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Online magnetic bead based dynamic protein affinity selection coupled to LC–MS for the screening of acetylcholine binding protein ligands

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ABSTRACT

A magnetic beads based affinity-selection methodology towards the screening of acetylcholine binding protein (AChBP) binders in mixtures and pure compound libraries was developed. The methodology works as follows: after in solution incubation of His-tagged AChBP with potential ligands, and subsequent addition of cobalt (II)-coated paramagnetic beads, the formed bead-AChBP-ligand complexes are fetched out of solution by injection and trapping in LC tubing with an external adjustable magnet. Non binders are then washed to the waste followed by elution of ligands to a SPE cartridge by flushing with denaturing solution. Finally, SPE-LC–MS analysis is performed to identify the ligands. The advantage of the current methodology is the in solution incubation followed by immobilized AChBP ligand trapping and the capability of using the magnetic beads system as mobile/online transportable affinity SPE material. The system was optimized and then successfully demonstrated for the identification of AChBP ligands injected as pure compounds and for the fishing of ligands in mixtures. The results obtained with AChBP as target protein demonstrated reliable discrimination between binders with pK_i values ranging from at least 6.26 to 8.46 and non-binders.

1. Introduction

Over the last decade, mass spectrometry (MS) has proved to be a valid technique for the screening of receptor ligands [1–3]. Despite the lower throughput when compared to platereader methodologies traditionally used in high throughput screening (HTS), MS offers several advantages: direct structure elucidation of unknown hits and analysis of bioactive mixtures. Furthermore, the ability to develop "label free" assays makes MS assays widely applicable in screening approaches. Indeed, ligand–receptor complexes can be detected directly, or indirectly after disruption of the ligand–receptor complexes, followed by analysis of the ligands released.

Several distinct MS approaches towards screening of bioactives have been developed allowing the evaluation of protein–ligand binding by MS [4]. (Pulsed) ultrafiltration technologies utilize a target protein in an ultrafiltration chamber [5]. Injection or infusion of ligands results in binding to the target. Subsequent washing, disruption and direction to MS allows analysis of the binders [6]. Separation of ligands bound to a target protein can also be accomplished by rapid size exclusion chromatography [7–9]. After the separation step, a disruption step follows, allowing LC–MS analysis of the released ligands [10]. In direct methods, distinction between ligands and non-binders is monitored directly in the MS by measurement of the protein–ligand complex in the gas phase [11]. Powerful approaches are based on immobilization of the target protein onto a solid support, e.g., affinity chromatography or affinity selection MS. In affinity chromatography, the target protein is immobilized onto a chromatographic column to allow retention of ligands based on their affinity [12–14]. Detection of eluting ligands usually occurs with MS. In affinity selection MS screening techniques, the binding of ligands occurs to immobilized protein targets on a solid support. After washing away non binders, the protein–ligand complex is disrupted and the ligands

Abbreviations: AChBP, acetylcholine binding protein; nAChRs, nicotinic acetylcholine receptors; IMAC, immobilized metal affinity chromatography; HTS, high throughput screening; PBS, phosphate buffered saline; BR, ELISA blocking reagent; ESI, electrospray ionisation; HOAc, acetic acid; TFA, trifluoroacetic acid; EIC, extracted ion chromatogram; LC, liquid chromatography; HPLC, high performance liquid chromatography; SPE, solid phase extraction; NMR, nuclear magnetic resonance; MS, mass spectrometry.

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are analyzed by LC–MS analysis [15]. Modified affinity selection MS approaches developed in our group include dynamic affinity selection MS where the target-ligand incubation occurs in solution followed by capturing of the His-tagged target protein by an immobilized metal affinity column (IMAC) [16]. After immobilization, the procedure is similar to traditional affinity selection MS methodologies. The advantages here are that the incubation occurs under native target conditions in solution and that new target protein is used for every measurement. In recently published work, we utilized the last mentioned approach with immobilized metal affinity chromatographic (IMAC) paramagnetic beads (referred to as 'magnetic beads') as solid support material and the His-tagged estrogen receptor (ligand binding domain) as the target protein [4]. The methodology employs in vial and solution based formation of estrogen receptor–ligand complexes followed by addition of magnetic beads. This in turn results in binding of the (His-tagged) receptor–ligand complexes to the beads. The resulting suspension is injected into and transported online through the analytical system for analysis. The use of a strong movable magnet allows retention and washing of the beads in the tubing on demand and eluting the bound ligands by a disruption step.

