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A highly efficient preparative-scale generic immunoaffinity chromatography method for the purification of antibodies to hydrophobic haptens: Purification procedure and monitoring tests

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Abstract

A generic affinity chromatography purification protocol for the isolation of preparative quantities of pure and stable polyclonal antibodies to hydrophobic haptenic analytes is described together with a panel of tests to monitor the purification process and assess the functional and structural purity of isolated antibodies. The purification method is based on the use of a mixture of acetonitrile and propionic acid to elute bound antibodies from Sepharose 4B-based immunoabsorbent gels. Highly specific and pure antibodies to steroid estrogens, pentachlorophenol and Irgarol 1051 were isolated in 50–150 mg quantities per preparation in a batch-wise method using appropriate ligands linked to the solid phase via a hydrophilic chemical arm, tetraethylene pentamine. The panel of ELISA tests together with SDS–PAGE enabled the monitoring of the absorption and elution steps and provided data relevant to the assessment of the degree of structural and functional purity of the isolated antibody preparations. The study demonstrates that the affinity purification procedure is practical, simple, generic for antibodies to hydrophobic haptens and suitable for scaling up. In addition, the study showed that the functional properties of the affinity-purified antibodies indicated improvements on the operational properties (specificity and assay detection limits) of the source antisera. The isolated IgG antibodies showed near 100% functional and structural purity and no deterioration of activity on storage for long periods. The method provides critical reagents for labelled-antibody immunoassays and immunosensors and antibody-dependent sample purification techniques.

Keywords: Immunoaffinity chromatography; Hydrophobic haptens; Generic elution methods

1. Introduction

The increasing wider use of immunochemical techniques in larger numbers of application areas and the pressure to continuously develop improved more convenient diagnostic test formats require the development of appropriate key reagents to meet emerging needs. This is particularly true for the analysis of haptenic analytes in clinical, toxicology and environmental diagnostics. The emergence of new technologies such as immunosensors, sensing arrays and tandem techniques with rapid sample purification steps require cost-effective sourcing of purified antibody reagents in amounts appropriate for product manufacture and long-term planning. Immunochemical analysis of haptenic analytes involve either immobilisation of antibodies to solid phases or more frequently assays are based on immobilised antigens (haptenic species) in which case antibodies are conjugated to detectable labels. Labelling of functionally pure antibodies and antibody fragments with enzymes [1–4], fluorescent substances [5–11] and nanoparticles [12,13] provide convenient strategies for most of the mainstream immunoassay formats. In addition to requirement of purified antibodies by analytical techniques, the reagents are also needed for the preparation of sample extraction mini columns and in even larger quantities for therapeutic purposes [14–16].

Polyclonal antisera are currently the main source of antibodies to organic haptenic analytes. This is due to partly cost consideration, advancement in the technology of the generation of polyclonal antisera but mainly due to performance features.

Isolation of functionally pure antibodies from antisera requires use of affinity chromatographic techniques based on the

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functional specificity of antibodies. The process involves several steps including treatment of source antisera, preparation of solid phase ligand species, absorption (extraction) of target antibodies, removal of unwanted proteins and other substances, elution of bound antibodies, removal of elution agents and recovery of re-natured products. Several aspects of the purification process are considered to be important: the capacity of the extraction solid phase matrix, the ease of eluting bound antibodies rapidly and with minimum damage to antibodies and sorbent matrix and the scalability of the process. However, the elution method is the critical element of the process. Several reports have described different methods for the elution of antibodies to hydrophobic haptenic substances. The methods involve use of mixtures of acids, organic solvents and chaotropic salts which varied considerably in their efficiency of recovering purified antibodies [17–20]. Fully optimised generic techniques for the isolation of preparative quantities of functionally pure and stable antibodies to priority haptenic analytes would contribute to the advancement of the art, this currently is not available. In addition, monitoring and characterisation tests for the quantitative assessment of both the isolation process and purity of products would provide critical enabling tools that perhaps are lacking at present.

In this report, a generic method for the elution of antibodies to hydrophobic haptens [21–23] is applied to the isolation of polyclonal antibodies to three haptens (estrogens, pentachlorophenol and Irgarol 1051) and the details of ELISA-based monitoring and assessment tests are given.

2. Materials and methods

2.1. Materials

Buffer salts, bovine serum albumin, sodium azide, thimerosal, Tween-20, fish gelatine, porcine gelatine, goat anti-sheep-horse radish peroxidase, hydrogen peroxide solution (30%), ABTS, cyanogen bromide, Sepharose 4B, DEAE-Sephadex A50, activated charcoal powder, dialysis tubing cellulose membrane (treated as recommended, retains MW greater than 10 kDa), acrylamide/bis-acrylamide 30% solution, *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED), Coomassie brilliant blue, bromophenol blue, glycerol and steroid compounds were obtained from Sigma Chemical Company, Poole, Dorset, UK. Full range rainbow molecular weight marker was obtained from Amersham Biosciences Ltd., Little Chalfont, Buckinghamshire, UK.

Semipermeable membranes (retention size 10 kDa) were obtained from Schleicher and Schuell UK Ltd., Brunswick Industrial Estrate, London, UK.

Propionic acid, acetonitrile, glacial acetic acid, methanol, mecaptoethanol, triethylamine, tetraethylene pentamine, and *N*,*N*-bis(3-aminopropyl)ethylenediamine were purchased from Aldrich Chemical Company, Gillingham, Dorset, UK. ELISA plates (highbind flat 96-wells) were from Greiner bio-one, GmbH, Gloucestershire, UK. Triazine reference compounds were from Reidel-de-Haën (Sigma Chemical Company). Chlorophenols were obtained from Lancaster Synthesis, Morecambe, Lancashire, UK. TMB ready-to-use substrate was purchased form MicroImmune Ltd., Brentford, Middlesex, UK.

2.2. Hapten derivatives and coupling to carrier protein

Preparation of hapten derivatives for general estrogens, pentachlorophenol and Irgarol 1051, coupling to carrier proteins and generation and assessment of sheep antisera have been described previously [24–26].

2.3. Preparation of immunoabsorbents

The immunoabsorbent gels (affinity gels) were prepared by attaching the hapten derivatives to Sepharose 4B, via a relatively long hydrophilic linkage arm, tetraethylene pentamine. The Sepharose 4B-*arm*-NH_2 matrix was prepared using CNBr-activated Sepharose according to described procedures [22,27]. To a suspension of 20 mL of the Sepharose 4B-*arm*-NH_2 preparation (suspended in 20 mL 1% $NaHCO_3$, 10 mL DMF and 0.5 mL of TEA), about 80 mg of hapten derivative *N*-hydroxysuccinimide active ester (8 mg/mL in DMF) was added gradually over 30 min while mixing. Mixing was continued for 16 h at room temperature before the gel was transferred to a 100-mL glass sintered funnel and washed (under suction with vacuum) with 600 mL of 50% methanol, 80 mL of 50% DMF, 120 mL of elution mixture (20% acetonitrile and 1% propionic acid), 1 L of water, 0.5 L of 3% $NaHCO_3$ and finally with 0.5 L of phosphate buffer. The washed affinity gels were mixed with 10 mL of serum (taken from un-immunised sheep) diluted in 40 mL of phosphate buffer followed by washing cycles using buffer and elution mixture. The affinity gels were stored in 50% methanol at 8 °C.

