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Systematic development and validation of sanitization protocols for a chromatographic system designed for biotherapeutics purification*

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Abstract: Production of protein therapeutics through the application of genetic engineering and biotechnology techniques requires comprehensive attention to good manufacturing practice and good laboratory practice (GMP/GLP) guidelines for product recovery and purification. Validated clean-in-place procedures are part of the master method and require analysis of microbial bioburden to assess the efficacy of cleaning protocols. This article describes the extensive microbial challenge of a chromatography system, the use of membrane filtration methods for high sensitivity microbial contamination measurement, and the effectiveness of sodium hydroxide and ethanol solutions in achieving multilog reduction of microbial contamination.

Keywords: *Clean-in-place; sanitization; FPLC; bioburden analysis; microbial challenge.*

Introduction

The development of protein derived therapeutic drugs through the application of genetic engineering and biotechnology techniques has resulted in the design of multi-step and multi-technology processes for producing the target products. Considerable development time must be invested to optimize the fermentation, harvesting and purification steps to yield reproducible, high yield, economical products. Once developed, implementation of the procedures requires comprehensive attention to good manufacturing practices (GMP), good laboratory practices (GLP), and other guidelines specified by regulatory agencies. These regulations and guidelines address all aspects of the production process including facility design, equipment selection, personnel training and process validation, as well as specific testing protocols.

Many of the process requirements for product recovery and purification are met by combinations of tangential flow filtration and chromatography separation techniques. Consequently, increasing emphasis being placed on the development of separation techniques that meet the specifications for final product release. Table 1 summarizes the types of final product release tests needed and identifies their dependence on the substrate from which the product is derived [1]. Key components of the validation process for chromatographic separations are equipment design and operation certification, column packing certification and standard operating procedures (SOP).

Clean-in-place procedures (CIP) become part of the master method and require validation as part of the entire process. Consequently, validation of CIP procedures requires definition of what 'clean' is for a given process [2]. Documentation of sanitization effectiveness, the chemical process of killing vegetative microbial cells, on microbial contaminants is also necessary for certain types of equipment. It is critical that the analytical techniques used in the validation exercise for sanitization procedures are sensitive, accurate and reproducible. The elements of the production process addressed in this paper are the detection and removal of microbiological contamination from the chromatographic system.

Two main sources of microbial contamin-

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Table 1
Summary of final product release testing

Tests	Biological products derived from: Monoclonal* antibodies	Human cell	Mouse/hamster cell	Bacteria	Yeast
General safety	X	X	X	X	X
Sterility	X	X	X	X	X
Rabbit pyrogen/LAL	X	X	X	X	X
Mycoplasma	†	†	†	NR	NR
Contaminating DNA	X	X	X	X	X
Viral contamination	†	†	†	NR	NR

*Current FDA recommendations for Phase I studies. Actual requirements should be discussed with FDA.
†Depends on findings of unprocessed bulk.
X = required; NR = not required.
This table originally appeared in *Lot Release — Final Product Safety Testing* by L.J. Schiff *et al.* Reprinted from *BioPharm*. Vol. 5, No. 5, pp. 36–39 (1992) with permission of Advanstar Communications.

ation of chromatography equipment are the outside environment and the host cells. Therefore, validated sanitization procedures should involve microbial bioburden analysis. In this study, two challenge organisms were selected as representative of the primary contaminants to be dealt with by sanitization protocols. *Pseudomonas aeruginosa* was chosen because its capsular glycocalyx is more resistant to sanitization than other gram negative bacteria and because it is of water and soil origin. *Acholeplasma laidlawii* was selected as the second test organism because bacteria in the class *Mollicutes* are a primary concern for contamination in tissue and cell culture, animal serum and nutritive additives in which rapid proliferation is supported by the highly nutritive media.

The most critical part of this experiment was the quantitative analysis of the test effluent. The test was designed so that low levels of vegetative micro-organisms (<10 cfu) in the effluent could be detected. The most sensitive analysis technique for low numbers of micro-organisms is the membrane filter method [3, 4]. In this method, the entire effluent was passed through a microporous membrane filter disc (0.45 or 0.22 μm) which was then plated on to a suitable agar substrate and incubated under specific conditions. This method allowed evaluation of 100% of the effluent and direct enumeration of the microbial colonies.

