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QUANTITATIVE AFFINITY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NEUROENDOCRINE POLYPEPTIDES USING POROUS AND NON-POROUS GLASS DERIVATIVES

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SUMMARY

Analytical affinity HPLC was developed to isolate and characterize neuroendocrine peptide/protein components. Bovine neurophysin II (NP-II) was covalently immobilized on succinamidopropyl derivatives of both controlled-pore glass (CPG) and non-porous glass (NPG). These derivatives were packed into 25 × 0.46 cm I.D. stainless-steel columns and incorporated into a high-performance liquid chromatograph. Interaction of [^{3}H]Arg^8-vasopressin ([^{3}H]AVP) with NP-II was examined by chromatography of AVP on both CPG and NPG affinity matrices. Zonal elution profiles of [^{3}H]AVP on NPG matrix showed, as predicted theoretically, a linear dependence of retardation on the concentration of hormone injected. The data permit calculation of the equilibrium dissociation constant for the NP-II/AVP interaction. Elution characteristics also were measured by frontal analysis of large-zone chromatography experiments, the results of which were in good agreement with the zonal elution analysis. Affinity resulting from dimerization also was studied by chromatography of [^{125}I]NP-II on the NPG matrix. In this case, concentration dependence of retardation was non-linear, again as predicted theoretically. Off-rate kinetic constants for dissociation of the mobile interactant from the stationary phase also were obtained. The studies illustrate the utility of analytical affinity HPLC on non-porous beads for measuring relative affinities for various soluble ligands with small amounts of material. Chromatography on the CPG column proved useful for purification of microscale amounts of [^{3}H]AVP.

INTRODUCTION

The utility of biospecific interactions between an immobilized interactant and soluble substances for isolating biological molecules is well established[1]. It also has been established that such affinity chromatography, used analytically, can provide significant information pertaining to the thermodynamic and kinetic characteristics

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of the biomolecular interactions[1]. For example, Dunn and Chaiken[2] derived expressions for zonal elution chromatography in the presence of soluble competing ligand, and they examined the interaction of staphylococcal nuclease with immobilized and soluble nucleotides. Other bimolecular systems also have been evaluated in several laboratories by the zonal elution method (refs. 1 and 3 and references therein). Nichol *et al.*[4] expanded the theoretical treatment to encompass many possible equilibria and applied their relationships to frontal analysis of large-zone chromatography. The method of frontal analysis of large-zone chromatography was further developed and applied to studies of trypsin/ligand interactions by Kasai and Ishii[5,6]. More recently, it has been suggested that the kinetic off-rate constants for the biospecific interaction could be obtained from an analysis of the zone spreading[7,8]. It was also suggested that use of impenetrable beads may allow measurement of the true chemical off-rate constant by eliminating the effect of mass transfer[3,9].

The neuroendocrine peptide/protein system of hormones and neurophysin, encompassing a complex set of interrelated interaction equilibria[10], is an excellent candidate for testing the limits of analytical affinity chromatography. Previous affinity chromatographic analysis of neuroendocrine polypeptides, as generally for other peptide and protein systems, has utilized compressible porous gels, in this case mainly agarose (*e.g.*, refs. 11 and 12). The purpose of the present study was to develop porous and non-porous rigid glass (silica) chromatographic media with the macromolecule in the matrix phase and to test their use in analytical high-performance affinity chromatography (analytical HPAC). Use in microscale preparative isolation also has been evaluated.

THEORETICAL

General

Immobilization of bovine neurophysin II (NP-II) in the stationary phase permits affinity chromatography of both soluble neurophysin and hormone. In the case of NPG, assuming local equilibrium, a partition coefficient can be defined as

$$\sigma = \frac{Q_s}{V_0 C} \tag{1}$$

where Q_s is the amount of solute adsorbed to the matrix, V_0 is the elution volume of an unretarded molecule, and C is the solute concentration in the mobile phase. The elution volume of solute, V, can then be expressed as

$$V = V_m + \sigma V_0 \tag{2}$$

where V_m is the mobile phase volume. When there are no biospecific interactions, $\sigma_0 V_0 = V_0 - V_m$. Thus, when no non-specific adsorption occurs ($V_0 = V_m$), we have $\sigma_0 V_0 = 0$. Therefore

$$\sigma = \frac{V - V_0}{V_0} \tag{3}$$

Chromatography of hormone

If the hormone is chromatographed on immobilized NP-II, then the corresponding partition coefficient is