2.4. Treatment of antisera and batch wise loading and elution

Antisera, 50 mL, were diluted in phosphate buffer to 250 mL, mixed with 1.5 g of activated charcoal powder and filtered to remove fines and charcoal. The treated antisera solutions were mixed with about 15 mL of the appropriate immunoabsorbent for about 10 h before the gel and antiserum mixture were transferred to a sintered glass funnel and the process was continued with the washing and elution steps as described previously [21,22], using 0.5 M NaCl in 50 mM sodium bicarbonate, followed by washing with deionised water. The elution was carried out with an ice-cold mixture of 20% acetonitrile and 1% propionic acid in deionised water, using 15 mL batches. The elution mixture was left in contact with the gel for about 10 min before draining into 1 mL of 100 mM sodium phosphate buffer pH 8 (kept on ice). After taking absorbance reading at 280 nm, 100 μL of 1% organic base (*N*,*N*-bis(3-aminopropyl)ethylenediamine), diluted in phosphate buffer) were added to the eluate to neutralise the acid. After taking aliquots for assessment tests, the fractions were dialysed against 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 0.5% activated charcoal powder. Antibody solutions were recovered and volumes reduced using

an Amicon ultrafiltration stirred-cell (Amicon, Beverly, MA, USA), that was fitted with a semipermeable membrane that had a retention size of 10 kDa, equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 150 mM NaCl, passed through 3-mL mini-columns of DEAE-Sephadex A50 ion exchanger and the antibody proteins were precipitate by the addition of equal volumes of saturated ammonium sulfate and stored in the cold until assessment.

2.5. Monitoring of the purification process

Absorption of antibodies by the appropriate immunoabsorbent gels was measured by solid phase ELISA in which antibody activity was titrated using samples taken before and after mixing with the affinity gels. Antibody activity measurement was carried out using ELISA plates coated with appropriate plate coating antigen (PCA) preparations. The antibody dilution response curves covering dilution range of 1/200 to 3.2×10^6, were carried out according to the previously described procedures [24–26].

The elution process was also monitored by measuring absorbance at 280 nm and by solid phase ELISA in which antibody binding activity was assessed using samples taken from washing and elution fractions. Samples were first diluted to appropriate levels ranging from 1/400 to 1/2000 before 150 μL aliquots were added to PCA-coated plates. Alternatively, this was easily performed by diluting samples directly in assay plates because it was impossible to predict in advance the appropriate dilution suitable for different preparations. After incubation for 1 h, the plates were washed and the signal was developed using secondary antibody HRP conjugate as described previously [24–26]. The absorbance at 280 nm and the ELISA activity at 405 nm were plotted against fraction numbers.

2.6. Assessment of purified antibodies

Samples of antibodies stored under the ammonium sulphate were collected by centrifugation, dialysed against 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and the protein content was adjusted to 5 mg mL^{-1}. For comparison, IgG fractions of the antisera were prepared by ion exchange chromatography on DEAE Sephadex A50 [28] and samples were also adjusted to about 5 mg mL^{-1}. The purified and un-purified antibody samples were assessed in terms of antibody functional purity, IgG structural purity, gel electrophoresis protein patterns, analyte dose response curves by competitive ELSA, minimum detection limits (MDL) and antibody binding specificities.

2.6.1. Assessment of antibody functional purity

Antibody functional purity testing refers to determining the degree of purity of specific antibodies present in the given antibody samples, or the proportion of specific antibody protein to the total IgG content. This parameter was assessed by mixing antibody samples (taken from purified antibody samples or from IgG preparations isolated from source antisera) with aliquots of the appropriate immunoabsorbent gels or with equal amounts of control gel, Sepharose 4B-*arm*-NH_2 gel without any ligand attached. In order to ensure excess immunoabsorbent gel capacity (0.5 g of wet cake gel) antibody samples were adjusted to 200 μg of protein in 1.5 mL. Pure antibody and IgG samples from each antiserum were mixed with both types of gels for 10 h at 4 °C before centrifugation at 2000 RPM for 30 min. The supernatants were recovered and the gels were washed three times with 1.5 mL of phosphate buffer. The supernatant and the washing fractions were assessed in terms of antibody binding activity (using antibody dilution response tests) and in terms of sheep IgG content.

The IgG content was estimated by analysis of the supernatant and washing fractions with an antigen-drop-down ELISA in which the sheep IgG protein acts as an antigen. Therefore, the tests provided data on the proportion of IgG that is actually specific antibody. The IgG-class ELISA (IgG drop down) was carried out by coating serially diluted samples of the test solutions followed by signal development using goat anti sheep-HRP conjugate. The ELISA absorbance results (of unbound proteins) were used to estimate the proportions of IgG bound to the various Sepharose gels.

2.6.2. Assessment of IgG structural purity

Analysis of purified antibodies by non-denaturing and denaturing gel electrophoresis was perfumed using a Bio-Rad mini protein III system, according to the basic SDS–PAGE protocol [29] for analysis of immunoglobulin proteins under non-reduced [30] and reduced conditions [31]. Briefly samples of the purified antibodies and IgG preparations were adjusted to 2 mg mL^{-1} and 5 μL were added to 15 μL of either nondenaturing buffer (0.625 m Tris pH 6.8 with 2% SDS, 10% glycerol and 0.001% bromophenol blue) or denaturing buffer (above buffer with 5% mecaptoethanol) and the samples were vortexed briefly. Samples to be denatured were boiled for 5 min and centrifuged for 2 min. The entire volume of samples was loaded on 10% polyacrylamide gels and run at 80 mV using a discontinuous Tris-glycine buffer system for about 2 h. After which time they were stained for 2 h with Coomassie blue stain (0.2% Coomassie blue, in 50% methanol with 1% glacial acetic acid), while gently shaking, followed by washing twice in de-stain (0.05% methanol with 0.075% glacial acetic acid in distilled water) before de-staining overnight.

2.6.3. Analyte dose response curve and assessment of minimum detection limits

Analyte dose response curves, standard calibration graphs, were constructed as previously described using plate coating antigen-coated ELISA methods [24–26]. Assays for the individual analytes using either purified antibody samples or unpurified antisera were optimised using respective analyte heterologous PCA preparations. Appropriate dilutions were made from stocks of purified antibody preparations adjusted to 5 mg mL^{-1}.

The dose response graphs are shown as normalised curves in which binding at zero analyte is taken as 100%. The assessment of minimum detection limits was carried out according to previously described protocols being taken as concentration

of analyte that was found at three times the standard deviation of the mean of six replicas of zero analyte [25,26,32,33]. All three analytes were assessed using buffer and river (Thames) water matrices, however, irgarol 1051 was also analysed under seawater conditions [25].

