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Pharmaceutical Container/Closure Integrity II: The Relationship Between Microbial Ingress and Helium Leak Rates in Rubber-Stoppered Glass Vials

LEE E. KIRSCH*, LIDA NGUYEN, CRAIG S. MOECKLY, and RONALD GERTH

Division of Pharmaceutics, College of Pharmacy and the State Hygienic Laboratory The University of Iowa, Iowa City, Iowa

ABSTRACT: Helium leak rate measurements were quantitatively correlated to the probability of microbial ingress for rubber-stoppered glass vials subjected to immersion challenge. Standard 10-mL tubing glass vials were modified by inserting micropipettes of various sizes (0.1 to 10 μm nominal diameter) into a side wall hole and securing them with epoxy. Butyl rubber closures and aluminum crimps were used to seal the vials. The test units were sealed in a helium-filled glove bag, then the absolute helium leak rates were determined. The test units were disassembled, filled with media, resealed, and autoclaved. The test units were thermally treated to eliminate airlocks within the micropipette lumen and establish a liquid path between microbial challenge media and the test units' contents. Microbial challenge was performed by immersing the test units in a 35°C bath containing magnesium ion and 8 to 10 logs of viable P. diminuta *and* E. coli *for 24 hours. The test units were then incubated at 35°C for an additional 13 days. Microbial ingress was detected by turbidity and plating on blood agar. The elimination of airlocks was confirmed by the presence of magnesium ions in the vial contents by atomic absorption spectrometry. A total of 288 vials were subjected to microbial challenge testing. Those test units whose contents failed to show detectable magnesium ions were eliminated from further analysis. At large leak rates, the probability of microbial ingress approached 100% and at very low leak rates microbial ingress rates were 0%. A dramatic increase in microbial failure occurred in the leak rate region $10^{-4.5}$ to 10^{-3} std cc/sec, which roughly corresponded to leak diameters ranging from 0.4 to 2 μms. Below a leak rate of $10^{-4.5}$ std cc/sec the microbial failure rate was <10%. The critical leak rate in our studies, i.e. the value below which microbial ingress cannot occur because the leak is too small, was observed to be between 10^{-5} and $10^{-5.8}$ std cc/sec, which corresponds to an approximate leak diameter of 0.2–0.3 μm.*

Introduction

Parenteral vial quality assurance depends, in part, on demonstrating the ability of the container/closure system to prevent microbial ingress. This can be accomplished by challenging the container/closure system with microorganisms (1); however, physical methods of measuring the system's integrity are frequently desirable because they are more reproducible, faster, less expensive, more reliable, and quantitative (2). The use of physical methods for container/closure integrity quality assurance depends on establishing a correlation between microbial failure and physical measurements.

Our studies were undertaken to investigate the utility of mass spectrometry-based helium leak detection for parenteral product container/closure integrity quality assurance. These studies included the development of a pharmaceutical test unit based on a glass vial that contained a known leak, the development of standard operating procedures for leak rate quantitation using mass spectrometry, the establishment of a correlation between microbial ingress and leak rate, and the validation of the mass spectrometry-based leak rate procedure vis-à-vis microbial challenge methods on a significant scale. We have previously reported on the development of the test unit and operating procedures for leak rate quantification using mass spectrometry (3). The purpose of the studies described herein was to correlate microbial ingress to helium leakage rate.

Ideally, a meaningful correlation can be constructed if the controlling mechanism for microbial ingress and helium leakage rate is the same: namely, the size of the leak. The dependence of helium leak rate measurements on leak size and other experimental variables has been previously described (3).

The detection of microbial ingress depends on the probability that the challenge organism can find a container/closure leak, the organism's ability to traverse the leak, and its ability to grow in the internal container environment. The probability that the challenge organism can find the leak is controlled experimentally by choosing mobile organisms, using adequate concentrations of them, and allowing sufficiently long container exposure to them. The ability of the organism to traverse the leak is dependent not only on leak size but also the mobility of the organism, the absence of leak surfaces that adsorb the organism, and the existence of a

*Author to whom correspondence should be addressed: 118 Pharmacy Building, Iowa City, IA 52242-1112.

fluid path throughout the leak (i.e. the absence of an air lock). Mobility can be assured by organism choice. Adsorption can be avoided by using leaks that are composed of non-adsorptive materials (e.g. glass). The elimination or detection of air locks requires test unit manipulations and the use of a soluble tracer. Finally, the ability of the organism to thrive in the container can be assured by media choice and incubation following container exposure.