$$\sigma_L = \frac{[ML]}{[L]} \tag{4}$$

where M represents the immobilized molecule and [L] is the concentration of hormone in the mobile phase. Since the dissociation equilibrium constant, $K_{M/L}$, for NP-II–hormone equilibrium is given by

$$K_{M/L} = \frac{[M][L]}{[ML]} \tag{5}$$

and the total immobilized NP-II concentration, $[M]_T$, is

$$[M]_T = [M]\,(1 + [L]/K_{M/L}) \tag{6}$$

then, provided the concentration of soluble ligand is equal in the stationary and mobile phase, we obtain

$$\frac{V_0}{V - V_0} = \frac{K_{M/L}}{[M]_T} + \frac{[L]}{[M]_T} \tag{7}$$

Chromatography of neurophysin

Owing to the biospecific dimerization of neurophysin under suitable conditions, chromatography of soluble neurophysin reflects the affinity of soluble monomer for the immobilized monomer. The equilibria occurring are:

$$MP \leftrightharpoons M + P;\ K_{M/P} = \frac{[M][P]}{[MP]} \tag{8}$$

and

$$P_2 \leftrightharpoons 2\,P;\ K_{P/P} = \frac{[P]^2}{[P_2]} \tag{9}$$

where P is soluble monomer and P_2 is soluble dimer of neurophysin. As above, the partition coefficient is given by

$$\sigma_P = \frac{[MP]}{[P]_T} = \frac{V - V_0}{V_0} = \frac{[M][P]/K_{M/P}}{[P]_T} \tag{10}$$

Since $[M] = [M]_T/(1 + [P]/K_{M/P})$, we have

$$\frac{V_0}{V - V_0} = \frac{K_{M/P}\,[P]_T}{[M]_T\,[P]} + \frac{[P]_T}{[M]_T} \tag{11}$$

Since the concentration of soluble monomer neurophysin is given by

$$[P] = \frac{K_{P/P}}{4}\left[\left(1 + \frac{8[P]_T}{K_{P/P}}\right)^{1/2} - 1\right] \tag{12}$$

and since

$$[P]_T = [P] + 2[P]^2/K_{P/P} \tag{13}$$

where the total neurophysin concentration, $[P]_T$, is expressed in terms of the monomer, combination of these three equations gives

$$\frac{V_0}{V - V_0} = \frac{K_{M/P}}{2[M]_T} + \left[\frac{K_{M/P}}{2}\left(1 + \frac{8[P]_T}{K_{P/P}}\right)^{1/2} + [P]_T\right]\frac{1}{[M]_T} \tag{14}$$

From this result, we note that under the condition that $[P]_T \ll K_{P/P}$ and $K_{M/P}$, the quantity in brackets becomes $K_{M/P}/2$; consequently

$$\frac{V_0}{V - V_0} = \frac{K_{M/P}}{[M]_T} \text{ when } [P]_T \ll K_{P/P} \text{ and } K_{M/P} \tag{15}$$

MATERIALS AND METHODS

Purification of neurophysin II

Acetone powders of bovine pituitaries were extracted with dilute hydrochloric acid and fractionated by Sephadex G-75 chromatography. NP-II was obtained by chromatography on DEAE-Sephadex A-50, as previously described[13], and was further purified by preparative reversed-phase HPLC[14]. Aliquots of protein were injected onto a 25 × 0.92 cm I.D. Zorbax (DuPont) ODS column, equilibrated with triethylamine phosphate buffer (pH 3.0), containing 23% acetonitrile. NP-II was eluted with a 60-min 23–25% acetonitrile gradient and a flow-rate of 3.2 ml/min. The eluted protein, after removal of acetonitrile, was adsorbed by biospecific interaction on a column of Met-Tyr-Phe-Sepharose[15], equilibrated with 0.4 *M* ammonium acetate at pH 5.7 and eluted with 0.2 *M* acetic acid.

