Annexure 2(c)

AFLATOXINS

Determination of aflatoxin in herbal drugs

Introduction

Aflatoxins are a group of poisonous chemicals that are produced by certain species of molds that can grow on food. Aflatoxins are naturally occurring mycotoxin produced mainly by Aspergillus flavus and Aspergillus parasiticus. These fungi are common and widespread in nature and are most often found when certain grains are grown under conditions of stress such as drought. The mould occurs in soil, decaying vegetation, hay, and grains undergoing microbial spoilage, and invades all types of organic substrates whenever and wherever the conditions are favourable for its growth. Favourable conditions include high moisture content and high temperature. Aflatoxins are very toxic and carcinogenic.

There are four main types of aflatoxins: B_1, B_2, G_1, and G_2. In addition, there are other types of aflatoxins, namely M1 and M2 have been reported as contaminants of food, especially of milk. Aflatoxin B_1 is the major toxin produced.

The clinical effects of aflatoxins may include death, liver cancer, reproductive problems, anaemia, immune system suppression, and jaundice.

Herbal drugs contain not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2.

[CAUTION—Aflatoxins are highly potent, and extreme care should be exercised in handling aflatoxin materials. Aflatoxins are highly toxic. Handle with care. The presence of unexpected contamination with aflatoxins is to be considered in determining compliance.]

Methods to determine aflatoxins in plant origin drugs:

Method A

The Aflatoxin is determined by HPTLC (Appendix 2.4.17)

This HPTLC test is provided to detect the possible presence of AFB_1, AFB_2, AFG_1, and AFG_2 in any material of plant origin.

Test solution 1

Grind about 200.0 g of plant material to a fine powder. Transfer about 50.0 g of the powdered material, accurately weighed, to a glass-stoppered flask. Add 200 ml of a mixture of methanol and water (17:3). Shake about 30 min, and filter. [NOTE—if the solution has interfering plant pigments, proceed as directed for Test Solution 2.] Discard the first 50 ml of the filtrate, and collect the next 40-ml portion. Transfer the filtrate to a separating funnel. Add 40 ml of Sodium Chloride Solution and 25 ml of hexane, and shake for 1 min. allow the layers to separate, and transfer the lower aqueous layer to a second separating funnel. Extract the aqueous layer in the separating funnel twice, each time with 25 ml of methylene chloride, by shaking for 1 min. allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 125 ml conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for

Cleanup procedure in Test Solution 2. Otherwise, dissolve the residue obtained above in 0.2 ml of a mixture of chloroform and acetonitrile (9.8: 0.2), and shake well.

Test solution 2

Collect 100 ml of the filtrate from the start of the flow, and transfer to a 250 ml beaker. Add 20 ml of Zinc Acetate–Aluminium Chloride Reagent and 80 ml of water. Stir, and allow to stand for 5 min. Add 5.0 g of a suitable filtering aid, such as diatomaceous earth, mix, and filter. Discard the first 50 ml of the filtrate, and again further collect 80 ml. Prepare Test Solution 1.

Cleanup procedure: Place a medium porosity sintered glass disk or a glass wool plug at the bottom of a 10mm × 300mm chromatographic tube. Prepare slurry of 2.0 g of silica gel with a mixture of ethyl ether and solvent hexane (3:1), pour the slurry into the column, and wash with 5 ml of the same solvent mixture. Allow the absorbent to settle and add to the top of the column a layer of 1.5 g of anhydrous sodium sulphate. Dissolve the residue obtained above in 3 ml of methylene chloride and transfer it to the column. Rinse the flask twice with 1 ml portions of methylene chloride, transfer the rinses to the column and elute at a rate not more than 1 ml/min. Add successively to the column 3 ml of solvent hexane, 3 ml of ethyl ether, and 3 ml of methylene chloride; elute at a rate not more than 3 ml/min; and discard the elutes. Add to the column 6 ml of a mixture of methylene chloride and acetone (9:1) and elute at a rate not more than 1 ml/min. Collect this elute in a small vial and evaporate to dryness on a water bath. Dissolve the residue in 0.2 ml of a mixture of chloroform and acetonitrile (9.8: 0.2) and shake well.

