Several tricyclic compounds and benzodiazepine derivatives were chromatographed in the selection of the internal standard necessary for the quantitation of amitriptyline hydrochloride and chlordiazepoxide, but a stable material with a suitable retention time was not found. Sulfanilamide eluted before amitriptyline hydrochloride and chlordiazepoxide and was well separated from their respective impurities, so it was chosen as the internal standard. Separation of the compounds including sulfanilamide is shown in Fig. 1, and the calculated chromatographic characteristics are listed in Table I.

Linearity—A plot of peak height *versus* the amount of the two components injected was linear between 0.3 and 11 μg of amitriptyline hydrochloride and 0.13 and 4 μg of chlordiazepoxide. The working concentration was ~5.7 μg of amitriptyline hydrochloride and 2 μg of chlordiazepoxide for the content uniformity test and assay. For dissolution measurements, the working concentration was 0.6 μg of amitriptyline hydrochloride and 0.2 μg of chlordiazepoxide.

Precision—Chromatographic system reproducibility was determined by injecting six 20-μl aliquots of a sample solution containing 0.1 mg of internal standard/ml, 0.3 mg of amitriptyline hydrochloride/ml, and 0.1 mg of chlordiazepoxide/ml as well as corresponding amounts of the reference standards. The relative standard deviation was ±0.16 and ±0.18% for amitriptyline hydrochloride and chlordiazepoxide, respectively. Six replicate samples of composite tablet mass also were assayed. The relative standard deviations were 0.96% for amitriptyline hydrochloride and 0.36% for chlordiazepoxide.

Recovery—A known amount of both active components was added to an assayed sample, and 97.7% of the amitriptyline hydrochloride and 100.2% of the chlordiazepoxide were recovered.

Figure 2 shows a typical chromatogram of the tablet assay. No interference due to a placebo was found. The completeness of the extraction of the tablet mass during sample preparation was checked; no active components remained in the tablet mass after filtering. Good agreement was obtained between the HPLC method and the spectrophotometric procedures, *i.e.*, measurements of the bromcresol green complex of amitriptyline hydrochloride and of the diazotization and coupling product of chlordiazepoxide (Table II).

For the dissolution test, external rather than internal standards were used since, at low concentrations, the sensitivity had to be increased and the blank interfered in the region of the internal standard. A typical chromatogram is shown in Fig. 3.

The described procedure subsequently was used for experimental tablet and capsule formulations containing only amitriptyline hydrochloride. The assay and content uniformity results correlated well with the data obtained using the pharmacopeial (5) procedures.

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Validation of Washing Procedures for Maintaining a Microbiologically Clean Gel Filtration Column

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Abstract □ Chromatographic gel filtration matrixes used in various separation techniques are subject to microbial contamination. The need for a microbe-free column is critical when preparing materials that require a low or zero microbial count. This report proposes two alternative washing systems: 0.02 *N* HCl containing 0.81% NaCl, and 0.1 *M* tromethamine–hydrochloride buffer (pH 7.0) containing 0.81% NaCl and 0.02% thimerosal. These washing systems were validated using a 100 × 2.6-cm column packed with a modified dextran gel slurry previously inoculated with known counts of USP test organisms. After each wash, the column separation characteristics were verified further with appropriate test proteins.

Keyphrases □ Chromatography—gel filtration, washing procedures for columns contaminated with microorganisms □ Contamination—microorganisms in gel filtration columns, washing procedures for column disinfection

Chromatographic column packing materials such as gel filtration and ion-exchange matrixes used in separation and the buffer systems usually employed with such matrixes are subject to microbial contamination and proliferation. Excessive microbial growth obstructs the flow, affects the chromatographic properties of these columns, and contaminates material that is affected adversely by microorganisms.

