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Research paper

Impact of extractables from rubber closures on protein stability under heat stress

Carolin Richter[a,b], Cornelia Lipperheide[c], Uwe Lipke[c,*], Alf Lamprecht[b]

[a] WESSLING GmbH, Münster, Germany
[b] Department of Pharmaceutics, Institute of Pharmacy, University of Bonn, Germany
[c] Federal Institute for Drugs and Medical Devices (BfArM), Bonn, Germany

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ABSTRACT

Commercially available, uncoated elastomeric closures were examined in regard to a potential contribution of extracted compounds from the rubber stoppers to protein aggregation under worst-case conditions. All rubber stoppers were confirmed to comply with Ph. Eur. quality requirements. Extraction with 2-propanol under reflux-conditions for 3 h led to closure-specific extraction profiles of the tested samples. One type of rubber stopper exhibited a considerably greater number and higher content of extractables. Four extracted compounds were identified as trialkyl benzene-1, 2, 4–tricarboxylates (trivial name: trimellitates), a substance class which is increasingly established as an alternative to phthalates. A highly concentrated aqueous solution of total extractables from this rubber stopper facilitated the formation of soluble and non-soluble high-molecular aggregates when incubated with model biopharmaceuticals (recombinant human immunoglobulin G (IgG) and recombinant erythropoietin (EPO)) under stress conditions (IgG: 60–64 °C for 130 min, EPO: 55 °C for 8 days). Furthermore, it was shown that the surfactant concentration (polysorbate 20, 0.1 m/v% vs. 1.0 m/v%) decisively influenced the formation of high-molecular aggregates. In case of EPO, the 10 fold increased concentration of surfactant was sufficient to prevent the aggregate formation completely.

This study suggests the necessity of revisiting the current test system of Ph. Eur. monograph 3.2.9 for appropriate rubber stopper quality evaluation.

1. Introduction

In recent years the development and thereby the number of marketing authorizations for biopharmaceuticals increased rapidly. Currently, more than 400 recombinant biopharmaceuticals (peptides and proteins) were already approved worldwide by May 2015. At the same time around 1300 additional recombinant biopharmaceuticals were counted as very promising candidates, of which around 80% already had entered the stage of pre-clinical studies respectively clinical trials [1]. Because of their size and structural properties protein molecules tend to aggregate, which involves a loss of biological activity and pharmacological efficacy [2] combined with an increased risk of causing undesirable immunogenic reactions [3]. This was shown for recombinant human growth hormone (hGh) [4], Willebrandt factor VIII [5] and interferon beta [6].

For enhancing their chemical and physical stabilization, biopharmaceutical formulations usually contain various excipients like buffer salts, antioxidants, chelating agents, and surfactants. Common surfactants in marketed biopharmaceuticals are polysorbate 20 and 80 (PS 20 and 80), which are used in a concentration range of 0.001–0.1% [7]. The choice of surfactant and the optimum concentration depend on e.g. formulation ingredients, protein concentration and the type of container [7].

Biopharmaceuticals are mainly offered as liquid or lyophilized formulations in glass containers that are closed with elastomeric closures. Synthetic rubber closures are the first choice because of their higher purity and the remarkable material properties, particularly their elasticity. This elasticity leads to a tighter contact with the glass container and thus ensures integrity and sterility of the parental product. Furthermore, rubber has a sufficient resealing capability that allows repeated piercing by a needle [8]. Elasticity of the material is obtained by “vulcanization”, a complex chemical procedure at high temperatures and pressure in the presence of various reagents like curing agents, activators, and accelerators. This vulcanization process leads to the desired cross-linked structure of the rubber. Further additives like plasticizers, fillers, antioxidants, and pigments are needed to achieve additional necessary material properties. These additives do not have any covalent binding to the polymer chains and subsequently may leach

* Corresponding author.
E-mail address: uwe.lipke@bfarm.de (U. Lipke).

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from the rubber stopper material into the medicinal product during its shelf life. Leaching should be minimized as leachables from the container closure system can negatively affect the medicinal product's safety, stability, and efficacy.

Extraction studies are the first step for evaluating potential leachables from a container closure component. Samples of the component are exposed to appropriate solvents at extreme conditions in order to maximize the extraction of all compounds capable to migrate. Ding et al. propose various solvents for the examination of the extractables from the elastomeric parts of single-use systems for biomanufacturing applications [9]. Since leachables are largely a subset of the extractables, obtaining the complete extraction profile enables an early evaluation of potential leachables and their risk assessment [10].

Earlier work underlined the impact of the formulation on the potential leaching behaviour from rubber closures [11]. Especially the group of non-ionic polysorbates are suspected to force leaching out of the primary packaging due to their ability for changing the extraction behaviour of the aqueous drug formulation [11]. Accordingly, the compatibility of each parenteral drug product and its selected primary packaging system is verified within the marketing authorization procedure by conducting leachables studies at recommended storage temperature according to the current EMA [12] and FDA [13–15] guidelines.

Ph. Eur. 3.2.9 [16] and USP ⟨3 8 1⟩ [17] describe quality requirements for elastomeric closures intended to be used as a component of the container closure system for parenteral products. The chemical, biological (only USP) and functional tests requested are aimed to confirm the desired quality of elastomeric closures. The chemical quality parameters are studied with an aqueous test solution prepared by boiling a specific number of stoppers in water. This test solution is subsequently used to determine physicochemical parameters, as e.g. "Acidity/Alkalinity" or "Reducing Substances", and to perform tests on "Ammonium" and "Extractable Zinc". No other solvents with different polarities are requested by the pharmacopoeia although recently published recommendations underline the need of different extraction solvents for obtaining a complete summary of extractables and potential leachables from drug product packaging components [18].