In the present study, we applied the magnetic beads based approach to the acetylcholine binding protein (AChBP), a structural analog of the extracellular ligand binding domain of the α7 nicotinic acetylcholine receptor (nAChRs) [17,18] originating from the snail *Lymnaea Stagnalis*. After its crystal structure was published [19], it has become a model for nAChRs to efficiently screen compound libraries for potential ligands and for structure based synthetic approaches in medicinal chemistry [20]. The nAChR family is intensively studied in relation to its potential as pharmaceutical target against epilepsy, Alzheimer's disease, pain relief, Parkinson's disease, anxiety and cognitive and attention deficits [17,18,21–23].

For our ligand fishing approach towards the AChBP, ligands were first incubated with His-tagged AChBP. Cobalt (II) coated paramagnetic (IMAC) affinity beads were then added and allowed to bind to the AChBP. The samples were subsequently injected in an integrated solid phase extraction liquid chromatography mass spectrometry (SPE LC–MS) system in order to perform the online isolation of the AChBP-ligand complex, removal of non-binders, and the elution of the ligands from the magnetic beads for analysis by SPE LC–MS. Finally, the magnetic beads were released to the waste prior to analysis of the next sample.

2. Experimental

2.1. Chemical and biological reagents

AChBP (from species *Lymnaea stagnalis*) was expressed from baculovirus using the pFastbac I vector in Sf9 insect cells and purified from the medium as described by Celie et al. [18]. ELISA blocking reagent (BR) was obtained from Hoffmann-La Roche (Mannheim, D). Glycine·HCL, dextromethorphan hydrobromide, desipramine hydrochloride, cetirizine dihydrochloride, sulfamethoxypyridazine, phenacetin, (±)epibatidine dihydrochloride hydrate, diclofenac sodium salt, potassium dihydrogen phosphate, disodium monohydrogen phosphate, acetic acid (HOAc), trifluoro acetic acid (TFA) and trizma base were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Sodium chloride and ammonium hydrogen carbonate were from Riedel de Haen (Seelze, Germany). LC–MS grade methanol (MeOH; 99.95%) was purchased from Biosolve (Valkenswaard, The Netherlands). respectively. HPLC grade water was produced using a Milli-Q purification system from Millipore (Amsterdam, The Netherlands). 1 μm Cobalt loaded Dynabeads TALON (His-Tag Isolation & Pulldown) were purchased from Dyanal, Invitrogen (Breda, The Netherlands) and supplied in an ethanol/water (20:80) solution. All ligands were synthesized in house by the VU University Medicinal Chemistry department [20] and are detailed in the Supporting Information. All in house synthesized ligands used are depicted as compound (cmpd) **1**–**7** while the non-binders are depicted by their trivial name. Only, the high affinity ligand epibatidine used for the displacement reactions is also named by its trivial name.

2.2. Instrumentation

2.2.1. SPE symbiosis

All SPE experiments were carried out on a Symbiosys Pharma (Spark Holland, Emmen, The Netherlands) sample pre-treatment system. The SPE cartridge used was a Hysphere C18HD (Silica based C18 with high density end-capping, 2 mm × 10 mm; particle size 7.5 μm, adsorbent mass 18.5 mg, carbon content 14.5%) from Spark Holland. The cartrigde was systematically and preventively replaced after 30 injections. In these conditions, no change in the recovery was observed.