Although the membrane filter method has been used extensively to detect micro-organisms in parenteral drug products and water [4, 5], use of the membrane filter method for mycoplasma analysis of cell culture fluids has only recently been documented [3, 6]. Roche and Liss extended the technique to mycoplasma by incorporating a post-incubation staining procedure that did not interfere with the growth of colonies [3, 6]. The Dienes stain is used to differentiate mycoplasma from other microbial stains and to enhance the visualization of the colonies on the nitrocellulose substrate [7]. Mycoplasma colonies will stain blue because *Mollicutes* cannot reduce the Methylene Blue incorporated in the Dienes stain; other classes of bacteria can reduce Methylene Blue. By this method, a single mycoplasma colony can be located quickly under 40× magnification.

Sodium hydroxide and aqueous ethanol solutions are two common agents used for chromatographic system sanitization. The effectiveness of these solutions in removing microbial contamination is dependent on a number of factors including system materials of construction, contact time with contaminated fluids, contact time with cleaning fluids, and system storage conditions. This article describes the microbial challenge of a Waters™ 650 advanced protein purification system and the sanitization effectiveness of both sodium hydroxide and ethanol solutions in achieving multilog reduction of microbial contamination.

Experimental

Apparatus

All experiments were conducted on Waters 650 advanced protein purification system equipped with a 5.1-ml loop manual injector and a 486 tunable absorbance detector equipped with a non-metallic flow cell (Milli-

pore, Milford, MA). All fluid connections were made with the standard Tefzel (ethylene-tetrafluoroethylene) tubing and fittings provided by the manufacturer. Chromatography columns were excluded from this study. The sample collection site was located at the system outlet port downstream of the absorbance dector. The collection site was fitted with a Tefzel luer slip fitting to facilitate sample collection (see Fig. 1).

Reagent preparation

Prepared 70% ethanol (Ethanol, Quantum Chemical Corporation, Newark, NJ) and deionized water, (Milli-Q®, Millipore, Bedford, MA) were sterile filtered through a 0.22-μm hydrophobic polyvinylidene fluoride membrane (Millipore, Bedford, MA). Fortified commercial broth (FCB) and fortified commercial agar (FCA) were prepared according to the method of Macy [8] and filter sterilized through a 0.22-μm hydrophilic polyvinylidene fluoride membrane (Millipore, Bedford, MA). Dulbecco's phosphate buffered saline (PBS) [9], 0.1% peptone water (Difco, St Louis, MO), saline lactose broth (SLB) [10] and soybean casein digest broth (TSB; Difco, St Louis, MO) were sterilized in an autoclave at 121°C, 15 psig for 30 min. Trypticase soy agar and slants (TSA) were purchased pre-prepared from Northeast Labs (Waterville, ME) and 1.0 N sodium hydroxide was purchased from J.T. Baker (Phillipsburg, NJ).

Challenge organisms and culture conditions

Pseudomonas aeruginosa No. 9027 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and rehydrated according to the method recommended by the manufacturer. *P. aeruginosa* was stored on prepared TSA slants at 4°C ± 2°C for a maximum of 30 days. Forty-eight hours prior to the challenge test, an initial culture of *P. aeruginosa* was initiated by inoculating 30 ml of TSB with two loopfuls (approximately 20 μl) of bacteria from a prepared slant and incubating for 24 h at 30°C ± 2°C. Samples of the culture were prepared by Gram Stain and by streak plate for isolation and contamination determination. A working culture was then prepared by inoculating 4 μl of the initial culture into 250 ml of SLB. The working culture was allowed to multiply for 24 h at 30°C ± 2°C. Samples of the culture were prepared by Gram Stain and by streak plate for isolation and contamination determination. The culture was diluted 1:10 with sterile SLB to obtain a 10^7 colony forming unit per millilitre (cfu ml^{-1}) challenge in the test system.