Gel filtration matrixes can be sterilized in the wet or dry state by autoclaving (1, 2), but such techniques are not feasible for a working column. Gaseous sterilization with formaldehyde or ethylene oxide is ineffective and unsafe due to the limited sterilant penetration and the residual toxic effects (2, 3). Organic solvents such as chloroform and toluene are incompatible with the matrixes and some column components. Several antimicrobial agents are compatible with the column packing matrixes. Thimerosal is one of the most commonly used preservatives because of its highly biostatic effect (2, 4). Its routine use as an applicable and effective antimicrobial agent in chromatographic columns has to be evaluated.

The present investigation was undertaken to develop effective methods to minimize or eliminate microbial contamination in a gel filtration column and to validate the antimicrobial effectiveness and applicability of these methods.

EXPERIMENTAL

Microbial Inoculum Suspension Preparation—The bacterial cultures, *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC

0022-3549/80/0200-0140$01.00/0

8739), and *Pseudomonas aeruginosa* (ATCC 9027), were grown for 24 hr at 35° on soybean–casein digest agar[1] slants. The yeast culture, *Candida albicans* (ATCC 10231), was grown for 48 hr at 23° on Sabouraud dextrose agar[2] slants. On the slurry inoculation day, individual culture suspensions were prepared in 0.9% NaCl[3] contained in sterile colorimeter tubes (150 × 15 mm). Each culture was scraped aseptically with a loop from the agar slants and carefully emulsified in colorimeter tubes until the uniform turbidity, as determined at 420 nm[4], equaled an approximate viability count of 1–5 × 10^8 organisms/ml of suspension.

The bacterial culture, *Bacillus subtilis* (ATCC 9372), and the mold culture, *Aspergillus niger* (ATCC 16404), were prepared as spore suspensions in 0.9% NaCl solution, with the later suspension containing 0.05% polysorbate 80[5]. These spore suspensions had viability counts of ~1–5 × 10^8 organisms/ml.

Quantitative Determination of Microbial Viability—The individual inoculum suspensions and the initially inoculated samples of slurry and effluents were diluted serially in 10-fold increments to 10^{-9} in pH 7.2 phosphate buffer[6]. All other samples of effluents and slurry were similarly diluted to 10^{-5}. Then, in duplicate, 1.0 ml of each diluted sample was placed in sterile plastic petri dishes (150 × 25 mm) with a sterile glass serological pipet (1 in 1/100 ml). In addition, 1.0- and 0.1-ml quantities of each undiluted sample were placed aseptically in plastic petri dishes with glass serological pipets (1 in 1/100 ml and 1/10 in 1/100 ml, respectively).

For samples without thimerosal[7], Sabouraud dextrose agar was added to one set of plates to enumerate molds or yeasts, and soybean–casein digest agar was added to the other set of plates to enumerate bacteria. In the samples containing thimerosal, the appropriate media were supplemented with sodium thioglycolate[8] (500 mg/liter). After incubation of the soybean–casein digest agar plates for 3–5 days at 35° and the Sabouraud dextrose agar plates at room temperature for 7–11 days, colony-forming units were counted.

Qualitative Determination of Microbial Viability—To monitor the viability of *S. aureus*, *E. coli*, and *P. aeruginosa*, 0.1 ml of each sample was placed onto mannitol salt[9], MacConkey[10], and cetrimide[11] agar plates, which were incubated for ≥3 days at 35° and checked by Gram-stain or appearance for characteristic growth. In addition, the viability count plates were checked for colonies characteristic of each inoculated contaminant.

pH Determination—The pH of each sample was determined with a digital pH meter[12].

Mercury Determination—To monitor mercury as thimerosal in the effluents and slurries treated with the tromethamine–thimerosal wash solution, flameless atomic absorption spectroscopy was used.

Reagent blanks were prepared by pipetting water, 3 ml, into 300-ml bottles[13]. A working standard solution of 0.5 ppm of mercury was prepared by diluting a 1000-ppm mercury standard solution. Then 1.0, 2.0, and 3.0 ml of the working standard were pipetted into one of three 300-ml bottles[13].