Test solution 3

If interferences still exist in the residue, and prepared with with IAC (Immunoaffinity Column) in Test Solution in Method B.

Aflatoxin solution

Dilute the Aflatoxins RS with acetonitrile in 1:5 ratio to obtain a solution having a concentration of 0.4 µg/ml each of AFB_1 and AFG_1, and 0.1 µg/ml each of AFB_2 and AFG_2.

Procedure:

Determine by High Performance Thin Layer Chromatography (2.4.17), coating the plate with silica

gel GF254. (0.25mm layer) Mobile phase. 85 volumes of chloroform, 10 volumes of acetone, and 5

volumes of isopropyl alcohol

Test solution. Separately apply 2.5, 5, 7.5, and 10 µl of the Aflatoxin Solution and three 10 µl applications of either Test Solution 1, Test Solution 2, or Test Solution 3 to a suitable thin-layer chromatographic plate coated with a of chromatographic silica gel mixture. Superimpose 5 µl of the aflatoxin Solution on one of the three 10 µl applications of the Test Solution. Allow the spots to dry, and develop the chromatogram in an unsaturated chamber containing a solvent system until the solvent front has moved not less than 15 cm. Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry.

Reference solution. Aflatoxin solution

Determination. By UV light at 365 nm.

System suitability: The four applications of the Aflatoxin Solution appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the position with those of the Aflatoxin Solution. Any spot obtained from the Test Solution with the superimposed aflatoxin Solution is not less intense than that of the corresponding aflatoxin Solution.

Acceptance criteria: No spot from any of the other applications of the Test Solution corresponds to any of the spots obtained from the applications of the aflatoxin Solution. If any spot of aflatoxins is obtained in the Test Solution, match the position of each fluorescent spot of the Test Solution with those of the aflatoxin Solution to identify the type of aflatoxin present. The intensity of the aflatoxin spot, if present in the Test Solution, when compared with that of the corresponding aflatoxin in the Aflatoxin Solution will give an approximate concentration of aflatoxin in the Test Solution. For aflatoxins, the limits are not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2.

Method B

This HPTLC test is provided to detect the possible presence of AFB_1, AFB_2, AFG_1, and AFG_2 in any material of plant origin.

Immunoaffinity Column (IAC):

Before conditioning, adjust the IAC to room temperature. For conditioning, apply 10 ml of Phosphate Buffered Saline Solution onto the column and let it flow through the column by gravity force at a rate of 2 to 3 ml per minute. Leave 0.5 ml of the Phosphate Buffered Saline Solution on top of the column until the Test Solution is applied.

Test solution:

Sample extraction: Transfer about 5.0 g of a powdered sample, accurately weighed, to a glass-stoppered flask. Add 20 ml of a mixture of 17 volumes of methanol and 3 volumes of water. Shake vigorously for not less than 30 min, and filter. Discard the first 5 ml of the filtrate, and collect the next 4-ml portion. Transfer the filtrate to a separating funnel. Add 4 ml of Sodium chloride solution and 2.5 ml of hexane, and shake for 1 min. allow the layers to separate, and transfer the lower aqueous layer to a second

separating funnel. Extract the aqueous layer in the separating funnel twice, each time with 2.5 ml of methylene chloride, by shaking for 1 minute. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for cleanup procedure with IAC. Otherwise, dissolve the residue obtained above in 200 µl of acetonitrile, and shake well.

Cleanup Procedure with IAC: The residue is dissolved in 5 ml of a mixture of 60 volumes of methanol and 40 volumes of water and then diluted with 5 ml of water. This extract is applied onto a conditioned IAC. The IAC is rinsed twice with 10 ml of Phosphate Buffered Saline Solution, and the elution is performed slowly with 2 ml of methanol. Evaporate the elute with nitrogen, and dissolve the residue in 200 µl of acetonitrile.

Aflatoxin solution:

Dilute quantitatively the Aflatoxins RS with acetonitrile in 1:50 ratio to obtain a solution containing 0.04 µg/ml each of AFB_1 and AFG_1, and 0.01 µg/ml each of AFB_2 and AFG_2

Analysis:

Determine by HPTLC (2.4.17), coating the plate with silica gel GF254.

