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Preparative electrophoresis: a general method for the purification of polyclonal antibodies

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Abstract

Antibodies were purified from normal rabbit, sheep, goat, rat, human and bovine serum using preparative electrophoresis on a Gradiflow in a single-step process using an asymmetrical cartridge with three different pore size polyacrylamide membranes. Recoveries in each case were over 80% and were higher than those obtained using affinity chromatography on protein A, protein G or protein L. Degree of purity was at least comparable with these methods. These results suggest that preparative electrophoresis can be considered a general method for the purification of research quantities of antibodies from multiple serum sources and may be particularly useful where the reactivity with protein A, G or L is unknown.

Keywords: Preparative electrophoresis; Instrumentation; Gradiflow; Antibodies; Immunoglobulins

1. Introduction

The use of serum antibodies for most applications requires some degree of antibody purification. It is generally accepted that purities of >80% are required for many common applications of these antibodies, for example as a coating reagent or label in an immunoassay or for the construction of an affinity column. To achieve this degree of purity a variety of chromatographic and precipitation techniques have been developed for this purpose. The preferred method often involves ammonium sulfate precipitation [1,2] and some form of column chromatography such as ion-exchange on a DEAE [3,4] or cation-exchange resin [5], hydroxyapatite chromatography [6], specific immunoaffinity columns [7,8], immobilized metal affinity chromatography [9], or affinity chromatography on protein A [10], G [11–13], or L [14].

Affinity chromatography on protein A, G, or L has become increasingly common for antibody purification by commercial suppliers and academic laboratories [15] and there are a number of commercially available reagents and columns. Protein A [16,17] and protein G [18–20] are cell wall proteins originally isolated from staphylococci and group G streptococci, respectively, that exhibit specificity for the Fc regions of mammalian immunoglobulins. In contrast, the more recently identified protein L was isolated from the cell wall of the anaerobe *Peptostreptococcus magnus* and has strong binding affinity for the mammalian immunoglobulin κ-light chain [21–23].

Despite the variety of available antibody purification methods, the efficient purification of the polyclonal immunoglobulin fraction from serum does present challenges. The charge heterogeneity of

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antibodies makes it difficult to purify this class of molecules under a single set of conditions. Precipitation and chromatography methods are always a compromise between yield and purity. For example, precipitation procedures utilise the slightly increased hydrophobicity of the antibody molecules compared to other serum proteins. Localised high precipitant concentrations can cause significantly increased contamination [24]. Ion-exchange columns rely on the antibody charge, and as a result there are usually broad bands generated upon elution [3,5,25]. Optimisation of these types of methods is required and the purification process usually takes several days as multiple purification steps are necessary [4,6].

For protein A, G and L chromatography, the binding and elution behaviour of serum antibodies with the affinity ligands is species and isotype dependant and can be hard to predict. Determination of correct binding and elution conditions is required to produce a reasonable yield [24]. Affinity chromatography resins can also exhibit limited capacity [9], and ligand leakage during elution is a common source of contamination [26,27]. The application of serum subjects the adsorbants to contamination by lipids, nucleic acids and other proteins. Cleaning procedures, especially necessary when different serum types are applied to the column, eventually degrade the column and increase the amount of ligand leakage [28,29].

The use of monoclonal antibodies, rather than polyclonal serum antibodies, has often proved to be a more predictable alternative for purification. However the generation of monoclonal antibodies can be time consuming and relatively expensive when compared to the simple production of serum antibodies [2,11].

Gradiflow (Fig. 1) is a preparative electrophoretic system that uses a combination of porous polyacrylamide membranes and recirculation of sample and product to provide an improved methodology for protein separation [30,31]. The electrophoretic buffer has the dual functions of cooling the electrophoresis and setting the pH for the separation [30,32]. The polyacrylamide membranes, sandwiched together in a three-membrane cartridge (Fig. 2) act as barriers to protein movement rather than as a media for separation. The configuration of the cartridge combined with the properties of the circulating electrophoretic

