Pergamon

Tetrahedron 55 (1999) 11653–11667

TETRAHEDRON

Iterative size-exclusion chromatography coupled with liquid chromatographic mass spectrometry to enrich and identify tight-binding ligands from complex mixtures

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Received 23 June 1998; accepted 13 November 1998

Abstract

A method is described for the enrichment of tight-binding ligands from complex mixtures. The mixture is equilibrated with a protein or receptor, and protein:ligand complexes are separated from unbound ligands by size-exclusion. If the resulting mixture is allowed to re-establish its equilibrium and then passed again through the size-exclusion column, significant enrichment of tight-binding ligands is realized, even in cases where the weak-binders are present in large excess. Ligands are identified by liquid chromatography and mass spectrometry following the size-exclusion step. The process can be repeated multiple times to further enrich ligands. Analysis of pools of ligands generated by combinatorial chemistry is an obvious application of the technique.

Keywords Chromatography, combinatorial chemistry, mass spectrometry, proteins

1. Introduction

The development and use of combinatorial chemistry has radically changed the way diverse chemical compounds are synthesized as potential drug candidates [1-3]. A new drug discovery paradigm of screening hundreds of thousands of small molecules against a biological target has become the norm in many pharmaceutical companies. One screening approach involves assaying compounds as mixtures, and then re-synthesizing and re-assaying smaller and smaller sub-pools in an iterative fashion, until the active component/s is/are identified. While

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0040-4020/99/$ - see front matter
PII: S0040-4020(99)00685-7

this approach is cost-effective from an assay standpoint, the synthetic deconvolution to determine active components is time- and labor-consuming. More efficient ways to distinguish molecules with a desired biological activity from inactive compounds in the library would be desirable.

Bioaffinity selection has been proposed as a means of achieving the separation of active compounds from inactive. In its simplest form, this might involve attaching the target protein to a column and pouring a mixture of ligands through it, to see which ones are 'selected' by adsorbing to the column [4-6]. Alternatively, the target protein and the ligand mixture can be incubated together, and protein:ligand complexes can be separated by a fast size-exclusion chromatography (SEC) column, such as a "spin column". Spin columns based on SEC have been widely used in receptor binding assays. Ligands that bind to the receptor move through the column with the protein, while unbound ligands move more slowly. Usually a binding assay measures the competition of the test ligand with a radioactive ligand of known binding affinity. This technology has been incorporated by several groups into a method of selecting tight-binding ligands from mixtures, with the additional step of liquid chromatographic mass spectrometry (LC/MS) as a means of identifying the ligands [7]. The complexes of protein and tight-binding ligands elute first from the SEC column, and that fraction is analyzed by LC/MS. The harsh conditions of the HPLC separation dissociate the complexes, leading to a separation of protein and ligand(s), both of which can be detected by the mass spectrometer [7]. More recently, methods have been reported that use ultrafiltration coupled with liquid chromatography-mass spectrometry (LC/MS) for screening libraries [8-9]. As with the spin-column method, the general strategy is to use a receptor or target molecule to isolate the active components as a receptor-ligand complex from a library typically containing 20-30 components, with the aid of an ultrafiltration device. As before, the active compounds are dissociated from the complex, separated from the receptor, and subsequently identified by LC/MS.

While these methods are very useful when the mixture of ligands is approximately equimolar, if the tight-binding ligands are present in very low concentration relative to weaker-binding ligands, the method is less successful. The latter situation might occur, for example, when a tight-binding component is part of a very large library of compounds (e.g. a combinatorial library) or when the ligand is a minor component in, for example, a tissue extract in which there might be much higher concentrations of weaker-binding ligands. In a biological assay, this same difficulty manifests itself in an inability to differentiate between the response of a single tight-binding ligand and an equivalent response caused by many weak-binding ligands. In the former case, time spent in deconvolution of the library would be useful; in the latter case it would probably not be. The fundamental difficulty is that there is little

discrimination in the method between strong- and weak-binding ligands, beyond some initial threshold of K_D.

The SEC separation is based on the fact that once equilibrium is established between the protein and all ligands in the solution, the time required for separation is relatively fast, and will be over before the perturbed equilibrium, resulting from the separation of bound and unbound ligands, can be re-established. If this were not the case, ligands would be dissociating from the protein during the course of the separation and would not elute with the protein, but at some later time. We reasoned that if the material eluting from one of these SEC columns was allowed to re-establish its equilibrium, ligands would dissociate based on the equilibrium constant of the protein:ligand complex. Weak-binding ligands would dissociate to a greater extent than tight-binding ligands, however, if a second stage of SEC was conducted after equilibrium had re-established, a relative enrichment of the tight-binders would result. In principle, this process could be repeated a number of times, each time with a steady increase in the relative amount of tight-binding ligand until one could isolate essentially pure tight-binding ligand, even from a very complex sample.

Both theoretical calculations and experimental data suggest this to be the case; and, to our surprise, two previously undetected tight-binding ligands were identified using the technique.

