Sanitization and endotoxin clearance in AxiChrom columns

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Sanitization and endotoxin clearance in AxiChrom™ columns

Columns used in cGMP environments need to maintain very high standards of hygiene. AxiChrom chromatography columns were tested for the efficiency of microbiological sanitization and endotoxin clearance by challenge testing using 1 M sodium hydroxide (NaOH) as the clearance/ sanitization agent. A selection of small and large columns from the AxiChrom platform (including both steel and plastic bed supports), packed with Sepharose™ Fast Flow chromatography media (resins) were challenged and incubated for 16 to 20 h at room temperature before being treated with 1 M NaOH and then sampled.

Results showed highly effective sanitization and endotoxin clearance, with all acceptance criteria fulfilled. Despite high levels of microbiological contamination, no challenge organisms were found after treatment with 1 M NaOH. In addition, 1 M NaOH gave a 6-log reduction of endotoxin concentration. The final level in the column flowthrough was less than 0.05 EU/mL, which is below the United States Pharmacopeia (USP) recommendation for water for injection (WFI).

The results show that the AxiChrom column design allows operation at high standards of hygiene and confirm that 1 M NaOH is an effective clearance/santization agent.

Fig 1. Three columns from the AxiChrom range.

Introduction

AxiChrom is a range of low-pressure chromatography columns suitable from pilot to process scale (Fig 1). As products processed on AxiChrom columns might be used in clinical applications, stringent levels of purity and microbiological presence are required. Sanitization and endotoxin clearance are two techniques used to achieve such high hygienic standards.

This application note describes sanitization and endotoxin clearance studies on AxiChrom columns using NaOH, a widely used and inexpensive cleaning agent. The studies were based on microbiological and endotoxin challenge tests. Based on the results from these studies, calculations to assess the sanitization properties of AxiChrom 1200 to 1600 were made.

Sanitization

Sanitization is the use of a chemical agent to reduce a microbial population to an acceptable, predetermined level. Efficient sanitization of chromatography columns and systems is required to minimize the risk of contaminating the product. Regulations require the efficiency of sanitizing agents to be evaluated, and this is generally performed using microbiological challenge tests.

Endotoxin clearance

Endotoxins are heat-stable lipopolysaccharides located in the outer membrane of the cell wall of Gram-negative bacteria. Pharmaceutical products contaminated with released endotoxins can cause severe complications in patients. For this reason, the USP recommends a maximum endotoxin level of 0.25 EU/mL for WFI.

Endotoxins cannot be removed by conventional filtration systems. They are, however, susceptible to agents like NaOH, which efficiency can be tested by endotoxin challenge testing.

Challenge testing

The principle of challenge testing is to introduce high concentrations of microorganisms or endotoxins into the equipment (e.g., the column) to be investigated. The equipment is then left to stand for a specified time, after which it is treated with the sanitizing/clearance agent. Following treatment, samples are taken at predetermined sites, and the numbers of viable cells or levels of endotoxins remaining are measured. The objective of these studies is to achieve a result that fulfills acceptable, predetermined criteria.

Materials and methods

Microbiological challenge tests

The materials used for the microbiological challenge test are listed in Table 1. In the studies described here, microbiological challenge tests were performed on AxiChrom 70, 140 PE, 200, 600, and 600 PE columns packed with Sepharose 6 Fast Flow or Sepharose 4 Fast Flow media. The challenge organism was *E. coli* (Gram-negative bacteria) diluted to a concentration of approximately 10^6 viable organisms/mL. The infected columns were incubated for 16 to 20 h at room temperature before being treated with 1 M NaOH.