This report describes the studies involved in the development of an appropriate microbial challenge testing protocol and the application of that protocol, in combination with the helium leak rate procedures previously reported, to establish a correlation between microbial ingress and helium leak rates for glass parenteral vials. The correlation was used to identify the critical absolute helium leak rate that indicated the lack of microbial ingress.

Materials and Methods

Design of Test Units

Standard 10-mL tubing glass vials were modified by drilling a 2-mm hole in the side wall (supplied by Wheaton and Comar Glass companies). Micropipettes of various sizes (0.1-, 0.2-, 0.3-, 0.4-, 0.5-, 1-, 2-, 5-, and 10-µm nominal diameter, World Precision Instruments) were inserted into the side wall holes and secured with epoxy. Butyl rubber closures (West S-127 4432/50) and aluminum crimps were used to seal the vials.

Development of Procedures for Eliminating Air Locks from the Micropipette Lumens

Since microbial ingress can be prevented by an air lock regardless of leak dimensions, efforts were made to eliminate air locks and demonstrate the existence of a fluid path between external challenge media and the container contents. The existence of the fluid path was demonstrated by measuring the diffusion of magnesium ions through the test units by atomic absorption spectrometry. The basic procedure was to place water-filled vials with known leaks into a bath containing magnesium ions. At various times, aliquots of the vial contents were removed, and tested for the presence of magnesium.

A bath was prepared by dissolving magnesium sulfate (10 mg/mL) in filtered (0.2 µm) double-distilled water. Modified vials (with embedded 1-µm micropipettes) were filled with water, stoppered and sealed with a West gas-powered crimper at 40 psi.

Four methods were evaluated to eliminate airlocks.

1) In the first method, the vials were filled with room temperature water and then immersed in a 60°C water bath for 2 hours. After this, the vials were transferred to a room temperature magnesium solution bath where they were immersed to the neck.
2) In the second method, the vials were filled with 60°C filtered water, stoppered and sealed. The vials were then transferred to a room temperature magnesium bath where they are immersed to the neck.
3) In the third method, the vials were filled with room temperature water and then immersed in a room temperature magnesium bath. The bath was placed in a pressure vessel which was evacuated to 11 psi for 30 minutes.
4) The fourth method was the similar to the third, except pressure was applied at 20 psig for 30 minutes.

Samples were taken at 1 and 24 hours post immersion. In order for the water level inside the vials to remain above the level of leak, 100 µL samples were withdrawn. This volume was then immediately replaced with 100 µL of filtered water. In an attempt to eliminate any pressure change that could occur within the vial due to this sampling procedure, an additional syringe needle was placed through the vial septum as an outlet port.

The samples were analyzed for magnesium content using a Varian AA-1475 atomic absorption spectrophotometer set at a wavelength of 285.2 nm and a lamp current of 4 mA. Prior to testing, the samples were mixed with 100 µL of 1% nitric acid (1:1) to increase the output signal generated. With such small samples being analyzed, a microinjection system was used in which peak height values were output from the instrument.

Detection sensitivity was determined by preparing a calibration curve of AA peak height versus standard concentrations. Linear calibration curves were obtained from standard solutions in the range 0 to 5 ppm magnesium. The minimum detectable magnesium level corresponded to an absorbance of 0.01.

Development of Immersion Microbial Challenge Procedure

Pseudomonas diminuta (*Brevundimonas diminuta*) and *Escherichia coli* were chosen as challenge organisms. *P. diminuta* is an asporogenous, Gram-negative rod. It has a rod length of 1 µm, diameter of 0.3 µm, and flagella length of 0.6 µm. *P. diminuta* is capable of penetrating a 0.45 µm-rated filter under high organism challenge conditions but retained by a 0.22 µm-rated membranes. *E. coli* has a variable rod length of 1.0–3.0 µm and diameter of 0.5 µm. Additionally, the *E. coli* mechanism of mobility differs from *P. diminuta*. The latter possesses polar flagella, whereas in the former, smaller flagella are dispersed over its cell wall. (4).

The challenge media composition included components to support organism viability, metal ion (magnesium) tracer was used to demonstrate the absence of airlock in container leaks, and a surfactant was tested to improve container surface wetability.

Saline lactose broth is a nonproteinaceous broth and the absence of proteins in the broth reduces the likelihood of clogging test unit leaks. Furthermore, this broth is not associated with cell aggregation that increases the effective size of challenge organisms (5). Microbial growth is evenly distributed throughout the media.

Studies were conducted to assess the compatibility of the test organisms in challenge media, magnesium ion, and Tween 80. The surfactant was tested as a possible aid wetting the leak and thereby improving airlock elimination.

