A New Assay for Molecular Recognition

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Summary: Only specifically binding species appear as off-diagonal spots using 2D TLC with ligand affixed on the second of two similar stationary phases.

Molecular recognition by small synthetic receptors has become a field of intense activity in recent years.[1] Our own efforts in this area are directed toward peptide-like molecules which are rapidly synthesized and whose structures may easily be perturbed slightly for optimization of binding selectivity. In connection with this work, we needed a method of rapidly screening many molecules for binding to a specific ligand. We report the development of such an assay, which promises to identify those components of a mixture that specifically bind to the desired ligand. Our method is a novel application of two-dimensional thin-layer chromatography[2] that avoids several of the disadvantages of the usual affinity chromatography modes for this purpose. Typically, a crude mixture containing the potential binding site is applied to the lower left of the square plate shown in Figure 1 and eluted in the vertical direction on the first of two domains of equal polarity. Now a second elution with the same eluant, perpendicular to the first, carries the analyte onto the second domain, which is coated with ligand. A specific association[3] between the analyte and ligand retards only the second migration and is thus recognizable as an off-diagonal deviation of the spot. In this way retention by normal adsorption modes may be distinguished from a more specific association. One-dimensional TLC has been elegantly used to measure ligand binding semiquantitatively[4] but is less applicable to screening of binding sites as it must be used with single standardized, purified materials to be interpretable.

We chose to demonstrate our method using vancomycin (Figure 2), a molecule which binds acylated D-alanyl-D-alanines, but not the analogous L-alanyl compounds, in aqueous solution with dissociation constants in the range of 10^{-3}–10^{-6} M.[5,6] Vancomycin thus has selective binding properties similar to those we want to develop.

Preparation of the Ligands. Treatment of a suspension of D-alanyl-D-alanine (101 mg, 0.63 mmol) in CH_2Cl_2 (5 mL) with trimethylsilyl chloride (0.5 mL, 3.9 mmol) and pyridine (1.0 mL, 12.4 mmol) gradually caused silylation and dissolution of the dipeptide. After 8 h, oleoyl chloride (175 mg, 0.60 mmol) was added, and the mixture was stirred at 23 °C until acid chloride was consumed. The reaction mixture was diluted with ethyl acetate, washed six times with 0.1 N HCl and then saturated NaCl, and dried over $MgSO_4$, and the solvent was removed at reduced pressure to yield oleoyl-D-alanyl-D-alanine (DD-OAA, 231 mg, 94%), contaminated with a trace of oleic acid. The oleic acid could be easily removed by silica gel chromatography (1:1:0.2 CCl_4–EtOAc–*i*-PrOH) but was usually left in as the compound was further diluted with oleic acid as used. No evidence of epimeric oleoylalanylalanines was observed in the 300-MHz ^1H NMR spectrum; 5% would have been readily detected (standard prepared analogously). This convenient in situ carboxyl protection[7,8] avoided the frequently encountered[9] racemization, incomplete coupling, and oligomerization observed under heterogeneous conditions.

Preparation of Stationary Phases. The first domain must have polarity similar to that of the second domain with its bound ligand. For this demonstration, we have used the enantiomer of the ligand to ensure that any difference between the two phases must be the result of a specific interaction, but we have found that oleic acid, for example, can be substituted for the oleoyl-L-analyl-L-alanine without affecting the separation. These ligands were affixed to their respective domains on a C-18 thin-layer chromatography plate (Whatman) by the simple expedient of eluting the plate with a solution containing 10 mM DD-OAA/40 mM oleic acid in a solvent in which it has an R_f of 1 (30% $CHCl_3$, 1% pyridine, in acetone). Subsequent use of the plate was restricted to solvents in which OAA has an R_f of 0 on C-18 silica—almost any partially aqueous solvent. This impregnation method affords even distribution and allows efficient use of ligand.

