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Rediscovery and Revival of Analytical Refractometry for Protein Determination: Recombining Simplicity With Accuracy in the Digital Era

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ABSTRACT

Among "vintage" methods of protein determination, quantitative analytical refractometry has received far less attention than well-established pharmacopoeial techniques based on protein nitrogen content, such as *Dumas* combustion (1831) and *Kjeldahl* digestion (1883). Protein determination by quantitative refractometry dates back to 1903 and has been extensively investigated and characterized in the following 30 years, but has since vanished into a few niche applications that may not require the degree of accuracy and precision essential for pharmaceutical analysis. However, because high-resolution and precision digital refractometers have replaced manual instruments, reducing time and resource consumption, the method appears particularly attractive from an economic, ergonomic, and environmental viewpoint. The sample solution can be measured without dilution or other preparation procedures than the separation of the protein-free matrix by ultrafiltration, which might even be omitted for a constant matrix and excipient composition.

In the present study, therapeutic human plasma protein concentrates of albumin obtained by Cohn fractionation, and of intravenous IgG as further purified by chromatography, were investigated at a concentration range up to 30%. Based on *Kjeldahl* or *Dumas* protein concentration data, the specific refractive index increments dn_D^{20}/dc was recalibrated to ~0.000183 mL/mg. The response remained linear across the concentration range. Statistical comparison by Bland-Altman data analysis showed negligible bias and practical equivalence. Comparison with established methods for albumin and IgG concentrates demonstrates that refractometry is a feasible alternative that fulfills both the stringent methodologic requirements and economic and environmental standards for a state-of-the-art, "green," biopharmaceutical analytical technique.

Introduction and Objective

Reviving an apparently antiquated analytical method such as quantitative refractometry for protein determination to make it compete with current pharmacopoeial methods may appear daunting, especially in view of the scientific debate on its comparison with other, well-established and characterized total serum protein quantitation methods in clinical and pharmaceutical analysis. However, scrutinizing review of the technique, its basics, historical development, and current technology, reveals the method's potential in modern biotechnology of therapeutic protein fractionation and purification.

Current digital and thermostatized instruments warrant hitherto unmet measurement condition constancy and consistency, allowing unparalleled accuracy and precision in determining the refractive index. Both instrument and sample handling circumvent all past mechanical and manual pitfalls and ensure extraordinarily easy and rapid measurement. In conjunction with centrifugal ultrafiltration to obtain a protein-free matrix permeate as the sample blank, quanitative refractometry enables net refractive index measurement and calculation of the protein concentration independent of sample matrix composition. For high-purity and high-concentration protein biotherapeutics, such as blood plasma-derived albumin and immunoglobulin solutions and recombinant mAb concentrates, quantitative refractometry thus appears a method-of-choice for rapid, accurate, and economic concentration measurement without dilution.

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Instrumentation

For refractive index measurement, various types of refractometers have been developed, based on the critical limiting angle for internal total reflection, such as the familiar *Abbe* refractometer,[1] and the *Pulfrich-Reiss* immersion (dipping) refractometer.[2,3] These instruments require only a few drops of the sample solution to measure the critical (internal total reflection) angle on exit of the light beam from the optically thicker medium (glass, sapphire, or garnet prism) to the optically thinner medium, namely the sample solution. The boundary mark between the light and the shadow zone (due to total reflection) is detected either visually or, in digital refractometers, with a spatially resolving charge-coupled device photodiode array.

Although earlier instruments were thermostated with water, modern refractometers use Peltier elements for temperature control. In digital benchtop refractometers, the measured value is displayed numerically with at least 0.0001 Δn_D resolution; it is no longer necessary to estimate the value from interpolated scale readouts. High-resolution instruments resolve at least up to 0.00001 Δn_D (claimed even to 0.000001 Δn_D) with an accuracy and reproducibility of ~0.00002 Δn_D. Such refractometers do not contain moving parts and can be constructed as relatively compact and robust devices. In contrast to the highly dispersive but soft flint glass of the original dipping refractometers, garnet and sapphire prisms are scratch-resistant during sample loading and cleaning. For compensation of tolerances, for example, via thermal expansion, a “zero” calibration routine to reset to the refractive index of distilled water is included.

