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# Experimental proof of contamination of blood components by (1→3)-β-D-glucan caused by filtration with cellulose filters in the manufacturing process

**Abstract** The level of (1→3)-β-D-glucan in blood is a diagnostic index of fungal infection because it is released from the fungal cell wall. However, high levels of plasma (1→3)-β-D-glucan in patients administered blood components may give false positive results. High levels of (1→3)-β-D-glucan have been detected in blood components. We suspected that (1→3)-β-D-glucan from cellulose filters had been eluted into blood components by filtration in the manufacturing process. To investigate the contamination of blood components by (1→3)-β-D-glucan from cellulose filters, in vitro experiments were performed by using six cellulose filters and a nylon filter. Human serum albumin (HSA) solution (100 ml) was flowed through each filter after rinsing with 100 ml of distilled water, and (1→3)-β-D-glucan in each fraction was determined by Fungitec G test MK. The concentration of (1→3)-β-D-glucan eluted from cellulose filters in 100-ml distilled water fractions ranged from 6 to 207 pg/ml, and that of HSA fractions ranged from 33 to 20,784 pg/ml. These data showed that remarkably higher (1→3)-β-D-glucan levels were detected in HSA fractions flowed through cellulose filters in spite of advance rinsing with 100 ml of distilled water. In the case of a nylon filter, (1→3)-β-D-glucan was not eluted in either fraction. These results indicate that (1→3)-β-D-glucan contamination in blood components is caused by filtration with cellulose filters in the manufacturing process.

**Key words** (1→3)-β-D-glucan · Human serum albumin · Blood components · Cellulose · Fungal infection

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## Introduction

(1→3)-β-D-glucan is a glucose polymer with a main chain composed of β-1,3 linkage. (1→3)-β-D-glucan is a structural polysaccharide of the cell wall and occurs in fungi such as molds and mushrooms, higher plants, and algae. (1→3)-β-D-glucans isolated from the fruiting body, mycelium, and products in the medium of fungi show antitumor activity.[1–6] Curdlan, a nonbranching (1→3)-β-D-glucan produced by *Alcaligenes faecalis var. myxogenes*, is a food additive used for thickening and gel formation.[7]

(1→3)-β-D-glucan in the blood is a diagnostic marker of fungal infection, because it is released from the fungal cell wall. Recently, a method of measuring (1→3)-β-D-glucan for the diagnosis of fungal infection was developed.[8–11] The level of (1→3)-β-D-glucan in a healthy person is usually under 10 pg/ml. When the level is over 20 pg/ml, a fungal infection is diagnosed.

However, false positive results may occur. An increase of the (1→3)-β-D-glucan level in the blood after administration of blood components or hemodialysis using cellulose membranes has been reported.[12–15] High levels of (1→3)-β-D-glucan were in fact detected in blood components. Usami et al. reported that (1→3)-β-D-glucan levels in blood components ranged from 0 to 6930 pg/ml.[16] (1→3)-β-D-glucan levels over 1000 pg/ml were detected in 28% (14 of 50) of samples of blood components (24 albumin and plasma protein fractions, 10 blood coagulation fractions, and 16 immunoglobulin solutions).[16] Furthermore, release of (1→3)-β-D-glucan from cellulose filters was also shown during in vitro filtration.[16] It is strongly suggested that the false positive is caused by contamination of blood components with (1→3)-β-D-glucan released from cellulose mate-

rials. Because cellulose materials are made from higher plants containing (1→3)-β-D-glucan in the cell wall, contamination of blood components with (1→3)-β-D-glucan in filtration processes using cellulose filters is conceivable.

We performed an in vitro experiment to test the hypothesis that (1→3)-β-D-glucan contamination of blood components was caused by filtration with cellulose filters in the manufacturing process.

## Materials and methods

### Materials

Zetaplus-LA (LA) filters and Zetaplus-LP (LP) filters were provided by Cuno (Yokohama, Japan). EKSP filters, EK1P filters, and EK filters were provided by U.S. Filter Japan (Tokyo, Japan). Milligard CWSS (CWSS) was provided by Millipore (Billerica, MA, USA). Ultipor N66 (N66) was provided by Nihon Pall (Tokyo, Japan). Otsuka Distilled Water (distilled water for injection) was purchased from Otsuka Pharmaceutical (Tokyo, Japan). Buminate 25% (25% human serum albumin [HSA]) was provided by Baxter (Deerfield, IL, USA). Fungitec G test MK was provided by Seikagaku (Tokyo, Japan).

