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Molecular Diagnostics Using Analytical Immuno High Performance Liquid Affinity Chromatography

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ABSTRACT

Analytical immuno high performance liquid affinity chromatography (analytical immuno HPLAC) was evaluated as a molecular diagnostic tool. Antibodies raised in rabbits against bovine neurophysin II were immobilized through Protein A crosslinking onto coated silica. Interaction of immobilized antibody with mobile antigen was characterized by zonal and frontal elutions of ^{14}C-labeled bovine neurophysin II under isocratic, nondenaturing conditions. The chromatographic behavior shows that analytical immuno HPLAC with immobilized antibodies can be used to detect the number and functional nature of matrix-interacting antigens in mixtures, thus providing a quantitative chromatographic technology for "antigen mapping."

Index Entries: Antigen mapping; microscale analytical immunoaffinity chromatography; neurophysin ELISA; [antiNeurophysin II-Protein A] silica.

ABBREVIATIONS

HPLAC	high performance liquid affinity chromatography;
IgG	immunoglobulin G;
BNPII	bovine neurophysin II;
HRP	horseradish peroxidase;

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acim	acetimidyl;
PBS	phosphate buffered saline;
HPLC	high performance liquid chromatography;
WS-DCC	water soluble cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho paratoluensulphonate

INTRODUCTION

Affinity chromatography has become widely used as an analytical tool to detect and measure molecular interactions (*1,2*). Moreover, the introduction of rigid, high performance affinity supports (*3*) has expanded the versatility of the technique substantially, allowing characterization of biomolecular interactions on a microscale with improved resolution (*4,5*). The resultant analytical HPLAC[1] thus can be used potentially as a flow-through biosensor technology, to detect interacting substances in biological, environmental, and other samples, and also to measure their functional binding characteristics.

We were interested to test the applicability of analytical HPLAC for diagnostic mapping of antigenic substances. The specificity of immobilized antibodies often has been used in affinity chromatography to isolate and concentrate antigens (*6,7*). The efficiency of immunoaffinity supports improved when the disadvantage of random orientation of antibodies during immobilization, often leading to antibody inactivation, was overcome by first coupling Protein A to the solid support. Using the propensity of Protein A to bind the Fc portion of the IgG antibodies (*8*), antibodies could be crosslinked to [Protein A]matrix (*9,10*) so as to leave the antigen combining sites in the Fab domains unblocked and available to interact with soluble antigens. Such immobilized antibodies on rigid matrices could be used chromatographically for molecular analysis of antigens.

In the current study we prepared anti-BNPII covalently crosslinked through Protein A onto coated silica and characterized its interaction properties with mobile BNPII. The usefulness of this matrix as a microanalytical tool to measure various forms of mobile neurophysin was evaluated by analytical immuno HPLAC.

MATERIALS AND METHODS

Tween 20, o-dianesidine, and WS-DCC were purchased from Sigma Chemical Corporation (St. Louis, MO). Protein A was from Pharmacia Fine Chemicals (Piscataway, NJ) and styrene cuvets from Fisher Scientific Company (Washington, DC). Anti-IgG conjugated to HRP was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and [1-^{14}C]ethyl acetamidate was from Amersham Corporation (Arlington Heights, IL). Accell silica-based affinity medium was kindly provided by Waters Chromatography Division (Milford, MA).

^{14}C Acetimidation of Bovine Neurophysin II

BNPII, isolated from neurointermediate pituitary as before (*11*), was dissolved in 0.5 M sodium bicarbonate (pH 10.3) at 1 mg/mL. [^{14}C]Ethyl acetimidate (0.2 mg/mg protein) was added and the mixture incubated for 1 h at 0°C. Unlabelled ethyl acetimidate (3 mg/mg protein) then was added to assure complete protection of α and ϵ amino groups. After 1 h further reaction at 0°C, the mixture was acidified to pH 5.4 with glacial acetic acid. [^{14}C]Triacetimidyl BNPII was separated from residual reagents by gel filtration on Sephadex G-25 in 0.2 M acetic acid and then by biospecific adsorption on Met-Tyr-Phe-Affigel-102, as described before (*11,12*).