Refractometry in Biotechnology: History and Historical Data

The concept of differential refractometry, based on the refractive index difference between the protein-containing solution and the dissolved nonprotein solids in the solution, was initially conceived by Reiss.[4,5] The method was unanimously welcomed for its simplicity, ease of use, and consumption of moderate sample volumes, especially compared with the time-consuming *Kjeldahl* method.[6] The question of the non–protein-contributed refractive index was solved 20 years later with the separation of the protein-free matrix fluid by (gravity-driven) ultrafiltration of serum through a “collodion” (nitrocellulose) membrane[7]. An overview of the historical development since then is presented in the Supplementary data, as well as a compilation of specific refractive index increment (d*n*/d*c* for 1 mg/mL) data for human and animal plasma, serum, and the most abundant proteins (Supplementary Table S1).

Current dn/dc Data

As laid out in the Supplementary data, the necessity of calibrating valid d*n*/d*c* data experimentally appears as crucial to reestablish refractometry as a technique equivalent to the accepted nitrogen-based methods. A software tool SEDFIT was developed to calculate d*n*/d*c* values from the amino acid composition,[8] however, without considering glycans or protein-bound ligands.

Materials and Methods

All protein solutions (unpasteurized albumin bulk concentrate, pasteurized 20% and 25% albumin concentrate in flexible bags, 10% intravenous [i.v.] and 16% subcutaneus [s.c.] IgG) obtained for the present study were either plasma fractionation intermediates or final products from licensed process schemes, and thus represent actual manufacturing conditions and compositions. For linearity assessment, the 10% i.v. IgG solution was further concentrated in the laboratory by centrifugal ultrafiltration. Formulation-grade reagents (NaCl, glycine, sucrose, maltose, and sorbitol) and reverse-osmosis purified water were used throughout the study. To determine the net refractive index contributed by the protein, the technique by Neuhausen and Rioch[7] has been modernized by the use of centrifugal ultrafiltration tubes to obtain the protein-free permeate.

Preconditioning of Centrifugal Ultrafiltration Tubes

Amicon Ultra-4 centrifugal filter units with a 3 kDa or 10 kDa Ultracel (regenerated cellulose) membrane filter insert required a prewash centrifugation run (at 4500 or 6000 rpm) with 4 mL water to remove the stabilizer glycerol. The wash permeate was discarded, and collection tube and membrane inserts blown dry with compressed air.

Albumin Bulk and FLEXBUMIN Concentrate

Human serum albumin intermediates and concentrates, as obtained from *Cohn* fraction V and stored refrigerated as clear solutions with a slightly olive-green to amber color, were at least 96% pure according to pharmacopoeial specifications.

Albumin ultrafiltration bulk solutions, partially stabilized with N-acetyltryptophan and caprylate, were obtained in capsulated 5-mL aliquots. Four milliliter sample solution was centrifuged in a fixed-angle rotor for 60 min at 6000 rpm and +15°C setpoint through a 3-kDa membrane filter unit, and the permeate (about 0.3 mL) was kept for refractometric measurement. The retentate was discarded. The bulk solution and the permeate were stored refrigerated in the dark and allowed to equilibrate at least 30 min before measurement to room temperature (RT). For a dilution series, residual bulk solutions were pooled, and the density and the refractive index measured. The pool was weighed into volumetric flasks and diluted with 0.1 M NaCl/0.05% NaN_3.

Fully N-acetyltryptophan–stabilized and caprylate-stabilized and pasteurized 20% and 25% albumin concentrates in flexible bags (FLEXBUMIN) were obtained filled and labeled. An aliquot of the sample solution was drawn by punctuation of the bag’s nozzle. Sample processing and centrifugation was done as for the bulk concentrate.

Total protein data from *Dumas* combustion[9] in a LECO analyzer were provided by the Baxalta Vienna QC department, based on a nitrogen content of 16%.[10]

Plasma-Derived Immunoglobulin G Concentrates

Ten percent i.v. IgG (KIOVIG/GAMMAGARD LIQUID),[11] formulated in 0.25 M glycine, pH = 4.6-5.1, is obtained from a precipitated subfraction of the ethanol fractionation precipitate and purified after solvent-detergent treatment by cation and anion exchange chromatography to an at least 98% pure IgG solution, which appears as an essentially clear and only slightly opalescent liquid. Total protein data from *Kjeldahl* digestion were provided by the Baxalta Lessines QC department.

Sixteen percent s.c. IgG (SUBCUVIA), formulated in 0.3% NaCl/0.3 M glycine, pH ~ 7, containing at least 95% IgG, is manufactured essentially from redissolved Cohn II precipitate, which is solvent-detergent treated for virus inactivation, purified by cation exchange chromatography, freeze-dried, and reconstituted. PEG 3350 may be present at not more than 8 mg/mL. The solution appears distinctly more opalescent than the 10% i.v. concentrate. Total protein data from *Dumas* combustion in a VarioMax analyzer were provided by the Baxalta Vienna QC department.