Immobilization of neurophysin II

NP-II was covalently immobilized on 200-nm pore diameter controlled-pore glass (CPG-2000, Electro-Nucleonics, Fairfield, NJ, U.S.A.) and on non-porous glass beads (Type 1-W, 75–150 μm diameter, Sigma, St. Louis, MO, U.S.A.). The protein was attached to the porous glass using a thionyl chloride-activated, succinamidopropyl-glass with a 1:100 dilution of succinamidopropyl sites with unreactive glycerolpropyl sites, as described by DuVal *et al.*[16]. Immobilization was performed by recirculation of 6 ml of 0.5 mg/ml solution of NP-II in phosphate buffer (pH 7.0) through 2.5 g of the activated glass. Following immobilization, the beads were thoroughly washed in the fixed-bed reactor with 1 *M* sodium chloride (pH 7.0) and 0.4 *M* sodium

acetate (pH 5.7) before storage in the pH 7.0 phosphate buffer. All solutions used in the immobilization reaction were filter-sterilized using 0.2-μm Millipore filters.

For immobilization of NP-II on NPG, the beads were acid-cleaned and allowed to react with 20% (v/v) aqueous aminopropyltriethoxysilane, adjusted to pH 8, at 70°C for 3 h. After the excess liquid has been decanted, further polymerization was induced by incubation at 100°C overnight. The aminopropyl-glass beads were succinylated by incubation at 4°C in 20 ml of distilled water, containing 15 g of succinic anhydride, while the pH was maintained at 6 with 40% sodium hydroxide. NP-II, purified as described above but with the preparative HPLC fractionation omitted, was covalently immobilized by using a simultaneous activation/immobilization procedure[17]. A 6-ml volume of beads was allowed to react with 6 ml of a 1 mg/ml NP-II solution in 0.05 *M* sodium chloride (pH 7.0) and 0.01 *M* 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide to activate the surface carboxyl groups. The immobilization was performed in a fixed-bed reactor after which the beads were exhaustively washed with 1 *M* sodium chloride and 0.4 *M* ammonium acetate (pH 5.7). All solutions were filter-sterilized, as described above.

Analytical high-performance affinity chromatography

The immobilized NP-II glass beads were packed in a 0.46 cm × 25 cm stainless-steel HPLC column (25 × 0.46 cm I.D.) by introducing a slurry into the top of the column and packing with vibration and a flow-rate of 0.8 ml/min. For chromatography, the columns were connected to a Varian 5000 HPLC system with the exit tubing leading to a fraction collector equipped for collection directly into scintillation vials. In the case of zonal elution chromatography, a 200-μl sample was injected with a standard sample loop, and two- or three-drop fractions were collected. Zero time was taken as the time when half the sample had entered the column. For large-zone chromatography, a 3-ml or 5-ml loop was constructed from polyethylene tubing for introduction of the sample and three-drop fractions were collected. In both cases, all solutions were degassed under vacuum and filter-sterilized prior to use. When not in use, the columns were stored in the cold-room with buffer containing 0.02% sodium azide.

RESULTS AND DISCUSSION

Micropurification of [^{3}H]arginine vasopressin by chromatography on NP-II immobilized on porous glass

Commercially obtained [^{3}H]Arg^8-vasopressin ([^{3}H]AVP) was found to contain a labelled component that does not bind to neurophysin. The elution profile for [^{3}H]AVP and resulting purification of microscale amounts is illustrated in Fig. 1. Roughly 0.23 nmol (10 μCi) of [^{3}H]AVP was applied and 0.07 nmol was obtained, in Fractions 38–60, which was homogeneous when chromatographed immediately on [NP-II] NPG. However, after storage for several weeks, labeled material again appeared in the void volume of HPAC elutions (see below); the amount of radioactivity in the void volume increased with age. In any case, microscale amounts of [^{3}H]AVP were readily purified by NP-II porous-bead chromatography, the complete elution requiring less than 80 min.

