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Journal of Chromatography A, 925 (2001) 89–97

JOURNAL OF CHROMATOGRAPHY A

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Efficient enantioselective separation of drug enantiomers by immobilised antibody fragments

Tarja K. Nevanen[a,*], Lotta Söderholm[a,1], Kaija Kukkonen[b], Tapani Suortti[a], Tuija Teerinen[a], Markus Linder[a], Hans Söderlund[a], Tuula T. Teeri[a,2]

[a]*VTT Biotechnology, PO Box 1500, FIN-02044 VTT, Espoo, Finland*
[b]*Orion Pharma, PO Box 65, FIN-02101 Espoo, Finland*

Received 28 March 2001; received in revised form 6 June 2001; accepted 6 June 2001

Abstract

There is an increasing need for methods for efficient enantioselective separation and purification of chiral drugs. Genetic engineering provides the means for generating recombinant antibodies exhibiting extremely high specificity for even small molecular mass compounds. Here, recombinant antibody fragments have been generated for the drug diarylalkyltriazole that contains two chiral centres. Immobilised antibody fragments has been used successfully for efficient, step-wise separation of two enantiomers of the drug. Owing to the antibody specificity, one enantiomer came out in the flow-through, while the bound enantiomer could be specifically eluted. One of the antibodies tolerated solvents required both for dissolving the target molecules and for their elution for extended times and was shown to function over multiple cycles of the separation process.

Keywords: Enantiomer separation; Immunoaffinity adsorbents; Immobilised antibody fragments

1. Introduction

Within the field of pharmaceuticals strict legislation has been implemented concerning chiral compounds as drugs. While the use of racemic mixtures as drugs is permitted, the pharmacokinetic and pharmacodynamic properties of the different enantiomers/stereoisomers need to be studied individually to prevent unintended and potentially harmful side effects. In spite of the increased need for efficient enantiospecific separation and purification of drug molecules, method development in general has not quite been able to raise to the challenge. Most of the current methods are empirical and rely on selective crystallisation or chiral separations. Different chiral selectors have been used as stationary phases [1]. Although no truly general methods are currently available, chiral stationary phases based on different immobilised proteins are increasingly used because of their versatility [2–5]. The proteins used do not usually bind the given drug in a specific manner and their separation properties must be determined empirically for each different compound. Some of the many available chiral protein selectors can separate a large number of enantiomeric pairs and modifying parameters of the mobile phase can often extend

*Corresponding author. Fax: +358-94552103.
[1]Present address: University of Helsinki, Division of Biochemistry, PO Box 56, FIN-00014 Helsinki, Finland.
[2]Present address: Royal Institute of Technology, Department of Biotechnology, S-10691 Stockholm, Sweden.

0021-9673/01/$ – see front matter
PII: S0021-9673(01)01021-4

their use. However, enantiospecific chromatographic separation using non-specific proteins is based on low-affinity partitioning which consumes solvents and causes substantial dilution of the eluting enantiomers. The protein phases often suffer from poor efficiency and low loading capacity, and cross-reactivity can become a problem if the unwanted enantiomer is in great excess compared to the wanted one. Proteins can also be relatively unstable in organic solvents required to dissolve many of the target molecules.

Antibodies are molecules generated by the immune system of higher animals to recognise many different kinds of molecules with high specificity. Immunisation with small organic molecules coupled to carrier proteins is routinely used to generate antibodies even for small organic compounds such as hormones and drugs. In addition, the affinity [6–8], specificity [9] and stability [10,11] of the antibody fragments can be improved by genetic engineering or using combinatorial mutagenesis coupled with the phage display technology [12,13]. Antibody affinity and specificity can be fine-tuned to recognise even minor differences in small organic molecules [14]. The production of antibody fragments can be carried out in a relatively large scale using microbial production systems [15–17], and various tags can be added to the engineered antibody fragments for efficient, pre-oriented immobilisation on desired matrices [18–20]. Despite the current rapid progress in antibody engineering immunoaffinity chromatography with antibody fragments has not previously been generally used for enantiospecific fractionation. In the few cases described in the current literature, enantiomeric separation has been attempted using polyclonal antiserum [21,22] or monoclonal antibodies [23,24]. Here we describe the development of specific recombinant antibody fragments for the chiral drug molecule, diarylalkyltriazole.

