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Affinity purification of novel bispecific antibodies recognising carcinoembryonic antigen and doxorubicin

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Abstract

We have developed a method which combines Protein A affinity chromatography and HPLC analytical and semi-preparative hydroxyapatite affinity chromatography to purify bispecific antibodies (BsMabs) from hybrid-hybridomas secreting antibodies recognising carcinoembryonic antigen (CEA) and the chemotherapeutic drug doxorubicin (Dox). Elution of the HPLC hydroxyapatite columns with a 60–360 m*M* phosphate buffer gradient was found to give better separation than elution with a 60–180 m*M* phosphate buffer gradient. Careful monitoring of HPLC fractions by enzyme linked immunosorbent assays for anti-CEA, anti-Dox and dual anti-CEA/anti-Dox activity, and pooling of fractions on the basis of these results, enabled the purification of novel BsMabs for use in in vitro and preclinical in vivo experiments. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Bispecific antibodies; Doxorubicin; Carcinoembryonic antigen

1. Introduction

Antibodies have been used as carriers of radioisotopes for immunodiagnosis [1] and radioimmunotherapy [2], or conjugated to drugs [3] or toxins [4]. In a complementary approach to direct conjugation of the toxic agent to antibodies, bispecific antibodies (BsMabs) have been produced for targeted therapy which recognise a tumour-associated target with one antigen binding site and a variety of molecules with the other antigen binding site, including molecules on cells of the immune system [5–8], toxins [9,10] and cytokines [11]. We have previously reported the production of immunoconjugates of the chemotherapeutic drug doxorubicin (Dox) with an anti-carcinoembryonic antigen (CEA) monoclonal antibody [12,13] and the production of BsMabs recognising both CEA and Dox [14] for site-specific drug delivery. Allelic exclusion ensures the expression of only 1H and 1L chain in normal antibody producing cells. However, hybrid-hybridomas secreting BsMabs codominantly express both parental H and L chain genes. Since, theoretically, any L chain can combine with any H chain there are 10 H and L chain combinations in the secreted product of a BsMab hybridoma, only one of which is the BsMab

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of interest [15]. The objective of the present study was to develop a method to purify BsMabs recognising CEA and Dox by affinity chromatography for their later in vitro and preclinical in vivo evaluation.

2. Experimental

2.1. Cell lines

Seven hybrid-hybridomas secreting BsMabs to CEA and Dox; the parental hybridoma (11-285-14) secreting monoclonal anti-CEA antibody which was used to produce the hybrid-hybridomas [14]; P3x63Ag8 myeloma which secretes an IgG1 of unknown specificity. All these were grown in RPMI1640 with 10% foetal bovine serum (Gibco-BRL, Life Technologies, Paisley, UK) supplemented with L-glutamine, penicillin and streptomycin to final concentrations of 2 mM, 105 units and 105 μg ml^{-1}, respectively (all supplements from Gibco-BRL).

2.2. Production of ascitic fluid

Balb/c mice were primed with an intraperitoneal injection of pristane 1–3 weeks prior to the injection of 1×10^6 viable hybrid, or myeloma, cells intraperitoneally (i.p.) and ascitic fluid was obtained after 2–4 weeks. Ascitic fluid was removed using a syringe and needle. This was repeated up to four times on individual animals over a period of weeks and then they were terminated.

2.3. Protein A affinity chromatography

All procedures were carried out at 4°C and all buffers and solutions were degassed.

Protein A Sepharose Fast Flow (Amersham Pharmacia Biotech, Piscataway, NJ, USA; Glyfada, Greece) was equilibrated with 0.1 M phosphate-buffered saline (PBS), pH 8.0. After pooling from individual mice the ascitic fluids were stored at −20°C. These were thawed, centrifuged at 20 000 g for 30 min, the supernatant diluted with two volumes of phosphate buffer, pH 8.0, filtered through glass wool and then through a 0.45-μm filter (Millipore, Bedford, MA, USA), the pH adjusted to 8.0 and then aliquoted into 50-ml sterile tubes and stored at 4°C.