2.6.4. Assessment of specificity

The cross reactivity levels of both the antisera and the respective purified antibody samples were assessed using competitive ELISA. The measurement of the cross reactivity and the calculation of results were carried out as previously described [24–26]. The calculation of the cross reactivity levels was performed by the 50% inhibition method [34,35].

3. Results

3.1. Preparation of immunoabsorbent gels

The affinity ligands, hapten derivatives, for the purification of the three antisera were linked to the Sepharose-4B through a relatively long hydrophilic arm, tetraethylene pentamine, to improve accessibility of ligands and reduce hydrophobic non-specific adsorption. The attachment of ligands was carried out using excess active ester intermediates to ensure preparation of high capacity affinity gels. Tests with lower amounts of active esters usually resulted in reduced capacity and that affected the efficiency of the purification process. The washing of the affinity gels before use was found to be quite an important phase probably due to trapping of water-insoluble ligands. The inclusion of organic solvents in the washing process was essential to remove all traces of uncoupled ligands. In addition to extensive washing with solvent-water mixture, the gels were treated with the elution mixture and with non-immune serum to remove all traces of weakly linked or adsorbed substances.

3.2. Uptake, elution and monitoring of the purification process

The binding of antibodies from the treated source antisera solutions was carried out by mixing with the immunoabsorbent gels for long periods of time at low temperature, 4–8 °C. The proportion of antibodies absorbed by the gels were assessed by titrating activity in the supernatants and compared with the total activity levels using samples of starting antisera solutions. The antisera dilution response graphs indicated virtual complete uptake of anti-general estrogens and anti-pentachlorophenol antibodies. However, the irgarol immunoabsorbent gel showed only about 80% uptake, Figs. 1A–3A. The elution of bound antibodies was performed in a batch-wise manner in order to avoid labour-intensive and slower column chromatographic procedure.

Monitoring of antibody activity and protein levels during the washing and elution phases indicated that wash fractions contained considerable amounts of proteins but much reduced antibody activity as expected. It should be added that the volumes of the wash fractions were about 50 mL and thus the actual protein content is not truly reflected in the absorbance bar charts shown in Figs. 1B–3B. Addition of elution mixture at fraction 15 led to immediate appearance in the eluate of considerable amounts of protein coupled with substantial levels of antibody activity. The elution of the general estrogens antibody showed a slightly different profile to the other two models. The protein elution profile (absorbance at 280 nm) showed a distinctive peak and continued to remain high for a considerably larger number of fractions. In all three cases the antibody activity profile (absorbance at 405 nm) remained higher than the corresponding protein absorption (280 nm). The estimated levels of recovery of eluted antibodies from 50 mL of source antisera were 155, 56 and 50 mg for the general estrogens, pentachlorophenol and irgarol, respectively. These, by any

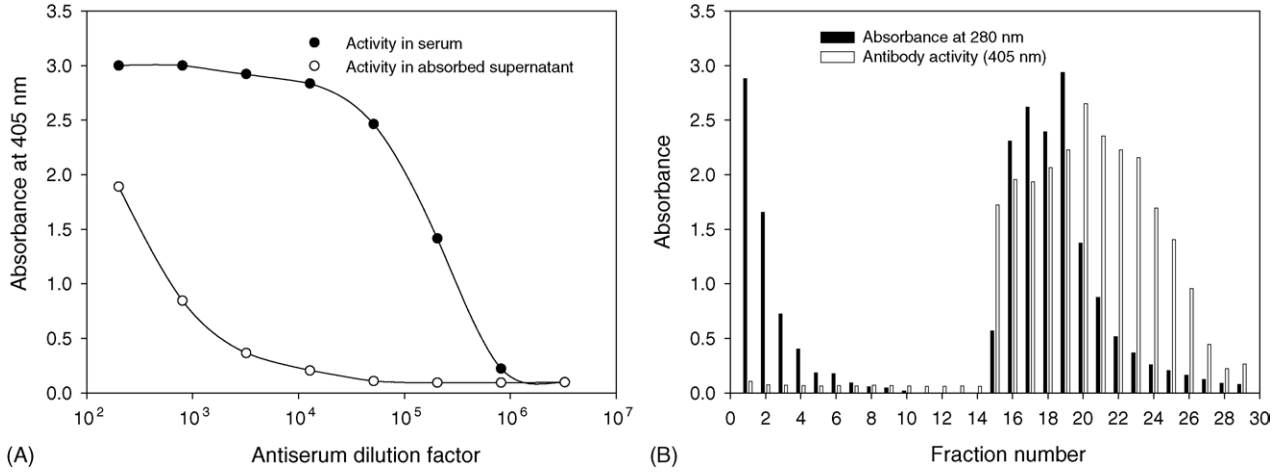

Fig. 1. The uptake of the anti-general estrogens antibody by the immunoabsorbent gel and a chromatogram of the affinity purification process: (A) shows antiserum dilution response curves before and after absorption by E_1-*O*-HG-Sephasrose 4B gel and (B) the levels of protein and antibody activity in wash and elution fractions. The elution profile of bound antibodies appears after the application of the elution mixture at fraction 15. The antiserum dilution response graphs in (A) were generated using estrone-17-carboxmethyl oxime-gelatine conjugate (PCA) at 1/100,000 and the antibody activity profile in (B) was assessed using the same PCA at 1/1600 and fraction samples diluted to 1/2000.

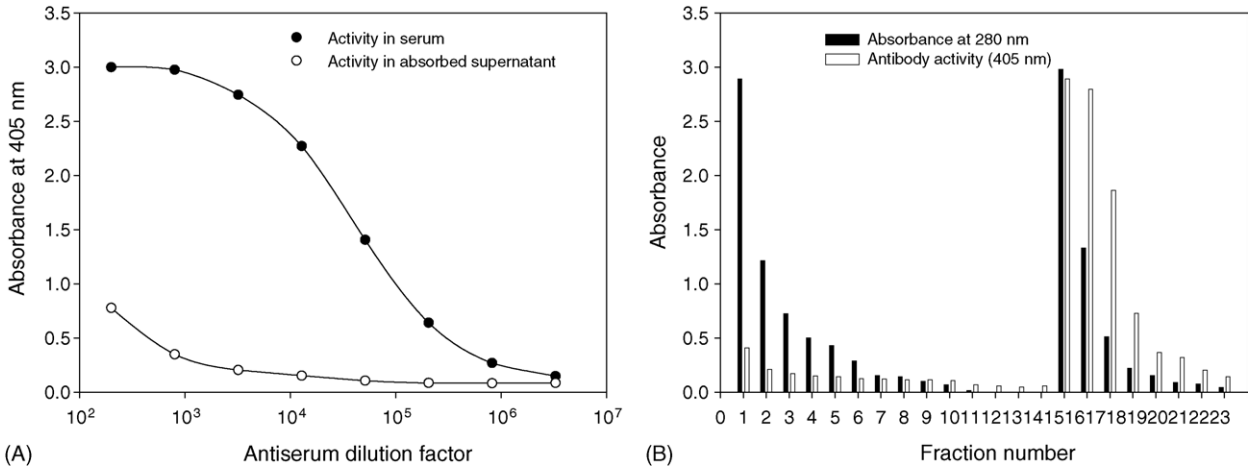

Fig. 2. The uptake and affinity chromatography of the anti-pentachlorophenol antibody: (A) shows antiserum dilution response curves before and after absorption by pentachlorophenoxy-butyric acid-Sepharose 4B gel and (B) wash and elution profile of proteins and bound antibodies after the application of the elution mixture at fraction 15. The antiserum dilution response graphs in (A) were generated using pentachloro-amido-butyric acid gelatine conjugate (PCA) at 1/3000 and the antibody activity profile in (B) was assessed using the same PCA and fraction samples diluted to 1/500.

standard of affinity chromatography, are respectable preparative quantities of products that were shown to be pure fully active antibodies.