2. Experimental

2.1. Chemicals

EDC (*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodi-imide hydrochloride, Fluka, Switzerland), S-NHS (*N*-hydroxysulfosuccinimidi, Fluka), DMSO (dimethylsulfoxide, Merck, Germany) BSA (bovine serum albumin, Sigma, St. Louis, MO, USA) KLH (keyhole limpet hemocyanin, Fluka), Freund's adjuvant (Difco Laboratories, USA), PEG-4000 (polyethylene glycol, Gibco, UK), PBS (20 m*M* Na-phosphate, 150 m*M* NaCl, pH 7.4, Merck), anti-mouse, Vector ABC kit (Vector Laboratories, USA) Protein G-Sepharose and Chelating Sepharose Fast Flow (Amersham–Pharmacia, Sweden), goat anti-mouse κ-alkaline phosphatase (Sigma), PNPP [2 mg *p*-nitrophenylphosphate (Orion, Finland)/ml diethanolamine–$MgCl_2$ buffer (Reagena, Finland)], RNAgents®Total RNA isolation system (Promega, USA), Oligotex-dT mRNA Mini Kit (Qiagen, Germany) reverse transcription system (Promega), Dynazyme polymerase (Finnzymes, Finland), L-Broth medium (Difco) IPTG (isopropyl-β-D-thiogalactopyranoside, Promega), BCA Protein assay (Pierce, USA).

2.2. Production of hybridomas

In order to facilitate immunisation, a three-carbon linker (2-carboxyethane) with a free COOH group was linked to the racemic mixture of the diarylalkyltriazole (Fig. 1). A total of 5.8 mg of the linkered drug dissolved in 0.4 ml of 100% DMSO

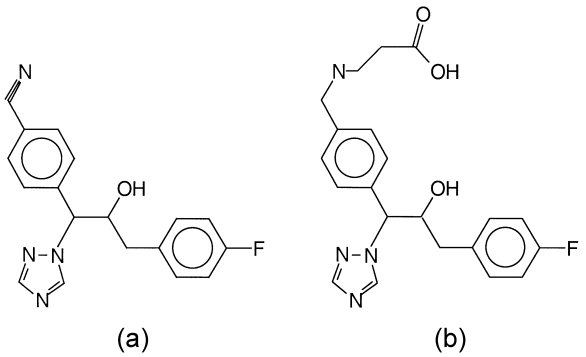

Fig. 1. (a) The target molecule, diarylalkyltriazole, has two chiral centres and thus four stereoisomers. (b) For the immunization a linkered version of the drug was used. The linker was prepared in three steps: diarylalkyltriazole has an aromatic nitrile group, which was first reduced to a primary amine with lithiumaluminiumhydride. This amine was then reacted with 2-bromopropionic acid ethyl ester in ethanol together with sodium carbonate to afford the end product as ethyl ester which was hydrolysed with potassiumhydroxide to the corresponding acid.

was activated for conjugation using 0.26 *M* EDC and 0.26 *M* S-NHS. Half of the mixture was incubated overnight at +4°C together with 5 mg BSA and the other half with 5 mg KLH, both in 50 m*M* phosphate buffer, pH 7.5. The KLH-conjugate was used to for immunisation of mice and the BSA-conjugate for screening the subsequent monoclonal antibodies.

2.3. Screening of monoclonal antibodies

Enzyme-linked immunosorbent assay (ELISA) was used as a standard assay to screen and analyse the binding properties of the enantioselective monoclonal antibodies. To screen for positive hybridomas, 96-well EIA plates (Dynatech, USA) were coated with 400 ng/well of the hapten–BSA conjugate in PBS, pH 7.4, overnight at +4°C. The plates were washed four times with PBS and blocked with 1% BSA in PBS for 30 min. The serum samples were applied and incubated for 60 min, washed with PBS, and the bound antibody was detected using anti-mouse Vector ABC kit. Media of the positive clones were collected and the monoclonal antibodies were purified by Protein G-Sepharose affinity chromatography according to the manufacturer's instructions.

Specific binding of the monoclonals obtained was verified using competitive ELISA. The plates were coated, washed and blocked as described above. The antibody samples were then applied to the plates with or without the non-conjugated enantiomer using final concentrations between 0.1 n*M* and 1 m*M*, incubated for 2 h, washed with PBS, whereupon the bound antibody was detected using goat anti-mouse κ-alkaline phosphatase. The substrate used was PNPP detected at 405 nm.

