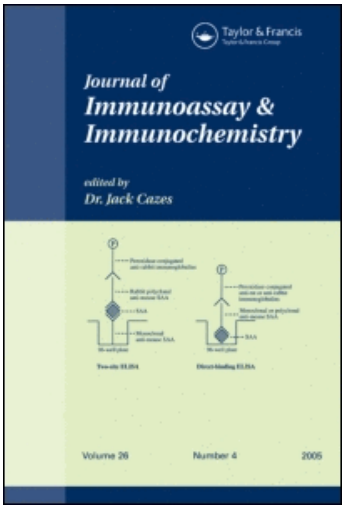

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DEVELOPMENT OF A SPECIFIC FLUOROIMMUNOASSAY FOR OVINE ALBUMIN

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ABSTRACT

A fluoroimmunoassay has been developed to measure serum levels of albumin in sheep. It employs ovine albumin labelled with fluorescein as the tracer and a rabbit antiserum raised against ovine albumin. Separation of the antibody bound and free fractions is achieved using a second antiserum directed against the Fc of rabbit immunoglobulin G and, to simplify the assay, the two antisera are premixed prior to use. Assay validation parameters are satisfactory and the reagents are predicted to be stable for at least one year at 4°C. In contrast to the bromocresol green method, the assay is unaffected by immunoglobulins.

A reference range for serum albumin levels has been established in lambs, normal ewes and ewes undergoing immunisation. Mean serum levels were 38.8, 51.3 and 37.8g/l respectively. The sensitivity of the assay also enabled its use to monitor albumin levels at various stages during the production of specific antibody fragments from ovine antisera for therapeutic purposes.

(KEY WORDS: Fluoroimmunoassay, Ovine Albumin, Premix)

INTRODUCTION

Serum albumin levels can provide a useful indication of the general health and nutrition of an individual sheep once a reference range has been established. However, the reported values in normal healthy sheep vary widely with mean

serum levels ranging from 24g/l [1] to over 40g/l [2]. These differences may reflect, in part, the age or breed of the sheep being studied but, most probably, are also due to differences in the analytical method employed.

Assays for serum levels of albumin can be direct or indirect. In the latter the albumin is separated then measured by a general protein assay such as that described by Lowry or by a microKjeldahl technique. Direct determination involves the use of affinity dyes or antibodies [3] . Probably the most commonly used method for albumin determination, because of its ease of automation, is the dye binding assay using bromocresol green(BCG) [4] , first proposed by Rodkey [5] . Unfortunately, such methods are relatively insensitive and lack specificity because the dye also binds to immunoglobulins. A wide range of immunological methods, including radioimmunoassay (RIA) [1] have also been introduced and offer considerable advantages in terms of specificity and sensitivity.

Albumin is the most abundant plasma protein and is likely therefore to be the major contaminant in other serum or blood derived products. Thus we also require an assay specific and sensitive enough for quality assurance purposes during every stage of a process designed to produce specific ovine immunoglobulin G(IgG) derived Fab for such purposes as the treatment of snake envenomation or drug overdose.

A fluoroimmunoassay (FIA) was developed in preference to a RIA because it offered the necessary sensitivity and specificity while avoiding the limited shelf life and potential hazards associated with radioisotopes. In the FIA, fluorescein

labelled ovine albumin is used together with a rabbit antiserum raised against ovine albumin and separation of the bound and free fractions is achieved using donkey anti-rabbit Fc. The assay has been used to establish reference ranges for serum albumin in healthy, non-immunised ewes and lambs, and in ewes that are being immunised monthly with one of a variety of immunogens. The assay was also used to monitor albumin levels during the manufacture and purification of specific ovine Fab directed against the tricyclic antidepressants and intended for the treatment of drug overdose.

MATERIALS AND METHODS

Reagents

Fluorescein isothiocyanate isomer I (FITC), ovine albumin, IgG, bovine gamma globulin and BCG were obtained from Sigma Chemical Co., Poole, Dorset, and Sephadex G-50 from Pharmacia, Milton Keynes, Bucks. Disodium hydrogen phosphate, methanol, potassium chloride, sodium azide, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide and Triton X-100 were all ANALAR grade where possible and obtained from BDH Chemicals, Dagenham, Essex.