2. Theory

If a receptor (R) is incubated with a mixture of two ligands (W and T, for weak- and tight-binder, respectively), an equilibrium will be established between the bound and unbound forms of each ligand with receptor. If K_{weak} and K_{tight} are the binding constants of the weak and tight-binding ligand, respectively, the ratio of receptor complexed to weak binder (RW) and tight binder (RT) can be expressed:

$$[RT]/[RW] = K_{weak}\,[T]/K_{tight}\,[W] \qquad \text{eq. (1)}$$

It is only the bound forms RW and RT that are collected after the spin column. It follows from eq. 1 that the enrichment of the tight-binding ligand is dependent on the balance of the ratio of the binding constants of the weak- and tight-binding ligands and their relative concentrations.

If the receptor:ligand complexes are allowed to re-equilibrate after the spin column, some fraction of each ligand will dissociate to unbound ligand. The total amount of ligand (bound and unbound) after the first spin column cannot exceed the amount of receptor. The weak-

binding ligand will dissociate to a greater extent than the tight-binding ligand. If the solution is passed through the spin column again, separating the bound and unbound forms of the ligands, a further enrichment of the tight-binding ligand is realized (depicted in Fig. 1). This process can, in principle, be repeated iteratively to obtain any desired enrichment of tight-binding ligand. In practice, however, some losses of protein at each step and dilution with each pass through the spin column lead to a limit of diminishing returns.

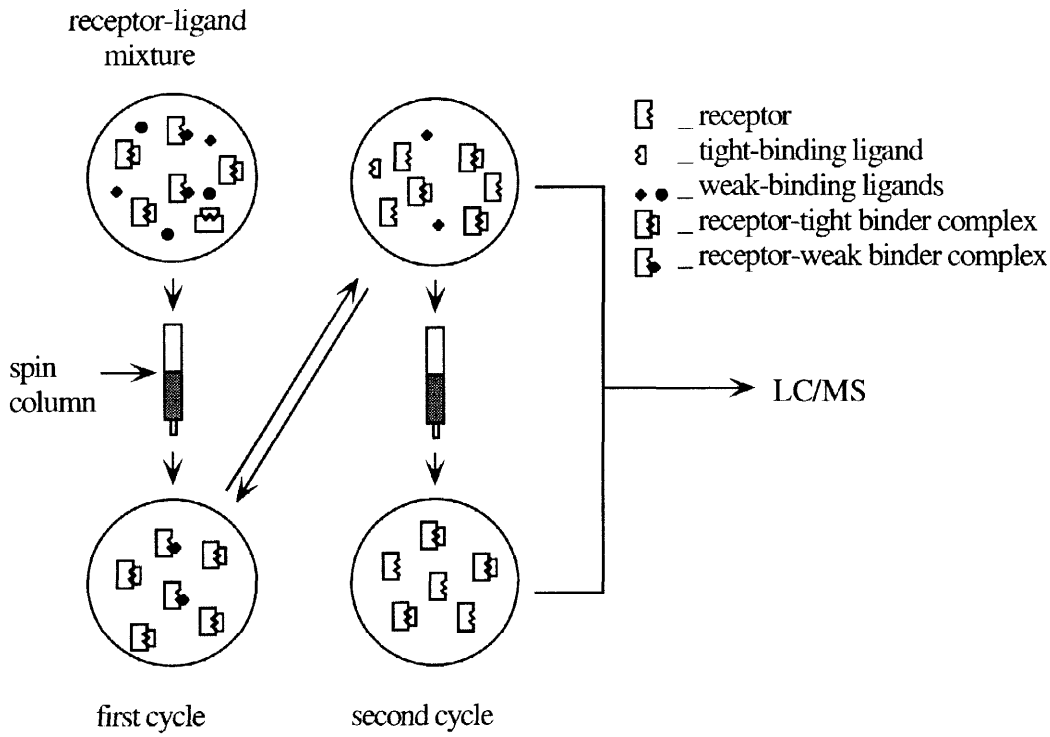


Figure 1. Spin column enrichment scheme. After one cycle, the spin column effectively retains any unbound ligand allowing only receptor:ligand complexes to pass through. When the equilibration is re-established, complexes begin to dissociate to a degree dictated by their binding constants, i.e., the weaker complexes dissociate more readily thereby freeing the weaker-binding ligands for retention during a second cycle through a column.

We have created a spreadsheet (available upon request) to calculate the concentrations of ligands (weak- and tight-binding) at each stage of SEC based on the multiple equilibria, given receptor concentration, binding constants, and starting ligand concentrations. It makes some simplifying assumptions, for example, no loss of protein and no dilution of receptor in eluting from the column. Fig. 2 shows the predicted concentrations of tight and weak binding ligands of a hypothetical situation where the starting concentrations are: receptor: 5 μM; tight-binding ligand: 1 μM; weak-binding ligand: 100 μM; K_{tight} = 50 nM; and K_{weak} = 50 μM . It is evident that the weak-binder is rapidly depleted from the solution, while the tight binder is

relatively enriched. From a starting ratio of 1:100 (tight:weak), the ratio improves to 1 : 2.8 after one pass through the spin column; 1 : 0.2 after two passes; and 1 : 0.02 after three passes! Over the same three steps, the theoretical recovery of tight-binding ligand (assuming no loss of protein and no dilution) remains above 90 %. This represents a 36-fold enrichment of the tight-binding ligand after one pass through the spin column, and a 460-fold enrichment after two passes.