Acholeplasma laidlawii No. 23206 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and rehydrated in FCB according to the method recommended by the manufacturer. *A. laidlawii* stocks were stored in 2-ml aliquots at −70°C ± 2°C for a maximum of 6 months. Forty-eight hours prior to the challenge test, an initial culture of *A. laidlawii* was initiated by inoculating 100 ml of FCB with 4 ml of the bacteria and incubating for 48 h at 37°C ± 2°C, 7% CO_2. Samples of the culture were prepared by Gram Stain and by stab plate for isolation and contamination determination. The culture was diluted 1:100 with sterile PBS to obtain a 10^8 colony forming unit per millilitre (cfu ml^{-1}) challenge in the test system.

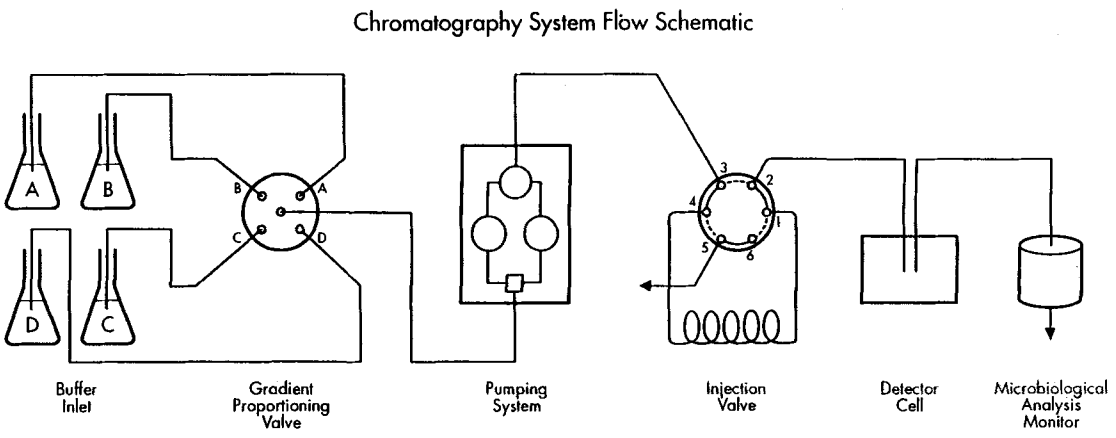

Figure 1
Chromatography system flow schematic.

Effect of sanitizers on liquid cultures

To determine whether or not the selected sanitizing agents had bactericidal properties, a study was designed to screen the sanitizers for their ability to destroy *P. aeruginosa*. A working culture of *P. aeruginosa* was prepared as described above. Forty millilitres of the diluted working culture was aseptically transferred into separate 50-ml Oak Ridge centrifuge tubes (Sorvall-Dupont, Wilmington, DE). One tube was prepared for each sanitizer to be evaluated and one tube was reserved as a bacterial culture control. The cells were harvested in a Sorvall RC5C centrifuge fitted with an SS-34 fixed angle 34° rotor (Sorvall-Dupont, Wilmington, DE) at 9750*g*, 4°C, for 15 min. The supernatant was discarded. The bacterial pellet was resuspended in 20 ml of 0.22 μm filtered sanitizer and mixed by vortex for 60 s. The control sample was resuspended in 20 ml of sterile 0.76% saline (0.76% saline is the concentration of sodium chloride in SLB). The assay was started immediately following the resuspension of the bacterial pellet to determine the effect on the cells at 'time zero'. Samples were enumerated as described in the *Microbiological analysis* section at various time periods (including 'time zero'). The samples were held at ambient temperature.

