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High-throughput immunoturbidimetric assays for in-process determination of polyclonal antibody concentration and functionality in crude samples☆

H. Bak[a,b,1], J. Kyhse-Andersen[b,2], O.R.T. Thomas[a,c,*]

[a] Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, Building 223, Søltofts Plads, DK-2800 Lyngby, Denmark, UK
[b] DakoCytomation A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark, UK
[c] Department of Chemical Engineering, School of Engineering, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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Abstract

We present fast, simple immunoturbidimetric assays suitable for direct determination of antibody 'concentration' and 'functionality' in crude samples, such as in-process samples taken at various stages during antibody purification. Both assays display excellent linearity and analytical recovery. Possible influences from commonly employed buffers and salts (present in samples at various concentrations), and of pH variations, were studied for both assays. Interference effects were shown to be negligible for the 'concentration' assay, such that sample pre-treatment prior to assay is unnecessary. The 'functionality' assay displayed concentration dependent sensitivity to interference for ammonium sulphate and Tris-(hydroxymethyl)-amino-methane, but was essentially unaffected by all other salts and buffer combinations tested. The immunoturbidimetric assays described here are generically applicable to polyclonal antibodies, require only basic laboratory equipment, are robust, fast, cheap, easy to perform, and readily adapted to automation.

Keywords: Immunoglobulins; Nephelometry; Precipitin reaction; Titre; Turbidimetry

1. Introduction

Traditional quantitative and semi-quantitative assays for antibodies, such as SDS-PAGE, ELISA, HPLC, or in-gel precipitation techniques such as single radial immunodiffusion (SRI) or 'Rockets', are commonly employed, but time-consuming techniques. This makes them poorly suited for 'in-process' monitoring during fermentative and/or downstream processing of monoclonal and polyclonal antibodies. In stark contrast, the somewhat less commonly employed technique of immunoturbidimetry can deliver results within a matter of just few minutes, an advantage that makes it an altogether more promising starting point for the development of rapid 'in-process' antibody monitoring techniques.

The classical theory of the quantitative precipitin reaction was first described by Heidelberger and Kendall [1], and the resulting technique immunoturbidimetry (or nephelometry) has been employed extensively within clinical biochemistry and diagnostics applications [2–4]. In immunoturbidimetry, a soluble antigen (Ag) and its corresponding antibody (Ab) form immune complexes when mixed in suitable proportions, with the size of the complexes being dependent on the ratio of Ab to Ag [5].

Immunoturbidimetric assays are often performed on expensive, purpose-built machines, which require extensive training of personnel in order to perform the required programming and control of the equipment. In this work, we show how immunoturbidimetry can form the basis of two quick, reproducible, and robust methods—one for the determination of Ab 'concentration' (the C-assay), and the other, for Ab 'functionality' (the F-assay) measured by its titre (i.e. presence of intact antigen binding sites). Whereas the F-assay is only suitable for determining the titre or functionality of polyclonal antibodies, the C-assay could be applied to measure the concentration of any

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* Corresponding author. Tel.: +44 121 414 5278; fax: +44 121 414 5377.
E-mail address: o.r.t.thomas@bham.ac.uk (O.R.T. Thomas).
[1] Present address: Regeneron Pharmaceuticals Inc., Preclinical Manufacturing and Process Development, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA
[2] Present address: Dalsø Park 8, DK-3500 Værløse, Denmark, UK

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multi-epitope antigen (e.g. monoclonals, polyclonals and other proteins). A particular advantage of the assays, we describe here, is that both require only very simple and low cost standard equipment, typically found in most biotech laboratories. In this study, we have developed methods specifically for the determination of rabbit immunoglobulin (RbIg) concentration (using goat anti-rabbit immunoglobulins), and titre of rabbit anti-human transferrin antiserum against human serum, and have employed both of these successfully for the analysis of a laboratory scale antibody purification process [6], running from serum through to the Ig-fraction.

2. Materials and methods

2.1. Materials

Antisera pools from Danish White rabbits were supplied by DakoCytomation A/S (Glostrup, Denmark), as were all of the following: purified immunoglobulin (X0903) from non-immunised rabbits; dilution buffer 1 (S2005); reaction buffers 1 (S2007) and 2 (S2008); goat anti-rabbit immunoglobulins (Z0421); delipidated human serum (X0908); and antibody standard (Q0327). Dialysis tubing (12–16 kDa MWCO) was obtained from Viskase (Willowbrook, IL, USA), and DEAE-Sephadex A-50 was bought from GE Healthcare (Uppsala, Sweden). Ammonium sulphate and sodium chloride were purchased from Brenntag Nordic, Glostrup, Denmark and Brøste A/S (Lyngby, Denmark), respectively, and acetate buffer was obtained from Merck (Whitehouse Station, NJ, USA). All other chemicals were of AnalaR or equivalent grade, and were obtained from registered suppliers.

2.2. Protocol for purification of immunoglobulins

Ig-fractions were purified according to the method originally described by Harboe and Ingild [6], a brief description of which is presented below.

