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Development of Downstream Processing to Minimise Beta-Glucan Impurities in GMP-Manufactured Therapeutic Antibodies

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Abstract *(245 words, 250 allowed)*

The presence of impurities or contaminants in biological products such as monoclonal antibodies (mAb) could affect efficacy or cause adverse reactions in patients. ICH guidelines (Q6A and Q6B) are in place to regulate the level of impurities within clinical drug products. An impurity less often reported and therefore lacking regulatory guideline is beta-glucan. Beta-glucans are polysaccharides of D-glucose monomers linked by (1-3) beta-glycosidic bonds, and are produced by prokaryotic and eukaryotic organisms, including plants. They may enter manufacturing processes *via* raw materials such as cellulose-based membrane filters or sucrose. Here we report the detection of beta-glucan contamination of a monoclonal IgE antibody (MOv18), manufactured in our facility for a first-in-human, first-in-class clinical trial in patients with cancer.

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Since beta-glucans have potential immunostimulatory properties and can cause symptomatic infusion reactions, it was of paramount importance to identify the source of beta-glucans in our product and to reduce the levels to clinically insignificant concentrations. We identified beta-glucans in sucrose within the formulation buffer and within the housing storage buffer of the virus removal filter. We also detected low level beta-glucan contamination in two of four commercially available antibodies used in oncology. Both formulation buffers contained sucrose. We managed to reduce levels of beta-glucan in our product 10-fold, by screening all sucrose raw material, filtering the sucrose by Posidyne® membrane filtration, and by incorporating extra wash steps when preparing the virus removal filter. The beta-glucan levels now lie within a range that is unlikely to cause clinically significant immunological effects.

Keywords: Beta-glucan, biotherapeutics, monoclonal antibodies, downstream processing, Good Manufacturing Practice

1. Introduction:

Monoclonal antibodies (mAbs) are increasingly used as therapeutic agents in the treatment of patients with a range of diseases and medical conditions, including cancer, auto-immune, allergic and dermatological diseases, osteoporosis, hypercholesterolemia and transplantation. Development of mAbs and their derivatives is an area of great interest in the pharmaceutical, biotech and academic field due to the ability of these agents to target specific antigens on malignant and other cells, to modulate the body's immune system, and to deliver other therapeutic modalities, including radio-isotopes (eg. ibritumomab-tiuxetan [ZEVALIN®]) and cytotoxic drugs (eg. trastuzumab-emtansine [KADCYLA®]).

Therapeutic monoclonal antibody manufacture using mammalian cell culture has been developed and established for many years and can now be considered routine in pharmaceutical production. Improvements in cell line generation and large-scale production technologies have led to high antibody harvest titres (grams per liter), increasing overall productivity and making production of mAbs economically viable. However, many challenges remain for the manufacture of mAbs, as for other biologic products, due to their inherent complexity, heterogeneity and presence of impurities derived from raw materials, the host cells or the manufacturing process.

Depending on the type and amount, impurities in medicinal products may be a serious concern, potentially leading to unwanted immunological and other reactions or even reduced efficacy. Good Manufacturing Practice (GMP) guidelines are in place concerning the acceptable level of impurities in a pharmaceutical product (ICH Q3A and Q3B Impurities). Currently, endotoxins are strictly regulated in terms of maximum permitted levels (ICH Q6A and Q6B Specifications, 1999 [1, 2]). Advisable limits are also in place for residual proteins and polypeptides in biological products (ICH Q6B Specifications, 1999 [2]). However, there are currently no clear specifications available for acceptable levels of beta-glucans.

Beta-glucans are polysaccharides of D-glucose monomers linked by (1-3) beta-glycosidic bonds. Their molecular weight can vary from approximately 6800 Da to over 2 million Da, and they are structurally diverse [3, 4]. Beta-glucans are known to bind to immune cells and are generally considered pro-inflammatory and immunostimulatory molecules [5]. However, depending on their chemical and physical properties, different forms of beta-glucans can elicit different biological reactions [4], and anti-inflammatory properties have also been reported [6, 7]. Beta-glucans are known to have immunologically-mediated anti-cancer properties and they have been shown to enhance the anti-tumor effects of tumor-directed antibodies in vivo. This adjuvant effect is mediated by a complex cascade of events including macrophage-mediated cleavage of beta-glucans to release bioactive fragments which bind to neutrophil C receptor 3 (CR3) and enhance neutrophil cytotoxicity [8, 9]. Beta-glucans are produced by a range of prokaryotic and eukaryotic organisms, and both insoluble and soluble forms have been used therapeutically for many years, especially in oriental medicine [10-12]. Beta-glucans have also been reported to originate from cellulose-based filters [13-117], cell culture raw materials [18], and formulation buffers used in pharmaceutical processing [15,17].

Beta-glucan contamination of pharmaceutic products may be suspected when an “out of specification” result is received following endotoxin testing. Falsely-high endotoxin levels have been reported in the Limulus amebocyte lysate (LAL) assay. Beta-glucans have been shown to cause endotoxin test interference, seen as an enhancement effect. Although endotoxins and beta-glucans follow a different enzymatic pathway in the LAL assay, both activate a clotting cascade which results in the production of the coagulin protein detected in this assay [3, 16]. The potency of beta-glucan in the clotting cascade varies depending on the molecular weight and on the helical formation it takes, with a single helix conformation being more potent than the triple helical form in activating the LAL assay [19]

During manufacture of a novel IgE mAb which recognizes a tumor-associated antigen, folate receptor-alpha (FRα), we obtained variable in-process endotoxin results. The cause was found to be beta-glucan contamination. The antibody, MOv18 IgE, is intended for evaluation in a first-in-human, first-in-class clinical trial in cancer patients (CRUKD/14/001; EudraCT number 2014-000070-19). Since the desired therapeutic effects of MOv18 IgE are immunologically mediated [20-25] and since rapid intravenous administration of beta-glucans is known to provoke infusion reactions [26, 27] which symptomatically could be hard to distinguish from an early IgE-mediated anaphylactic response, we felt it was imperative to find the source of beta-glucan contamination of the MOv18 IgE product and to eliminate it if possible. In this paper, we report our investigations into the causes of the beta-glucan contamination in our GMP manufacturing process. We also describe the GMP downstream process steps developed to minimize beta-glucan contamination in the final MOv18 IgE product. In addition, we measured beta-glucan levels in mAbs already in clinical use, for comparative purposes.

