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Rapid Communication

Protein-Excipient Interactions Evaluated via Nuclear Magnetic Resonance Studies in Polysorbate-Based Multidose Protein Formulations: Influence on Antimicrobial Efficacy and Potential Study Approach

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A B S T R A C T

Preservatives are excipients essentially needed in pharmaceutical multidose formulations to prevent microbial growth. Among available substances, phenol is widely used for parenterals; however, it is known to interact with nonionic surfactants like polysorbate and potentially with the active pharmaceutical ingredient. Although the need for combinations of surfactants and preservatives is growing, to date possible molecular interactions which can eventually weaken the stability and antimicrobial activity of the formulation are not yet well understood and properly investigated. In the current study, the binding of phenol to a model fusion protein as well as to polysorbate 20 was investigated. For this purpose, the fraction of bound phenol was successfully quantified via diffusion ordered nuclear magnetic resonance spectroscopy. The binding of phenol to the surfactant is negligible in pharmaceutically relevant polysorbate concentrations, but the binding to the employed active pharmaceutical ingredient was relevant and concentration dependent. The resulting consequence of this interaction was the decrease of the antimicrobial efficacy. As a final outcome of this study, nuclear magnetic resonance analysis is proposed as a material saving method to be used in combination with the antimicrobial activity testing described in the Pharmacopeias.

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Introduction

Antimicrobial preservatives are necessary for multidose injections because they ensure the sterility of a product upon first use. Among suitable substances, the most commonly applied preservatives for parenterals are phenol and m-cresol.[1,2]

Generally, the influence of preservatives on the stability of drug products was reported contradictorily. On the one hand, they can destabilize the active pharmaceutical ingredient (API),[3] albeit in some cases more stable conformational states can be induced, as for instance observed with phenolic compounds employed in insulin formulations.[4]

Since the activity of the named substances is pH dependent[5] and further several phenolic preservatives are not fully compatible with widely used excipients such as polysorbate (PS), the pH and the quali-quantitative composition of the final formulation need to be carefully evaluated.

The selection of the preservative and its optimal concentration is usually conducted by testing the stability of the API under accelerated storage and stress conditions.[2,6] In addition, the antimicrobial activity needs to be evaluated according to the criteria

Abbreviations used: API, active pharmaceutical ingredient; D_2O, deuterated water, heavy water, deuterium oxide; JP, Japanese Pharmacopeia; NMR, nuclear magnetic resonance; Ph. Eur., European Pharmacopeia; PS, polysorbate; USP, United States Pharmacopeia.

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given in the United States Pharmacopeia (USP) and European Pharmacopeia (Ph. Eur.).[7,8]

The test consists of challenging the preparation with a prescribed inoculum of suitable microorganisms (i.e., *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus brasiliensis,* and *Candida albicans*), storing the preparation at 20°C-25°C, withdrawing samples at specified intervals of time, and counting the organisms. The properties of the preservatives are adequate if there is a significant fall in the number of microorganisms after the specific times.

According to the Ph. Eur., the most strict acceptance criterion A is achieved if there is at least a 2 log reduction of the bacteria after 6 h and no recovery after 28 days. For fungi, there must be a 2 log reduction after 7 days and no increase after 28 days.

To meet criterion B of the Ph. Eur., a 1 log reduction after 24 h, a 3 log reduction after 7 days and no increase after 28 days must be achieved for bacteria, whereas for fungi a 1 log reduction after 14 days and no increase after 28 days must be measured.[7,8]

The antimicrobial testing despite being highly useful requires large volumes (about 60 mL) of the formulated API. This might represent a limitation during early-stage pharmaceutical development of biologics, where the concentration of the active ingredient is generally high (>50 mg/mL), posing problems of material availability.

In this study, we investigated the interaction between phenol and a model fusion protein formulated in the presence of PS, using nuclear magnetic resonance (NMR) spectroscopy. Our results suggest that the preservative strongly interacts with the API, whereas the interaction with the surfactant is negligible in pharmaceutically relevant PS 20 concentration, that is, up to 2000 ppm (equal to 2 mg/mL and 0.2%). Preliminary results of the antimicrobial activity of solutions, formulated in the presence or absence of the API, are in agreement with NMR results and suggest that the antimicrobial activity is reduced as a consequence of the phenol-protein interaction.

Based on the obtained results, the quantification of the protein-preservative interaction via NMR is a useful technique for the prediction of the antimicrobial activity of multidose protein therapeutics using low volumes.

Materials and Methods

Materials

PS 20 (Ph. Eur., Japanese Pharmacopeia [JP]) was purchased from Dr. W. Kolb Nederland B.V. (Klundert, The Netherlands).