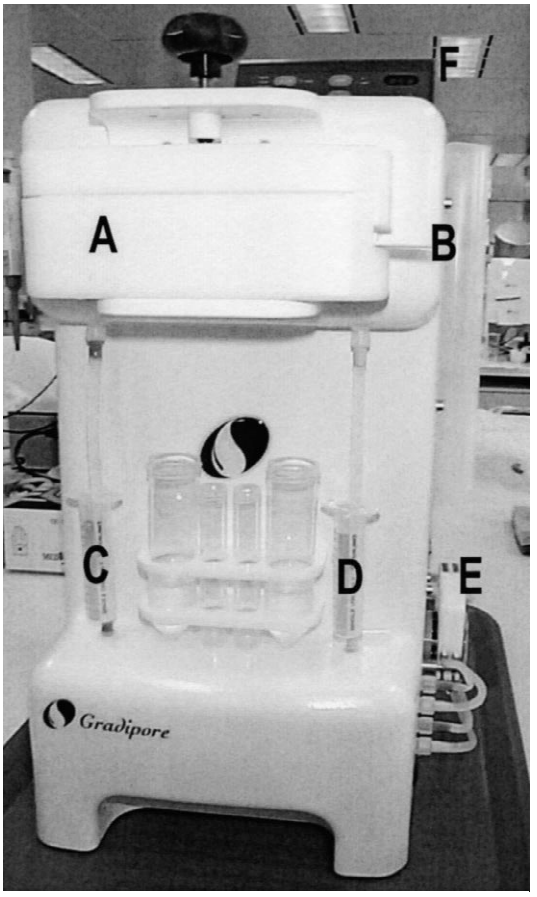

Fig. 1. Front view of the Gradiflow BF200 Instrument. A= Separation unit; B=cartridge; C=upstream reservoir; D= downstream reservoir; E=the upstream/downstream pump; F= power supply. The buffer reservoir is housed directly behind the front panel, alongside the upstream/downstream pump.

buffer allows a mixture of proteins to be separated on the basis of size and isoelectric point simultaneously [33]. Applications for the Gradiflow include separation of a proteins from plasma and algal extracts [30], egg white [34], milk whey [35], a recombinant protein from *Dictyostelium discoideum* [35], and prefractionation of human serum proteins prior to two-dimensional gel electrophoresis [36]. Impregnation of the polyacrylamide membranes with an affinity ligand can further extend the applications of the Gradiflow instrument [37].

Previously, we used Gradiflow preparative electrophoresis to purify monoclonal antibodies from mu-

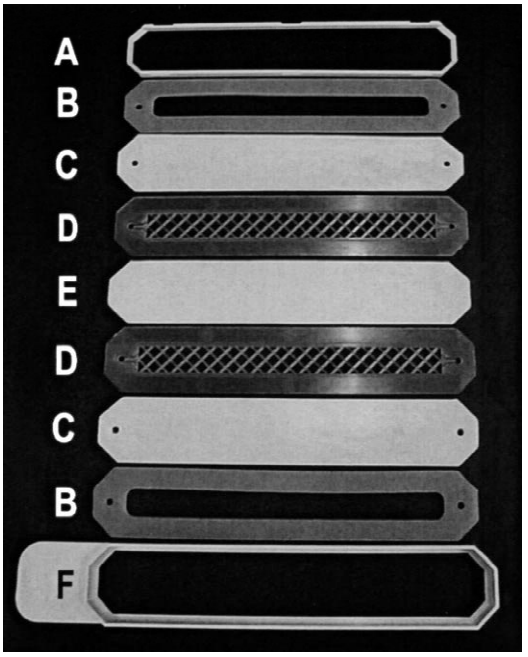

Fig. 2. Exploded view of the membrane cartridge. A=Retaining clip; B=gaskets; C=restriction membranes; D=grids; E=separation membrane; F=cartridge housing. The retaining clip sits nearest the cathode, whilst the cartridge housing is nearest the anode. The protein solution is contained between the separation and restriction membranes.

rine ascitic fluid [38]. We used a size- and then charge-based strategy determined by the isoelectric point of the antibody, which meant that the purification required two separations on the Gradiflow. Here we use a combination of different sized ''restriction'' membranes to simplify the purification of serum antibodies and enable each of these to be separated in a single step.

2. Experimental

2.1. Materials

Serum from the different animal species and all chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. HiTrap protein A and protein G Sepharose 4 Fast Flow were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). ImmunoPure immobilised protein L was obtained from Pierce (Rockford, IL, USA).

