rial after the step are +a, the 95 percent confidence limits for the reduction factor are

$$\pm\sqrt{S^2 + a^2}$$

B. Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$$p = ((V-v)/V)^n$$

where V (L) is the overall volume of the material to be tested; v (L) is the volume of the sample; and n is the absolute number of infectious particles statistically distributed in V.

If V >> v, this equation can be approximated by the Poisson distribution:

$$p = e^{-cv}$$

where c is the concentration of infectious particles per L.

$$\text{or, } c = \ln p/-v$$

As an example, if a sample volume of 1 mL is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

c	10	10	1,000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

APPENDIX 4

Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material: vol v′; titer $10^{a'}$;
virus load: $(v')(10^{a})$,
Final material: vol v″; titer $10^{a''}$;
virus load: $(v'')(10^{a''})$,
the individual reduction factors Ri are calculated according to

$10^{Ri} = (v')(10^{a'}) / (v'')(10^{a''})$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

APPENDIX 5

Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

I. Assumptions

Measured or estimated concentration of virus in cell culture harvest = 10^6/mL

Calculated viral clearance factor = > 10^{15}

Volume of culture harvest needed to make a dose of product = 1 L (10^3 mL)

II. Calculation of Estimated Particles/Dose

$$\frac{\left(10^6 \text{ virus units/mL}\right)\times\left(10^3 \text{ mL/dose}\right)}{\text{Clearance factor} > 10^{15}}$$

$$= \frac{10^9 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}}$$

$$= < 10^{-6} \text{ particles/dose}$$

Therefore, less than one particle per million doses would be expected.

⟨1051⟩ CLEANING GLASS APPARATUS

Success in conducting many Pharmacopeial assays and tests depends upon the utmost cleanliness of the glassware apparatus used. For example, the accuracy of the assays of heparin sodium and vitamin B_{12} activity, as well as the pyrogen and total organic carbon tests, are particularly dependent upon scrupulously clean glassware.

One effective method used in the past for cleaning glassware is the application of hot nitric acid. A second traditional method for removing organic matter that does not require heat is the use of a chromic acid–sulfuric acid mixture. However, the chromic acid wash is not recommended because of the hazardous and toxic nature of the material.

Several safer alternatives, including the use of cleansing agents, such as trisodium phosphate and synthetic detergents, have proven highly useful, but require prolonged rinsing. It may be useful to rinse with diluted nitric or sulfuric acid prior to rinsing with water. This operation will facilitate removal of residual alkaline material.

For optical measurements, special care is required for cleaning containers, but the use of both chromic acid and highly alkaline solutions should be avoided.

Effective removal of organic matter is very important for testing pharmaceutical waters in accordance with the general test chapter *Total Organic Carbon* ⟨643⟩. It has been demonstrated that an alkaline detergent with potassium hy-

droxide as the primary ingredient* leaves the least amount of organic matter residuals. Heating in a muffle furnace produces comparable results and is the least labor-intensive procedure; however, it requires specialized equipment.

In all cases, it is important to verify that the cleaning procedure is appropriate for the particular test or assay being undertaken. This can be accomplished via blank runs, scientific judgments, residuals data from cleansing agent and detergent manufacturers, or other controls. Specifically, special care is required for cleaning containers for optical measurement applications; the use of highly alkaline and the no longer recommended chromic acid solutions should be avoided. Finally, a statement should be included in the cleaning protocol describing how the success of the cleaning procedure will be assessed.

⟨1052⟩ BIOTECHNOLOGY-DERIVED ARTICLES—AMINO ACID ANALYSIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by amino acid analysis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ✦. The footnote below is in the *USP* but is not in the *EP* or *JP*. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩, and *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩.

INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

*CIP 100; available from Steris Corporation, Mentor, Ohio, 44060-1824.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available* for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino

*✦Suitable standards may be obtained from NIST (Gaithersburg, MD), Beckman Instruments (Fullerton, CA), Sigma Chemical (St. Louis, MO), Pierce (Rockford, IL), or Agilent (Palo Alto, CA).✦