Table 1. Materials used for microbiological challenge tests

Column	AxiChrom 70, 140 PE, 200, 600*, and 600 PE
Challenging organism	*E. coli* ATCC 8739 (approx. 10^6 viable organisms/mL)
System	ÄKTApilot™ (AxiChrom 70, 140 PE, 200) ÄKTAprocess™ (AxiChrom 200, 600, 600 PE)
Medium	Sepharose 6 Fast Flow (AxiChrom 70, 200) Sepharose 4 Fast Flow (AxiChrom 140 PE, 600, 600 PE)
Sanitizing agent	1 M NaOH
Solutions	Ethanol (70%), sterile water, sterile NaCl (9 mg/mL), sterile 50 mM NaCl
Sampling materials	Peptone water (pH 7.2) Tryptic soy broth (TSB) Tryptic soy agar (TSA) Melted tryptic soy agar Alginate swabs in isotonic solution Sterile 0.45 µm cellulose nitrate membrane filters Sterile NaCl (9 mg/mL)

* In addition to the main study, two complimentary studies were performed on the column adapter sealing and bottom valve.

Test methods

Each microbiological challenge test was performed in duplicate.

Method 1. Direct filtration

Sample solutions (minimum 10 mL) collected in sterile tubes were filtered through a 0.45 µm cellulose nitrate membrane filter. The filter was washed with 100 mL sterile NaCl solution (9 mg/mL) and incubated on TSA-plates at 30°C to 35°C for 5 days, after which colony forming units (CFU) were counted and the number of viable microorganisms in the sample calculated.

Method 2. Agar plate

A sample of the chromatography medium taken after sanitization was mixed with 30 mL of molten TSA and allowed to solidify in petri dishes. After 5 days incubation at 30°C to 35°C, CFU were counted and the number of viable microorganisms in the sample calculated.

Method 3. Swab

Surface samples were taken with alginate swabs that were then inserted into tubes containing isotonic swab rinse solution. After the samples dissolved, the whole solution (including the swab tips) was poured into molten TSA and solidified in petri dishes. After 5 days incubation at 30°C to 35°C, CFU were counted and the number of viable microorganisms in the sample calculated.

Method 4. Peptone water filtration

Detachable column parts were aseptically removed, placed in 50 mL sterile peptone water, and vigorously shaken for at least 20 min. The solutions were filtered through a 0.45 µm cellulose nitrate membrane filter, which was then washed with 100 mL sterile NaCl solution (9 mg/mL) and incubated on TSA-plates at 30°C to 35°C for 5 days, after which CFU were counted and the number of viable microorganisms in the sample calculated.

Method 5. Contact plates

TSA-contact plates were put directly on a surface for sampling. The plates were then incubated at 30°C to 35°C for 5 days. CFU were counted and the number of viable microorganisms in the sample calculated.

Method 6. Viable count

Samples of the challenging organism suspension were diluted and incubated on TSA-plates at 30°C to 35°C for 1 to 2 days, after which CFU were counted and the number of viable microorganisms in the samples calculated.

Preparing the challenge organism suspension

E. coli ATCC 8739 (stored deep-frozen) was grown on a nutrient agar plate at 30°C to 35°C for 18 to 24 h. Organisms were inoculated in 20 mL TSB-medium and incubated at 30°C to 35°C for 18 to 24 h. The bacterial culture was centrifuged (10 min at 4500 rpm) and the pellet suspended in sterile peptone water. The suspension was diluted in sterile peptone water to obtain 10^6 CFU/mL (sterile 50 mM NaCl was used in the runs on AxiChrom 600 columns). Microorganism concentration was determined by viable count (see "Method 6. Viable count").

Preparing the column

As a precleaning process, all small column parts were disassembled and soaked in 1 M NaOH solution for 18 to 24 h, rinsed with sterile water and reassembled. For most column sizes, the column tube and adapter rod were sprayed with 70% ethanol in a laminar flow (LAF) hood before assembly. The AxiChrom 600 acrylic column tube was sprayed with 20% ethanol outside of the hood before assembly.

Packing the column

After assembly, the column was packed with chromatography medium with sterile water as packing buffer. The target bed height was 10 cm and the flow during packing was 60 cm/h. The column volumes (CV) were approximately 400 mL for AxiChrom 70, 1.6 L for AxiChrom 140, 3.1 L for AxiChrom 200, and 40 L for AxiChrom 600.