Each sample from the tromethamine–thimerosal wash solution was acidified with an equal volume of concentrated nitric acid to kill any microorganisms. All samples were stored at 5° prior to the atomic absorption analyses. At that time, each sample was diluted with double-distilled water to obtain ~1 ppm of mercury, and 1-ml samples were pipetted into 300-ml bottles[13].

To each blank, standard, and sample solution were added 5 ml of 35% HNO_3, 5 ml of 50% (v/v) H_2SO_4, and 5 drops of 5.0% potassium permanganate to produce a purple color. After standing for 30 min, each preparation was diluted to volume (100 ml) with distilled water. Hydroxylamine hydrochloride (1.5%), 5 ml, was added to decolorize each solution, the solution was treated with 5 ml of 10.0% stannous chloride, and the flask was stoppered immediately with the aspirator tube of the instrument[14]. The enclosed atmosphere of each solution was circulated through the apparatus drying train using the electric pump. The maximum absorbance was recorded when the mercury vapor concentration reached an equilibrium value. Between runs of each solution, the vapor was circulated through the mercury scrubber to clean the system.

The average blank absorbance was subtracted from the standard and sample absorbances. The quantity of mercury in each diluted sample was read from a calibration curve prepared by plotting the mercury quantities in the standards in micrograms against the corrected standard absorbances. The mercury concentration in the original sample was calculated by:

$$\text{concentration (ppm) of mercury} = \frac{C \times D}{S} \qquad \text{(Eq. 1)}$$

where C is the amount of mercury (parts per million) in 1 ml of diluted sample, D is the dilution factor, and S is the amount of sample (milliliters).

Antimicrobial Activity Determination of Acid Wash Solution—Dry modified polymeric dextran gel[15] (~50 g) was added to ~1500 ml of sterile distilled water. After 3 days of equilibration at room temperature, the distilled water in the slurry was decanted and replaced with sterile 0.81% NaCl. Following an additional 2-day equilibration at room temperature, the supernate was decanted, leaving ~1000 ml of slurry. This slurry was inoculated with either 10 ml of each inoculum suspension (*S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*) or 10 ml of each spore suspension (*A. niger* and *B. subtilis*).

Over 24 hr, the inoculated slurry was packed by gravity into the column[16] to a height of ~95 cm. The column was maintained at 5° by circulating controlled-temperature water through the column jacket. The slurry and column effluent were sampled immediately and tested for microbial count and pH.

The packed column, continuously maintained at 5°, was washed with ~3–4 liters of the sterile, filtered, pH 1.8 acid wash solution at a flow rate of ~0.8 ml/min. Column effluent samples were collected in sterile screw-capped test tubes (150 × 20 mm) after 1 and 4 days to determine the microbial count and pH. The remaining effluent was pooled for determining the total acid wash elution volume.

After this acid wash treatment, the column was rinsed by washing with 1.5–2.5 liters of 0.1 *M* tromethamine–hydrochloride buffer (pH 7.0) containing 0.81% NaCl and 0.02% sodium azide (rinsing buffer) at the same flow rate.

After 1 and 2 days of rinsing, the column effluent samples were collected in sterile screw-capped test tubes for microbial counting and pH determination. The remaining effluent was pooled for determination of the total rinsing buffer elution volume. Five days after the rinse termination, a sample of the effluent was aseptically collected for microbial counting and pH determination. The slurry was removed immediately from the column and sampled aseptically from the top, middle, and bottom for the microbial count and pH determination.

Antimicrobial Activity Determination of Tromethamine–Thimerosal Wash Solution—The described washing and rinsing protocol and the time schedule were followed in using the tromethamine–thimerosal wash solution to clean the inoculated columns. In addition to the microbial count and pH determination, column effluents and slurry were assayed for mercury by flameless atomic absorption spectroscopy to monitor the thimerosal concentration.