3.3. Characterisation of the purified antibodies

The purified antibody preparations were assessed to determine the degree of purity in terms of specific antibody content, functional purity tests, and in terms of purity of IgG class protein, structural purity tests. In addition, the performance in competitive assays for the respective analytes and levels of cross reactivity with relevant compounds were measured. In all cases the results obtained with the purified antibodies were compared with values obtained with the un-purified IgG preparations isolated from the same batches of source antisera. Purified antibody samples were prepared and stored under 50% ammonium sulphate at 6–8 °C. During the generation of data for this study, antibody samples were used several months after the purification period. Titration tests on purified anti-Irgarol antibody carried out immediately after purification and 1 year later, under the same conditions, indicated titres of 1/40,000 and 1/39,000, respectively. In general no deterioration of antibody activity was observed over up to 3 years when stored under the conditions specified.

3.3.1. Binding of antibody preparations to affinity and control gels

Equal amounts of protein of both the purified antibodies and the IgG preparations (not affinity purified) were first mixed with samples of excess affinity gels or with equal amounts of control Sepharose 4B, before functional ELISA tests (measurement of antibody activity) were carried out on the supernatant and wash samples from each group. The tests showed general trend features that demonstrated the valuable aspects of the assessment

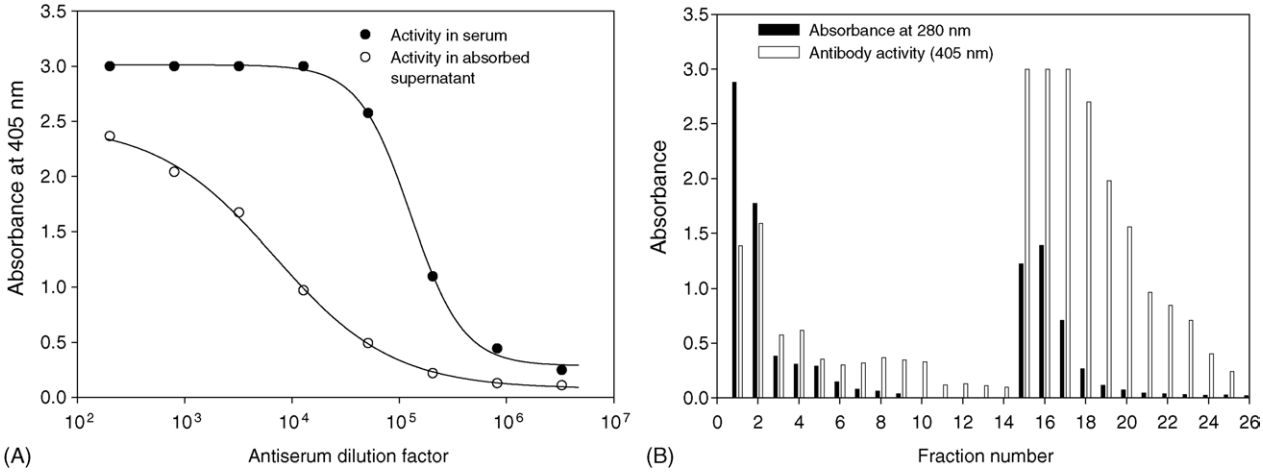

Fig. 3. The extraction and affinity chromatography of the anti-irgarol antibody: (A) shows antiserum dilution response curves before and after absorption by irgarol-thio-propionic acid Sepharose 4B gel and (B) wash and elution profile of proteins and bound antibodies after the application of the elution mixture at fraction 15. The antiserum dilution response graphs in (A) were generated using irgarol-*O*-phenoxybenzoic acid-gelatine conjugate (PCA) at 1/2500 and the antibody activity profile in (B) was assessed using the same PCA and fraction samples diluted to 1/2000.

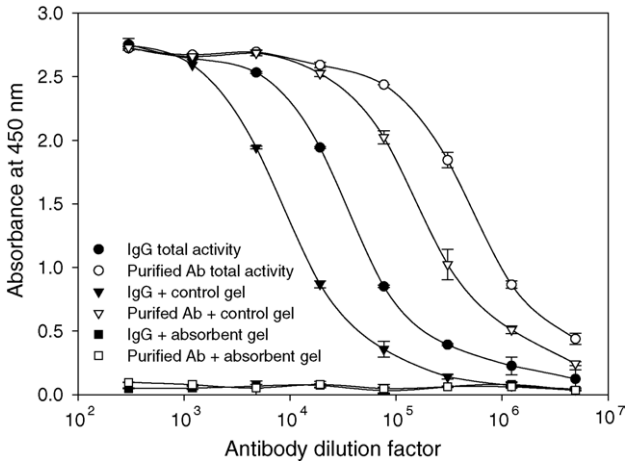

Fig. 4. Assessment of the binding of the general estrogens purified and IgG antibody with the immunoabsorbent and control gels. The figure shows antibody dilution response graphs of the total applied activities, IgG (●), purified antibody (○), IgG after mixing with control gel (▲), purified antibody after mixing with control gel (△), IgG (■) and purified antibody (□) after mixing with the immunoabsorbent gels. The total removal of detectable activity is observed when both antibody preparations were mixed with the immunoabsorbent gels.