2.2.2. LC–MS

The LC–MS system consisted of a high-pressure gradient LC system (Shimadzu LC20, 's Hertogenbosch, the Netherlands) coupled to a Thermo Electron LCQ Deca ion trap MS (Breda, The Netherlands) equipped with an electrospray ionization (ESI) probe. The LC–MS system was operated at 200 μL/min. Solvent A consisted of water/MeOH 99:1 and 0.1:0.02% HOAc/TFA. Solvent B consisted of water/MeOH 1:99 and 0.1:0.02% HOAc/TFA. For separation, an XBridge 3.5 μm particle C18 analytical column (100 mm × 2.1 mm i.d.; Waters, Milford, MA, USA) was used. Gradient LC elution was applied by running a 1.5 min isocratic elution at 30% B, then rising to 95% B in 10 min. The autosampler was set to 4 °C. The MS was operated in positive electrospray ionization mode. N_2 was used as a sheath gas (60 psi) and auxiliary gas (20 psi), the needle voltage was 5000 V and the heated capillary was at 250 °C with the capillary voltage set at 17 V.

2.3. Procedures

2.3.1. Receptor ligand incubation

The composition of the binding buffer used for receptor ligand reaction was 1 mM KH_2PO_4, 3 mM Na_2HPO_4, 0.16 mM NaCl and 20 mM Trizma base pH 7.5 with 0.5 mg/mL ELISA BR. The binding experiments were performed by incubating 20 μL of test compound (10^{-6} M) with 15 μL of AChBP (321 ng/μL, ∼12 μM) in 185 μL of binding buffer for 15 min at room temperature. A 10 μL magnetic bead suspension (20 mg/mL) was added to the mixture. After 5 min, the resulting suspension was placed in the autosampler of the symbiosis at 4 °C for further analysis. The same procedure was followed for the competitive experiments, except that the 15 min incubations were done with 20 μL of the potent AChBP ligand epibatidine (10^{-5} M; pK_i ∼ 9.00), 20 μL of test compound (10^{-6} M) and 15 μL of AChBP (321 ng/μL; ∼12 μM) in 185 μL of binding buffer.

2.3.2. Magnetic beads handling

A 20 mg/mL magnetic bead suspension was prepared according to the recent paper of Jonker et al. [4]. In brief, 120 μL of bead suspension was washed three times with 400 μL binding buffer and finally resuspended in 240 μL of binding buffer resulting in a concentration of 20 mg/mL. The trapping of the magnetic beads was achieved in PEEK tubing (0.25 mm internal diameter) by a 50 N 1.4 T permanent neodymium magnet with dimension of 7 cm × 4 cm × 3.5 cm. In order to trap and release the beads, the PEEK tubing was attached to an in house built, pneumatically driven aluminum arm. By moving the arm either in close proximity of or 2.6 cm away from the magnet, the setup allowed to trap and