Test Preparation

Ten test solutions were prepared with varying concentrations of magnesium sulfate and Tween 80 (Table I). All test

TABLE I
Growth Promotion of Microorganisms in Media Containing Saline Lactose Broth and Magnesium Ion and/or Tween 80.
"−": No Growth, "+": Slight Growth, "++": Moderate Growth

[Tween 80] (mg/200cc)	Magnesium Sulfate (mg/cc)	AN	CA	BS	EC	SE	BP	PA	PD
0	0	++	+	+	++	−	++	++	++
7.85	0	++	+	+	++	−	++	+	+
3.14	0	++	+	+	++	−	+	+	+
1.57	0	++	+	+	++	+	+	+	+
0	5	++	−	+	++	+	−	++	++
0	10	++	−	+	++	+	−	++	++
1.57	5	++	+	+	++	++	−	+	+
3.14	10	++	−	−	++	+	−	+	+
7.85	5	++	−	+	++	++	−	++	++
7.85	10	++	+	+	++	+	−	++	++

Test organisms: AN = *Aspergillus niger*, BS = *Bacillus subtilis*, SE = *Staphylococcus epidermis*, PA = *Pseudomonas aeruginosa*, CA = *Candida albicans*, EC = *Escherichia coli*, BP = *Burkholderia peckettii*, PD = *Pseudomonas diminuta*.

solutes were dissolved in a 7.6 g/970 mL NaCl solution. A 6-mL solution of lactose broth, composed of 1.3 g of dehydrated lactose broth medium in 100 mL of water, was added to the mixture. A sufficient amount of the NaCl solution was added to bring the total volume of each test solution to 200 mL. Solutions were autoclaved at 121°C for 20 minutes using a slow exhaust liquid cycle and allowing them to cool to room temperature prior to use.

Organism Preparation

Twenty-four hours prior to the challenge, three subcultures of each organism were prepared making a slant of trypticase soy agar in a 5-inch large bore screw cap tube. The tubes were melted, slanted to give the largest surface area and allowed to cool to room temperature to harden. One of the aliquots of the frozen bacteria was removed from −70°C storage, thawed and washed down the face of each slant. Care was taken to not damage the face of the slant and to cover as much of the surface of the slant as possible. The caps were loosened and the tubes were incubated at 35°C overnight until ready for use. After incubation each tube was examined to ensure that good growth and that no obvious signs of contamination existed. The organisms were washed off the surface of each slant using sterile normal saline and a 10 mL pipette. Care was taken to not damage the surface of the agar and to wash as many of the cells as possible off of the agar. Once each of the tubes had been harvested, the organism suspension was used to spike the challenge broth. The organisms used in the growth promotion studies were *Aspergillus niger* (AN), *Candida albicans* (CA), *Bacillus subtilis* (BS), *Escherichia coli* (EC), *Staphylococcus epidermis* (SE), *Burkholderia peckettii* (BP), *Pseudomonas aeruginosa* (PA), and *Pseudomonas diminuta* (PD).

Methods for the Correlation of Microbial Ingress and Helium Leak Rate

The general procedure is described in the flow chart labeled Figure 3.

Determination of the Absolute Leak Rate

Modified vials were prepared by inserting and affixing glass micropipettes of various nominal diameters (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 5, and 10 μm) into side wall holes. The test units were then filled with tracer (helium) using the following procedure: test units, stoppers, crimp seals, oxygen monitor, and sealing equipment were placed inside an inflatable glove bag. The glove bag was inflated and flushed with helium. Residual air was monitored by continuously measuring the oxygen level using a galvanic electrochemical oxygen monitor. When the residual oxygen level was <2%, the helium flow was turned off, and the helium outlet was sealed. An aluminum crimp was placed on top of the rubber stopper and secured with an air crimper. The test units were removed from the glove bag. The test units were exposed to atmosphere for >12 hours prior to leak testing in order to allow helium desorption from container surfaces.

The absolute leak rates were determined using a helium leak rate detector (3). The test units were then opened by removing the rubber stopper and aluminum crimp seal.

Microbial Challenge Testing

A saline lactose broth was prepared. Sodium chloride in the amount of 7.6 g was dissolved in 970 mL of reagent grade water. 30 mL of lactose broth (a solution of 1.3 g of dehydrated lactose broth medium in 100 mL of reagent grade water) was added. The pH of the resulting mixture was adjusted to 6.9 ± 0.1 with sodium hydroxide.