Since two previous experiments examined sanitizer effects on suspended cells, a second set of experiments was designed to examine sanitizer effects on cells attached to a surface. Forty-eight hours prior to the test, an initial culture of *P. aeruginosa* was initiated by inoculating 30 ml of TSB with two loopfuls (approximately 20 μl) of bacteria from a prepared slant and incubating for 24 h at 30°C ± 2°C. To increase the surface area and to promote cell attachment, a sterile dissecting pick was used to aseptically scratch the interior walls of sterile polystyrene 16 × 100 mm screw top test tubes (VWR, Boston, MA). The test tubes were rinsed with sterile water to remove excess polystyrene. Next, 15.0 ml of sterile SLB was added to each tube. Each test tube was then inoculated with 0.1 ml of the TSB culture and incubated for 24 h at 30°C ± 2°C. After 24 h, a 0.1-ml aliquot was removed from each test tube for enumeration and purity determination as described above. The liquid in each test tube was removed by pipette and the test tubes were gently rinsed with approximately 3 ml of 0.76% saline. Fifteen millilitres of sanitizer or 0.76% saline (control) was added to each rinsed tube and inverted 2–3 times. The assay was started immediately following the inversion of the test tubes to determine the effect on the cells at 'time zero'. Samples were enumerated as described in the *Microbiological analysis* section at various time periods (including 'time zero'). The test tubes were held at ambient temperature.

System challenge and sanitization procedures

All challenge and sanitization procedures were conducted at ambient temperature. The chromatography system was challenged with *P. aeruginosa* suspended in SLB by introducing the bacteria through the fluid handling system which consisted of the four elution buffer inlet lines, the injector sample loop and bypass flow path, and the detector flow cell (see Fig. 1). The bacterial suspension was pumped through the system at 1.5 ml min^{-1} until a constant 280 nm UV absorbance was achieved. The challenge suspension was allowed to stand in the system for 16–18 h prior to initiating the sanitization procedure. The system outlet port was submerged in 95% ethanol during the static flow periods to inhibit the introduction of microbial contaminants.

After the *P. aeruginosa* 16–18 h static period, the chromatography system was sanitized. The selected sanitizer was introduced into the fluid handling system and pumped through the system at various flow rates until a constant 280 nm UV absorbance was achieved. The sanitizer was allowed to remain in contact with the fluid path for various time periods. (Refer to Table 2 for a summary of sanitizers, flow rates and time periods examined.) Following the sanitization period, sterile 0.1% peptone was pumped through the system to remove residual sanitizer. Next, a 0.45-μm mixed esters of cellulose microbial analysis monitor (Millipore, Bedford, MA) was attached to the system outlet port and 100 ml of 0.1% peptone was processed at 1.5 ml min^{-1}. After sample collection, the chromatography system was stored in 70% ETOH until the next use to prevent micro-organism contamination.

In a separate set of experiments, the chromatography system was challenged with *A. laidlawii* suspended in PBS by introducing the bacteria through the chromatography system as described above. All challenge and sanitization procedures were conducted at ambient temperature. The bacterial suspension

was pumped through the system at 1.5 ml min^{-1} until a constant 280 nm UV absorbance was achieved. The challenge suspension was allowed to stand in the system for 16–18 h prior to initiating the sanitization procedure. The system outlet port was submerged in 95% ethanol during the static periods to inhibit the introduction of microbial contaminants.

After the *A. laidlawii* 16–18 h static period, sanitizer was introduced into the fluid handling system at various flow rates until a constant 280 nm UV absorbance was achieved. The sanitizer remained in contact with the fluid path for various time periods. (Refer to Table 3 for a summary of sanitizers, flow rates and time periods examined.) Following the sanitization period, sterile 0.1% peptone water was pumped through the system to remove residual sanitizer and a 100 ml peptone sample was aseptically collected in a sterile flask at 1.5 ml min^{-1} for microbiological analysis. After sample collection, the chromatography system was stored in 70% ETOH until the next use to prevent micro-organism contamination.