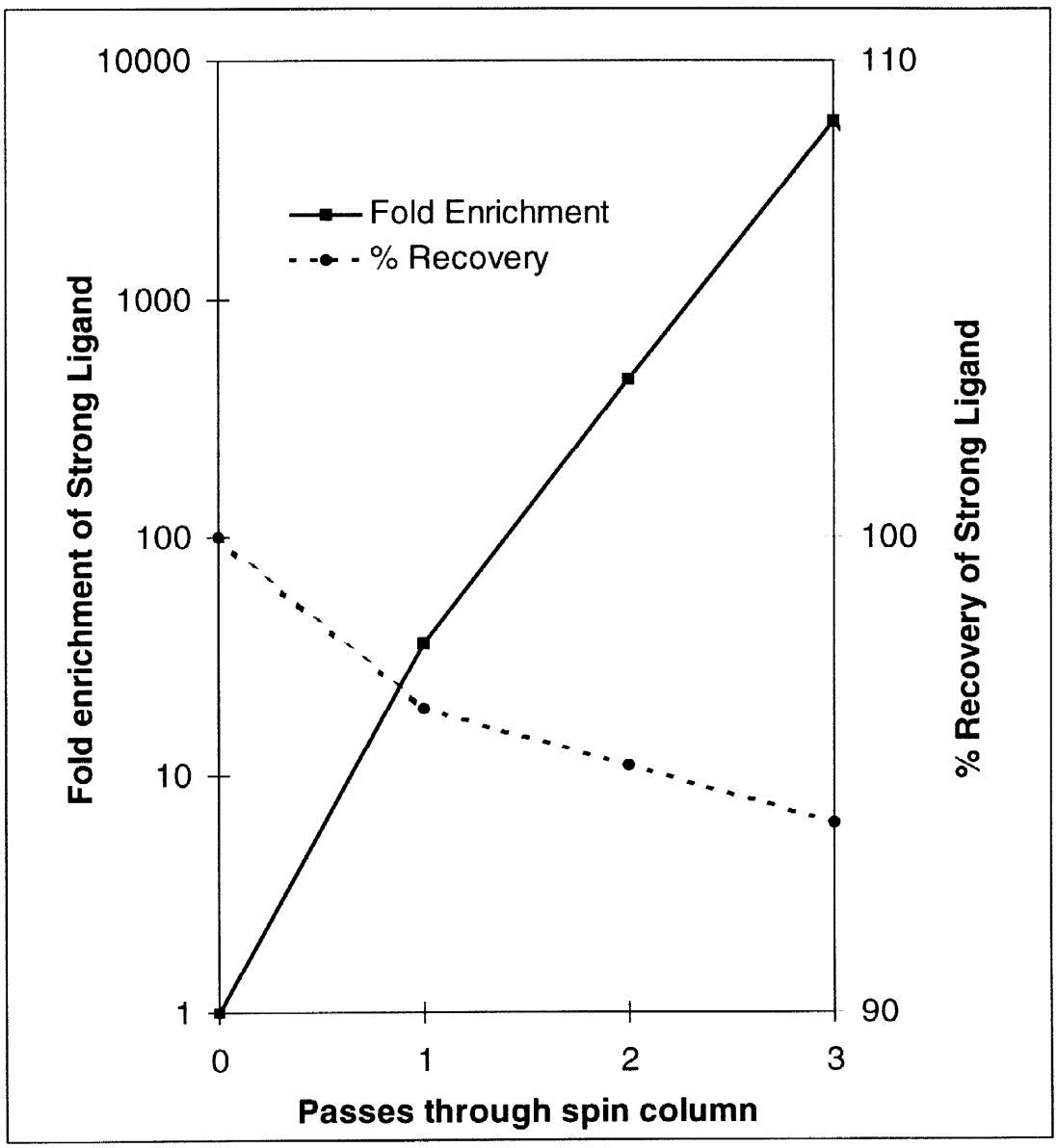


Figure 2. Hypothetical enrichment of strong binding ligand. Complete recovery of protein and no dilution of sample are assumed.

3. Results and Discussion

The spin column enrichment scheme that we developed is illustrated in Fig. 1. It is a modification of binding assay protocols that are widely used in the pharmaceutical industry. After the initial equilibration period, the receptor:ligand mixture will consist of bound and unbound ligands and protein complexes. Three situations (tight-binding, weak-binding, non-binding ligands) are represented in Fig. 1. When the mixture elutes from the spin column, only complexes of protein and bound ligands remain. Upon equilibration, these complexes begin to dissociate in accordance with their equilibrium binding constants. When equilibrium is re-established, the mixture is passed through another spin column, and the separation proceeds. Multiple cycles of spin and re-equilibrate are possible.