The test units were each filled with 5 mL of saline lactose broth, then sealed with butyl rubber stoppers and aluminum crimps using a gas-powered crimper at 40 psi. The broth filled vials were autoclaved in a 20 minute liquid cycle. Five milliliters of the broth was placed into the 16 mL capacity modified vials. Following autoclaving a second coat of epoxy was applied with care to the outer surface of the leak to avoid occluding the leak. The broth-filled vials were immersed in a 60°C waterbath for one hour. Then they were removed, inverted, and immersed in a 25°C saline lactose broth containing 10 mg/mL magnesium. The inversion of the vials allowed the broth level to be above the level of micropipettes. This was essential in order to eliminate airlocks. The broth temperature inside the vial was allowed to stabilize to 25°C for 1 hour. The broth bath was then spiked with 8–10 logs of viable *P. diminuta* and *E. coli* and mixed to disperse the bacteria. The bath was placed in a

TABLE II
The Mean Magnesium Concentration of Leaky Vials Contents after Immersion (1 and 24 Hour) in a 10 mg/mL Magnesium Sulfate Bath. The Methods Correspond to the Experimental Procedures Used to Eliminate Airlocks and Are Described in the Report Text

		1 Hour		24 Hour	
Method	Number of Vials Tested	Mean [Mg] ppm	Number of Vials with No Detectable Magnesium	Mean [Mg] ppm	Number of Vials with No Detectable Magnesium
1: 60° C/2 hour immersion	8	0.00	8	1.61	0
2: Filled with 60° solvent	5	0.01	4	4.56	2
3: Vacuum/30 minutes	4	0.00	4	0.00	4
4: Pressure/30 minutes	5	0.22	2	0.71	2

35°C incubator for 24 hours. The test units were removed from the challenge broth and incubated in an upright position at 35°C for an additional 13 days.

Following the 14-day incubation period, the vials were removed from the incubator and visually inspected for the presence of bacteria indicated by turbidity. When visual inspection did not detect microbial growth, the test unit contents were streaked on a blood agar plate for verification of the absence of microorganisms.

Sterilization of Challenged Vials

Five mL of saline lactose broth was withdrawn from each vial by a 20-gauge needle and syringe in order to perform atomic absorption analysis for verification of a fluid path. These samples were autoclaved using a 20-minute liquid cycle. The challenged test units were chemically sterilized by injecting 5 cc of Lysol disinfectant into the vial. This method of sterilization method was used to minimize damage to the integrity of the test unit. Following injection of disinfectant, the vials were soaked in a bath of disinfectant. After a 5-minute soak, the vials were removed for storage.

Flame Atomic Absorption Spectroscopy Analysis

Flame atomic absorption spectroscopy was used for the detection of magnesium which was atomized by a high temperature flame (1700–3200°C) and then detected spectrometrically. A Varian AA-1475 atomic absorption spectometer was used to determine if the autoclaved broth solutions withdrawn from the challenged vials contained magnesium ion. Samples were analyzed using a Varian magnesium cathode lamp containing neon gas.

Standards were prepared and mixed with equal amounts of 1% nitric acid to increase the output signal generated resulting from standard solutions of 0.5, 1, 1.5, 2, 3, 4, and 5 ppm. A blank solution was prepared with double-distilled water mixed 1:1 with 1% nitric acid. Additionally, 1 mL of each sample was mixed with 1 mL of 1% nitric acid. Using an integration time period of 5 seconds, at least five absorbance readings of each standard and sample were taken.

Logistical Regression Analysis of the Relationship between Microbial Ingress and Leak Rate

Logistical regression was used to predict the probability of microbial ingress as a function of the absolute helium leak rate. In this technique, the negative log likelihood attributed to the following model was minimized:

$$\text{Probability of ingress} = (1 + \exp(\log \text{leak rate} \times \beta)^{-1}$$

where β is estimated by model fitting (6).

Results

Development of Procedures for Eliminating Air Locks

The mean magnesium concentrations of vial contents for each method are shown in Table II. Method 3 (vacuum chamber) was clearly ineffective in eliminating airlocks. Of the remaining methods, only method 1 (2 hour 60°C water bath exposure) consistently eliminated airlocks. Detectable magnesium levels were found in all test vials after 24 hour immersion in the magnesium bath. Thus, this method was used for subsequent microbial challenge testing.

Development of Immersion Microbial Challenge Procedure

The growth results varied by media for most organisms with the exception of the *Escherichia coli* and the *Aspergillus niger* for which moderate growth was observed in all test media. Since the *Aspergillus niger* is a non-motile spore it was not considered for use as a test agent for this study. *P. diminuta* viability was well promoted in 5 and 10 mg/mL of magnesium with no Tween 80 solutions and 5 and 10 mg/mL magnesium with 7.85 mg/200 mL Tween 80, as indicated by moderate growth in those solutions. *E. coli* and *P. diminuta* were found to be compatible and well supported in saline lactose broth with magnesium. Tween 80 was deemed unnecessary based on the successful elimination of airlock by thermal manipulation of the test units.

