BIOSPECTRA
100 Majestic Way, Bangor, PA 18013 / www.biospectra.us

HEPES TESTING METHODS

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1. PURPOSE:

1.1. To provide the Laboratory personnel with procedures for testing of HEPES raw materials (RM), in-process (IP), finished goods (FG), and stability.

2. SCOPE:

2.1. Applies to the testing of HEPES in the Laboratory. Methods include testing for all types of HEPES sold by BioSpectra; only the specific tests required for requested type must be tested for.

3. RESPONSIBILITIES:

3.1. The Laboratory Manager is responsible for the control, training, maintenance and implementation of this procedure.

3.2. The Laboratory technicians are responsible for compliance with the terms of this procedure. This includes notifying the Laboratory Manager and Quality Assurance Managers, or designees, if any analyses fail to meet their respective specifications.

4. REFERENCES:

4.1. BSI-ATM-0054, Analytical Method of Analysis: HEPES via ICP-MS
4.2. BSI-ATM-0089, Analytical Method of Analysis: Trace Metals in Finished Goods Products by ICP-OES
4.3. BSI-ATM-0121, HEPES Identity by HPLC with UV Detection
4.4. BSI-ATM-0131, Analytical Method for the Determination of Trace Metals in BioTech Products
4.5. BSI-PRL-0338, Analytical Method Validation Protocol: HEPES Identity via HPLC
4.6. BSI-PRL-0353, Analytical Method Validation Protocol: Residual Solvents USP 1467: HEPES - Methanol
4.7. BSI-PRL-0508, Analytical Method Validation Protocol: Determination of ICH Q3D Elemental Impurities (Class 1, 2A, 2B, 3 & 4) by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) in HEPES
4.8. BSI-RPT-1758, Analytical Method Transfer Report: HEPES Identity by HPLC with UV Detection
4.9. BSI-SOP-0090, Lambda 25 UV/Vis Operation and Calibration
4.10. BSI-SOP-0094, Muffle Furnace SOP and Calibration
4.11. BSI-SOP-0095, DNase (Endonuclease) Assay
4.12. BSI-SOP-0096, RNase (Ribonuclease) Assay
4.13. BSI-SOP-0098, Balance SOP
4.14. BSI-SOP-0126, Laboratory Notebooks
4.15. BSI-SOP-0138, DNase (Exonuclease) Assay
4.16. BSI-SOP-0139, Protease Assay
4.17. BSI-SOP-0140, Standardization of Titrants
4.18. BSI-SOP-0143, Metrohm Titrando 907 Auto-Titrator SOP
4.19. BSI-SOP-0242, Portable Turbidimeter Operation and Calibration
4.20. BSI-SOP-0254, Spectrum Two UATR SOP
4.21. BSI-SOP-0255, XL200 pH/mV/Conductivity Meter SOP
4.22. BSI-SOP-0303, NexION 350X ICP-MS SOP
4.23. BSI-SOP-0345, Endosafe nexgen-PTS Endotoxin Reader SOP

4.24. BSI-SOP-0422, Empower 3 General Procedure
4.25. ACQUITY UPLC Quaternary Solvent Manager PLUS Series
4.26. ACQUITY UPLC TUV Detector Operator's Overview and Maintenance Guide
4.27. ACS, Reagent Chemicals, current edition
4.28. Current USP

5. EQUIPMENT:

5.1. PerkinElmer Lambda 25 UV/Vis Spectrophotometer
5.2. Optically matched set of UV quartz cells, 10 mm path length
5.3. Analytical Balance
5.4. Endosafe nexgen-PTS Endotoxin Reader
5.5. Waters Acquity UPLC
5.6. PerkinElmer Spectrum Two UATR
5.7. PerkinElmer NexION 350X ICP-MS
5.8. PerkinElmer Avio 500 ICP-OES
5.9. XL200 pH/Conductivity Meter or equivalent pH / mv / Conductivity Meter
5.10. Muffle Furnace
5.11. Blue M Oven, or equivalent
5.12. Metrohm 907 Auto-Titrator
5.13. Hach 2100Q Portable Turbidimeter, or equivalent
5.14. Shimadzu GC-2010, FID detector