Chromatography. A mixed sample containing (1–5 × 10^{-10} mol each of histidine methyl ester, vancomycin, methionine methyl ester, homocysteine thiolactone, and alanine, was applied to the narrow L,L-OAA-coated domain of a square TLC plate, dried in vacuo, and eluted on the L,L-OAA domain with 7:3 H_2O–CH_3CN buffered to pH 5 with 50 mM pyridine/50 mM acetic acid. Removal of eluant in vacuo, coating of the large domain by elution of D,D-OAA as described above, drying in vacuo, and elution with the same aqueous buffer onto the large domain in a direction perpendicular to the first completed the process. Ninhydrin visualization of the spots clearly shows all components on a diagonal line of slope 1, except for the vancomycin spot which remains near the edge of the D,D-OAA domain.[10] All compounds we have investigated have migrated to the diagonal, with the exception of ristocetin, which has binding properties similar to those of vancomycin.

Conclusions. Advantages of the technique include its simplicity and low cost, making it conducive to screening large numbers of compounds. In contrast to a highly retained species in a one-dimensional affinity analysis, interpretation of an off-diagonal spot is fairly unambiguous as a specific interaction, as long as the possibility of de-

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(3) By "specific association" we designate those interactions between the two molecules of interest, in contrast to general modes of association with a chromatographic support which do not *require* the presence of the specific ligand molecule, even though they may involve the same forces of hydrophobic interaction, hydrogen bonding, or ion pairing.

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(10) R_f values observed (first direction/second direction), ± represents size of spot on a single plate, not replicant variability: vancomycin (0.21 ± 0.06/0.02 ± 0.01), histidine methyl ester (0.04 ± 0.04/0.04 ± 0.04), methionine methyl ester (0.33 ± 0.04/0.30 ± 0.06), homocysteine thiolactone (0.40 ± 0.05/0.38 ± 0.07), alanine (0.86 ± 0.05/0.82 ± 0.08).

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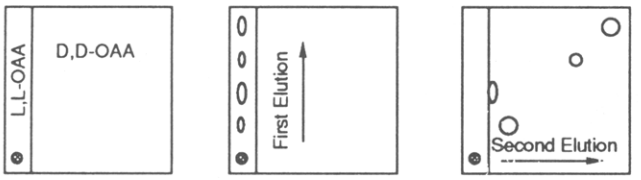

Figure 1.

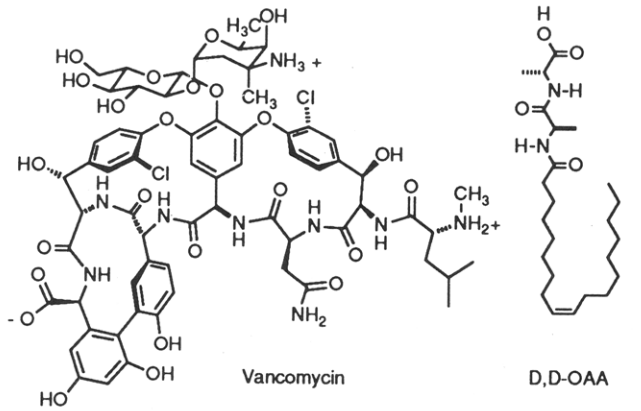

Figure 2.

composition of a component to a slowly migrating species is excluded by two-directional TLC on a single chromatographic phase. High-resolution chromatography and quantitative measurement are not required, as they are for a recent procedure[11] which allows simultaneous measurement of binding constants to several ligands. Even if the resolution of the chromatographic separation is insufficient for separation of all components in our procedure, an off-diagonal deviation is evidence of a specific interaction deserving more careful study. Special techniques[12,13] allow high-resolution in the two-dimensional mode.

The major disadvantage of the method is that solvent in which the binding is to take place must be determined to some extent by the chromatographic requirements. Solvent can have a large effect on binding affinities.[14-16] This is not so limiting as it might seem because many separation modes are compatible with this basic idea (normal- and reverse-phase separations on a variety of stationary supports, ion exchange, size exclusion, electrophoresis) and even within the purview of this implementation, the stationary-phase polarity may be readily modified by admixture of the OAA with other amphiphiles to vary R_f with a given eluant.

The method is not intended to supplant current methods[1,17] for determining binding affinities and geometries. It is intended as a first screen to identify, from a large group, those molecules on which to focus study. For this purpose it seems well suited.

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