Methods for the Correlation of Microbial Ingress and Helium Leak Rate

A total of 288 vials were subjected to microbial challenge testing. Of these vials, 114 were visually observed to fail. Of those test units in which visual failure could not be detected, another two vials were shown to contain *P. diminuta* by plating. The number of microbial failures (column 4) is shown in Table III as a function of log leak rate ranges (column 3).

Those test units whose contents failed to show detectable magnesium ions were eliminated from further analysis because a fluid path in the leak had not been demonstrated. Additionally, the contents of a group of 24 test units with

TABLE III
Summary of Microbial Challenge Testing and Helium-filled Test Unit Leak Rate Measurements for Modified SVPs Containing Affixed Micropipettes of Nominal Diameters 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 5.0, 10.0 Microns

Group	Number of Units	Mean Nominal Leak Diameter (Microns)	Log Leak Rate Range[a]	Microbial Failure Frequency	Test Units with Detected Magnesium Ion[b]	# of Vials with Damaged Leaks [c]	Corrected # Units with Positive Microbial Growth/ Corrected # Tested
A	29	8	−1.9–1.3	28/29	29	not tested	28/29
B	45	6	−2.5–1.9	36/45	43	not tested	36/43
C	35	2	−3.3–2.7	23/35	33	not tested	23/33
D	37	0.7	−4–3.4	16/37	25	not tested	16/25
E	60	0.4	−5.4–4.7	9/60	42	5	4/37
F	19	0.3	−6.0–5.4	2/19	16	1	1/15
G	17	0.2	−6.8–6.5	1/17	8	1	0/7
H	46	0.1	−7.2–6.8	1/46	29	1	0/28

[a] Absolute leak rates were measured in standard cubic centimeters per second.
[b] Detected atomic absorbance was greater than background upon repeated measurement.
[c] Helium leak rates measured after microbial challenge testing was determined to be >ten-fold in excess of prechallenge results (see text for methods description).

low absorbance (<0.4) and high log leak rates (>−4) were retested for the presence of magnesium. In all cases, the presence of magnesium was reconfirmed.

Twelve test units with log leak rates <−4 that were positive for microbial ingress were investigated to confirm that leak integrity was unchanged during microbial ingress testing. These test units were bombed in a helium containing pressure vessel for 2 hours at 15 psig. After a 12 hour desorption period, the leak rates were measured and compared to predicted results based on the absolute leak rates (determined prior to microbial testing). Leak rates (Q_m) after charging were predicted with the following equations:

$$Q_m = \frac{Q_a}{P_a} f_{HE} (P_o + [P_{BC} - P_o]Ae^{-(Q_a t_{desorb})/V_{HS}})$$

where Q_a and P_a are the absolute leak rate and test unit headspace pressure during absolute leak rate measurement, P_O is the headspace pressure at the beginning of charging, P_{BC} is the charging chamber pressure, t_{desorb} is the duration of desorption, V_{HS} is the test unit headspace volume, A is the accumulation of tracer in the test unit during charging and is given by

$$A = 1 - e^{-(t_{bomb}Q_a/V_{HS})}$$

where t_{bomb} is the duration of charging and V_{HS} is the vial headspace volume, and f_{HE} is the fraction of helium and given by

$$f_{HE} = \frac{P_{BC} - P_o}{([P_{BC} - P_o]A + P_o)} A$$

Previous studies have demonstrated the accuracy of the prediction procedure based on the absolute leak rate (3).

For three of the questionable test units from microbial challenge testing, the predicted and observed log leak rates post bombing were identical. For two other units, the observed leak rates were much less than the predicted leak rate. It is likely that the leaks in these two test units became obstructed during microbial testing by microorganism growth. The results from these five retested units were included in the final estimates of microbial failures. The observed leak rates after bombing for seven other retested units were >10-fold higher than the predicted leak rates. This was likely due to by a change in leak integrity during the microbial testing procedure. These test units were eliminated from further analysis (Table III, column 6).