Antisera

Rabbit anti-ovine albumin and donkey antibodies directed against the Fc segment of rabbit IgG were obtained from Polyclonal Antibodies Ltd., Llandysul, Dyfed, Wales. These were premixed prior to use in the assay by the addition of 50µl of the rabbit first antiserum and 850µl of the donkey second antiserum to 24ml of assay buffer. After gentle mixing, this reagent was stored at 4°C. prior to use.

An immunoelectrophoretic evaluation [6] was carried out with rabbit anti-whole ovine serum and the rabbit anti-ovine albumin to demonstrate the specificity of the latter.

Fluorescein-Labelled Ovine Albumin (OA-FTC)

OA-FTC was prepared by reacting FITC, dissolved in a minimum volume of methanol, with ovine albumin dissolved in sodium bicarbonate buffer (pH 9.0, 50 mmol/L) in a molar ratio of 6:1. The product was purified on Sephadex G 50 following an established procedure [7] and the entire labelled protein peak, which was well separated from unreacted FITC and identified by its colour, was collected.

Fluorescence Measurement

A Perkin-Elmer LS-5 Luminescence Spectrometer with a Perkin-Elmer 3600 Luminescence Data Station or a Perkin-Elmer LS-20 Filter Fluorimeter with Epson HX-20 computer control were used.

Assay Buffer

Phosphate buffered saline (PBS), pH 7.4, containing 0.5g/l bovine gamma globulin and 0.5g/l sodium azide.

Assay Standards

Assay standards comprising 0, 10, 20, 50, 100, 200 and 500g/l in assay buffer were prepared for the FIA, and standards comprising 0, 5, 10, 15, 20 and 25g/l were prepared for the BCG assay.

Samples

Serum samples were collected from 46 non-immunised ewes, 46 immunised ewes (producing large quantities of specific antibodies against a broad range of immunogens) and 19 lambs. The samples were diluted 1000 fold in buffer prior to assay. Samples obtained during Fab production and purification comprised the whole antiserum, IgG before and after papain digestion, and affinity purified Fab.

Quality Control: Samples

Three quality control(QC) samples were prepared from a previously assayed sample of non-immunised sheep serum, and diluted in assay buffer to give concentrations of 18, 55 and 85mg/l (low, medium and high QC's).

Stability Studies

Both the antibody premix and the tracer were tested for stability using bulk stock reagents and individual standards which were assayed at intervals of 0,1,2 and 5 days. When not in use, all reagents were stored at 4°C. In addition, a sample of the label and premix were stored at 37°C for one week separately and then run with individual standards following the assay protocol.

Procedures

Assay Protocol:

Assays were performed in duplicate at room temperature. Tracer(200µl,0.5µg) was added to 25µl of standard or sample in a 3.5ml polystyrene tube(Sarstedt Ltd., Leicester, Cat No: 55.484)followed, after vortex mixing, by 200µl of the premixed antibodies. The tubes were again mixed and after incubation for two hours in the dark, centrifuged for 1 h at 3000rpm at room temperature. The supernatant was aspirated to waste and the pellet (containing the bound fraction) dissolved in 1.5ml of 0.1M sodium hydroxide, vortex mixed and its fluorescence measured.

BCG Assay Protocol:

Assays were performed at room temperature and in duplicate. All serum samples were first diluted 1 in 5 with PBS containing 0.5g/l sodium azide, and 10µl of sample or standard were placed into wells in a microtitre plate. To each sample well, 200µl of Sigma BCG reagent was added and absorbance at 628nm was measured immediately, using an ELISA microtitre plate reader.