Pretreatment

The column was washed with 1 CV sterile water and 2 CV 1 M NaOH (both up flow) followed by 2 CV 1 M NaOH (down flow), after which it was left overnight. Before challenge, the column was washed with sterile water (4 CV, up flow) to achieve neutral pH. Fluid velocity during pretreatment was 60 cm/h.

Challenging the column

The column was challenged with 1.5 CV of challenge organism suspension at a down-flow velocity of 20 cm/h, after which the column was incubated for 16 to 20 h at room temperature.

Sanitization procedure

The sanitization run started by rinsing with 2 CV sterile water for 20 min followed by 2 CV 1 M NaOH for 20 min (both up flow) and 2 CV 1 M NaOH (down flow) for 20 min. Fluid velocity was 60 cm/h throughout the sanitization procedure. Approximately 5 CV 1 M NaOH was recirculated (up flow) for 4 h. Sanitization ended by washing (up flow) with 4 CV sterile NaCl (9 mg/mL) for 40 min to achieve neutral pH prior to sampling. Sterile water was used in the runs on AxiChrom 600 columns. To sanitize the bottom valve, 1 M NaOH was added until the flowthrough reached pH 14. The equipment was then left to stand for about 5 h before neutralization (wash with sterile water). Table 2 summarizes the general sanitization procedure.

Microbiological testing

Microbiological samples (including a sample of the chromatography medium) were taken at several predetermined sites in a complete column study (Fig 2, 3, and 4). The challenge organism concentrations in the flowthrough and in the bottom valve were determined by viable count (see "Method 6. Viable count") post-challenge, both before and after the sanitization procedure. In the studies of the two column sizes with plastic bed supports, the sampling focused on the new design components.

Samples of the challenge organism and an outlet sample from the mobile phase were also taken.

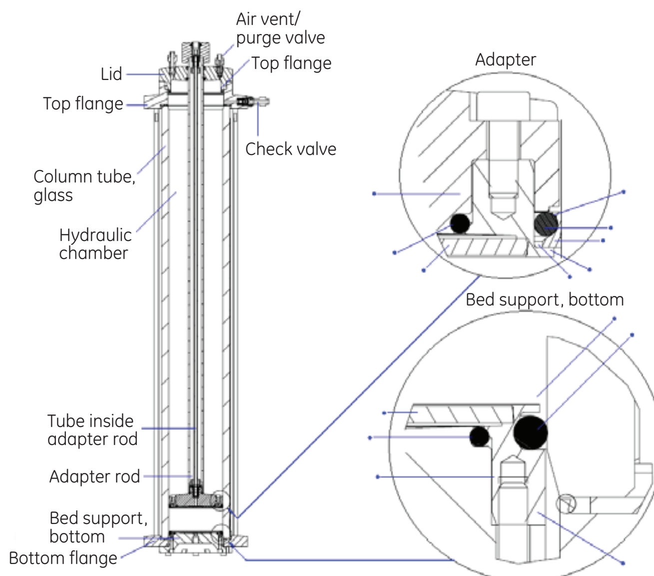


Fig 2. General locations of the predetermined sites sampled in AxiChrom 70 and 200 columns.

Endotoxin test method

Endotoxin samples were analysed using Limulus amebocyte lysate (LAL) reagent according to the kinetic-chromogenic method, which is a highly sensitive and specific way to detect and measure endotoxin. In this method, the presence of endotoxin causes a yellow color to develop in a synthetic chromogenic substrate present in LAL reagent. This color can be detected and measured with precision using a spectrophotometer. Collected samples were stored at 2°C to 8°C prior to analysis.