6. REAGENTS:

6.1. **Barium Chloride Dihydrate:** purchased commercially.
6.2. **Barium Chloride TS:** Dissolve 12 grams of barium chloride dihydrate in purified water. Filter and dilute to make a total volume of 100 mL with purified water.
6.3. **Composite 5:** purchased commercially
6.4. **Hydrochloric Acid, Concentrated:** purchased commercially.
6.5. **Hydrochloric Acid, 0.02N:** Dilute 10mL of 1N HCl to 500mL with purified water.
6.6. **Hydrochloric Acid 3N:** Pipette 25.75 mL of concentrated hydrochloric acid and transfer to a 100 mL volumetric flask that contains a small amount of purified water. Dilute to volume with purified water.
6.7. **LAL Water:** purchased commercially.
6.8. **Nitric Acid:** purchased commercially.
6.9. **Platinum Cobalt Color Standard (10 APHA):** Dilute 2mL of Platinum Cobalt Color Standard 500 to 100mL with purified water.
6.10. **Potassium Hydrogen Phthalate (KHP) Standard:** Prepare a vial at 120°C for 30 minutes. Allow to cool in a desiccator and weigh a maximum of 10.0g of NIST Potassium Hydrogen Phthalate. Dry at 120°C for 2 hours. Cool and store in a desiccator in a closed container. Stable for 3 months.
6.11. **Silver Nitrate, 0.1N:** purchased commercially.
6.12. **Sodium Hydroxide 1N:** purchased commercially.
6.13. **Sodium Hydroxide 0.1N:** purchased commercially
6.14. **Sulfuric Acid:** purchased commercially
6.15. **Sulfuric Acid 0.02N:** Slowly add 20 mL of 0.1N Sulfuric Acid to 80 mL purified water to

make a total volume of 100 mL.

7. PROCEDURE:

7.1. MOTHER LIQUOR ABSORBANCE:

7.1.1. Prepare 10 mL of a 1:1 dilution by pipetting 5 mL of purified water and 5 mL of the Mother Liquor into an LOD vial or small beaker.

7.1.2. Swirl to homogenize the solution.

7.1.3. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the absorbance of the sample. Record results at specified wavelengths in the appropriate laboratory documentation and Batch Record.

7.2. MOTHER LIQUOR ASSAY:

7.2.1. Standardize Metrohm pH electrode as per Metrohm Titrando 907 Auto-Titrator SOP.

7.2.2. Standardize or perform a daily check of 0.1N NaOH as per Standardization of Titrants.

7.2.3. Accurately weigh 0.8 g of sample and transfer to a beaker.

7.2.4. Dissolve in a suitable amount of purified water. Determine the Assay concentration using the Metrohm Auto titrator.

$$\% \text{ HEPES} = \frac{(\text{mL x N of NaOH})(23.831)}{\text{Sample Weight (g)}}$$

7.2.5. Record results in the appropriate laboratory documentation and Batch Record, if applicable.

7.3. ABSORBANCE (0.1M):

7.3.1. Accurately weigh 0.60 g of sample.

7.3.2. Transfer accurately weighed sample to a graduated cylinder and dilute to 25 mL with purified water.

7.3.3. Swirl to dissolve completely.

7.3.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

7.4. ABSORBANCE (0.05M):

7.4.1. Accurately weigh 0.30 g of sample.

7.4.2. Transfer accurately weighed sample to a graduated cylinder and dilute to 25 mL with purified water.

7.4.3. Swirl to dissolve completely.

7.4.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

7.5. ABSORBANCE (1M):

7.5.1. Accurately weigh 6.0 g of sample.

7.5.2. Transfer accurately weighed sample to a graduated cylinder and dilute to 25 mL with purified water.

7.5.3. Swirl to dissolve completely.

7.5.4. Refer to Lambda 25 UV/Vis Operation and Calibration to determine the Absorbance of the sample.

7.6. APPEARANCE AND COLOR:

7.6.1. Place 25-50 g of sample in a clean, dry glass beaker.

7.6.2. In an area with sufficient lighting, view the sample from all angles.

7.6.2.1. The sample should be white in color and characteristic of powder. If the sample does not conform to the specifications, notify a supervisor immediately.