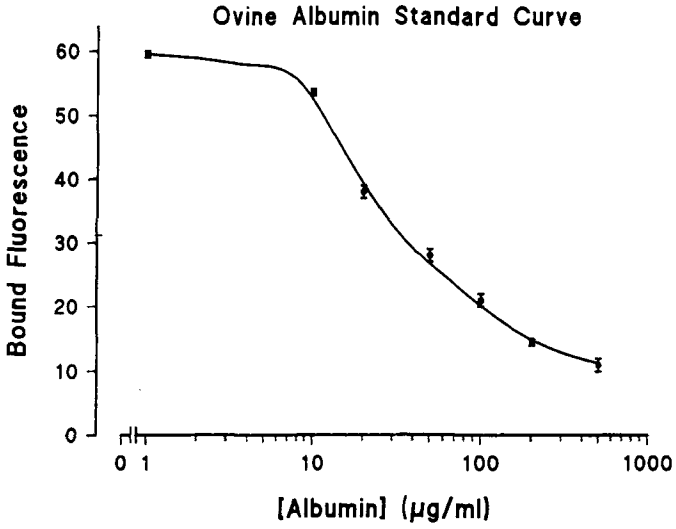

Figure 1. A Typical Standard Curve with Albumin Concentrations (mg/ml)Plotted Against Bound Fluorescence.

RESULTS

Standard Curve

Using an incubation time of 2h, a centrifugation time of 1h, and 25µl of standard or buffer, a typical standard curve shown in Figure 1 was obtained. The sensitivity was such that serum samples from sheep had to be diluted 1000 fold prior to assay, while the IgG fraction obtained during antibody purification required a 200 fold and the papain digest, containing Fab, a 20 fold dilution. The final purified Fab was dissolved in assay buffer at the required concentration.

Reference Ranges in Sheep Sera and Antibody Products

The reference ranges, mean values and standard deviations for the three sheep populations were determined by FIA. Values in lambs ranged from 26 to 54 with a mean of 38.8±7.3g/l. The levels in non-immunised ewes ranged from 34 to 73

with a mean of 51.3±11.5g/l and were significantly higher than those from immunised ewes with values from 23 to 60 and a mean of 37.8±12.0g/l. When examining the albumin content of samples from each stage of processing from serum(43g/l), salt fractionation(1.0g/l), papain digestion(0.24g/l) and, finally, affinity purified Fab(0.01% w/w) it was found that approximately 98%(w/w) of the albumin content of the serum is removed during the IgG salt fractionation step. After the papain digestion step there is less than 1%(w/w) remaining and this is further reduced to less than 0.1% after affinity purification.

Stability studies

Both the label and the premix were found to be stable when stored at 37°C for one week, predicting their stability for one year at 4°C [8].

Assay validation

The within assay coefficient of variation(CV) was found to be less than 10% at various points of the standard curve. Between assay imprecision was calculated from the results of the three QC samples and gave CV's of 13.2%(low), 9.1%(mid) and 8.2%(high). The sensitivity was determined to be 3.0mg/l with a minimum detectable dose of 75ng [9]. Serially diluted serum from two sheep gave curves parallel to the assay standards. Recovery results were 87% and 111% when albumin was added to the low QC.

The immunoelectrophoretic evaluation of the rabbit anti ovine albumin confirmed its specificity, giving a single arc of precipitation against whole sheep serum, in contrast with the multiple arcs generated by rabbit anti whole sheep serum.

Comparison with BCG

The correlation of albumin levels in the serum samples determined by the FIA and by the BCG method was poor(r=0.23,Fig.2a). The mean serum albumin value