2. Materials and Methods

GMP Manufacture of MOv18 IgE

The chimeric anti-FRα monoclonal IgE antibody, MOv18 IgE, was manufactured to GMP at the Biotherapeutics Development Unit, Cancer Research UK. All batches of the mAb used in this investigation were produced using a master cell bank of a mammalian SP2/0 cell line [28]. The cell culture was expanded to 200 L in a 250 L bioreactor (Thermo Fisher Scientific Massachusetts, US) in ADCF-mAb HyClone media (GE Healthcare, Uppsala Sweden) supplemented with L-Glutamine (Sigma Aldrich, St Louis, US) and grown for six to seven days in a fed-batch process using glucose feed (Fluka Sigma Aldrich, St. Louis, US). The harvest material was filtered using a MilliStak+ DOHC P0D filter (Millipore, Massachusetts, US) and 0.2 µm Sartopore 2 Maxicap (Sartorius Stedim, Goettingen, Germany) filter. Following filtration, the clarified harvest was purified using a succession of chromatography steps comprising KappaSelect capture chromatography (GE Healthcare, Uppsala, Sweden), Sartobind Q anion exchange filtration (Sartorius Stedmin, Goettingen, Germany), followed by SP Sepharose FF polishing chromatography (GE Healthcare, Uppsala, Sweden). The purified material was then passed through a Planova™ 20N virus removal filter (0.3m2) (Asahi Kasei Medical Co. Ltd, Tokyo, Japan).The purified IgE material was formulated by concentration/diafiltration through a hollow fibre cartridge (30 kDa, 0.2 m^2) (GE Healthcare, Uppsala, Sweden) into formulation buffer containing (sodium citrate; Sigma Aldrich, St. Louis, US), L-arginine (Sigma Aldrich, St. Louis, US), sucrose (VRW Pennsylvania, US), and polysorbate 20 (Sigma Aldrich, St. Louis, US). Before sterile filling, the formulated drug product was filtered again through a 0.2 µm Sartopore 2 150 filter (Sartorius Stedim, Goettingen, Germany).

Endotoxin Test Method

The Limulus Amebocyte Lysate (LAL) Kinetic QCL assay kit™ from Lonza (Basel, Switzerland) was used according to the manufacturer's instruction, to determine endotoxin levels as part of the in-process and drug product GMP testing regime. Briefly, five endotoxin standards in a series of 10-fold dilutions (from 50 to 0.005 EU/mL) were added to a 96-well plate, each concentration in quadruplicate. Limulus Amebocyte Lysate reagent water (LRW) was used as a negative control. Samples were diluted with LRW in a range from 1/5 to 1/40 depending on their interference with the assay, and each was tested in duplicate. A parallel series of the same samples was spiked with 1 EU/mL endotoxin standard to assess percent recovery. The plate was incubated in the dark at 37^{o}C for 10 min, followed by addition of 50 µL of Kinectic QCL reagent to each well. The plate was analysed using a PHERAstar plate reader (BMG Labtech, Germany) and the absorbance at 405 nm was measured every 4.5 seconds for 90 minutes. The endpoint absorbance of the endotoxin standard concentration was plotted to generate a standard curve using the Omega software (BMG Labtech, Germany). This curve was used to determine endotoxin concentration within the samples.

For samples where beta-glucan was thought to be causing interference in the LAL assay, a β-G-Blocker kit from Lonza (Basel, Switzerland) was used, as per manufacturer's instructions. Samples to

be tested were diluted half in LRW and half in the β-G-Blocker. For example, instead of 1/10 dilution in LRW, the sample would be diluted 1/5 in LRW and 1/5 in the β-G-Blocker.

Beta-Glucan Test Assay

The Glutacell™ chromogenic test kit from Cape Cod Inc (East Falmouth, MA) was used to determine the level of (1,3)-β-D-glucan within in-process and final drug product samples. The test was performed following manufacturers instruction. All reagents and consumables used were part of the kit. Briefly, four beta-glucan standards in a series of 2-fold dilutions (from 40 pg/mL to 5 pg/mL) were added to a 96-well plate in triplicate. LRW was used as a negative control. Samples were diluted with LRW in a range from 1/5 to 1/80 depending on their interference with the assay and tested in duplicate. A parallel series of the same samples were spiked with 20 pg/mL beta-glucan standard to assess percent recovery. All wells received 50 µl of reconstituted Glutacell. The plate was incubated in the dark at 37^{o}C for 30 minutes. To stop the reaction and allow chromogenic detection of the beta-glucan, 50 µL of sodium nitrate reconstituted in HCl was added to all wells, followed by 50 µL of ammonium sulfamate and 50 µL of N-(1-napthyl) ethylenediamine HCl. The absorbance was read at 540 nm using the PHERAstar plate reader (BMG Labtech, Germany) and, the beta-glucan standard concentration was plotted against the absorbance to generate a standard curve using the Omega software (BMG Labtech, Germany).

