Pharmaceutical Analysis for Small Molecules

Pharmaceutical analysis is integral for the determination of the quality – including identity, purity, and strength – of drugs. In addition, related studies and programs are needed to assure the performance of the drug products.

Addressing these challenges requires analysts with an understanding of analytical chemistry and a thorough appreciation of pharmaceutical requirements.

Based on analytical results, product quality at each stage – whether this be raw material, in-process material, intermediate, drug substance, or drug product – determines the next course of action. In broader terms, the analytical results determine product quality in terms of a drug's safety and efficacy.

From a good manufacturing practice (GMP) perspective, product quality, and the delivery of expected quality attributes, depends on the quality of analysis.

Quality of the analytical results depends on various factors, the most important of which are:

- Analyst
- Instrument
- Method

Here we present the most important techniques used for the characterization of products, the parameters of weighting balance to be considered during qualification, and their importance.

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High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is the most widely used instrumental technology (35%) in pharmaceutical industry analytical laboratories.[1-5]

HPLC is most suitable for analysis of polar, water-soluble with relatively larger molecular weight, compounds. HPLC with polar mobile phase (e.g. water/acetonitrile) is used for analysis of most pharmaceutical products. [6]

The use of detectors, including: UV, fluorescence, refractive index, electrochemical, laser light scattering, aerosol-based detector, conductivity, and mass spectrometer, has extended the applicability of HPLC for characterization of pharmaceutical products.

The coupling of HPLC with other spectroscopic techniques such as FTIR and NMR has also provided an excellent combination of separation and structure elucidation of compounds of interest.[2]

The four major modes of HPLC are:

- Normal-phase chromatography (NPC)
- Reversed-phase chromatography (RPC)
- Ion-exchange chromatography (IEC)
- Size-exclusion chromatography (SEC)

NPC is also known as adsorption chromatography. In this mode, the stationary phase (typically silica and alumina) is more polar than the mobile phase. Therefore, the polar compounds are absorbed more strongly by the stationary phase and elute later than the nonpolar compounds.

RPC is more common than NPC. In this mode, the stationary phase (typically octadecylsilane or shorter alkyl chains) is less polar than the mobile phase. Therefore, the nonpolar compounds are adsorbed (retained) more by the stationary phase and elute later.

In IEC, the stationary phase contains ionic groups such as sulfonate (SO_3^-) or quaternary ammonium (NR_3^+), which are bonded to silica or resin.

The mechanism of separation is based on exchange (displacement) of the counter ions (e.g., Na^+ or OH^-) with the ionic analyte (A^+ or A^-) in the mobile phase as follows:

Cation exchanger:

Resin S – + O N a A
Resin S – + O A Na
– + + – + + 3 3 (3.1)

Anion exchanger:

Resin – + NR OH A
Resin – + NR A OH
+ – – + – – 3 3 (3.2)

SEC separates the compounds based on their molecular sizes. A porous material is used for the stationary phase. The larger analytes elute first because these are excluded from the pores. On the other hand, the smaller molecules diffuse into the pores and thus elute later.

Other chromatographic methods for specific applications or separation include:[2, 4, 7]

- **Chiral chromatography:** For the separation of enantiomers based on chiral stationary phase or chiral mobile phase.
- **Ion-pair chromatography:** For the separation of both ionic and neutral compounds by using ion-pairing reagents to make the samples suitable for common reverse-phase HPLC analysis.
- **Affinity chromatography:** Separation is based on a stationary phase containing a receptor specific for certain samples such as proteins and lipids.

The most recent trend in the development of HPLC instrumentation is Ultra High-Performance Liquid Chromatography (UHPLC), which provides faster analysis and higher resolution.

Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical tool and has had significant impact on the analysis of pharmaceutical products.[8, 9] The principle of MS is based on production of gas-phase ions from the sample in an electric or magnetic field and separation of ions due to the mass-to-charge ratio. It is used for both quantitative and qualitative analysis and is excellent for structure elucidation.