2.4. Cloning of the enantiospecific Fab-fragments

Standard methods were used for all recombinant DNA work [25]. Total RNA was isolated from the hybridoma cell lines (10^8 cells each) using the extraction protocol provided by Promega. The mRNA fraction was purified using the Oligotex-dT polyA+ purification kit and the cDNA synthesis was performed with the AMV reverse transcriptase system. The cDNAs were then subjected to PCR amplification using primer mixtures specific for each antibody group, nine for heavy chains and four for light chains [26]. The PCR reaction was carried out using Dynazyme polymerase. After the amplification, both the heavy and light chains were cloned as a dicistronic operon under the *tac* promoter controlled by the *lac*I^q repressor present in the expression vector pTI8 [27]. For secretion, the PelB signal sequence of pectate lyase of *Erwinia carotovora* [27] was linked for both the heavy and the light chains. During cloning a tag of six histidines was added to the C-termini of the light chains of both Fab-fragments for subsequent purification and immobilisation using the Chelating Sepharose matrix loaded with nickel for purification and copper for immobilisation. The resulting plasmids were called pENA5His and pENA11His and corresponding proteins ENA5His and ENA11His, respectively.

2.5. Production and purification of Fab-fragments

The plasmids pENA5His and pENA11His were transformed to the production host *Escherichia coli* RV308 (ATCC 31608) for high cell density cultivation. Ten to 20 single colonies for each construction were first cultivated in a small scale to screen for the best producers. Fed-batch cultivations were then performed for both Fab-fragments using a 1-litre laboratory fermenter (Chemap, Switzerland). An exponentially grown seed culture, in 100 ml of L-Broth medium, was added into 800 ml of mineral salt medium [16] containing 0.5 g/l glucose and 100 mg/l ampicillin. The pH of the medium was controlled at 6.8 with 12.5% NH_4OH. During growth at 28°C, additional glucose, trace elements, Mg_2SO_4, ammonia and ampicillin were fed to the growth medium. Following parameters were continuously monitored during the fermentation: pH, dissolved oxygen, temperature, composition of exhaust gas and the cell density (A_{600}). Sixty μ*M* IPTG were added to induce the protein production at the late-log phase of the growth (A_{600}=20–40), whereafter the cultivation was continued overnight. Substantial cell lysis occurred during the production phase releasing the Fab fragments to the culture supernatant. The cell debris was removed by centrifugation (5000 rpm, 20 min), whereafter the liberated DNA was removed by adding 2 mg DNAse to the culture supernatant (0.5–1 l) and stirring gently 1–2 h at room temperature. The pH of the recovered cell supernatant was

adjusted to 7.5–8.0 with NaOH and centrifuged (12 000 rpm, 30 min, +4°C). The supernatant was recovered and filtered through an HVLP 04700 filter (Millipore, USA) prior to application onto the Ni^{2+}-NTA Chelating Sepharose Streamline-column (Amersham–Pharmacia). Immunoblotting was used to follow the amount of the Fab fragments retained on the absorbent. The column was then washed with PBS, 50 m*M* imidazole, pH 7.0, until the baseline was achieved. Fab-fragments were eluted with linear imidazole gradient (50–500 m*M* imidazole in PBS, pH 7.4). Metal affinity chromatography was followed by ProteinG affinity chromatography. Purifications were performed according to the manufacturer's instructions. ELISA was used to confirm the binding activities of ENA5His and ENA11His antibody fragments, SDS–PAGE and immunoblotting were used to confirm their purity and absorbance at 280 nm and BCA Protein assay to determine their protein concentration.

2.6. Determination of the solvent concentrations used for elution

ELISA was used to evaluate the effects of solvents on the antigen recognition by ENA5His and ENA11His, and to screen for optimum elution conditions for the two enantiomers. In these experiments the ELISA plates were coated, blocked and washed as described above. A total of 250 ng of purified Fab fragments (see below) was preincubated with one of the three solvents where diarylalkyltriazole is soluble (ethanol, methanol and DMSO) at concentrations varying from 0 to 100% for 1–48 h. The samples were then added to the microtiter plates and the ELISA was performed as described above. Plain PBS was used as a control. Concentrations of each solvent leading to the lowest response in ELISA were used as an initial solvent concentration for elution of bound enantiomer in column format.

