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Original research paper

Size exclusion chromatography as green support for forced degradation study of adalimumab

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ABSTRACT

Size exclusion chromatography (SEC) has become a powerful tool for analysing size variants of biologic drugs in their native form. Modern SEC can be defined by the use of chromatographic columns packed with sub-3 μm particles, allowing an increase in method throughput compared to that of conventional SEC.

We performed the forced degradation study of adalimumab, the first genetically engineered fully humanised immunoglobulin G1 monoclonal antibody, and evaluated tha possibilities of an advanced SEC column packed with sub-3 μm particles for elucidation of the degradation pathway. Our results revealed the main adalimumab degradation products to be antibody fragments. Acidic and basic conditions had the most intensive effect on the degradation of the adalimumab while the drug exhibits relative stability under thermal and photolytic stress conditions.

The AGREE and AGREEprep calculators were used for the evaluation of the environmental performance of the forced degradation procedure. The results of the green score evaluation are presented as round pictograms with a circle in the centre that shows the overall score of 0.81 and 0.61, respectively. Both pictograms are highlighted in green, indicating the eco-friendly conditions.

Keywords: inflammatory bowel disease, biopharmaceuticals, adalimumab, size exclusion chromatography, forced degradation study

INTRODUCTION

The treatment of immune-mediated inflammatory diseases has been revolutionised over the last few decades because of a substantial increase in the knowledge of the underlying pathogenesis of these conditions. This has opened the door for the development of targeted therapeutic solutions in the form of biopharmaceutical agents, well-recognized as

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biologic drugs, for a variety of conditions, such as inflammatory bowel disease (IBD). This group of chronic idiopathic inflammatory gastrointestinal disorders is mainly represented by Crohn's disease and ulcerative colitis (1).

Since their introduction in the 1990s, the use of monoclonal antibodies as biologic--based therapy in the treatment of IBD has improved outcomes for patients. Adalimumab (Humira®, AbbVie Corporation, USA) is the first genetically engineered fully humanised immunoglobulin G1 (IgG1) monoclonal antibody binding with high affinity to the first cytokine fully validated as a therapeutic target tumor necrosis factor-alpha (TNF-α). It inhibits the binding of both soluble and transmembrane forms of TNF-α with receptors on the cell surface and thus reducing TNF-α receptor-mediated cell signalling (2–4).

In 2018, product-specific patent protection on Humira® came to an end in most European Union countries, 15 years after the initial marketing authorization. With the loss of exclusivity for the originator molecule, we have witnessed the introduction of adalimumab biosimilar drugs into clinical practice. Advances in the development, process, quality control, regulation, and application of biological drugs have facilitated biosimilar drug development. Analytical characterization of these complex molecules is pivotal at all stages of compound development, from research and development phases to final batch release. Up-to-the-minute reliable instrumental techniques of analysis are needed to support a comprehensive characterization of those highly complex biologic drugs. Moreover, the recent trends in analytical method development focus on reducing the environmental impact of analytical practises, making them more sustainable and eco-friendlier by using renewable resources, eliminating hazardous chemicals, and generating less waste (5).

Size exclusion chromatography (SEC) has become a powerful tool for analysing size variants of biologic drugs in their native (physiological-like) form. With its mild chromatographic conditions, SEC permits the analysis of biologic drugs with minimal impact on the conformational structure and local environment of a molecule. Thus, it is used in the manufacturing process to guide the cell-line selection and development of the purification process, formulation development, routine analysis of aggregation of biotherapeutic proteins, and stability studies (6). It is included in the list of the typically used tests in the European Pharmacopoeia guidance document entitled "Technical Guide for the Elaboration of Monographs on Synthetic Peptides and Recombinant DNA Proteins" (7).

In recent years, the need for fast and reliable methods for the analysis of biologic drugs has renewed interest in SEC technology. The opportunities to improve the resolution and throughput of SEC methods were of particular interest to the scientific community. Modern size exclusion high-performance liquid chromatography can be defined by the use of chromatographic columns packed with sub-3 μm particles, allowing a 3- to 5-fold increase in method throughput compared to that of conventional SEC. These innovative columns with inert stationary phases with a carefully controlled pore size and distribution may offer the analyst the opportunity to control the environmental impact of newly developed methods (8).

