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Review

# Antibody–antigen binding study using size-exclusion liquid chromatography

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## Contents



## 1. Introduction

Size-exclusion HPLC (SE-HPLC) separates molecules based primarily on molecular mass. Changes in antibody (Ab) and antigen (Ag) elution profiles due to Ab–Ag complex formation can provide valuable information about complex formation and binding characteristics. Principles of Ab–Ag complex formation and analysis using SE-HPLC will be discussed in this review with primary reference to literature published since 1989 with occasional reference to relevant earlier works. Earlier works have been reviewed by Stevens [1]. Reference to SE-HPLC used solely for antibody, antigen, or complex isolation has not been included.

## 2. Basic principles

Procedures using SE-HPLC in antibody–antigen binding studies include separation, detection and quantitation of Ab–Ag complexes, Ab, and Ag. Ab–Ag complexes have greater mass then either Ab or Ag. The separation of components is simple and involves using an appropriate SE-HPLC column and elution buffer. Recommended procedures for the selection and use of SE-HPLC columns can be obtained from the manufacturers. Detection of components in the sample depends on the complexity of the mixture. Table 1 summarizes various detection methods used

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Table 1
Detection methods used in SE-HPLC studies of antibody–antigen interactions

| | Ref. |
|---|---|
| *In-line detection* | |
| Light scattering/refractive index | [4,5] |
| Absorbance | |
| 280 nm | [5,11,13–15,17,21] |
| 243 nm[a] | [6] |
| 220 nm | [15] |
| 214 nm | [12,13] |
| Fluorescence detection of enzyme activity using enzyme-labeled antibody | [24] |
| *Analysis of fractions*[b] | |
| Radiometric | |
| $^{125}$I-Ab | [13,23] |
| $^{125}$I-Ag | [6,22] |
| $^{111}$In-Ab | [13,23] |
| SDS–PAGE/autoradiography | [6] |

[a] The mobile phase contained 0.5% Triton-X 100. Proteins could be detected at 243 nm within the detergent spectrum.

[b] Samples from SE-HPLC fractions could be analyzed by most immunological methods.

in SE-HPLC studies of antibody–antigen interactions. UV or fluorescence detection may be adequate for samples without interfering substances (e.g., mixtures of isolated antibody and antigen). Samples with either a low concentration of antibody or antigen or samples which contain other interfering substances may require other detection methods such as radioisotope labeling, enzyme activity, enzyme immuno assays, and ELISA. Certain detection methods are suitable for in-line detection, whereas others require collection of fractions prior to analysis. Quantitation is achieved using appropriate standards. Other immunological detection methods including chromatographic-based immunoassays have been reviewed by Hage [2].

## 3. Elution profile analysis

The main factors in the analysis of SE-HPLC elution profiles are (1) elution time (or elution volume) and (2) peak area (or peak height). Elution time is related to the relative molecular mass, $M_r$, as determined from a series of standards [3]. The hydrated volume and shape of the molecules influence the elution time; thus, non-ideal elution profiles may result. Methods such as low angle light scattering [4,5], or SDS–polyacrylamide gel electrophoresis [6,7] may be useful to determine the $M_r$ of components with atypical (non-ideal) elution profiles. Peak area (or height) is related to component concentration. Practical methods for determining peak areas have been described by Dyson [8]. Multiple-peak detection algorithms have been described [9] and software is available commercially to assist in peak identification and quantitation.

The appearance of peaks with elution times shorter than either the antibody or antigen is indicative of complex formation. Fig. 1 illustrates the elution profiles of mouse anti-human serum albumin (mIgG) and human serum albumin (HSA) at varying mole fractions of antibody. Fig. 1A presents the predicted elution profiles for no interaction between mIgG and HSA, whereas Fig. 1B presents elution profiles of reaction mixtures. Stevens [1] and Stevens et al. [10] have described the analysis of antibody–antigen interactions using ''delta'' profiles obtained by subtracting ''synthetic'' profiles (representing the sum of control elution profiles) from reaction mixture profiles. Changes in ''delta'' profiles may indicate changes in antibody–antigen complex composition as well as changes in reactant and product concentrations. Fig. 2 illustrates control, reaction, ''synthetic'', and ''delta'' profiles for representative data from Fig. 1. The major regions of interest in elution profiles are (1) regions of high molecular mass Ab–Ag complexes, and (2) regions of non-reacted antibody or antigen.