Due to the wide application of MS during the last couple of decades, the databases of spectra for many compounds of interest are available and easily used for comparison. The coupling of MS detector with other separation techniques such as GC, HPLC, SFC, and CE has also increased the versatility and application of this technique.

Loss on Drying (LOD)

This procedure determines the amount of volatile matter driven off under specified conditions.[10] This simple procedure involves heating the sample under specified conditions (temperature, pressure, time).

The sample is weighed before and after. The difference in sample weight is the volatile content. The volatile compounds include water and potential volatile organic compounds such as solvents that may have been used in the manufacturing of the drug substance or products.

The limit of water in pharmaceutical substances is normally controlled to minimize the product stability or microbial growth. The residual solvents in pharmaceutical products are also controlled due to the toxic nature of certain solvents. This determination is also needed to calculate, as appropriate, the results for assay on the dried basis for comparison to the corresponding acceptance criteria.

Loss on Ignition (LOI)

The procedure determines the amount of test material volatilized under specified condition of temperature.[11] The sample is ignited for a period of time to reach a constant weight. The difference in weight before and after the period is the loss due to ignition. If needed, the assay result needs to be corrected for the percentage of LOI to be consistent with the corresponding acceptance criteria.

Water Determination

The amount of water (moisture) is commonly determined and controlled in the pharmaceutical ingredients for product quality and stability.[12, 13] The most common procedure for the determination of the amount of water is the Karl Fischer Titration. Both volumetric and coulometric Karl Fischer titrations are used with the latter procedure providing more sensitivity for the determination of trace amounts of water.

Residue on Ignition (ROI) or Sulfated Ash

Here, the amount of residual material not volatilized from a sample is determined when it is ignited at a specified high temperature in the presence of sulfuric acid.[14, 15] The sample is ignited at a high temperature to a constant weight.

The difference between the weight before and after this ignition period corresponds to the amount of residue or sulfated ash. This amount usually corresponds to the content of inorganic impurities in an organic substance.

Analytical Instrument Qualification

The main goal in qualifying laboratory equipment is to ensure the validity of data. This calls for a robust instrument qualification program. Qualification involves a science-based approach to provide documented evidence that the instrument is capable of consistently operating within established limits and tolerances.

Here, we present the importance of a weighing balance and the parameters that should be considered during its qualification.

Laboratory Balances

LEVELING

Ensures that the balance is stable from all four corners as well as the center. The readings observed should become stabilized within the typical time required.

REPEATABILITY

The closeness of agreement for successive measurements of the same mass.

PRECISION

The smallest amount of mass difference that a balance is capable of resolving.

REPRODUCIBILITY

The closeness of agreement of measured values when measurements of a given mass are repeated over a period of time. Reproducibility may be affected by hysteresis, for example.

INTERNAL CALIBRATION/ ADJUSTMENT

Motordriven comparison with built-in calibration weights. Internal calibration is more important when there are frequent condition changes (temperature variation, power failure, etc.) where the balance is installed.

HYSTERESIS

Any difference in the measured mass as a function of the history of the balance operation, e.g., difference in measured mass when the last measured mass was larger than the present measurement vs. the measurement when it was smaller.

ACCURACY

The degree to which a measured value agrees with the true value.

VERIFICATION

External verification with calibrated weights ensures the displayed weight value, calibrated weight, and internal balance weights are in line – thus ensuring accuracy of balances during daily usage.

ECCENTRICITY

Weighing at least 30% of maximum weight capacity of the balance, or maximum weight to be used, on the balance on different locations of the weighing pan. This ensures weight values obtained are within the acceptance criteria during routine usage.

LINEARITY

Degree to which measured values of a successive set of standard masses – weighed on the balance across the entire operating range of the balance – approximates a straight line. Some balances are designed to improve linearity by operating in two or more separately calibrated ranges. The user selects the range of operation.

DRIFT TEST

Drift is a progressive change in display (continuously upward or downward) of the digital readouts of balance, which means that weight readouts are not stable.