2.2. Gradiflow BF200 and cartridge configuration

All separations were carried out on a Gradiflow BF200 instrument (Gradipore, Sydney, Australia) illustrated in Fig. 1. For the separations described in this paper a cartridge (Fig. 2) was employed that had reinforced polyacrylamide membranes of three different pores sizes (supplied by Gradipore). The membrane near the anode was a restriction membrane preventing the entry of molecules with molecular masses less than 5000. The centre (separation) membrane had a large pore size to allow rapid transfer of proteins with molecular mass up to $1 \cdot 10^6$. The third membrane nearest the cathode was also a restriction membrane, restricting proteins of molecular mass 150 000 and over. For the separation of human IgG the cathode restriction membrane contained an M_r 5000 restriction.

2.3. Antibody separation conditions

A 1-ml volume of goat, sheep, rat, rabbit, human or bovine serum was diluted with 9 ml of a pH 5.6 (at 20°C) buffer consisting of 46 m*M* MES [2-(*N*-morpholino)ethanesulfonic acid], 12 m*M* L-histidine, and 2 m*M* acetic acid (MHA). The pH of the diluted serum was adjusted to pH 5.6 (at 20°C) with acetic acid if required and loaded into the downstream compartment of the Gradiflow nearest the anode. The asymmetrical cartridge described in Section 2.2 was placed into the Gradiflow separation chamber. A 10-ml volume of the MHA buffer was circulated through the upstream. A 1.6-l volume of MHA buffer was placed into the Gradiflow buffer reservoir, cooled during the separation with an ice-filled stainless steel beaker. The pH of the buffer and sample increased to around 5.8 once chilled to between 10 and 15°C for the separation. A potential of 250 V was applied across the separation chamber for up to 60 min.

Following the separation step, the serum sample was removed from the downstream, and the downstream flushed with buffer several times to remove traces of the serum. A 10-ml volume of fresh buffer was then circulated through the downstream and the

pH of the upstream and downstream was adjusted to about 5.0 with acetic acid. The voltage was reapplied for an additional 30 min to remove any remaining proteins away from the antibody.

2.4. Scale up of antibody separation

For scale up experiments conditions were essentially identical to the 1 ml separation procedure. A 10-ml volume of serum was diluted to 50 ml with the MES–histidine–acetic acid buffer, pH 5.6 and placed in the downstream. Initially, 25 ml of MHA buffer was circulated through the upstream. The separation was conducted at 250 V for a total of 4 h. The upstream containing antibody was harvested at 1 h and 3 h, and replaced with 15 ml and 10 ml fresh buffer, respectively. At the end of the separation the harvested fractions were pooled (total volume 50 ml) in the upstream and the pH adjusted to around 5.0. The downstream was emptied, rinsed and replaced with 10 ml fresh buffer. Separation was then allowed to continue for a further 30 min at 200 V.

2.5. Affinity chromatography

Affinity chromatography was carried out on 1 ml columns of protein A, protein G and protein L according to instructions recommended by the manufacturers. A separate column was utilised for each animal species. Briefly, a 20 m*M* phosphate binding buffer, pH 7.0 was utilised for proteins A and G, and a buffer of 0.1 *M* phosphate, 0.15 *M* NaCl, pH 7.2 was used for binding of antibody to protein L. The breakthrough and wash fractions were discarded. Antibody was eluted at pH 3.0 using 0.1 *M* citric acid for protein A, and 0.1 *M* glycine for protein G and protein L. The eluted antibody was immediately neutralised by addition of Tris base.

2.6. Antibody immunoassay

Antibody recovery was determined by a series of sandwich immunoassays using unlabelled anti immunoglobulin (IgG) antibody as the capture antibody and a labelled anti-IgG antibody as a label tag essentially as described previously [12] Antibodies used in sandwich immunoassays were purchased from Dako (Carpinteria, CA, USA) or Sigma.

The following combinations were used: rat: rabbit anti-rat IgG (Dako) and rabbit anti-rat IgG horseradish peroxidase (HRP) (Dako). Rabbit: goat anti-rabbit IgG (Sigma) and goat anti-rabbit IgG peroxidase conjugate (Sigma). Goat: rabbit anti-goat IgG (Sigma) and rabbit anti-goat IgG peroxidase conjugate (Sigma). Sheep: rabbit anti-sheep IgG (Sigma) and rabbit anti-sheep HRP (Dako). Bovine: rabbit anti-bovine IgG (Sigma) and rabbit anti-bovine IgG peroxidase conjugate (Sigma).

Human IgG recovery was estimated using nephelometry (Dade-Behring, Marburg, Germany) according to the manufacturers instructions.