To test our hypothesis, we selected a nuclear receptor as the target protein. The nuclear receptors are a family of intercellular, ligand-activated transcription factors, many of which are of interest as drug targets [10-11]. One of these, a peroxisome proliferator activated receptor (PPARγ), was chosen, in part because we had a variety of ligands whose binding to the protein had been characterized, and in part because protein was readily available. PPARγ has been shown to be the molecular target of the thiazolidinedione anti-diabetic drugs such as troglitozone [12, 13]. The PPARγ construct used in these experiments was the ligand-binding domain of the receptor and had a molecular weight of 32,537 Da.; the molecular weights of the ligands ranged from 283 to 587 Da. Therefore, a spin column with a molecular weight cutoff of 6000 Da. was found to be well suited for retaining small molecules while allowing the receptor and receptor:ligand complexes to pass through relatively unhindered. After passing through the spin column, an aliquot of the mixture was analyzed by LC/MS using a fast, perfusive chromatographic medium. This chromatography allowed us recover and quantitate (by mass spectrometric response) both the protein and ligands in a single LC/MS analysis. The chromatographic conditions were, however, harsh enough to dissociate any receptor:ligand complexes, so what was observed both in the chromatograms and in the mass spectra, was free receptor and free ligands.

To characterize the behavior of the spin columns and to gain an understanding of the levels at which they effectively retain small molecules, a mixture of two ligands (**A** and **B**, representing a tight- and weak-binder, respectively) was prepared in a molar ratio of 1:100 and taken through the enrichment scheme. A labeled form of ligand **A** is used as the radioligand in one of our binding assays for PPARγ. Fig. 3A (pre-column) shows the LC/MS response for each compound prior to fast SEC. (Note: the response of the weak-binder is so large as to be distorted and non-linear.) After one pass through the spin column (no receptor present) the response of both ligands is much diminished. The response for the **A** has decreased to 4% of its initial value. Since the pre-column response for **B** is off-scale, it is difficult to obtain an

accurate value for its recovery after one pass, however it is estimated to be around 10%. These results indicate that the free ligands are, for the most part, retained in the column. After passing through the column again (second pass), neither ligand elutes to a significant extent.

When receptor (PPARγ) is present, a different result is expected. A new mixture was prepared that contained the same concentration of tight- and weak-binder indicated above, but also with 5 μM PPARγ. Figure 4 shows the LC/MS response of the receptor and ligands **A** and **B** before passing through the column, after one pass through, and after a 30 minute re-equilibration followed by a second pass through a spin column. Some protein is lost at each step (78 % and 38 % of pre-column levels for the first pass and second pass, respectively), so the recovery of ligands is also diminished, but the tight-binder (**A**) diminishes only slightly, while the weak binder (**B**) goes from 100-fold excess at the start to approximately 3.8-fold excess over the strong-binder after one pass, to about one-seventh of the amount of the strong-binder after the second pass through the column. The ***relative*** enrichment of the strong-binder is clear.

The experiment above was modeled using the spreadsheet, and is the situation represented in Fig. 2. After correcting for the amount of ligand that breaks through the spin column, the observed enrichment was about 26.5, reasonably close to the predicted enrichment of 36. The loss of protein on the column causes the overall recovery of both ligands to suffer somewhat, but that loss seems to affect both ligands to the same degree, and so there is no adverse consequence on relative enrichment of the tight-binder. The decrease in recovery does impose a limit to the number of cycles through the spin column, however, before all of the tight-binding ligand disappears.

In practice, we have not needed more than two or three passes through the spin column to observe the enrichment. Nor is it necessary to continue the analysis until pure tight-binding ligand is obtained. It is only necessary to see the relative enrichment beginning to happen. Once the trend is clear, tandem mass spectrometric studies can be initiated to further characterize the tight-binding ligands, even from an unresolved mixture. If applied in a library deconvolution scheme, this spin column technique could save valuable time in re-synthesis and re-screening of sub-pools.

There are two scenarios where this technique might be important. One is a situation where one is interested in pulling strong-binding ligands out of a pool of ligands, such as a library. The other is in finding tight-binding ligands in a tissue or other complex mixture. To model the first of these situations, we prepared a mixture of ten ligands, including two known strong-binders and eight weaker-binders (Table 1). Each component was present at 5 μM. Like the experiment depicted in Fig. 3, without receptor present, when the ten component mixture was passed through a spin column, little of any of the ligands was detected after one cycle and none were detected after the second cycle (data not shown).