The literature review showed that SEC was used for the investigation of binding characteristics between adalimumab and its target TNF (9). It was crucial for the demonstration of the physicochemical and functional similarity of adalimumab and its biosimilar throughout the evaluation of size-related impurities (10), size heterogeneity (11), and heat stress degradant pathway (12). A U.S. Pharmacopeia chapter provides the panel of analytical procedures for adalimumab analysis. The SEC and capillary gel electrophoresis are recommended for high-molecular-weight species assessment. Capillary electrophoresis or

liquid chromatography coupled with fluorescence detection are recommended for oligosaccharides analysis and liquid chromatography with amperometric detection for sialic acid determination.

Our research aimed to evaluate the possibilities recently introduced SEC chromatographic column packed with sub-3 μm particles for the forced degradation study of adalimumab. Considering the increasing demand for sustainable analytical methods AGREE and AGREEprep calculators based on the conversion of principles of green analytical chemistry into variables were used for evaluation of the environmental impact of the developed analytical procedure.

EXPERIMENTAL

Chemicals and reagents

Adalimumab-certified reference material (10 mg mL^{-1} in 12.5 mmol L^{-1} buffer) used for optimization and validation of the method was purchased from Cerilliant Analytical Standard (USA). Adalimumab dosage form (Humira®, AbbVie Corporation, USA) was supplied as a sterile, prefilled syringe composed of 40 mg adalimumab in 0.4 mL of a solution containing mannitol, polysorbate 80, and water for injections. Drug samples were maintained following the manufacturer's instructions and evaluated within the shelf life specified. Phosphate buffer solutions were prepared using di-sodium hydrogen phosphate dihydrate (buffer substance for chromatography) and sodium dihydrogen phosphate dihydrate (EMSURE® reagent Ph. Eur.) both by Merck KGaA, Germany. Sodium hydroxide pellets (ACS reagent, 97.0 %) were supplied by Sigma-Aldrich (USA), while hydrochloric acid (37 %, for analysis) by Carlo Erba (France). All studies used ultrapure water from the MilliQ UF-Plus water system Millipore (Germany) with a resistivity of 18.2 MΩ cm (25 °C) and a total carbon value < 5 ppb.

Preparation of stock solution, quality control samples, and drug samples

The stock solution of adalimumab (2 mg mL^{-1}) was prepared by diluting an appropriate volume of certified reference material in water for injection. Further dilutions of the stock solution produced a series of standard working solutions in the concentration range of 5–1000 μg mL^{-1}. Quality control (QC) samples for method validation were independently prepared in the water for injection at low (QC_1, 25 μg mL^{-1}), middle (QC_2, 250 μg mL^{-1}), and high (QC_3, 500 μg mL^{-1}) concentrations representative of the range of calibration curves.

Humira® sample solutions were made by diluting the drug solution with water for injection in a 1:200 (*V*/*V*) ratio.

All solutions were kept in the dark at 4 °C until used.

Chromatographic analysis

For the SEC assay, the chromatographic system was an Agilent 1260 series UHPLC system with a diode array detector (DAD) by Agilent Technologies (USA). Data acquisition and processing were carried out using OpenLab ChemStation (Agilent Technologies). Quality control, drug, and stress samples were analysed using size exclusion chromatographic

column AdvanceBio SEC (7.8 × 150 mm), 2.7 μm particle size with a pore size of 130 Å supplied from Agilent Technologies with an operating temperature of 25.0 ± 0.1 °C. A mobile phase of 0.1 mol L^{-1} sodium phosphate buffer (pH 7.4 ± 0.05) was used under isocratic elution conditions. Before use, the mobile phase was filtered through a membrane filter with a particle size of 0.45 μm (Sartorius, Germany) using the system for the filtration of the mobile phase (Supelco, USA). The flow rate during the analysis of 15 minutes was 0.5 mL min^{-1} while the injection volume was 10 μL. The DAD quantitation of adalimumab was performed at 280 nm. The absorbance of the analytes during a chromatographic run was collected in the spectral range of 190–400 nm. During analysis, all samples were stored in amber vials at 4 °C.