Separation Properties of Columns Treated with Acid and Tromethamine–Thimerosal Wash Solution—Two milligrams each of cytochrome c[17], bovine serum albumin[18], and a dextran indicator[19] were added separately to 2-ml aliquots of the rinsing buffer. The test solution was prepared by combining 1.0 ml of each of these solutions. This test solution was fractionated on the same uninoculated column after each of the following procedures: (*a*) rinsed and equilibrated with ~2 liters of rinsing buffer over 24 hr (control run); (*b*) rinsed and equilibrated with

[1] Bacto-tryptic soy agar, Difco Laboratories, Detroit, Mich.
[2] Bacto-Sabouraud dextrose agar, Difco Laboratories, Detroit, Mich.
[3] Saline TS, USP XIX.
[4] Spectronic-20 colorimeter, Bausch & Lomb, Rochester, N.Y.
[5] Saline TS, USP XIX, as specified for preparation of mold spore suspensions.
[6] Butterfield's phosphate buffer.
[7] Ethylmercurithiosalicylic acid sodium salt, item 11228, Eastman Kodak Co., Rochester, N.Y.
[8] Difco Laboratories, Detroit, Mich.
[9] BBL Division, Becton, Dickinson & Co., Cockeysville, Md.
[10] Bacto-MacConkey agar, Difco Laboratories, Detroit, Mich.
[11] Pseudosel agar, BBL Division, Becton, Dickinson & Co., Cockeysville, Md.
[12] Corning digital 112 research pH meter, Corning Scientific Instruments, Medfield, Mass.
[13] Biological oxygen demand bottles.
[14] Model AA5, Techtron atomic absorption spectrophotometer, Varian, Palo Alto, Calif. The instrument conditions include a mercury tube and the following settings: wavelength, 253.65 nm; current, 3.5 mamp; and slit, 100 μm. The absorption cell was obtained from Perkin-Elmer Corp., Instrument Division, Norwalk, Conn. A mercury analysis kit, model 303-0830, with a drying train was also used.
[15] Sephadex G-100, Pharmacia Fine Chemicals, Piscataway, N.J.
[16] Column type K26/100 (100 × 2.6 cm i.d.), Pharmacia Fine Chemicals, Piscataway, N.J.
[17] Type VI, from horse heart, 95–100%, Item C-7752, Sigma Chemical Co., St. Louis, Mo.
[18] Crystallized and lyophilized, item A-4378, Sigma Chemical Co., St. Louis, Mo.
[19] Blue dextran 2000, Pharmacia Fine Chemicals, Piscataway, N.J.

Table I—Acid Wash Treatment of a Column Slurry Contaminated with Nonspore-Forming Bacteria and Yeast

Sampling Time, days	Column Eluting Solution Used between Sampling	Total Effluent Volume between Sampling, ml	Sample Analyses: Nature of Sample	pH	Microorganism Count per Milliliter: Total Bacteria	Yeast
0	Acid wash solution	0	Slurry	6.0	8.0×10^6	5.2×10^6
			Effluent	6.7	5.0×10^6	3.0×10^6
1	Acid wash solution	800	Effluent	1.8	0	1.8×10^4
4	Rinsing buffer	2325	Effluent	1.8	0	1.1×10^2
5	Rinsing buffer	640	Effluent	7.0	0	4.2×10^1
6	Rinsing buffer[a]	575	Effluent	7.0	0	2.2×10^1
11	Rinsing buffer[a]	0	Effluent	7.0	0	3.5×10^3
			Slurry from top	7.0	0	1.8×10^2
			Slurry from middle	7.0	0	6.0×10^2
			Slurry from bottom	7.0	0	2.2×10^4

[a] Standing in the slurry.