### 3.1. Antibody–antigen complexes

Evaluation of Ab–Ag complex elution profiles can provide information on the stoichiometry of Ab–Ag binding and the distribution of different complexes in reaction mixtures. Fig. 1B illustrates the concentration-dependent appearance of two high molecular mass peaks (C1, C2) with concurrent decrease in antibody (mIgG) and antigen (HSA) peak area (compared to controls). The $M_r$ of each complex suggests a binding stoichiometry of $Ab_1Ag_1$ for C1 and $Ab_2Ag_2$ for C2 with a transition of C1 to C2 at higher Ab concentration [11]. The concentration-dependent formation of multiple forms of human

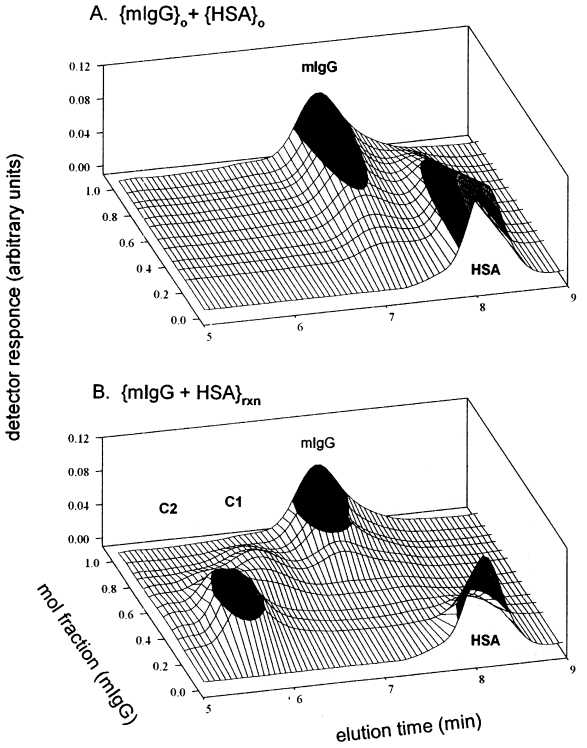



Fig. 1. Mesh plot of elution profiles of human serum albumin (HSA) and mouse monoclonal anti-HSA (mIgG) at increasing mole fractions of mIgG. (A) Predicted elution profiles of HSA and mIgG if there was no reaction. (B) Observed elution profiles of reaction mixtures. C1 and C2 are high molecular mass complexes. The elution profile data have been normalized to 1 mg mIgG at mole fraction (mIgG) = 1.0 and 1 mg HSA at mole fraction (mIgG) = 0. SE-HPLC procedures have been described in Ref. [11].

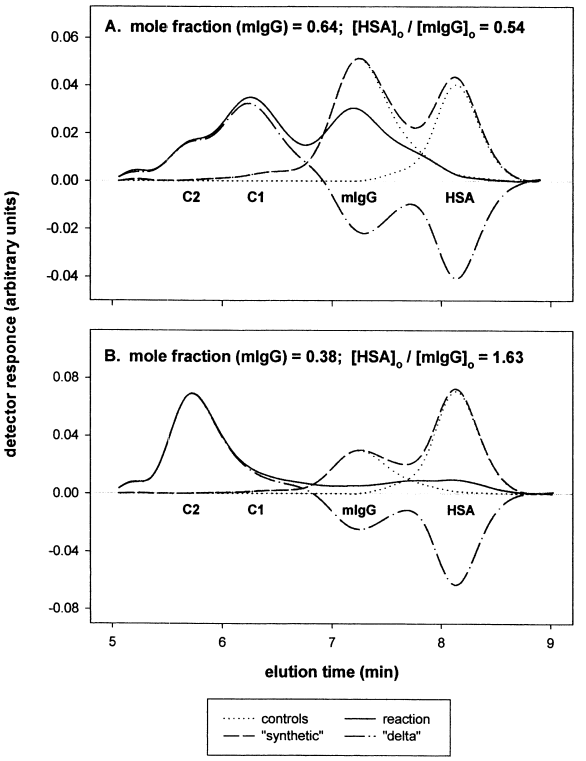
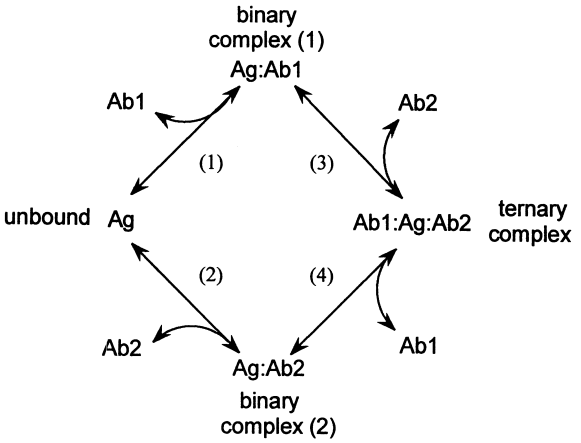