Analytical method validation

The SEC method was validated per the International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2 (R2) on validation of analytical procedures (13).

Forced degradation study

A forced degradation study of adalimumab (final concentration of 500 μg mL^{-1} in stressed samples) was performed under the following conditions: pH, temperature, light, and freeze-throw. Acidic and basic hydrolysis were investigated by stressing adalimumab with 10 μL of hydrochloric acid (0.1 mol L^{-1}) or sodium hydroxide (0.1 mol L^{-1}) solutions. Stressed samples were prepared separately into amber screw cap vials and left at room temperature. The thermal stress testing was carried out by keeping adalimumab solution in amber screw cap vials in a thermostatically controlled orbital shaker incubator (ES-20/60 by Biosan, Latvia) maintained at 60 °C. Photostability was investigated by exposing adalimumab solution (500 μg mL^{-1}) stored in transparent screw cap vials to direct sunlight. Samples were withdrawn at regular intervals (1, 2, 4, 12, 24, and 48 hours), to monitor the degradation process using the optimized SEC method. Comparison between the stressed and reference samples was used to follow a decrease in the main peak area and an increase in the number and peak area of degradation products. The forced degradation procedure was carried out until the limit of degradation of 10–20 % was reached. Lastly, samples were stored at –80 °C for 24 hours and then thawed unassisted at room temperature for three cycles.

Greenness score evaluation of forced degradation study supported by SEC method

The platforms Analytical GREEnness metric approach software (AGREE) and Analytical GREEnness sample preparation metric approach software (AGREEprep) were used for the analytical method greenness score evaluation (14).

RESULTS AND DISCUSSION

Method optimisation

Molecular size-based separation methods are the long-standing standard in the biopharmaceutical industry for quality control strategies of therapeutic antibodies such as adalimumab. This IgG1 antibody with a total molecular weight of 148 kDa is composed of

two kappa light chains each with a molecular weight of approximately 24 kDa and two IgG1z, heavy chains each with a molecular weight of approximately 49 kDa. To evaluate the possibilities recently introduced SEC column packed with sub-3 μm particles for the forced degradation study of adalimumab column with a pore size of 130 Å was selected for optimization of the stability-indicating method. It allows the separation of proteins with molecular weights between 10 and 500 kDa and is thus well suited for the separation of adalimumab, its trimers, dimers, and monomers as well as fragments having sizes of 25–100 kDa. Furthermore, to increase the method throughput without sacrificing performance SEC column with smaller dimensions and the 2.7 μm packing was selected for method optimisation (8).

Mobile phase optimisation is an important element of method development to create procedures that effectively minimize secondary interactions between proteins and the column, which can cause peak tailing, peak shape distortion, poor resolution, and poor assay reproducibility. It also minimises aggregation of the proteins, which causes erroneous results. The SEC assay with 0.1 mol L^{-1} phosphate buffer as mobile phase showed a high peak for adalimumab standard samples. The chromatogram of the certified reference material solution (concentration level QC_3 – 500 g mL^{-1}) recorded at 280 nm shows the adalimumab peak at a retention time of 5.17 minutes (Fig. 1a). To assess the reliability of the analytical method, the suitability of the chromatographic system was tested according to the United States Pharmacopeia & National Formulary (15). Results are displayed in Table I, for a set of parameters: retention time, resolution, peak area, number of theoretical plates, retention factor, peak purity, and peak symmetry. The test was performed in six replicates and all data are within the acceptance criteria (Relative Standard Deviations (RSDs) were lower than 5.0 %), revealing complete baseline separation of adalimumab and adjacent peaks and high selectivity of the optimized method.

Method validation

The validation of the SEC method was performed as per approved guidelines of the ICH for selectivity, linearity, precision, accuracy, limits of detection (*LOD*) quantification (*LOQ*), and stability (13).

Table I. System suitability (concentration level 500 μg mL^{-1}; n = 6) of SEC method

Parameter	Symbol	Value	RSD[a] (%)
Retention time (min)	t_R	5.17	0.01
Resolution	R_s	1.89	0.10
Peak area (mAU)	A	930.2	0.38
Peak purity	P	999.99	0.01
Retention factor	k	9.33	0.01
Symmetry	A_s	0.78	4.33
Theoretical plate count	N	3699	0.03

[a] RSD – Relative Standard Deviation

Selectivity of the method was confirmed in the forced degradation study with a resolution between a critical pair of peaks (degradant 2 and degradant 3) of 1.5 (Fig. 1c). Moreover, the peak purity of adalimumab in the stressed samples was higher than 999.