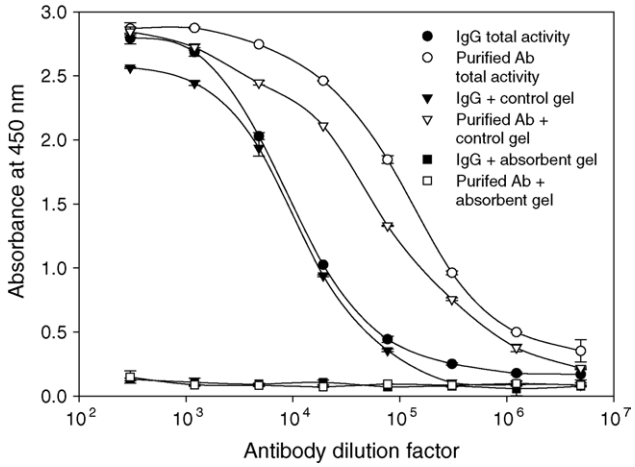

Fig. 6. Assessment of the uptake of the irgarol purified and IgG antibody with the immunoabsorbent and control gels. The figure shows antibody dilution response graphs of the total applied activities, IgG (●), purified antibody (○), IgG after mixing with control gel (▲), purified antibody after mixing with control gel (△), IgG (■) and purified antibody (□) after mixing with the immunoabsorbent gels. Removal of all detectable activity is seen when both irgarol antibody preparations were mixed with the immunoabsorbent gels.

tests (Figs. 4–6). The ELISA tests for antibody activity showed total disappearance of detectable activity in the supernatants after mixing with the affinity gels (obviously none was detected in subsequent wash liquids). This was observed both in the case of purified antibody and IgG preparation samples. Some losses (much less than after mixing with the affinity gels) were observed in antibody samples mixed with the control gels. The latter was due to the exaggerated non-specific adsorption to Sepharose beads because the tests were carried out without inclusion of any carrier proteins for operating reasons. The evidence for this was obtained by recovering substantial antibody activities in subsequent washing cycles of the control gels (not shown).

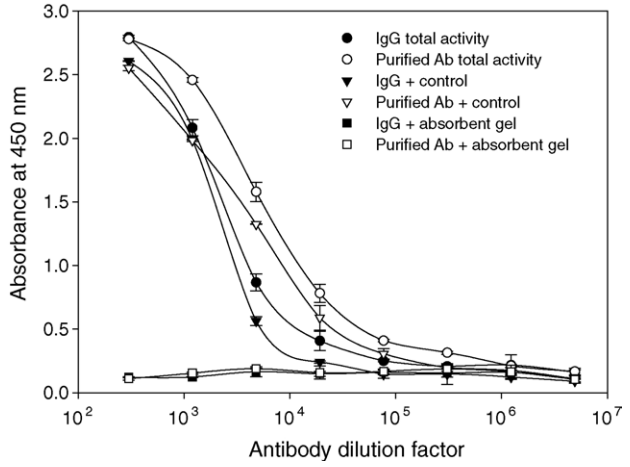

Fig. 5. Assessment of the absorption of the pentachlorophenol purified and IgG antibody with the immunoabsorbent and control gels. The figure shows antibody dilution response graphs of the total applied activities, IgG (●), purified antibody (○), IgG after mixing with control gel (▲), purified antibody after mixing with control gel (△), IgG (■) and purified antibody (□) after mixing with the immunoabsorbent gels. Complete removal of detectable activity is observed when the antibody preparations were mixed with the immunoabsorbent gels.

In the second panel of functional tests the antibodies under testing were measured as IgG antigens using the goat anti-sheep-peroxidase conjugate as the primary antibody. Therefore, the test provides information on the amounts of IgG left in the supernatant and washing samples. The results were used to estimate the proportion of IgG bound to the various gels. These antigen-drop-down tests demonstrated that mixing of the purified antibody samples with the control gels showed losses of about 10% protein whereas mixing with the affinity gel samples resulted in an uptake of about 99.8% of the starting material. The losses of proteins from the IgG preparation samples after mixing with the control gels were between 6 and 8%. The uptake of the protein from the IgG preparation with the affinity gel was around 20%. Since both the purified antibody and IgG preparations (200 μg per test) were used without carrier proteins to enable the direct antigen-drop-down ELISA tests to be carried out, the non-specific adsorption was noticeably high and therefore in order to recover all of the unbound proteins subsequent washing of the gels was necessary. The estimated bound materials after the washing steps were in the same order with all three antibodies and corresponding affinity gels (Fig. 7). The bar chart in the figure shows the average percentage of IgG bound taken from the three model systems (which were very similar).

3.3.2. SDS–PAGE tests

SDS–PAGE tests carried out on samples of purified antibodies and corresponding IgG preparations were performed to demonstrate the structural purity of samples in terms of immunoglobulin class. The tests were run under reduced and non-reduced conditions and the results confirmed the expected picture (Fig. 8). Both the IgG preparations and purified antibody series were of IgG class and contained no detectable contaminant

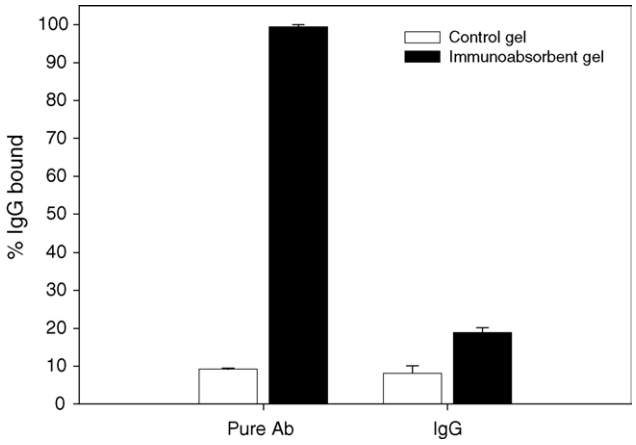

Fig. 7. Absorption of purified antibody and IgG preparations to the three test analytes by control and by the corresponding immunoabsorbent gels. The absorption was measured in this case by the antigen drop down assay in which the assay detected sheep IgG proteins as *antigens* and therefore the uptake percentage is independent of the individual binding activities of the tested antibody proteins. Unlike the activity absorption tests, this test is an accurate measurement of the functional purity of the antibody preparations.

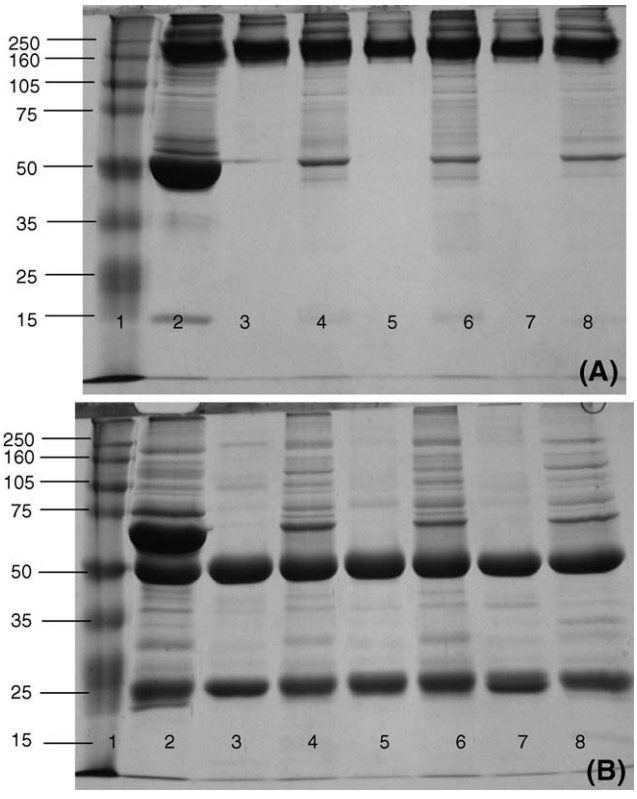

Fig. 8. Images of SDS–PAGE stained gels of non-reduced (A) and reduced (B) samples of purified antibodies and source antisera. Lane 1 shows the profile of the full range molecular weight markers, lane 2 shows protein stains of an untreated sheep serum sample, lanes 3, 5 and 7 show the profiles of purified antibodies to general estrogens, pentachlorophenol and irgarol, respectively. Lanes 4, 6 and 8 show the profiles of the IgG preparations.

