Validating a Reversed-Phase HPLC Method to Determine Residual Nonoxynol-9 on Pharmaceutical Process Equipment Using a Nonporous Silica Column

PAC-710 is a solution used in the pharmaceutical industry to clean process equipment. One of its active ingredients, nonoxynol-9, is a nonionic surfactant comprising nonylphenoxypolyethoxyethanol oligomers. To support their company's cleaning validation program, the authors' laboratory developed and validated a high performance liquid chromatography method that used a 1.5-μm d_p nonporous silica column to test for residual nonoxynol-9 on stainless steel, polytetrafluoroethylene, and acrylic substrates. They designed method validation experiments to ensure that the analytical method could determine PAC-710 residue accurately in a solution concentration range of 10–210 μg/mL. In this article, the authors compare their nonporous silica analytical method with the *U.S. Pharmacopeia* nonoxynol-9 assay test.

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Cleaning validation is an important part of any pharmaceutical manufacturing activity, as documented elsewhere (1). Pharmaceutical manufacturers are responsible for ensuring that product contact surfaces are free of residues from the previous product, as well as any cleaning agents, before starting a new product batch. Many commercially available cleaning solutions of various compositions — acidic, basic, and ionic and nonionic surfactants — have been developed for pharmaceutical manufacturing equipment. PAC-710 (Thermo-cote Inc., Patterson, New Jersey) is an effective cleaning solution that contains several ingredients, including tripolyphosphates, 2-butoxyethanol, and the nonionic surfactant nonoxynol-9 (Figure 1).

Our laboratory previously analyzed pharmaceutical surfaces for residual PAC-710 by testing for phosphates through a colorimetric comparison based on the molybdenum blue reaction (2). Although this method was validated, it was nonselective and only semiquantitative. The disadvantages inherent in the use of this method include the ubiquity of phosphate in the environment, which could result in false positive reactions, and the ability of several other ions to yield positive or negative interference (for example, Cr^{6+}, Fe^{3+}, Cu^{2+}, NO_3^-, and SiO_4^{2-}). FDA guidelines recommend that analytical methods be selective and quantitative (see http://www.fda.gov/ora/inspect_ref/igs/valid.html). Therefore, we decided to develop and validate a method to test for residual PAC-710 on pharmaceutical surfaces that would be both selective and quantitative. 2-Butoxyethanol, with a reported boiling point of 171–172 °C (3), appeared promising as an analyte using simple gas chromatography methodology; however, the

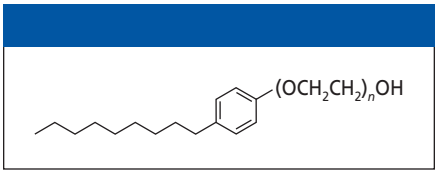

Figure 1: General structure for nonoxynol oligomers.

volatilization of 2-butoxyethanol from substrate surfaces, as evidenced by low recovery results (much less than 50%) obtained during recovery feasibility experiments, precluded it from further consideration. We shifted our focus to the development and validation of an analytical method for nonoxynol-9 using a nonporous silica column.

We used a nonporous silica C18 column and an isocratic mobile phase of 60:40:0.05 (v/v/v) water–acetonitrile–85% *o*-phosphoric acid. At a flow rate of 1.0 mL/min and a temperature of 40 °C, the total run time was approximately 4 min. A variable-wavelength UV–vis detector was set at 210 nm. Although the nonporous silica method provided chromatography that was qualitatively similar in appearance to that obtained with the *U.S. Pharmacopeia* (*USP*) method, the nonporous silica method has the advantage of a 4-min run time as compared with approximately 20 min for the *USP* method.

We conducted experiments to determine the specificity of the method (as potential chromatographic interferences from swab materials), linear range, system and method precision, method accuracy, solution stability, and limits of quantitation and detection. The method could accurately determine residual quantities of nonoxynol-9 on pharmaceutical process equipment. During validation of the method, we discovered that the recovery of nonoxynol-9 from stainless steel was dependent on the length of time it was in contact with the substrate.

Why Nonporous Silica?

Cleaning validation methods must be designed to detect low levels of analytes with analytical concentrations centered on an acceptable residual limit. The acceptable residual limit of a substance is based on several factors, including the size of the equipment train (4–6). When we considered cleaning validation methods for equipment trains that differed in total area, we obtained markedly different acceptable residual limits — 8.0 μg/cm^2 and 120 μg/cm^2 of nonoxynol-9 — for PAC-710.