Table 2. Summary of sanitization procedure

Step	Solution	CV	Flow direction	Fluid velocity (cm/h)	Time (min)
Post-infection rinse	Sterile water	2	Up	60	20
Sanitization	1 M NaOH	2	Up	60	20
		2	Down	60	20
		5	Up (circulate)	60	240
Post-sanitization rinse	Sterile NaCl (9 mg/mL) or sterile water	4 or more until pH neutral	Up	60	40 or more until pH neutral

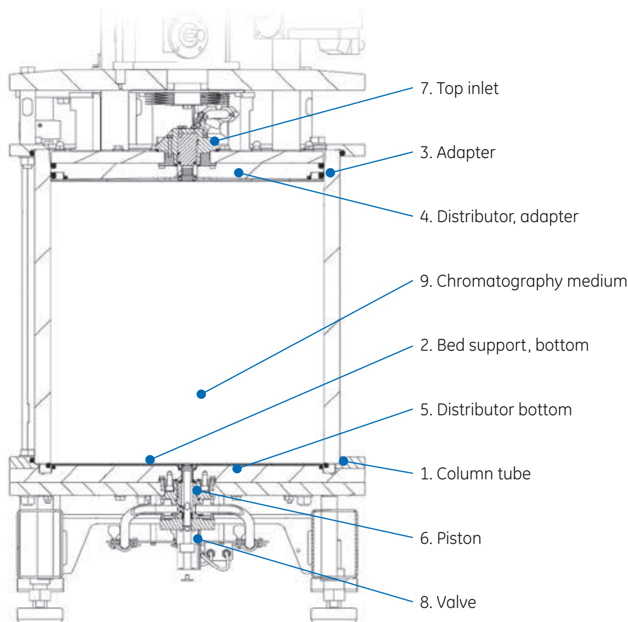


Fig 3. General locations of the 53 predetermined sites sampled in the AxiChrom 600 column.

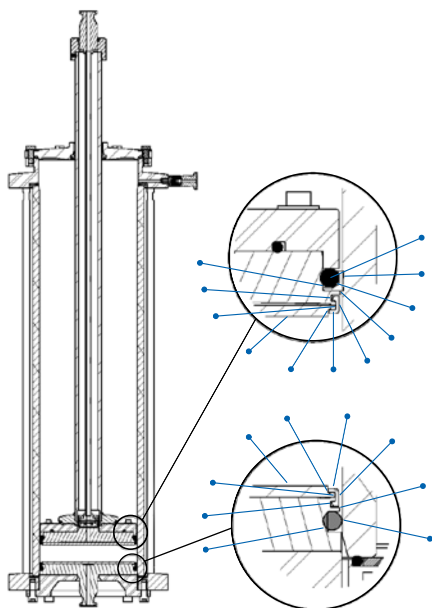


Fig 4. Detailed locations of the adapter and bottom sites sampled in the AxiChrom 140 PE column study.

Endotoxin challenge test on AxiChrom 50 column

The endotoxin challenge test was performed on an AxiChrom 50 column packed with Sepharose 6 Fast Flow medium. Challenging endotoxins were diluted to a concentration of approximately 50 000 EU/mL. The column was incubated for 16 to 20 h at room temperature before being treated with 1 M NaOH. The materials used for the endotoxin challenge test on AxiChrom 50 column are listed in Table 3.

Table 3. Materials used for endotoxin challenge test on AxiChrom 50 column

Column	AxiChrom 50
Challenging endotoxin	Endosafe™ endotoxin indicator
System	ÄKTApilot
Medium	Sepharose 6 Fast Flow
Clearance agent	1 M NaOH
Solutions	Ethanol (70%), sterile water, LAL-negative water

Preparing the endotoxin solution

Two endotoxin vials were each dissolved in 1 mL LAL-negative water and shaken for 7 min. The solutions were mixed with 398 mL LAL-negative water to give a final concentration of approximately 50 000 EU/mL. A sample (endotoxin challenge solution, start material) was taken for analysis.

Preparing the column

All small column parts were disassembled and soaked in 1 M NaOH solution for 18 to 24 h after which they were rinsed with sterile water and reassembled. The column tube and the adapter rod were sprayed with 70% ethanol in a LAF-hood before assembly.

Packing the column

The reassembled column was packed with Sepharose 6 Fast Flow medium. Sterile water at a fluid velocity of 60 cm/h (17.7 mL/min) was used as packing solution. The final packed bed height was 10.5 cm (CV 206 mL).