To 200 mL volumes of antisera pools (from Danish White rabbits that had been immunised with human transferrin, human IgM or human albumin), ammonium sulphate was added to a final concentration of 250 g ammonium sulphate per litre of antiserum, and the resulting precipitate was collected by centrifugation at ~5000 × g_{av} in a swing out rotor (JS-4.2, JS-4.2A, JS-4.2SM, or JS-4.2SMA) of a Beckman J-6B or J-6M high spin centrifuge (Beckman Coulter Inc., Fullerton, CA, USA) operated at 4 °C for 0.6 h. Subsequently, the precipitate was resuspended in 233 g L^{-1} ammonium sulphate, and re-centrifuged (as described above). The supernatant obtained was then dialysed (12–16 kDa MWCO dialysis membrane) extensively (at 4–8 °C), first, against distilled water, and then against an acetate buffer, in order to prepare the feedstock for subsequent batch adsorption of contaminants on to DEAE Sephadex A-50. The unbound Ig-containing fraction was subjected to a further round of precipitation with ammonium sulphate (250 g L^{-1}), before centrifugally collecting (5000 × g_{av}, 0.25 h, 4 °C) the Ig precipitate, resuspending and dialysing it against distilled water, and then finally versus 0.1 M NaCl.

2.3. Standard C-assay: ommunoturbidimetric determination of immunoglobulin concentration

Purified Ig from non-immunised rabbits was diluted with dilution buffer 1 to generate a series of Ag standards with concentrations between 6.6 and 500 mg L^{-1}, and unknown samples were appropriately diluted with the same buffer so as to lie within this range. Duplicate portions (50 μL) of diluted samples and standards were pipetted into the wells of a microtitre plate (96-well Polysorb, Nalgene Nunc Int., Rochester, NY, USA), followed by 90 μL aliquots of reaction buffer 2. After 5 s of brief mixing, the plate was incubated for ~300 s at 30 °C in a pre-heated $Thermo_{max}$ or Spectra Max 250 microtitre plate reader (Molecular Devices, Sunnyvale, CA, U.S.A) and then read at 340 nm. Next, 210 μL aliquots of an Ab mixture consisting of two-fold diluted goat anti-rabbit immunoglobulins (GoaRbIg) mixed in a 4:3 ratio with reaction buffer 2 were added rapidly to each well with a BioHit Proline 12-channel automated pipette (50–1200 μL, Helsinki, Finland). After incubating at 30 °C for 300 s, the plates were re-read at 340 nm.

2.4. Standard F-assay: immunoturbidimetric determination of antiserum titre

Delipidated human serum was diluted with dilution buffer 1 to generate a series of 19 Ag standards between 0.113 and 33.33% (v/v). Duplicate portions (50 μL) of diluted Ag standards were pipetted into the wells of a microtitre plate, followed by 90 μL aliquots of reaction buffer 1. After 5 s of brief mixing, the plate was incubated for ~300 s at 30 °C in a pre-heated microtitre plate reader and then read at 340 nm. Next, 210 μL aliquots of an antibody mixture consisting of either diluted Ab sample or Ab standard mixed in a 4:3 ratio with reaction buffer 1, were added rapidly to each well. After incubating at 30 °C for 420 s, the plates were re-read at 340 nm. The data points were fitted to an eigth order polynomial as per an internal DakoCytomation A/S Excel routine, which calculated the maxima of the polynomial and hence the Ab titres.

3. Results

3.1. General immunoturbidimetric theory

Turbidimetry is the measurement of the decrease in light transmitted through a suspension of particles [5], and in immunoturbidimetry it is immune complexes that scatter light. The reaction of an antibody (Ab) and its corresponding antigen (Ag) results in the formation of immune complexes, the sizes of which being strongly dependent on the ratio of Ab to Ag numbers. Fig. 1a shows a schematic illustration of the classical precipitin curve first described by Heidelberger and Kendall [1]. At low Ag/Ab ratios, i.e. in the 'antibody excess' zone, reaction results in the formation of simple complexes, consisting of two Ag and one Ab [5]. As the Ag to Ab ratio is raised, lattice formation (achieved by a rearrangement of binding sites, allowing for cross-linking of Ab and Ag) commences. With increasing time these lattice-like complexes aggregate and form a visible

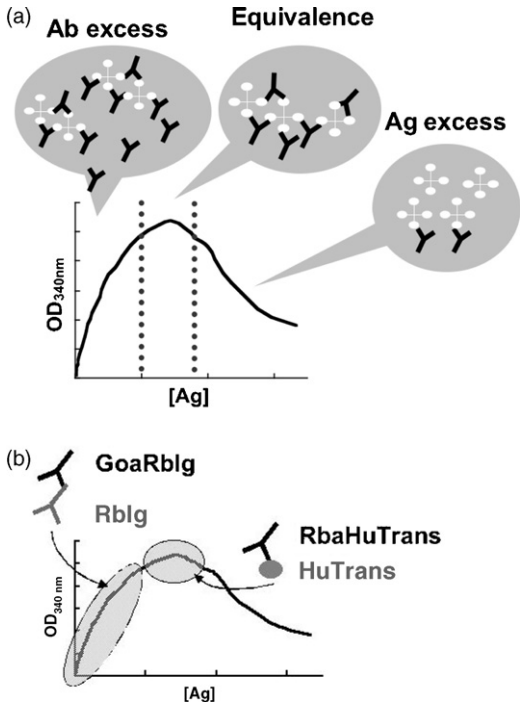

Fig. 1. When a constant quantity of Ab is added to an increasing amount of Ag, the classical precipitin curve is generated (a). It may be divided into three zones: (i) the Ab excess zone, where the supernatant still contains free Ab; (ii) the equivalence zone, where the supernatant contains neither free Ag nor free Ab; and (iii) the Ag excess zone, where the supernatant contains free Ag, adapted from Blirup-Jensen [2]. In this work (b) when determining [Ab], rabbit immunoglobulins (RbIg) are the antigens and goat anti-rabbit immunoglobulins (GoaRbIg) are the antibodies; only the first part of the precipitin curve, shaded in grey, is used. The titre of rabbit anti-human transferrin (RbaHuTrans) antibodies is determined using human transferrin (HuTrans) as the Ag and determining the maxima of the precipitin curve (shaded in grey).