The *USP* contains a monograph for nonoxynol-9 raw material, including a reversed-phase high performance liquid chromatography (HPLC) method that is performed at ambient conditions and uses a 250 mm × 3.9 mm, 10-μm d_p octadecylsilane-bonded silica column, an 80:20 (v/v) methanol–water isocratic mobile phase, UV detection at 280 nm, and a nonoxynol-9 concentration of 25 mg/mL. Our evaluation of this method demonstrated that it failed to provide the necessary sensitivity for determining nonoxynol-9 at the lower concentrations required for this cleaning validation method.

One reason for its failure is the wavelength chosen for the analysis; experiments in our laboratory showed that the absorbance of nonoxynol-9 is approximately 5.5 times higher at 210 nm than at 280 nm. Another reason is the column. The *USP* method uses a 250 mm × 3.9 mm octadecylsilane–silica column and a run time of approximately 20 min for a single chromatographic injection (see Figure 2). The silica used in this type of column is of a porous nature, and perfusion of the pores, as well as interaction with the derivatized outer surfaces of the particles, occurs as analytes traverse the column. The perfusion and interaction cause an increase in retention time, which is attributable to an increase in the multiple path term of the van Deemter equation. The silica used in nonporous silica chromatographic columns has no pores; therefore, the analyte interacts with only the stationary phase on the surface of the particle, and the multiple path term is minimized. The 1.5-μm particle size of the packing material also enables very short column lengths that can yield chromatography similar to that obtained with longer columns that contain larger porous particles (see Figures 2 and 3). The result is that chromatograms obtained using a nonporous silica column typically exhibit shorter retention times than those obtained with traditional porous silica HPLC columns (7,8). Because of the short retention times, the band spreading in the column is minimized; thus analyte concentration is enhanced, which provides lower limits of quantitation.

We expected other more general advantages from the nonporous silica columns. Mobile-phase additives often are used in reversed-phase chromatography systems with porous silica–based columns to reduce nonspecific interactions with the analyte. Because of both the nonporous nature of the silica in the column used and the extent of surface derivatization, analytes experience fewer nonspecific interactions; therefore, mobile phases on nonporous silica columns in general can be prepared without these additives. The nonporosity and extent of derivatization also lend great pH stability to these columns. In our laboratory, we have used mobile phases of pH 1–12 with these columns with no perceptible deleterious effects to subsequent chromatographic analyses. Finally, nonporous silica columns have been used successfully to analyze environmental and pharmaceutical analytes (9–12). Therefore, we chose a nonporous silica column for our development and validation efforts.

The nonoxynol-9 chromatographic profile obtained with the nonporous silica method (see Figure 3) was comparable to the chromatographic profile obtained from the method outlined in the *USP* for nonoxynol-9. The nonoxynol-9 chromatographic peak obtained with each method has front and back shoulders. The nonporous silica and *USP* methods both achieve quantitation through summation of the area of all peaks from nonoxynol-9, including shoulders and bumps. The results of the validation study presented below demonstrate that the method developed in our laboratory can determine residual levels of nonoxynol-9 on stainless steel, polytetrafluoroethylene (PTFE), and acrylic precisely and accurately

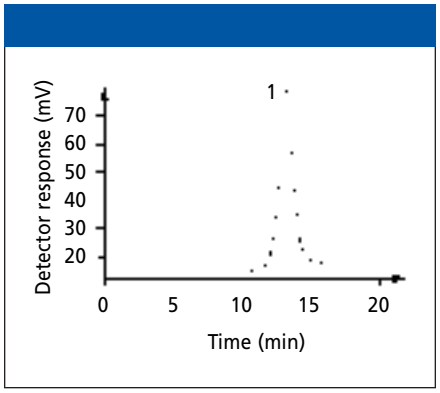

Figure 2: *USP* chromatogram of nonoxynol-9 (0.25 mg/mL). Peak 1 is nonoxynol-9.