(200 mm layer) Mobile phase. 14 volumes of chloroform, 2 volumes of

acetone, and 0.03 volumes of water.

Test solution. Separately apply 5, 7.5, and 10 µl of aflatoxin Solution and three 10 µl applications of the test Solution to a suitable HPTLC plate. Superimpose 5 µl of aflatoxin Solution on one of the three 10 µl applications of the test Solution. Allow the spots to dry, and develop the chromatogram in a saturated chamber containing a solvent system until the solvent front has moved not less than 72 mm from the origin (80 mm from the lower edge of the plate). Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry for 5 minutes. Compare each fluorescent spot of the test solution with those of aflatoxin solution to identify the type of aflatoxin present. The concentration of aflatoxins in the test solution can be calculated from the calibration curve obtained from the scan data with aflatoxin solution.

Reference solution. Aflatoxin solution

Determination. By scanning fluorescence density (>400 nm) under UV light at 366 nm.

System suitability:

The four applications of aflatoxin solution appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the Test Solution that coincides in position with those of Aflatoxin Solution. Any spot obtained from the Test Solution with the superimposed aflatoxin solution is not less intense than that of the corresponding Aflatoxin Solution. The mean recovery of spiked AFB_1 and AFG_1 is not less than 70 per cent.

Acceptance criteria:

The limits are not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2, except when otherwise indicated.

Method C

This test method is provided for the detection of the possible presence of AFB_1 and total aflatoxins (AF: sum of AFB_1, AFB_2, AFG_1, and AFG_2).

Working aflatoxin standard solutions:

Prepare six solutions in separate 10 ml volumetric flasks according to Table 1. Dilute with methanol and water (1:1, v/v) to volume.
Store in a refrigerator, and equilibrate to room temperature before use. Always prepare fresh solution.

Table 1- Working aflatoxin standard solutions

Working Aflatoxin Standard Solutions	Aflatoxins RS (µl)	Aflatoxin Final Concentration of Working Standard Solution (ng/ml)				Aflatoxin ∑AF
		AFB_1	AFB_2	AFG_1	AFG_2	
1	0	0	0	0	0	0
2	12.5	0.25	0.0625	0.25	0.0625	0.625
3	25	0.5	0.125	0.5	0.125	1.25
4	50	1	0.25	1	0.25	2.5
5	100	2	0.5	2	0.5	5
6	200	4	1	4	1	10

Immunoaffinity Column (IAC)[2]

Use an immunoaffinity column that contains monoclonal antibodies cross reactive toward AFB_1, AFB_2, AFG_1, and AFG_2. The immunoaffinity columns have a minimum capacity of not less than 100 ng of total aflatoxin and give a recovery of not less than 80 per cent for AFB_1, AFB_2, AFG_1, and AFG_2 when 5 ng of each AFB_1, AFB_2, AFG_1, and AFG_2 is applied in 10 ml of 10 per cent methanol in Phosphate Buffered Saline Solution (v/v).

Test Solution

Extraction:

Weigh 5.0 g of a sample in a centrifuge tube. Add 1.0 g of sodium chloride and 25 ml of a mixture of methanol and 0.5 per cent sodium bicarbonate in ratio of 7:3. Shake at 400 rpm for 10 min. Centrifuge for 10 min at 7000 rpm (g value = 5323 mm/s^2) or at a

speed that can result in a firm pellet of residues. Immediately pipette 7 ml into a centrifuge tube, add 28 ml of 0.1 M Phosphate Buffer Solution, mix, and filter through glass microfiber paper. Collect 25 ml of filtrate (equivalent to 1 g of test sample) into a 25 ml graduated cylinder, and proceed immediately with IAC chromatography.

IAC clean up:

Remove the top cap from the column, and connect it with the reservoir. Remove the end cap from the column, and attach it to the column manifold (the fit must be tight). Let the liquid in the column pass through until the liquid is about 2 to3 mm above the column bed. Pass 25 ml of filtrate into the reservoir.

Let the column run dry. To start the flow again easily, remove the column from the manifold, add about 2 ml of Phosphate Buffered Saline Solution into the column, reattach the column to the reservoir, and wash the column with an additional 3 ml of Phosphate Buffered Saline Solution and then with 5 ml of water (the 5 ml of Phosphate Buffered Saline Solution can be added directly to the column reservoir if other techniques are used to dislodge the air bubble at the end of the column and to start flow again easily).