Table 2
Pseudomonas aeruginosa bioburden assessment

Cleaning method	Initial challenge concentration (cfu ml^{-1})	Day 1	Day 2	Day 3	Day 4	LRV
1 N NaOH, static, 60 min	4.98×10^7	0	TNTC	TNTC	*	N/A
1 N NaOH, 10 ml min^{-1}, 95 min	5.50×10^7	0	0	TNTC	TNTC	N/A
1 N NaOH, 1.5 ml min^{-1}, 60 min	3.98×10^7	*	0	80	*	5.69
70% ETOH, static, 60 min	6.75×10^7	8	TNTC	TNTC	*	N/A
70% ETOH, static, 16–18 h	7.50×10^7	0	3	*	TNTC	N/A
70% ETOH, static, 16–18 h Replicate experiment No. 1	1.06×10^8	0	0	*	0	8.02
70 ETOH, static, 16–18 h Replicate experiment No. 2	8.47×10^7	0	0	*	0	7.93
70% ETOH, static, 16–18 h Replicate experiment No. 3	9.38×10^7	0	0	*	0	7.97
70% ETOH, static, 16–18 h Replicate experiment No. 4	6.03×10^7	0	0	*	0	7.78

The presence of *P. aeruginosa* contained in 100 ml peptone water samples was assessed both prior to and after system challenge and cleaning was assessed by TSA plating.

TNTC denotes 'too numerous to count'.

* A sample was not taken on that day.

N/A = Non applicable. The LRV could not be calculated because no real number exists.

Table 3
Acholeplasma laidlawii bioburden assessment

Cleaning method	Initial challenge concentration (cfu ml^{-1})	Day 1	Day 2	Day 3	Day 4	LRV
70% ETOH, static, 16–18 h	3.96×10^8	0	0	*	0	8.59
95% ETOH, static, 16–18 h	2.62×10^7	0	0	*	75	5.54
1 N NaOH, static, 16–18 h	1.47×10^8	0	0	*	0	8.17
1 N NaOH, static, 16–18 h Replicate experiment No. 1	1.19×10^9	0	†	*	0	9.07
1 N NaOH, static, 16–18 h Replicate experiment No. 2	1.18×10^9	0	38	*	>100 <300	6.59
1 N NaOH, static, 16–18 h Replicate experiment No. 3	2.60×10^8	0	0	*	>100 <300	5.94
1 N NaOH, static, 16–18 h Replicate experiment No. 4	3.48×10^8	0	†	*	0	8.54
1 N NaOH, static, 16–18 h Replicate experiment No. 5	3.85×10^8	0	†	*	0	8.58
1 N NaOH, static, 16–18 h Replicate experiment No. 6	3.37×10^8	0	0	*	0	8.53

The presence of *A. laidlawii* contained in 100 ml peptone water samples was assessed both prior to and after system challenge and cleaning was assessed by FCA plating.

* A sample was not taken on that day.

† *Staphlococcus* contamination was detected pre-sanitization but not post-sanitization.

Microbiological analysis

An aliquot of the *P. aeruginosa* challenge suspension was reserved for initial concentration determination via the dilution and spread plate method [11]. Samples were serially diluted in 0.1% peptone, plated on TSA, and incubated at 30°C ± 2°C for 48 h. Post-incubation, colonies were enumerated and reported as colony forming units per millitre (cfu ml^{-1}).

The 100 ml peptone test sample was collected using the membrane filter method for microbial recovery. A sterile 37 mm 0.45 μm bacteriological analysis monitor (Millipore, Bedford, MA) containing a 0.45-μm gridded mixed esters of cellulose membrane filter was attached to the system outlet port. After sample collection, the membrane filter was aseptically removed from the bacteriological monitor with flamed forceps and plated upon TSA. The sample was incubated at 30°C ± 2°C for 7 days. Post-incubation, colonies were enumerated and reported as colony forming units per sample (cfu/100 ml).

An aliquot of the *A. laidlawii* challenge suspension was reserved for initial concentration determination via the drop and stab method [12]. Samples were serially diluted in PBS, plated on FCA, and incubated at 37°C ± 2°C, 7% CO_2 for 3–5 days. Post-incubation, colonies were enumerated using a stereomicroscope at 40× power and reported as colony forming units per millilitre (cfu ml^{-1}).