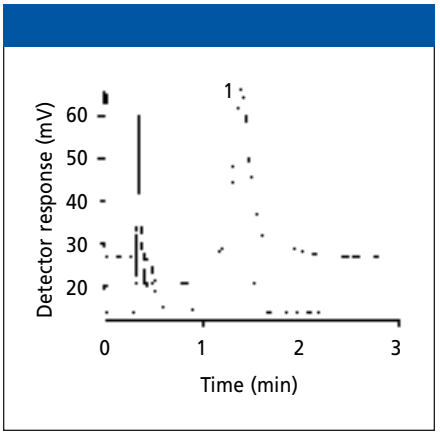

Figure 3: Nonporous silica chromatograms of nonoxynol-9 (0.040 μg/mL), diluent, and swab blank (upper, middle, and lower chromatograms, respectively).

at two distinctly different acceptable residual limits — 40 and 140 µg/mL nonoxynol-9 in 5.0 mL of recovery solution — and also achieve marked time efficiency.

Experimental

Reagents: All reagents were of analytical grade; deionized water was prepared in-house. We used 0.2-µm PTFE Acrodisc syringe filters (Pall Gelman, Ann Arbor, Michigan) to filter samples and standards, and 0.2-µm polycarbonate disk filters (Osmonics, Minnetonka, Minnesota) to filter mobile phase; both types of filters were purchased from Fisher Scientific (Fairlawn, New Jersey). Pure nonoxynol-9 was purchased from Sigma (St. Louis, Missouri) and as a compendial reference standard from the USP. Recovery experiments were performed with swabs purchased from Coventry Clean Room Products (Kennesaw, Georgia). Stainless steel, PTFE, and acrylic substrates of approximately 20 cm × 30 cm were prepared by demarcating them into 5 cm × 5 cm squares, as outlined in Figure 4.

Equipment: We used a 33 mm × 4.6 mm, 1.5-µm d_p nonporous silica C18 column (Micra Scientific Inc., Northbrook, Illinois). For quantitative work, we used Agilent 1100 and 1090 HPLC systems (Agilent Technologies, Palo Alto, California) with UV detectors. Comparative chromatographic results were generated using a 300 mm × 3.9 mm, 10-µm d_p µ-Bondapak C18 column (Waters Corp., Milford, Massachusetts).

Experimental conditions: We used an isocratic mobile phase of 60:40:0.05 (v/v/v) water–acetonitrile–phosphoric acid. The flow rate was set to 1.0 mL/min with a temperature of 40.0 °C. The system routinely operated at a back pressure of approximately 180 bar. We used a variable-wavelength UV–vis detector set at 210 nm for detection.

Each 5 cm × 5 cm pharmaceutical process substrate subdivision was swabbed twice: once with a swab wetted with 50:50 (v/v) water–methanol (diluent) and then with a dry swab. Both swab tips were placed in a tube that contained 5.0 mL of diluent. Samples that were expected to lie within the linear range — solutions I and II in the recovery study — were analyzed without modification. For samples that contained higher concentrations of nonoxynol-9 — solutions III and IV in the recovery study — 3.0 mL was diluted to 10.0 mL with diluent before analysis. Solutions III and IV contained a high concentration of nonoxynol-9, representing the concentration expected if nonoxynol-9 were present on the surface at 100% and 150% of the higher acceptable residual limit, respectively. Quantitation of the sample solutions was accomplished using a standard prepared at 40 µg/mL nonoxynol-9.

Swab extractables and specificity: Analysts can choose from many different ways to swab a pharmaceutical surface. One method uses a cotton pledget wetted with recovery solvent and held in the tip of a pair of forceps to wipe the surface. We have observed that chromatographic interference often is reduced when the swab material is cotton. This swabbing procedure can be inconvenient, however, for pharmaceutical technologists who would be suspended in a process tank while wiping the underside of a mixing blade. Fortunately, many companies sell premade swabs that have a plastic applicator with a section of cloth-like material attached to one end. Although easier to use, these swabs occasionally have caused interfering chromatographic peaks in undesirable areas of the sample chromatograms when organic recovery solvents are used.

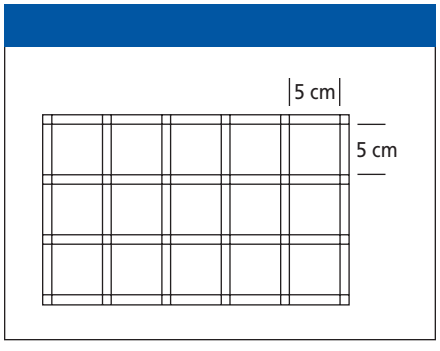

Figure 4: Example of a substrate plate used for swab recovery experiments. Detergent-fortified solutions are deposited within the 5 cm × 5 cm squares and allowed to dry before swabbing.