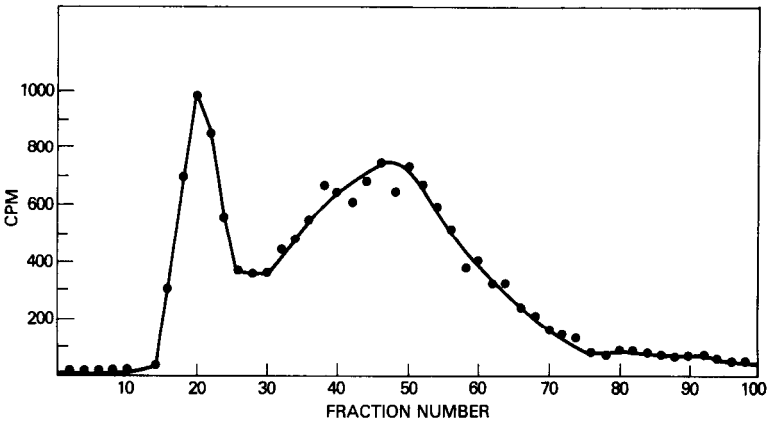

Fig. 1. Elution profile for commercial [^{3}H]Arg-vasopressin obtained by chromatography with NP-II immobilized on porous glass (CPG-2000). The column (25 × 0.46 cm I.D.) was equilibrated and eluted with 0.4 *M* sodium acetate (pH 5.7) at room temperature using a flow-rate of 0.2 ml/min. A 200-μl sample containing *ca.* 0.23 nmol (10 μCi) of [^{3}H]AVP was injected and 1-min fractions were collected; 20-μl portions were removed from even-numbered fractions and added to scintillation fluid for counting.

Zonal elution chromatography with NP-II immobilized on non-porous glass beads

The chromatography of both AVP and NP-II was examined with [NP-II]NPG. In all of these experiments, the same concentration of labeled compound was used, whereas the total concentration was varied by addition of unlabeled AVP or NP-II.

The zonal elution profile for [^{3}H]AVP at a total AVP concentration of 0.155 μM is shown in Fig. 2. The hormone sample applied contained a non-binding [^{3}H]AVP fraction. We found it convenient in these studies to use the non-binding

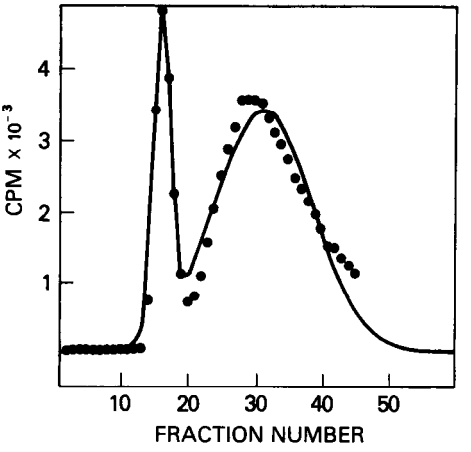

Fig. 2. Zonal elution profile for [^{3}H]Arg-vasopressin at a total AVP concentration of 0.155 μM, obtained by chromatography with NP-II immobilized on non-porous glass beads. The column (25 × 0.46 cm I.D.) was equilibrated and eluted with 0.4 *M* ammonium acetate (pH 5.7) at room temperature and a flow-rate of 0.2 ml/min. A 200-μl sample containing 0.016 nmol of [^{3}H]AVP and 0.015 nmol of AVP was injected and three-drop fractions (0.135 ml) were collected, starting at 0.5 min. The fractions were collected directly in scintillation vials for counting. The line represents a computer-fit to Gaussian equations.

material as an internal marker for the unretarded elution volume. The elution position was obtained by computer-fitting to Gaussian curves; a typical fit is illustrated in Fig. 2. Evaluation of numerous elutions indicated that the position given by the Gaussian fit differed from an estimate of that for the maximum ordinate by a fraction or less since the amount of tailing in these elutions was very small. Data given in Table I indicate the precision of this method, *e.g.*, less than 2% S.D. The fact that the elution position of the non-binding [^{3}H]AVP fraction is independent of total AVP concentration, and that it is the same as that found for [^{3}H]ribonuclease, substantiates the conclusion that this material is unretarded during chromatography.

TABLE I

DETERMINATION OF THE UNRETARDED ELUTION VOLUME, V_0, FOR THE COLUMN CONTAINING NP-II IMMOBILIZED ON NON-POROUS GLASS BEADS

Method	V_0 *(two-drop fractions)**	V_0 *(ml)***
Zonal elution:		
Homogeneous non-binding [^{3}H]AVP	23.8	2.142
[^{3}H]Ribonuclease	23.4	2.107
Non-binding [^{3}H]AVP included as	24.2	2.179
an internal marker	24.4	2.201
	24.3	2.187
	24.2	2.180
	23.9	2.148
Frontal analysis of large-zone chromatography:		
Homogeneous non-binding [^{3}H]AVP	24.8	2.232
Mean ± S.D.	24.13 ± 0.42	2.172 ± 0.039

* Determined by computer-fitting to Gaussian curves as illustrated by the fit given in Fig. 2.