Table II—Acid Wash Treatment of a Column Slurry Contaminated with Bacterial and Mold Spores

Sampling Time, days	Column Eluting Solution Used between Sampling	Total Effluent Volume between Sampling, ml	Sample Analyses: Nature of Sample	pH	Microorganism Count per Milliliter: Bacteria Spores	Mold Spores
0	Acid wash solution	0	Slurry	6.4	2.2×10^6	3.0×10^6
			Effluent	6.3	1.9×10^6	1.6×10^4
1	Acid wash solution	1000	Effluent	1.9	2.1×10^2	3.0×10^0
4	Rinsing buffer	2150	Effluent	1.8	3.7×10^1	4.0×10^0
5	Rinsing buffer	1130	Effluent	7.0	2.3×10^2	8.0×10^1
6	Rinsing buffer[a]	930	Effluent	7.0	8.0×10^1	4.0×10^1
11	Rinsing buffer[a]	0	Effluent	7.1	1.6×10^4	8.5×10^2
			Slurry from top	7.1	3.8×10^5	8.0×10^5
			Slurry from middle	7.0	3.8×10^5	1.2×10^6
			Slurry from bottom	7.0	3.8×10^5	1.4×10^6

[a] Standing in the slurry.

~2 liters of rinsing buffer and treated with ~1 liter of the acid wash solution over 24 hr followed by rinsing and equilibration with ~2 liters of rinsing buffer over 48 hr (acid wash run); and (c) rinsed and reequilibrated with ~2 liters of rinsing buffer and treated with ~1 liter of the tromethamine–thimerosal wash solution over 24 hr followed by rinsing and equilibration with ~2 liters of rinsing buffer over 48 hr (tromethamine–thimerosal wash run). These solutions were allowed to flow through the slurry in the column by gravity at 0.8 ml/min.

After each of the three column treatments, the test solution was added to the column and was allowed to flow ~2.5 cm into the slurry before initiation of the rinsing buffer eluent, which flowed by gravity at a similar rate. Six-milliliter fractions were collected[20], and each fraction was monitored visually and/or spectrophotometrically[21] for components of the test solution.

The column void volume was estimated from the elution volume of the blue dextran, which was detected spectrophotometrically by its absorbance at 625 nm and visually by its blue color. The elution volume of cytochrome c was determined spectrophotometrically from its absorbance at 407 nm and visually by its red color. The elution volume of bovine serum albumin was determined by its absorbance at 280 nm. Then the relative elution volumes were calculated after each run by dividing the elution volume of each test protein, V_e, by the respective void volume, V_0, for each respective run. In addition, the Ouchterlony double-diffusion technique was used to confirm the separation of bovine serum albumin from cytochrome c.

Ouchterlony Double-Diffusion Technique—In the Ouchterlony technique, 50 μl from each of the three fractions of the bovine serum albumin peak was added to each of three outer wells of an Ouchterlony plate[22]. Fifty microliters from each of the two fractions of the cytochrome c peak was added to each of two additional outer wells on the same Ouchterlony plate. Fifty microliters of a control solution (2 mg of bovine serum albumin/ml) was added to the sixth outer well of the Ouchterlony plate. Fifty microliters of rabbit antibovine serum albumin[23] (2 mg/ml) was added to the center well of the plate, which was incubated for 24 hr in a moisture chamber at room temperature and subsequently examined for precipitin bands.

[20] LKB ultrorac fraction collector, instrument group 7000, LKB-Produktor AB, S-16125, Bromma 1, Sweden.

[21] Model 25 spectrophotometer, Beckman Instruments, Fullerton, Calif.

[22] IDF cell 1, Cordis Laboratories, Miami, Fla.

[23] Antibovine serum albumin (rabbit), code 65-111, Miles–Yeda Ltd.; distributed by Research Division, Miles Laboratories, Kankakee, Ill.

RESULTS

Validation of Antimicrobial Activity of Column Wash Solutions—The results indicate that both the acid wash and the tromethamine–thimerosal wash solutions are capable of eliminating microbial contamination from the gel filtration slurry packed into a column maintained at 5°. The acid wash was more effective than the tromethamine–thimerosal wash solution against both the nonspore-forming bacteria and the spores of *B. subtilis* (Tables I–IV). The acid wash solution was extremely active against the nonspore-forming bacteria since the combined bacterial count of *S. aureus* (Gram-positive cocci), *E. coli*, and *P. aeruginosa* (both Gram-negative bacilli) was reduced from 5.0×10^6 bacteria/ml of effluent to zero in 1 treatment day (Table I).

