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GUIDELINE FOR THE DETERMINATION OF RESIDUAL MOISTURE IN DRIED BIOLOGICAL PRODUCTS

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Comments regarding these analytical methods should be submitted to Docket Management Branch (HFA-305), Food and Drug Administration, Rm 4-62, 5600 Fishers Lane, Rockville, MD 20857. Comments should be identified with [Docket No. 89D-0140]

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

Guideline for the Determination of Residual Moisture in Dried Biological Products

I. Introduction

This guideline is issued under 21 CFR 10.90, and as such, it states principles and practices of general applicability that are not legal requirements but are acceptable to the Food and Drug Administration (FDA). A person may rely upon this guideline with the assurance of its acceptability to FDA, or may follow different procedures. When different procedures are chosen, a person may, but is not required to, discuss the matter in advance with FDA to prevent the expenditure of money and effort on activity that may later be determined to be unacceptable.

The guideline is issued concurrently with a final rule that amends the test for residual moisture in dried biological products found in 21 CFR 610.13 of the biologics regulations. The guideline may be revised and updated after the agency reviews and evaluates comments submitted by interested persons.

Freeze-drying (lyophilization) has been used successfully for the preservation and storage of many vaccines, microbial cultures and other labile biological products. Certain biological preparations are lyophilized in order to maintain integrity, potency and other properties of the product, when for that particular product other methods of preservation, such as freezing alone or addition of a preservative, have not been found to provide sufficient stability. Residual moisture has been the term used to describe the low level of surface water, usually from less than 1% to 5%, remaining in a freeze-dried vaccine or other biological product after the bulk of the aqueous solvent has been removed during the freeze-drying (vacuum sublimation) process. Levels of residual moisture should be sufficiently low so that, where applicable, the viability, immunologic potency and integrity of the product are not compromised over time. However, levels of residual moisture for certain products should not be so low that the properties of the product, i.e., viability, are compromised by overdrying (Greiff and Rightsel, Applied Microbiology, 16, 835-840, 1968). Overdrying may cause living cells to lose viability, cause the tertiary molecular structure of complex proteins to be altered with subsequent loss of activity, or remove monolayers of water from active sites on molecules which then can react with traces of oxygen and thus degrade. Each product needs to be evaluated on a case by case basis to determine the optimum residual moisture level.

Examples of freeze-dried biological products include Antihemophilic Factor (Human), Measles Virus Vaccine Live, Streptokinase, Alfa Interferon, Typhoid Vaccine, Meningococcal Polysaccharide Vaccine Groups A and C Combined, and Wasp Venom Allergenic Extract.

In practice, the freeze-dried product contains not only the freeze-dried biologic material, for example, attenuated measles virus in Measles Virus

Vaccine Live, but other residuals of the manufacture of the product such as media, buffer and any vehicle such as lactose, sucrose or sorbitol that has been added to the product to facilitate the freeze-drying process and cake formation, and to preserve the product's activity. Freeze-dried biological products may be heterogeneous mixtures that contain a mixture of proteins, polysaccharides, inorganic salts, water and other material. The water retention properties and types of water present may be different for each product. The different types of water have been referred to as bound, surface and trapped water with many other variations defined and alternate terms used. The term "moisture content" of the sample may represent water in different states because of its different physical and chemical interactions with the material and also because of the geometric configuration of the space it may occupy. Because of the different types of water that may exist in a freeze-dried biological product, different moisture results may be found when different methods are used for the determination of the moisture content of the sample.

Freeze-dried preparations are usually packaged in flame sealed glass ampoules or glass containers with rubber closures. These preparations are usually sealed under vacuum or nitrogen.

II. Testing Procedures; Results; Standards for Determining Residual Moisture

A. Testing Procedures

As specified in 21 CFR 610.13 of the biologics regulations, each lot of dried product shall be tested for residual moisture and shall meet and not exceed established limits as specified by an approved method on file in the product license application. As also specified in 21 CFR 610.13 the test for residual moisture may be exempted by the Director, Center for Biologics Evaluation and Research, when deemed not necessary for the continued safety, purity and potency of the product.

The contents of the freeze-dried final container of the biological product should be tested for residual moisture. The test methods that have been used and approved are the following:

1. Gravimetric Method (Loss on Drying)

The gravimetric or loss on drying test for residual moisture (Code of Federal Regulations, 21 CFR 610.13 (a), p. 52. U.S. Government Printing Office: Washington, D.C. , 1988; May, Wheeler and Grim, Cryobiology, 26,277-284, 1989) in freeze-dried biological products measures the maximum loss in weight of a weighed sample equilibrated to constant weight over anhydrous phosphorus pentoxide at a pressure of not more than 1 mm of mercury and a temperature of 20 °C to 30 °C for as long as it has been established is sufficient to result in a constant weight. The optimum sample size is 200 milligrams and is obtained from the contents of one or a pool of several final containers, depending upon the amount of sample per original sample. Samples are prepared for analysis in a low humidity glove box. The test is performed in a temperature and humidity controlled environment to prevent ambient humidity from interfering in the test procedure.