Fig. 2. Examples of control, reaction, ''synthetic'', and ''delta'' SE-HPLC elution profiles for the interaction of HSA and mIgG (Fig. 1). ''Synthetic'' and ''delta'' profiles have been described by Stevens [1] and Stevens et al. [10].

Cu/Zn superoxide dismutase and murine monoclonal antibody complexes ($Ab_1Ag_1$, $Ab_1Ag_2$, $Ab_2Ag_2$) [12] and interleukin-2 receptor alpha chains (Tac) and anti-Tac antibody complexes ($Ab_1$:$Ag_1$, $Ab_1$:$Ag_2$) [13] have been characterized by SE-HPLC. Two forms of human IgE and human IgE receptor α-chain IgG chimera complexes [4] and three forms ($Ab_1Ag_1$, $Ab_1Ag_2$, $Ab_2Ag_2$) of peroxidase:anti-peroxidase complexes in commercial preparations [14] have also been characterized.

Analysis of changes in complex $M_r$ and composition can be used for epitope mapping (typing) as described by Stevens [1] and Stevens et al. [10]. The method is based on binding of antigen to intact anti-antigen monoclonal antibodies or antigen-binding monoclonal antibody fragments from different cell lines, as illustrated in Scheme 1. The pattern of Ab–Ag complex elution profiles or ''delta'' profiles can be used to differentiate between independent (non-competitive) binding to spatially distinct epitopes and competitive binding to the same or to spatially restricting epitopes. Nakamura et al. have described epitope typing of two anti-human serum albumin monoclonal antibodies [15].

Stable Ab–Ag complexes are relevant to immunotherapies and in vivo antibody–antigen interactions. Stable Ab–Ag complexes have been identified by SE-HPLC for monomeric and dimeric forms of single-chain anti-carcinoembryonic antigen fragments and carcinoembryonic antigen [7], and an active peptide derived from activity-dependent neurotrophic factor (ADNF) and anti-ADNF [16]. Stable complexes of snake venom components and antibodies have been reported. Stable complexes have




$$M_{r,\,\text{unbound}} < M_{r,\,\text{binary}} < M_{r,\,\text{ternary}}$$

| binding: | reactions: |
|---|---|
| non-competitive | (1), (2), (3), and (4) |
| competitive or spatially restrictive | (1) or (2) only |

Scheme 1.

been reported for *Crotalus atrox* (Western diamondback rattlesnake) venom and Antivenin (Crotalidae) Polyvalent (equine origin antivenin) [11,17], β1-bungarotoxin (a presynaptic neurotoxin), A chain and B chain, and non-precipitating antibodies or antibody fragments [18], and β1-bungarotoxin and a bispecific anti-β1-bungarotoxin A chain antibody [19].

SE-HPLC has been used to identify and characterize stable Ab–Ag complexes and reactive antibodies or antigens in sera. Molecular masses of IgA-circulating immune complexes (CIC) in serum of patients with IgA nephropathy were estimated by SE-HPLC [20]. SE-HPLC was used to detect circulating immune complexes in serum of rats during infection with *Plasmodium berghei* (a malarial protozoon) [21]. Elution profile differences were observed between complexes of human insulin and polyclonal and monoclonal anti-insulin antibodies from patients with insulin autoimmune syndrome [22]. Immune complexes of murine monoclonal antibody (or antibody fragments) and human anti-murine monoclonal antibodies were detected in sera of cancer patients who had received repeated infusions of murine monoclonal antibodies (or antibody fragments) [23]. A method using SE-HPLC developed to determine antibody bioactivity during antibody immunotherapies was used to examine the bioactivity and concentration of free antibody in 14 patients undergoing therapeutic trial with Humanized anti-Tac antibody in leukemia and lymphoma [13].