### Sterilization of filters

A filter was autoclaved in a sterilization bag (121°C for 20 min) and incubated at 50°C for 24 h in a dryer.

### Elution of (1→3)-β-D-glucan

Each filter was fitted on an autoclaved filter holder in aseptic condition, and 100 ml of Otsuka Distilled Water was flowed through each filter, using a Perista Pump (Atto, tube 3 mm ϕ) at flow rate of 2.5 ml/min. The flow rate was decided according to the filter rinsing protocol of Cuno. The eluate was divided into 25-ml fractions. Then 5% HSA solution (100 ml) prepared by diluting 25% HSA (Buminate 25%) with distilled water was flowed through in the same way, and divided into 25-ml fractions. The filtration process was performed three times for each filter, and the mean ± SD levels of (1→3)-β-D-glucan were determined from three experiments.

### Determination of (1→3)-β-D-glucan

(1→3)-β-D-glucan was determined by the Fungitec G test MK.[11] A sample solution (25 μl) was added to a Multiplate 96F (Sumitomo bakelite), and 100 μl of enzyme solution, a mixture of limulus coagulation factor G system and synthetic substrate (p-nitroaniline-peptide conjugate), was added. A two-step enzyme reaction is induced in the presence of (1→3)-β-D-glucan, and p-nitroaniline is released by hydrolysis of the synthetic substrate. Detection of the released p-nitroaniline was carried out by reading the absorbance at 405 nm and 490 nm with incubation at 37°C. The reaction was monitored by Microplate Fluorescence Reader FL600 (Bio-Tek), and the concentration of (1→3)-β-D-glucan was calculated by the kinetic measurement method. When the samples eluted by 5% HSA were determined, the (1→3)-β-D-glucan concentration in 5% HSA for the blank value was subtracted from each value.

## Results

### (1→3)-β-D-glucan levels of solutions to elute

In this study, Otsuka Distilled Water was used for the dilution and rinsing of filters. The concentration of (1→3)-β-D-glucan in the distilled water was under the limit of detection (less than 1.5 pg/ml), and that of 5% HSA was 23.7 ± 0.9 pg/ml ($n$ = 5).

### Material and measured value of each filter

We used six cellulose filters (LA, LP, EKSP, EK1P, EK, and CWSS) and a nylon filter (N66). All of the filters are used in the manufacture of blood components, according to a questionnaire survey of pharmaceutical corporations. The size and weight of the filters are shown in Table 1. CWSS and N66 were light and thin compared with other filters. In the experiment using CWSS, two pieces of filter were used at the same time (CWSS RW03 and CWSS RW06).

### (1→3)-β-D-glucan level of each fraction eluted from LA filter

Figure 1 shows the (1→3)-β-D-glucan level of each 25 ml of fraction filter with the Zetaplus-LA filter. The data are

**Table 1.** Material and measured values of each filter[a]

| Filter | Material | Diameter (mm) | Thickness (mm) | Weight (g) |
|---|---|---|---|---|
| Zetaplus-LA | Cellulose | 47 | 3.10 | 2.192 |
| Zetaplus-LP | Cellulose | 47 | 3.66 | 2.465 |
| EKSP | Cellulose | 47 | 3.38 | 2.497 |
| EK1P | Cellulose | 47 | 3.52 | 2.459 |
| EK | Cellulose | 47 | 3.62 | 2.337 |
| Milligard CWSS | Cellulose | 47 | 0.35 (2 pieces) | 0.254 |
| Ultipor N66 | Nylon | 47 | 0.24 | 0.189 |

[a] The thickness ($n$ = 15) of each filter was measured by a micrometer

shown as means ± SD ($n$ = 3). In the first 25 ml of the distilled water fraction, over 50% of the total (1→3)-β-D-glucan content in 100 ml of fraction was eluted. As the volume of the flow increased, the level decreased little by little. The same tendency was recognized in the 5% HSA fraction.

The (1→3)-β-D-glucan level in the last 25 ml of the distilled water fraction was 14 ± 14.1 pg/ml ($n$ = 3). Although a flow of more than 200 ml of distilled water was desirable to decrease the elution of (1→3)-β-D-glucan to under the limit of detection, about 95% of the total (1→3)-β-D-glucan content in the 200 ml of distilled water fraction was eluted by flowing 100 ml of distilled water. Consequently, it is concluded that the LA filter was washed enough by flowing 100 ml of distilled water.