2.7. Immunoaffinity chromatography for enantiospecific separation

Chelating Sepharose Fast Flow was loaded with copper ions and mixed with 5–6 mg of a Fab fragment (ENA5His or ENA11His) per 1 ml gel according to the manufacturer's instructions. The Fab fragments were applied in batch to ensure even distribution of the protein upon immobilisation to the matrix. The 300–500 μl gel were then loaded into small glass capillaries with a diameter of 3-mm containing glass wool at the bottom as a filter. Any unbound Fab was washed with ca. five bed volumes of PBS. The racemate (5 mg/ml in 100% DMSO) was diluted with 2% DMSO–PBS and an amount corresponding to the molar amount of the Fab binding sites was applied to the column. For control, the same amount of racemate was applied in similar conditions to a stripped column matrix lacking the immobilised Fab fragment. The columns were washed with 18–19 bed volumes of PBS–2% DMSO. The bound enantiomer was eluted using a high pH buffer (PBS (pH 11.6)–2% DMSO), and fractions of 0.8–1.4 ml were collected through the whole chromatographic run. After the elution the columns were re-equilibrated with PBS. The possible leakage of the Fab fragments during enantiomeric separation was monitored by measuring the protein concentration of the fractions by BCA Protein Assay according to manufacturer's instructions using BSA or purified ENA5His or ENA11His as a standard.

For automated analysis of enantioseparation, 18.9 mg of purified ENA11His was immobilised through its histidine tag to 1.1 ml of Chelating Sepharose Fast Flow according to manufacturer's instructions and packed in HR5/5 columns (Amersham–Pharmacia). Chromatographic separation was performed with 10S ÄKTA-system (Amersham–Pharmacia) equipped with a UV–Vis detector. Standard chromatography conditions were as follows: flow-rate, 1 ml/min; injection volume, 2 ml; room temperature. Columns were first equilibrated with PBS before applying the racemate ad in PBS–2% DMSO. Two parallel columns were used for optimisation of conditions and two intact columns were used for reusability studies. First the columns were overloaded with the racemate in order to determine the capacity of the column in efficient enantioseparation. The subsequent runs were performed at this upper limit of capacity in order to see immediate changes in column capacity because of the possible inactivation of Fab fragments during several cycles of elution.

After application the racemate columns were

washed with PBS (pH 8.5)–2% DMSO followed either by elution with 15 bed volumes of a 0–40% methanol gradient in PBS, pH 7.4, or by stepwise elution with 40% methanol–PBS, pH 7.4. After elution the columns were first washed with 10 bed volumes of 40% methanol–PBS, pH 7.4, to ensure the release of any bound a-enantiomer and then equilibrated to PBS, pH 7.4, with 10 bed volumes. Leakage of the protein from the matrix during runs was determined from every fraction by BCA-method either from crude or 10 times concentrated samples.

2.8. HPLC analysis for the detection of enantiomers

The presence of the drug enantiomers in fractions was analysed by an HPLC system (Waters, USA) consisting of a Waters 2690 separation module and a M996 diode array detector. The column used for separation was Ultron ES-OVM (4.6×150 mm) (Hewlett-Packard, USA), at 30°C and the mobile phase 0.02 *M* ammonium phosphate (pH 5.0)–20% methanol (v/v) at a flow-rate of 1.0 ml/min. The detection wavelength was 230 nm. The limit of determination was 0.5 mg/l ($S/N=10$).

3. Results

3.1. Preparation of enantiospecific antibodies

The compound diarylalkyltriazole is an inhibitor of the aromatase enzyme. The chemically synthesized molecule has two asymmetric centres and subsequently four stereoisomers SS (designated d), RR (a), SR (b) and RS (c). While the diastereomers b and c can be separated from the enantiomeric pair a and d by selective crystallisation, fractionation of the a and d has proved to be a more difficult task. In order to obtain specific antibodies for the separation of the a- and d-enantiomers the racemic mixture of the drug was coupled to the KLH-carrier protein and used for immunisation of mice. Four monoclonal antibodies giving a positive signal were identified when screened by ELISA using a racemic drug–BSA conjugate on the microtiter plates. These four monoclonal antibodies were then subjected to competitive ELISA carried out with a- and d-enantiomers. Based on different affinities and specificities, two hybridoma cell lines producing antibodies were chosen for further work. The result shows that a racemic mixture can be used for immunisation to obtain antibodies against both components of the mixture.