Anti-BNPII Purification

Antibodies against BNPII were purified from crude serum by HPLAC on [BNPII[Accell (1.4 μmol BNPII in 7.5 mL bed vol, column dimension 95 x 10 mm I.D.) prepared as described before (*5*). Rabbit antiserum was treated with Tween 20 (3.0 μL/mL serum) and filtered (0.45 filter, Amicon). Aliquots of 1.0 mL treated serum were injected onto the column and eluted with 0.4 M NH_4Ac, pH5.7, at a flowrate of 1.0 mL/min. Bound antibody was removed from the matrix by changing the elution buffer to 0.2 M HAc. Collected fractions were dialyzed in visking #16 Nojax tubing (molecular weight cutoff about 10 kD) against 0.4 M NH_4Ac, pH 6.5, for 2 d, then frozen, and lyophilized.

ELISA Assay of Anti-BNPII

An ELISA test was devised to detect the presence of BNPII antibodies. Square, 2.9 mL styrene cuvets, previously washed with 95% ethanol and dried, were filled with 1 mL of a 3 μg/mL BNPII solution in 0.05 M Tris HCl, 2 mM EDTA, 0.3 M KCl, pH 8.0, and the filled cuvets incubated for 1 h at 37°C, then washed several times (over a 20-min period) with PBS in 0.1% Tween 20 buffer, and finally dried. Freeze dried fractions to be tested for anti-BNP content were reconstituted in 1.2 mL PBS-0.1% Tween 20 buffer and assayed either directly or diluted further up to 1:10,000 with the same buffer. Aliquots of 1 mL were incubated at 37°C for 1 h in the BNPII-pretreated cuvets. Cuvets were then washed with PBS-Tween buffer (for 20 min) and air dried. Then, 1 mL of 1:2000 diluted commercial anti-IgG conjugated to HRP (0.021 U/mL final concentration of HRP, dilution with PBS-0.1% Tween 20) was added to each cuvet and incubated at 37°C for 1 h. After washing for 20 min with PBS-Tween buffer, 1 mL of HRP substrate was added and incubated for 40 min. The HRP substrate was prepared by dissolving 3.0 mg of o-Dianesidine (3,3-dimethoxybenzidine dihydrochloride) in 66 μL of a 1:1 mixture of MeOH/50 mM NaAc, pH 5.0, and then adding 933 μL of 50 mM NaAc, pH 5.0, and 4 μL of 30% H_2O_2. The HRP reaction in the cuvette was quenched by adding 20 μL 5 N HCl. Absorbance values of cuvette contents then were measured at 400 nm.

Preparation of [Protein A]Accell

Protein A (15 mg) was dissolved in 15 mL of coupling buffer (0.1 M $NaHCO^3$-0.5 M NaCl, pH 8.0) and shaken for 2 h at room temperature with 2 g of Accell affinity medium (Polyacrylamide-coated silica, 55-100 μm particles size, with a six carbon spacer and a terminal carboxyl activated with N-hydroxysuccinimide). The silica support was previously washed twice with 200 mL deionized water and 200 mL coupling buffer on a sintered glass funnel. The supernatant was decanted and 600 mg of glycinamide in coupling buffer (15 mL) was added to the resin and shaken at room temperature for 1 h. The resin was then washed twice with each of coupling buffer (200 mL) and deionized water (100 mL).

Preparation of [Anti-BNPII Protein A]Accell

[Protein A]Accell (2g), prepared as described above, was incubated, with agitation, with affinity-purified anti-BNPII (5mg) in 10 mL PBS buffer for 4 hrs at room temperature. After buffer decanting, 10 mL of 0.1 M $NaHCO_3$ 10 mM WS-DCC, pH 9.0, were added and the suspension was shaken for 2 h at 4°C. The resin then was washed extensively with deionized water and packed in a 100×6.6 mm I.D. HPLC glass "Omni" column (Rainin).

Performic Acid Oxidation

[^{14}C]Triacim-BNPII was performic acid oxidized similarly as for BNPII (*13*). Briefly, [^{14}C]triacim-BNPII (10 μg) was dissolved in 0.5 mL performic acid (1.8 mL of 98% formic acid added to 0.2 mL of 30% H_2O_2) in an ice bath and left at 0°C for 3 h. Deionized water (1 mL) was then added and the solution was frozen and lyophilized.