In comparison, the combined viability count for a mixture of the same three nonspore-forming bacteria was reduced from 3.8×10^6 to 1.6×10^3 organisms/ml of effluent sample following 4 days of treatment with the tromethamine–thimerosal wash (Table III). Visual (qualitative) examination of the viability count plates indicated that, after 4 days of treatment, *P. aeruginosa* was eliminated completely, that *E. coli* contamination was reduced significantly, and that most of the remaining nonspore-forming contamination was *S. aureus*.

The acid wash produced almost a 100,000-fold reduction in the number of *B. subtilis* spores per milliliter of effluent with 4 days of treatment (Table II). On the other hand, only a 1000-fold reduction in the number of *B. subtilis* spores per milliliter of effluent was produced with 4 days of treatment with the tromethamine–thimerosal wash solution (Table IV).

The tromethamine–thimerosal wash solution was more effective than the acid wash solution against yeast and the *A. niger* mold spores. *A. niger* spores, initially at concentrations of 1.5×10^6/ml of slurry and of 1.5×10^4/ml of effluent, were reduced to zero per milliliter of effluent with 1 day of treatment with the tromethamine–thimerosal wash solution (Table IV). This reduction was permanent since no mold spores were recovered from the treated slurry. Similarly, this same wash produced a 100,000-fold reduction in the *C. albicans* count per milliliter of effluent (Table III).

Table III—Tromethamine–Thimerosal Wash Treatment of a Column Slurry Contaminated with Nonspore-Forming Bacteria and Yeast

Sampling Time, days	Column Eluting Solution Used between Sampling	Total Effluent Volume between Sampling, ml	Sample Analyses				
			Nature of Sample	pH	Mercury Content, %	Microorganism Count per Milliliter	
						Total Bacteria	Yeast
0	Tromethamine–thimerosal wash solution	0	Slurry	6.6	—[a]	3.8×10^6	3.5×10^6
			Effluent	6.4	—[a]	3.8×10^6	2.4×10^6
1	Tromethamine–thimerosal wash solution	1000	Effluent	7.0	0.01	3.8×10^4	4.0×10^3
4	Rinsing buffer	2720	Effluent	7.1	0.01	1.6×10^3	3.5×10^1
5	Rinsing buffer	1000	Effluent	7.0	—[a]	7.6×10^2	1.0×10^1
6	Rinsing buffer[b]	1560	Effluent	7.0	—[a]	6.0×10^2	0
11	Rinsing buffer[b]	0	Effluent	7.0	—[a]	1.2×10^3	0
			Slurry from top	7.0	—[a]	1.6×10^3	0
			Slurry from middle	7.0	—[a]	3.2×10^3	0
			Slurry from bottom	7.0	—[a]	3.2×10^4	0

[a] Negligible mercury content. [b] Standing in the slurry.

Table IV—Tromethamine–Thimerosal Wash Treatment of a Column Slurry Contaminated with Bacterial and Mold Spores

Sampling Time, days	Column Eluting Solution Used between Sampling	Total Effluent Volume between Sampling, ml	Sample Analyses				
			Nature of Sample	pH	Mercury Content, %	Microorganism Count per Milliliter	
						Bacteria Spores	Mold Spores
0	Tromethamine–thimerosal wash solution	0	Slurry	6.4	—[a]	1.5×10^6	1.5×10^6
			Effluent	6.5	—[a]	2.0×10^6	1.5×10^4
1	Tromethamine–thimerosal wash solution	825	Effluent	7.0	0.01	5.5×10^3	0
4	Rinsing buffer	2250	Effluent	7.0	0.01	2.4×10^3	0
5	Rinsing buffer	1000	Effluent	7.0	—[a]	1.2×10^3	0
6	Rinsing buffer[b]	1150	Effluent	7.0	—[a]	8.0×10^2	0
11	Rinsing buffer[b]	0	Effluent	6.8	—[a]	9.0×10^4	0
			Slurry from top	6.8	—[a]	3.5×10^5	0
			Slurry from middle	6.8	—[a]	5.6×10^5	0

[a] Negligible mercury content. [b] Standing in the slurry.