Pretreatment

The column was washed with 2 CV sterile water followed by 2 CV 1 M NaOH (both up flow) and with 2 CV 1 M NaOH (down flow) before being left overnight. Prior to the endotoxin challenge, the column was washed with sterile water (4 CV, up flow) to achieve neutral pH at a fluid velocity of 60 cm/h (19.6 mL/min). A sample (prechallenge sample) was taken at the column flowthrough.

Challenging the column

The column was challenged with 1.5 CV (approximately 300 mL) endotoxin suspension at a fluid velocity 20 cm/h (6.5 mL/min) down flow. A post-challenge sample was collected at the flowthrough directly after. The challenged column was incubated for 16 to 20 h at room temperature.

Clearance

Before NaOH treatment began, a sample (pre-NaOH treatment sample) of the flowthrough was taken. Treatment involved rinsing with 2 CV sterile water followed by 2 CV 1 M NaOH (both up flow for 20 min) and 2 CV 1 M NaOH (down flow), also for 20 min. Five CV (approximately 1 L) 1 M NaOH was circulated with up flow for 1 h. The procedure ended by washing (up flow) with at least 4 CV sterile water to achieve neutral pH before a post-NaOH treatment sample of the flowthrough was taken. Fluid velocity was 60 cm/h (19.6 mL/min) during the whole clearance procedure, which is summarized in Table 4. Finally, the bed was unpacked and a post-NaOH treatment chromatography medium sample was taken.

Table 4. Summary of the clearance procedure following endotoxin challenge in AxiChrom 50 column

Step	Solution	CV	Flow direction	Fluid velocity (cm/h)	Time (min)	Solution (mL)
Post-challenge rinse	Sterile water	2	Up	60	20	400
Clearance	1 M NaOH	2	Up	60	20	400
		2	Down	60	20	400
		5	Up (circulate)	60	60	1000
Post-clearance rinse	Sterile water	4 or more until pH neutral	Up	60	40 or more until neutral pH	800 or more until neutral pH

Results

Sanitization

Despite high initial levels of challenge bacteria, the sanitization procedures described resulted in no surviving *E. coli* in the main studies, nor in the complementary study on AxiChrom 600. The overall AxiChrom column design, therefore, allows thorough and effective column sanitization.

Table 5 shows the typical levels of microbiological infection in AxiChrom 70 columns at different phases of the sanitization procedure. Note the high initial levels prior to sanitization. The results were similar over the column size range and in the complementary study.

Tables 6 to 10 show the number of CFU remaining after sanitization for each column size tested.

Table 5. A typical challenge organism concentration (CFU/mL) at different phases of a sanitization procedure in an AxiChrom 70 column

Phase in sanitization	Sampling method	Run 1 (CFU/mL)	Run 2 (CFU/mL)
Inoculum sample (start concentration)	6	1.7×10^6	1.7×10^6
Post-challenge (flowthrough sample after challenge)	6	1.4×10^6	1.4×10
Presanitization (flowthrough sample before sanitization)	6	5.5×10^7	5.8×10
Post-sanitization (flowthrough sample after sanitization)	1	0*	0

* Sample was contaminated (1 CFU/mL) by an organism other than *E. coli*

Table 6. Number of *E. coli* CFU remaining after sanitization in AxiChrom 70 column

Sampling site	No. of sampling points	Sampling method	Run 1 (CFU/mL or sampled unit)	Run 2 (CFU/mL or sampled unit)
Hydraulic chamber[1]	1	1	0[4]	0
Lid[1]	3	3	0	0
Top flange[1,2]	1	3	0	0
Column tube, glass	4	3	0	0
Check valve[1]	1	4	0	0
Air vent/purge valve[1]	1	5	0[5]	0
Adapter	13	3, 4	0	0
Adapter rod[1]	2	3, 4	0[6]	0
Tube inside adapter rod	2	5	0	0
Bed support, bottom	11	3, 4	0[6]	0
Bottom flange	1	3	0	0
Top flange[3]	1	3	0	0
Chromatography medium	1	2	0	0