However, the level of (1→3)-β-D-glucan in the first 25 ml of the 5% HSA fraction (205 ± 26.6 pg/ml, $n$ = 3) was about 15 times the value in the last 25 ml of the distilled water fraction ($P < 0.01$, $t$-test). Although the cellulose filters had been washed with 100 ml of distilled water, much higher (1→3)-β-D-glucan levels were detected in the 5% HSA fractions. Other filters also showed the same tendency.

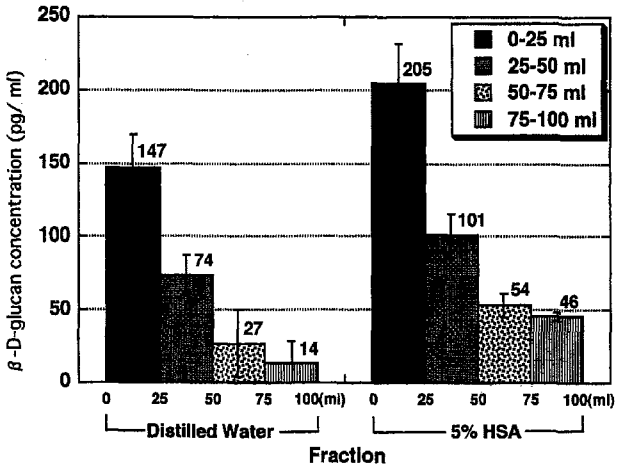


**Fig. 1.** (1→3)-β-D-glucan levels in distilled water fraction and 5% HSA fraction: each 25-ml fraction filtered with Zetaplus-LA filter

## Elution of (1→3)-β-D-glucan from filters

Figure 2 shows the (1→3)-β-D-glucan levels in each total fraction (100 ml) of distilled water and 5% HSA. The (1→3)-β-D-glucan levels in distilled water fractions filtered with LA, LP, EKSP, EK1P, EK, CWSS, and N66 filters were 65 ± 22.7 pg/ml, 207 ± 52.0 pg/ml, 6 ± 3.3 pg/ml, 23 ± 5.8 pg/ml, 150 ± 51.2 pg/ml, 39 ± 20.2 pg/ml, and under the limit of detection, respectively. The levels in 5% HSA fractions filtered with LA, LP, EKSP, EK1P, EK, CWSS, and N66 filters were 101 ± 9.1 pg/ml, 165 ± 21.2 pg/ml, 4,848 ± 288.8 pg/ml, 215 ± 28.3 pg/ml, 20,784 ± 1990.6 pg/ml, 33 ± 7.0 pg/ml, and under the limit of detection, respectively. In cellulose filters, much more (1→3)-β-D-glucan was eluted in the 5% HSA fraction than in the distilled water fraction. The tendency was remarkable in EKSP, EK1P, and EK. These data show that a high level of (1→3)-β-D-glucan is