** Based on a fraction volume of 90 μl (two drops) determined by weight analysis of larger (*e.g.* 80-drop) fractions.

Eqn. 7 indicates that unless the concentration of soluble ligand is small relative to the amount of immobilized interactant, a significant concentration dependence of elution volume is to be expected. The concentration dependence for [^{3}H]AVP was examined, the data obtained being plotted according to eqn. 7 in Fig. 3. Since the mobile phase concentration of AVP is not constant and is unknown, $1/(V - V_0)$ was plotted as a function of the initial concentration, $[AVP]^0$. The results in Fig. 3 indicate that the relationship is linear in the concentration range studied. Extrapolation of $K_{M/L}/V_0[M]_T$ to $[L] = 0$ permits $K_{M/L}$ to be obtained by using the experimentally determined value for $V_0[M]_T$ (total amount of NP-II). Amino acid analysis of the column contents at the conclusion of these experiments indicated that 6.3 nmol of NP-II were immobilized in the total column volume. This result yields a value of *ca.* 3 μM for the dissociation constant $K_{M/L}$, compared to a value of *ca.* 7 μM for dissociation of lysine vasopressin from soluble NP-II[10]. It should be noted that these calculations assume all of the immobilized NP-II is active; therefore, if some of the protein is inactive, the actual dissociation constant would be lower than that obtained chromatographically.

A zonal elution profile for [^{125}I]NP-II, at an initial zonal concentration of

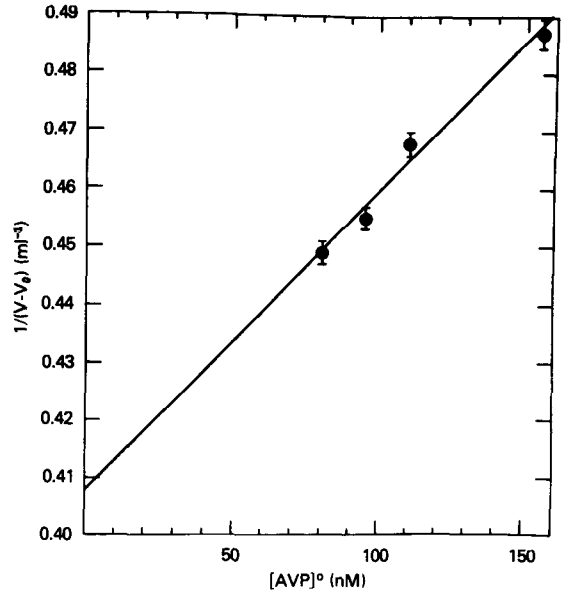

Fig. 3. Concentration dependence of the elution parameters obtained by zonal elution chromatography of [^{3}H]Arg vasopressin with NP-II immobilized on non-porous glass beads. The data are plotted according to eqn. 7 as a function of the concentration in the applied sample. The parameters were obtained from experiments as described in Fig. 2. The data fit the line given by $y = 0.00051x$, correlation coefficient = 0.991. [AVP]° is the initial concentration of [^{3}H]AVP in the zone applied to the affinity column.

NP-II of 75 μM, is illustrated in Fig. 4. As for the case of AVP chromatography, the elution position of NP-II is expected to exhibit a concentration dependence. Moreover, according to eqn. 14, the dependence is unlikely to be linear unless $[P]_T \ll K_{P/P}$.

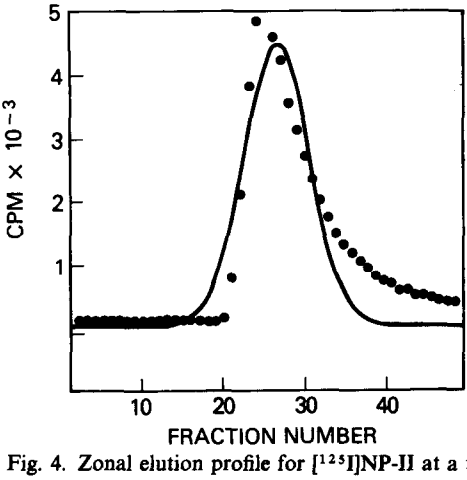

Fig. 4. Zonal elution profile for [^{125}I]NP-II at a total NP-II concentration of 75 μM obtained by chromatography with NP-II immobilized on non-porous glass beads. The column (25 × 0.46 cm I.D.) was equilibrated and eluted with 0.4 M ammonium acetate (pH 5.7) at room temperature and a flow-rate of 0.2 ml/min. A 200-μl sample containing 7.5 pmol of [^{125}I]NP-II and 0.15 nmol of NP-II was injected and two-drop fractions (0.090 ml) were collected directly into scintillation vials, starting at 0.5 min. The line represents a computer-fit to a Gaussian curve.