Instrumentation for Analytical HPLAC

Derivatized silica supports were dry-packed into 100×6.6 mm I.D. glass "Omni" columns (Rainin). The packed columns were installed in an LKB high performance liquid chromatograph, equipped with a Model 2150 HPLC pump (with ceramic heads), a Model 2151 variable wavelength absorbance monitor and a Model 2211 fraction collector. All solutions for chromatographic elutions were filter-sterilized and degassed under vacuum prior to use. When not in use, the columns were stored at 4°C in elution buffer containing 0.02% sodium azide.

RESULTS

Immuno HPLAC Purification and Immobilization of Anti-BNPII

Fractionation of BNPII antiserum on [BNPII]Accell was used to separate a protein peak which as eluted by HAc (Fig. 1A). The identity of this

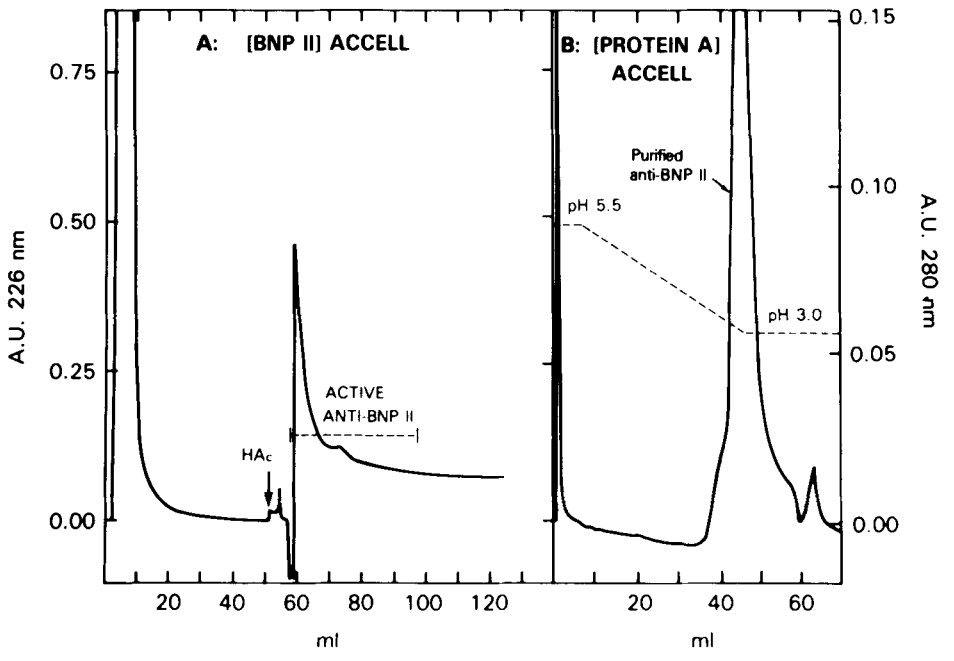

Fig. 1. (A) Purification of IgG anti-BNPII from crude rabbit serum (1 mL) by biospecific adsorption on [BNPII]Accell (95×10 mm I.D) equilibrated with 0.4 M NH_4Ac, pH 5.7, at a flowrate of 1.0 mL/min. At the arrow position, the eluent was changed to 0.2 M HAc. Effluent from the column was monitored continuously by U.V. absorbance, at 226 nm, 1.26 absorbance units (A.U.) full scale. Active antibody was identified by ELISA monitoring of eluted fractions. (B) Elution of retained peak from [BNPII] Accell on [Protein A]Accell.

second peak as active anti-BNPII was verified by ELISA assay. The population of anti-BNPII so affinity-purified was composed predominately of material which could bind to [Protein A]Accell (Fig. 1B) and be eluted as a single peak with a pH gradient from 5.5 to 3.0. The strong non-covalent binding of purified anti-BNPII to [Protein A]Accell formed the basis for its oriented immobilization to the latter matrix.

Analytical HPLAC Characterization of BNPII Binding to [Anti-BNPII-Protein A]Accell

The characteristics of frontal and zonal elution affinity chromatography of different concentrations of [^{14}C]triacim-BNPII were used to characterize [anti-BNPII-Protein A]Accell. Frontal chromatography was performed using as eluent solutions different concentrations of BNPII dissolved in PBS buffer and monitoring eluate by absorbance at 226 nm. The unretarded elution volume, V_o, was determined to be 1.7 mL by frontal elution with 1 mg/mL BSA. Void volume, V_m, was found to be 1.1 mL as determined by elution with 1 mg/mL blue dextran. Experimental elution volumes of BNPII were found to increase as the concentration of BNPII is decreased.