The calibration curve was prepared to demonstrate the relationship between the nominal analyte concentration and the response of the analytical platform to the analyte. A calibration curve was created using six concentration levels (5, 25, 50, 250, 500, and 1000 μg mL^{-1}) using three separately prepared standard solutions. It was found to be linear with a good

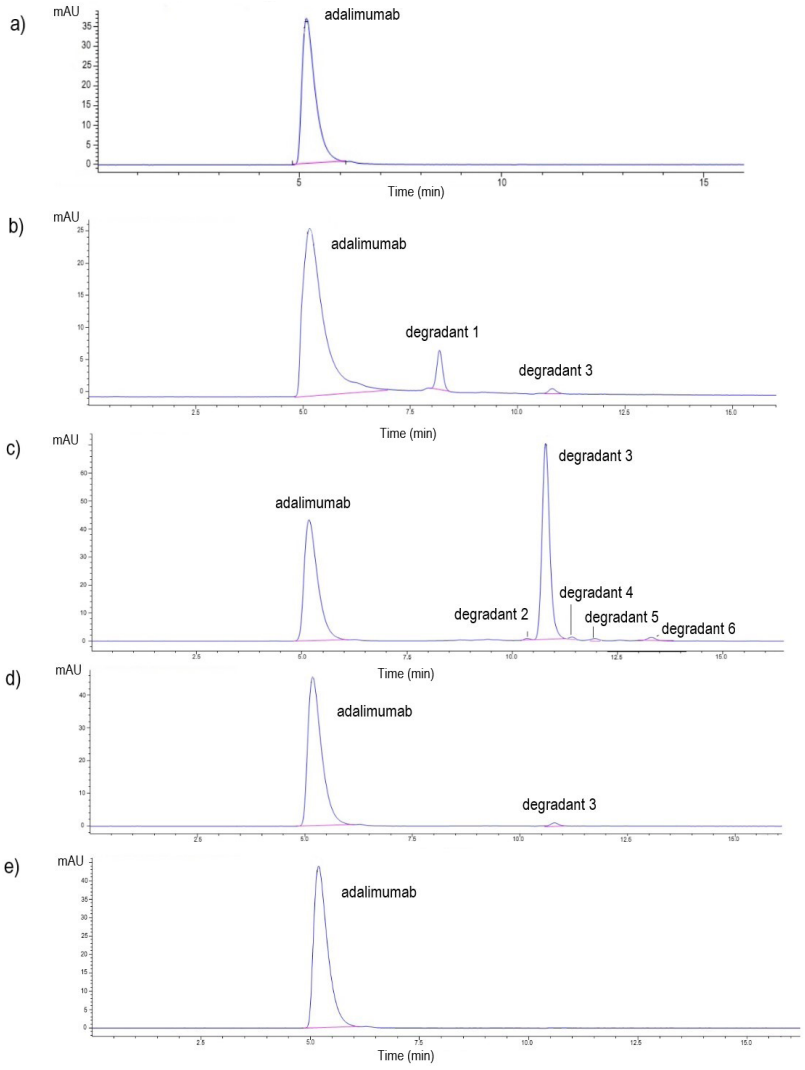

Fig. 1. Forced degradation chromatograms of adalimumab: a) non-stressed sample, b) acidic hydrolysis, c) basic hydrolysis, d) thermal stress, and e) photolytic stress.

regression coefficient value ($r > 0.999$) (Table II). The accuracy of the back-calculated concentrations of each calibration standard was found to be within ± 5.2 % of the nominal concentration.

The limit of detection (*LOD*) was obtained by diluting the standard solution with water for injection based on a 3:1 signal-to-noise ratio, and the limit of quantification (*LOQ*) was obtained based on a 10:1 signal-to-noise ratio (Table II).

