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▲⟨1430.7⟩ ANALYTICAL METHODOLOGIES BASED ON SCATTERING PHENOMENA—NEPHELOMETRY AND TURBIDIMETRY

1. INTRODUCTION

Nephelometry and turbidimetry are based on the direct (nephelometry) or indirect (turbidimetry) measurement of the scattered light intensity of an incident light beam from a monochromatic light source. In the rate nephelometry technique, the change of the scattered light intensity is measured as a function of time. Turbidimetry or nephelometry may be useful for the measurement of precipitates formed by the interaction of highly dilute solutions of reagents or other particulate matter such as suspensions of bacterial cells. All variables must be carefully controlled to achieve consistent results. Where such control is possible, extremely dilute suspensions may be measured.

2. THEORY

2.1 Nephelometry and Turbidimetry

For all relevant terms and definitions see *Nephelometry and Turbidimetry* ⟨855⟩. Both techniques are governed by the same equation, which describes the (time-averaged) intensity of the light beam as it interacts with matter along its path, i.e., when the incident light detected at an angle of zero decreases exponentially with the thickness, x, of the layer of material. See *Equation 1*. (For more information, see *Analytical Methodologies Based on Scattering Phenomena—General* ⟨1430⟩ and ⟨855⟩.)

$$I = I_0 e^{-\tau x} \quad (1)$$

2.2 Rate Nephelometry

As discussed above, the scattered light intensity when a sample is submitted to a constant incident monochromatic light beam depends on the number and size of the particles that scatter the light. If either or both of these factors change over time as a result of a chemical or physical process, the cumulative scattered light intensity at a given direction (usually 90° to the incident light direction) will change accordingly. Thus, rate nephelometry measures the change, or rate of change, in the intensity of light scattered during the formation of complexes (e.g., antigen-antibody) in solution. A correlation between the physicochemical changes and the scattered light intensity can be established and used to evaluate the property changes in the samples under test.

The extent of formation of light scattering complexes depends on concentrations of complex formation entities, e.g., antibodies and antigen molecules for immunonephelometry. The maximum rate response occurs when the antibody and antigen are in optimal proportions. Generally a fixed amount of antibody is used. Standard applications typically use 670-nm lasers with the detector aligned at 90° to the incident light direction, and the scattered signal is measured at 5-s intervals. System software calculates the rate of change at the end of reaction. The rate of change is converted to concentration by the system software.

2.3 Rate Turbidimetry

Rate turbidimetric methods measure the decrease in intensity of light as it passes through a solution while complexes form. The light source is a light emitting diode (LED) at 940 nm. Turbidimetric measurements are made at 0° from the incident beam. The intensity decrease is converted to an increasing scatter signal by *Equation 2*.

$$\text{Scatter signal} = -\log_{10}\left|\frac{\text{light intensity}}{\text{initial light intensity}}\right| \quad (2)$$

3. INSTRUMENTATION

A schematic diagram and principles of operation of a nephelometric (turbidimetric) instrument are shown in *Nephelometry and Turbidimetry* ⟨855⟩, *Figure 1*. A typical instrumental design dedicated for rate nephelometric measurement is shown below in *Figure 1*. The main components are: a light source that is usually a laser (635 nm) with a highly collimated beam to avoid stray light, a sample cell, and a light detector. The laser beam passes through the sample well into a scattered light detector assembly. Typically an integrating sphere (Ulbricht sphere), which helps improve the signal-to-noise ratio and enhance the signal for very low concentrations, is attached to a photodiode and used as the detector in rate nephelometry instruments. The unscattered light (light not deflected by particles) passes straight through the sphere and no signal is generated. Light scattered by particles is reflected around the interior of the sphere and then detected by the photodiode detector. The angle of detection of the scattered light is usually 90°, which is, in general, suitable for smaller aggregates ($d \leq \lambda/20$). A light trap is attached to the Ulbricht sphere at the opposite side of the light beam entrance window to absorb the unscattered light. The amount of scattered light is proportional to the concentration of particles. Some instruments can perform both rate nephelometry and rate turbidimetry measurements. These instruments are equipped with a laser source at 670 nm with 90° scatter detection for nephelometric measurements and an LED source at 940 nm with 0° scatter detection for turbidimetric measurements.