The PS 20 employed was either freshly opened or from storage, light protected with nitrogen overlay.

Phenol (Ph. Eur., USP, JP) was purchased from Merck (Darmstadt, Germany).

Deuterated water, heavy water, deuterium oxide (D_2O) was obtained from Eurisotop (Saarbrücken, Germany).

Histidine hydrochloride (Ph. Eur., British Pharmacopeia, JP), sodium chloride (Ph. Eur., USP, JP), and mannitol (Ph. Eur., USP) used for buffer preparation were from Merck (Darmstadt, Germany), Südsalz GmbH (Regensburg, Germany), and Cargill Deutschland GmbH (Krefeld, Germany), respectively.

Sample Preparation: Formulation and Placebos for Antimicrobial Testing

The API used in this study was a fusion protein, provided at 60 mg/mL in a buffered solution containing 0.2 mg/mL PS 20.

Formulation and placebos were prepared in 2R glass type I vials (filling volume 2 mL).

Based on previous formulation studies, a lead candidate formulation containing 2.7 mg/mL phenol in 10-mM histidine buffer pH 6.5, 2% mannitol, and 100-mM NaCl was identified. Hence, the formulation and placebos summarized in Table 1 were prepared.

Phenol was used at a final concentration between 2.7 and 3.5 mg/mL, based on the concentration of phenolic preservatives in marketed parenteral multidose formulations.[1,9]

To obtain the final protein formulation containing phenol, 35 mL of the received sample were first dialyzed against 10-mM histidine buffer pH 6.5, containing 0.2 mg/mL PS 20, using Slide-A-Lyzer G2 cassettes (10 kDa, 30-70 mL; Thermo Fisher Scientific, Dreieich, Germany). Dialysis was performed protected from light exposure for 1 day at room temperature, exchanging 2.5 L of buffer after 2 and 4 h, for a total of ~5 L to ensure a ratio of about 150:1 between the volume of the dialysis buffer and the volume to dialyze.

Next, the protein sample was concentrated ~1.7-fold using 15 mL Turbo Spin centrifugal filter units (Sartorius, Göttingen, Germany) with a 10 kDa cutoff, to about 100 mg/mL, using a swinging rotor centrifuge (Thermo Fisher Scientific) and a centrifugal speed of $2000 \times g$ for 30 min at room temperature.

Protein concentration was constantly monitored by UV measurements using an Agilent 8453 UV-Vis spectroscopy system (Waldbronn, Germany). To this end, a small aliquot was diluted 200-fold in 10-mM histidine buffer pH 6.5, containing 0.2 mg/mL PS 20 (i.e., the dialysis buffer), in order to be within 0.5-1 absorbance units. Measurements were carried out at room temperature in 1-cm quartz cuvettes.

The concentration step was needed to prevent API dilution during the addition of the excipients, which were prepared as stock solutions in 10 mM histidine buffer pH 6.5 (12% [w/w] mannitol, 3-mol/L NaCl, 16-mg/mL phenol, and 20-mg/mL [2%] PS 20). This latter surfactant stock solution was added to compensate the PS 20 dilution induced by the addition of the other excipients, prepared in the absence of PS 20.

After the dialysis and concentration steps, before adding the stock solutions, the PS 20 concentration was confirmed to be 0.2 mg/mL (assessed during a pretest to implement the procedure with HPLC coupled to evaporative light scattering, data not shown).

This is not surprising and in agreement with what shown by Lei et al., where the recovery of PS 20 in the retentate remains low in the initial filtration steps with centrifugal filter units.[10]

Placebos were prepared by diluting the above mentioned phenol and PS 20 stock solutions to obtain a final preservative concentration of 2.7 and 3.5 mg/mL.

Antimicrobial Testing

This antimicrobial testing was performed according to Ph. Eur. Eighth Edition, 5.1.3. Efficacy of antimicrobial preservation.[7]

Briefly, samples of the preparations were inoculated separately with 10E5-10E6 CFU/mL of a suspension from *S aureus*, *E coli*, *P aeruginosa*, *A brasiliensis*, *C albicans,* and stored at 20°C-25°C. After 6 and 24 h as well as after 7, 14, and 28 days, samples were withdrawn, and the number of colony forming units (CFU) were determined and compared with the inoculum.

Nuclear magnetic resonance

NMR measurements were performed using an Avance II 600 MHz spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) with a triple resonance room temperature probe. All experiments were run at 300 K (26.85°C) in triplicate measurements (3 measurements from the same sample preparation). The standard BRUKER stimulated echo diffusion ordered pulse sequence

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