READABILITY

Value of the smallest mass unit that can be read without estimation. In digital instruments, the smallest displayed digit does not always have a unit increment. Some balances increment the last digit. Other balances incorporate a vernier or micrometer to subdivide the smallest scale division. In such cases, the smallest graduation represents the balance's readability.

Weighing Solutions by Sartorius

Weighing in a regulated environment can be complex, with stringent controls required to deliver the accuracy and reliability of results required by the pharmaceutical industry. One of the technologies helping to achieve this accuracy is the Cubis® II balance series from Sartorius.

All weighing modules are designed for intuitive operation, aided by intelligent diagnostic systems, guaranteeing a higher degree of repeatability for different workflows while lowering the probability of human error. It offers modern interfaces, pharmaceutical and GxP compliance, including data handling, data integrity and connectivity, ergonomic sample handling, easy process integration, and unlimited communication at the highest level of accuracy and precision.

Find out more:
https://www.sartorius.com/en/products/weighing/laboratory-balances/cubis-ii

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Microbiological Methods

Determining the purity and safety of nonsterile and sterile pharmaceutical products is related to the Federal Food, Drugs, and Cosmetics Act – which defines adulteration of a product that does not conform to microbiological tests included in the US Pharmacopeia[16] – as well as the concept of mislabeling.

Additional microbiological tests can be operational if they are included in approved new drug applications (NDAs) or biologics license applications (BLAs).

Microbiological testing in pharmaceutical products is different from physicochemical tests in terms of accuracy and precision. Accuracy of a physicochemical method, such as HPLC, is typically less than 5% as molecules are uniformly dispersed in products.

Because microorganisms are not distributed uniformly in pharmaceutical products, most microbiological tests are estimates of the number of microorganisms in a sample, as the conditions for microbiological tests are compromised.

There are two types of microbial limit tests: one designed to determine the enumeration of microorganisms in a sample, and another to determine the presence/absence of specified microorganisms.

Microbial Limit Tests

Enumeration via a Plate Count

Purpose of the Test: To determine the quantitative enumeration of mesophilic bacteria that may grow under aerobic conditions.[17-19]

Approach Used: The ability to detect bacteria in the presence of the product must be established – requiring antimicrobial substances to be removed, inactivated, or neutralized. Capabilities of the media used to support growth of bacteria must be determined by testing the growth promotion using test microorganisms such as Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans, and A. brasiliensis.

Two media are used:

1. Soybeanecasein digest agar incubated at 30–35°C for 3–5 days for the determination of the total aerobic microbial count (TAMC).
2. Sabouraud dextrose agar at 20–25°C for 5–7 days for the determination of the total yeast and mold count (TYMC).

The preparation of samples depends on the physical characteristics of the product.

Limitations of the Test: TAMC and TYMC are obtained under standardized temperature and time – excluding microorganisms that cannot be detected under specified conditions. TAMC is the count of CFUs that will grow in the soybeancasein digest agar, even if they are yeasts and molds. TYMC is the count of colony-forming units (CFUs) that will grow in the Sabouraud dextrose agar, even if they are bacteria. This double counting is a limitation.

Interpretations of Results: Since counts are accepted as microbiological quality of the raw material and final products – and taking into consideration the inherent variability of microbial counts – a limit of 10 cfu/g will be extended by a factor of 2 to a count of 20 cfu/g and still be acceptable as being within the limit.[20]

Membrane Filtration Method

Purpose of the Test: To estimate the microbial count of products that could not be estimated using the membrane filtration procedure or the plate count procedure.

Approach Used: At least three serial 10-fold dilutions of the samples prepared as earlier. For each dilution, add 1g or 1mL to 9–10mL of soybeancasein broth and incubate at 30–35°C for NMT 3 days.

Limiations of the Test: Not an accurate estimate of TYMC and less precise than membrane filtration and plate count procedures. Surface active and inactivators of antimicrobial agents need to be added. For some products, visual determination of growth could be difficult. In this case, subculture the content of the doubtful tubes in broth or on plate agar incubated for 1–2 days at the same temperature. The MPN test is generally used when the bioburden of the product is very low.