haze. At the 'equivalence point' the antibody titre of the sample is determined. With yet further increase in Ag over Ab, the 'Ag excess' zone is reached. According to Whicher et al. [5] all of the Ab binding sites are occupied, and most Ab molecules exist as binary Ab–Ag complexes. Because these small complexes scatter light to a much lesser extent *cf.* the larger complexes observed in the 'equivalence' and 'Ab excess' zones, a reduction in optical density results. It is important to note that immunoturbidimetry can only be applied using polyclonal antibodies for 'detection' (see Fig. 1a and b). For lattice formation to occur, the 'detecting' antibody must recognise more than one epitope on the antigen. In Fig. 1b we have annotated the precipitin curve to highlight the regions that are particularly pertinent to the C- and F-assays detailed in this work. We have made use of the first part of the precipitin curve (the Ab excess zone) only for determination of Ig concentration, and the central peak region (equivalence zone) for the determination of functionality (or titre). For the C-assay in this work rabbit Ig (RbIg) represents the Ag and goat anti-rabbit Ig (GoaRbIg) the Ab. In the F-assay rabbit anti-human transferrin (RbaHuTrans) serves as the Ab to the Ag, human transferrin (HuTrans). Although we have developed both assays specifically for concentration and titre determination of rabbit antibodies, in principle they could be applied to any polyclonal antibody population, and in the case of the C-assay, to any monoclonal antibody or antigen carrying multiple epitopes as well.

3.2. Time dependency of Ab–Ag complex formation

Preliminary investigations showed that the Ab–Ag complex formation for both C- and F- assays (2.3 and 2.4) was dynamic (i.e. no end-point reached) for at least 0.4 h. Given our desire to produce a fast assay, the identification of a suitable times for reading the optical density at 340 nm was chosen in each case, in preference to the determination of a 'pseudo' end point.

3.2.1. Time dependency in the C-assay

Dilutions of the Ag, ranging from 3.9 to 1.1 g L^{-1} were prepared, and the general procedure described in Section 2.3 was followed. At each Ag concentration, immediately following Ab addition, the optical density at 340 nm was read 12 times (in a plate reader at 30 °C) at regular intervals between 30 and 840 s. The results obtained are shown in Fig. 2. At incubation times greater than 180–240 s after Ab addition, the data sets collected at all subsequent times were superimposable. For the sake of increased assay robustness, an $OD_{340\,nm}$ reading time of 300 s after Ab addition was chosen.

3.2.2. Time dependency in the F-assay

Dilutions of the standard Ag (0.133–33.3%, v/v) and 6.45% (v/v) Ab standard were prepared, and the protocol described under Section 2.4 was pursued. At each Ag concentration, immediately after Ab addition, the optical density at 340 nm was read 10 times (in a plate reader at 30 °C) at regular intervals between 30 and 600 s. Fig. 3 shows the results obtained. A plateau was reached after incubation times of >360 s after Ab addition. In order to minimize assay variation an $OD_{340\,nm}$ reading time of 420 s after Ab addition was selected. The consequent titre of 2350 mg L^{-1} ±1.1% RSD determined in the assay for the standard Ab, was in good agreement with the manufacturers quoted

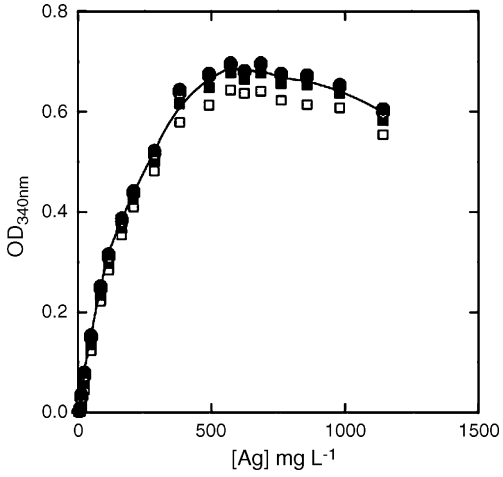

Fig. 2. Effect of time after Ab (GoaRbIg) addition to Ag (RbIg) on development of C-assay precipitin curves. GoaRbIg was added to various concentrations of RbIg, and the $OD_{340\,nm}$ were then recorded after 30 s (□), 60 s (■), 120 s (◯), 180 s (●), and 240–840 s (—, mean values). The SD determined was <0.02 $OD_{340\,nm}$.

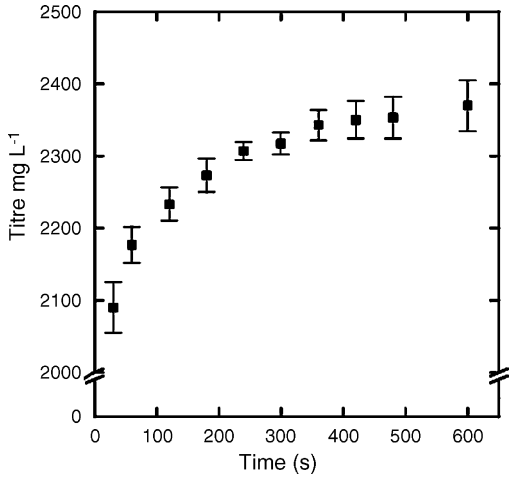

Fig. 3. Effect of time after Ab (RbaHuTrans) addition to Ag (HuTrans) on the value of Ab titre determined in the F-assay. After 420 s the titre measured 2350 mg L^{-1} ±1.13% RSD. Three independent dilutions of standard Ab (6.45%, v/v) were employed and the error bars represent 1 SD (<1.5% RSD).