The column run dry and then forces 3 ml of air through the column with a syringe. Elute with 1 ml of methanol, and collect the analytes in a 3 ml volumetric flask. The column run dry. Stand for 1 min, then elute with an additional 1 ml of methanol, and collect in the same volumetric flask. Let the column run dry, and force 10 ml of air through the column. Dilute the elute with water to volume. Use this as the Test Solution, and perform the analysis of aflatoxins immediately.

System suitability solution: Prepare a spiked sample by adding 5 ml of working aflatoxin standard solution 5 to a 5 g sample and repeating the procedure for the Test Solution, using 20 ml instead of 25 ml of the mixture of methanol and 0.5 per cent sodium bicarbonate in ratio of 7:3.

Chromatographic system

Flow rate: 0.8 ml/min

Detection. Fluorescence detector set at excitation wavelength (Ex) 362 nm and emission

wavelength (Em) 440 nm Column: 4.6 mm × 15 cm containing 3-mm packing C18

Mobile phase: Isocratic

For post-column derivitization with PHRED cell—6 volumes of Water, 25 volumes of methanol, and 15 volumes of acetonitrile [Post-column derivitization (PCD) systems PHRED cell—Post-column photochemical derivitization cell]

Analysis:

Post-column derivatization for aflatoxins: Use UV. Inject 50 µl of reagent blank (Working Aflatoxin Standard Solution 1), Working Aflatoxin Standard Solutions 2–6, or the Test Solution into the LC column.

Identify the aflatoxin peaks in the Test Solution by comparing the retention times with those of the working standards. The aflatoxins elute in the order AFG_2, AFG_1, AFB_2, and AFB_1. After passing through the PHRED, the AFG_1 and AFB_1 have been derivatized to form AFG_{2a} (derivative of AFG_1) and AFB_{2a} (derivative of AFB_1). [NOTE—the chemical structures of the derivatives resulting from electrochemical bromination and photolysis are not the same.] The retention times of AFG_2, AFG_{2a}, AFB_2 and AFB_{2a} are between about 14 and 27 min using the PHRED cell. The peaks should be baseline resolved. Construct standard curves for each aflatoxin. Determine the concentration of each aflatoxin in the Test Solution from the calibration curve.

Aflatoxins calibration curves: Calibration curves are prepared for each of the aflatoxins using the Working Aflatoxin Standard Solutions containing the four aflatoxins described. These solutions cover the

range of 0.25–4 ng/ml for AFB_1 and AFG_1, and the range of 0.0625 to 1 ng/ml for AFB_2 and AFG_2. Make the calibration curves before analysis according to Table 1, and check the plot for linearity. If the test portion area response is outside (higher) the calibration range, then the Test Solution should be diluted with a mixture of methanol and water (1:1, v/v) and reinjected into the LC column.

Quantitation of aflatoxins:

Quantitation of aflatoxins is performed by measuring peak areas at each aflatoxin peak retention time and comparing them with the corresponding calibration curve.

System suitability

The mean recovery of spiked AFB_1 (2 µg/kg) and the total of aflatoxins (5 µg/kg) is not less than 68 per cent and 70 per cent, respectively. The relative standard deviation (RSD) is not more than 10 per cent for AFB_1 and for the total of aflatoxins.

Calculations

Plot the peak area (response, y-axis) of each of the toxin standards against the concentration (ng/ml, x-axis) and determine the slope

(S)and y-intercept (a). Calculate the level of toxin in the sample by the

following formula: Toxin (mg/kg) = {[(R − a)/S] × V/W} × F

Where,

R is the Test Solution peak area;

V is the final volume of the injected Test Solution (ml) which is 3 ml

F is the dilution factor. So, F = 1

W is 1 g of test sample passed through the immunoaffinity column.
The total of aflatoxins is the sum of AFG_2, AFG_1, AFB_2, and AFB_1.

Acceptance criteria
Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are not more than 5 ppb for AFB_1 and not more than 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2, except when otherwise indicated.

