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Tutorial Article

Chemometric analysis of attenuated total reflectance infrared spectral data for quantitation of immunoglobulin G in equine plasma and serum

S. Hou [a,d,*], R.A. Shaw [b], C.B. Riley [c,d]

[a] ChemoBio Data Analysis Technologies, Charlottetown, Prince Edward Island, Canada
[b] National Research Council of Canada, Medical Devices Portfolio, Winnipeg, MB, Canada
[c] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand
[d] Department of Health Management, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada

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ABSTRACT

Immunoglobulin G (IgG) is a crucial antibody to protect animals from invasion by microorganisms. Although there exist several methods in veterinary medicine to measure IgG levels for diagnostic and therapeutic purposes, these methods suffer from various weaknesses. Infrared (IR) spectroscopy coupled with chemometric tools such as principal component regression has been widely employed for the measurement of compounds in mixtures with the advantages that include simplicity, quickness and low test cost. Earlier investigation for IgG assay based on transmission IR spectroscopy using laboratory grade equipment has been conducted, but it is not readily transferrable to the clinic, hospital or small laboratory setting. More robust attenuated total reflectance (ATR) IR spectroscopy platforms have recently been developed for a range of roles in the field. This study investigated the possibility of using ATR-IR spectroscopy to determine the IgG concentrations in foal serum and adult horse plasma samples. The results of this work showed that immunoglobulin G concentrations predicted by ATR-IR spectroscopy with chemometric analysis had good agreement with those obtained from the radial immunodiffusion (RID) reference method. The precision of this approach was most compatible to RID method when the IgG concentration was high, but poorer for lower IgG concentrations. It was also showed that building a united calibration model for serum and plasma samples is likely. The results of this work indicate that ATR-IR spectroscopy coupled with chemometric analysis is a promising technique to measure the equine serum and plasma IgG concentrations in the veterinary clinical or hospital environment.

1. Introduction

As an important antibody in mammal body fluids, immunoglobulin G (IgG) plays a critical role in protecting the animals from harmful invasion of viruses, bacteria, and fungi [1]. Insufficiency of IgG is often associated with high morbidity and mortality of animals and this is especially important for neonates of many ungulate species as they lack immunoglobulins at birth. These newborn animals (e.g. foals) acquire maternal immunoglobulin (predominately IgG) from the intake of colostrum, with an increased risk of neonatal sepsis and infectious disease if they fail to absorb sufficient maternal antibody. The concentration of IgG in body fluids can be measured for diagnostic and therapeutic purposes [2,3]. For example, IgG levels increase in most infections, hyperimmunizations, hepatic disease, severe malnutrition, dysproteinemia, hypersensitivity granulomas, dermatologic disorders, IgG myeloma, and rheumatoid arthritis [2,3]. In contrast, IgG levels may decrease for conditions such as agammaglobulinemia, lymphoid aplasia, severe combined immunodeficiency, Bence Jones proteinemia, chronic lymphoblastic leukemia, and failure of transfer of passive immunity [2–4].

In veterinary medicine, several tests are commonly used for the clinical measurement of IgG. Radial immunodiffusion assay (RID) has been considered the "gold standard" test for the quantitative measurement of IgG in horses and other domestic species [5–9]. While RID generates quantitative IgG data, it has significant drawbacks in many clinical and laboratory environments [5–9]. It typically takes 18 to 24 h to obtain results, requires technical skill to interpret, is not amenable to automation, utilizes reagents with a limited shelf life, and is often more expensive than alternatives. A variety of enzyme-linked immunosorbent assay (ELISA) immunoglobulin tests using species-specific anti-IgG antibody have also been used as neonatal IgG screening, but unfortunately these tests suffer from poor sensitivity [5]. Other screening tests including the glutaraldehyde coagulation test, zinc sulfate turbidity test, and serum total protein or globulin tests are non-specific [5,7,8]. In response to the limitations imposed by the poor sensitivity and/or poor specificity, and other challenges posed by these approaches to IgG

* Corresponding author.
E-mail address: houssnn@gmail.com (S. Hou).

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measurement, earlier efforts to determine IgG based upon Fourier transform infrared spectroscopy have been reported [9–11].

In brief, infrared (IR) spectroscopy is a widely used technique in analytical chemistry to qualitatively and quantitatively determine the chemical compounds present in many different types of samples [12, 13]. The coupling of IR spectroscopy and chemometrics tools such as partial least squares regression and principal component regression has been widely used to build multivariate calibration models that form the basis for quantitative analytical methods [12–14]. However, depending on the compounds to be tested, the sample matrix, and the specific spectroscopic methods, the accuracy of IR-based analytical methods may vary dramatically [12–15]. While the early proof-of-concept transmission-IR spectroscopy-based methods to quantify IgG concentrations in equine and camelid serum samples hold promise, a concern for transmission IR spectroscopy is that the thickness of the sample is difficult to control because of the small volume of samples, typically from 5 to 10 μl. Furthermore, these works were performed using laboratory grade equipment that may not be readily accessible or affordable, thus hindering transfer to the small diagnostic laboratory or veterinary hospital environment.