Experimental evidence indicates that this gravimetric method measures surface moisture and loosely bound water of hydration (May, et.al., Journal of Biological Standardization, 10, 249-259, 1982). Surface moisture is the classical definition of residual moisture.

Alternate methods to the gravimetric method have been approved for the determination of residual moisture.

2. Karl Fischer Methodology

The Karl Fischer method for moisture determination is one method that has been approved for the assay of some biologics. Iodine together with pyridine, sulfur dioxide and methanol form the Karl Fischer reagent which reacts quantitatively with water.
There are several traditional approaches to the use of the Karl Fischer reagent for the determination of moisture.

If sample size is large enough (approximately 40 mg), a sample dissolved in methanol may be titrated to a visual endpoint with Karl Fischer reagent by using a burette (United States Pharmacopoeia XXII/National Formulary XVII, 22th rev., p. 1619, Mack Publishing Co.: Easton, Pennsylvania, 1989).

Various electrochemical methods have been devised that combine titration with a burette with electrometric endpoint detection. An amperometric instrument introduced by Wernimont and Hopkinson (Ind. Eng. Chem., Analytical Ed., 15, 272-274, 1943) autotitrates the sample until an excess of iodine is sensed by the polarized electrodes and a "dead-stop" endpoint detection concludes the titration. With residual moisture values in biological products ranging from less than 1% to 5%, approximately 40 milligrams of a freeze-dried sample is required for titration using this type of instrumentation.

Instruments using coulometry eliminate the burette. Iodine is generated coulometrically to react with the water in the added sample in the presence of pyridine, sulfur dioxide and methanol in the reaction vessel (Meyer and Boyd, Analytical Chem., 31, 215-219, 1959). The ability of the coulometric Karl Fischer method to measure residual moisture in about 10 milligrams of a freeze-dried biological sample makes it the most practical Karl Fischer method for the determination of residual moisture in freeze-dried biological products in single dose final containers (May, et al., Journal of Biological Standardization, 10, 249-259, 1982). Manufacturers of Karl Fischer instruments are also providing a heating unit accessory in which the sample is heated and the moisture given-ff is carried by a gas from the sample and into the Karl Fischer vessel solution for titration. Care must be taken that the sample is not heated excessively as the decomposition of the sample also yields water and this would give a falsely high value for moisture content.

Special sample handling procedures should be employed. Samples sealed under vacuum should be opened to atmospheric pressure in a dry box before being weighed. Failure to release the vacuum would cause a false weight due to buoyancy when the vial or ampoule contains vacuum instead of air. Errors as large as 46 milligrams have been encountered in determining the weight of the final container plus sample when the vacuum was not released in a 50 mL vial before weighing.

The Karl Fischer apparatus should be enclosed in a dry box or other apparatus to prevent contamination of the sample and reagents by moisture in the surrounding air. Phosphorus pentoxide is used as a desiccant in the box. A small hygrometer is used to monitor the humidity within the dry box.

In one alternate technique to using a dry box to minimize interference from ambient humidity, a known amount of anhydrous methanol is added with a syringe to the freeze-dried biological product in a final container. The methanol should dissolve the sample. Known amounts of sample and methanol are withdrawn and added to the Karl Fischer titration vessel for moisture determination. The moisture content of the anhydrous methanol is determined as the blank. After the weight of sample in the final container is determined, the percent moisture in the biological product is calculated.

In another alternate technique "pyridine free" Karl Fischer reagent is used instead of the Karl Fischer reagent that contains pyridine. In the "pyridine free" reagent another amine is substituted for pyridine. This type of reagent is specified when it is used as part of a Karl Fischer alternate procedure.

Freeze-dried biological products cannot be analyzed by the Karl Fischer methodology when:

a.) materials that interfere with the Karl Fischer reagents, such as substances that bind iodine, are present in the matrix of the biological product, or

b.) the sample does not adequately dissolve in the Karl Fischer reagent, methanol or other solvent or

c.) the sample moisture does not adequately extract into these solvents.

Other methods must be used to determine residual moisture in such biological products.

3. Thermogravimetry and Thermogravimetry/Mass Spectrometry

Thermogravimetry (TG) has also been applied to the determination of residual moisture in freeze-dried biological products (May, et. al., Journal of Biological Standardization, 10, 249-259, 1982). The thermogravimetric method measures both surface and bound moisture in freeze-dried biological products. It is applied to the determination of moisture in samples that are small and below the sample sizes required for the gravimetric (200 mg) and Karl Fischer (approximately 10 mg) methods. The TG method can determine the moisture content in freeze-dried samples as small as two milligrams. TG methodology is used at the Center for Biologics Evaluation and Research as a second method to confirm Karl Fischer test results for freeze-dried products that have been tested especially when the Karl Fischer test results indicate that the sample has a failing moisture content.