The corrected microbial failure frequency is summarized in Table III (column 7). The probability of microbial failure as a function of the log leak rate was estimated using the logistical regression platform in JMP (6). The curve in Figure 1 represents the regression model from which the probabilities of ingress at various log leak rate values were estimated. Thus, the logarithm of the absolute leak rate (std cc/sec) corresponding to probabilities of microbial failure at the 50%, 10%, 5%, 1%, 0.1% were estimated to be −3.7,

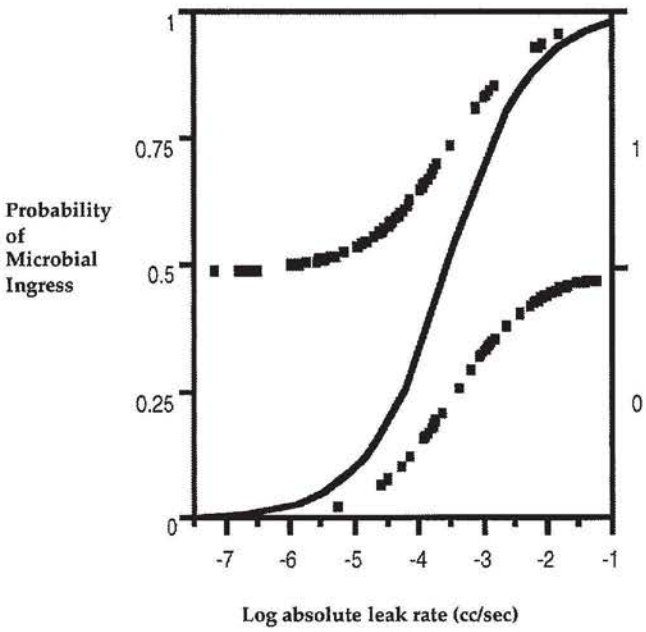

Figure 1—The correlation of microbial ingress probability versus the logarithm of the absolute leak rate using logistical regression. The estimated probability is given by the value of the curve at specific log leak rate values. The distribution of failures is represented by the points scattered below the curve. The distribution of test units that showed no signs of ingress (by observation and plating) are shown above the curve, whereas those units that failed microbial challenge are distributed below the curve.

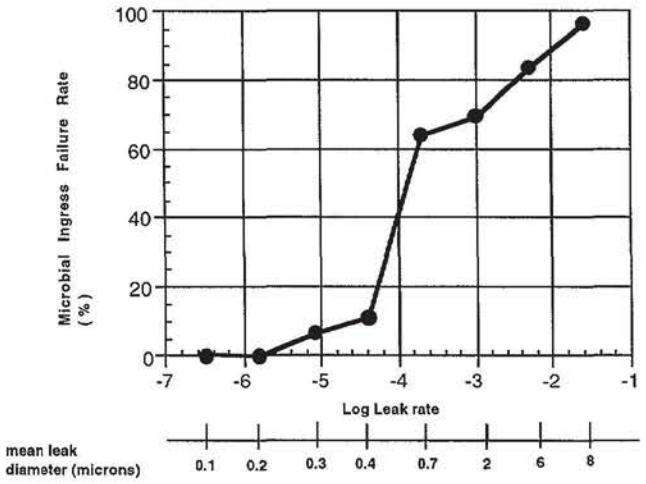

Figure 2—The correlation of microbial failure rate (%) and the mean logarithm of the absolute leak rate and nominal leak diameter for modified SVPs. The absolute leak rate (standard cubic centimeters per second) was determined by mass spectrometry-based helium leak rate detection. Microbial failure was measured by microbial ingress after 24 hour immersion in a bath (37°C) containing 10^8 to 10^{10} *P. diminuta* and *E. coli* organisms/mL and a 13 day, 35°C incubation.

−5.2, −5.7, −6.9, and −8.6, respectively. The data above the curve shows the distribution of test units that did not show ingress, and the data below the curve represents the test units in which microbial ingress was present. Although this model was useful for prediction purposes, it represented a continuous function that may not represent a physical reality. In other words, it is likely that a critical leak rate value exists, below which organisms are unable to invade the container because the leak constriction is simply too small. Therefore, the probability of ingress as a function of gas leak rate is probably discontinuous and cannot be accurately represented by a function that asymptotically approaches a zero probability.

The relationship between microbial failure and log leak rate can be more simply represented by plotting the microbial failure rate versus the mid-value of each of the groups represented in Table III (Figure 2). The critical leak rate region is between 4×10^{-5} and 2.5×10^{-4} std cc/sec. This region corresponds to nominal leak diameters of 0.4 to 1.0 µm. Of the 66 test units with leak rate $<4 \times 10^{-5}$ std cc/sec, three failed the microbial challenge. Thus, the likelihood of microbial failure at log leak rates ≤ -5 was remote.