The precision of an analytical method is defined as the closeness of agreement among individual test results from repeated analyses of a homogeneous sample. The intra-day precision or repeatability was performed by preparing and analysing QC_2 solution (250 μg mL^{-1}) within the same day in six replicates. The inter-day precision or intermediate precision was performed at the same quality control level (QC_2) on three consecutive days in three replicates. The data expressed as RSDs are presented in Table II. The values lower than 0.48 % demonstrate that the results were highly reproducible.

The accuracy of the method explained the closeness of the experimental results to the actual value. It was evaluated by recovery assay at three concentration levels (QC_1 – 25 μg mL^{-1}, QC_2 – 250 μg mL^{-1}, and QC_3 – 500 μg mL^{-1}) in triplicate. The results are presented

Table II. SEC method validation data

Validation parameter	Value
Linearity	
Range (μg mL^{-1})	5–1000
Equation	$y = 1.8771x - 12.378$
Standard deviation of slope	0.02
Standard deviation of the intercept	11.1
Correlation coefficient (r)	0.9994
Sensitivity	
Limit of detection (μg mL^{-1})[a]	3.0
Limit of quantitation (μg mL^{-1})[b]	5.0
Precision	
Repeatability (n = 6; RSD, %)[c]	0.37
Intermediate precision (n = 9; RSD, %)[d]	0.48
Accuracy	
QC_1 (n = 3; mean recovery, % / RSD, %)[e]	104.3/0.91
QC_2 (n = 3; mean recovery, % / RSD, %)[f]	105.2/0.99
QC_3 (n = 3; mean recovery, % / RSD, %)[g]	94.6/0.18

[a] The limit of detection (*LOD*) was obtained by diluting the standard solution based on a 3:1 signal-to-noise ratio.
[b] The limit of quantification (*LOQ*) was obtained by diluting the standard solution based on a 10:1 signal-to-noise ratio.
[c] The repeatability was performed by analysing QC_2 (250 μg mL^{-1}) within the same day in six replicates.
[d] The intermediate precision was performed by analysing QC_2 (250 μg mL^{-1}) on three consecutive days in three replicates.
[e] Accuracy at low concentration levels was performed by analysing QC_1 (25 μg mL^{-1}) in three replicates.
[f] Accuracy at medium concentration level was performed by analysing QC_2 (250 μg mL^{-1}) in three replicates.
[g] Accuracy at high concentration level was performed by analysing QC_3 (500 μg mL^{-1}) in three replicates.

Table III. Forced degradation study results

Stress type	Degradation condition	Degradation (%)	Peak area (mAU/s)					
			Degradant 1 (t_R = 8.19 min)	Degradant 2 (t_R = 10.42 min)	Degradant 3 (t_R = 10.79 min)	Degradant 4 (t_R = 11.35 min)	Degradant 5 (t_R = 11.98 min)	Degradant 6 (t_R = 13.30 min)
Acidic hydrolysis	0.1 mol L^{-1} HCl, 1 h	9.88	59.3	ND[a]	11.1	ND	ND	ND
Basic hydrolysis	0.1 mol L^{-1} NaOH, 1 h	17.02	ND	11.2	900	13.9	12.5	17.7
Thermal	60 °C, 48 h	10.12	ND	ND	16.8	ND	ND	ND
Photolytic	Daylight, 48 h	14.94	ND	ND	ND	ND	ND	ND

[a] ND – not detected

as recovery (%) and RSD (%) values (Table II). It was satisfactory at all three QC levels (the recoveries ranged from 94.6 to 105.2 % with corresponding RSD values of less than 0.99 %).

Finally, the adalimumab proved to be stable for 48 hours and stored in an amber vial in a refrigerator at 4 °C (recovery was 99.8 %).

Assay determination in pharmaceutical formulation (Humira®)

SEC assay was used for the determination of adalimumab in Humira® 40 mg/0.4 mL solution for injection. The measurements were carried out in triplicate and satisfactory recoveries were obtained (from 100.2 to 101.0 %) with high reproducibility of the analytical procedure (RSD value was 0.33 %) with peak purity higher than 999.9. The deviations from the declared content were lower than 0.5 %.