7.7. **APPEARANCE OF SOLUTION (1% WATER)** :

7.7.1. Prepare a 1% solution of the sample.
- 7.7.1.1. Weigh 1.0g of sample and transfer to a 100mL volumetric flask.
- 7.7.1.2. Dissolve sample in purified water and dilute to 100mL with purified water.
- 7.7.1.3. Swirl to dissolve completely.

7.7.2. Solution must be clear and colorless when compared to a clear and colorless reference standard.

7.8. **ASSAY AND pKa** :

7.8.1. Standardize Metrohm pH electrode as per Metrohm Titrando 907 Auto-Titrator SOP.
- 7.8.1.1. For pKa, ensure that the slope of the standardization is 99.3-101.0% and the pH (0) is between 6.8-7.2

7.8.2. Standardize 0.1N NaOH as per Standardization of Titrants.

7.8.3. Accurately weigh 0.8g of sample dried per LOD method and transfer to a beaker.
- 7.8.3.1. Raw material may be analyzed as-is. Refer to the assay requirement of the code being tested.

7.8.4. Dissolve in a suitable amount of purified water. Determine the assay concentration using the Metrohm Auto Titrator.

7.8.5. The pKa should be reported as the Assay printout from the Metrohm Auto Titrator.

$$\% \text{ HEPES} = \frac{(mL\ of\ N\ of\ NaOH)(23.831)}{Sample\ Weight\ (g)}$$

7.9. **CHLORIDE** :

7.9.1. Weigh 2.0g of sample and dissolve sample in 40mL of purified water in a Nessler Color Comparison Tube. If necessary, neutralize the solution with nitric acid to litmus.

7.9.2. Pipette 0.141mL of 0.02N HCl and dilute to 40mL with purified water in a Nessler Color Comparison Tube.

7.9.3. Add to each solution, 1mL of concentrated nitric acid and 1mL of 0.1N Silver Nitrate.

7.9.4. Dilute to 50mL with purified water. Cover with parafilm and mix by inversion.

7.9.5. After 5 minutes, the turbidity of the sample prep does not exceed that produced by the standard when viewed against a dark background.

7.9.6. If a visible difference in the turbidity is not observed, then utilize the Turbidimeter to measure the turbidity of the standard and the sample solutions. Follow Bangor Portable Turbidimeter SOP and Calibration.

7.10. **ENDOTOXINS** :

7.10.1. Accurately weigh 0.100g of sample into a sterile tube. Add 170µL of 1N NaOH. Dilute to 10mL with LAL reagent water, dissolve, and mix thoroughly for a final concentration of 0.0100 g/mL.

7.10.2. Refer to Endosafe NexGen-PTS Endotoxin Reader SOP for further instrument instructions and sample analysis.

7.11. **ENZYME ACTIVITY** :

7.11.1. DNase: Refer to DNase (Exonuclease) Assay (BSI-SOP-0138) and DNase Endonuclease Assay (BSI-SOP-0095) for sample preparation and analysis.

7.11.2. RNase: Refer to RNase (Ribonuclease) Assay (BSI-SOP-0095) for sample preparation and analysis.

7.11.3. Protease: Refer to Protease Assay (BSI-SOP-0139) for sample preparation and analysis.

7.12. **HEAVY METALS** :

7.12.1. Refer Section 7.26 Trace Metals for sample preparation and analysis.

7.13. **IDENTIFICATION TEST (UATR)** :

7.13.1. Follow Spectrum Two UATR SOP (BSI-SOP-0254).

7.14. **IDENTIFICATION (TLC equivalent)** :

7.14.1. Refer to BSI-ATM-0121 for Primary Method via HPLC

7.15. **INSOLUBLE MATTER** :

7.15.1. Accurately weigh 20.00 g of sample and transfer to a 600 mL beaker.

7.15.2. Add 200 mL of purified water. If necessary, utilize a Teflon encapsulated magnetic stirring bar and electric stir plate to dissolve the sample.