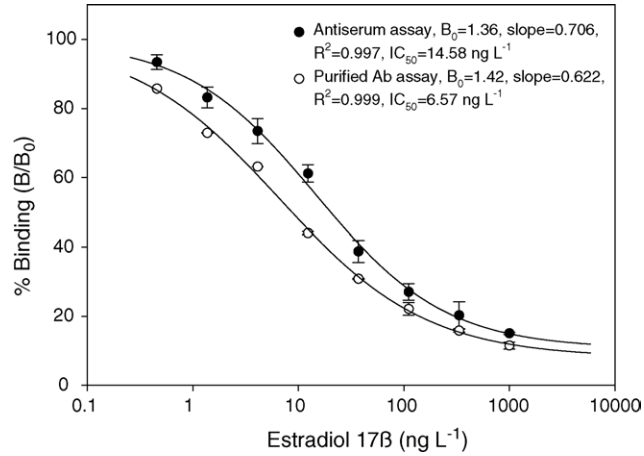

Fig. 9. Normalised calibration graphs of competitive ELISA for estradiol-17β using the source antiserum (●) and the purified antibody (○). The assay was carried out using estrone-17-*O*-carboxymenthyl oxime-gelatine as PCA diluted to 1/1600. The antiserum and the purified antibody were used at 1/30,000 and 1/190,000, respectively.

proteins that could have conceivably been concentrated during the affinity purification process.

3.3.3. Analyte dose response graphs using competitive ELISA

Individually optimised antigen-coated competitive ELISAs were used to construct analyte standard curves with the corresponding purified antibody samples and IgG preparations. Response by the general estrogens antibody showed significant improvements after the purification step where a reduction of IC_{50} value from 14.5 to 6.57 pg mL^{-1} when using the purified antibody (Fig. 9). This was accompanied by an equal improvement in the MDL (as calculated by the 3 × S.D. method) of 0.15 (±0.017) pg mL^{-1} under buffer conditions and about 0.2 (±0.025) pg mL^{-1} in river water, for the unpurified antibody, which were reduced to 0.059 (±0.030) and 0.028 (±0.067) pg mL^{-1}, respectively using the purified antibody.

The pentachlorophenol competitive ELISA also showed substantial improvement in sensitivity with the purified antibody where a decrease in IC_{50} from 1.67 to 0.079 ng mL^{-1}. This was also accompanied by significant reduction in MDL from 0.07 (±0.02) (analyte buffer) and 0.105 (±0.01) (river water) to 0.009 (±0.006) and 0.0302 (±0.005) ng mL^{-1}, respectively with the purified antibody (Fig. 10).

The Irgarol 1051 assays showed slight decreased in sensitivity with the purified antibody. A shift of IC_{50} from 28 to 40 pg mL^{-1} was seen when the purified antibody was used (Fig. 11). The MDL value was also adversely affected, however, not quite to the same extent as the IC_{50}. Values of 2.45 (±0.84) in analyte buffer and 3.1 (±0.53) in river water were observed with unpurified antibody whereas 2.05 (±0.55) and 5.98 (±0.34) were found using pure antibody samples. Assays of Irgarol 1051 under seawater conditions showed a similar level of change in MDL values: 3.68 (±0.69) and 5.74 (±0.61) pg mL^{-1}, respectively for the unpurified and pure antibody samples.

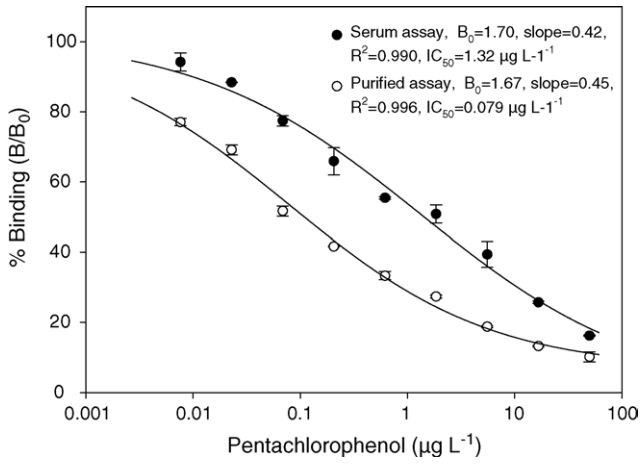

Fig. 10. Competitive ELISA normalised calibration graphs for pentachlorophenol using the source antiserum (●) and the purified antibody (◯). The assay was carried out using was pentachlorophenyl amido butyric acid-gelatine as PCA diluted to 1/3000. The antiserum and the purified antibody were used at 1/30,000 and 1/35,000, respectively.

3.3.4. Assessment of specificity

Specificity of the individual antisera and purified antibody samples was assessed using selected compounds appropriate for each analyte system. The general trend held throughout using purified antibody preparations was a decrease in cross reaction levels with the majority of the tested compounds indicating improvement in specificity of the purified antibodies toward the target analytes (Tables 1–3). The modest improvement in specificity of the general estrogens antibody found after purification (Table 1) is only of academic significance because the antibody was meant for use as a generic anti-estrogens (generic anti-class antibody) reagent for the analysis of mixtures of estrogen steroids that share the A, B and C rings [26]. Of particular note is the improvement in specificity of the anti-irgarol antibody, which although showed some loss of sensitivity, it showed substantial improvement of specificity toward

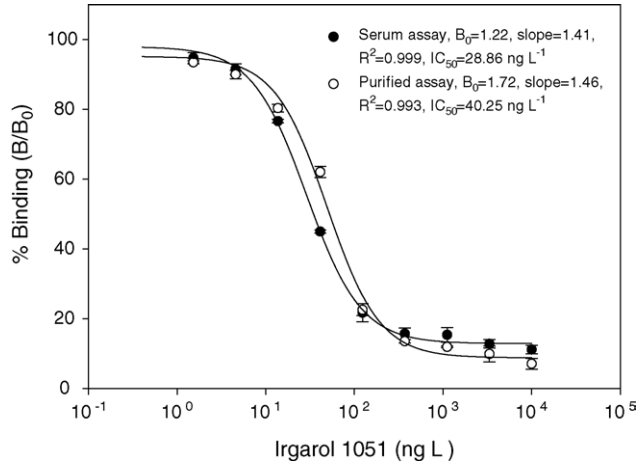

Fig. 11. Normalised calibration graphs of competitive ELISA for Irgarol 1051 using the source antiserum (●) and the purified antibody (◯). The assay was carried out using was irgarol-*O*-phenoxy benzoic acid-gelatine as PCA diluted to 1/2500. The antiserum and the purified antibody were used at 1/30,000 and 1/75,000, respectively.