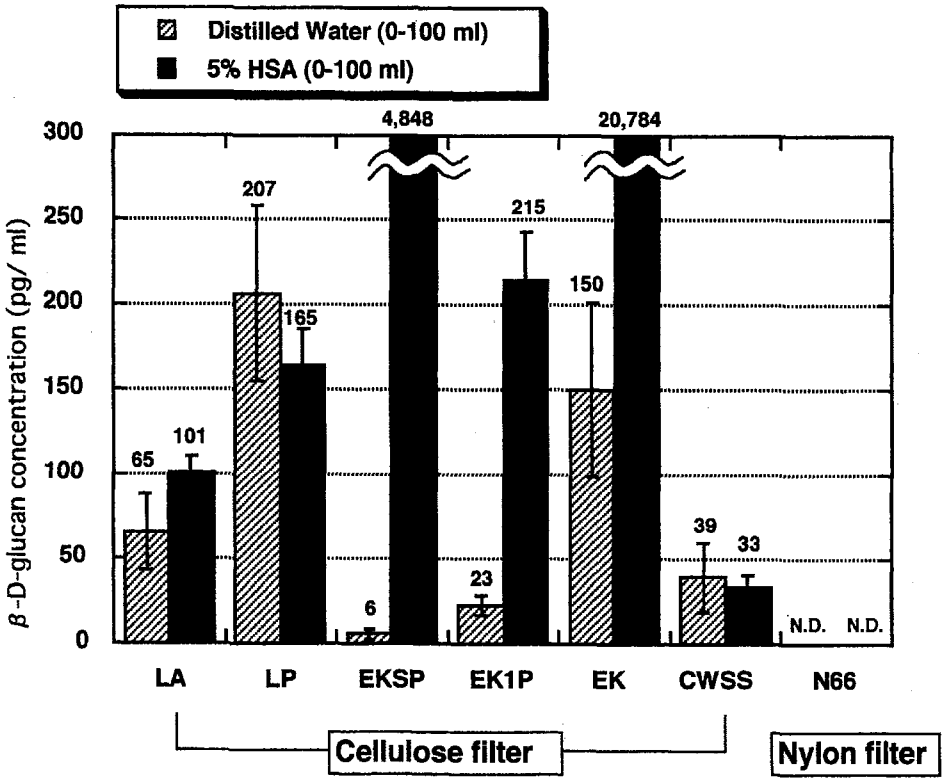


**Fig. 2.** Elution of (1→3)-β-D-glucan to 100 ml of distilled water fraction and 100 ml of 5% HSA fraction from each filter

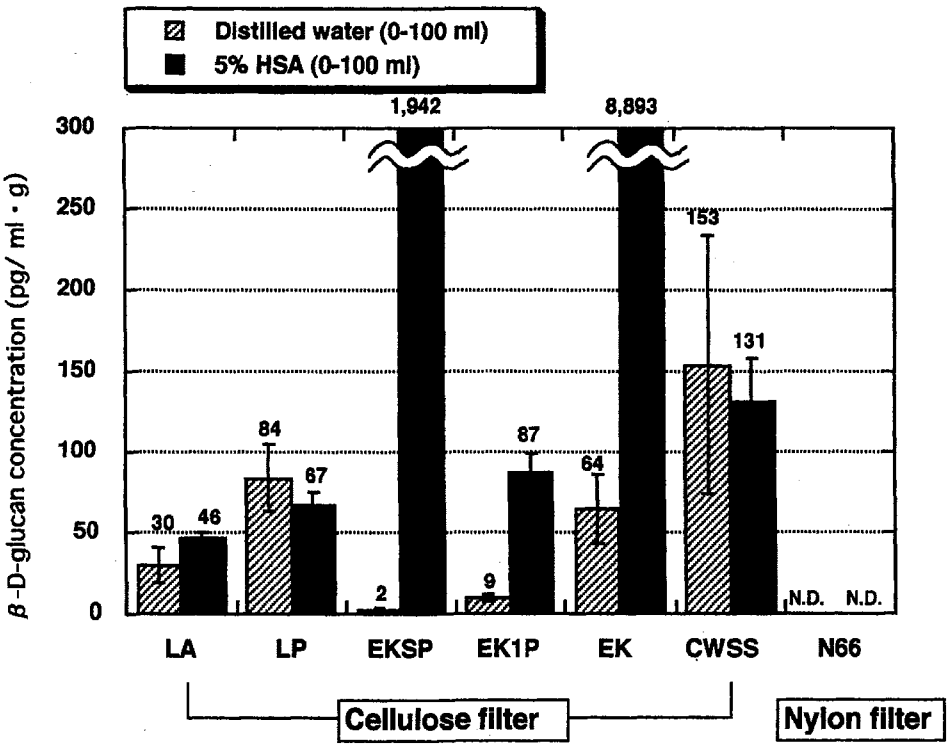


**Fig. 3.** (1→3)-β-D-glucan level per unit weight of each filter in 100 ml of distilled water fraction and 100 ml of 5% HSA fraction

eluted by a flow of 5% HSA in spite of advance rinsing with 100 ml of distilled water. The level of (1→3)-β-D-glucan in 100 ml of 5% HSA fraction filtered with CWSS was remarkably low (33 ± 7.0 pg/ml), as compared with the levels in the fractions filtered with other cellulose filters. In the case of the nylon filter (N66), (1→3)-β-D-glucan did not elute (under the limit of detection) in either fraction.