Method D
This test method is provided for the detection of the possible presence of AFB_1 in plant material by HPLC (2.4.14)

Aflatoxins are subject to light degradation. Carry out the determination protected from daylight by using UV-absorbing curtains or blinds in combination with artificial light (fluorescent tubes are acceptable). Protect aflatoxin-containing solutions from daylight.

Rinse glassware before use with a 10 per cent v/v solution of sulphuric acid and then rinse carefully with distilled water until no more acid is present.

Test solution: Use an immuno affinity column containing antibodies against aflatoxin B_1 with a capacity of not less than 100 ng of aflatoxin B_1 and which gives a recovery of not less than 80 per cent when a solution of 5 ng of aflatoxin B_1 in a mixture of 12.5 ml of methanol and 87.5 ml of water is passed through. Allow the immuno affinity column to reach room temperature. To 5.00 g of the powdered drug add 100 ml of a mixture of 30 ml of water and 70 ml of methanol and extract by sonication for 30 min. Filter through folded filter paper. Pipette 10.0 ml of the clear filtrate into a 150 ml conical flask. Add 70 ml of water. Pass 40 ml through the immuno affinity column at a flow rate of 3 ml/min (not exceeding 5 ml/min). Wash the column with 2 volumes, each of 10 ml, of water at a flow rate not exceeding 5 ml/min and dry by applying a slight vacuum for 5-10 sec or by passing air through the immuno affinity column by means of a syringe for 10 sec. Apply 0.5 ml of methanol to the column and allow to pass through by gravity. Collect the elute in a 5 ml volumetric flask. After 1 min, apply a 2nd portion of 0.5 ml of methanol. After a further 1 min, apply a 3rd portion of 0.5 ml of methanol. Collect most of the applied elution solvent by pressing air through or applying vacuum to the column. Dilute to 5 ml with water and shake well. If the solution is clear, it can be used directly for analysis. Otherwise, pass it through a disposable filter unit prior to injection. Use a disposable filter unit (e.g. 0.45 µm pore size polytetrafluoroethylene filter) that has been shown not to cause loss of aflatoxin by retention.

Aflatoxin B_1 primary stock solution: Dissolve Aflatoxin B_1 in a mixture of 2 ml of acetonitrile and 98 volumes of toluene to give a 10 µg/ml solution. To determine the exact concentration of aflatoxin B_1 in the stock solution, record the absorbance between 330 nm and 370 nm.

Calculate the aflatoxin B_1 mass concentration, in micrograms per millilitre, using the following expression:

$$\frac{A \times M \times 100}{\varepsilon \times l}$$

A = Absorbance determined at the maximum of the absorption curve M = Molar mass of aflatoxin B_1 (312g/mol)

$\in$ = Molar absorptivity of aflatoxin B_1 in the toluene-acetonitrile mixture (1930 m^2/mol)

Aflatoxin B1 secondary stock solution. Prepare a secondary stock solution containing 100 ng/ml aflatoxin B_1 by diluting aflatoxin B_1 primary stock solution with a mixture of 2 ml of acetonitrile and 98 ml of toluene. Wrap the flask tightly in aluminium foil and store it below 4 °C. Before use, do not remove the aluminium foil until the contents have reached room temperature.

Aflatoxin B1 standard solutions. Place the volumes of aflatoxin secondary stock solution indicated in Table in separate 250 ml volumetric flasks. Pass a stream of nitrogen through at room temperature until the solvent has just evaporated. To each flask, add 75 ml of methanol, allow the aflatoxin B_1 to dissolve and dilute to 250 ml with water.

Table 2 - Aflatoxin B_1 standard solutions

Standard solution	Volume of secondary stock solution (µl)	Final concentration of standard solution (ng/ml)
1	125	0.05
2	250	0.1
3	500	0.2
4	750	0.3
5	1000	0.4

Calibration curve: Prepare the calibration curve using aflatoxin B_1 standard solutions 1 to 5, which cover a range equivalent to 1-8 µg/kg of aflatoxin B_1 in the herbal drug. Check the plot for linearity. If the content of aflatoxin B_1 in the sample to be examined is outside of the calibration range, the test solution must be diluted to an aflatoxin content that should be distinguish calibration curve.

