19	73.3	17.2	5.8	3.7	62	52
20	13.7	70.0	8.5	7.8	113	114
21	21.0	25.0	50.0	4.1	40	40
22	40.6	37.0	17.4	5.2	59	59
23	27.4	44.5	23.8	7.0	87	87
24	36.9	32.4	25.9	4.9	64	70
25	37.3	41.7	15.8	5.3	62	64
26	33.1	42.1	19.0	5.8	82	72
27	54.1	28.9	12.8	4.3	66	66
28	32.2	32.7	30.3	4.9	59	55
29	50.1	29.8	15.7	4.4	73	67

In order to propose a mathematical model for the assessment of filgrastim potency, the first step was to evaluate chromatographic methods used in this research. As it was expected, RP-HPLC was the only analytical method able to sufficiently separate all three filgrastim related impurities mixed in one sample (representative chromatogram presented in Fig. 5).

Twenty-nine samples with different volume fractions (%) of filgrastim related impurities were prepared according to the procedure described in Section 2.5 and analyzed by RP-HPLC. For quantitative RP-HPLC analysis of samples with a different content of filgrastim related impurities, peak area of a given peak was expressed as a percentage of a sum of the areas of all the peaks (peak area normalization). These results were than fitted to mixed quadratic polynomial function given by Eq. (1) [29]. Y_c represents calculated potency, and X represents peak area percent.

$$Y_c = a_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4}\sum_{j=1}^{4} c_{ij} X_i X_j \quad (1)$$

The values for calculated coefficients of quadratic polynomial function are given in Table 2.

Twenty-nine prepared samples were tested in parallel by *in vitro* filgrastim biological assay. Results obtained for each filgrastim impurity determined by RP-HPLC along with experimentally obtained and calculated (according to Eq. (1)) potency values for filgrastim samples are presented in Table 3.

Correlation between results of experimentally obtained and calculated potency values for filgrastim samples is presented in Fig. 6.

The biggest difference observed between estimated and measured value was 16.1%. Coefficient of determination (R^2) for polynomial fit was 0.92 which confirmed correlation between experimentally obtained and calculated potency values. We found this result to be acceptable and in line with inherent variability of *in vitro* bioassay. These findings point out towards RP-HPLC as a chromatographic method suitable for prediction of filgrastim potency.

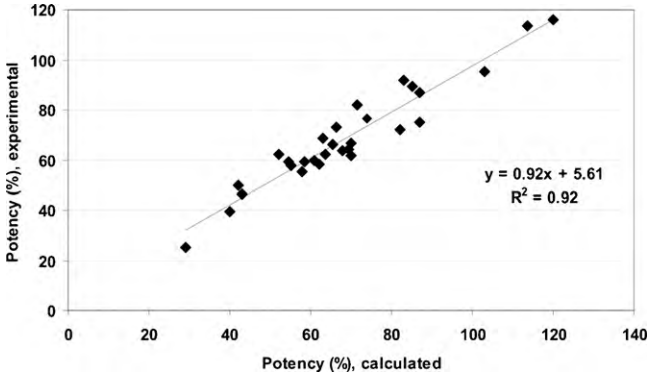

Fig. 6. Correlation between calculated and experimentally determined potency of filgrastim samples of different purity.

4. Conclusion

Obtained results show that determination of filgrastim related impurities content in filgrastim samples by RP-HPLC enables prediction of its potency according to a simple mathematical model described by Eq. (1). Such an alternative approach enables replacement of a complex and time consuming biological assay with more robust and precise instrumental method, therefore providing improvement in quality control of a biological product.

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