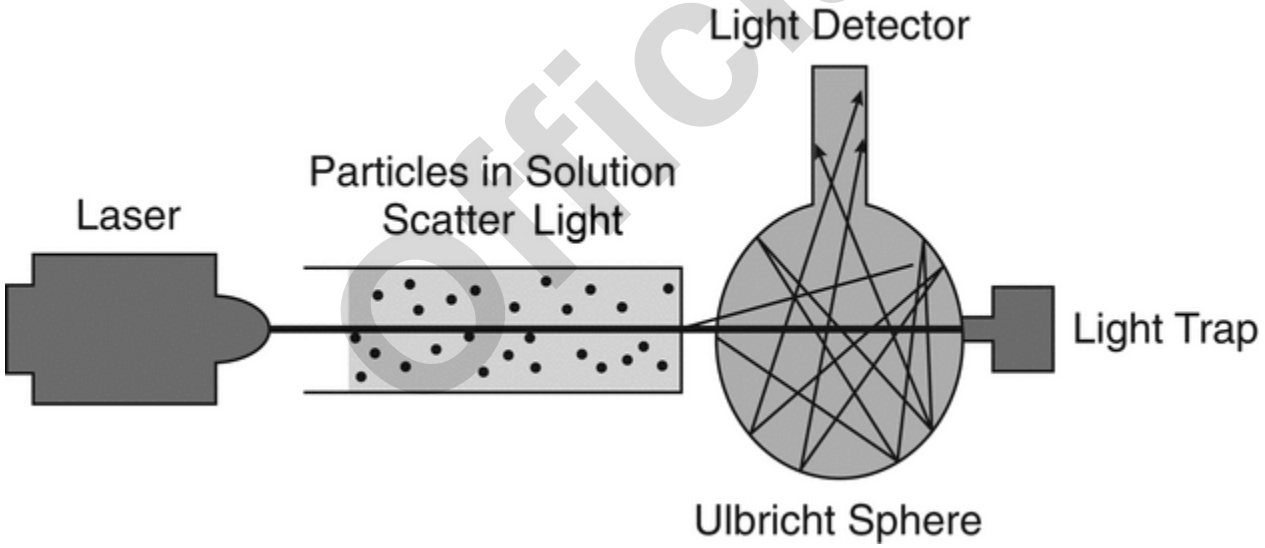

Figure 1. Schematic of a typical nephelometer.

3.1 Rate Nephelometry Instrumental Parameter Settings

The most important rate nephelometric system parameter settings are:

- **Acquisition time:** Reaction cuvettes, housed on a reaction wheel, are scanned in cycles of 5 s, for total reaction times ranging from 1.5 to 10 min. Acquisition time commences upon completion of a 5-min preset baseline acquisition period, also in 5-s cycles. Most reactions in nephelometric applications achieve peak rates within 2 min, whereas those used in turbidimetric applications require longer reaction times (3 min or longer).
- **Gain settings:** These settings are related to signal amplification. Generally, decreasing analyte concentration requires a concomitant increase in gain to maintain sensitivity.
- **Onboard compartment temperatures:** Reaction wheel temperature is maintained at 37° to enhance aggregate formation. The reagent compartment is cooled to extend stability of antibody reagents, whereas the sample compartment temperature is ambient.
- **Laser beam width and intensity adjustment:** If applicable, the laser beam width and intensity are adjustable in some instruments and can be optimized to obtain the best scattered signal response.

3.2 Calibration of Rate Nephelometric Instruments

Calibration of the rate nephelometer is typically a multitier process consisting of preprogrammed and user-defined components. The preprogrammed component is executed with each instrument run and is comprised of measurements of an onboard reference cuvette of known scatter values. The user-defined component relies on protein controls. Certified protein controls are routinely used for initial instrument calibration and may be utilized for periodic instrument performance qualification. For method calibration and performance monitoring, specially formulated product-relevant reference materials are used.

Data assessment is accomplished by comparison against the control manufacturer's published range, peer data, and/or internally established acceptance criteria. Each analytical run typically includes a calibration curve of reference material and, where applicable, a single concentration of control, each selected to represent the product of interest. The validity of an instrument run is dependent on satisfaction of acceptance criteria established through method development and statistical analysis of temporal method performance.

4. APPLICATIONS

⟨855⟩ gives a general overview of the applications of turbidimetric and nephelometric techniques including the rate nephelometry and rate turbidimetry (see *Nephelometry and Turbidimetry* ⟨855⟩, *3. Applications*). The following discussions represent additional and more detailed information on the applications of rate nephelometry and rate turbidimetry in biopharmaceutical industry and clinical laboratories, which are summarized in *Table 1*.