For separation, a 50-ml aliquot was loaded onto Protein A in a XK26/40 jacketed 40 cm×26 mm I.D. column (Amersham Pharmacia Biotech) which had been equilibrated with phosphate buffer at pH 8.0. The column was then washed with several column volumes of buffer to remove nonbound material and antibody was eluted using a citrate–phosphate buffer at pH 6.5. Following this, an elution with citrate–phosphate buffer at pH 3.0 was performed to remove all other bound immunoglobulins and the column was then re-equilibrated with loading buffer.

2.4. High-performance liquid chromatography (HPLC) purification

All HPLC procedures were carried out at room temperature (~22°C). Prior to HPLC purification, the Protein A, pH 6.5 purified material was thawed and concentrated using an Amicon ultrafiltration nitrogen pressure system (Amicon, Lexington, MA, USA) at 4°C and a membrane with an exclusion limit of 10 000 molecular mass. After ultrafiltration the antibody was filtered through a 0.22-μm filter (Millipore).

Affinity purification was achieved with analytical (HPHT 100×7.8 mm, bed volume 4.8 ml, particle size 10–90 μm) and semi-preparative (CHT20-1 ceramic, 113×15 mm, volume 20 ml, particle size 10 μm) hydroxyapatite columns with guard columns (Bio-Rad Labs., Mississauga, Canada; Hemel Hempstead, UK). The initial work was carried out in Canada using a System Gold Model 460AT with HPLC analog interface module 406, programmable detector module 166 set to 280 nm, autosampler 507, 110B solvent delivery module, computer controlled with System Gold software (Beckman Instruments, Mississauga, Canada). Work carried out in Kuwait utilised a GBC LC 1445 system organiser, LC 1150 multisolvent pump, UV–visible LC 1210 detector set at 280 nm, LC 1460 solvent degasser and computer controlled GBC WinChrom Chromatography Data Management System version 2.1 (GBC Scientific Equipment, Victoria, Australia).

A 60–180 mM sodium phosphate gradient was used for separation initially and this was changed to a 60–360 mM gradient after analysis of the preliminary results indicated that this gradient gave a better separation. The gradient was generated as follows: buffer A (60 mM) 100% and buffer B (360 mM) 0%

at time zero; buffer A 0% and buffer B 100% at 100 min. Flow-rates were 1 ml min^{-1} with the HPHT column and 1.5 ml min^{-1} with the CHT20-1 column. 1 ml min^{-1} fractions were collected from both columns.

2.5. Enzyme linked immunosorbent assays (ELISAs)

These have been published previously [14]. Very briefly, for the anti-CEA and anti-Dox assays microplates were coated with CEA, Dox (Adria Labs., Columbus, OH, USA) or Dox-BSA, respectively, and a rabbit anti-mouse Ig linked to horseradish peroxidase (RAM-HRP) (DAKO Diagnostics, Mississauga, Canada) was used for detection using 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS; Sigma, St. Louis, MO, USA; Deisenhofen, Germany) as the substrate.

Absorbance at 405 nm was measured using a Spectra Rainbow ELISA plate reader (SLT Instruments, Salzburg, Austria). For the dual specificity assay 1 mg of CEA was labelled with HRP using our previously published procedures [14] and the conjugate was purified on a G75 chromatography column. Activity of the CEA-HRP conjugate was assessed with microplates coated with either anti-CEA (11-285-14, IgG1 isotype) or Ag8 (IgG1 antibody of unknown specificity secreted by the P3x63Ag8 myeloma) as a control. For the dual specificity assay, microplates were coated with Dox (or Dox-BSA), the HPLC fractions added and then after incubation at 37°C for 2 h, CEA-HRP was used for detecting those fractions containing antibodies binding to both Dox and CEA. For the anti-Dox and dual specificity assays plates were read after 1 h at room temperature and after overnight storage at 4°C to increase sensitivity.