Since sterile microbial analysis monitors were not available in a 0.22-μm configuration, the membrane filter technique was modified for test sample collection. The sample was collected in a sterile 100 ml graduated cylinder and filtered through a 47 mm 0.22 μm mixed esters of cellulose membrane filter (Millipore, Bedford, MA) seated in a borosilicate glass vacuum filter funnel (Millipore, Bedford, MA). The membrane filter was aseptically removed from the filter funnel with flamed forceps and plated upon FCA. The sample was incubated at 37°C ± 2°C, 7% CO_2 for 7 days. Post-incubation, colonies visualized by staining the membrane filters with 33% Dienes Stain [12]. Colonies were enumerated using a stereomicroscope at 40× power and the results were reported as cfu/sample (cfu/100 ml).

Scanning electron microscopy

Scanning electron microscopy was used to evaluate the lumen walls of the chromatography test system before and after sanitization. Samples included tubing excised from the chromatography system fluid path. Tubing samples were immersed in 5% glutaraldehyde (Sigma Chemical, St Louis, MO) buffer at pH 7.2 for 30 min, transferred to 7% sucrose (Sigma Chemical, St Louis, MO) buffer for 30 min; and immersed in 2% osmium tetroxide (Polysciences, Warrington, PA) buffer for 30 min. The buffer base was Dulbecco's PBA [8]. Samples were washed three times in deionized water prior to being sequentially dehydrated in ethanol starting at 35% with exchange every 5 min in 10% increments to 95%. The 95% dehydration step was repeated three times followed by one 5-min exchange in 100% ethanol. Post-fixation, the tubing sections were cut sagitally to expose the lumen wall and then sputter coated with gold palladium (Structure Probe Inc. West Chester, PA) and examined with a scanning electron microscope (Topcon Corp., Tokyo, Japan).

Results and Discussion

Both 1.0 N NaOH and 70% aqueous ethanol demonstrated bactericidal properties by their ability to destroy *P. aeruginosa* colonies in both working cultures (Fig. 2) and adsorbed to polystyrene (Table 4). No microbial contamination, representing a 7–8 log reduction value, was measured after 1 h of contact time with the sanitizing agents. Therefore, either agent can be considered a viable alternative for sanitizing the chromatography system.

Challenge conditions were chosen to duplicate the systematic application of a chromatography system in biomolecule purification procedures. In order to do this, the liquid flow path was chosen so it included each of the four buffer inlet lines, the gradient proportioning valve, the buffer delivery system, both flow paths of the injector valve, and the detector flow cell. It was also important that all peptone flushing and sanitization steps followed the same liquid flow path.

A worst case challenge was achieved in the test system by allowing the test bacteria to stand in the system overnight so that colonization of the lumen walls could occur. Due to complex cell-to-cell and cell-to-substrate attachments, sessile cells tend to be more resistant to sanitization as compared with planktonic or free living cells which are less metabolically active and are easier to destroy

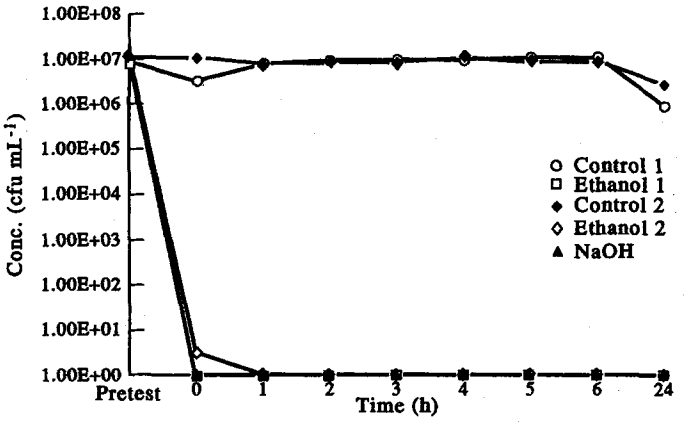

Figure 2
Concentration of *Pseudomonas aeruginosa* in colony forming units per millilitre as a function of time in 70% ethanol, 1.0 N sodium hydroxide and 0.76% saline.