Fab fragments of antibodies contain their specific antigen-binding domains but lack the effector functions needed for the cellular immune response. When antibodies are used in affinity chromatography, only the antigen-binding domains are needed. cDNA copies of the Fab fragments of both monoclonal antibodies were therefore cloned and their nucleotide sequences determined. Comparison of the two sequences revealed no apparent sequence similarity in the complementarity determining regions (CDRs) of the two Fab fragments: both the length and the amino acid composition of the hypervariable loops responsible for antigen binding were different. Total identity of the sequences at amino acid level was 36% for the heavy variable domains and 53% for the light variable domains. Both fragments were efficiently expressed in *E. coli*. Immunoblot analysis of the culture supernatant analysed on a non-reducing protein gel revealed that most of the protein produced was correctly assembled as Fab fragments, but small amounts of the free chains were detected. A two-step purification protocol removed contaminating proteins as well as free light chains. The final purification yield was repeatedly 100–150 mg Fab fragments/1 l culture medium. SDS–PAGE analysis in both reducing and non-reducing conditions revealed that most of the Fab fragments contained disulphide bridge covalently joining the two chains together (data not shown).

3.2. Small scale enantioseparation

The enantioselective properties of both Fab fragments were first examined in small non-automated columns. No separation of enantiomers was observed in control experiments using the matrix without antibody. The antibody fragments were then immobilised in an oriented manner with 95–98% efficiency to the metal affinity matrix via their polyhistidine tags, and manually packed into small experimental columns as described in the experimental section. The gravity flow-rates measured for these columns were 2.5–6 ml/h, depending on the volume

of the matrix. The drug racemate is not soluble in water but can be maintained in solution for several hours by addition of 2% DMSO. In order to evaluate the impact of prolonged exposure to the DMSO, the immunoaffinity column containing immobilised antibody was washed with 250 column volumes of 2% DMSO in PBS, pH 7.4, prior to applying racemate. No loss of capacity of the column pre-exposed to the solvent could be observed as compared to the column flushed with PBS alone.

The analysis of the flow-through fractions of the immunoaffinity column containing the immobilised ENA5His showed specific binding of the d-enantiomer while the a-enantiomer was detected in the flow-through fractions (Fig. 2a). Immobilised ENA11His retained specifically the a-enantiomer, while the d-enantiomer was found in the flow-through fractions (Fig. 2b). Both bound enantiomers were quantitatively eluted from corresponding columns at pH 11.6. Both Fab fragments bound only the b-enantiomer when the other enantiomeric pair of the drug, b and c, was applied. These results indicate that the specificity of the immobilised antibody fragment is based on the configuration of the different chiral centres of diarylalkyltriazole.

No protein leakage from the columns was detected upon extensive washing with buffers containing 2% DMSO in PBS, indicating good stability of both the immobilisation and the antibody structure in these conditions. However, the high pH required for the elution of the bound enantiomer was apparently not tolerated. Determination of the protein concentration of the eluting fractions indicated a leakage of 4–5% of the immobilised Fab at pH 11.6. Upon the second round of enantioseparation by ENA5His only 10–15% of the enantiomer binding capacity was remained in the column, suggesting that most of the antibody fragments had been inactivated during the previous elution. Therefore, alternative means were sought for the hapten elution.

Disruption of the antibody–hapten complex is most commonly performed by using high or low pH but can also be achieved by altering the buffer composition, temperature or by adding different solvents. Here we used ELISA for preliminary screening of the conditions for elution of the specifically bound d-enantiomer from ENA5His column using DMSO, ethanol or methanol. Every solvent