When this variation is plotted in Fig. 2 as $1/(V-V_o)$ vs $[P_o]$ (initial concentration of mobile interactor) according to Eq. [1],

$$\frac{V_o - V_m}{V - V_o} = \frac{K_{M/P} + [P]}{[M]_T} \quad [1]$$

graphical analysis yielded values of $K_{M/P}$, the equilibrium dissociation constant of the complex of mobile protein (P) and matrix-immobilized interactor, (M), and $[M]_t$, the concentration of functional immobilized molecules. The calculated values are given in Fig. 2.

Zonal elution was performed by injecting small aliquots containing different quantities of [^{14}C]triacim-BNPII in PBS-glycine buffer (0.5 mg/mL) and continuing elution with PBS. An increase in elution volume was observed with decreasing zonal concentration of BNPII (Fig. 3). Elutions were followed by radioisotope monitoring, by adding 3 mL Aquasol to each eluted fraction of 0.5 mL followed by scintillation counting. The values of elution volumes plotted as $1/(V-V_o)$ vs $[P_o]$ according to Eq. [1]

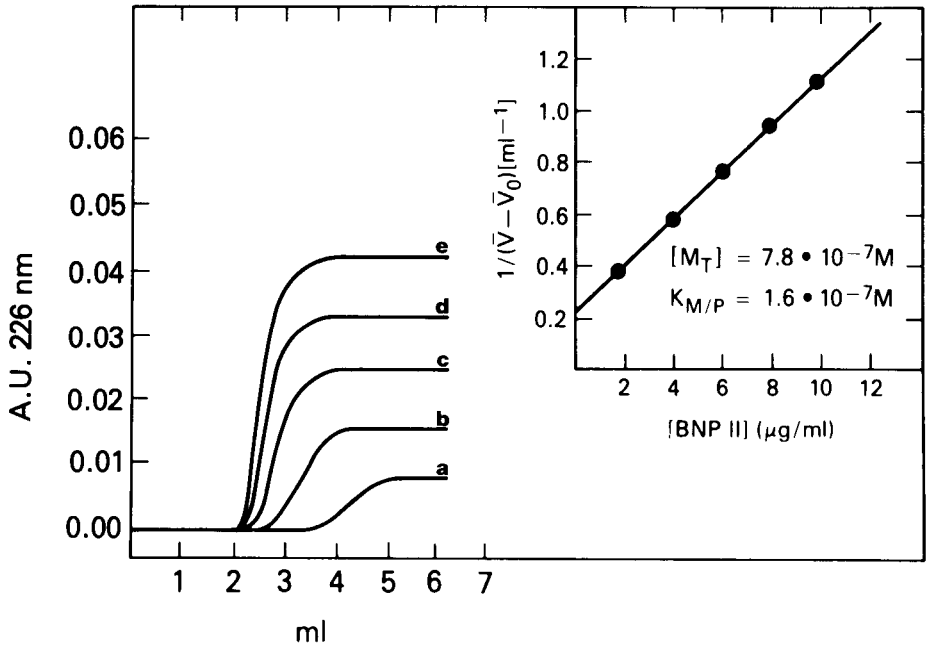

Fig. 2. Frontal elutions of BNPII on [anti-BNPII-Protein A]Accell and determination of column capacity. The column (100×6.6 mm I.D.) was equilibrated with PBS buffer, at a flow rate of 1.0 mL/min. Solutions containing varying amounts of BNPII were pumped continuously to the column, and the effluent was monitored continuously by U.V. absorbance at 226 nm, 0.04 absorbance units (A.U.) full scale. Protein concentrations were as follows: a, 2 μg/mL; b, 4 μg/mL; c, 6 μg/mL; d, 8 μg/mL; e, 10 μg/mL. Inset: Dependence of the elution volume (V) on BNPII concentration. Data are plotted according to Eq. [1], except that elution volumes are designated $\bar{V}$ and $\bar{V}_o$ when obtained by frontal analysis.