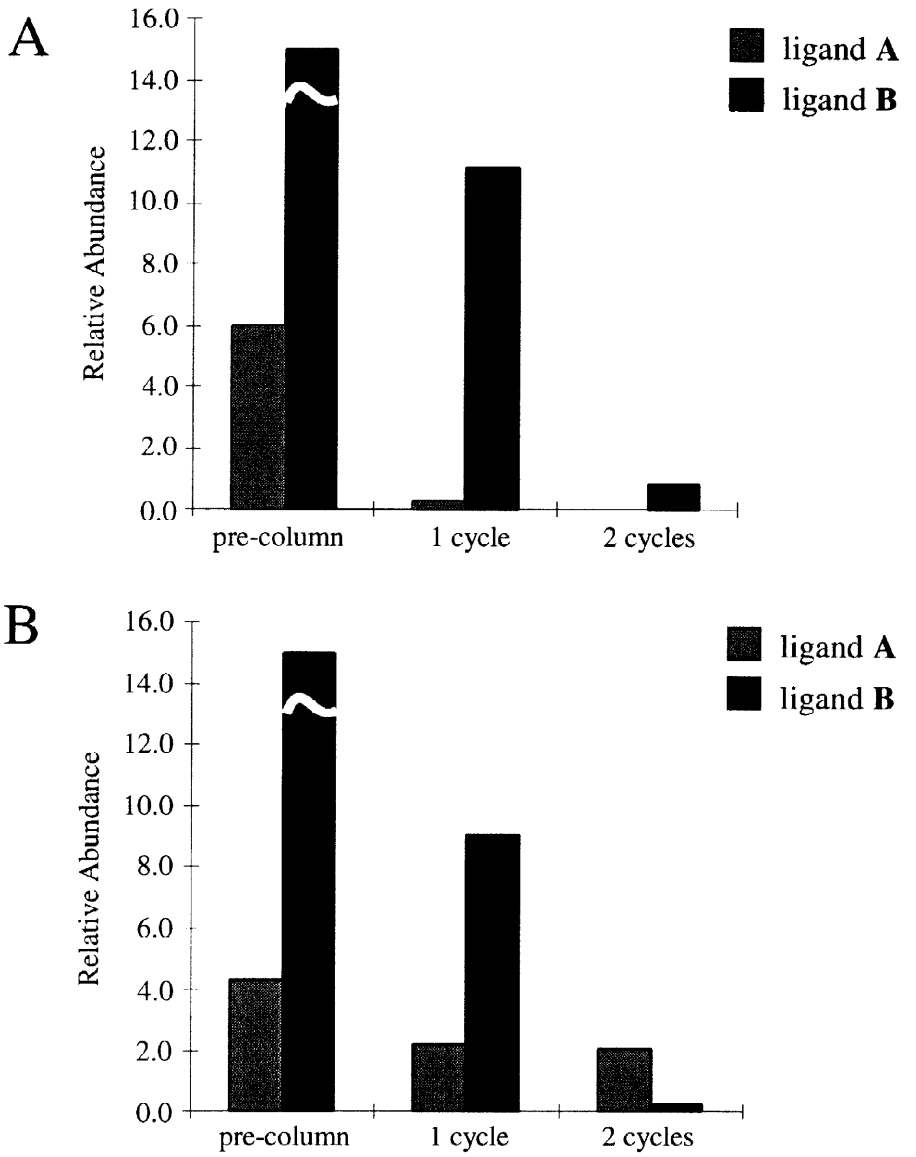
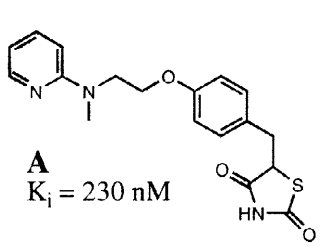


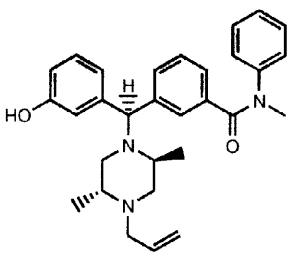
Figure 3A. LC/MS responses of ligands **A** and **B** before and after spin-column cycling (without receptor). The pre-column response is for 80 pmol of **A** and 8 nmol of **B**. The pre-column response for **B** was unmeasurable (saturated signal); its bar is shown for illustration purposes only. Figure 3B. LC/MS responses of ligands **A** and **B** before and after spin-column cycling (with 5 μM PPARγ). The mixture contained the same concentration of **A** and **B** as in 3A. The "breakthrough" that was observed in 3A has been subtracted from the responses shown in 3B. The first cycle and second cycle response for PPARγ was 78% and 38%, respectively, of its pre-spin level. (n = 3; the standard deviation was typically less than 10%).

Table1.

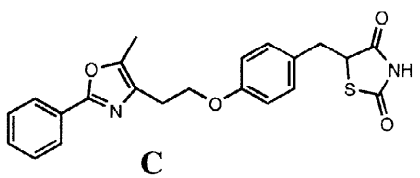
Structures and binding constants (K_i's) of the ligands used in this study. **A** and **C** are considered tight-binders of PPARγ.