Table 4
Concentration of *Pseudomonas aeruginosa* adsorbed to polystyrene in colony forming units per millilitre as a function of time in 70% ethanol and 1.0 N sodium hydroxide

Time (h)	0.76% Saline control	70% Ethanol	1.0 N NaOH
Pretest	9.00E + 07	6.00E + 07	1.03E + 08
0	TNTC	NG	NG
1	TNTC	NG	NG
2	TNTC	NG	NG
3	TNTC	NG	NG
4	TNTC	NG	NG
5	TNTC	NG	NG
6	TNTC	NG	NG
24	TNTC	NG	NG

TNTC denotes 'too numerous to count'; colony counts were in excess of selected dilutions. The information does indicate there was a rapid kill of the *P. aeruginosa* in the sanitizers and that viability of the bacteria was maintained to some degree in the control, but was non-quantitable.

NG denotes 'no growth', indicates that the presence of *P. aeruginosa* was not detected.

by sanitization. Monitoring the sanitization by conducting peptone water flushes at various times post-sanitization demonstrated whether or not sessile cells were completely destroyed. (Refer to Fig. 3 for an example of the lumen wall of the chromatography system before and after sanitization.)

Table 2 shows that sanitization of *P. aeruginosa* depended upon the choice of sanitizer and the contact time with the system. Initial data show that NaOH purging resulted in the removal of seven logs of bacteria from the system. However, the follow-up monitoring of the system, a 24-h static peptone soak, indicated that the sanitization was incomplete as evidenced by the presence of *P. aeruginosa* colonies on the membrane filters from samples obtained after the peptone soak. Sanitization conditions were adjusted to evaluate sanitizer contact time in both static and constant flow modes, but the level of sanitization achieved was not consistent. While sanitization with NaOH may be sufficient for systems with low level contamination, it was not found to completely destroy *P. aeruginosa* in a highly contaminated system. The manner in which the bacteria interact with the internal surfaces of the instrument may explain the unsuccessful sanitization procedure of *P. aeruginosa* with 1 N NaOH.

A strongly hydrophobic solution, 70% aqueous ethanol, was selected as a second sanitizing agent. Following a *P. aeruginosa* challenge, the system was treated with static ethanol sanitization cycles. Ethanol coupled with an overnight static soak was more effective in removing the bacteria from the system than the 1 N NaOH procedure. After an initial episode of Gram Positive cocci (*Staphylococcus* spp.) contamination attributed to operator handling of the system, the overnight (16–18 h) static soak in 70% ethanol post *P. aeruginosa* challenge on the system resulted in reproducible sanitization of the system (see Table 2).

Similar experiments were conducted to develop an effective sanitization procedure for *A. laidlawii* challenges on the system. Ethanol was not found to be an effective sanitizer for mycoplasma. However, a similar sanitization procedure was developed which involved a 1 N NaOH static 16–18 h system soak. This protocol resulted in an effective and reproducible