Forced degradation study

Degradation studies, performed on 500 μg mL^{-1} solution of adalimumab, were carried out to demonstrate the suitability of the SEC analytical method to investigate the degradation pathways of this humanised IgG1 monoclonal antibody and its stability under stressful conditions: pH, temperature, light, and repeated freeze-thaw. The goal of the forced degradation study was to identify optimal conditions that result in meaningful levels of degradation. Thus, the degradation level of 10–20 % was considered adequate for the achievement of significant degradants. Biopharmaceuticals having a complex physiochemical character with various functional groups are suspectable to instability through various degradation pathways. SEC method was optimized to monitor the formation of high-molecular-weight aggregates and low-molecular-weight fragments.

The results of the conducted forced degradation study are presented in Table III. Fig. 1 presents the change in the size profile of adalimumab under stress conditions. All stress samples showed a high purity of adalimumab peak (more

than 999.0). Acidic and basic conditions have the most intensive effect on the degradation of the adalimumab with the formation of low-molecular-weight fragments within the first hour (Fig. 1b,c). On the other hand, adalimumab exhibits relative stability under thermal stress conditions (at 60 °C for 48 h), as demonstrated by the reduction of the peak area of the adalimumab (t_R = 5.17 min) and the appearance of low-molecular-weight fragment degradation peak at the retention time of 10.79 min (Fig. 1d). As demonstrated in the previous research high temperature accelerated the fragmentation of adalimumab from peptide bond cleavage (16). Exposure to daylight had a weaker effect on the degradation of adalimumab, where no other peaks than the intact drug peak appeared in the respective chromatogram (Fig. 1e). The amino acid residues tryptophan, tyrosine, phenylalanine, cysteine, and peptide backbones are the most susceptible sites for photoinduced degradation (16). The obtained results show that adalimumab follows the first-order reaction kinetics as confirmed in the research conducted by Hassan and coworkers (17). It is worth highlighting that the major obtained stress products of adalimumab were low-molecular--weight fragments with different UV spectra compared to biopharmaceutic.

Shabestari and coworkers (18) also found fragments as stress products of Humira® and its biosimilar CinnoRA® using acid/base hydrolysis as well as thermal stress. Furthermore, Füssl and coworkers (19) performed forced degradation of adalimumab followed by highly selective cation exchange chromatography-based charge variant analysis with online Orbitrep mass spectrometric detection. Similarly, to our study, they identified multiple modifications on adalimumab, including lysine truncation, deamidation, N-terminal aspartic acid loss, and fragmentation along with the N-glycan distribution of each identified proteoforms. The major stress products of Humira® were low-molecular-weight fragments while high-molecular-weight aggregates were not detected. Based on the study of Hassan and coworkers (17) we consider this could be associated with the presence of polysorbate 80 in the formulation leading to a decreased risk of aggregate formation.

Freeze-thaw is often explored as a stage of forced degradation study to determine the susceptibility of a protein to temperature cycling. After three freeze-thaw cycles, the drug was stable with recovery above 98 %.

Greenness score evaluation of forced degradation study supported by SEC method

The pharmaceutical industries are concerned about environmental safety and green analytical method development as a value in the vision of the fourth pharmaceutical industrial revolution known as term called Pharma 4.0. The main aims of Pharma 4.0 are to increase efficiency, productivity, and responsiveness in the pharmaceutical industry using artificial intelligence as well as utilization of advanced environment-friendly techniques and procedures (20).

The environmental impact of an analytical procedure is a multidimensional and complex term that is not easy to define and assess. So far, several *in silico* approaches to evaluating analytical green method scores have been proposed. In our research, we used the Analytical GREEnness calculator (AGREE), an available, comprehensive, flexible, and straightforward assessment approach suitable for chromatographic analysis that provided an easily interpretable and informative result (21). This newest greenness assessment software was used for evaluating the overall forced degradation procedure of adalimumab from sample preparation to validated SEC analysis and data processing by converting all

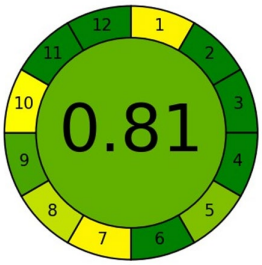

Fig. 2. Results of AGREE analysis for forced degradation study supported with validated SEC method. Legend: 1 – sample procedure, 2 – sample size, 3 – sampling, 4 – sample preparation steps, 5 – automatization, 6 – derivatization, 7 – waste, 8 – analysis throughput, 9 – energy consumption, 10 – renewable reagents, 11 – toxicity of reagents, 12 – operator's safety. Green metrics: green (high greenness of sample procedure), yellow/orange (moderate greenness of sample procedure) and red (low greenness of sample procedure).