The results obtained by computer-fitting of gaussian curves to elution profiles for a series of concentrations are shown in Fig. 5 as a function of the initial NP-II concentration, $[NP\text{-}II]^0$. As predicted, the variation of $1/(V - V_0)$ does not appear to be linear over a wide concentration range. In the region where $[P]_T \ll K_{P/P}$ ($K_{P/P}$ *ca.* 140 μM[10]), the data extrapolated to $[P]_T = 0$ give a value for $K_{M/P}/V_0[M]_T$. Using 6.3 nmol of NP-II as the amount of immobilized NP-II in the column gives a value of *ca.* 10 μM for $K_{M/P}$. This value is somewhat lower than the value of 120 μM obtained for soluble NP-I by equilibrium ultracentrifugation[18], but it agrees with a recent estimate of the dissociation constant (14 μM) for NP-II obtained by chromatography on Np-II-Sepharose[19].

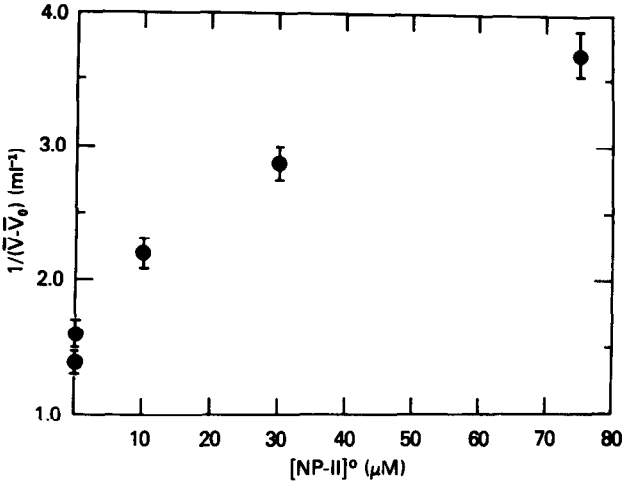

Fig. 5. Concentration dependence of the elution parameters obtained by zonal elution chromatography of [^{125}I]NP-II with NP-II immobilized on non-porous glass beads as the stationary phase. The data are plotted, as suggested by eqn. 14, as a function of the concentration of NP-II in the applied sample. The parameters were obtained from experiments as described in Fig. 4. $[NP\text{-}II]^0$ is the initial concentration of NP-II in the zone applied to the affinity column.

The above data emphasize the ease, speed, and accuracy with which the analytical HPAC method can be used to measure biospecific affinities of a molecule for a set of interactants. Moreover, elution times of less than 30 min could be achieved with sample sizes of mobile interactant as small as 16 ng of [^{3}H]AVP and 80 ng of [^{125}I]NP-II.

In addition to the equilibrium properties of the interaction, the kinetic off-rate constant for dissociation of the solute molecule from the immobilized phase can be determined from the degree of zone spreading[7–9]. For the case of impenetrable beads in the absence of soluble competing ligand, Hethcote and DeLisi have shown that (see ref. 8, p. 134):

$$k_{-3} = \frac{2F(V - V_0)}{W_e} \tag{16}$$

where F is the flow-rate and W_e is the variance of the elution profile. Using a different approach, Denizot and DeLaage[7] showed that

$$k_{-3} = \frac{2E^2(t_0)\,[E(t') - E(t_0)]}{\sigma'^2\,E^2(t_0) - \sigma_0^2\,E^2(t')} \tag{17}$$

where $E(t')$ and σ' and $E(t_0)$ and σ_0 are the elution times and standard deviations for interacting and unretarded molecules, respectively. Using the variance and peak widths obtained from computer-fitting of gaussian curves to elution profiles such as those given in Figs. 2 and 4, we have calculated the off-rate constants for dissociation of AVP and NP-II by both methods. Values obtained for AVP dissociation were 0.0070 sec^{-1} for the Hethcote and DeLisi method and 0.0069 sec^{-1} for the Denizot and DeLaage method. Thus, the two methods give identical results for non-porous beads, as predicted by Hethcote and DeLisi[9]. Similarly, the two methods gave off-rate constants of *ca.* 0.008 sec^{-1} for dissociation of NP-II. The meaning of these chromatographically obtained rate constants is currently being evaluated.