Typically, the TG balance is enclosed in a Plexiglas™ dry box which contains phosphorus pentoxide to maintain a low humidity. The humidity is monitored by a portable hygrometer. The sample, balance parts and furnace are manipulated within the dry box with the use of flexible rubber gloves. For this procedure, the electrobalance must first be modified to prevent charge buildup from interfering with balance operation during analysis within the low humidity glove box. The quartz tube surrounding the sample pan and the Pyrex tube surrounding the counter weights are painted with gold paint. Wires connect the gold layer

to the electrical ground of the TG balance. Residual moisture is calculated from the weight-loss profile thermogram or from isothermal TG data.

TG transitions attributed to residual moisture are verified for samples with complex thermograms by thermogravimetry/mass spectrometry (TG/MS). The combination of thermogravimetry and mass spectrometry has proven to be effective by providing precise TG heating conditions and weight loss information along with mass spectral identification of volatiles evolved during the weight loss process. Mass spectra are taken of the TG off-gases continuously while the weight loss and rate of weight loss (differential thermogram, i.e., DTG) scans are recorded. The ion intensities of mass peaks 18 and 44 are monitored to show the changes in the amounts of water and carbon dioxide in the TG off-gases. When superimposed on the respective TG data, the mass spectral ion intensities verify the transition caused by moisture in the freeze-dried sample by differentiating between the water content of the sample and the water evolved from thermal decomposition of the sample, which coincides with the evolution of carbon dioxide (May, Wheeler, and Del Grosso, In : Compositional Analysis by Thermogravimetry, American Society for Testing and Materials Special Technical Publication #997, pp. 48-55, C. M. Earnest, Ed., American Society for Testing and Materials: Philadelphia, 1988).

4. Other Alternate Methodologies

Techniques involving gas chromatography and the Moisture Evolution Analyzer (Jewell, Workman and Zeleznick, Developments in Biological Standardization, 36, pp.181-189, S. Karger: Basel, 1977) as well as several variations of the gravimetric or loss on drying method, for example, those involving different times or temperatures, have been approved for use as alternate methods for the determination of residual moisture in freeze-dried biological products. These methods are not discussed in great detail because they are used less frequently than the previous three methods.

B. Results; Standards to be Met

For most products levels of residual moisture should be low, usually from less than 1.0 percent to 3.0 percent, so that the viability, immunologic potency and therefore the stability of the product is not compromised over time. However, for certain products it can be demonstrated that levels of residual moisture should not be so low that viability or other characteristics are compromised by overdrying. For certain products the residual moisture should not be so low that living cells lose viability, that the tertiary structure of complex proteins is impaired with subsequent loss of activity, or monolayers of water are removed from active sites on molecules which then can react with traces of oxygen and degrade.

The gravimetric method most accurately measures the surface moisture of the freeze-dried product, the original definition of residual moisture. The gravimetric method will also measure some loosely bound water of hydration.

The general recommendation for most products is that the residual moisture should not exceed 1.0 percent by the gravimetric method.
Typical exceptions to the 1.0 percent recommendation by the gravimetric method are that the residual moisture content should not exceed:
1.) 1.5 percent for BCG vaccine

2.) 2.0 percent for Measles Virus Vaccine Live, Rubella Virus Vaccine Live and Antihemophilic Factor (Human)
3.) 3.0 percent for Thrombin and Streptokinase, and
4.) 4.5 percent for Antibody to Hepatitis B Surface Antigen for the Reversed Passive Hemagglutination Test.

The Karl Fischer method measures surface moisture and most types of bound water.
For some products this means that the Karl Fischer method will measure more moisture than the gravimetric method.

Typical exceptions to the 1.0 percent limit by the Karl Fischer method are that the residual moisture content and other volatile substances
1. should not exceed 3.0 percent for one manufacturer's Measles Virus Vaccine Live, Alfa Interferon, Rabies Vaccine, and
2. should not exceed 4.0 percent for one manufacturer's Asparaginase.

Limits other than 1.0 percent are set for residual moisture and other volatile substances when;
1. adequate stability data for the product are submitted that demonstrate that the safety, purity and potency of the product is maintained throughout the product's dating period at the alternate moisture limit, or
2. data are submitted comparing residual moisture results by the gravimetric method, already established for the product, to higher or lower residual moisture results obtained by a method other than the gravimetric method in accordance with 21 CFR 610.9, Equivalent Methods and Processes.

One freeze-dried product, Limulus Amebocyte Lysate, which is not an injectable biological product, has been exempted from the moisture regulation when the amount of sample in the final container is less than 3 milligrams. In this case, a potency test must be done every four months throughout the dating period and submitted to the Director, Center for Biologics Evaluation and Research. This exemption is provided for in the regulations for Limulus Amebocyte Lysate, 21 CFR 660.103 (g)(2)(i).

During validation studies for each product and changes such as vial size manufacturers should test several samples from each shelf in the lyophilizer and several positions on each shelf to determine that the lot meets the residual moisture specification for the product.