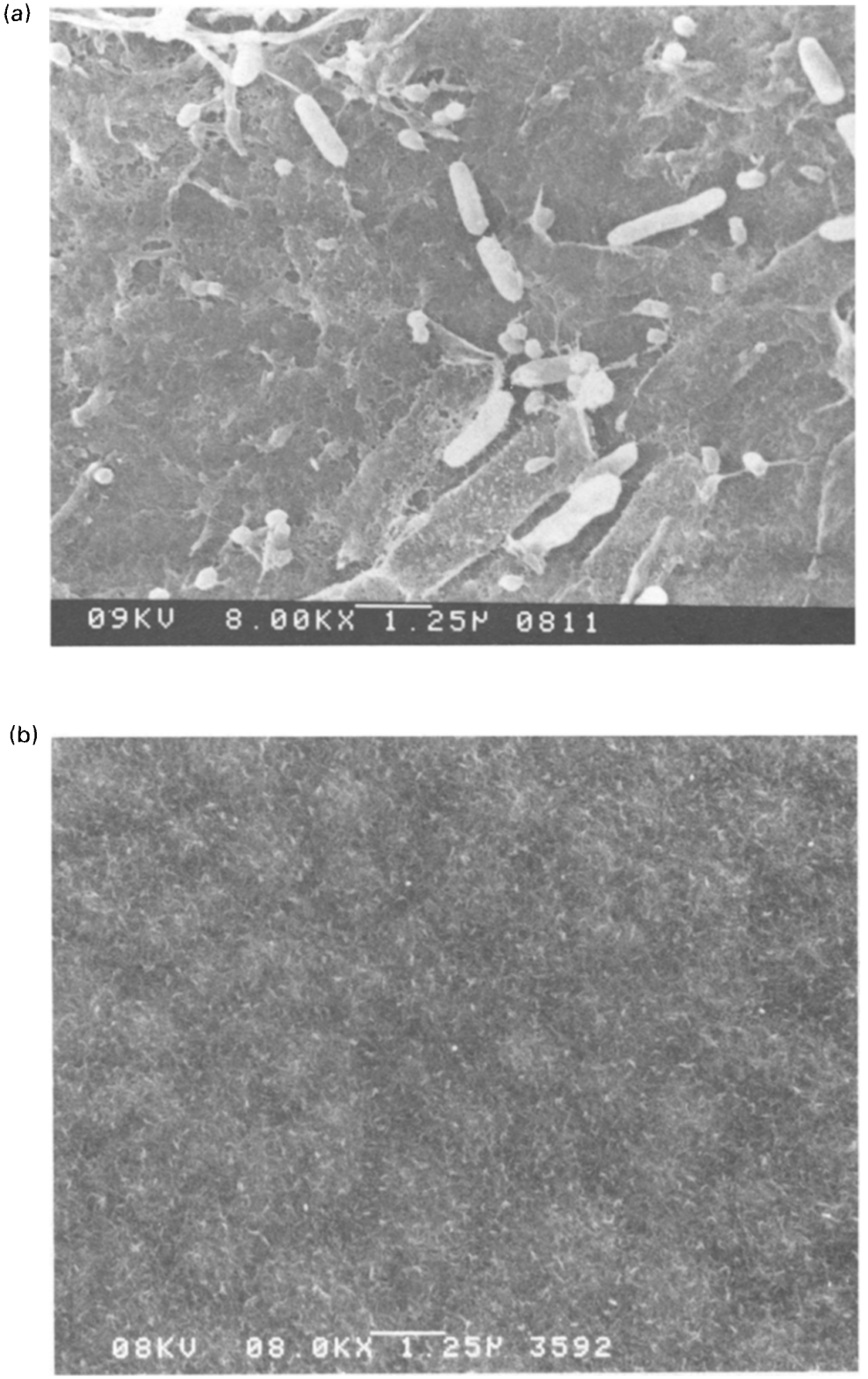

Figure 3
The lumen (a) before and (b) after sanitization.

method for destroying *A. laidlawii* contamination in the chromatography system (see Table 3).

Conclusions

The results of this study demonstrate that extensive microbial contamination can be eliminated from a chromatographic system. Log reduction values of 8–9 have been achieved using 1.0 N NaOH and 70% aqueous ethanol as sanitizing agents. It was also found that the type of sanitizer and its contact time with the system flow path was a critical parameter for successfully eliminating specific microbes. Seventy per cent ethanal was effective in the sanitization of *P. aeruginosa* and the environmental bioburden isolates but was not effective in the sanitization of *A. laidlawii*, one of the four most common contaminants of cell cultures. Conversely, 1.0 N NaOH was effective in the sanitization of *A. laidlawii* but was not effective in the destruction of *P. aeruginosa*. In both cases, extended sanitizer contact time with the system was required to destroy sessile microbes and eliminate the potential for renewed bioburden growth in a system over a multiple day period.

Validation of sanitization protocols requires that the microbial analysis be sensitive, accurate, reproducible and quantitative. The inclusion of the membrane filter method for large volume effluent collection enables high sensitivity detection by concentrating the samples, and provides an easily implemented, quantitative means for sample plating and colony enumeration. In addition, the validation process must address any bioburden anticipated in a given process and monitor sanitization effectiveness for several days following protocol implementation.

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