Large-zone chromatography of AVP with NP-II immobilized on non-porous glass beads

Using homogeneous, chromatographically purified [^{3}H]AVP, elution volumes can be determined by frontal analysis of a large-zone elution profile[4–6]. It can be shown that the equations previously derived apply (*e.g.*, see Nichol *et al.*[4] with the elution volume given by the first moment of the elution boundary. Thus, eqn. 7 can be written as

$$\frac{1}{V - V_0} = \frac{K_{M/L}}{V_0[M]_T} + \frac{[L]}{V_0[M]_T} \tag{18}$$

which corresponds to the more statistically accurate Hanes plot form of the Michaelis–Menten equation for enzyme kinetics. In the case of large-zone chromatography, [L] is constant if a plateau region is achieved in the elution and is equal to the initial concentration of mobile interactant applied.

The elution profile is given in Fig. 6 for a large-zone experiment in which 5 ml of 0.91 μM AVP were applied to the non-porous bead column. For such profiles, the first moment of the boundary curve, $\bar{V}$, was obtained from the relationship

$$\bar{V} = na - a \sum_{i=1}^{n} \mathrm{cpm}_i/\mathrm{cpm}_0 \tag{19}$$

where n is a fraction in the plateau region, a is the fraction volume, and cpm_i and cpm_0 are the counts per minute in the ith fraction and the applied solution, respectively[6]. Data in Table I show that the first moment for the elution of an unretarded molecule is an excellent agreement with V_0 determined from zonal elution experiments. Data, obtained in large-zone experiments similar to that in Fig. 6 and at varying [AVP], are plotted according to eqn. 18 in Fig. 7. The results indicate that

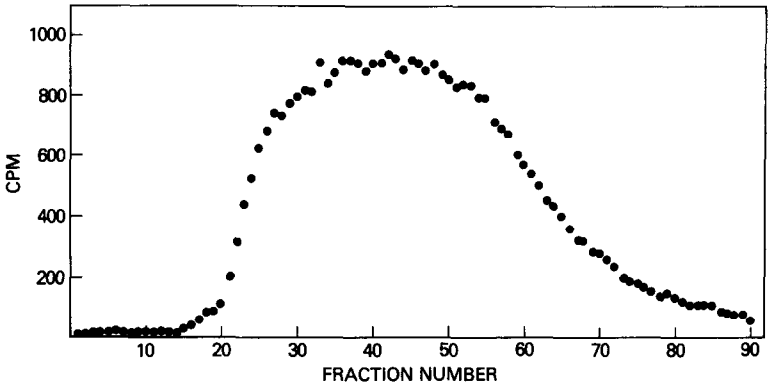

Fig. 6. Large-zone elution profile of [^{3}H]Arg-vasopressin at a total AVP concentration of 0.91 μM obtained by chromatography with NP-II immobilized on non-porous beads. The column was equilibrated and eluted with 0.4 M ammoniumm acetate (pH 5.7) at room temperature using a flow-rate of 0.2 ml/min. A 5-ml sample containing 4.54 nmol AVP and affinity-purified [^{3}H]AVP was injected and three-drop fractions (0.135 ml) were collected, starting at 0.5 min. This 5-ml sample contained 910 cpm per 135-μl aliquot, indicating that a plateau concentration equal to the original sample concentration was achieved in the elution. The fractions were collected directly into scintillation vials for counting.

a linear relationship is obtained, as theoretically predicted. Furthermore, a value of *ca.* 2 μM is obtained, for the dissociation constant of AVP, $K_{M/L}$, when the resulting intercept and $V_0[M]_T$ = 6.3 nmol are used. The value of $K_{M/L}$ obtained by large/frontal analysis is in reasonable agreement with that obtained by zonal elution analyses.