As an alternative to transmission IR spectroscopy, the authors are presently exploring the suitability of attenuated total reflectance (ATR) infrared spectroscopy [16] for quantitative analysis of serum/plasma. This technique maintains the benefits of economic, environmentally sustainable and reagent free testing, yet provides an approach that is more readily translatable to the end user. The ATR-IR technique involves an optical arrangement that sends the IR beam through an IR-transparent crystal. The crystal is cut in such a fashion that when a beam of infrared light passes through it, the beam is reflected a few times off the internal surface of the crystal. If a sample is placed into contact with a surface from which internal reflections take place, an evanescent wave extends into the sample. Some of that radiation is absorbed by the sample and the internally reflected radiation is attenuated specifically at those frequencies where sample absorption occurs.

Although ATR-IR spectroscopy sounds to have a variety of advantages, its practical use requires advanced processing and analysis of the spectral data. In this work, chemometric analysis was performed on the ATR-IR spectra. It is hypothesized firstly that ATR-IR spectroscopy could be used to determine the IgG concentrations in adult equine plasma and neonatal equine serum, and secondly that a single multivariate calibration could provide the basis for the analysis of both serum and plasma samples. Principal component regression [14] (an adaptation of principal component analysis to regression analysis) was applied to develop multivariate calibration models from which IgG levels could be determined. Monte Carlo cross validation was used to determine the optimal number of principal components for each model [10]. The performances of the calibration models and model adequacy were tested using independent samples with the scatter plot, concordance correlation coefficient, and Bland-Altman plot employed as model evaluation tools [19–21]. The results of this study indicated that ATR-IR spectroscopy coupled with chemometrics tools is promising as a simple approach to measure IgG concentrations in horse serum and plasma samples in a clinical setting.

2. Materials and methods

2.1. Samples

Neonatal serum and adult equine plasma samples were used in this study. Foal serum samples ($n = 89$) were collected from 24 to 72 h old foals in the Maritime Provinces of Canada following a protocol approved by the Animal Care Committee of University of Prince Edward Island. Adult horse plasma samples ($n = 105$; age unknown) were donated by Lake Immunogenics Inc. (Ontario, NY). The foal serum and adult plasma samples were stored in a −80 °C freezer before use.

2.2. Reference data by radial immunodiffusion assay

Concentrations of IgG were determined in the foal serum and adult horse plasma samples in accordance with the manufacturer's instructions using the corresponding RID (the "gold standard" reference method for horse serum and plasma samples) kits from Triple J Farms (Bellingham, WA) [10]. Samples were diluted in a ratio 1:1 (v/v) by 0.85% (w/v) sterile aqueous saline solution so that IgG concentrations fell within the range for which the kit is certified. Five-microliter aliquots of each diluted sample were applied to the RID test plates, with 5 replicates for each sample, and the diameter of the precipitation rings read after 24 h. The corresponding reference standards provided by the manufacturer were tested in parallel in replicates of 5. The RID data were input into a spreadsheet and then imported into MATLAB® (R2011b, MathWorks, Natick, MA, USA). Calibration models based on the manufacturer's standards were built and then used to calculate the IgG concentrations in the foal serum and adult horse plasma samples. The average IgG concentrations from replicates were used subsequently as reference values in building the multivariate calibration models for the ATR-IR spectroscopic method.

2.3. Spectral data by attenuated total reflectance infrared spectroscopy

The foal serum and adult horse plasma samples were interrogated using a bench top IR spectrometer (Cary 630 Fourier transform IR spectrometer, Agilent Technologies, Dansbury, Connecticut; footprint of base 13 cm × 28 cm) with a custom made ATR accessory (3-bounce Diamond ATR Module, ZnSe element). Each sample was diluted (1:1, v/v) with 4 g/L of potassium thiocyanate (KSCN) solution, and 8 μl of this diluted sample was applied to the sample window in the ATR optics module of the spectrometer. After the sample was dried to a thin film, the mid-infrared spectra (4000–650 cm^{-1}) were acquired. For each acquisition, 32 interferograms with a nominal resolution of 8 cm^{-1} were collected and averaged. The interferograms were Fourier transformed and infrared spectra were saved. For each test, a new background measurement was collected. Five replicate tests were performed for each sample. This resulted in 445 (89 × 5) spectra for the foal serum samples and 525 (105 × 5) spectra for the adult horse plasma samples. One original spectrum from each of two adult plasma samples was excluded from further analysis because one original spectral file for each sample was corrupted; thus 523 adult spectra were remained. All the spectra were converted to printable (PRN) formatted data using GRAMS/AI software (ver. 7.02, Thermo Fisher Scientific Inc., Waltham, MA, USA), and then imported into MATLAB® (R2011b, MathWorks, Natick, MA, USA). Further analysis and multivariate calibration model development were then carried out using programs written by the authors in MATLAB®.

2.4. Data analysis

2.4.1. Spectrum pre-processing

The same spectrum pre-processing steps were used for serum and plasma samples. The collected infrared spectra were smoothed using the Savitzky-Golay method [22] (9-point, 2nd order polynomial function) followed by spectral sub-region selection at 3700–2600 cm^{-1} and 1800–1300 cm^{-1} [10]. To reduce the effect of light scattering that arises from variation in sample size, shape, surface and texture, standard normal variate transformation (SNV) [23] was applied to each spectrum. As replicate spectra were available, outlying spectra were detected and excluded by using Dixon's Q-test [24,25] for intensities of replicate spectra at each wavenumber. A spectrum which had over 50% of the wavenumbers found to be outliers at 95% confidence level was treated as an outlier and excluded [10]. The average of the replicate spectra (after removal of outliers, if applicable) for each sample was used in the subsequent analysis.