3. Results

3.1. Protein A and hydroxyapatite HPLC affinity chromatography

A typical Protein A elution profile from the initial Protein A purification step is shown in Fig. 1 for one of the BsMabs. After pooling and concentration of the pH 6.5 eluted material, trial comparison elutions

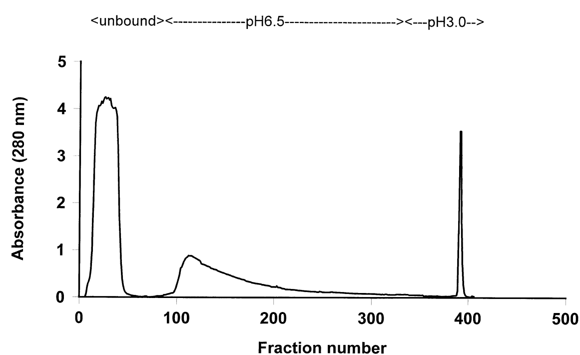

Fig. 1. Protein A elution profile of 26-61-10. A 50-ml aliquot of ascitic fluid loaded and eluted under conditions described in Experimental.

with 60–180 m*M* and 60-360 m*M* sodium phosphate gradients were undertaken with one of the BsMabs using the HPHT analytical HPLC column. Since a slightly better discrimination between peaks was achieved with the 60–360 m*M* sodium phosphate gradient (data not shown), this was used for all subsequent elutions and 50, 100, 250, 500, 1000 and 10 000 μl volumes were loaded onto the column in different runs and eluted using the 60–360 m*M* phosphate gradient. It became clear from the results that once the 1000 μl value was exceeded the elution profile became grossly distorted (data for 50–500 μl elutions shown in Fig. 2), therefore 1 ml of each Protein A purified ascitic fluid from the hybridomas underwent preliminary trial separations on the HPHT column (Fig. 3).

Purification of one of the BsMabs, 26-61-10, was proceeded with as a model for the others. The semi-preparative CHT20-1 column was brought into use and Fig. 4 shows a typical elution profile, with the parental 11-285-14 anti-CEA profile overlaid for comparison. To obtain the relatively large amounts of purified BsMab that are required for in vivo work we experimented with increasing the concentration of the sample that was loaded from 2.5 to 44.5 mg ml^{-1} and the effect of this on the retention time (t_R) is shown in Table 1. From this it can be seen that with some exceptions (e.g., peak 1 at 6.8 mg ml^{-1}) from 2.5 to 25 mg ml^{-1} the t_R values were similar. However, above 25 mg ml^{-1} the change in t_R was more pronounced and in view of this it was decided to work with Protein A purified antibody concentrated to a maximum of 25 mg ml^{-1}. Representative

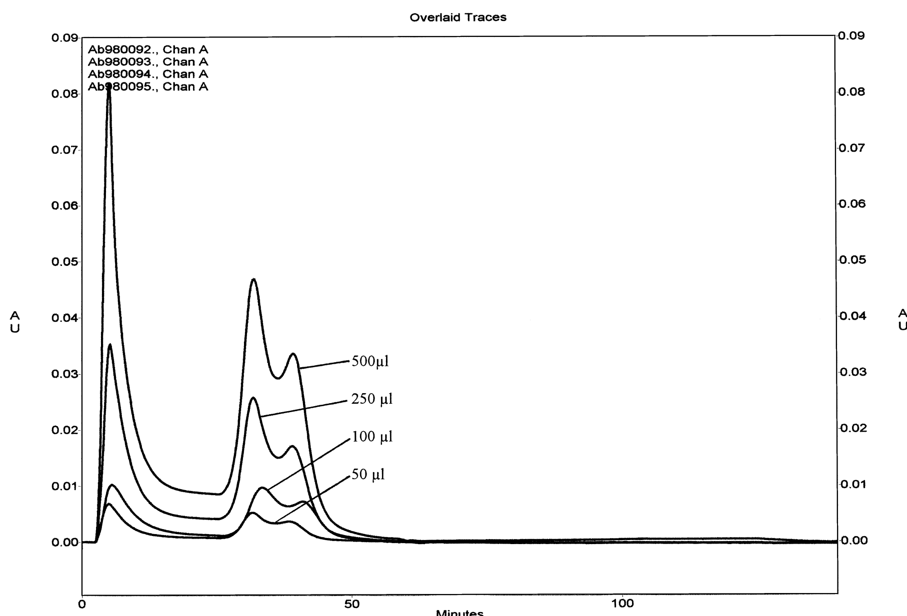

Fig. 2. HPLC elution profile of 26-61-10 (HPHT column) with increasing loading volumes from 50 to 500 µl and a flow-rate of 1 ml min^{-1}. AU=absorbance at 280 nm.

elution profiles from different separations are shown in Fig. 5 and clearly indicate excellent reproducibility from run to run.