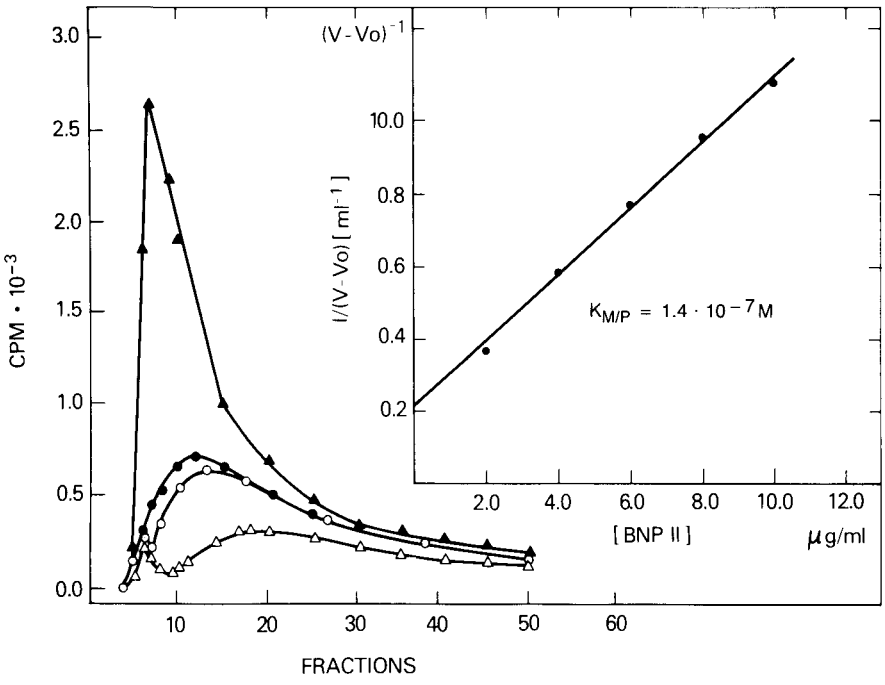

Fig. 3. Zonal elution profiles of ^{14}C-labeled BNPII on the [anti-BNPII-Protein A]Accell. The column was equilibrated with PBS buffer at a flow rate of 1.0 mL/min. Zones (20 µL) containing different amounts of ^{14}C-labelled BNPII were injected onto the column, and fractions of 500 µL were collected directly into vials for scintillation counting. Protein amounts were as follows; 3.5 µg (▲); 2 µg (●); 1.5 µg (○), and 1 µg (△). Inset: Dependence of the elution volume on [^{14}C]BNPII concentration, expressed as $1/(V-V_o)$ vs the amount of [^{14}C]BNPII in the initial 20 µL zone.

(*see* Fig. 3, inset) gave a $K_{M/P}$ value of 1.4×10^{-7} M from the *y*-intercept and assuming $[M]_t = 7.8\times10^{-7}$ M (based on calculation for frontal elution data).

Differential Elution of BNPII and Its Oxidized Derivative

By zonal elution, it was found that oxidized BNPII shows no significant affinity for [anti-BNPII-Protein A]Accell. Upon injecting a mixture of native and oxidized ^{14}C-labeled BNPII, two peaks were observed (Fig. 4). The first one, corresponding to V_o, can be assigned to oxidized BNPII, whereas the retarded second peak corresponds to native BNPII. This type of diagnostic elution allows not only the separation of the differentially antigenic proteins but also an assignment of apparent $K_{M/P}$ values based on extents of retardation at the concentrations in the initial sample zone. The $K_{M/P}$ value of the second peak agrees well with the values, 1.6×10^{-7} and 1.4×10^{-7} M, determined respectively by extrapolation of $1/(V-V_o)$ value to $[P_o]=O$ for multiple frontal and zonal elution analyses such as those in Figs. 2 and 3. Similar extrapolated values of $K_{M/P}$ can be obtained for unknowns by doing elutions of the latter with several dilutions.