In bacterial polysaccharide-containing vaccine analysis applications, the reaction of excess of (bivalent, polyvalent) monoclonal or polyclonal antibodies with high molecular weight bacterial polysaccharides—such as those from *Haemophilus influenzae* type b (Hib vaccine), *Neisseria meningitidis* (meningococcal vaccine) or *Streptococcus pneumoniae* (pneumococcal vaccine)—causes crosslinking and the formation of high molecular weight aggregates that scatter light. Therefore, the rate at which aggregates develop allows the polysaccharides, free or conjugated, to be quantified. Endpoint nephelometry is an alternative application in which the endpoint of the reaction is determined in the analysis.

In the case of multivalent vaccines, the specificity of the antibody used allows the selective detection and quantification of a specific polysaccharide, and the use of different antibodies with different specificities allows multiple components to be quantified.

Immunonephelometry for biologics analysis is based on antibody-antigen immune complex formation (immunoprecipitation). Formation of the immunoprecipitation is dependent on the presence of the antigen and antibody molecules in optimal proportions. Quantitative methods are based on the principle that when the amount of antibody is held at a constant excess, the degree of immunoprecipitation will increase with increasing antigen content up to a maximal amount. A standard curve can be generated by maintaining a constant level of antibody and measuring the light scatter with increased concentrations of antigen. Due to the reliance upon antibody and antigenic epitope recognition, the above applications are employed for characterization purposes, monitoring of functional group content, stability, and assessment of lot-to-lot consistency, i.e., for quantification of the antigenic saccharide content in components and/or products of vaccine manufacture. Such components and/or products include: (a) bulk monovalent glycoconjugate immunogens; (b) bulk polyvalent glycoconjugate immunogen blends; (c) bulk polyvalent polysaccharide blends; (d) glycoconjugate vaccine final fills; and (e) polysaccharide vaccine final fills. Rate nephelometric analysis of the multicomponent product is generally employed in conjunction with direct saccharide quantitation of the individual components by orthogonal methods.

Turbidimetry is widely used for measurement of activity in recombinant coagulation factor and other hemophilia replacement therapies, as well as for patient monitoring in clinical hemostasis laboratories. The turbidimeter monitors the transmittance of light at 670 nm as fibrin converts into fibrinogen during clot formation. Calibrators in the form of reference plasmas, generally traceable to World Health Organization standards, serve for instrument calibration and may be used for relative activity determination for the analyzed product.

Table 1. Examples of Analytical Applications

Turbidimetry	Nephelometry
Immunoagglutination assay: detection of Chagas disease	Polysaccharide quantitation: pneumococcal vaccine
Multiple latex immunoagglutination assays	Polysaccharide quantitation: meningococcal vaccine
Fibrinogen assays	Relative molecular size (nephelometry coupled with high pressure size exclusion chromatography)

5. METHOD DEVELOPMENT

Thorough method development encompasses designing the method to fit the application through assessing a complex set of conditions including choice of reagents, standards, and controls; sample handling; and instrument settings. The following areas should be considered during method development in order to determine operating ranges and limits.

5.1 Method Design

Competitive versus noncompetitive assay modes are available. The option chosen will be dictated by the sample molecular weight/size and concentration of analyte to be analyzed; where noncompetitive immunoprecipitation mode is preferred for

molecular weight (MW) > 10^4 Da. Sample concentration directs the selection of instrument gain, where lower concentrations require higher gain levels.

For each method, the optimal acquisition time, sequence of administration of components, and gain level must be determined.

Sample volumes will dictate the type and size of sample containers.

A precision study should be performed during development to determine the replication testing scheme for samples in order to obtain the desired method precision (see *Analytical Data—Interpretation and Treatment* ⟨1010⟩).

A response curve between the analyte concentration and measured light scattering unit (LSU) values (or antigenicities calculated from LSU values) is prepared from standard solutions at concentrations encompassing the anticipated concentration of the test solution. The standard curve is then evaluated using appropriate statistical methods, such as least-squares regression.

5.2 Sample Considerations

Care must be taken to ensure samples, as with all other components, are essentially free from particulates and air bubbles in order to minimize nonspecific contributions to light scattering and liquid level sensing errors, respectively. Analytes such as monovalent polysaccharide-protein conjugates are diluted in several steps to a single point targeting the midpoint of an established standard curve.