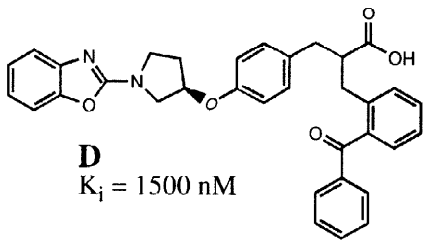
A
K_i = 230 nM



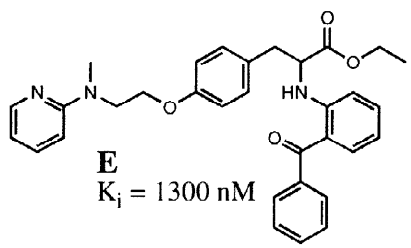
B
K_i ~ 50,000 nM



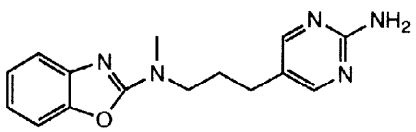
C
K_i = 9 nM



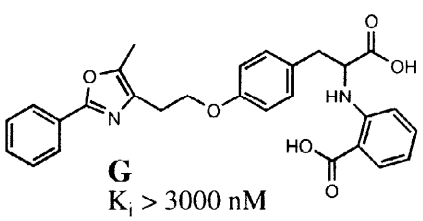
D
K_i = 1500 nM



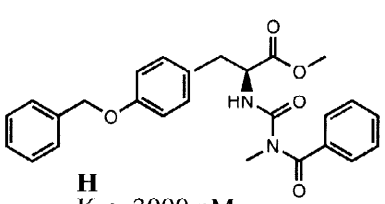
E
K_i = 1300 nM



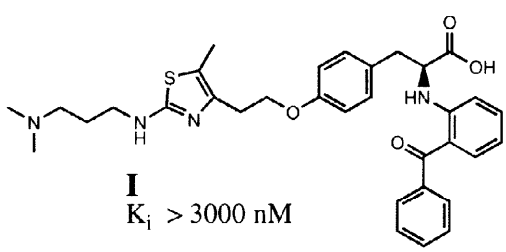
F
K_i > 3000 nM



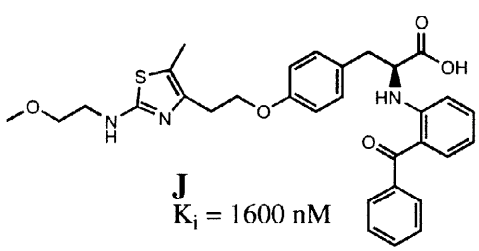
G
K_i > 3000 nM



H
K_i > 3000 nM



I
K_i > 3000 nM



J
K_i = 1600 nM

Note:

(a) > 3000 means less than 50% inhibition at a concentration of 3000 nM.

(b) For samples where K_i was measures multiple times, the precision was about +/- 60%.

Fig. 4A shows the LC-MS response of the ten compounds, in the presence of 5 μM receptor but without passing through a spin column. After one pass through the spin column, the chromatogram in Fig. 4B results. Several weak-binding ligands have disappeared altogether (**B, F, H**), several others have diminished in relative amount (**D, E, G**). The tight-binders (**A, C**) and, interestingly, two "weak-binders" (**I, J**) still produce strong signals indicative of tight-binding. After a second pass through the spin column (Fig. 4C), the three weak-binders (**D, E, G**) that were diminished in the first pass are further diminished, but the remaining four ligands persist. In fact, one of the "weak-binders" (**J**) appears to be recovered better than any of the other ligands in the mixture, indicating a very tight-binding ligand. If the anomalies of **I** and **J** are momentarily disregarded, the experimental results track well the predictions based on the measured binding constants. The tight-binders (**A** and **C**) are relatively enriched; intermediate binders (**D** and **E**) are enriched, but to a lesser degree; and most weak binders (**B, F, G, H**) disappear according to prediction. The results suggest that this could be a method of measuring relative binding affinities of ligands in mixtures by mass spectrometry; or, if the true binding constant of one ligand in the mixture was known, a method of measuring multiple binding constants in a single LC/MS experiment.

There are several possible explanations for the apparent contradiction in pulling out two "weak-binders" along with the tight-binding ligands in this experiment. We rule out non-specific binding, as that would be expected to bind all ligands to approximately the same degree. One might argue that if a ligand was particularly hydrophobic, it might selectively adsorb to the receptor; nuclear receptor ligand-binding domains are notoriously "sticky". However, the LC retention does not indicate any unusual hydrophobicity in the two anomalous ligands. A possible explanation was that there was specific binding to the receptor, but at a site other than the active site (the binding constants are determined in a competition assay). Alternatively, the possibility of experimental error in the determination of the initial binding for these two compounds exists.

At the same time we were performing these studies, X-ray crystallography was being conducted on the receptor:ligand complexes [14]. Interestingly, the X-ray results showed that the ligand binding site was very large, and that with tight-binding ligands in place, enough room remained in the binding pocket to accommodate another ligand. This could explain the anomalous observations in the spin column experiments. **I** and **J** were, in fact, tight-binding ligands, but we did not know it because they did not compete in the competition binding assay in the way we expected. When these compounds were tested in a cell-based, functional assay, they did behave as ligands of PPARγ.