2.7. Determination of antibody purity

Antibody purity was determined by non-denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 8–16% T gels (Gradipore)[1]. Under these conditions the antibody runs as a broad band of M_r 150 000–170 000. Wide range molecular mass markers (Sigma) consisted of myosin (205 000), β-galactosidase (116 000), phosphorylase B (97 000), fructose-6-phosphate kinase (84 000), bovine serum albumin (66 000), glutamic dehydrogenase (55 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (20 000), α-lactalbumin (14 200), and aprotinin (6500).

The protein content of the samples was determined by bicinchoninic acid (BCA) reagent (Pierce).

3. Results and discussion

3.1. Electrophoretic separation of polyclonal IgG from serum

A time course for the Gradiflow purification of IgG from 1 ml rabbit serum is demonstrated in Fig. 3. At pH 5.6–5.8 the majority of IgG molecules will be positively charged and progressively migrate through the M_r $1 \cdot 10^6$ separation membrane into the

[1] T=(g acrylamide+g *N,N′*-methylenebisacrylamide)/100 ml solution.

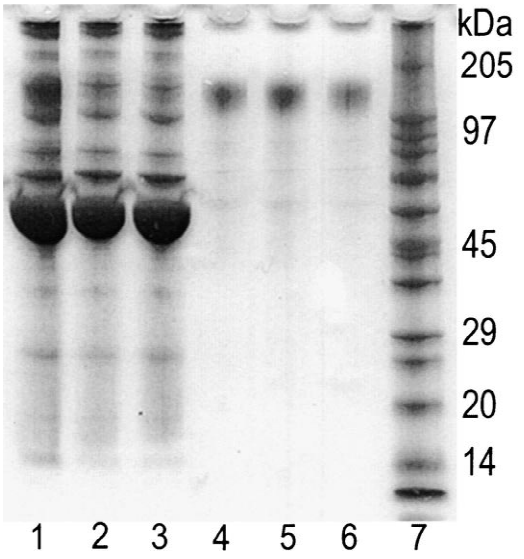

Fig. 3. Rabbit IgG purification from 1 ml serum. Rabbit serum downstream at 0, 30, and 60 min (lanes 1–3). Upstream IgG fraction at 30 and 60 min, and following the clean-up step at pH 5.0 (lanes 4–6). Molecular mass markers (lane 7). kDa= Kilodaltons.

upstream compartment nearest the cathode. By 30 min the majority of the antibody has transferred to the upstream (lane 4). Serum proteins with isoelectric points (p*I*) below 5.6 will be negatively charged, remaining in the downstream at the end of the separation (lane 3). Serum proteins with p*I* above 5.6 will transfer through the large pore size separation membrane with the antibody, but those of a lower molecular mass will continue through the M_r 150 000 restriction membrane into the buffer reservoir. The additional 30 min electrophoresis step following the replacement of the downstream with fresh buffer allows any lower-molecular-mass protein contaminants remaining in the upstream to continue moving through the restriction membrane into the running buffer, and to prevent any further proteins from the serum sample transferring into the antibody preparation.

This preparative electrophoresis procedure was found to be generally applicable to separating polyclonal antibodies from animal serum. IgG from sheep, goat and bovine could be recovered from serum without modification of the standard method.

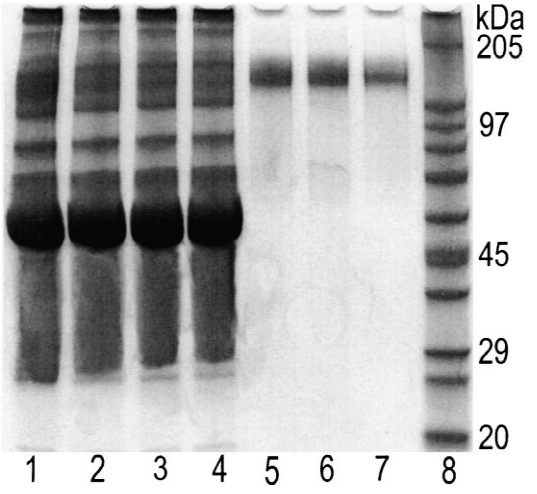

Fig. 4. Rabbit IgG purification from 10 ml serum. Rabbit downstream at 0, 2, 3, and 4 h (lanes 1–4). Rabbit IgG harvests at 2, 3, and 4 h (lanes 5–7). Molecular mass markers (lane 8).

The kinetics of purification in each case were very similar to rabbit serum. Rat IgG proved a little more difficult to separate with this method than IgG from other species. The reasons for this are unknown, but could possibly be related to the high amount of IgG in rat serum.