Posidyne® Membrane Filtration

To assess the capacity of the Posidyne® filter to remove beta-glucan from the sucrose buffer at a concentration of 250 g/L, a small scale study was performed using a Acrodisc® syringe filter with Posidyne® membrane (PALL Corporation, New York, US) attached to a 50 mL syringe (BD Biosciences New Jersey, US). A stock solution of sucrose at 250 g/L was prepared; 50 mL was passed through the Posidyne® filter and samples were taken at 5 mL, 10 mL and 20 mL. The samples were tested before and after Posidyne® filtration for the presence of beta-glucan and sucrose. For use in GMP manufacture of the IgE mAb, the filter was scaled up to the Posidyne® Kleenpak capsule filter (0.2 µm, 750 cm^2 nominal effective filter area) (PALL, New York, US). Sucrose samples were taken pre- and post- filtration to confirm removal of beta-glucan.

Sucrose Test Assay

The Sucrose Assay from Sigma Aldrich® (St Louis, US) was used to determine the level of sucrose in Posidyne®-filtered sucrose samples. The test was performed following manufacturers instruction. Briefly, five 100 µL sample dilutions of sucrose were prepared at concentrations ranging from 100–1,000 µg/mL. To each sample, 100 µl of sucrose assay reagent was added, mixed and incubated for 10 minutes at room temperature. Then, 2.0 mL of the glucose assay reagent was added, mixed and incubated for 15 minutes at room temperature. The absorbance was read at 340 nm using a PHERAstar plate reader (BMG Labtech, Germany). The sucrose standard concentration was plotted against the absorbance to generate a standard curve using Omega software (BMG Labtech,

Germany). This curve was used to determine sucrose concentrations within samples before and after filtration through the Posidyne® filter.

3. Results and Discussion

3.1. Identification of beta-glucans in in-process samples and in the final drug product

During the GMP manufacture of MOv18 IgE, an endotoxin out-of-specification result was reported in the 0.2µm filtered harvest sample (Figure 1 column A). By including a β-G-Blocker in the samples, the apparent level of endotoxins was greatly reduced, as shown in Figure 1 (column B). This suggested that the endotoxin out-of-specification results were caused by the presence of beta-glucans. Endotoxin results from samples taken following KappaSelect purification gave negligible endotoxin results (Figure 1 Column C), whereas in the MOv18 IgE final product 1 (FP1 Column D) endotoxin levels increased to 0.56EU/mg. This level, was still well within the endotoxin specification of 7EU/mg for MOv18 IgE, as calculated from the maximum dose likely to be administered to a patient within the trial and an average patient weight of 70 kg (Ph Eur 2.06.14). However, there was no clear reason why the endotoxin level was higher in the final product than in earlier stages of the purification process.

Testing of the samples using the Glutacell™ kit revealed levels of beta-glucan in the mAb harvest material which exceeded 25,000 pg/mL (Figure 2 column A). The source of the contamination was suspected to be the Millistak+ DOHC POD filter, a cellulose-based filter used for the removal of cell debris.

Following downstream purification steps which included: KappaSelect, Sartobind Q and SP Sepharose FF chromatography, the beta-glucan was effectively removed to barely detectable levels, as shown in Figure 2 (columns B, C and D). However, beta-glucan testing of batches1 and 2 of the MOv18 IgE final product (FP1 and FP2) revealed levels of beta-glucan of 4,822 pg/mL and 2,581 pg/mL, respectively (Figure 2, columns E and F). These findings suggested that beta-glucan was entering the product following the downstream purification steps and this coincided with the increased levels of endotoxin detected in the final product.

3.2. Beta-glucan analysis of the formulation buffer

We then investigated the sources of the later beta-glucan contamination. Firstly, we examined the formulation buffer (see Methods section for constituents), used to formulate MOv18 IgE following purification. Two batches of formulation buffer were examined; formulation batch 1 (FB1), which was used to formulate MOv18 IgE into final product batch 1 (FP1), and formulation batch 2 (FB2), which was used to formulate batch 2 of final product (FP2). The beta-glucan concentration was approximately 450 pg/mL in FB1, and 265 pg/mL in FB2 (Table 1).

Sucrose is a component of the formulation buffer for MOv18 IgE. Sucrose is commonly used in formulation buffers for mAbs due to its stabilizing ability and protein aggregation-reducing properties

[29]. However, it has been reported that when sucrose is harvested and refined, it is co-processed with beta-glucans [15] and previous reports have shown beta-glucans to have entered pharmaceutical products through the addition of sucrose [15, 18].

We therefore assessed a number of sucrose batches (five) and found beta-glucans in all, but with substantial batch-to-batch variation (Figure 3). Sucrose batch 1 (SB1) and sucrose batch 2 (SB2) were used for formulation buffer batch 1 and 2, and for the formulation of MOv18 IgE final drug product batch 1 and batch 2, respectively. The higher level of beta-glucans in sucrose batch 1 (327 pg/mL) than in sucrose batch 2 (228 pg/mL) is reflected in the higher level of beta-glucans in formulation buffer batch 1 versus 2, and in the MOv18 IgE mAb final product batch 1 versus batch 2 (Figure 2). However, absolute levels of beta-glucans were much higher in the final product than in the formulation buffer or sucrose solution. We therefore evaluated the processing steps following introduction of the formulation buffer.

During formulation of the purified material, the mAb is diafiltrated into approximately 5 L of formulation buffer and then concentrated down to 1 L using a 30 kDa hollow-fibre cartridge (GE Healthcare). As previously stated, beta-glucans vary in size, but are unlikely to pass through a 30kDa pore size membrane. Thus, one would expect the beta-glucans to be concentrated alongside the mAb product when concentrated in this way. Consistent with this, following concentration of the formulation buffers at small scale using a Vivaspin™ column with a 30 kDa membrane (GE Healthcare), levels of beta-glucan were found to have increased approximately 4-fold to 1,813 pg/mL and 1,178 pg/mL for formulation buffer batch 1 (FB1) and 2 (FB2), respectively (Table 1).