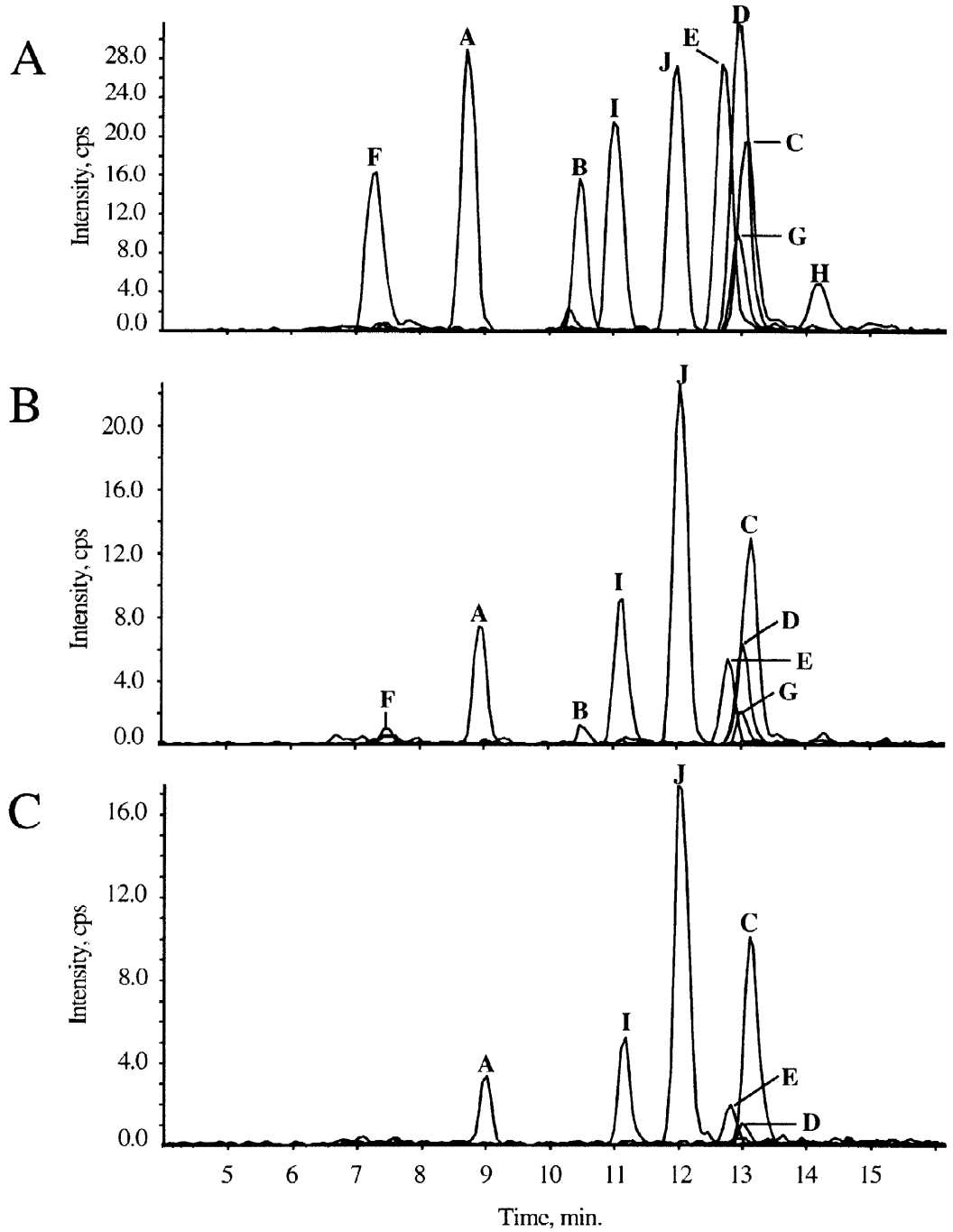


Figure 4. Extracted ion plots of a ten compound mixture in the presence of receptor (a) before passing through a spin column, (b) after one cycle, and (c) after two cycles.

4. Conclusions

Using bioaffinity selection with fast SEC spin columns in an iterative fashion provides a useful way to isolate and enrich tight-binding ligands in mixtures. This method is not restricted to any receptor type, and the only restriction on ligand type is that they be amenable to electrospray ionization. Although multiple cycles of the receptor:ligand mixture through spin-columns is the key feature of the method, it is not necessary to continue the process until only one ligand is obtained. Once the trend in the relative enrichment becomes clear, the next step to identify the enriched ligand can be started. The method is particularly useful in situations where tight-binding ligands are present at very low concentration relative to weaker-binding ligands, as may be encountered when working with combinatorial libraries or when attempting to isolate a tight binder from a tissue extract. In these complex systems, the binding of a tight-binder may be masked by either the large number or the high concentration of weak-binders. The method is also more efficient than synthetic deconvolution in decoding pools identified as being active from binding assays. The identification of strong-binding ligands does not require a radioligand, as do many other binding assays. Actual binding is measured with this method, but nothing can be determined about whether the binding is at the active site (i.e., competitive) or elsewhere.