3.2. Enzyme-linked immunosorbent assays

Initially all three ELISAs were performed on each HPLC run. However, to conserve HPLC fractions and the CEA-HRP conjugate, the initial screening was used to be more selective about which fractions to focus on, as follows. Preliminary anti-CEA assays showed that most if not all of the fractions had significant anti-CEA activity and therefore the anti-CEA assay was not used further, except when any doubt about anti-CEA activity occurred.

Also, after screening 19 of the HPLC separations for anti-Dox activity it became clear that there were two major anti-Dox peaks. One occurred early in the separation approximately at fractions 21–45 and the other later at approximately fractions 56–75. We now routinely screen only the fractions containing the anti-Dox peaks by ELISA.

Fractions were pooled on the basis of the ELISA results as follows: pool 1 (first anti-Dox peak fractions 21–45); pool 2 (part of second anti-Dox peak containing highest dual activity); pool 3 (corresponding to parental monoclonal anti-CEA peak). Table 2 summarises representative data on the ELISA results obtained with the HPLC fractions for 26-61-10.

After pooling, concentration, dialysis and estimation of protein concentration, BsMab 26-61-10 has been used for in vitro and in vivo experiments.

4. Discussion

Purification of BsMabs of interest from the other nine potential species of antibody secreted by hybrid-hybridomas [15] is essential for their subsequent use as immunoprobes [16] or for their evaluation as targeting agents [17]. Several approaches have been advocated but none are generally applicable. Hydroxyapatite is a calcium phosphate matrix which has been used for the purification of a variety of biologically important molecules including monoclonal [18,19] and bispecific [20,21] antibodies from mouse ascitic fluid. We have developed a method that uses a combination of Protein A affinity chromatography, hydroxyapatite HPLC separation and dual