[1] Note that this point is not in the sanitization process stream
[2] Sampling from locking bayonnet groove
[3] Sampling from o-ring facing column tube top
[4] Sample was contaminated (1 CFU/mL) by an organism other than *E. coli*
[5] Sample was contaminated (2 CFU/sampled unit) by an organism other than *E. coli*
[6] One sample was contaminated (1 CFU/mL) by an organism other than *E. coli*

Table 7. Number of CFU of *E. coli* remaining after sanitization in AxiChrom 200 column

Sampling site	No. of sampling points	Sampling method	Run 1 (CFU/mL or sampled unit)	Run 2 (CFU/mL or sampled unit)
Hydraulic chamber[1]	1	1	0	0
Lid[1]	1	3	0	0
Top flange	1	3	0	0
Column tube, glass	4	3	0[2]	0[3]
Check valve[1]	1	4	0	0
Air vent/purge valve[1]	1	3	0	0
Adapter	10	3	0[4]	0
End cell, adaptor	6	3	0	0
Tube inside adapter rod	5	3	0[2]	0
Bed support, bottom	5	3	0	0
Bottom flange	1	3	0	0
End cell, bottom	7	3	0	0
Chromatography medium	1	2	0	0

[1] Note that this point is not in the sanitization process stream
[2] One sample was contaminated (overgrown) with organisms other than *E. coli*
[3] One sample was contaminated (1 CFU/mL) with organisms other than *E. coli*
[4] Three of the samples were contaminated (1 CFU/mL, 3 CFU/mL, and one sample overgrown) with organisms other than *E. coli*

Table 8. Number of CFU of *E. coli* remaining after sanitization in AxiChrom 600 column

Sampling site	No. of sampling points	Sampling method	Run 1 (CFU/mL or sampled unit)	Run 2 (CFU/mL or sampled unit)
Column tube, acrylic	2	3	0	0
Bed support, bottom	6	3	0	0
Adapter, net	11	3, 4, 5	0	0
Distributor, adapter	7	3	0	0
Distributor, bottom	9	3, 4	0[2]	0
Top inlet	2	4	0[3]	0[4]
Chromatography medium	1	2	0[5]	0
Adapter, sealing[1]	4	3	0	0
Piston[1]	2	3	0	0
Valve[1]	9	3, 4	0[6]	0

[1] Complementary studies
[2] One sample was contaminated (11 CFU/sampled unit) with organisms other than *E. coli*
[3] One sample was contaminated (overgrown) with organisms other than *E. coli*
[4] One sample was contaminated (1 CFU/sampled unit) with organisms other than *E. coli*
[5] One sample was contaminated (1 CFU/mL) with organisms other than *E. coli*
[6] One sample was contaminated (1 CFU/sampled unit) with organisms other than *E. coli*

Table 9. Number of CFU of *E. coli* remaining after sanitization in AxiChrom 140 PE column

Sampling site	No. of sampling points	Sampling method	Run 1 (CFU/mL or sampled unit)	Run 2 (CFU/mL or sampled unit)
Hydraulic chamber[1]	1	1	0	0
Column tube, glass	2	3	0[2]	0
Top lid[1]	1	3	0	0
Adapter	11	3	0	0
End cell, adapter	4	3	0	0
Tube inside adapter rod	4	3, 4, 5	0	0
Bed support bottom	8	3	0	0[3]
End cell bottom	4	3	0	0
Chromatography medium	1	2	0	0

[1] Note that this point is not in the sanitization process stream
[2] One sample was contaminated (1 CFU/mL) with organisms other than *E. coli*
[3] Two of the samples were contaminated (1 CFU/mL and one overgrown) with organisms other than *E. coli*

Table 10. Number of CFU of *E. coli* remaining after sanitization in AxiChrom 600 PE column

Sampling site	No. of sampling points	Sampling method	Run 1 (CFU/mL or sampled unit)	Run 2 (CFU/mL or sampled unit)
Column tube	2	3	0[1]	0
Bed support bottom	11	3, 4	0[2]	0[3]
Adapter, bed support	16	3, 4	0[1]	0
Bottom valve	2	3, 4	0	0
Chromatography medium	1	2	0	0