3.2. Scale up of the Gradiflow IgG purification

Serum volumes of 2, 5, and 10 ml were applied to the Gradiflow to determine whether this polyclonal method could be used with larger volumes of serum. The time for completion of the separation was proportional to the total amount of protein, taking 4 h for complete separation of 10 ml serum.

The 10 ml scale up was attempted using two different species, rabbit and sheep. As demonstrated in Fig. 4, IgG could be recovered with comparable purity to the 1–2 ml examples. Table 1 shows

Table 1
Recovery of IgG from 10 ml serum using Gradiflow

Source	Initial (mg)	Recovered (mg)	Recovery (%)
Rabbit serum	38	34	88
Sheep serum	69	54	78

Table 2
Recovery of IgG from Gradiflow and affinity chromatography

Source	Initial (mg)	Gradiflow recovery		Protein G recovery		Protein A recovery		Protein L recovery	
		mg	%	mg	%	mg	%	mg	%
Rabbit	4.5	4.0	90	1.0	22	3.2	71	2.4	54
Sheep	5.7	5.1	90	1.4	25	1.5	27	0.02	0.4
Bovine	4.6	4.0	86	2.4	52	1.9	41	0.01	0.3
Goat	13.5	11.7	87	5.5	41	1.4	10	0.01	0.1
Rat	13.9*	12.8	92	4.0*	78	1.1	8	1.8*	36
Human	8.8	7.8	89	6.0	68	4.5	51	nd	nd

*Initial mg for rat serum loaded onto protein G and protein L was 5.1 mg. Only 1/2 ml rat serum could be applied to these columns to avoid overloading.
nd: Not determined.

average recoveries of IgG from 10 ml sheep and rabbit serum to be 80–90%, comparable to recoveries from the small scale separations (Table 2). The kinetics of the separations were very similar suggesting that larger volumes of serum from other species can also be processed using this method on the Gradiflow.

3.3. Comparison of polyclonal IgG preparation methods

A comparison of the recovery of polyclonal IgG obtained from 1 ml serum using the preparative electrophoresis method and affinity chromatography is presented in Table 2. Figs. 5–7 demonstrate the purity of IgG obtained from each procedure.

As expected from the literature, there was wide variation in the efficacy of affinity chromatography separations with different animal species (Table 2). Rabbit IgG recovery was highest from protein A chromatography at 71%, whereas protein G produced the highest recoveries for human, rat, goat and bovine IgG. Protein L is claimed to be less selective and thus to have a wider application than proteins A and G [21,22,39], but this affinity matrix is still species specific. Protein L is able to bind most antibody classes, subclasses and fragments of human, mouse and rat [40], but is not widely applicable to polyclonal IgG from the different species. Protein L did not bind sheep, bovine or goat IgG, and gave only moderate recoveries of rat and rabbit IgG. The recombinant functional hybrids protein LA and protein LG exhibit some affinity for various serum antibodies [41,42], but these matrices are not readily available and would still require optimisation of the purification protocol for each individual species.

Table 2 indicates that the Gradiflow method consistently gave better recoveries than proteins A, G and L for all polyclonal antibodies, independent of species. Enzyme immunoassay of the antibody fractions indicated recoveries were 80–90% from the Gradiflow preparation, compared with the variable yields obtained with the affinity procedures. Protein recoveries were consistent with the expected amount of IgG present in serum.

The results suggest that preparative electrophoresis has a number of advantages over affinity chromatog-

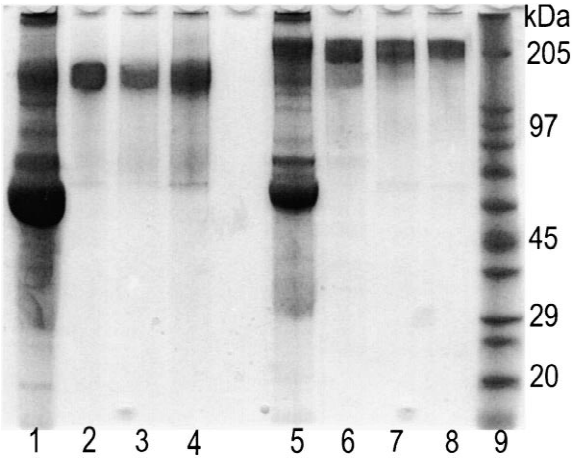

Fig. 5. Comparison of purification procedures for rabbit and rat IgG. Rabbit serum (lane 1). Rabbit IgG purified by Gradiflow (lane 2), protein G (lane 3) and protein A chromatography (lane 4). Lane 5, rat serum. Rat IgG purified by Gradiflow (lane 6), protein G (lane 7) and protein L chromatography (lane 8). Molecular mass markers (lane 9).