Figure 3 shows the (1→3)-β-D-glucan concentration per unit weight of each filter. To allow for the difference in weight among the filters, the value shown in Fig. 2 was divided by the weight of each filter. The levels in 100 ml of distilled water fractions filtered with LA, LP, EKSP, EK1P, EK, and CWSS filters were 30 ± 10.4 pg/ml·g, 84 ± 21.1 pg/ml·g, 2 ± 1.3 pg/ml·g, 9 ± 2.4 pg/ml·g, 64 ± 21.9 pg/ml·g, and 153 ± 79.6 pg/ml·g, respectively. The levels in 100 ml of 5% HSA fractions filtered with LA, LP, EKSP, EK1P, EK, and CWSS filters were 46 ± 4.2 pg/ml·g, 67 ± 8.6 pg/ml·g, 1942 ± 115.7 pg/ml·g, 87 ± 11.5 pg/ml·g, 8893 ± 851.8 pg/ml·g, and 131 ± 27.6 pg/ml·g, respectively. The (1→3)-β-D-glucan levels in fractions filtered with CWSS were relatively high, because this filter is very thin (Table 1). To a greater or lesser degree, contamination with (1→3)-β-D-glucan in both fractions from all cellulose filters was shown.

## Discussion

Although the cellulose filters had been washed with 100 ml of distilled water, much higher (1→3)-β-D-glucan levels were detected in HSA fractions (Figs. 1 and 2). The tendency was particularly remarkable in EKSP, EK1P, and EK. It is noteworthy that a (1→3)-β-D-glucan level of 20,784 ± 1990.6 pg/ml ($n$ = 3) was detected in 100 ml of 5% HSA fraction flowed through EK. The (1→3)-β-D-glucan level released from CWSS was relatively low, as shown in Fig.2, because of the difference in thickness. It is possible to decrease the degree of contamination of blood components by (1→3)-β-D-glucan by the use of a thin cellulose membrane such as CWSS. Judging by the elution of (1→3)-β-D-glucan in 100 ml of both fractions per unit weight of each filter (Fig. 3), relatively high (1→3)-β-D-glucan levels were found in both fractions filtered with CWSS, compared with the results with other cellulose filters. Consequently, elution of (1→3)-β-D-glucan from cellulose filters is inevitable.

It is thought that the relation between elution of (1→3)-β-D-glucan from cellulose filters and the difference in the species of cellulose filter depends on the degree of purification of cellulose material or additives.[16] Cellulose filters usually contain diatomaceous earth or acid-washed diatomaceous earth as an additive to enhance filtration efficiency. Diatoms, the source of diatomaceous earth, store (1→3)-β-D-glucan (chrysolaminaran) produced by photosynthesis. It is suspected that (1→3)-β-D-glucan from diatomaceous earth might be eluted by filtration, although there is no information on (1→3)-β-D-glucan levels in diatomaceous earth.

According to the data in the Seitz-filter-Werke catalog, characteristic retention rates of EKSP, EK1P, and EK for practical purposes are 0.1–0.4 μl, 0.2–0.4 μl, and 0.4–0.6 μl, respectively. The bacteria retention capacities of EKSP, EK1P, and EK tested with *Escherichia coli* in physiological NaCl solution are under $10^9$ CFU/cm$^2$, $10^8$ CFU/cm$^2$, and

$10^7$ CFU/cm$^2$, respectively. These data indicate that the pore size decreases in the order EKSP, EK1P, and EK. The parallel relation between the difference in (1→3)-β-D-glucan levels in 100ml of distilled water fractions eluted from EKSP, EK1P, and EK and the difference in pore sizes of EK filter series was recognized. However, there was no relation between the difference in (1→3)-β-D-glucan levels in 100ml of 5% HSA fractions eluted from EKSP, EK1P, and EK and the difference in pore sizes.

We demonstrated the release of high levels of (1→3)-β-D-glucan from cellulose filters by flowing HSA through them. Further, Usami et al. reported that (1→3)-β-D-glucan levels of 0–6930pg/ml were detected in blood components.[16] However, the mechanism of contamination of blood components by (1→3)-β-D-glucan is not clear. In the manufacture of blood components, the influence of ethanol addition and pH adjustment has been suspected. HSA has been prepared by ethanol fractionations[17,18] under the 8%–40% ethanol concentrations based on the method of Cohn et al.[19] To investigate the effect of ethanol on the elution of (1→3)-β-D-glucan from LA, we performed the same filtration process as mentioned above using 100ml of 10% and 40% ethanol solutions. Each 100ml of ethanol solution was flowed through LA after rinsing with 100ml of distilled water, and the level of (1→3)-β-D-glucan was determined in each ethanol fraction. The (1→3)-β-D-glucan levels in 100ml of both ethanol fractions were extremely low (under the limit of detection). This result suggests that the elution of (1→3)-β-D-glucan from cellulose filters is not affected by the addition of ethanol in the process of manufacturing blood components.