7.15.3. Dry a filter crucible and filter paper at 105°C ± 2°C for 1 hour. Cool in ambient air for 15 minutes and weigh.

7.15.4. Filter sample solution through the filter crucible using a suitable vacuum pump.

7.15.5. Rinse sample vessel and filter crucible with 100 mL of purified water.

7.15.6. Dry the filter crucible and filter paper at 105°C ± 2°C for 1 hour. Cool in ambient air for 15 minutes and weigh.

$$\%\ Insolubles = \frac{residue\ weight\ (g)}{sample\ weight\ (g)}\ x\ 100$$

7.16. **LOSS ON DRYING (LOD) (MOISTURE)** :

7.16.1. Dry a Loss on Drying (LOD) vial in an oven at 105°C ± 2°C for 30 minutes. Cool for 15 minutes in a desiccator, weigh on the analytical balance, and record results.

7.16.2. Tare the dried vial and weigh 1 – 2 g of sample and record the weight.

7.16.3. Dry for 3 hours at 105°C ± 2°C. Cool for 15 minutes in a desiccator.

7.16.4. Reweigh and calculate the % LOD.

7.16.5. Retain sample for Assay, dried basis.

$$\%\ LOD = \frac{initial\ sample\ weight\ (g) - final\ sample\ weight\ (g)}{initial\ sample\ weight\ (g)}\ x\ 100$$

7.17. **MICROBIAL CONTENT** :

7.17.1. Microbial analysis will be performed by an outside testing laboratory.

7.17.1.1. Primary Provider: Mary Paul Laboratories

7.17.1.2. Package and send NLT 35 grams of sample to Mary Paul Laboratories with a purchase order and analysis request form.

7.18. **pH OF A 5% SOLUTION** :

7.18.1. Prepare a 5% solution of the sample.

7.18.1.1. Accurately weigh 5.0 g of sample.

7.18.1.2. Transfer accurately weighed sample to a beaker and dissolve in 100 mL of purified water.

7.18.1.3. Swirl to dissolve completely.

7.18.2. Follow the appropriate SOP to measure and record the pH at 25 ± 2°C.

7.19. **pH OF A 1% SOLUTION** :

7.19.1. Prepare a 1% solution of the sample.

7.19.1.1. Accurately weigh 1.0 g of sample.

7.19.1.2. Transfer accurately weighed sample to a beaker and dissolve in 100 mL of purified water.

7.19.1.3. Swirl to dissolve completely.

7.19.2. Follow the appropriate SOP to measure and record the pH at 25 ± 2°C.

7.20. **RESIDUAL SOLVENTS (Methanol)** :

7.20.1. Refer to Analytical Method Validation Protocol: Residual Solvents by Head Space GC-FID HEPES: Methanol (BSI-PRL-0353) for instrument parameters and sample analysis.

7.21. **RESIDUE ON IGNITION/SULFATED ASH** :

7.21.1. Turn on muffle furnace and allow temperature to stabilize at 600°C. Follow Muffle Furnace SOP and Calibration for operation of the muffle furnace.

7.21.2. Utilize forceps to insert and remove crucible into the furnace.

7.21.3. Ignite the quartz crucible at 600 ± 50°C for 30 minutes. Cool in a desiccator for one hour and 30 minutes and weigh.

7.21.4. Weigh 1.0 g sample in the previously ignited quartz crucible. Moisten the sample with 0.5 mL of sulfuric acid.

7.21.5. Volatize the sample until the sample is thoroughly charred. Heat the sample slowly, so that the sample does not boil over and sample is not lost.

7.21.5.1. The rate of heating should be such that from ½ to 1 hour is required to volatilize the sample.

7.21.5.2. Continue to heat the sample until all the excess sulfuric acid has been volatilized.

7.21.6. Ignite the quartz crucible in a muffle furnace at 600 ± 50°C for 15 minutes or until all carbon has been removed.

7.21.7. Gently remove the ignited crucible with forceps from the furnace.

7.21.8. Inspect the crucible for cracks, chips, or signs of damage such as discoloration- the muffle furnace insulation is made of rough ceramics and metal, care must be taken to not crack, chip, or rub the crucible against the lining.