Table 1
The cross reactivity values found for the selected steroids and derivatives using the general estrogens antiserum and purified antibody

Compound	Precentage cross-reactivity	
	Serum sample	Purified Ab
Estradiol-17β (E_2)	100.0 (±0)	100 (±0)
Estradiol-17α ($E_2\alpha$)	98.6 (±1.0)	98.1 (±1.5)
Ethynylestradiol (EE)	68.6 (±2.3)	15.37 (±1.2)
Estrone (E_1)	96.78 (±3.1)	99.25 (±1.6)
Estriol (E_3)	18.8 (±3.1)	5.9 (±1.6)
Equilin	<0.1	<0.01
Estrone-3-β-D-glucuronide	<0.01	<0.001
Estrone-3-sulphate	<0.01	<0.001
E_2-17-hemiglutarate	118.0 (±4.2)	92.99 (±3.1)
E_1-17-carboxymethyl oxime	52.3 (±2.1)	41.0 (±3.5)
E_1-3-carboxymethyl ether	3.0 (±0.5)	2.53 (±0.8)
E_2 17α-ethinyl estradiol 3 methyl ether	0.1 (±0.6)	<0.01
Progesterone	<0.1	<0.001
4-Androstene-3,17-dione	<0.01	<0.001
Testosterone	<0.01	<0.001
E2-3-Humonic acid	–	<0.001

Table 2
The cross reactivity values found for the selected chlorophenols and derivatives using pentachlorophenol antiserum and purified antibody

Compound	Precentage cross-reaction	
	Serum sample	Purified Ab
Pentachlorophenol	100 (±0)	100 (±0)
Pentachlorothiophenol acetic acid	39 (±1.5)	23.2 (±1.1)
Pentachlorothiophenol	55.5 (±0.5)	<0.01
Pentachlorothiophenol butyric acid	70.5 (±1.3)	35.9 (±2.3)
Pentachlorophenoxy acetic acid	359.2 (±2.6)	355.9 (±1.5)
Pentachlorophenoxy valeric acid	161.9 (±1.8)	170.2 (±0.6)
Pentachlorophenoxy butyric acid	56.5 (±0.4)	35.9 (±1.2)
Pentachloronitrobenzene	175.7 (±1.6)	75.8 (±1.1)
Pentachloroaniline	22.8 (±0.2)	3.95 (±0.5)
2,3,4,6-Tetrachlorophenol	6.5 (±1.0)	2.08 (±0.2)
Pentachlorobenzene	2.7 (±1.4)	<0.01
Tetrachloropthalic acid	2.5 (±0.5)	2.4 (±0.4)
Hexachlorobenzene	19.8 (±1.1)	<0.01
3,5-Dichloro-4-hydroxyanaline	<0.01	<0.001
2,4,5-Trichlorophenol	<0.01	<0.001
2,4,6-Trichlorophenol	<0.01	<0.001
2-Methyl-4-chlorophenoxy acetic acid (MCPA)	<0.01	<0.001
2,4-Dichlorophenoxy acetic acid(2,4-D)	<0.01	<0.001

Irgarol 1051, Table 3. The pentachlorophenol purified antibody ELISA also showed a general trend of increased specificity, Table 2.

4. Discussion

The antibodies that have been used for the assessment of purification parameters are for analytes found as organic contaminats in river and drinkable waters. However, the assay methods and the purification protocol are generic in nature and may be applied to antibodies with specificity to other types of analytes.

Table 3
The cross reactivity values found for a selection of triazine compounds using the irgarol antiserum and purified antibody

Compound	Precentage cross-reaction	
	Serum sample	Purified Ab
Irgarol 1051	100 (±0)	100 (±0)
Cyanazine	<0.01	<0.001
Desethylatrazine	<0.01	<0.001
Simazine	<0.1	<0.001
Atrazine	3.2 (±0.7)	1.0 (±0.3)
Tertbutylatrazine	4.4 (±0.1)	3.2 (±0.05)
Propazine	6.4 (±0.7)	4.5 (±0.1)
Simetryn	18.0 (±0.9)	13.5 (±0.2)
Ametryn	56 (3.5)	46.5 (±0.8)
Prometryn	60 (±2.1)	37.7 (±01.3)

The study concerns the construction and evaluation of a generic affinity chromatography method for the purification of polyclonal antibodies to hydrophobic haptenic analytes. The main objectives of the study were to demonstrate the efficiency and general applicability of an elution system made up of 20% acetonitrile and 1% propionic acid for the desorption of bound antibodies and the usefulness of a panel of ELISA-based tests for monitoring of the purification process and for assessing the properties of the isolated antibody preparations.

The principal operative features that are considered ideal for antibody affinity chromatography methods include treatment steps of source antisera, solid phase capacity and stability, degree of non-specific adsorption, speed and efficiency of the elution process, re-naturation steps and removal of eluting agents, degree of purity and stability of the isolated antibodies. In addition to these basic features, it is suggested that the scalability of the process, availability of practical monitoring and assessments tests and the generic nature of the elution steps are critical to the wider use of a purification process.

The preparation of the immunoabsorbent gels in this study involved the use of *N*-hydroxysuccinimide active esters and effecting peptide bonds with the terminal amino group of the linkage arm, Fig. 12. The reaction of the active esters with the solid phase linked amine was noticeably inefficient because relatively large amounts of the esters needed to be used to achieve reasonable capacity of the gels (unreported observations). This is not of a great consequence except when the active ester for a particular analyte is in short supply. The need to use relatively large amounts of active esters of hydrophobic analytes for linkage to Sepharose required the inclusion of high proportions of organic solvents. Triethylamine base was added to the reaction mixtures to promote the coupling step in preference to using higher aqueous pH conditions (which would encourage break down of the active esters). The capacity of the various gels was indicated by the degree of uptake of antibodies from the various source antisera. For example the general estrogens gel yielded up to 150 mg of pure antibody. It is not possible to provide figures for the maximum capacity for the various gels from data in this study, however, the near total extraction of the specific antibodies from both the general estrogens (Fig. 1A) and the pentachlorophenol gel (Fig. 2A) indicated reasonably high levels in both of these cases. By contrast the capacity of the irgarol immunoabsorbent gel was lower as indicated by the reduced antibody uptake level as shown in Fig. 3A.

The removal of the unbound proteins from the immunoabsorbent gels did not require particularly complex steps or regimes (except the use of 0.5 M NaCl) as indicated by the rapid decrease in protein levels detected in the wash fractions (Figs. 2B–4B). It is considered that this was the result of not so much the structure of the immunoabsorbent gels but rather the treatment steps of the source antisera prior to mixing with various gels. The treatment steps, dilution with buffer followed by mixing with activated charcoal, achieved removal of small molecular weight compounds, including natural analytes where relevant [36], but more importantly the treatment step enabled the removal of fine serum protein aggregates which would normally become trapped within the gel matrix and appear as non-specifically adsorbed proteins [22].