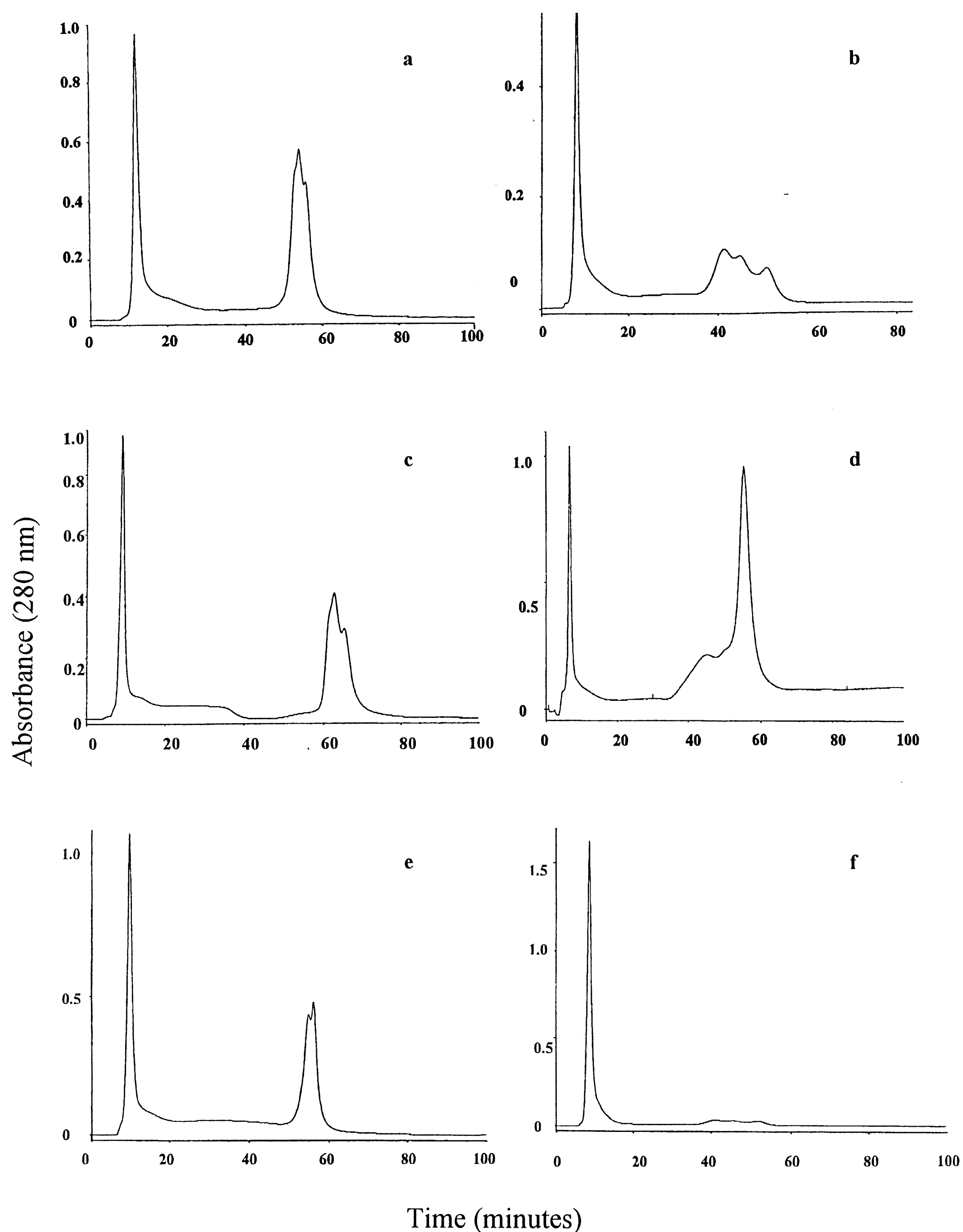

Fig. 3. HPLC elution profiles of six BsMabs (HPHT column). A 1-ml volume of sample loaded. a=26-61-1; b=26-61-2; c=26-61-4; d=26-7-35; e=57-11-25-17; f=57-9-6-4. Flow-rate 1 ml min^{-1}.

specificity monitoring of HPLC fractions to purify BsMabs recognising CEA and Dox.

The Protein A purified ascitic fluid from the hybrid-hybridomas shown to be secreting BsMabs [14] were initially evaluated using the analytical HPHT hydroxyapatite column. As expected [15] all had different elution profiles (Fig. 3). In order to test the method we focused on the BsMab which had the