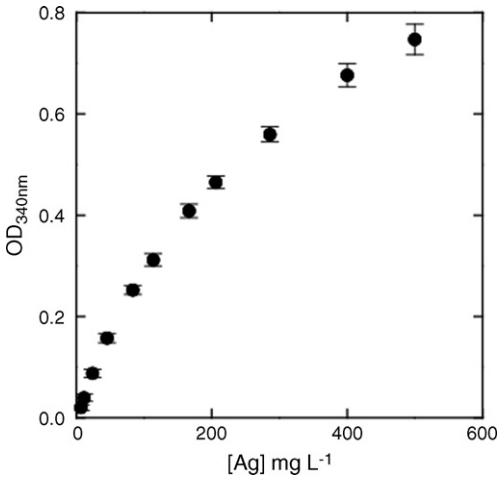

Fig. 4. Demonstration of the reproducibility of the C-assay. The average $OD_{340\,nm}$ density from 30 standard curves performed on 30 different days are plotted against Ag concentration. The error bars represent one standard deviation.

value for the product of 2600 mg L^{-1} ±10% RSD (product insert of Q0327, DakoCytomation A/S).

3.3. Reproducibility of C-assay standard curves

The standard curve shown in Fig. 4 is the average of 30 standard curves prepared on 30 different days. It should be noted that five different dilutions, all originating from the same manufacturing batch of goat anti-rabbit concentrate, and one lot number of rabbit immunoglobulin standard was used to generate all of the standard curves. Clearly, the reproducibility of the standard curve is very high.

3.4. [Ab] detection limit in the C-assay

In this study we employ the definition of detection limit given by Blirup-Jensen [2], i.e. the lowest signal of a standard, where the standard deviation (SD) around that signal multiplied by three, is less then the signal itself, i.e. $OD_{340\,nm} \geq 3 \times SD_{OD340\,nm}$. The two lowest standards used (6.6 and 11.2 mg L^{-1}) in the C-assay gave average $OD_{340\,nm}$ (±SD; $n = 30$) measurements of 0.020 ± 0.006 and 0.039 ± 0.007, respectively. Consequently, the standard curve can be used in the range covered (6.6–500 mg L^{-1}), resulting in a preliminary detection limit of 6.6 mg L^{-1} (corresponding to 0.05% of the Ig concentration in rabbit serum). It is interesting to note that the detection limit identified here is in good compliance with that quoted by Galvin [7] for light scattering immunoassays.

3.5. Imprecision in the C- and F-assays

The intra-, inter-, and total-assay imprecision was determined independently for both assays, using unconditioned rabbit serum for the C-assay, and neat rabbit anti-human transferrin (RbaHuTrans) antiserum for the F-assay. The samples were tested five times on three different days. One set of standard dilutions was used for all samples tested within one day, and fresh standard dilutions were made every day. The relative standard deviation (RSD) within one day (Table 1) ranged from 2.2 to 3.8% for the C-method, and from 1.9 to 2.4%

Table 1
Imprecision of the F- and C-assays tested on rabbit serum and RbaHuTrans antiserum, respectively

Assay	Measurement	Day	Titre or [Ig] (%)					Mean ± %RSD[a]
			Test 1	Test 2	Test 3	Test 4	Test 5	
F	Titre	1	98.9	100.9	98.6	103.2	100.1	100.3 ± 1.9
		2	93.9	97.8	96.2	95.4	100.1	96.7 ± 2.5
		3	101.7	103.2	100.1	105.5	104.4	103.0 ± 2.1
		All						100 ± 3.3
C	[Ig]	1	100.0	101.3	96.8	94.3	93.0	97.1 ± 3.7
		2	107.0	103.0	105.5	101.7	97.1	102.9 ± 3.7
		3	98.1	98.9	101.1	98.7	103.4	100.1 ± 2.2
		All						100 ± 3.9

On three consecutive days, five independent concentration and titre determinations were made. The total average concentration and titre were set to 100 and all results are written as percentages there of.

[a] %RSD = SD × 100/mean.

for the F-assay, whereas the overall relative standard deviation for all determinations ($n = 15$ for both assays) came to $\leq$3.9% and $\leq$3.3% for the C- and F-methods, respectively. These values of imprecision compare very favourably with those reported previously for manual light scattering immunoassays [8].

3.6. Linearity in the C-assay

The linearity of the method for quantifying Ig concentration, i.e. achieving the same result with a sample, irrespective of where on the standard curve it falls, was tested with numerous samples taken at various stages during an Ab purification run (performed at DakoCytomation following the method of Harboe and Ingild [6]). Seven main product stream fractions (numbered 1, 2, 3, 4, 5, 7, and 8), covering the entire purification scheme (Fig. 5) from sample to product, were assayed. A master dilution of a sample was prepared to be within the range of the standard curve. This diluted sample was subsequently diluted 1 in 2, 1 in 4, and 1 in 8, to generate three additional samples. The results obtained were normalised with respect to the master dilution (a value of unity was assigned to the master dilution) and plotted against the expected values. Regression analysis performed after pooling all the data gave the equation $y = 0.995x + 0.001$ and an R^2 value of 0.999, i.e. very close to unity ($x = y$).