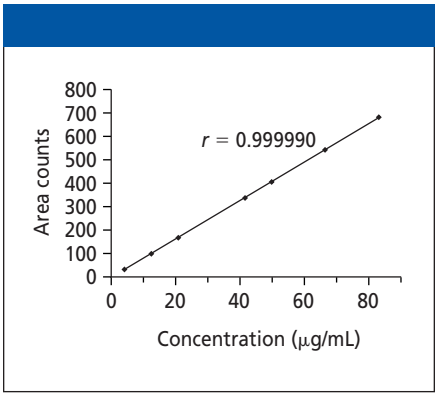

Figure 5: Plot of nonoxynol-9 area counts versus the concentration of nonoxynol-9 in solution.

Our evaluation of the specificity obtained with the 50:50 (v/v) water–acetonitrile solvent demonstrated chromatographic interference for nonoxynol-9 (greater than 0.5% area with respect to the area obtained from a standard at 40 µg/mL). A recovery solvent of 50:50 (v/v) water–methanol yielded acceptable specificity (chromatographic interference approximately 0.1% area with respect to the 40 µg/mL nonoxynol-9 standard; refer to Figure 3). Therefore, we used this diluent as the standard preparation solvent and recovery solvent–diluent in all subsequent experiments. Please note that the small peak appearing in both the diluent and swab blank chromatograms in Figure 3 at the retention time of nonoxynol-9 are attributable to less than 0.2% carryover.

System suitability: Before performing any validation experiments, we made replicate injections of a 40-µg/mL working standard. Then we determined the total peak area caused by nonoxynol-9 and the percent relative standard deviation (RSD, $n = 5$). The RSD of replicate injections always was less than 2.0%, which demonstrated that the method could provide precise results. We observed a consistent chromatographic profile throughout the entire validation.

Linearity: We injected seven linearity standards, prepared to cover a 4.15–82.9 µg/mL range (approximately 10–200% of the 40-µg/mL acceptable residual limit solution) in triplicate. The correlation coefficient of the linear regression plot of the average responses was 0.999990, as Figure 5 demonstrates.

Limits of quantitation and detection: The limit of quantitation was established by injecting the lowest linearity standard solution (4.15 µg/mL) six times onto a suitable HPLC system. We measured the total peak area of the nonoxynol-9 chromatographic peak, including the shoulders, and obtained an RSD of 0.9% for the six injections. We calculated the concentration of a limit-of-detection solution that would yield a signal-to-noise ratio (*S/N*) of approximately 3 based on the observed *S/N* of the limit of quantitation preparation. This concentration was determined to be 0.06225 µg/mL nonoxynol-9. The limit of detection was established by conducting a single analysis of the 0.06225-µg/mL nonoxynol-9 standard. An *S/N* of 4 was measured and recorded.

Method accuracy and method precision: Four nonoxynol-9 solutions (solutions I, II, III, and IV) were prepared at different concentration levels. We deposited and recovered these solutions from each of the three substrates (stainless steel, PTFE, and acrylic). Equal volumes of each of the solutions were deposited evenly on five separate 25-cm^2 areas of each substrate. After drying, we recovered residual nonoxynol-9 using a swab wetted with diluent from a centrifuge tube prefilled with 5.0 mL of diluent. Before the residual diluent could evaporate completely, we repeated the recovery process with a dry swab. The tips of both swabs were cut off, placed in the same centrifuge tube that we used to wet the initial swab, and agitated to extract the nonoxynol-9 from the tips. For solutions I and II, we transferred an aliquot of sample solution to an HPLC vial and analyzed it for nonoxynol-9. To ensure that the final analytical concentrations would lie within the previously qualified linear range, we further diluted 3.0 mL of solutions III and IV to 10.0 mL with diluent before analysis for nonoxynol-9.

As Table I shows, all mean recoveries were greater than 70% and all relative standard deviations were less than 6.0%. The recovery experiment was designed so that the solution concentrations would cover the range of 25% of the lowest solution's acceptable residual limit to 150% of the highest solution's acceptable residual limit, or approximately 10–210 µg/mL nonoxynol-9, in the sample solutions. The volatility of nonoxynol-9 from pharmaceutical surfaces became an issue during the execution of the validation experiments (see the Discussion section below).