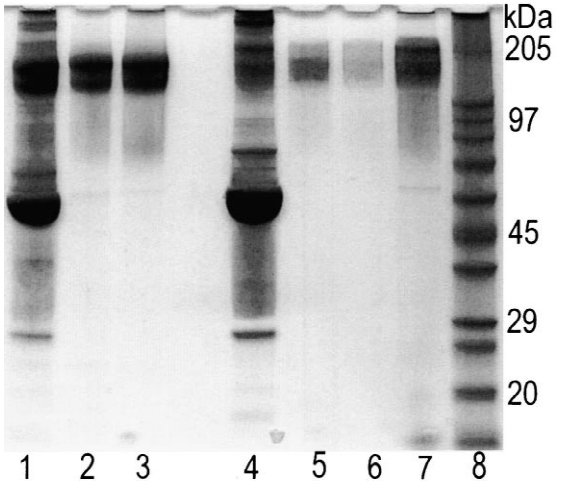

Fig. 6. Comparison of purification procedures for goat and human IgG. Goat serum (lane 1). Goat IgG purified by Gradiflow (lane 2) and protein G chromatography (lane 3). Human serum (lane 4). Human IgG purified by Gradiflow (lane 5), protein G (lane 6) and protein A chromatography (lane 7). Molecular mass markers (lane 8).

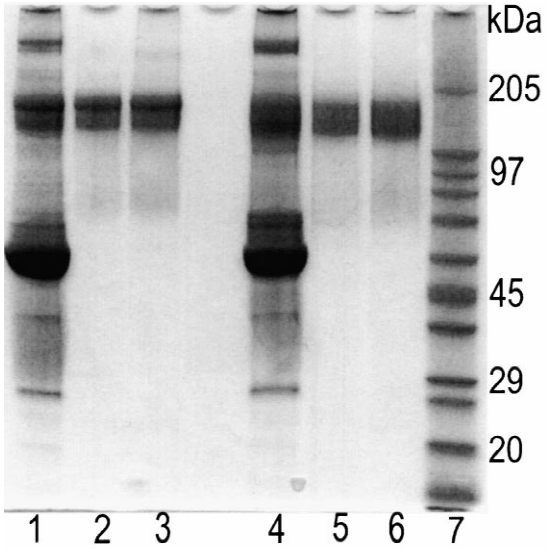

Fig. 7. Comparison of purification procedures for ovine and bovine IgG. Sheep serum (lane 1). Ovine IgG purified by Gradiflow (lane 2) and protein G chromatography (lane 3). bovine serum (lane 4). Bovine IgG purified by Gradiflow (lane 5), and protein G chromatography (lane 6). Molecular mass markers (lane 7).

raphy for the purification of serum antibodies. This method is not dependent on a variable binding interaction and exhibits a broad species applicability. A similar method can be used for all species and there is little chance that the purified product will be devoid of a particular isotype, a possibility when either protein G or A [43] are employed. Up to 50 mg of antibody can be purified on a single run on a disposable cartridge minimising the possibility of cross-contamination. The high cost of most affinity matrices makes the re-use of the resin common and there is the possibility of the leaching of the affinity ligand into the antibody fraction [27]. Cross-contamination from repeated use of an expensive affinity resin for different antibodies is avoided as the Gradiflow uses a fresh disposable cartridge for each separation. Mild purification conditions in the Gradiflow replace the low pH conditions required for elution of antibody from affinity chromatography reducing potential losses due to denaturation and aggregation. Finally, the procedure is simple and there is no column manipulation required. The antibody can be purified from 1 ml of serum within an hour, and from 10 ml of serum within 4 h.

4. Conclusion

IgG from sheep, human, rabbit, rat, bovine and goat serum could be recovered using the preparative electrophoresis apparatus, Gradiflow, at a comparable purity and a greater yield when compared with conventional affinity chromatography. An average of 8 mg antibody could be obtained within 1 h from 1 ml serum, and up to 50 mg was separated from 10 ml serum in 4 h. The described single-step, scalable procedure appears to be generally applicable to the purification of serum antibodies.

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