Table V—Relative Elution Volumes of the Test Solution Components after Each Run

Test Solution Components	Relative Elution Volumes (V_e/V_0)[a]		
	Control Run	Acid Wash Run	Tromethamine–Thimerosal Wash Run
Bovine serum albumin[b]	1.04	1.05	1.00
Cytochrome c[b]	2.07	2.56	2.60

[a] The column void volume (V_0) was determined using blue dextran; V_e is the test protein elution volume. [b] Fractions were verified further by the Ouchterlony double-diffusion technique.

At the conclusion of this experiment, no viable *C. albicans* cells were recovered from the slurry (Table III).

The acid wash solution produced a less dramatic reduction in the number of yeast and mold spores. The yeast spore count per milliliter of effluent was reduced 10,000-fold with 4 days of treatment, although the count increased after the treatment ended (Table I). The mold spore count per milliliter of effluent was reduced almost 10,000-fold with 1 day of treatment, and as with the *C. albicans*, the number of colony-forming units of *A. niger* increased following termination of the 4-day flow of the acid wash solution (Table II).

Each wash solution was eliminated from the column within ~24 hr by continuous elution with the rinsing buffer, as shown by the pH or mercury determination (Tables I–IV). Only a trace of mercury was detected in the gel slurry after rinsing the column previously treated with the tromethamine–thimerosal wash (Tables III and IV).

Validation of Separation Properties of Treated Column—The test protein elution patterns (bovine serum albumin and cytochrome c) obtained from the control run, the acid wash run, and the tromethamine–thimerosal run showed no significant differences, indicating that the column separation properties were not affected by either washing procedure. The relative elution volumes of the test proteins after both the acid wash run and the tromethamine–thimerosal wash run were similar to the relative elution volumes obtained after the control run (Table V). In addition, the Ouchterlony double-diffusion technique verified that there was no cross-contamination of the cytochrome c fraction with bovine serum albumin.

DISCUSSION

Selection of Suitable Wash Solutions—Several disinfectant solutions other than the tromethamine–thimerosal and the acid wash solutions were considered, but they were not studied because of their undesirable characteristics. Formaldehyde (3–8%) and glutaraldehyde (2%) were reported to be sporocidal (4), but formaldehyde is corrosive and damaging to the plastic parts of the column equipment, and glutaraldehyde is expensive and unstable upon storage (4). Other substances, such as chloroform, butanol, and toluene, cause the gel particles of certain gel filtration matrixes to shrink (2), are effective only at high concentrations (2, 5), and are incompatible with the plastic parts of the column equipment (2).

Thimerosal, a well-known antimicrobial agent (4), commonly is used as a preservative in multiple-dose containers and in topical preparations (4, 6). Furthermore, it is compatible with gel filtration and cation-exchange matrixes (2, 5). Dilute hydrochloric acid was examined since its acidity is expected to be antimicrobial and since gel filtration matrixes can be exposed to 0.02 *N* HCl for at least 6 months without deleterious effects (2). The 0.02% sodium azide included in the rinsing buffer does not interact with proteins or carbohydrates or affect their chromatographic behavior (2). The inclusion of isotonic sodium chloride concentrations in the wash solutions and rinsing buffer keeps the ionic strengths constant to avoid gel volume shrinkage or swelling upon changes in the eluents.

Antimicrobial Activity Determination and Monitoring of Wash Solution Removal—The washing procedures were examined under working conditions in a typical gel filtration column maintained at 5°. To investigate their antimicrobial effectiveness, the gel slurry was inoculated with high counts of the five test microorganisms recommended by USP XIX for antimicrobial effectiveness testing (7). To ensure meaningful results with the tromethamine–thimerosal wash, the static effects of mercury (7, 8) were neutralized by the addition of sodium

thioglycolate to a final concentration of 500 mg/liter (7) to all culture media possibly containing thimerosal. To substantiate the microbial counts and pH or mercury concentrations of effluent samples taken at the end of each wash and rinse cycle, column slurry samples were also analyzed at the conclusion of each antimicrobial effectiveness test.