3.3. Beta-glucan removal from sucrose

We then explored the use of Posidyne® filters as a method of removing beta-glucan contaminants from the formulation buffer. Posidyne® filters are nylon-based filters used for the removal of endotoxins in the manufacturing process of biologics. They have recently been reported to also remove beta-glucan contaminants in small scale manufacturing [15]. Filtration of 50 mL of 250 g/L sucrose through a small scale Posidyne® filter resulted in efficient removal of beta-glucans, with levels reduced from 1740 pg/mL to below 25 pg/mL (Table 2). Filtration through the Posidyne® filter had no impact on sucrose levels (Table 2). These data show that when used on a small scale, the Posidyne® filter was able to effectively remove beta glucans from 250 g/L sucrose solutions without affecting sucrose concentration.

3.4. Beta-glucan analysis of the virus filtration step

We also investigated the virus removal filtration step as another possible source of beta-glucan contamination. The filter used in our process was the Planova™ 20N, a cellulose-based filter and therefore a potential source of leachable beta-glucans. However, Planova™ 20N has been validated by the supplier (Asahi Kasei) to leach ≤200 pg/mL of beta-glucan. Furthermore, a study by Gefroh et al [15] comparing virus filters, identified the Planova™ 20N filter as one that shows minimal leaching of beta-glucans.

Nevertheless, we evaluated beta-glucan levels in water flush samples from the Planova™ 20N filter and found unexpectedly high levels of beta-glucans – up to 7,000 pg/mL (Figure 4). Interestingly, we observed that beta-glucan levels varied markedly from sample to sample, and there was no obvious decline in beta-glucan levels with consecutive flushes. These findings were at odds with those reported in the literature and with the manufacturer's validation documents for the Planova™ 20N filter. We therefore evaluated the effect of further washes, beyond that recommended by the supplier, and also took samples from the filter housing storage buffer (water for irrigation [WFI], Asahi Kasei), and wash samples following rinsing of the housing unit with WFI (Baxter UK). Our results clearly showed the filter housing storage buffer to be the source of the impurity, with a beta-glucan level in excess of 10,000 pg/mL (Figure 5). Samples from the housing rinse and the filter flush contained negligible levels of beta-glucan following thorough rinsing.

Since the filter housing storage buffer is WFI, it seems likely that the beta-glucans within it originated from the cellulose membrane of the Planova™ 20N filter, leaching slowly from it over time. Removing the housing buffer and rinsing the housing unit with beta-glucan-free WFI, effectively reduced the beta-glucan levels in the Planova™ 20N filtrate to negligible levels.

These findings indicate that the Planova™ 20N filter housing unit needs to be washed carefully prior to use to remove the housing storage buffer, in addition to the recommended flushing of the filter, in order to effectively remove any residual beta-glucan that has leached from the filter.

3.5. Optimisation of GMP 250 L process

Following these findings, the GMP process for manufacturing MOv18 IgE at 250 L scale was optimized. Key process steps added were as follows. Firstly, all sucrose batches were analysed to identify the batch with the lowest levels of beta glucan. Secondly, 250 g/L of sucrose solution was filtered through a Posidyne® filter (Posidyne® Kleenpak capsule filter). The filtered sucrose was then diluted into the formulation buffer. Thirdly, the housing storage buffer of the Planova™ 20N filter was completely removed and the housing was rinsed with WFI before use. By adding these steps, the beta-glucan levels in the formulation buffer and rinse samples were greatly reduced (Table 3). This led to a significant reduction in beta-glucan levels in the final product. Before optimisation, beta-glucan levels in the final product were measured at 4,822 pg/mL in FP1 and 2,581 pg/mL in FP2. With implementation of the extra processing steps, the beta-glucan levels were greatly reduced to 411 pg/mL in FP3, and to 243 pg/mL in FP4 of the final product (Figure 6).

These results show that the steps taken to remove beta-glucan at a small scale can be translated effectively to large scale GMP processes. The approach to filter the sucrose solutions at the higher concentrations of 250 g/L and then dilute in the formulation buffer to reach the required concentration was taken as filtration of the entire formulation buffer could potentially affect other constituents of the buffer.

3.6. β-glucan analysis of commercial mAbs

The processes used in the manufacture of MOv18 IgE for administration in a clinical trial setting are essentially the same as generally used for the manufacture of mAbs with the exception of the capture step. In particular, Planova™ 20N filters are commonly used for virus removal, and sucrose is commonly used for buffer stabilisation in commercial production processes. It therefore seemed likely that beta-glucans could be present in other mAb products.

Accordingly, we tested four commercially-produced mAbs for the presence of beta-glucans. We selected four mAbs, Herceptin® (trastuzumab), Avastin® (bevacizumab), Perjeta® (pertuzumab) and Kadcyla® (trastuzumab-emtansine) that are used to treat cancer patients in routine practice, and that are formulated in sucrose (Perjeta® and Kadcyla®) or trehalose (Herceptin® and Avastin®), according to their respective Summary of Product Characteristics. Trehalose is another sugar which may be co-processed with beta-glucans. Beta-glucan levels were undetectable or almost undetectable in Herceptin® and Avastin®, which are formulated in trehalose. However, Perjeta® and Kadcyla®, which include sucrose as a constituent of the formulation buffer, were found to contain beta-glucan levels of 195 pg/mL and 1,007 pg/mL respectively (Table 4).

Based on an average adult patient weight of 70 kg, 12.5 ng of beta glucans could be co-administered to patients with each infusion of Kadcyla®, and 5.4 ng of beta-glucans with the first dose of Perjeta®. These doses are considerably lower than the doses of beta-glucans administered intravenously to patients treated with therapeutic beta-glucan products, such as lentinan (2 mg doses commonly administered intravenously in trials and routine practice; see for example [30]). Such products are generally well tolerated except when administered as rapid infusions and/or at relatively high doses. For example, doses of lentinan up to 10 mg were well tolerated when given over 30 minutes but were associated with infusion reactions when given over 10 minutes [26]). The experimental beta-glucan product BTH1677 (Imprime PGG®), has been given as single doses of up to 6 mg/kg (ie. 420 mg for a 70 kg adult) or as 4 mg/kg daily doses (280 mg/day for a 70 kg adult) [27]. Infusion-related symptoms such as dyspnea, flushing, headache, nausea, paraesthesia, and rash, commonly occurred ($\geq$10% of participants) when given as intravenous infusions over one hour but were reduced when infusion times were increased (to 2 or 3 hours).