Fig. 3. Results of AGREEprep analysis for forced degradation study supported with validated SEC method. Legend: AGREEprep: 1 – sampling, 2 – hazardous materials, 3 – sustainability, renewability, and reusability of materials, 4 – waste, 5 – economy of sample, 6 – sample throughput, 7 – automatization, 8 – energy consumption, 9 – analytical instrumentation, 10 – operator's safety. Green metrics: green (high sample preparation greenness), yellow/orange (moderate sample preparation greenness) and red (low sample preparation greenness).

12 principles of green chemistry into analytical procedure variables: sampling procedure, sample size, *in situ* measurement, steps in the process, miniaturization, derivatization, waste, number of analytes, energy, type of reagents, the toxicity of reagents, and operator safety.

The result of the green score evaluation of the forced degradation procedure of adalimumab supported with SEC analysis is presented as a round pictogram with a circle in the centre that shows the overall score of 0.81, highlighted in green, indicating the eco-friendly conditions (Fig. 2). The performance of the procedure in each of the 12 criteria is reflected by the colour in the segment with the number corresponding to each criterion. The performance is excellent in the case of principles 2, 3, 4, 6, 11, and 12 highlighted in dark green with a score of 1.0. The calculator indicated principles 1, 7, and 10 as the weak points of our procedure highlighted in yellow with a score of 0.5. The first principle of

green analytical chemistry states that sample treatment should be avoided. Direct analysis is, however, not possible for a forced degradation study, since the samples should be treated to stress conditions such as acidic and basic hydrolysis. Moreover, the validation of the analytical procedure includes the preparation of standard and quality control solutions. The seventh principle states that prevention of analytical waste generation would be ideal from an environmental and economic point of view. Unfortunately, analytical waste is produced in all liquid chromatographic methods even though we used a small dimension SEC column packed with sub-3 μm particles and achieved a short total analysis time. The tenth principle promotes the use of chemicals derived from renewable resources. While the SEC method mobile phase was aqueous based, we needed to use acid and base to indicate hydrolysis of adalimumab.

Furthermore, we used the Analytical GREEnness Sample Preparation calculator (AGREEprep) the metric approach adopted to assess the environmental impact of sample preparation methods (Fig. 3). The approach is based on ten consecutive steps of assessment that correspond to the ten principles of green sample preparation. This approach was selected as the ten individual steps of assessment in AGREEprep gave special attention to the sample preparation step of the forced degradation procedure identified as a weak point of the analytical protocol using the AGREE approach. The results of the AGREEprep assessments highlighted a strong point of our procedure's small sample volume (criterion 4, 5, and 6), whereas the weak points were related to the use of chemicals such as acid, basis, and oxidant (criterion 2, 3, and 10). As those chemicals cannot be avoided in the forced degradation study we used as low as possible amounts of toxic chemicals to lower the ecological impact of the sample preparation procedure and improve analyst safety.

Taken together, these calculations highlight that SEC chromatography is a green analytical technique useful for the forced degradation study of adalimumab.

CONCLUSIONS

This research describes the usefulness of the validated SEC method for the eco-friendly forced degradation study of biopharmaceutical adalimumab. Our results revealed the main adalimumab degradation products to be antibody fragments. Acidic and basic conditions had the most intensive effect on the degradation of the adalimumab while the drug exhibits relative stability under thermal and photolytic stress conditions. The newest greenness assessment software AGREE and AGREEprep calculator was used for the evaluation of the environmental performance of the validated forced degradation procedure. The most prominent weak point of the procedure was sample preparation due to the hydrolytic stressing of adalimumab. To sum up, these findings highlight the role of SEC chromatography as green support for the forced degradation study of adalimumab.

Conflicts of interest. – The authors declare no conflict of interest.

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