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⟨341⟩ ANTIMICROBIAL AGENTS—CONTENT

An essential component of Injections preserved in multiple-dose containers is the antimicrobial agent or antimicrobial agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopeial requirement that the presence and amount added of such antimicrobial agent(s) be declared on the label of the container. This general chapter provides methods for the most commonly used antimicrobial agents. These methods or other suitably validated methods are to be used to demonstrate that the declared antimicrobial agent is present but does not exceed the labeled amount by more than 20%.

The concentration of an antimicrobial preservative added to a multiple-dose or single-dose parenteral, otic, nasal, and ophthalmic preparation may diminish during the shelf life of the product. Therefore, the manufacturer shall determine the lowest level at which the preservative is effective, and the product should be so formulated as to assure that this level is exceeded throughout the product's shelf life. At the time of its manufacture, the product should contain the declared amount of antimicrobial preservative (within ±20% to allow for manufacturing and analytical variations). The quantitative label statement of the preservative content is not intended to mean that the labeled quantity is retained during the shelf life of the product; rather, it is a statement of the amount added, within process limits, and which is not exceeded by more than 20%. An example of such a label statement is "____(unit) added as preservative". [Note—"____(unit)" would be a number followed by the unit of measurement, e.g., 0.015 mg/mL or 0.1%.]

The most commonly used antimicrobial agents include benzyl alcohol; chlorobutanol; phenol; the four homologous esters of *p*-hydroxybenzoic acid (methyl, ethyl, propyl, and butyl parabens); and the two mercurials, phenylmercuric nitrate and thimerosal. The method used for phenylmercuric nitrate is polarographic, whereas quantitative liquid chromatography was used for thimerosal and the four homologous esters of *p*-hydroxybenzoic acid. Gas chromatography is used in the determination of phenol, benzyl alcohol, and chlorobutanol.

GENERAL GAS AND LIQUID CHROMATOGRAPHIC METHODS

The general gas chromatography procedures set forth in the following paragraphs are applicable to the quantitative determination of benzyl alcohol, chlorobutanol, and phenol. Prepare the *Internal standard solution* and the *Standard solution* for each antimicrobial agent as directed below. Unless otherwise directed by the individual monograph, prepare the *Sample solution* from accurately measured portions of the sample under test and the *Internal standard solution* such that the concentration of the antimicrobial agent and the composition of the solvent correspond closely to the concentration and composition of the *Standard solution*. Suggested operating parameters of the gas chromatograph are provided in this section.

The general high-pressure liquid chromatography (HPLC) procedures set forth in the following paragraphs are applicable to the quantitative determination of parabens and *Thimerosal*. Prepare the *Internal standard solution* and the *Standard solution* for each antimicrobial agent as directed below. Unless otherwise directed, prepare the *Sample solution* from accurately measured portions of the sample under test and the *Internal standard solution*, if applicable, such that the concentration of the antimicrobial agent and the composition of the solvent is about the same as the concentration and composition of the *Standard solution*. Suggested operating parameters of the liquid chromatograph are provided in this section.

- **Benzyl Alcohol**

Diluent: Methanol and water (20:80)

Internal standard solution: 3.8 mg/mL of phenol prepared as follows. Dissolve a suitable amount of phenol in 10% of the flask volume of methanol, and dilute with water to volume.

Standard solution: 1.8 mg/mL of USP Benzyl Alcohol RS and 1.5 mg/mL of phenol prepared as follows. Dissolve 180 mg of USP Benzyl Alcohol RS in 20 mL of methanol contained in a 100-mL volumetric flask. Add 40.0 mL of *Internal standard solution*, and dilute with water to volume.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 30-m × 0.32-mm fused-silica; bonded with a 0.5-µm film of phase G16

Temperatures

Injection port: 200°

Detector: 310°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	0	150	5
150	10	230	7

Carrier gas: Helium

Flow rate (constant): 2 mL/min

Injection volume: 1 µL

Split ratio: 10:1

Run time: 20 min

System suitability

Sample: *Standard solution*

[Note—The relative retention times for benzyl alcohol and phenol are about 1.0 and 1.25, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the benzyl alcohol and phenol peaks

Tailing factor: NMT 2.0 for the benzyl alcohol peak

Relative standard deviation: NMT 2.0% for the peak response ratio of benzyl alcohol to phenol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of benzyl alcohol (C_7H_8O) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of benzyl alcohol to phenol from the *Sample solution*

R_S = peak response ratio of benzyl alcohol to phenol from the *Standard solution*

C_S = concentration of USP Benzyl Alcohol RS in the *Standard solution*

C_U = nominal concentration of benzyl alcohol in the *Sample solution*

- **Chlorobutanol**

Diluent: Methanol and water (50:50)

Internal standard solution: 10 mg/mL of 2,2,2-trichloroethanol in *Diluent*

Standard stock solution: 5 mg/mL of USP Chlorobutanol RS in methanol

Standard solution: 1.25 mg/mL of USP Chlorobutanol RS and 2 mg/mL of 2,2,2-trichloroethanol prepared as follows. Transfer 2.5 mL of *Standard stock solution*, 2.0 mL of *Internal standard solution*, and 0.5 mL of methanol to a 10-mL volumetric flask. Dilute with water to volume.