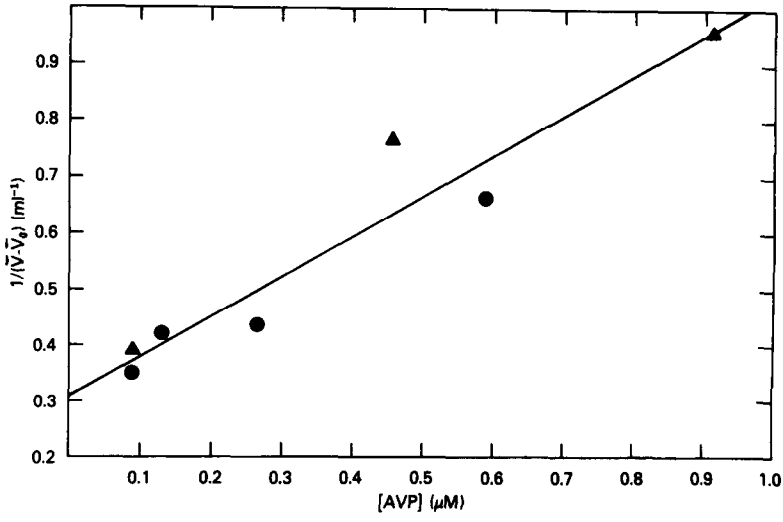

Fig. 7. Concentration dependence of the elution parameters using the first moment of the frontal boundary obtained by large zone chromatography of [^{3}H]AVP with NP-II immobilized on non-porous glass beads. The data are plotted according to eqn. 18. Data were obtained by injection of either a 3-ml sample (●) or a 5-ml sample (▲). The line is given by the equation $y = 0.31 + 0.72x$ (correlation coefficient 0.957).

CONCLUSION

Using the neurophysin–hormone interactions as a model system, this study has shown the utility of analytical affinity chromatography with non-porous beads for evaluation of thermodynamic and possibly kinetic properties of biospecific interactions. With zonal elution chromatography on such beads, these characteristics can be determined rapidly with very small amounts of material. Analytical affinity chromatography can be useful for accurate determination of the relative affinities of a molecule for a series of interactants. By incorporation of this technique into HPLC methodology, the precision of the measurements is likely to be greatly increased and the range of useful matrices broadened. By development of smaller diameter beads, it is anticipated that smaller columns, shorter analysis times, and greater precision in analysis of biomolecular binding properties will result. The application of HPLC to analytical affinity chromatography makes it possible for HPLC to move beyond the microscale isolation and chemical structure determination of biological molecules to the microscale characterization of their functional binding properties.

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REFERENCES

1 I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, Orlando, FL, 1983.
2 B. M. Dunn and I. M. Chaiken, *Proc. Nat. Acad. Sci. U.S.*, 71 (1984) 2382.
3 I. M. Chaiken, *Anal. Biochem.*, 97 (1979) 1.
4 L. W. Nichol, A. G. Ogston, D. J. Winzor and W. H. Sawyer, *Biochem. J.*, 143 (1974) 435.
5 K. Kasai and S. Ishii, *J. Biochem.*, 84 (1978) 1061.
6 K. Kasai and S. Ishii, *J. Biochem.*, 84 (1978) 1051.
7 F. C. Denizot and M. A. DeLaage, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 4840.
8 H. W. Hethcote and C. DeLisi, *J. Chromatogr.*, 248 (1983) 183.
9 H. W. Hethcote and C. DeLisi, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, Orlando, FL, 1983, p. 119.
10 I. M. Chaiken, D. M. Abercrombie, T. Kanmera and R. P. Sequeira, in M. T. W. Hearn (Editor), *Peptide and Protein Reviews*, Vol. 1, Marcel Dekker, New York, 1983, p. 139.
11 S. Angal and I. M. Chaiken, *Biochemistry*, 21 (1982) 1574.
12 D. M. Abercrombie, T. Kanmera, S. Angal, H. Tamaoki and I. M. Chaiken, *Int. J. Peptide Protein Res.*, 24 (1984) 218.
13 E. A. Fischer, J. G. Curd and I. M. Chaiken, *Immunochemistry*, 14 (1977) 595.
14 I. M. Chaiken, T. Miller, R. P. Sequeira and T. Kanmera, *Anal. Biochem.*, 143 (1984) 215.
15 I. M. Chaiken, *Anal. Biochem.*, 97 (1979) 302.
16 G. E. DuVal, H. E. Swaisgood and H. R. Horton, *J. Appl. Biochem.*, 6 (1984) 240.
17 V. G. Janolino and H. E. Swaisgood, *Biotechnol. Bioeng.*, 24 (1982) 1069.
18 P. Nicolas, G. Batelier, M. Rholam and P. Cohen, *Biochemistry*, 19 (1980) 3565.
19 I. M. Chaiken, H. Tamaoki, M. J. Brownstein and H. Gainer, *FEBS Lett.*, 164 (1983) 361.