Overall, from a safety perspective, these findings are reassuring for the administration of MOv18 IgE to patients in a clinical trial situation. Although levels of beta-glucan in MOv18 IgE final product (now consistently $\leq$ 10 ng/mL) remain above the range found in commercially available mAbs that are used in routine clinical practice in cancer patients (195-1007 pg/mL based on Perjeta® and Kadcyla®), the maximum dose of beta-glucans likely to be administered to any patient within the MOv18 IgE clinical trial (calculated as 500 ng) is considerably lower than doses of beta-glucans widely used to treat patients therapeutically in routine practice in parts of Asia (2 mg/dose of lentinan), and in clinical trials in the USA (up to 6 mg/kg/dose). Whether the low levels of beta-glucan found in commercially available mAb products and in our MOv18 IgE final product could have an immunostimulatory or other immunomodulatory effect is currently unknown. However, based on the concentrations of beta-

glucans reported to produce immunostimulatory effects in in vitro studies, this seems unlikely. These data are extensively reviewed in a separate publication from our group [31].

4. Conclusion

During the manufacture of a novel mAb for clinical trial use, we encountered falsepositive endotoxin test results that turned out to be caused by presence of beta-glucan. Our investigations into the source of the impurity revealed that the downstream purification steps (which included KappaSelect chromatography, Sartobind Q filtration and SP Sepharose FF chromatography) were effective at removing beta-glucans from the supernatant harvested from cell culture. However, beta-glucans were re-introduced during subsequent processing steps. In particular, we found variable levels of beta-glucans in the sucrose used to prepare the formulation buffer, and we found high levels of beta-glucans in the filter housing storage buffer provided by the manufacturer for the Planova™ 20N virus filter. Levels of beta-glucans originating from the formulation buffer were further increased by the mAb concentration step, during which a 30 kDa pore size filter was used. Since beta-glucans are too large to pass through this filter, they were concentrated along with the MOv18 IgE antibody.

We were able to successfully reduce the levels of beta-glucans in subsequent batches of the MOv18 IgE final product by testing and selecting sucrose batches for low levels of beta glucan contamination and by filtering the sucrose solutions through a nylon Posidyne® filter. We also introduced additional rinsing steps to ensure complete removal of the housing storage buffer for the Planova™ 20N filter. However, levels of beta-glucan in subsequent batches of MOv18 IgE final product were still higher than those we detected in commercially available mAbs used for cancer patients that are formulated in sucrose. Nevertheless, based on a review of the literature, beta-glucans levels in the MOv18 IgE final product up to 10 ng/mL (which could potentially result in administration of up to 500 ng in an infusion of MOv18 IgE in the CRUKD/14/001 clinical trial) are of no safety concern and are thought unlikely to result in any clinically significant immunological effect (reviewed in [31]). A maximum beta-glucan level of 10 ng/mL has been accepted by the UK Medicines and Healthcare Products Regulatory Agency for the MOv18 IgE final product, suggesting a specification that could be applied to other products affected by beta-glucan contamination in the future.

Control of beta-glucan levels in antibody therapeutic products is critical as differences could cause variability in the efficacy of the antibody. Our experiences highlight the need for manufacturers to be aware of potential beta-glucan contamination at all stages of the mAb manufacturing process. In particular, we recommend testing sucrose raw material using the Glutacell™ kit to identify sucrose batches with the lowest levels of beta-glucans.

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Figures and Tables

Figure 1. Detection of beta-glucan impurities following endotoxin testing of MOv18 IgE mAb harvest material. The 0.2µm filtered harvest material (Column A) appeared to show high levels of endotoxin. Following the addition of β-G-Blocker, levels were reduced by over half (Column B), indicating beta-glucan interference. Endotoxin levels in the KappaSelect eluate (Column C) were undetectable, suggesting the beta-glucan had been removed. Endotoxin levels appeared to be slightly higher in the mAb final product (FP1) (Column D), although remaining well within our endotoxin specification limit of 7 EU/mg. (Error bars represent SD with n=4)