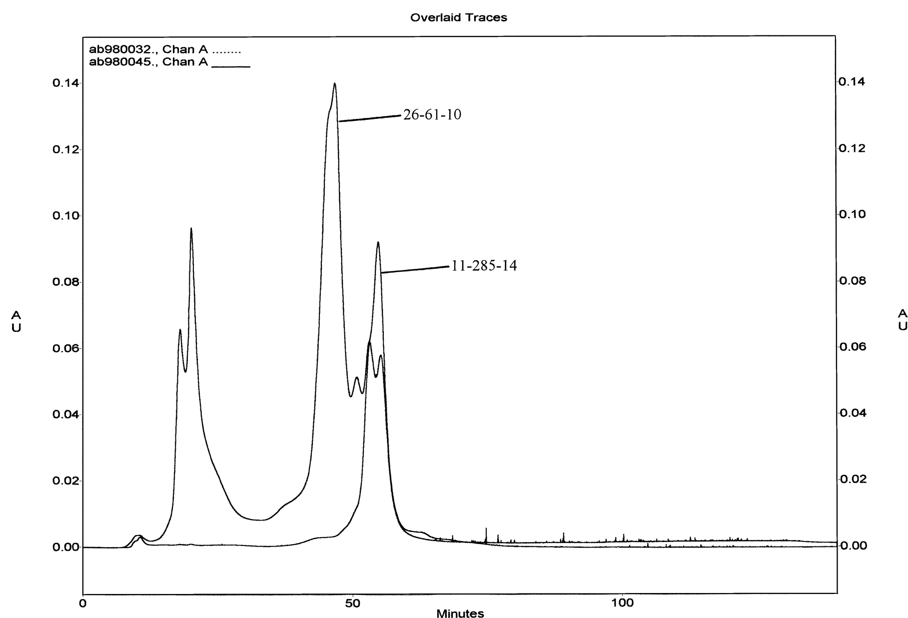

Fig. 4. HPLC (CHT20-1 column) elution profile of 26-61-10 (1 ml @ 2.5 mg ml^{-1} loaded) with overlay of ''parental'' monoclonal anti-CEA (11-285-14) antibody (1 ml @ 1.08 mg ml^{-1}). AU=absorbance at 280 nm. Flow-rate 1.5 ml min^{-1}.

Table 1
Retention time in relation to concentration (CHT20-1 column)

Concentration of Protein A purified antibody loaded (mg ml^{-1})[a]	Retention time (min)[b]					
	1[c]	2	3	4	5	6
2.5	10.44 (0.289)	18.51 (0.704)	46.48 (1.0)	50.41 (0.66)	52.71 (0.92)	54.75 (0.94)
6.8	12.24 (0.144)	18.56 (0.150)	44.66 (0.672)	48.18 (0.955)	50.37 (1.13)	52.37 (1.28)
25	10.24 (0.363)	18.95 (0.841)	44.23 (0.861)	48.93 (0.707)	50.60 (0.753)	53.35 (0.750)
42	12.70 (0.527)	19.20 (0.705)	44.21 (0.657)	48.43 (0.789)	50.55 (0.816)	52.92 (0.968)
44.5	12.82 (0.562)	19.00 (0.696)	42.84 (0.556)	46.73 (0.318)	48.90 (0.793)	51.46 (0.571)

[a] 1 ml loaded/run.
[b] Mean of 10–34 separate runs/concentration with SDs in parentheses.
[c] 1–6=elution peaks (maximum absorbance at 280 nm).

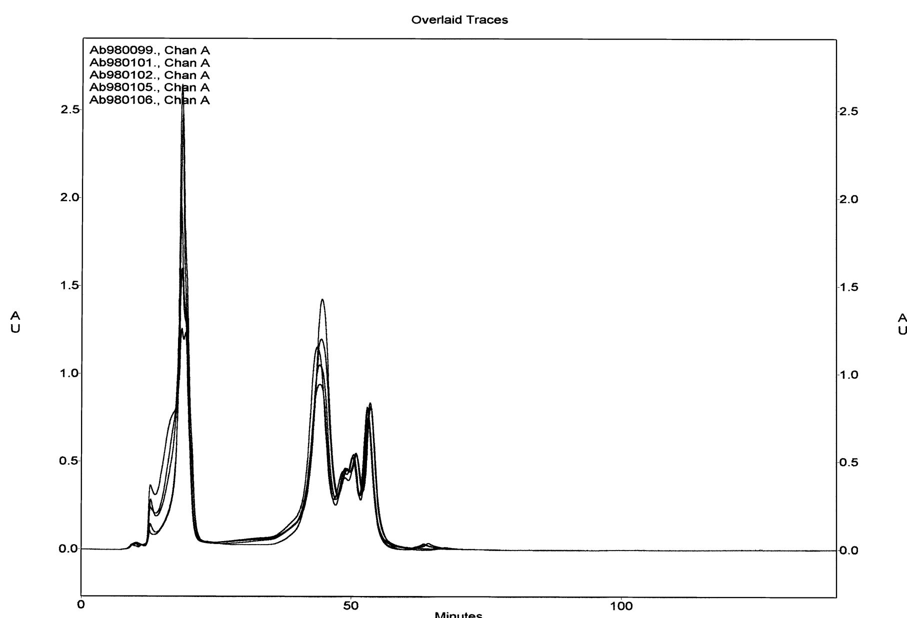

Fig. 5. Reproducibility of HPLC elution profiles of BsMab 26-61-10 (1 ml @ 25 mg ml^{-1} loaded; CHT20-1 column). AU=absorbance at 280 nm. Flow-rate 1.5 ml min^{-1}.

highest dual reactivity in ELISA (26-61-10) and purification was successfully scaled up to a semi-preparative CHT20-1 hydroxyapatite column. The HPLC elution profiles obtained (Fig. 3 and Fig. 4) clearly illustrate the value of hydroxyapatite HPLC purification as it can be seen that the secreted products from all seven hybridomas resolve into more than one peak, reflecting the fact that although the cell lines are hybridomas, they are secreting at least three species of antibody, i.e., parental anti-CEA, parental anti-DOX and bispecific.