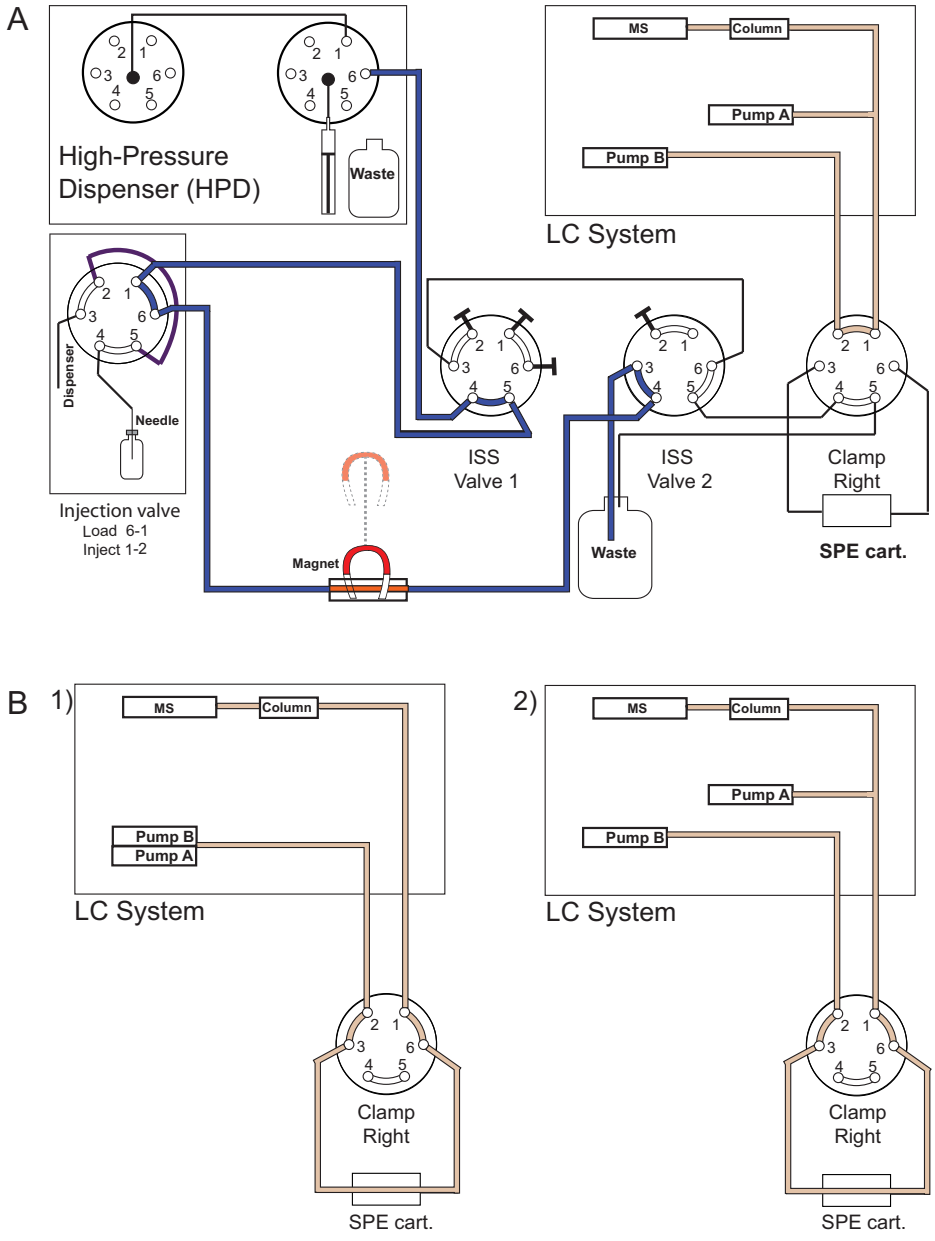

Fig. 1. (A) System setup used for the ligand affinity selection. For illustration purposes, the solvent flows and the valve settings shown represent the step where the beads (orange) are washed with NH_4HCO_3 (blue) to remove non-binders (see Fig. 2B). (B) Connection between HPLC and SPE cartridge holder (before (1) and after (2) optimization). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

release the beads, respectively. In trapping mode, the retractable aluminum arm pushes the tubing onto the magnet. The movement of the arm was triggered by a contact closure signal provided by the symbiosis system.

2.3.3. *Affinity selection procedure*

The setup of the affinity selection part of the system, which allows the isolation of ligands, is presented in Fig. 1A and the fully described method is published in the Supporting Information. The SPE cartridge was first solvated and conditioned with 1 mL of MeOH followed by 1 mL of H_2O at a flow rate of 4 mL/min. The injection loop and the tubing attached to the pneumatically driven aluminum arm were then flushed with 2.5 mL of the binding buffer (without BR) at a flow rate of 4 mL/min. Before loading the sample into the injection loop, the suspension of beads was resuspended by aspirating and dispensing 100 μL of the sample in the autosampler vial three times. Then, 100 μL of sample was loaded on the injection loop (inj valve, load; see Fig. 1A). The loaded sample was subsequently transported with 400 μL binding buffer (without BR) at a flow rate of 200 μL/min to the magnet in order to trap the beads in the PEEK tubing near the magnetic field (inj valve, inject; ISS valve 1, 6–1; ISS valve 2, 1–2; clamp right valve, 1–2; see Fig. 1A). The beads were then washed with 800 μL of an ammonium carbonate buffer (10 mM, 10% MeOH, pH 7.4) at a flow rate of 200 μL/min to remove the non-binders (inj Valve, load; ISS valve 1, 6–1; ISS valve 2, 1–2; clamp right valve, 1–2; see Fig. 1A). Dissociation of ligands bound to the AChBP was performed by flushing 400 μL of a glycine buffer (10 mM, pH 2) at a flow rate of 200 μL/min over the trapped beads (inj valve, load; ISS valve 1, 6–1; ISS valve 2, 6–1; clamp right valve, 1–2; see Fig. 1A). In this step, the glycine buffer denatured the AChBP and released the ligands, which were subsequently trapped by an upstream positioned SPE cartridge. The SPE cartridge was