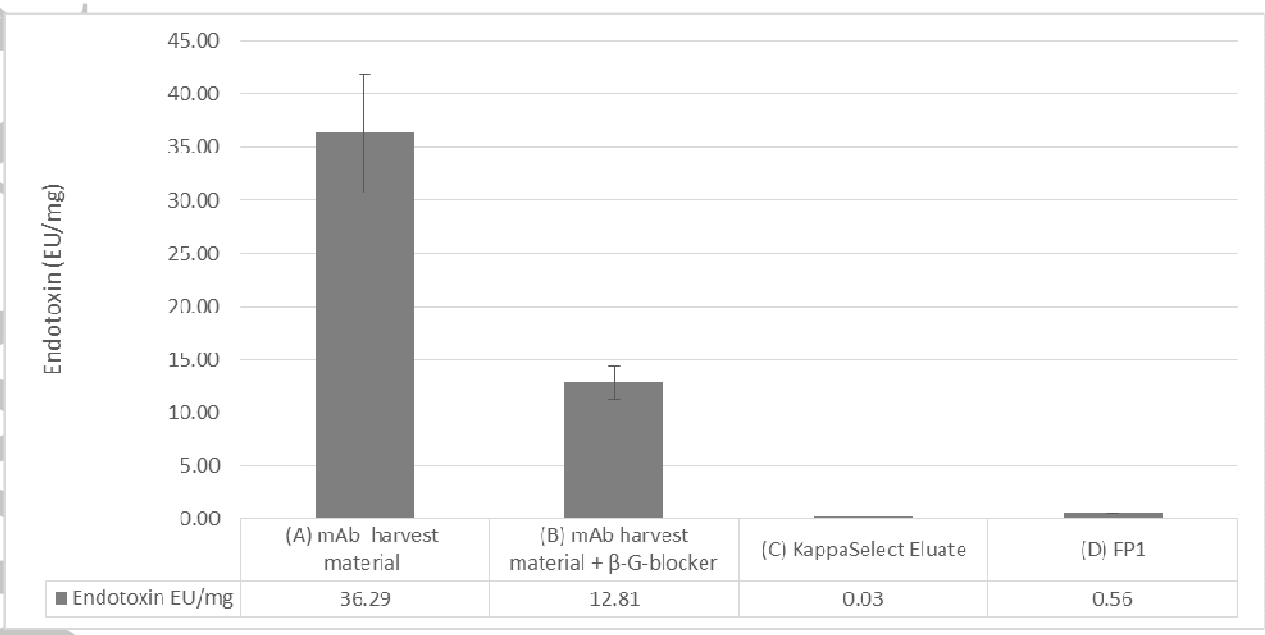

Figure 2. Beta-glucan clearance patterns throughout the downstream antibody purification process. A high level of beta-glucan was detected in the harvest material (Column A). The majority of beta-glucan was removed following Kappa Select affinity purification (Column B) and levels remained very low following two subsequent steps (Columns C and D). However, beta-glucan was detectable again in the final product batch 1 (FP1) (Column E). Final product batch 2 (FP2) was also tested and found to contain similar levels of beta-glucan (Column F). (Error bars represent SD, with n= 3)

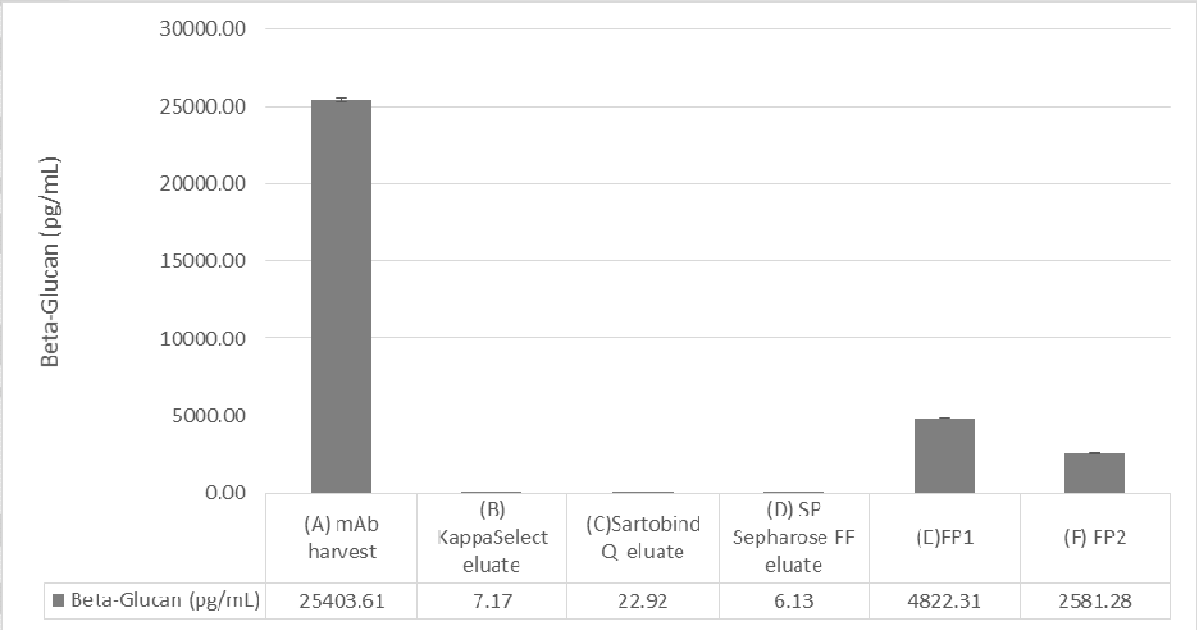

Table 1. Beta-glucan levels measured in formulation buffer batch 1 (FB1) and batch 2 (FB2), before and after small scale concentration using a Vivaspin™ column with a 30 kDa membrane (GE Healthcare) (n=2)

Samples	Beta-glucan (pg/mL)
FB1 Pre-Concentration	451 (446-455)
FB1 Concentrated 5x	1813 (1690-1935)
FB2 Pre-Concentration	266 (253-278)
FB2 Concentrated 5x	1178 (1162-1195)

Figure 3. Beta-glucan levels measured in 5 different batches of sucrose (SB1-5) prepared at a concentration of 50 g/L. A large difference in beta-glucan levels between the 5 batches of sucrose was observed. (Error bars represent SD, with n= 3)