Discussion

The study results detailed herein are pivotal for the establishment of non-microbial methods for container integrity quality assurance. 288 test vials (containing leaks with nominal pore diameters of 0.1 to 10 µms) were subjected to microbial challenge testing using *E. coli* and *P. diminuta* and to helium leak rate analysis. A strong correlation between microbial ingress and helium leak rates was observed (Figures 1 and 2). The critical log leak rate region was between −4.4 and −3.8 (leak rate in std cc/sec), which roughly corresponds to nominal leak diameters of 0.4 to 1.0 µm (Figure 2). Although the relationship between microbial ingress and nominal leak diameter makes sense in terms of the size of the test organisms, it should be noted that the nominal leak diameters are inferred from physical measurements (modified bubble tests) and not estimated by direct dimensional measurements (7).

Of the 66 test units with log leak rates <-4.5 std cc/sec, only three failed the microbial challenge. Thus, the likelihood of microbial failure at log leak rates ≤ -5 is remote. These results are consistent with the range reported by Morton-Guazzo *et al.* of 10^{-4} to 10^{-5} std cc/sec for *Pseudomonas aeruginosa* egress through defective vial seals (8).

The conditions for microbial challenge testing were extreme: test units were subjected to a 24 hour immersion challenge using 10^8 to 10^{10} viable *P. diminuta* and *E. coli* organisms preceded by manipulations designed to eliminate airlocks in the leaks and allow a fluid pathway from the challenge media to the vial contents. Moreover, the presence of a fluid pathway was confirmed by chemical tracers, and test units that failed to demonstrate such a pathway were eliminated from the analysis. Many more test units with very small leaks were eliminated than large leak units; e.g. half of the 0.1 µm leak test units failed to demonstrate chemical tracer ingress whereas none of the 10 µm leak units failed. However, this action was appropriate in that none of the test units that failed chemical tracer ingress showed microbial ingress. In other words, an airlock was an effective barrier to prevent microbial (and chemical) ingress.

It may be argued that the microbial challenge conditions did not reflect actual or even extreme shelf-life exposures of typical pharmaceutical container/closure systems to microbial ingress. In fact, the experimental procedures reported herein do not represent the best designs for simulating shelf-life, shipping or use conditions. But the purpose of these studies was to establish a correlation between microbial ingress and helium leak rates, and for that purpose, the procedures were appropriate. Microbial ingress is largely a probabilistic series of events. It depends on the probability of an organism finding a leak, the probability that the conditions in the leak allow it to be traversed by the organism, and that the leak is of sufficient dimensions to allow ingress. On the other hand, helium leak rates are deterministic measurements of a physical event that is governed by experimentally-controlled factors (e.g. tracer concentration, temperature, pressure differential, etc.) and the dimensions of the leak. Successful correlations between helium leak rates and microbial ingress depend on minimizing the importance of the stochastic events that control the latter. For instance, if the frequency of microbial ingress is largely dependent on the probability of an organism finding a leak, than a correlation with a physical measure of leak size will likely not be successful even if extremely large numbers of test units of varying leak size are used. Likewise, if small leaks have a high probability of airlocks and no experimental measures are taken to eliminate airlocks, then the apparent correlation between leak size and microbial ingress may, in fact, be more dependent on the surface tension of the challenge media than the dimensional characteristics of the leak. Although the presence of airlocks may be relevant to shelf-life simulation studies, it is detrimental to the establishment of a correlation between helium leak rates (which are