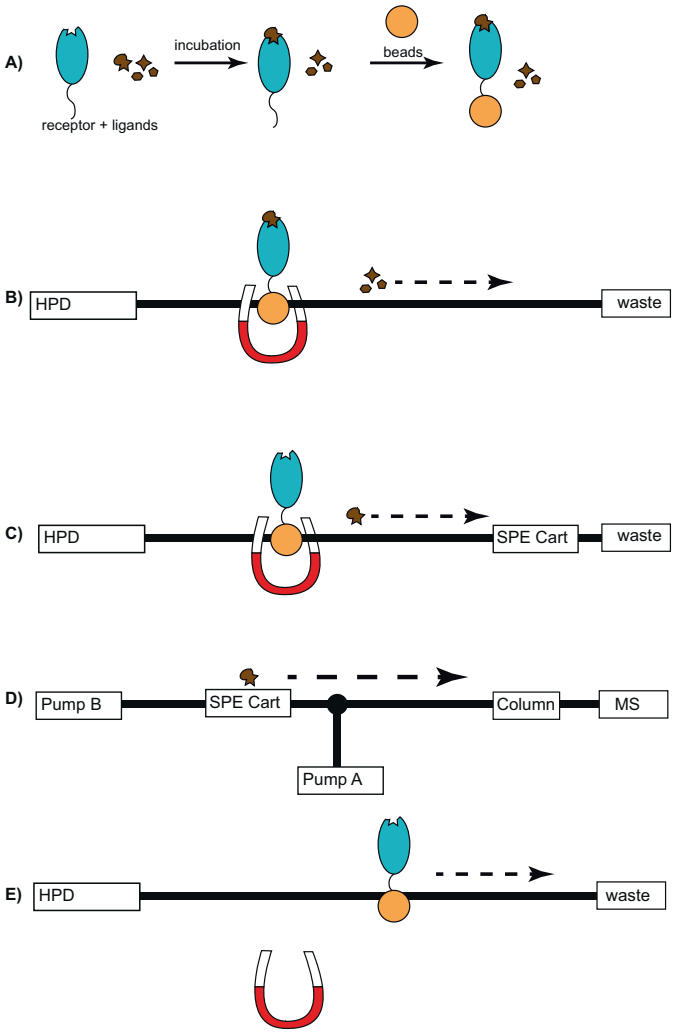

Fig. 2. Schematic overview of the online magnetic bead based dynamic protein affinity selection. (A) Receptor ligand incubation followed by addition of the magnetic beads. (B) Beads trapping after injection and elimination of non-binder by the washing step. (C) Dissociation of the ligand by the denaturing solution and its trapping on the SPE cartridge. (D) Ligand elution by solvent B and post SPE mixing with solvent A followed by LC–MS analysis. (E) Elimination of the beads.

then washed with 1 mL of water/MeOH 70:30 including 0.1:0.02% HOAc/TFA (SPE solvent) at a flow rate of 1 mL/min (ISS valve 1, 1–2; ISS valve 2, 1–2; clamp right valve, 1–2; see Fig. 1A). The ligands were eluted from SPE cartridge and directed to the analytical column by 100% of solvent B (clamp right valve, 6–1; see Fig. 1A). The gradient required for the LC–MS analysis is formed by a post SPE mixing with solvent A (Fig. 1). Finally, the magnetic beads were flushed from the system to the waste by moving the pneumatic arm away from the magnet. The tubing was washed with 4 mL of 100% MeOH at a flow rate of 1 mL/min (inj valve, load; ISS valve 1, 6–1; ISS valve 2, 1–2; clamp right, 6–1; see Fig. 1A).