7.21.9. Cool in a desiccator for an hour and a half and reweigh.

$$\% \, ROI = \frac{Residue\ weight\ (g)}{Sample\ weight\ (g)}\ x\ 100$$

7.22. **SULFATE** :

7.22.1. Sample Preparation:

7.22.1.1. Weigh out 2.0 g of sample and transfer to a 50 mL Nessler Color Comparison Tube. Dissolve in 40 mL purified water. If necessary, neutralize the solution with hydrochloric acid to litmus.

7.22.2. 50 ppm Standard Preparation:

7.22.2.1. Prepare a standard solution by pipetting 0.1 mL of 0.020 N Sulfuric Acid in a 50 mLNessler Color Comparison Tube. Dilute to 40 mL with purified water.

7.22.3. Procedure:

7.22.3.1. To both solutions add 1 mL of 3 N HCl and 3 mL of Barium Chloride TS. Dilute to 50 mL with purified water.

7.22.3.2. Cover with parafilm and mix by inversion.

7.22.3.3. Compare turbidity 10 minutes after addition of the barium chloride to the sample and standard solutions.

7.22.4. Any turbidity produced in the sample solution should not exceed that produced by the standard when viewed from above against a black surface.

7.22.5. If turbidity of the sample solution exceeds that of the standard, notify the Laboratory Manager immediately.

7.23. **SOLUBILITY (1%)** :

7.23.1. Weigh 1.0 g of sample into a clean glass beaker.

7.23.2. Add 100 mL of purified water and swirl to dissolve.

7.23.3. View sample from all angles under sufficient lighting. Solution should be clear and complete.

7.23.3.1. Note any insoluble matter present, if any, and refer to material TUPP and insoluble matter specification for disposition.

7.24. **SOLUBILITY (5%)** :

7.24.1. Add 100 mL of purified water and swirl to dissolve.

7.24.2. View sample from all angles under sufficient lighting. Solution should be clear and complete.

7.24.2.1. Note any insoluble matter present, if any, and refer to material TUPP and insoluble matter specification for disposition.

7.25. **SOLUBILITY (0.05M)** :

7.25.1. Weigh 1.19 g of sample into a clean glass beaker.

7.25.2. Add 100 mL of purified water and swirl to dissolve.

7.25.3. View sample from all angles under sufficient lighting. Solution should be clear and complete.

7.25.3.1. Note any insoluble matter present, if any, and refer to material TUPP and insoluble matter specification for disposition.

7.26. **TRACE METALS** :

7.26.1. For full elemental impurities requirements, refer to the method DCN: BSI-ATM-0054 as primary method of analysis for instrument parameters, standard calibration, and sample analysis.

7.26.2. Available Methods dependent on Product Code Requirements:

7.26.2.1. Refer to Analytical Method of Analysis: Trace Metals in Finished Goods Products by ICP-OES, BSI-ATM-0089.

7.26.2.2. Refer to Analytical Method for Determination of Trace Metals in BioTech Products, BSI-ATM-0131.

7.27. **WATER (BY KARL FISCHER TITRATION)** :

7.27.1. Standardize Composite 5 as per Standardization of Titrants.

7.27.2. Grind the sample in a dry mortar into a fine powder utilizing a pestle.

7.27.3. Immediately weigh 2.0 g of sample into the glass weighing spoon and tare it.

7.27.4. Transfer the sample to the KF vessel by removing the rubber septum and adding the sample into the titration vessel.

7.27.4.1. Do not leave the rubber septum open for long periods of time as this will allow moisture to enter the titration vessel.

7.27.5. Return the weighing spoon to the balance, making sure not to lose any sample that was left behind. Once the weight stabilizes, record the sample weight and transfer to instrument.

7.27.6. Check to make sure there is no residual sample stuck to the sides of the titration vessel.

7.27.6.1. If there is any sample stuck to the side, stop the stir bead from spinning before swirling the vessel to rinse the sides.

7.27.7. Once the method begins, check to ensure the sample is fully dissolved before the titration begins (i.e. before the stir command completes).

7.27.8. The moisture content will then be determined by the Metrohm Auto Titrando 907.

$$\%\, Moisture = \frac{(mL\ of\ Composite\ 5)(\frac{mg}{mL}\ of\ Composite\ 5)(0.1)}{Sample\ Weight(g)}$$