Desorption of the bound antibodies was achieved using the same mixture of acetonitrile (20%) and propionic acid (1%) in

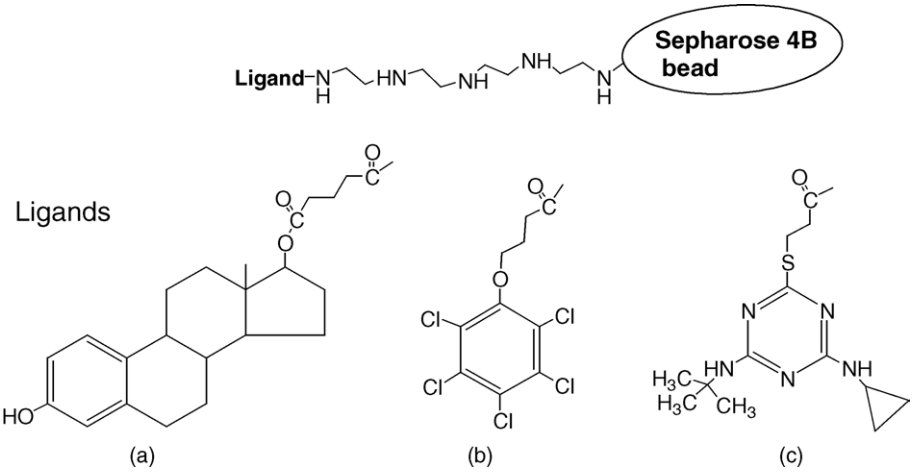

Fig. 12. The structures of the ligand derivatives used in the preparation of the immunoabsorbent gels: (a) estradiol-17-*O*-hemiglutarate; (b) pentachlorophenoxy butyric acid; and (c) irgarol-thio-propionic acid. The expected structure of Sepharose 4B-*ethylene pentamine*-NH_2 is also shown.

all three cases. This mixture has been used for the elution of antibodies to other hydrophobic or substantially so compounds with equal success [21–23]. It is therefore, proposed that this is a generic elution mixture for the isolation of antibodies to this broad class of analytes. The observed high efficiency and speed of elution of bound antibodies indicates that acetonitrile under acidic conditions is highly effective in disrupting antibody-hapten binding when the binding forces are substantially of hydrophobic interactions. The value of this generic elution protocol is in the fact that essentially a single procedure maybe applied to a host of analytes with predicable results. The use of the organic base (*N*,*N*-bis(3-aminopropyl)ethylenediamine) to neutralise the acid is significant in that fully active antibodies without precipitation have been recovered consistently regardless of antibody specificity. The minute amounts of organic base required to efficiently neutralise the organic acid avoided increasing volumes and possibility of foaming (when inorganic compounds are used).

Monitoring of the uptake of antibodies was a valuable test because it indicated progress or otherwise of absorption of the specific antibody prior to the multi step elution and recovery process. The antibody titration tests, before and after absorption steps, was simple to carry out and results can be obtained in a short time period before elution is initiated. The monitoring test is based on detecting antibody activity in the supernatant and therefore test samples are easily accessible and non-wasteful of the product. Rapid real-time monitoring of the washing and elution steps by 280 nm absorbance measurements provided a simple test that guided the elution process. However, the ELISA tests on the fractions were necessary to demonstrate binding activity in the recovered fractions. Although this monitoring ELISA test was not a real-time test, it provided valuable data and was carried out in all cases.

The characterisation of the recovered pure antibody crops was carried out using several tests to assess functional and structural purity, binding of authentic analyte (sensitivity limits) and specificity. In all cases the performance of purified material was compared with the source antisera.

The structural purity of the isolated products refers to the purity in terms of IgG protein without any reference to the antibody activity of the preparation. This was carried out with SDS–PAGE, the tests were useful but of limited value. The SDS–PAGE tests, carried out under reduced and non-reducing conditions, indicated the IgG preparations and the purified antibodies were pure IgG proteins (Fig. 8). The purified antibody samples appeared to be free from all traces of non-IgG proteins. The tests provided indication of the structural purity of purified antibody samples.

The functional purity tests results obtained by titrating antibody activity and measurement of IgG protein in the supernatant and washing samples after mixing with the corresponding immunoabsorbent gels and control Sepharose provided conclusive evidence for the very high purity of the isolated antibodies.

The antigen-drop-down assay was coupled with antibody uptake using excess corresponding immunoabsorbent gels and therefore the tests provided direct evidence on the proportion of pure antibody in the tested preparations. The results obtained by this test (Fig. 7) were interesting in view of the use of a sensitive drop down ELISA for IgG protein (antigen) that allowed the detection of very low levels of IgG in the supernatant and washing samples.

The structural and the functional panel of tests together provided practical, realistic, extremely sensitive and useful assessment methods that could be easily carried out without the need for reagents that are not already in use in the purification process. At variance with previously employed assessment methods [17–20] the described tests are practical and provide direct evidence on the purity of the isolated reagents.

The performance of the purified antibodies as reagents for subsequent competitive immunoassays was compared with the source antisera using standard analyte calibration curves. The estimated MDL and IC_{50} values showed significant improvements in two of the three model systems used in this study. The slight increase in IC_{50} for Irgarol 1051 cannot be explained at this point, but could well be due to the lower capacity and general lower qualities of the immunoabsorbent gel. However, the significant improvement in specificity of this antibody (Table 3) and the other two systems was an important feature of the purification methods because it demonstrated that the purification technique does not compromise the all important specificity of subsequent assays and indicates that the immunoabsorbent gels do not selectively enrich undesired antibody fractions, which may happen if the solid-phase ligand is not chosen carefully [37,38].

The panel of the described monitoring and assessment ELISA based tests provided a valuable new tool enabling the monitoring of the affinity purification procedure and the assessment of the purified preparations. At variance with previously proposed methods [17] the described tests are practical and provide essential useful data on the purified antibodies.

Methods for the purification of antibodies to haptenic analytes have not achieved the same level of refinement as those employed for the isolation of antibodies to protein antigens [39,40]. In this study a single protocol has been used to isolate preparative quantities of antibodies to three different typical hydrophobic haptens. The method is practical and by comparison with many of the published techniques is simple to carry out and scalable for the isolation of larger quantities of antibody proteins. The purified antibodies were shown to be pure by a panel of tests based on functional and structural properties of the isolated antibody proteins. Storage of the isolated antibodies for several months under ammonium sulphate did not alter any of the detectable properties.

It is proposed that the described immunoaffinity chromatography protocol is a generic technique suitable for the isolation of antibodies to hydrophobic haptenic substances. The importance of the described method is of technical as well as scientific reasons in view of the fact that polyclonal antisera remain to be the principle practical source of antibodies to haptenic analytes for use in immunochemical analysis. It is reasonable to suggest that, despite claims to the contrary, alternative more costly sources of antibodies to haptenic analytes for use in immunoassays have not demonstrated proportional improvements in assay performance [41,42].

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