Samples that contain adjuvants require solubilization of the adjuvant, carefully controlled and replicated with the reference material. Select applications, such as the specific analysis of adjuvant-bound antigenicity or the separation of conjugated antigenicity from free antigenicity, may require additional manipulations such as pH adjustment, preadsorption with Excel adjuvant, or temperature treatment prior to nephelometric analysis. If adjuvant solubilization is used for the sample, then the respective reference material must be subjected to the same treatment. Solubilization should be tightly controlled to minimize variability.

Specific pretreatments may need to be developed depending on the type of antigen. For example, polysaccharide-carrier protein conjugates may be analyzed for bound, conjugated, free, or total polysaccharide content. Example pretreatments include pH adjustment, adsorption with excess adjuvant, temperature treatment, and physical separation through centrifugation.

Cross-reactivity between component analytes may also need to be addressed during reagent selection (see the antibody discussion under *5.3 Reagents*).

5.3 Reagents

The choice of reaction buffer is dictated by compatibility with the analyte formulation buffer. Presence of aggregation enhancers or inhibitors should be considered. Interactions with containers such as glass test tubes may need to be considered. Detergent may be required for certain analytes, and the type and concentration must be determined experimentally.

Polyclonal antibodies are preferred for rate nephelometry as they are typically optimal for appropriate lattice formation; although monoclonal antibodies are frequently required for detection of specific functional groups. All antibodies must be sufficiently specific for the analysis of individual product components and of appropriate titer and avidity.

5.4 Method Specific Reference Standards

Reference materials are carefully selected from process representative batches, formulated, and packaged to optimally mimic the analyte of interest. Reference samples and standards must be treated equally.

5.5 Controls

Both positive and negative controls are likewise selected as product representative materials that may be formulated for extended stability via freezing or lyophilization.

5.6 Robustness

Method robustness assessments should include the following:

- Instrument parameters, e.g., gain (signal amplification), reaction time (interval between addition of last reagent and measurement)
- Concentration optimums
    - Antigen
    - Antibody
- Mix times
- Selectivity (lack of cross-reactivity) of antibodies
- Reagent use/hold times
- Type of ions, ionic strength of solution

6. METHOD VALIDATION

In addition to generally applicable validation characteristics as described in *Validation of Compendial Procedures* ⟨1225⟩, the following aspects are recommended for consideration in the context of nephelometry and/or turbidimetry:

- **Accuracy:** Quantitation of polysaccharides determined by assessment of known values generated with an orthogonal validated assay.
- **Linearity:** Should be assessed in both reference standards and analyte samples.
  - Standard curve required for each type of polysaccharide being assessed.
  - Standard polysaccharide concentration determined by alternative validated method (e.g., meningococcal serogroup C polysaccharide concentration determined by the sialic acid colorimetric method of Svennerholm.)
- **Lifecycle considerations:** Appropriate performance parameters are trended over time and utilized for bridging of new reference standards and for assessment of period of use of antibodies/reference standards/controls.
- **System suitability:** Positive control is assessed at the validated limits of adjustable instrument parameters. Results for the positive control must be within its defined range.

7. DATA ANALYSIS

A calibration curve is generated from the duplicate readings for the reference standard versus the standard concentration (from a validated orthogonal assay, e.g., nephelometric polysaccharide quantitation standard values from suitable sugar-specific colorimetric methods, i.e., hexose, uronic acid, sialic acid, or PABA assays.)

- Percent difference between duplicate readings for standards, controls, and samples is calculated.
- Antigenicity is calculated, in μg/mL, using sample LSU values and the slope and the intercept of the standard curve as shown in *Equation 3*:
  Antigenicity = $(\bar{A}_v - y_{int})/S$ (3)

$\bar{A}_v$ = average sample values (LSU)
y_{int} = *y*-intercept (LSU)
S = slope$\left(\frac{\text{LSU} \cdot \text{mL}}{\mu\text{g}}\right)$

Acceptance criteria for replicate measurements of each standard solution and/or sample (in LSU) should be developed such that valid results meet the desired accuracy and precision of the method, e.g., linearity, slope, and intercept may be monitored and assessed against acceptance criteria during routine laboratory use. Acceptance criteria shall be established for slope and linearity, satisfactory precision of replicates, minimum and maximum response for matching (positive control), and baseline (negative control).

8. ADDITIONAL SOURCES OF INFORMATION

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▲ (USP 1-Dec-2020)