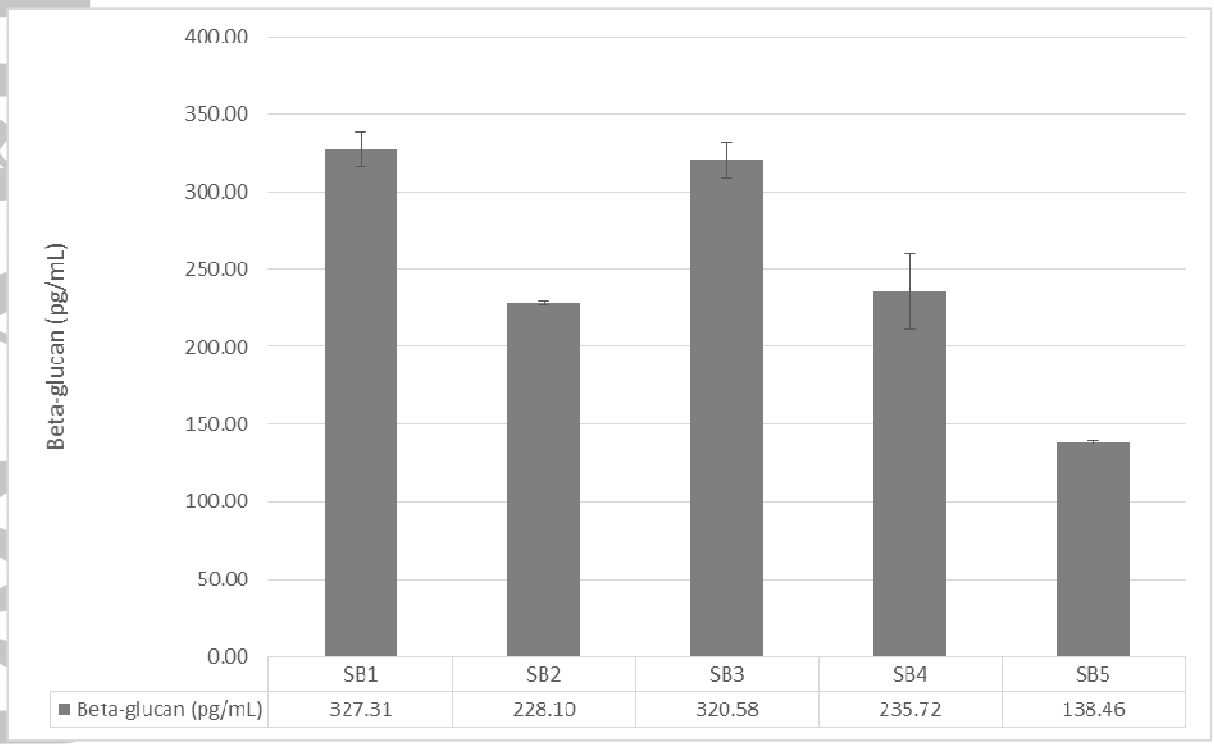

Table. 2. Beta-glucan and sucrose levels in 250 g/L sucrose buffer (SB3) before and after filtration through the Posidyne® filter. Removal of beta-glucan was observed with no impact on sucrose concentration as measured by Absorbance at OD 340nm. (n=2)

Sample	Beta-glucan (pg/mL)	Sucrose (OD 340 nm)
Unfiltered sucrose	1740 (1684-1795)	0.782
10 mL post filter	6 (5.7-6.4)	0.811
20 mL post filter	25 (23.2-25.8)	0.885
100 pg/mL beta-glucan standard/ no sucrose	100	-0.008

Figure 4. Beta-glucan levels in flush samples taken from the Planova™ filter. Levels of beta-glucan were found to be high in many samples with no clear downward trend in levels following repeat flushing. (Error bars represent SD, with n= 3)

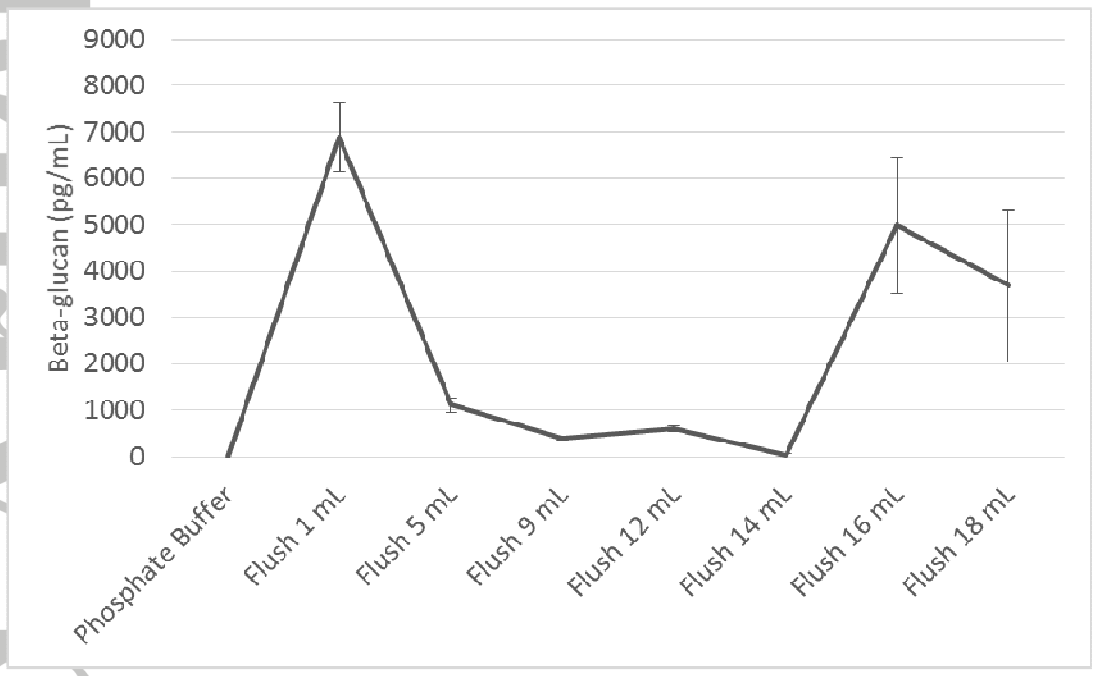

Figure 5. Beta-glucan levels in flush samples taken from the Planova™ filter. The filter housing storage buffer contained a large amount of beta-glucan. Complete removal of the storage buffer led to undetectable levels of beta-glucan in the Planova™ filter flush samples. (Error bars represent SD, with n= 3)