Sample stock solution: Quantitatively dilute, if necessary, an accurately measured volume corresponding to 2.5 mg/mL of chlorobutanol in water.

Sample solution: Combine 5.0 mL of *Sample stock solution* with 2.0 mL of *Internal standard solution* in a 10-mL volumetric flask, and dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 30-m × 0.32-mm fused-silica; bonded with a 0.25-µm film of phase G16

Temperatures

Injection port: 260°

Detector: 280°

Column: 135°

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 0.5 µL

Split ratio: 10:1

Run time: 12 min

System suitability

Sample: *Standard solution*

[Note—The relative retention times for chlorobutanol and 2,2,2-trichloroethanol are about 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 2.0 between chlorobutanol and 2,2,2-trichloroethanol

Relative standard deviation: NMT 1.0% for the peak response ratio of chlorobutanol to 2,2,2-trichloroethanol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of chlorobutanol ($C_4H_7Cl_3O$), on the anhydrous basis, in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of chlorobutanol to 2,2,2-trichloroethanol from the *Sample solution*

R_S = peak response ratio of chlorobutanol to 2,2,2-trichloroethanol from the *Standard solution*

C_S = concentration of USP Chlorobutanol RS in the *Standard solution*

C_U = nominal concentration of chlorobutanol in the *Sample solution*

- **Phenol**

Internal standard solution: 2 mg/mL of USP Benzyl Alcohol RS in methanol

Standard stock solution: 4 mg/mL of USP Phenol RS in water

Standard solution: 0.4 mg/mL each of USP Phenol RS and USP Benzyl Alcohol RS prepared as follows. Combine 5.0 mL of *Standard stock solution* with 10.0 mL of *Internal standard solution* in a 50-mL volumetric flask, and dilute with water to volume.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 30-m × 0.32-mm fused-silica; bonded with a 0.5-µm film of phase G16

Temperatures

Injection port: 200°

Detector: 310°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	0	150	5
150	10	230	7

Carrier gas: Helium

Flow rate (constant flow): 2 mL/min

Injection volume: 1 µL

Split ratio: 10:1

Run time: 20 min

System suitability

Sample: *Standard solution*

[Note—The relative retention times for benzyl alcohol and phenol are about 0.85 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between benzyl alcohol and phenol

Tailing factor: NMT 2.0 for the phenol peak

Relative standard deviation: NMT 1.0% for the peak response ratio of phenol to benzyl alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phenol (C_6H_6O) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phenol to benzyl alcohol from the *Sample solution*

R_S = peak response ratio of phenol to benzyl alcohol from the *Standard solution*

C_S = concentration of USP Phenol RS in the *Standard solution*

C_U = nominal concentration of phenol in the *Sample solution*

- **Methylparaben and Propylparaben**

Buffer: 7 g/L of monobasic potassium phosphate in water

Mobile phase: Methanol and *Buffer* (65:35)

Internal standard solution: 0.013 mg/mL of USP Ethylparaben RS in *Mobile phase*

System suitability solution: 0.01 mg/mL each of USP Butylparaben RS, USP Propylparaben RS, USP Ethylparaben RS, USP Methylparaben RS, and *p*-hydroxybenzoic acid in *Mobile phase*

Standard stock solution: 0.2 mg/mL of USP Methylparaben RS and 0.03 mg/mL of USP Propylparaben RS in *Mobile phase*

Standard solution: Combine 5 mL of *Standard stock solution* with 5 mL of *Internal standard solution*, and extract three times with 10-mL aliquots of diethyl ether. Filter the combined ether layers through anhydrous sodium sulfate. Evaporate the ether extract to dryness, and dissolve the residue in 50 mL of *Mobile phase*.