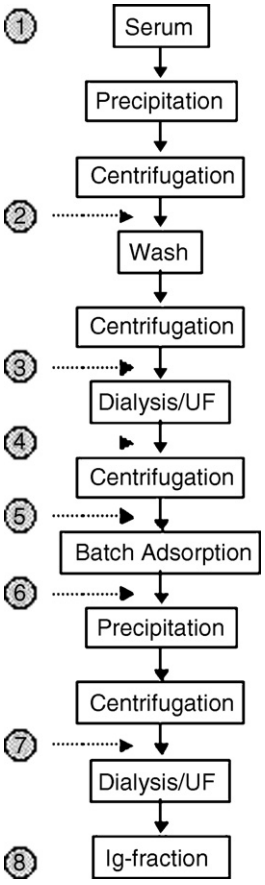

Fig. 5. Flow sheet of Ab purification process described by Harboe and Ingild [6]. The numbers refer to points where samples were taken.

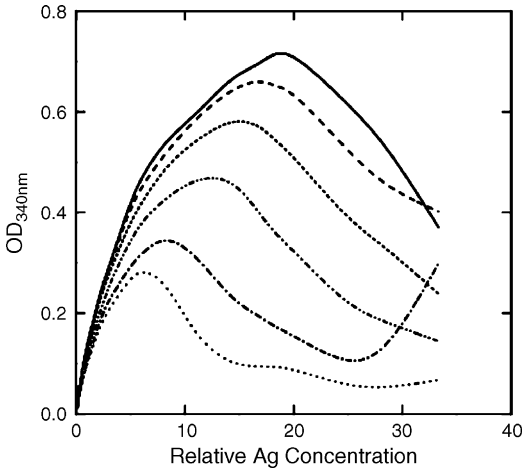

Fig. 6. F-assay precipitin curves determined at various Ab (RbaHuTrans) concentrations 420 s after addition to Ag (HuTrans). RbaHuTrans was diluted to: 8.62 (—); 7.52 (— —); 6.45 (– – –); 5.38 (– · · –); 4.10 (— · —); and 3.23 (· · · ·) % (v/v). An average titre of 2385 mg L^{-1} ±2.6% RSD was determined. When performing the same analysis with unconditioned RbaHuTrans antiserum a relative standard deviation of 2.4% was obtained.

3.7. Linearity in the F-assay

The linearity of the F-method for determining titre was evaluated with Ab standard and RbaHuTrans antiserum. Ab standard was analysed in six different dilutions (ranging from 8.62 to 3.23%, v/v, Fig. 6). An average titre of 2385 mg L^{-1} ±2.6% RSD was found, which lies within the relative standard deviation of 3.3% determined for the assay (see Table 1). Thus, samples with titres as low 76 mg L^{-1} (corresponding to 3% of the Ab standard) can be tested reliably. Although it is possible that more diluted samples could be analysed in the current system, precision in determining the maxima of precipitin curves decreases with fewer points in the Ab-excess zone (i.e. points at Ag concentrations greater than that at the maxima). The same analysis was performed with unconditioned RbaHuTrans antiserum, and a similar relative standard deviation of 2.4% was determined.

3.8. Matrix effects in the C- and F-assays

The sensitivity of the C- and F-assays to interference from components present in immunoglobulins' environment are commonly referred to as matrix effects. Matrix effects were tested in the C-assay by spiking known amounts of standards into five different samples of varying matrix complexity. These included diluted rabbit serum in filtered (0.45 μm) and unfiltered states, two diluted samples taken at intermediate stages during the Ab purification run (i.e. samples 3 and 5, Fig. 5), and the final product obtained (sample 8, Fig. 5). Fig. 7 shows plots of expected concentration versus those found during such spiking experiments. No adverse effects of any of the sample environments on measurements of Ig concentration were determined, as is evidenced by the fact that all of the data points were seen to collapse along a common straight line ($y = 0.96x + 14$, $R^2 = 0.998$) very close to unity ($y = x$).

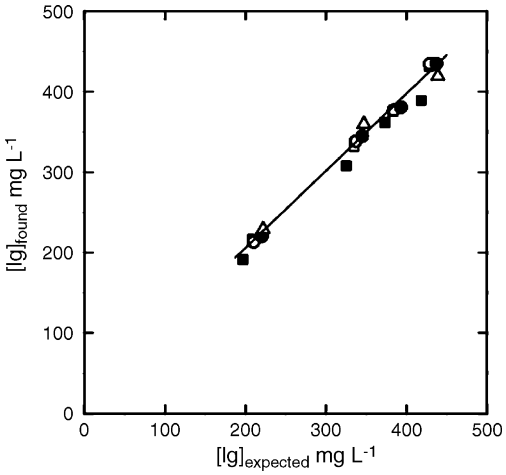

Fig. 7. Matrix effects in the C-assay. Standards were spiked into five different samples of varying complexity, and the Ig concentrations found were plotted against those expected. Key: (□) antiserum, sample 1; (■) Ig-fraction, sample 8; (◯) sample 3; (●) filtered antiserum; and (△) sample 5 (see text and Fig. 5 for sample identification).