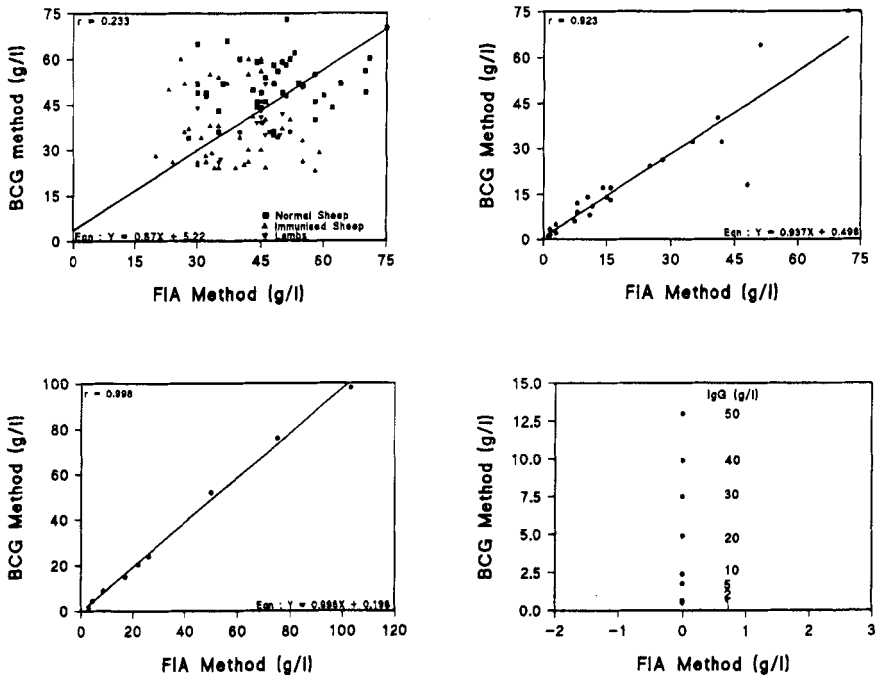

Figure 2. Correlation Between the FIA and BCG assays (a)The Correlation for All Samples from the Three Populations (n =111), (b)Serially Diluted Sheep Serum(c)Pure Ovine Albumin and (d)Pure Ovine IgG.

for the normal sheep and lambs were significantly different when determined by this specific FIA(51.3 and 38.8g/l respectively) and by the BCG assay(48.1 and 41.7g/l respectively). The mean value for the immunised sheep varied less markedly depending on the assay used, being 37.8g/l for the FIA and 39.6g/l for the BCG assay.

Further experiments were then performed, with a wider sample correlation range, to evaluate the effect of immunoglobulin interference in the BCG assay. Samples of serially diluted serum from three sheep with high levels of albumin showed a much improved correlation (r=0.923, Fig.2b) as did pure ovine albumin

samples diluted to 2 from 100 g/l (r=0.998,Fig.2c). Pure ovine IgG samples at least ranging from 1 to 50g/l gave positive values in the BCG assay while showing zero levels in the FIA (Fig.2d) clearly indicating that binding of the dye by immunoglobulin was leading to the inaccuracy of the BCG method.

DISCUSSION

Dye binding methods for albumin are popular in the clinical laboratory because they are easy to automate. The BCG method, however, as confirmed here, is prone to inaccuracy due to the dye binding with IgG as well as albumin. Although not automated, the FIA described in this paper was simple and easy to perform so that a large number of samples could be assayed accurately in one day. For example all our 119 serum samples were measured during the same day.

Fluorescein offers many advantages over radioactive isotopes and was used successfully to develop an assay for human albumin [7] , hence it was selected to develop the present assay. Premixing of the first and second antibody resulted in a stable premix reagent which could be used after 5 minutes from mixing. Premixing was possible because the second antibody in the premix does not interfere with the binding of albumin by the first antibody. This is because the second antibody was raised against the Fc part of the first antibody and not against the whole antibody.

The poor correlation(r=0.23)of the FIA with the BCG method(Fig.2a)was probably due to the susceptibility of the dye to bind to other proteins, especially IgG. The narrow range of ovine albumin levels in these samples also contributed to the poor correlation. Thus a good correlation between the two methods was achieved using pure ovine albumin, the correlation worsened (r = 0.920) upon using a serially diluted sheep sample(Fig.2b), and finally no correlation was found when pure ovine IgG was used in the absence of ovine albumin (Fig. 2d). In this

last experiment, the BCG assay gave positive albumin values while the FIA results were zero.

The present assay was used to establish a range of albumin levels in three populations of sheep. The mean results 51.3g/l for the non-immunised sheep, 38.8 for the lambs and 37.3 for the immunised sheep. These values differed from previously reported values described in the introduction depending on the technique and/or the reference materials used. Immunisation of sheep with drug-protein conjugates has been found to elicit antibody titres of between 5-10g/l(data not shown) against the drug. It is possible that high specific and non-specific antibody levels compensate for the low albumin levels in immunised sheep, thus keeping the total protein concentration in the blood fairly constant.

As albumin is present at high concentration in serum, it was necessary to demonstrate the absence of this protein in the final product of a sheep serum based therapeutic product. When applied to samples that had been affinity purified, the necessary increase in sensitivity for albumin was obtained by using less diluted Fab and albumin at 100ppm could be measured by using a 50g/l affinity purified specific Fab solution.

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