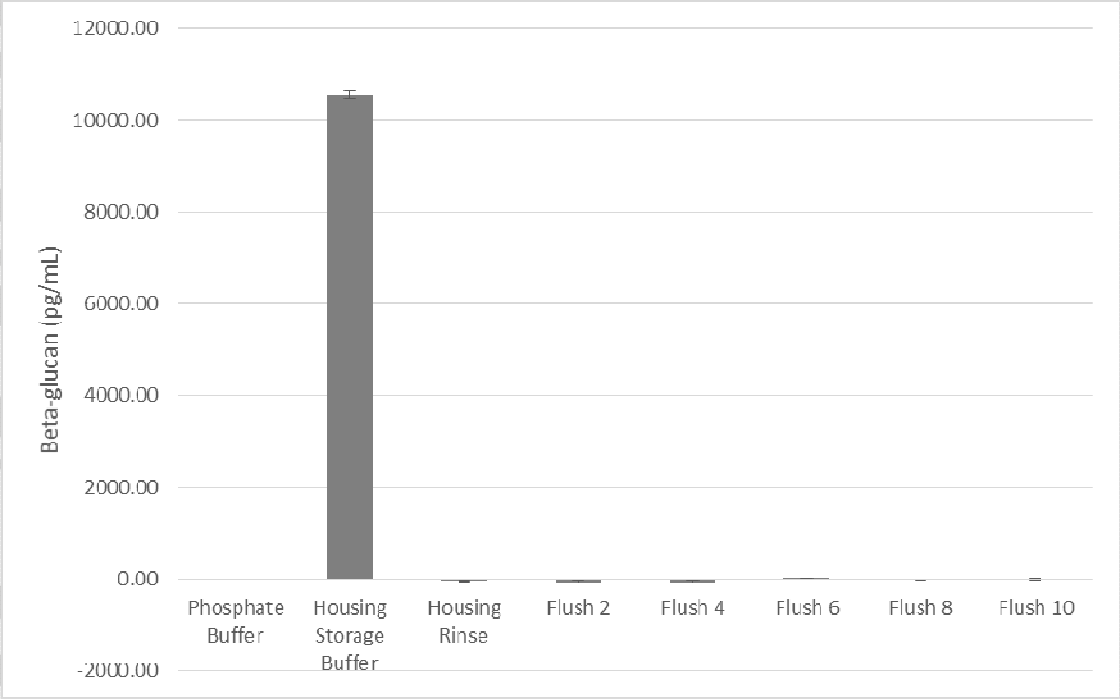

Table 3. Beta-glucan levels following the addition of downstream processing steps in the GMP manufacturing process. Posidyne® filtration, removal of the Planova™ 20N housing storage buffer and rinsing of the Planova™ 20N filter, produced a reduction in beta-glucan levels (n = 2)

Sample	Beta-glucan (pg/mL)
GMP Formulation buffer pre-Posidyne® filtration	549 (361-738)
GMP Formulation buffer post-Posidyne® filtration	42 (30-54)
Planova™ 20N housing buffer	21,985 (21,972-21,998)
Planova™ 20N housing rinse following removal of housing storage buffer and rinsing	801 (766-836)

Figure 6. Final product (FP) mAb batch 1 and batch 2 showing beta-glucan levels before optimization of downstream processing steps (high levels: 4,822 pg/mL and 2,581 pg/mL, respectively). Final product mAb batches 3 and 4 were obtained after optimization of downstream processing. Beta-glucan levels are significantly lower in these samples (411 pg/mL and 243 pg/mL, respectively). (The error bars represent SD, with n= 3).

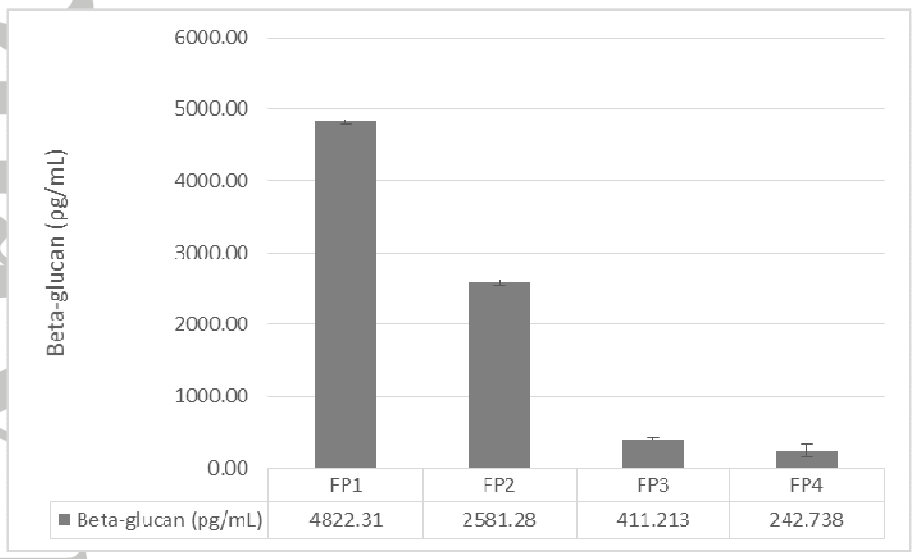

Table 4. Assessment of beta- glucan levels in four commercially-available mAbs used in patients with cancer. Based on an average body weight of 70 kg, patients may receive up to 12.6 ng of beta-glucan per infusion (n=3)

Product	mAb concentration (mg/mL)	Beta-glucan (pg/mL)	mAb dose (mg)	Beta-glucan dose (pg)	Beta-glucan dose (ng)
Herceptin® (trastuzumab)	21	below LOD*	560	0	0
Avastin® (bevacizumab)	25	1(-1.3 – 3.4)	1050	45	0.045
Perjeta® (pertuzumab)	30	195 (187-203)	840	5400	5.4
Kadcyla® (trastuzumab-emtansine)	20	1007 (997-1016)	252	12600	12.6

*LOD = Level of detection