Chromatographic system

- **Column: Size: 0.25m × 4.6 mm;**
- **Stationary phase: octadecylsilyl silica gel for chromatography (5 µm).**
- **Mobile phase:**
  Mobile phase A (for post-column derivitization with photochemical reactor or pyridinium bromide): acetonitrile, methanol, water (2:3:6 v/v/v);
  Mobile phase B (for post-column derivitization with electrochemically derived bromine): add 0.12 g of potassium bromide and 350 µl of dilute nitric acid per litre of mobile phase A
- **Flow rate: 1 ml/min.**
- **Detection: Fluorescence detector with a 360 nm excitation filter and a 420 nm cut-off emission filter, or equivalent. Recommended settings for adjustable detectors are 365 nm (excitation wavelength) and 435 nm (emission wavelength).**
- **Injection: 500 µl.**

Post-column derivitization with pyridinium hydro bromide perbromide (PBPB):

- **pulseless pump,**
- **T-piece with zero dead volume,**
- **polytetrafluoroethylene reaction tube, 0.45 m × 0.5 mm,**
- **mobile phase A,**
- **post-column derivitization reagent: dissolve 50 mg of pyridinium hydrobromide perbromide in 1000 ml of water (store protected from light and use within 4 days),**
- **flow rate of the derivitization reagent: 0.4 ml/min.**

Post-column derivitization with photochemical reactor (PHRED)

- **Reactor unit with one 254 nm low pressure mercury UV bulb (minimum 8 W), polished support plate,**

- **knitted reactor coil, polytetrafluoroethylene tubing knitted tightly around the UV bulb,**
- **size 25 m× 0.25 mm**
- **nominal void volume 1.25 ml;**
- **exposure time: 2 min;**
- **mobile phase A.**

Post-column derivitization with electrochemically generated bromine (KOBRA):

- **KOBRA-cell: electrochemical cell that generates a reactive form of bromine for derivitization of aflatoxins, resulting in enhanced fluorescence**
- **Derivation direct-current supply in series with the KOBRA-cell, providing a constant current of about 100 µA**
- **Polytetrafluoroethylene reaction tube, 0.12 m× 0.25 mm**
- **Mobile phase B.**

Elution order: aflatoxin G_2, aflatoxin G_1, aflatoxin B_2, aflatoxin B_1.

Calculation: calculate the calibration curve y = ax + b, with aflatoxin B_1 concentration (ng/ml) on the x-axis and the signal (S) on the y-axis. The aflatoxin B1 concentration (C) in a measured solution is equal to

$$\frac{S-b}{a}$$

Calculate the aflatoxin B_1 content of the herbal drug, in nanograms per gram, using the following expression:

$$\frac{V1 \times V2 \times C}{m \times Vi}$$

m =**mass of the herbal drug taken for analysis, in grams;**

V_1 = **volume of the solvent used for extraction, in millilitres;**

V_i = **aliquot taken for immunoaffinity clean-up, in millilitres;**

V_2 = **final volume of solution after elution from the immunoaffinity column and dilution, in millilitres;**

C =**measured aflatoxin B_1 concentration of the test solution, in nanograms per millilitre**

The presence of aflatoxin B_1 may be confirmed by recording the chromatogram without post-column derivitization, which leads to a large decrease (greater than 10-fold) in the response due to aflatoxin B_1.

Reagent list

Zinc acetate–aluminium chloride reagent: Dissolve 20.0 g of zinc acetate and 5.0 g of aluminium chloride in sufficient water to make 100.0 ml.

Sodium chloride solution: Dissolve 5.0 g of sodium chloride in 50.0 ml of water.

Phosphate buffered saline solution: Prepare 10 mM phosphate buffer solution containing 0.138 M sodium chloride and 0.0027 M Potassium chloride in water, and adjust with 2 M sodium hydroxide to a pH of 7.4

0.1 M phosphate buffer solution: Dissolve 8.69 g of anhydrous disodium phosphate and 4.66 g of anhydrous monosodium phosphate or 5.36 g of monosodium phosphate monohydrate in 800 ml water, adjust with 2 M sodium hydroxide to a pH of 7.4, add 10 ml of polysorbate 20, and dilute to 1000.0 ml.