1 One sample was contaminated (overgrown) with organisms other than *E. coli*
2 Three of the samples were contaminated (overgrown) with organisms other than *E. coli*
3 Two of the samples were contaminated (overgrown) with organisms other than *E. coli*

Endotoxin clearance

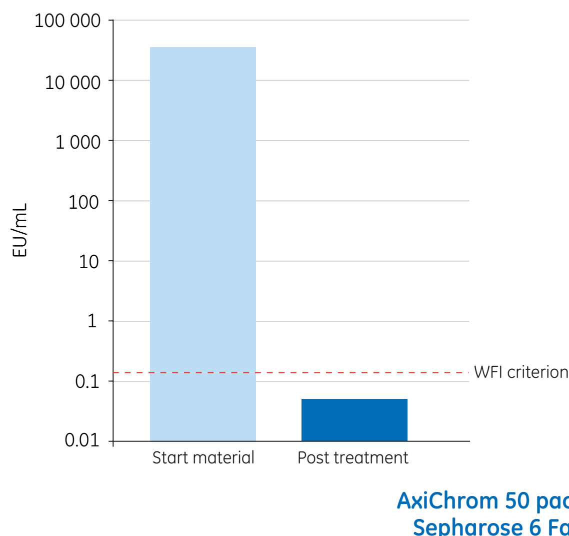
Table 11 shows the endotoxin concentration in samples taken in the clearance study. Treatment with 1 M NaOH effectively reduced the levels of endotoxins in the challenged column. A 6-log reduction of the introduced endotoxin concentration was achieved (Fig 5) and the final concentration in the column flowthrough after NaOH treatment was below 0.05 EU/mL. The endotoxin concentration is under the USP recommendation of less than 0.25 EU/mL for WFI.

The chromatography medium sample taken after NaOH treatment showed an endotoxin concentration of < 0.5 EU/mL, which is a 5-log reduction. This is the lowest limit possible to detect using medium as sample (medium samples should be diluted prior to analysis as the beads interfere with the method). Thus, it cannot be excluded that a 6-log reduction was achieved, or that the endotoxin concentration after treatment was below the USP-recommended 0.25 EU/mL.

Table 11. Endotoxin concentrations measured during the clearance study

Sample	Endotoxin concentration (EU/mL)
Chromatography medium, start material	< 0.50*
Sterile water	< 0.050
Prechallenge sample, column flowthrough	< 5.0
Endotoxin challenge solution, start material	3.2×10^4
Post-challenge sample, column flowthrough	1.2×10^4
Pre-NaOH treatment sample, column flowthrough	2.8×10^3
Post-NaOH treatment sample, column flowthrough	< 0.050
Post-NaOH treatment sample, chromatography medium	< 0.50*

* The detection limit for media samples is < 0.5 EU/mL. (To avoid beads interfering with the analysis, samples should be diluted first)



	AxiChrom 50 packed with Sepharose 6 Fast Flow
Endotoxin challange sample, start material	32 000 EU/mL
Post-NaOH treatment sample, column flowthrough	0.05 EU/mL

Fig 5. Graphical presentation of the logarithmic reduction of endotoxins in AxiChrom 50 column after treatment with NaOH for 1 h and 40 min.

Sanitization of AxiChrom 1200 to 1600

To evaluate the sanitization properties of the larger AxiChrom columns, the design of AxiChrom 1200, 1400, and 1600 was compared with the design of AxiChrom 600. Based on the results for the smaller columns and the similarities in design, it was concluded that AxiChrom 1200 to 1600 should also be possible to sanitize efficiently.

Summary

The performed studies showed efficient sanitization using 1 M NaOH in the tested AxiChrom columns. Despite the initially high levels of microbiological organisms, no surviving challenge microorganisms were found following sanitization. Endotoxin levels were reduced to concentrations below those recommended by regulatory authorities.

Note, however, that column design alone cannot guarantee good hygienic status in a chromatographic process. Methods like those described here should always be applied in conjunction with other well-designed and carefully controlled hygienic routines, together with rigorous control of buffers, water, and other input material.

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