To monitor the acid wash removal, pH determination was the most appropriate method. Since thimerosal contains ~50% mercury by weight (9), the tromethamine–thimerosal wash removal was monitored by cold vapor, flameless atomic absorption spectroscopy (10).

Washing Solution Effects on Treated Column Separation Properties—The proteins cytochrome c and bovine serum albumin were selected because, based on their respective molecular weights of 12,400 and 67,000, they can be readily fractionated by the slurry used in this investigation (2). Each protein, as well as the blue dextran void volume indicator, was readily assayed spectrophotometrically. In addition, the Ouchterlony double-diffusion procedure, based on a literature method (11), confirmed the clear separation of bovine serum albumin and cytochrome c in the control column and in treated columns.

It is feasible to apply both wash solutions, 0.02 *N* HCl containing 0.81% NaCl and the 0.1 *M* tromethamine–hydrochloride buffer (pH 7.0) containing 0.81% NaCl and 0.02% thimerosal, for disinfecting a modified gel filtration slurry under typical working conditions. Both wash solutions demonstrate antimicrobial activity against high concentrations of various microorganisms in the gel slurry packed into a column maintained at 5°.

Since the acid wash is more effective against nonspore-forming bacteria, including the more resistant species *P. aeruginosa* (4), and since the tromethamine–thimerosal wash is more active against mold and yeast, both wash solutions can be used routinely on an alternating basis or as needed. The wash solutions cannot be used in combination, however, since thimerosal is unstable in acidic solutions (4). In addition, both wash solutions can be removed from the slurry within 24 hr with no more than 1 liter of the rinsing buffer. Both wash solutions do not adversely affect the matrix separation properties and should be compatible with similar column packing materials.

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High-Performance Liquid Chromatographic Analysis of Trimethoprim and Sulfamethoxazole in Dosage Forms

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Abstract □ A rapid, sensitive, and automatable high-performance liquid chromatographic method is presented for the determination of sulfamethoxazole, trimethoprim, and a preservative in dosage forms in the presence of excipients and degradation products.

Keyphrases □ Trimethoprim—simultaneous analysis with sulfamethoxazole and methylparaben, high-performance liquid chromatography, oral suspension and solid dosage forms □ Sulfamethoxazole—simultaneous analysis with trimethoprim and methylparaben, high-performance liquid chromatography, oral suspension and solid dosage forms □ Antibacterial agents—trimethoprim and sulfamethoxazole, simultaneous high-performance liquid chromatographic analysis, oral suspension and solid dosage forms

The antibacterials sulfamethoxazole and trimethoprim are the active ingredients in several oral suspension and solid dosage forms. The official analyses of their dosage forms are spectrophotometric methods following extraction (1, 2). These methods are time consuming and relatively difficult. A high-performance liquid chromatographic (HPLC) method, presented for the analysis of various sulfonamides in combination with trimethoprim, does not separate the various degradation products and the active components and cannot be used as a stability-indicating assay (3).

This study was undertaken to establish a rapid, quantitative, and stability-indicating procedure for routine quality control testing of trimethoprim and sulfamethoxazole in dosage forms. Methylparaben, a commonly used preservative, also may be determined in the analysis of the oral suspension.

EXPERIMENTAL

Apparatus—A high-pressure liquid chromatograph[1] with a 254-nm detector and a stainless steel column (30 cm × 4 mm i.d.) was used. The column packing[2] was porous silica particles with an octadecylsilane-bonded coating. The system was operated at 2.0 ml/min with a column pressure of ~1800 psi for solid dosage forms and at 3.0 ml/min with a column pressure of ~2800 psi for oral suspensions.

[1] Model 6000A pump and 440 detector, Waters Associates, Milford, Mass.
[2] μBondapak C_{18}, Waters Associates, Milford, Mass.

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