3. Results and discussion

3.1. Methodology overview

The system presented in this article has been designed to screen for ligands towards His-tagged AChBP in pure compound libraries and in mixtures. The entire process can be subdivided in several steps (Fig. 2). First, AChBP is incubated with potential ligands. Magnetic beads are then added to bind the His-tagged moiety of the AChBP (Fig. 2A). The resulting suspension is subsequently injected

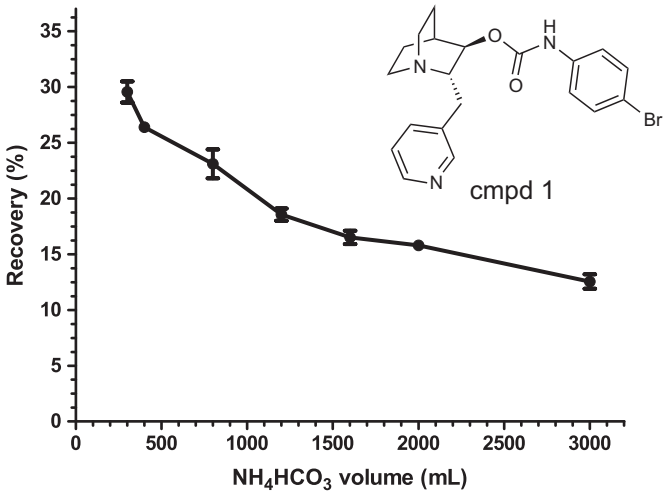

Fig. 3. Optimization of the washing volume of the beads.

into the online magnetic bead trapping SPE LC–MS system. In this system, the bead immobilized protein ligand complex is retained by a magnet and is washed with an appropriate buffer to remove non binders and non-specific binders (Fig. 2B). A second denaturing buffer solution is then flushed over the retained complex in order to dissociate the ligand(s) and direct them to the SPE cartridge (Fig. 2C). The SPE cartridge is washed and finally eluted to the LC–MS system. The gradient required for the LC–MS analysis is formed by a post SPE mixing of the elution solvent (solvent B) and a weaker solvent (solvent A) (Fig. 2D). While the ligands are analyzed, the beads are flushed with MeOH to waste after moving the tubing away from the magnet (Fig. 2E).

3.2. Evaluation of the technology for AChBP screening

The system was evaluated and tested for applicability towards screening AChBP ligands among a set of pure in house synthesized compounds. For evaluation and optimization of the procedure, including the LC–MS and SPE steps, compound (cmpd) **1** was used (pK_i 7.74) as test ligand. The structure of cmpd **1** is depicted in Fig. 3. Since the LC–MS procedure should be able to analyze ligands with varying affinities and polarities, a generic linear gradient from 2% to 95% solvent B was first evaluated. Then, the SPE step was incorporated into the system allowing the evaluation of the chemical trapping process on SPE followed by LC–MS analysis. This evaluation firstly mimics the ligand trapping process after disruption from the AChBP and secondly enables to study the removal of the glycine·HCL disruption buffer and any other non-volatile salts from the SPE cartridge prior to elution to LC–MS. For this evaluation, we developed a method derived from the affinity selection procedure described by Jonker et al. [4] in which the injected sample was directly sent from the autosampler to the SPE cartridge by the glycine buffer. Since a wide range of ligands in terms of polarity and affinity is expected, a generically applicable C18 SPE cartridge was desired. Several sorbents were tested (Hysphere C18 HD, Hysphere C18, Hysphere Resin SH, Hysphere C8, Hysphere C8 (EC)). Best results were obtained with the HySphere C18-HD cartridge in combination with an XBridge C18 column, although the chromatographic peaks exhibited strong tailing. The addition of a low concentration of TFA (0.02%) significantly improved the peak shape while maintaining a good sensitivity with the MS detector [24]. To further improve the peak shape, ligands were eluted from the SPE material with solvent B and then mixed with solvent A. For this, the methodology was modified in a way that the solvent A pump was connected after the SPE cartridge using an inverted