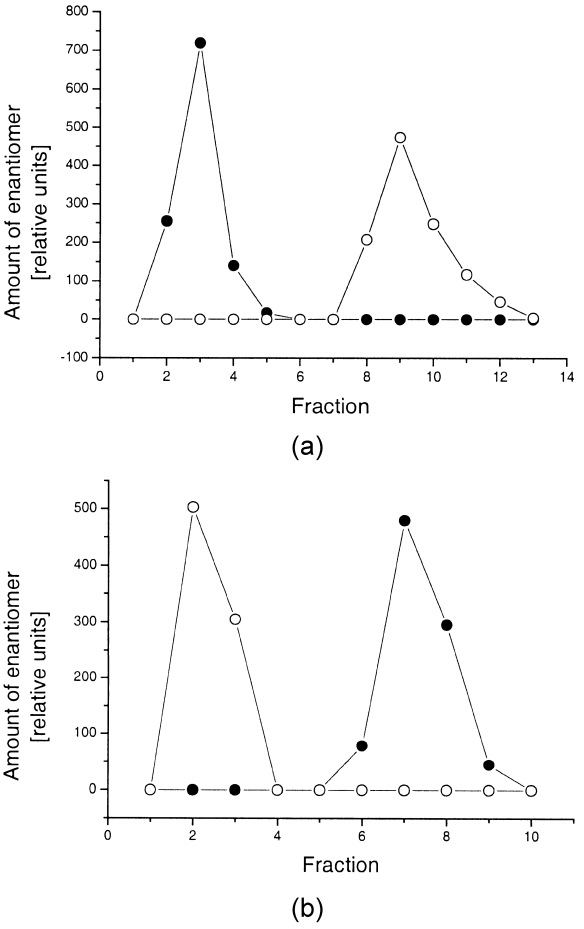

Fig. 2. Chromatograms of the performance of the ENA5His and the ENA11His antibody affinity columns in the enantioseparation of the a- and d-enantiomers of diarylalkyltriazole. (a) A chromatogram showing the ENA5His column with application of the racemate and washing of the unbound a-enantiomer (●) with 2% DMSO–PBS, pH 7.4 (fractions 1–5). Elution of the bound d-enantiomer (○) was with 2% DMSO–PBS, pH 11.6 (fractions 6–13). (b) A chromatogram showing the performance of the ENA11His in the same conditions as in (a). Note that this column retains the a-enantiomer, while the ENA5His column retains the d-enantiomer.

caused a reduction in the binding capacity as a function of solvent concentration and the lowest concentration of each solvent capable of elution was used as a starting point in the subsequent elution experiments (Table 1). In ENA5His columns, efficient elution was achieved using 55–75% methanol–PBS, pH 7.4. However, even the lowest effective methanol concentration of 55% caused antibody

Table 1
Performance of the ENA5His and ENA11His affinity columns during cycles of binding and elution using different solvents

Fab	Eluent	Eluent (%)	Separation cycle				
			1	2	3	4	5
ENA5His	DMSO	40	+				
	Ethanol	40	++				
		50	++				
	Methanol	20	−				
		50	++				
		55	+++	++			
		60	+++				
		65	+++	+			
		70	+++	+			
ENA11His	DMSO	20	++				
		30	+++	+++	+++	+++	+++
		40	+++	+++	+++		
	Ethanol	30	++				
		40	+++	+++			
	Methanol	30	+++				
		40	+++	+++	+++	+++	+++
		50	+++				
		60	+++	+++	+++	+++	

Repeated cycles were only performed with columns and elution conditions in which loss of binding capacity was not observed during the first one to two cycles The column performance, reflecting antibody stability, is given as the efficiency of the elution as a percentage of the bound enantiomer retrieved after each elution cycle: +++, 90–100%; ++, 60–90%; +, 20–60%; and −, <20%.

denaturation and only 70% of the Fab fragments were able to bind the d-enantiomer in the second round. In contrast, using the ENA11His column the bound a-enantiomer could be quantitatively eluted using 40% methanol–, 40% ethanol– or 30% DMSO–PBS, pH 7.4, with no apparent loss of antibody functionality during repeated cycles of binding and elution.

3.3. Continuous use of the immobilised ENA11His in enantioseparation

The performance of the ENA11His column in extended separation cycles was further studied with larger scale pre-packed columns in a more automated system. First the experimental binding capacity of the columns was determined by overloading racemate into the column, followed by elution with 40% methanol. During the first rounds of elution, about 20% decrease was observed in the maximum re-binding capacity of the antibody column. Some of the immobilised Fab fragments do not contain an interchain disulphide, but are instead connected by non-covalent forces. These interactions are probably sensitive to the 40% methanol used in elution, thereby resulting in a decrease in the amount of active antibody in the column. The ENA11His column stabilised at a binding capacity of 41–45% of the a-enantiomer of the theoretical maximum in the binding conditions used, reflecting the amount of stable antibody on the column.