### *3.2. Non-reacted antibody and antigen*

Non-reacted antibody and antigen can be identified from elution profiles and the concentrations determined from peak areas (or peak heights) using appropriate standards. Bound antibody and antigen can be calculated as $A_{\text{bound}} = A_0 - A$, where $A_{\text{bound}}$, $A_0$ and $A$ are the concentrations of bound antibody or antigen, total Ab or Ag, and free Ab or Ag, respectively. Ab or Ag may not be detected in the elution profiles of reactions that produce stable complexes unless that reactant is present in excess. The concentration of the limiting reactant that is bound would be approximately equal to the initial concentration of that reactant. Detection of low concentrations of free (unbound) antibody or antigen may require more sensitive methods of detection (e.g., radiolabeled antibody or antigen).

## 4. Profile areas, binding parameters, and the law of mass action

Evaluation of Ab, Ag, and Ab–Ag complex elution profiles have been used to determine binding parameters, including binding constants, reaction rates, pH effects, and temperature effects. Evaluation of binding parameters is illustrated in studies by Nakamura et al. for interactions between human serum albumin and anti-albumin monoclonal antibodies (or antibody fragments) [15] and between human chorionic gonadotropin (hCG) and peroxidase-labeled anti-hCG monoclonal antibody fragments [24]. The proportion of Ab, Ag, and two Ab–Ag complexes in elution profiles of mouse monoclonal anti-HSA and HSA was modeled in

terms of sequential binding, apparent association constants, initial concentrations of reactants, and peak area conversion factors (proportionality constants) [11].

The molar concentration of reactive components may not be known. Initial evaluation of elution profiles in mass units (i.e. g/L) may be useful. The total mass of the complexes can be estimated from $Ab_{bound} + Ag_{bound}$ in mass units and the mass fraction of bound antibody and antigen determined. The molecular mass of reactants and Ab–Ag complexes may be estimated from $M_r$ or determined by other methods. The mass and valence of antibody or antibody fragments can be determined by the structure of the molecule (e.g., valence of IgG = 2, Fab = 1) and the valence of the antigen may be predicted from complex $M_r$, $Ab_{bound}$ and $Ag_{bound}$.

Additional factors may need to be considered in elution profile analysis. The rates of association and dissociation of Ab–Ag complexes during SE-HPLC may influence elution profiles. Dissociation (and association) of Ab–Ag complexes during SE-HPLC may result in Ab–Ag peak ''tailing'', Ab or Ag peak ''fronting'', and decreased resolution of reaction components. Effects of association rate, dissociation rate, concentration of reactants and products, and run time have been described by Stevens [25] for simulated SE-HPLC elution profiles of protein–protein interactions. All of the components in a mixture may not be reactive, such as mixtures of antigens and polyvalent anti-sera; thus the maximum fraction of reactivity (reactive/total) may be less than 1. The maximum reactivity of Antivenin (Crotalidae) Polyvalent (equine origin antivenin) with *C. atrox* (Western diamondback rattlesnake) venom and with isolated *C. atrox* phospholypase $A_2$ was approximately 0.15 and 0.06, respectively (unpublished results), compared to 1.0 for mIgG and HSA [11]. The concentrations of reactive components may be calculated from the difference between the elution profile area and the fraction of the area that is non-reactive.

## 5. Conclusion

Size-exclusion HPLC is a simple method for separating antibody–antigen complexes from unreacted antibody and antigen. Evaluation of elution profiles provides information on the relative molecular mass of complexes, antibodies and antigens, the stoichiometry of binding, and the stability of complexes. Changes in complex $M_r$ and composition can identify multiple forms of complexes and can be used for epitope mapping (typing). Detection of stable complexes is relevant to immunoassays, immunotherapies and in vivo antibody–antigen interactions. Binding of antibodies and antigens to form complexes can be modeled mathematically using elution profile areas and equations derived from the law of mass action, thus providing information on reaction parameters.

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