Sample solution: Combine 5 mL of the specimen under test with 5 mL of *Internal standard solution*, and extract three times with 10-mL aliquots of diethyl ether. Filter the combined ether layers through anhydrous sodium sulfate. Evaporate the ether extract to dryness, and dissolve the residue in 50 mL of *Mobile phase*.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: UV 272 nm

Columns

Guard: 4.0-mm × 3-mm; packing L1

Analytical: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.3 mL/min

Injection volume: 10 µL

Run time: 10 min

System suitability

Samples: *System suitability solution* and *Standard solution*

[Note—The relative retention times for *p*-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben are about 0.58, 1.0, 1.4, and 2.1, respectively.]

Suitability requirements

Resolution: NLT 2.0 between *p*-hydroxybenzoic acid and methylparaben, NLT 2.0 between methylparaben and ethylparaben; *System suitability solution*

Tailing factor: NMT 2.0 for the methylparaben and propylparaben peaks, *Standard solution*

Relative standard deviation: NMT 2.0% for the peak response ratio of methylparaben to ethylparaben, NMT 2.0% for the peak response ratio of propylparaben to ethylparaben; *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of methylparaben ($C_8H_8O_3$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of methylparaben to ethylparaben from the *Sample solution*

R_S = peak response ratio of methylparaben to ethylparaben from the *Standard solution*

C_S = concentration of USP Methylparaben RS in the *Standard solution*

C_U = nominal concentration of methylparaben in the *Sample solution*

Calculate the percentage of the labeled amount of propylparaben ($C_{10}H_{12}O_3$) in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of propylparaben to ethylparaben from the *Sample solution*

R_S = peak response ratio of propylparaben to ethylparaben from the *Standard solution*

C_S = concentration of USP Propylparaben RS in the *Standard solution*

C_U = nominal concentration of propylparaben in the *Sample solution*

Ethylparaben and butylparaben may be determined in a similar manner using appropriate internal standard solutions. However, because the extraction recovery is matrix dependent, the user should verify the suitability of the procedure for their drug product and

for different product formulations.

- **Thimerosal**

Solution A: Trifluoroacetic acid and water (0.5:1000)

Mobile phase: Methanol and *Solution A* (60:40)

Standard solution: 25 µg/mL of USP Thimerosal RS in water

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: UV 222 nm

Column: 2.1-mm × 10-cm; 2-µm packing L1

Autosampler temperature: 4°

Flow rate: 0.35 mL/min

Injection volume: 2.5 µL

System suitability

Sample: *Standard solution*

Tailing factor: NMT 1.5

Relative standard deviation: NMT 1.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of thimerosal ($C_9H_9HgNaO_2S$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of thimerosal from the *Sample solution*

r_S = peak response of thimerosal from the *Standard solution*

C_S = concentration of USP Thimerosal RS in the *Standard solution*

C_U = nominal concentration of thimerosal in the *Sample solution*

POLAROGRAPHIC METHOD

- **Phenylmercuric Nitrate**

Standard stock solution: 0.1 mg/mL of phenylmercuric nitrate in sodium hydroxide solution (1 in 250). Warm, if necessary, to dissolve.

Standard solution: Pipet 10 mL of *Standard stock solution* into a 25-mL volumetric flask, and proceed as directed under *Sample solution* beginning with “add 2 mL of potassium nitrate solution (1 in 100)”.

Sample solution: Pipet 10 mL of the specimen under test into a 25-mL volumetric flask, add 2 mL of potassium nitrate solution (1 in 100) and 10 mL of pH 9.2 alkaline borate buffer (see in *Buffer Solutions* in the section *Reagents, Indicators, and Solutions*), and adjust to a pH of 9.2, if necessary, by the addition of 2 N nitric acid. Add 1.5 mL of freshly prepared gelatin solution (1 in 1000), then add the pH 9.2 alkaline borate buffer to volume.

Analysis: Pipet a portion of the *Sample solution* into the polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 min. Insert the dropping mercury electrode of a suitable polarograph (see *Polarography ⟨801⟩*), and record the polarogram from −0.6 to −1.5 volts versus the saturated calomel electrode.

Calculate the quantity, in µg/mL, of phenylmercuric nitrate ($C_6H_5HgNO_3$) in the portion of the sample taken:

$$\text{Result} = 2.5C[(i_d)_U/(i_d)_S]$$

C = concentration of phenylmercuric nitrate in the *Standard solution* (µg/mL)

$(i_d)_U$ = diffusion current of the *Sample solution*, as the difference between the residual current and the limiting current

$(i_d)_S$ = diffusion current of the *Standard solution*, as the difference between the residual current and the limiting current

- **USP Reference Standards ⟨11⟩**

USP Benzyl Alcohol RS
USP Butylparaben RS
USP Chlorobutanol RS
USP Ethylparaben RS
USP Methylparaben RS
USP Phenol RS
USP Propylparaben RS
USP Thimerosal RS

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