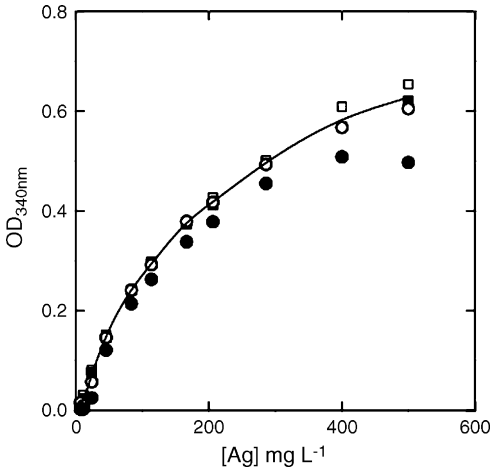

Fig. 8. Effect of ammonium sulphate concentration on determination of [Ig] in the C-assay. Key: (□) 0 M; (■) 125 mM; (◯) 250 mM; and (●) 500 mM. The line through the 0–250 mM ammonium sulphate data sets represents the average trace.

Matrix interference on the measured Ab titre in the F-assay, was also tested by spiking known amounts of Ab standard into RbaHuTrans antiserum. The plot of 'found' versus 'expected' titre ($y = 0.97x$; $R^2 = 0.998$) confirms the absence of significant matrix effects (data not shown).

3.9. Interference from reagents employed during Ig purification

In order to further assess the usefulness/robustness of the C- and F-immunoturbidimetric assays for their intended tasks, such as monitoring Ig concentration and functionality during Ig purification processes, it was important to establish the influence of various components featuring in these. Key to the success of Harboe and Ingild's [6] Ig purification procedure is the use of high concentrations of ammonium sulphate at several stages. Whicher et al. [5] describe Ag–Ab complex formation as being insensitive to changes in pH within the range 6–8, but highly sensitive to ionic strength. With this in mind, it was important to establish the influence of ammonium sulphate concentration on both assays.

For evaluating the impact of ammonium sulphate concentration of measurement of Ig concentration in the C-assay, four different standard curves were prepared by supplementing the standard dilution buffer 1 with various amounts of ammonium sulphate to yield a series with final concentrations of 0.125, 0.25, and 0.5 M. In the assay, the actual ammonium sulphate concentration was reduced by 1.9-fold following addition of other assay reagents, to give final ammonium sulphate concentrations of 64, 129, and 257 mM. Fig. 8 shows that even at the highest ammonium sulphate concentration employed (i.e. 257 mM final concentration), the assay signal was only slightly reduced. The highest ammonium sulphate concentration encountered in Harboe and Ingild's [6] purification procedure is 1.77 M, which for an undiluted sample after in-assay dilution yields a final ammonium sulphate concentration of 253 mM. Thus, significant interference arising from the presence of high concentrations of ammonium sulphate is unlikely.

Following assessment of assay sensitivity to ammonium sulphate, assay integrity was further studied with respect to a wide range of loading and elution buffers recommended for the purification of Ig by various chromatographic separation principles (e.g. affinity adsorption of rProtein A and synthetic mimics thereof, hydrophobic charge induction, and mixed mode). The following 10 buffers were tested: (i) 50 mM sodium acetate, pH 4.0; (ii) 50 mM sodium acetate + 150 mM NaCl, pH 5.5; (iii) 100 mM sodium citrate, pH 2.7; (iv) 100 mM sodium citrate, pH 3.5; (v) 100 mM sodium citrate, pH 5.5; (vi) 50 mM Tris–HCl, pH 8.0; (vii) 50 mM Tris–HCl, pH 9.0; (viii) 65 mM Bis-Tris, pH 6.0; (ix) 100 mM acetic acid; and (x) 20 mM sodium phosphate + 1 M NaCl, pH 6.5. Three or four dilutions of Ig standard were prepared in each buffer, and the Ig concentrations found in the C-assay were plotted against those expected (Fig. 9). Only one of the buffers showed significant interference in the assay, i.e. 20 mM sodium phosphate + 1 M NaCl, pH 6.5, reducing the Ig concentration 14% below the expected value. However, when analysing chromatographic runs, most of the fractions require at least a 10-fold dilution to bring them within the range of the assay. As a consequence of this, deviations observed in Fig. 9 from 20 mM sodium phosphate + 1 M NaCl, pH 6.5 are unlikely to be an issue of major concern, given that the buffering capacity of the dilution and reaction buffer ensures a pH of 6–8. The wide range of buffers studied here are representative of those most widely used in chromatographic Ig purification and serve to illustrate the general robustness and potential of the C-assay for routine monitoring of Ig purification processes.

The impact of ammonium sulphate on Ig titre determination in the F-assay was likewise evaluated. Six different standard Ab dilutions (diluted to 6.58%, v/v) were prepared by supplementing the standard dilution buffer 1 with various amounts of ammonium sulphate to yield a series with final concentrations of 0.25–1.5 M. In the assay, the actual ammonium sulphate concentration was reduced 3.1-fold following addition of other assay