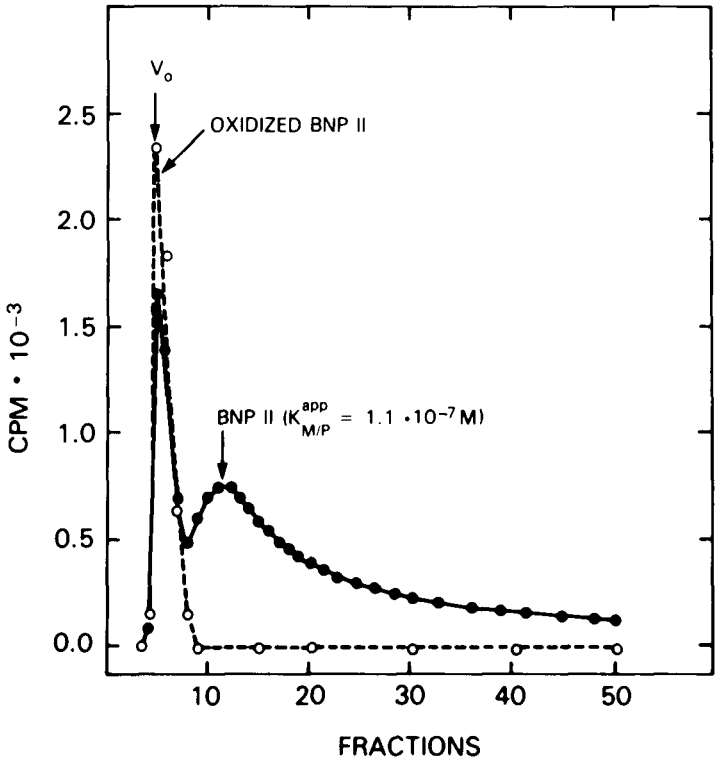

Fig. 4. Separation of oxidized [^{14}C]BNPII from intact [^{14}C]BNPII. A mixture (●) of oxidized (0.5 μg) and native (1.5 μg) [^{14}C]BNPII in a 20 μL zone was injected onto the [anti-BNPII Protein A]Accell column, equilibrated with PBS buffer at a flowrate of 1.0 mL/min. Fractions of 500 μL were collected for scintillation counting. Oxidized BNPII (○) was not retarded significantly on the column and eluted at the void volume.

DISCUSSION

The main goal of the present study was to determine whether antibodies, even of reasonably strong binding affinity, could be used in immobilized forms for quantitative chromatographic mapping of mobile antigens. In the test system used, polyclonal, affinity-purified anti-BNPII was immobilized, by crosslinking to Protein A, which itself was immobilized on a silica-based affinity matrix. Mobile BNPII, the antigen, was retarded specifically on this immobilized antibody and could be eluted by isocratic, nonchaotropic elution in practical experimental time. The elution position could be detected, elution volume measured, and the specific affinity of antigen to antibody calculated. Analytical chromatographic mapping was used to separate and characterize molecules with differential antigenicity, having K_a of up to 10^7 M^{-1}.

The data obtained here thus show that, with analytical immuno HPLAC, molecular interactions of antigenic molecules can be detected

and measured quantitatively. Multiple species with different affinities for immobilized antibody can be separated from one another diagnostically. When the molecular identity of eluting molecules can be defined by comparing to elution positions for standards, the elution profile of analytical immuno HPLAC provides a diagnositic map of antigen content. This potential has one caveat that must be remembered, namely, that elution volumes can have an appreciable dependence on amount of mobile interactor eluted (*see* Figs. 2 and 3 and Refs. (*4* and *5*). Thus, the assignment of molecular identity directly from elution position can only be approximated unless the amount of interactor is known. Alternatively, a limited set of elutions with different amounts of total sample can be made and the extrapolated value of $1/(V - V_o)$ at $[P] = o$ used in each case to determine a more reliable $K_{M/P}$ value. Another alternative would be to make the affinity matrix concentrated enough in immobilized ligand so that the elution position is minimally sensitive to amount of mobile interactor eluted. This latter situation has been observed previously with most soft gel matrices and some HPLAC matrices made and studied in this laboratory (e.g., Ref. (*15*)).

Immuno HPLAC generally can provide an important tool to purify antigens from complex biological fluids. No doubt, orientated immobilization through Protein A will yield affinity chromatographic matrices of increased functional efficiency. When used in the analytical mode, the same immobilized antibodies can be used as chromatographic biosensors. On the microscale, analytical immuno HPLAC may well be a valuable molecular diagnostic tool to identify and characterize limited amounts of biologically/clinically important molecules not easily differentiated, especially with quantitative characterization, by other techniques.

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