Adjustments of pH in the practical manufacturing process have been carried out. According to the method of Cohn et al.,[19] pH adjustments were performed ranging from 4.8 to 7.2. It is thought that the possibility of an increase in elution of (1→3)-β-D-glucan from cellulose filters is extremely low under this pH range, since (1→3)-β-D-glucan is usually soluble in alkaline solutions.

On the other hand, one of the functions of the HSA molecule is binding and transportation of various hydrophobic ligands. Part of the (1→3)-β-D-glucan molecule shows hydrophobicity, depending on the molecular weight and structural properties. For example, (1→3)-β-D-glucan in 5% HSA fraction filtrated with EKSP is almost insoluble in distilled water, judging from data shown in Fig.2. Accordingly, it is thought that HSA molecules bind to such hydrophobic (1→3)-β-D-glucan. Miura et al. strongly suggested the binding of serum or plasma proteins to particulate (1→3)-β-D-glucan.[20] Olson et al. reported that vitronectin, a glycoprotein in plasma, serum, and connective tissue, specifically binds to fungal (1→3)-β-D-glucan and augments macrophage cytokine release.[21] Vassallo et al. reported that vitronectin and fibronectin bind to fungal (1→3)-β-D-glucan and augment macrophage inflammatory responses.[22] The binding of crustacean plasma protein and (1→3)-β-D-glucan was also reported.[23] A relation between the binding of (1→3)-β-D-glucan to proteins and the structual properties was also reported. Osmond et al. reported that unbranched (1→3)-β-D-glucans showed tight binding to barley thaumatin-like proteins, and the (1→6)-β-linked branch made the binding weak.[24] These reports support the possibility of binding of (1→3)-β-D-glucan to plasma proteins such as HSA, vitronectin, and fibronectin, though the binding ability would depend on the structural properties of (1→3)-β-D-glucan. In this study, we determined the (1→3)-β-D-glucan level eluted by only one filtration. Since there are multiple filtration steps in the process of manufacturing blood components, the degree of contamination with (1→3)-β-D-glucan would clearly increase with increase in the number of filtration steps. It is quite possible that blood components are contaminated with more than 1000pg/ml of (1→3)-β-D-glucan, although the degree depends on the selection of the filter.

Removal of (1→3)-β-D-glucan from cellulose filters by washing with alkaline solution beforehand should be effective to avoid contamination, because (1→3)-β-D-glucan is soluble in alkaline solutions, and extraction with alkaline solutions has been carried out.[25,26]

The biological activity of (1→3)-β-D-glucan from cellulose materials is not clear. Antitumor-active (1→3)-β-D-glucans isolated from mushrooms are well known,[1–6] but inactive (1→3)-β-D-glucans also exist.[6] The degree of the activity is delicately related to the molecular weight, solubility, branching form, branching rate, and difference in helical conformation.[6,27–29] Furthermore, activation of (1→3)-β-D-glucan by chemical modifications such as Smith degradation, formolysis, and carboxymethylation has also been reported.[6,30–33] Hence, the activity of (1→3)-β-D-glucan seems to be influenced by its structural properties. We cannot predict the biological activity of (1→3)-β-D-glucan from cellulose materials, because its structural features are not clear. (1→3)-β-D-glucan remains in the organs because of the lack of a specific enzyme to digest it in the human body. Contamination with (1→3)-β-D-glucan, whose biological activity in the human body is unknown, is undesirable.

In summary, elution of (1→3)-β-D-glucan to HSA solution from cellulose filters, despite advance rinsing with 100ml of distilled water, was shown by experiment. It is evident that contamination of blood components by (1→3)-β-D-glucan is caused by filtration with cellulose filters in the manufacturing process. To eliminate false positive results due to elevated plasma (1→3)-β-D-glucan levels from administration of blood components, development of treatment to prevent (1→3)-β-D-glucan contamination of blood components and investigation of the biological activity of (1→3)-β-D-glucan in cellulose filters are needed.

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