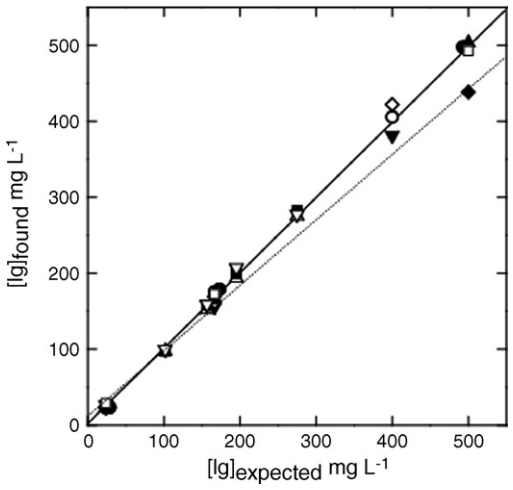

Fig. 9. Buffer interference on the C-assay. Three or four dilutions of standard rabbit Ig were prepared and assayed in the buffers: (□) 50 mM sodium acetate, pH 4.0; (■) 50 mM sodium acetate + 150 mM NaCl, pH 5.5; (◯) 100 mM sodium citrate, pH 2.7; (●) 100 mM sodium citrate, pH 3.5; (△) 100 mM sodium citrate, pH 5.5; (▲) 50 mM Tris–HCl, pH 8.0; (▽) 50 mM Tris–HCl, pH 9.0; (▼) 65 mM Bis-Tris, pH 6.0; (◊) 100 mM acetic acid; and (♦) 20 mM sodium phosphate, 1 M NaCl, pH 6.5. Linear regressions performed using the graphics program Microcal Origin software version 4.1 yielded the equations $y = 0.86x + 11.9$, $R^2 = 0.998$ (dashed line) for the '20 mM sodium phosphate + 1 M NaCl, pH 6.5' data set, and $y = 0.99x + 2.05$, $R^2 = 0.999$ (full line) for all of the other buffers examined.

reagents, to give final concentrations of 82–489 mM. In stark contrast to the C-assay's robustness towards ammonium sulphate, the F-assay (Fig. 10) is much less tolerant to its presence. At all concentrations employed the titre was underestimated and the kinetics of lattice formation were impaired. For example, after 420 s the presence of 82 mM ammonium sulphate reduced the apparent titre by 16% *cf.* the control case lacking added ammonium sulphate (i.e. from 2490 to 2090 mg L^{-1}). Following in-assay dilution, the highest ammonium sulphate concentration employed in Harboe and Ingild's [6] purification procedure, of 1.77 M reaches a final concentration of 616 mM. Thus, in contrast to the C-assay, the F-assay is likely to suffer very considerable interference arising from the presence of high concentrations of ammonium sulphate. For such samples the introduction of pre-assay conditioning measures would be a wise precaution.

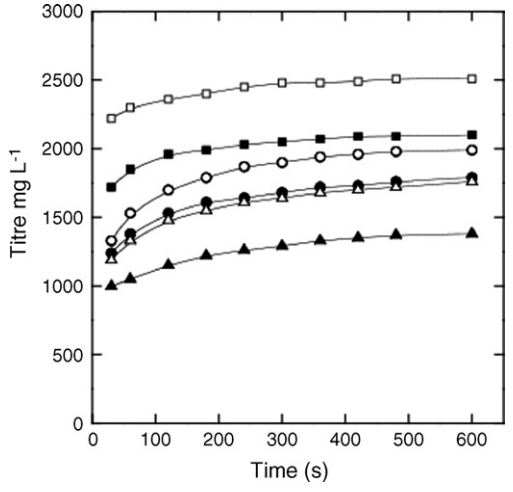

Fig. 10. Effect of ammonium sulphate concentration on determination of Ig titre in the F-assay. Standard Ab (6.58%, v/v) diluted ammonium sulphate containing buffer yielding a resulting concentration during reaction of: (□) 0 M; (■) 82 mM; (◯) 163 mM; (●) 245 mM; (△) 326 mM; and (▲) 489 mM. The titre determined at 420 s at 0 M ammonium sulphate was 2490 mg L^{-1}.

The F-assay's integrity was further studied with respect to pH variation and buffering capacity. For this, in addition to modifying the pH of the original phosphate based reaction buffer 1, two other buffer systems were investigated (at two different strengths, i.e. 10 and 100 mM), namely (*N*-morpholino)-ethane sulphonic acid (MES), pH 6, and Tris-(hydroxymethyl)-aminomethane (Tris), pH 8. Fig. 11 shows that substituting 10 and 100 mM Tris–HCl buffer, pH 8, for the unmodified reaction buffer 1 (pH 7.1–7.3) resulted in the titre being underestimated by 11.6% and 32%, respectively. In contrast, MES, pH 6 buffers exerted much less impact on the titre determined, i.e. resulting in 2.5% and 9% underestimation when used at strengths of 10 and 100 mM, respectively. Modification of the original reaction and dilution buffers by ±0.5 pH units from the normal pH = 7.2 did not change the expected titre significantly (Fig. 11), thus interference arising from fluctuations in pH (within the range 6.7–7.7) introduced by the sample matrix is unlikely to occur.

3.10. Impact of sample blanking

In most automated turbidimetric procedures, a sample blank is subtracted from the endpoint reading [2,4]. Control experi-

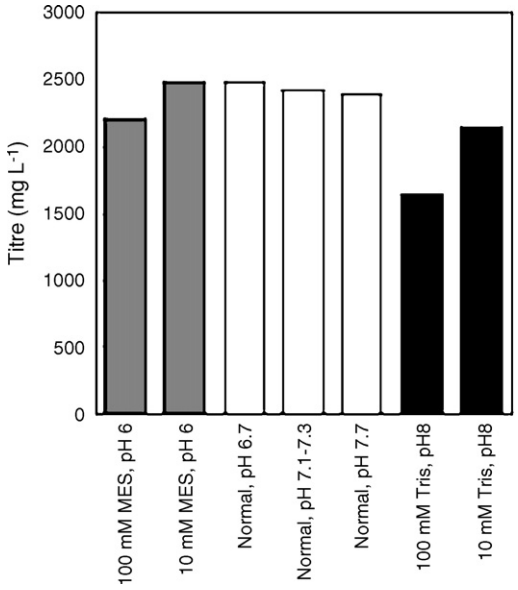

Fig. 11. Effect of reaction buffer composition and pH on titre determination of standard Ab (6.58%, v/v) in the F-assay. Key: White bars-standard (pH 7.2) and pH modified (pH 6.7 and 7.7) phosphate based reaction buffer 1 and dilution buffer 1 system; grey bars 10 and 100 mM MES-based reaction buffers, pH 6. All other constituents of the buffer were the same as those in standard reaction buffer 1. MES-based reaction buffer showed insufficient buffering capacity at 10 mM, yielding a final pH of the reaction mixture of 6.7; black bars 10 and 100 mM Tris–HCl based reaction buffers, pH 8. All other constituents of the buffer were the same as those in standard reaction buffer 1.