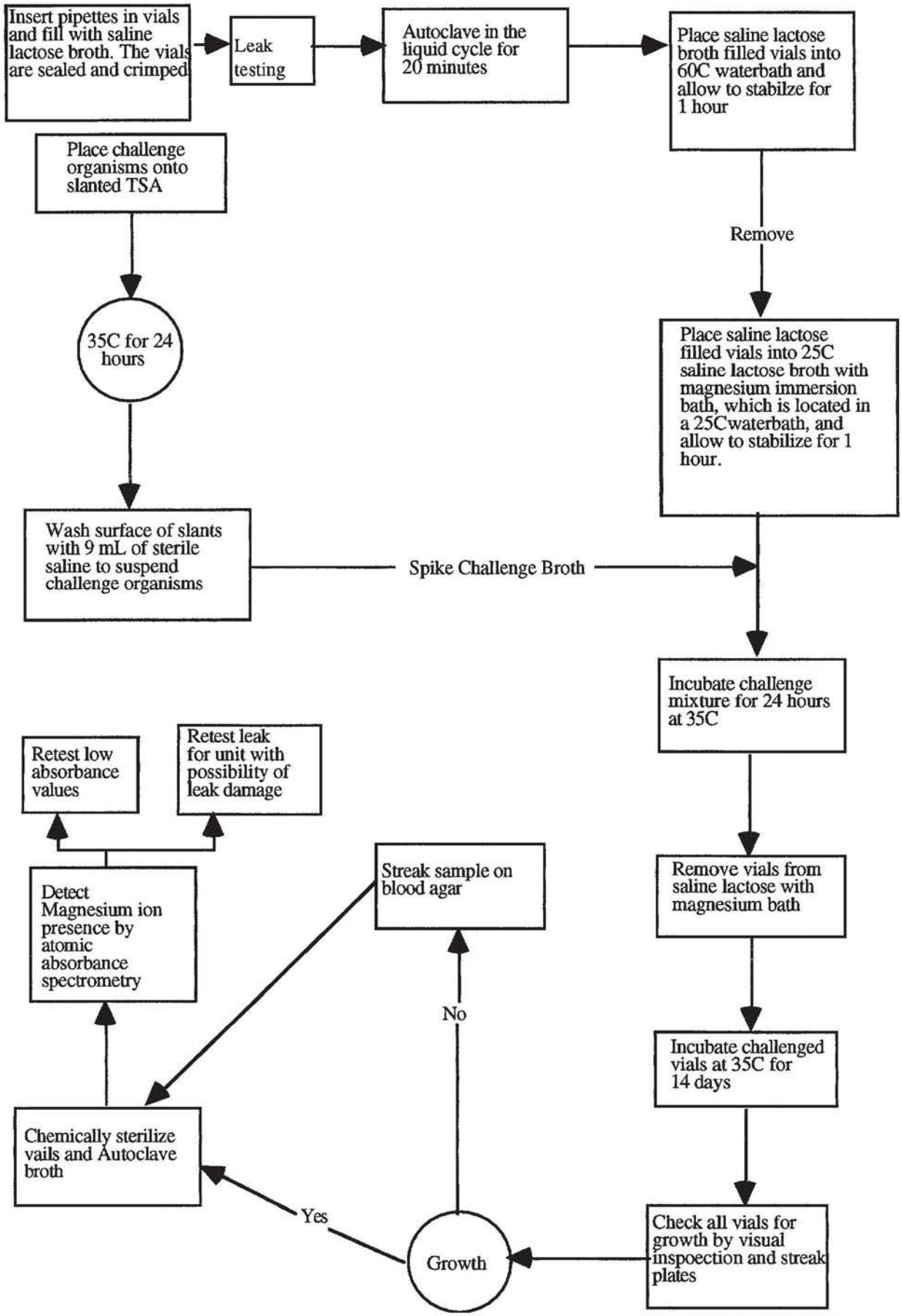

Figure 3—Flow diagram of procedures used to correlate microbial ingress to mass spectrometry-based leak rates in test units.

not dependent on airlocks or the surface tension of the microbial challenge media) and microbial ingress.

The rigorous microbial challenge conditions minimized the importance of the stochastic events that affect microbial ingress by allowing prolonged exposure of the leak to numerous challenge organisms and ensuring that a fluid path traversed the leak. Furthermore, the choice of an all-glass leak avoided the adsorptive retention of challenge organisms that is frequently associated with membrane filtration.

The critical leak rate was $10^{-5.2}$ std cc/sec. This was the leak rate associated with a low probability of microbial ingress. It is important to remember that this value was obtained under specific measurement conditions, namely the leakage of helium by convective flux at a pressure differential of one atmosphere. This leak rate value may be useful in establishing the critical values for other physical methods of pharmaceutical container/closure integrity if a meaningful and quantitative correlation between helium leak rate measurements and the alternate physical method can be developed. Clearly, if the alternate method measures a different property of the leaking container, e.g. diffusive flux of a tracer through a leak in the absence of a total pressure differential, then the value established herein should not be represented as the "critical" value without establishing a correlation between the two methods.

Conclusions

In short, microbial ingress was well-correlated to mass spectrometry-based leak rate measurements. Furthermore, the critical log leak rate value was -5.2, i.e. the absolute leak rate (helium-filled test units) for which the probability of microbial ingress was <0.10 was 6×10^{-6} std cc/sec. The fact that this leak rate value corresponds to a nominal leak diameter of between 0.1 and 0.3 μms is probably significant given the size of the smallest test organism, *P. diminuta*.

This correlation along with the standard methods for helium leak rate testing of pharmaceutical vials can be used to establish and qualify a mass spectrometry-based container integrity method. The design and validation of this method will be described in a future report.

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