Screening Mixtures by Affinity NMR

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The synthesis and screening of mixtures of compounds offers increased efficiency and throughput in biological testing as compared to making and testing single compounds. Mixtures of compounds can be prepared directly by synthesis or are made indirectly by combination of individual compounds. Screening compounds as mixtures requires a method to ultimately determine which molecule in the mixture is responsible for the desired biological effect.[1] A potential problem with many biological systems is that the activity observed can be the result of the sum total of weak, nonspecific binding of several mixture components in the assay. Several approaches to identify interesting components in a mixture have been described.[2] Methods which identify active components of mixtures directly could eliminate "false positives" and greatly reduce the effort required to analyze mixtures. One such method under investigation is affinity mass spectrometry.[3]

An NMR method for identifying active compounds from a library of low molecular weight ligands using ^{15}N labeled proteins has been recently reported.[4] The binding of a ligand is determined by chemical shift changes for the ^{15}N or ^{1}H NMR signals in the protein. This interesting method, which at present is limited to small, labeled biomolecular receptors, has promise to contribute a new approach to the drug discovery process.

Recently the use of pulsed field gradient (PFG) technology to obtain diffusion coefficients of molecules has been demonstrated as a useful technique for mixture analysis.[5] Size- or diffusion-resolved NMR assigns the resonances on the basis of the translational diffusion coefficient for each proton (or other spin) in the molecule. Since this value is an intrinsic property of a molecule as a whole, it can be used to distinguish resonances arising from different molecules.[6]

A new method, termed affinity NMR, has recently shown that the diffusion coefficient of a small molecule binding with a "receptor" in solution is significantly different from the small compound alone observed under PFG conditions.[7] Thus, molecules that are interacting with the "receptor" can be distinguished from noninteracting molecules in a manner reminiscent of physical separation of mixtures by affinity chromatography. Diffusion encoded spectroscopy (DECODES), which involves the use of PFG and TOCSY, simplifies the identification

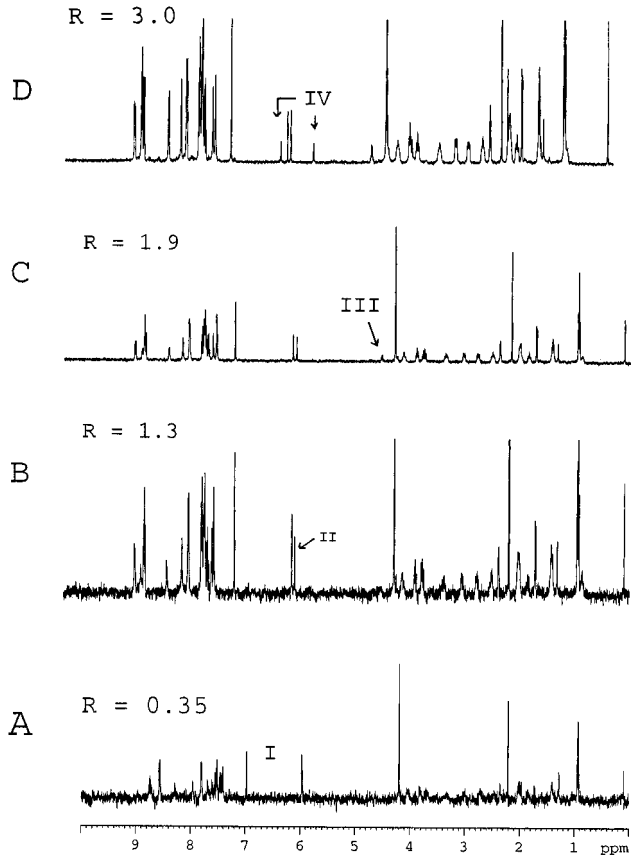

Figure 1. Titration of the 4-carboxylic acid mixture with hydoquinine 9-phenanthyl ether. R is the ratio of hydroquinine to the total equimolar mixture of acids. Key resonances arising from compounds **I**−**IV** are shown: **I,** dichloroacetic acid; **II**, *s*-(+)-*O*-acetylmandelic acid; **III**, 2-chloropropionic acid; and **IV**, methacrylic acid.

Table 1. pK_a of the Carboxylic Acids and Their Observed Binding Constants with Hydroquinine 9-Phenanthryl Ether

	pK_a	$K_d \times 10^{-5}$
I, dichloroacetic acid	1.26	0.9
II, s-(+)-*O*-acetylmandelic acid	2.09	1.3
III, 2-chloropropionic acid	2.84	5.0
IV, methacrylic acid	4.46	45

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of the interacting molecule.[8] This methodology has been applied successfully to a model system using hydroquinine 9-phenanthryl ether as a model receptor in a nine-component mixture containing six inert materials and two carboxylic acid ligands.[7]

A potential advantage of the PFG diffusion NMR method over the chemical shift method is that binding is detected by the observation of the ligand NMR spectrum and not by changes in the receptor NMR spectrum. This should allow the experiment to be "tuned" to the binding affinity of the ligand by changing the relative receptor concentration. We tested this premise by making a mixture of four carboxylic acids and using the hydroquinine 9-phenanthryl ether as the receptor. The binding constants for the ligands, and the pK_a's are given in Table 1.[9]

The NMR spectral tuning is obtained by changing the concentration of the hydroquinine 9-phenanthryl ether relative to the mixture of carboxylic acids. As can be seen in Figure 1A, when the concentration of hydroquinine 9-phenanthryl ether is only 0.35 equiv, only the strongest binding carboxylic acid **I**, dichloroacetic acid, is observed in the NMR spectrum.

As the concentration of hydroquinine 9-phenanthryl ether is increased, the other carboxylic acids, **II**−**IV,** sequentially begin to appear in their order of binding affinity. All four carboxylic acids are observable in Figure 1D. This demonstrates that the system can be tuned to a desired sensitivity level and that mixtures of compounds containing only a few components can be screened to select components which bind at a desired level of affinity. Alternatively, mixture components could be directly rank ordered by binding affinity. The application of this technique to biologically relevant systems is in progress.

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(9) All NMR experiments were carried out at room temperature on a Bruker DMX-500 NMR spectrometer equipped with a Acustar II pulse field gradient accessory. PFG-NMR spectra of the carboxylic acid mixture, containing 10 mM of each component, were acquired using LED pulse sequence.[6] The data were collected using 1.75 ms gradient pulses with a 39.4 G/cm gradient strength and a 0.15 s delay between the two gradient pulses. All compounds are commercially available and were used without further purification. The binding affinities of the carboxylic acids with hydroquinine 9-phenanthryl ether were obtained by NMR titration experiments. In each experiment, 10 mM quinine was titrated with the desired carboxylic acid and the chemical shifts of hydroquinine 9-phenanthryl ether were monitored at each titration point. The chemical shift verses concentration data was fitted with a nonlinear least-squares routine to calculate the dissociation constant shown in Table 1.