Interactions between container closure materials and biopharmaceuticals are of particular importance due to their potential impact on inducing protein aggregation: Silicone oil, a commonly used lubricant for glass vials and rubber stoppers, was discussed in the context of protein aggregation [19] as well as particles derived from equipment surfaces [7]. Also, soluble tungsten species, which originated from pins used for the production of prefilled syringes, was found to lead to an increased formation of aggregates of erythropoietin including both dimers covalently linked by disulphide bonds as well as higher-order aggregates. These tungsten-mediated erythropoietin aggregates were finally concluded as the potential root cause for increased immunogenicity observed during the investigational clinical trial with this medicinal product [20].

An increasing incidence of pure red cell aplasia (PRCA) in patients with chronic kidney disease was observed after subcutaneous use of Eprex® with erythropoietin alpha as active pharmaceutical ingredient in 1998. Leachables derived from the uncoated elastomeric plunger stopper were suspected to be primarily responsible for the formation of anti-erythropoietin antibodies. It was claimed, that the increase in immunogenicity of erythropoietin alpha was due to an adjuvant effect of the leachables from the rubber stopper by polysorbate 80 [21–23].

In view of these serious consequences of incompatibilities between container closure materials and susceptible biopharmaceuticals it is crucial to select suitable container closure components and materials when developing a medicinal product with a protein as active substance. The aim of this study was to examine if commercially available, uncoated rubber stoppers compliant with Ph. Eur. 3.2.9 show similar levels of extractables. Furthermore, it was the objective to investigate to what extent substances extracted by organic solvents from the stoppers are also capable to pass into a commonly used biopharmaceutical formulation consisting of an aqueous buffer solution with polysorbate. Finally, it was intended to develop an experimental setup to evaluate the potential impact of the extractables from a rubber stopper on protein stability. Therefore, a highly concentrated aqueous solution with extractables from rubber closures was generated, which was directly miscible with aqueous protein solutions. Because of their already well characterized aggregation behaviour human immunoglobulin G (IgG) [24] and erythropoietin (EPO) were used as model proteins.

2. Materials and methods

2.1. Materials

Human immunoglobulin BRP (cat. no. Y0001512) and erythropoietin BRP (cat. no. E1515000) were purchased from EDQM (Strasbourg, France). Pyrene (puriss., p.a.) and trioctyl trimellitate were obtained from Sigma-Aldrich (Steinheim, Germany). A protein mixture for the calibration of SEC chromatography, containing bovine thyroglobulin (670 kDA), IgA (300 kDa), IgG (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and uridine (0.244 kDa) was purchased from Phenomenex (cat. no. AL0-3042, Aschaffenburg, Germany).

Polysorbate 20 and polysorbate 80 were purchased from Caesar & Loretz (Hilden, Germany).

Five bromobutyl closures (BB-1, BB-2, BB-3, BB-4, BB-6) for injection from four different vendors and three chlorobutyl closures (CB-1, CB-2, CB-3) for injection from three different vendors were purchased. All elastomeric closures were non-coated, commercially available and intended for pharmaceutical use and complied with the acceptance criteria of Ph. Eur. 3.2.9 and USP ⟨3 8 1⟩. In case of absent certificates, the closures were tested for their compatibility with pharmacopoeias (data not shown).

2.2. Methods

2.2.1. Extraction studies

Extraction solvents were 2-propanol and water. One elastomeric closure was heated in a 20 mL extraction solvent (3h, reflux). The extraction solvents were heated without rubber stoppers for blank preparation. The extract was evaporated in a vacuum concentrator (S-concentrator BA-VC-300H, H. Saur Laborbedarf, Germany) with subsequent dilution in 5 mL 2-propanol, sonication, and filtration. The filtrates were subsequently analysed, Pyrene as Internal standard (IS) was added to two samples at final concentrations of 62.5 µg/mL and 1.25 µg/mL to allow for quantitative determination.

Samples were analysed with a gradient RP-HPLC UV method using different HPLC systems (Dionex and Agilent Technologies, column: Prodigy 3u ODS 3 (100A), 150 × 3.00 mm, Phenomenex, Germany; detection wavelengths: 220 and 254 nm (used for quantification); injection volume: 20 µL; column temperature: 40 °C). The analysis method was adapted from Andrikopolous et al. [25]: A ternary gradient of ammonium formate buffer pH 3.0 [A], ACN/MeOH (6:4 v/v) with 400 µL/L formic acid [B] and 2-propanol with 400 µL/L formic acid [C] was used:

Starting conditions were 48% A and 52% B held for 5 min, and then B was increased to 100% (ramp 1.6%/min) and held for 5 min. Subsequently, B was decreased to 40% while increasing C to 60% (ramp 7.5%/min). Conditions were held for 7 min. B was increased again to 100% in 1 min with a subsequent adjustment to start conditions within 4 min. The flow rate started at 0.5 mL/min and was held for 35 min, and then the flow rate was increased to 0.6 mL/min in 1 min held for 20 min. Finally, return to start conditions in 4 min.

This RP-HPLC UV method was also used for the forced migration studies, and also for structural identification but with slight modifications.