Several repeated rounds of enantioseparation were then done at the upper limit of column capacity in order to see even small changes in capacity caused by antibody denaturation/inactivation in the elution conditions. The unbound d-enantiomer was always washed from the column in 16 bed volumes of the binding buffer. The elutions were then performed either as a gradient of 0–40% methanol in PBS, pH 7.4, or stepwise with 40% methanol–PBS, pH 7.4. Elution volumes required for 100% recovery of the bound a-enantiomer were 25 and 10 bed volumes, respectively, indicating that the elution volume can be regulated by changing the elution procedure. The

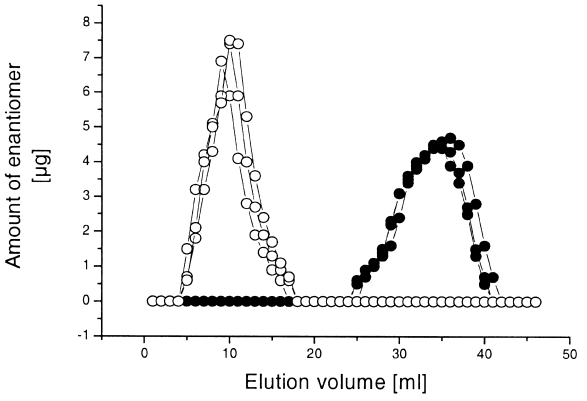

Fig. 3. Overlaid chromatograms showing repeated cycles (cycles 10, 15, 20) of the a/d-enantioseparation on a single ENA11His column. A dilution series of diarylalkyltriazole ad was used as a standard from which the amounts of enantiomers were calculated. After binding, the unbound d-enantiomer (○) was washed from the column (fractions 1–21) prior to elution. The bound a-enantiomer (●) was eluted by 40% methanol–PBS, pH 7.4 (fractions 22–46).

column runs subjected to gradient elution were repeated 20 cycles without loss in capacity when the detection limit was set 0.5 μg/ml, which corresponds to the 0.5% of the applied racemate. Column runs 10, 15 and 20 are shown in Fig. 3 as typical examples. However, in the case of the stepwise elution, the maximum binding capacity dropped about 10% during 20 elution cycles as indicated by leakage of bound enantiomer prior to elution (data not shown). No protein leakage was observed in any of the columns used, indicating that the loss of binding capacity is probably caused by antibody denaturation, not its detachment from the column matrix.

4. Discussion

We have shown here that enantiomeric separation of racemic compounds can be efficiently performed in one step with the aid of genetically modified antibody fragments. The utility of such columns in practical enantioseparation depends mainly on the efficiency of the enantioseparation and the stability of the column in repeated cycles of binding and elution. As shown in this work, antibodies recognising very small differences in the stereochemistry of an antigen can be obtained even if a racemic mixture has been used for the immunisation. In addition, protein engineering can be used to significantly improve the specificity of antibodies recognising small haptens [9]. The performance and the stability of the affinity columns also depend on the method of antibody immobilisation. In order to optimise the number of antibodies capable of antigen binding on the column, non-denaturing methods of immobilisation that allow orientation of the antibody fragments are preferred. Many different immobilisation methods are currently available for antibodies [28]. We have been experimenting with both covalent (via free cysteines, unpublished data) and non-covalent, directed immobilisation via histidine tag (this work) or cellulose-binding domains (CBD) [20]. In the conditions of this study, immobilisation using the histidine-tag onto a copper chelated affinity matrix was best in providing an efficient, oriented and stable antibody immobilisation. Finally, an economically feasible enantioseparation requires that the antibody affinity column is stable over repeated cycles of enantioseparation. In this case, the durability of the antibody columns largely depends on the antibody affinity, which in turn determines the harshness of the elution conditions required. The current work shows that, for an antibody with moderate affinity, it is possible to find conditions allowing at least 20 cycles of binding and elution, although an upper limit was not determined here. Moreover, protein engineering, and the phage display technology in particular, can be used to further optimise antibody stability, affinity, specificity and the elution conditions for almost any given application.

Acknowledgements

We are grateful to thank Arto Karjalainen, Arja Kalapudas and Arja Karjalainen (Orion Pharma, Finland) for providing diarylalkyltriazole. Jari Yli-Kauhaluoma is thanked for conjugating haptens to the carrier proteins and Arja Kiema, Tiina Liljankoski and Riitta Suihkonen for excellent technical assistance. Lennart Kågedal and Per-Mikael Åberg (Amersham–Pharmacia Biotech, Sweden) are thanked for their help in preparing columns for automated enantioseparation. The financial support

of the Orion Pharma, Danisco-Cultor, The National Technology Agency (Finland) and Eu-Project BE-1075 (Solid Matrices for Bioprocessing) is gratefully acknowledged.

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