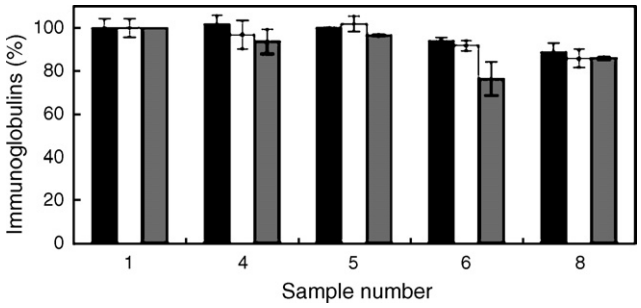

Fig. 12. Step yields from antibody purification runs conducted with rabbit anti-human IgM (black bars), rabbit anti-human transferrin (white bars), and rabbit anti-human albumin (grey bars). For sample number identity refer to the purification flow sheet depicted in Fig. 5.

ments proved this to be unnecessary for the assays described herein (data not shown). Provided that the non-specific optical density prior to reagent addition is reasonably low, the precision gained by using a sample blank is limited. Moreover, in its absence twice as many samples can be tested on a single microtitre plate, and the rate of consumption of reagents is halved.

3.11. Application of the C- and F-assays during an Ig purification process

The analysis of Ig purification runs performed on three different rabbit antisera (rabbit anti-human IgM, albumin and transferrin) using the C-assay, are summarised in Fig. 12. The overall mass yields obtained for rabbit anti-human IgM, albumin and transferrin were equivalent ($89 \pm 4\%$, $86 \pm 4\%$ and $86 \pm 1\%$, respectively); correlating to average step yields of >98%. These recoveries, based on mass, are in good agreement with SRI (data not shown) and with titre yields of the RbaHuTrans antiserum purification (Fig. 13) obtained with the F-assay. The overall titre yield was determined to 93% (*cf.* 86% based on mass, Fig. 12) corresponding to average step yields of 99%. These findings suggest that both assays give a good representation of the actual recovery profiles. Further validation of the C-assay for quantifying antibodies has recently been obtained in our laboratories [11,12] in the successful mass balancing chromatographic purifications of Igs from rabbit antisera feedstocks.

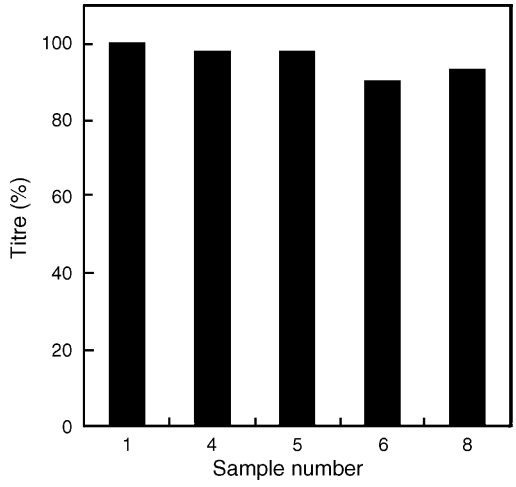

Fig. 13. Step yields from antibody purification run conducted with rabbit anti-human transferrin. The numbers identify the samples taken during purification (Fig. 5).

4. Conclusions

Although manual assays based on immunoturbidimetry have previously been described [8,9] they are significantly slower than those detailed in the present study. A recent development in turbidimetry has been the use of particles to enhance the signal sensitivity 10–100-fold, i.e. down to 15 $\mu g\,L^{-1}$. In this type of assay, the Igs are coupled to small (40–300 nm) polystyrene particles [2,3,7]. Commercial kits for measuring substances of clinical interest by particle-enhanced turbidimetry are available, and like the assay described in this work, some of these kits are designed for use in microtitre plates, allowing simultaneous measurement of multiple samples [10]. A major disadvantage of particle-enhanced immunoturbidimetric methods however, is non-specific agglutination of the polystyrene particles (induced by problem components present in samples being analysed). The latter phenomenon reduces the robustness of the assay significantly, making it unsuitable when the suspending phase composition is likely to change frequently, as it can during a purification process. The C-assay developed and validated in this work, although much less sensitive than particle enhanced turbidimetric assays, is nevertheless more than adequate for the task of measuring Ig concentrations during a purification process, given that the assay detection limit of 6.6 $mg\,L^{-1}$ corresponds to only 0.05% of the recognised value of IgG in rabbit serum of 13.6 $g\,L^{-1}$ [13]. Moreover, this method proved extremely tolerant to changes in suspending phase composition. Although somewhat more sensitive and time consuming, the assay for determining antibody functionality (F-assay) is an orthogonal tool, which enables selection of high titre fractions and monitoring of Ig functionality or loss thereof. The C- and F-assays can be performed manually with very basic laboratory equipment, or alternatively both methods are easily automated, e.g. with high throughput spectrophometric robot systems.

Acknowledgements

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