Method ruggedness and intermediate precision: A second analyst, working on a different day and a different column, repeated the method accuracy and method precision experiments using only the stainless steel substrate. As Table II shows, the second analyst obtained a minimum recovery of 81% and a maximum RSD of 10%. In addition, the average of the recovery percentages at each concentration level obtained by the second analyst was within 12% of the originating analyst's results on an absolute basis.

Table I: Average percentage of nonoxynol-9 recovered from surfaces by Analyst I*

	Solution I		Solution II		Solution III		Solution IV	
Substrate	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Stainless steel	83	3.1	87	2.3	93	1.4	92	2.9
PTFE	75	4.8	80	5.2	88	5.6	91	2.1
Acrylic	72	1.8	81	4.7	89	3.7	95	3.6

*$n = 5$.

Table II: Comparison of average percentage nonoxynol-9 recovered from stainless steel obtained by Analysts I and II*

Substrate	Solution I	Solution II	Solution III	Solution IV
Analyst I	83	87	93	92
Analyst II†	82 (3.3)	81 (9.7)	104 (2.2)	100 (5.3)
Relative percentage difference	−1.2	−6.9	11.8	8.7

*$n = 5$.
†RSD in parentheses.

Table III: Results of solution stability results

	Standard		Sample	
Time (h)	Concentration (ppm)	Difference from time 0 (%)	Concentration (ppm)	Difference from time 0 (%)
0	41.5	—	40.9	—
24	41.2	−0.7	40.2	−1.7
48	40.9	−1.4	39.9	−2.4
72	41.0	−1.2	40.0	−2.2
144	41.2	−0.7	40.3	−1.5

Solution stability: We prepared a standard solution and a sample solution, including the swab tips, both of which contained nonoxynol-9 at 40 μg/mL and stored them at room temperature. The solutions were periodically sampled and analyzed against a freshly prepared standard solution from time zero until and including 144 h. As Table III shows, the solutions were stable for 144 h when stored at room temperature.

Discussion

The 50:50 (v/v) water–methanol diluent used for samples and standards contained an organic modifier — methanol — that was different from the one used in the mobile phase — acetonitrile. As discussed previously, methanol was used in place of acetonitrile in the diluent because it provided for acceptable specificity with swab extractables. Because the final diluent and the mobile phase had similar solvent strengths, we observed no chromatographic changes compared with the use of a 50:50 (v/v) water–acetonitrile diluent.

The volume of the chromatography column used in this study is quite small; therefore, its manufacturer typically recommends an injection volume of 10 μL or less so that the injection solvent volume will not become too large a portion of the total solvent in the column at injection. However, we found in our studies that an injection volume of 25 μL yielded acceptable chromatography.

During the execution of the method ruggedness experiments, we discovered that both the recovery and reproducibility results obtained for solutions I and II — and, to a lesser extent, solutions III and IV — were adversely affected by the length of time the deposited solutions were allowed to dry. When we used a longer drying time, we obtained lower recoveries and greater relative standard deviations. Nonoxynol-9 is a liquid at room temperature. As the applied nonoxynol-9 solutions dried on the substrate surfaces, an initial wet sheen appeared and was quickly replaced by a thin film. The longer solutions I and II were allowed to dry on the surface of the substrates, the lighter the film became; eventually, the film disappeared completely. When we conducted the recovery immediately after the disappearance of the wet sheen, the recovery percentages were higher and the precision was better (lower RSD) than if the nonoxynol-9 was allowed to remain in contact with the substrate surface for longer periods of time. These experiments indicated that the inherent volatility of nonoxynol-9 could be an issue for accurate recovery determination. We subsequently recommended that samples from manufacturing equipment be obtained promptly after drying the manufacturing equipment.

Conclusion

We developed a nonporous silica, reversed-phase HPLC method in our laboratory to determine residual amounts of the cleaning agent PAC-710 and validated it to measure residual nonoxynol-9 from stainless steel, PTFE, and acrylic pharmaceutical surfaces. The chromatography obtained with the nonporous silica column is qualitatively similar to that obtained with the standard *USP* method, but the run time is one-fifth the length of the *USP* method's. The nonporous silica column provided consistent and reproducible chromatography with this method for more than 800 injections, even though the column also was used to evaluate other chromatographic systems with pH 1–12 mobile phases. Because the method uses common laboratory reagents and HPLC equipment and columns, the method is suitable for routine analysis.

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