Interpretation of the Test: The MPN per gram or milliliter is read in the appropriate MPN table.

Most Probable Number (MPN) Procedure

Purpose of the Test: To estimate the microbial count of products that could not be estimated using the membrane filtration procedure or the plate count procedure.

Approach Used: At least three serial 10-fold dilutions of the samples prepared as earlier. For each dilution, add 1g or 1mL to 9–10mL of soybean-casein broth and incubate at 30–35°C for NMT 3 days.

Limitations of the Test: Not an accurate estimate of TYMC and less precise than the membrane filtration and the plate count procedures. Surface active and inactivators of antimicrobial agents need to be added. For some products, visual determination of growth could be difficult. In this case, you can subculture the content of the doubtful tubes in broth or on plate agar incubated for 1–2 days at the same temperature. The MPN test is generally used when the bioburden of the product is very low.

Interpretation of Results: The MPN per gram or milliliter is read in the appropriate MPN table.

Tests for Specified Microorganisms

Purpose of the Test: Determine the presence or absence of specified microorganisms in raw materials or nonsterile finished products.

Approach Used: Samples are prepared as indicated in enumeration tests. Antimicrobial activity is removed or neutralized. Suitability of media used must be validated using American Type Culture Collection (ATCC) microorganisms: S. aureus, P. aeruginosa, E. coli, Salmonella enterica, Clostridium sporogenes, and C. albicans.[24-26] The test principle is to allow the growth of the specified microorganism, while inhibiting the growth of other microorganisms.

Limitations of the Test: Absence/presence of specified microorganisms is tested under the prescribed conditions of temperature, media used, and length of incubation. Some variants of the specified microorganisms might not be favored by the standardized conditions. Not all raw materials and nonsterile products need to be tested for the absence/ presence of all the specified microorganisms.

Interpretation of Results: Absence of specified microoorganisms per g or mL of product might require further identification of the isolated bacteria using commercially available systems.

Sterility Test

Purpose of the Test: This test fulfills the requirement that a product labeled sterile conforms to the label.[21-23]

Approach Used: A specified number of samples are tested, based on batch size or the type of product. Lack of growth indicates the sample is sterile. Fluid thioglycolate (which favors the growth of anaerobes and some aerobic microorganisms when incubated at 30–35 °C for 14 days) and soybeanecasein digest (which favors the growth of fungi and aerobic bacteria when incubated for 14 days at 20–25°C) are used as media.

Growth promotion testing of media needs to be validated using ATCC strains of S. aureus, B. subtilis, P. aeruginosa, C. sporogenes, C. albicans, and Aspergillus brasiliensis. Membrane filtration is recommended. Regardless of procedure, antimicrobial elements present in the sample before incubating must be neutralized/removed. The method of sample preparation varies depending on the characteristics and nature of the products.

Limitations of the Test: The test is a compliance test and will not indicate if the whole batch is sterile. Limitations of media compositions and preparations of samples, as well as the temperature of incubation, are a compromise that would result in some 'sterile' products to contain microorganisms that do not grow under test conditions.

Samples are observed at regular intervals during the 14-day incubation. As soon as a positive growth occurs, investigation is started to ascribe the cause of the positive growth. Identification of positive microorganisms is the first step to determine whether they are survivors of the sterilization process or a contamination during testing.

Another limitation is that the sample tested is not a statistical sample, meaning that it is not possible to infer sterility of the whole batch.

Interpretation of the Results: When the product tested interferes with the visual assertion of growth, it is necessary to subculture the positive samples to a fresh medium and incubate for no less than 4 days. Lack of evidence of growth indicates that the samples comply with the sterility test. If microbial growth is found, it does not pass the sterility test. There are no provisions for retesting the batch unless the first sterility test is invalidated. If invalidated, the test should be started using the same number of samples as original testing.