In conjunction with ELISA monitoring of HPLC

Table 2
ELISA results – pooled HPLC fractions

Pool (HPLC fractions)	Absorbance (405 nm)[a]				
	Anti-CEA	Anti-Dox[b]		Dual[b]	
		1 h	O/N	1 h	O/N
Pool 1 (21–45)	1.495 (0.1069)	0.14216 (0.0641)	0.25164 (0.115)	0.05972 (0.0055)	0.0746 (0.009)
Pool 2 (56–68)	1.3748 (0.06876)	0.08346 (0.0187)	0.1414 (0.0371)	0.05892 (0.0066)	0.1935 (0.0469)
Pool 3 (69–85)	1.4348 (0.0998)	0.10235 (0.022)	0.1887 (0.056)	0.0621 (0.007)	0.0757 (0.01)
Controls	0.0675 (0.00450)	0.05267 (0.0010)	0.06133 (0.005)	0.05633 (0.0026)	0.0622 (0.0058)

[a] Mean values with standard deviations in parentheses.
[b] 1 h, Incubation for 1 h at room temperature; O/N, overnight incubation for ~14 h at 4°C.

fractions, those fractions containing BsMab were identified and pooled. Since the dual specificity assay relies on only one antigen binding site being available to bind to Dox coated on the microplate, and only one antigen binding site being available for binding to the CEA-HRP conjugate, this is probably why the absorbance values were much lower than in the "conventional" anti-CEA and anti-Dox assays (Table 2).

Recently efficient bispecific antibody purification has been reported using gradient thiophillic affinity chromatography and this has been used successfully to purify BsMabs from parental monoclonal antibodies in rat/mouse hybrid-hybridomas [16]. However, as pointed out by the authors, their method may not be so useful for the purification of BsMabs derived from two monoclonals of the same species and subclass. In our case the parental anti-CEA is an IgG1 and preliminary evidence suggests that BsMab 26-61-10 is also an IgG1 (data not shown).

We have developed a method for the purification of BsMabs and demonstrated that it was successful in separating a BsMab recognising CEA and Dox from the parental anti-CEA and anti-Dox antibodies. The method will be applied to the purification of the secreted products of the other six hybrid-hybridomas. After concentration and dialysis the purified BsMab (26-61-10) has been found to be effective in reducing the IC_{50} values for Dox with CEA expressing human colon cancer cell lines in vitro and to be able to inhibit growth of CEA expressing colonic cancer xenografts in vivo [22,23].

5. Conclusion

The optimal conditions for achieving purification of a BsMab recognising CEA and Dox, which is showing promising targeting potential in vitro and in vivo, are as follows: (1) preliminary purification of 50-ml aliquots of ascitic fluid on a Protein A Sepharose 40 cm×26 mm column equilibrated with phosphate buffer, pH 8.0; elution of bound immunoglobulins with a citrate–phosphate buffer, pH 6.5; regeneration of the column with citrate–phosphate buffer, pH 3.0. (2) Concentration of the Protein A purified antibody to 25 mg ml^{-1} by ultrafiltration. (3) Loading 1 ml of this onto a semi-preparative hydroxyapatite CHT20-1 ceramic (113×15 mm, volume 20 ml, particle size 10 μm) HPLC column followed by elution with a 60–360 m*M* sodium phosphate buffer gradient at a flow-rate of 1.5 ml min^{-1}. (4) Monitoring the collected 1 ml min^{-1} fractions for anti-CEA, anti-Dox and dual bispecific activity by ELISA and pooling and concentration of those fractions with the highest dual reactivity.

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