5. Experimental Section

Materials. Human PPARγ (206- 477) ligand-binding domain (LBD) construct with a histidine tag was expressed in *E. coli* and purified at Glaxo Wellcome. The concentration of the stock protein solution was determined by amino acid analysis to be 9.53 mg/mL (293 μM). Test compounds were also synthesized in-house and had been previously tested for binding against PPARγ in a binding assay [12, 14]. The binding constant (K_i) was the basis for their selection for use in these experiments (see Table 1). Stock solutions were prepared in methanol, typically at a concentration of 200 μM. The incubation buffer consisted of 50 mM KCl, 20 mM CHAPS, 2 mM EDTA, and 10 mM DTT, pH 8.0. Spin columns with Bio-Gel P-6 packing (BIO-RAD, Hercules, CA) and a 6000 Da. exclusion limit were used for all experiments. Prior to use, the storage buffer was removed according to the manufacturer's instructions. The snap-off tip was removed and the storage buffer was allowed to gravity drain. The spin-column was then centrifuged for two minutes. A Centra GP8R centrifuge (IEC) was used to induce fast size-exclusion chromatography.

Affinity Selection and Enrichment Scheme. Binding of ligand(s) to the receptor was initiated by combining aliquots of PPARγ and ligand(s) in the incubation buffer. In all experiments

with receptor, the concentration of PPARγ was 5 μM which we have found to be the lowest receptor concentration that could be used initially and still get sufficient protein recovery after a second pass through a spin column. Also, without CHAPS in the incubation buffer, when starting with 5 μM PPARg, little (< 5%) of the protein was recovered after two cycles. The reaction volume was typically 200-300 μL. Depending upon the experiment, the ligand(s) concentration was either 1, 5, or 100 μM, as described in the text. Because of concerns that including too much methanol, which was used as the solvent for the test ligands, in the reaction mixture would lessen receptor:ligand binding, the percentage of methanol was kept below about 10% of the total reaction volume. The concentration of the stock ligand solutions was adjusted accordingly. The aliquot of receptor was always added last, and from that point the receptor-ligand mixture was equilibrated for two hours at room temperature with gentle mixing.

In order to enrich the tight-binding ligands relative to the weak-binders, 80 μL of the receptor:ligand mixture was loaded onto a spin column, centrifuged at 1100 x g for four minutes and collected in a microcentrifuge tube containing 5 μL of acetonitrile, added to deter adsorption of protein to the walls of the tube. The column was then washed by loading 40 μL of the incubation buffer and spinning again at 1,100 x g for four minutes. Both filtrates were collected in the same centrifuge tube. Ideally, only those ligands that bound to the receptor will pass through the spin column and into the collection vessel. For those experiments involving two cycles through a spin column, the filtrate from the first spin was allowed to equilibrate at room temperature for 30 min or 2 hr. After this second equilibration period, an 80-μL aliquot from the first filtrate was loaded onto a new spin column and centrifuged again, as described above.

Levels of analytes in the receptor:ligand mixture were determined by ion abundance in the LC/MS analysis.

Chromatography. HPLC separations were carried out with a reversed-phase column, packed in our laboratory, with POROS® R2/H material (Perceptive Biosystems; Framingham, MA). Column dimensions were 500 μm (i.d.) x 30 cm. The mobile phases were (A) water with 0.05 % trifluoroacetic acid (TFA) and (B) 90% aqueous acetonitrile with 0.035% TFA. Analytes were eluted with a gradient from 2% B to 65% B in 10 min, followed by a hold at 65% B for 6 min. The mobile phases were delivered with a HP 1090 (Hewlett Packard, Palo Alto, CA) LC with a pump flow rate of 0.15 ml/min, which was split to give a flow rate through the column of 45 μL/min. A UV detector was placed in-line but it was primarily used for diagnostic purposes.

Mass Spectrometry. The HPLC was coupled to a API I mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a pneumatically assisted electrospray (ion spray) interface. Spectra were acquired in positive ion mode. Full scans were obtained over the mass range 250-1650 Da scanned with a 0.3 Da step and a dwell time of 0.7 ms. This scan range enabled detection of the protonated ions for all of the ligands used in the study, as well as detection of almost all charges states for PPARγ that were observable under these conditions. Operating voltages were 5000 V needle voltage (ISV), 80 V orifice voltage (OR) and 30 V Q_0 voltage (RO).

Data was processed with the BioMultiview application supplied as part of the Sciex software package. Protonated molecular ions of the test compounds were used to generate extracted ion chromatograms (XIC's), from which the corresponding peak areas were determined. PPARγ was quantitated similarly using all observable charge states in the scan range to generate XIC's from which the peak area for each charge state was taken and summed.

After the protein:ligand mixture had equilibrated but just prior to the first cycle through a spin column, the initial responses for the receptor and the ligands were determined by LC/MS. A 10 μL aliquot containing 50 pmol of PPARγ was injected onto the POROS®R2/H column. After each cycle through the spin column, LC/MS analysis was repeated. Sample dilution occurs after each cycle. This dilution was accounted for by increasing the injection volume of the filtrates taken after each cycle in their LC/MS run. Assuming complete recovery of the protein, an aliquot corresponding to 50 pmol of protein was injected at each stage, which required injection volumes of 10, 16, and 26 μL for the pre-spin, first cycle and second cycle, respectively.

6. Acknowledgements

Protein was provided by Chris Mohr and Ann Miller. Technical assistance of Derek Parks, and helpful discussions with Mil Lambert, Mike Milburn, and Tim Willson (all of Glaxo Wellcome) are gratefully acknowledged.

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