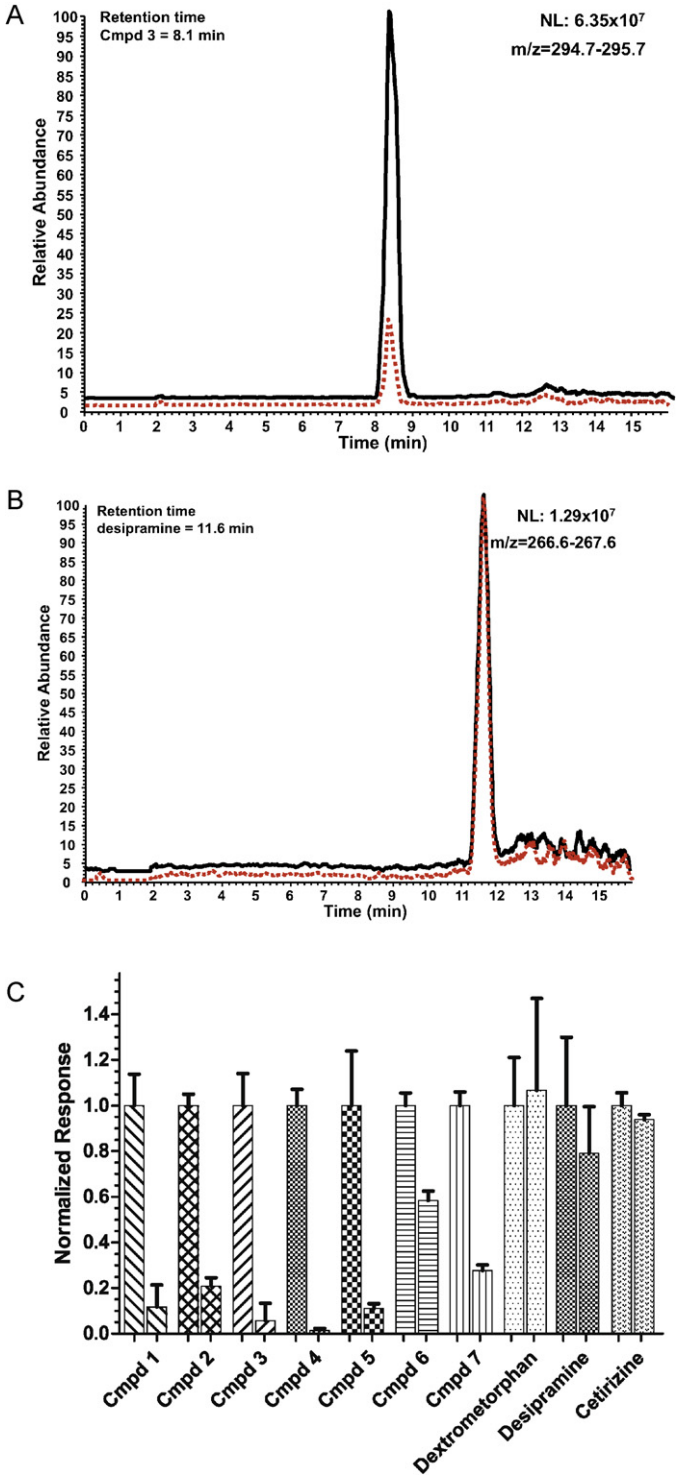

Fig. 4. Overlaid EIC chromatograms obtained for (A) a typical screening experiment with the binder cmpd **3** and (B) for the non-binder desipramine (plain black curve) is without epibatidine and (red dotted line) is with epibatidine. (C) Results of the compounds tested for the screening experiments with pure compounds. For each compound, the left bar represents the normalized response of the binding experiment and the right bar the normalized non-binding displacement or competition experiment with epibatidine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Y-piece and the solvent B pump before the SPE cartridge (Fig. 1B). This allowed elution of ligands from the SPE with 100% solvent B followed by post SPE mixing of solvent A to result in a final concentration of 30% solvent B to the LC column at the start of the gradient. This setup gave efficient ligand elution from SPE and subsequent trapping at the top of the LC column. This setup gave a good chromatographic resolution and peak shapes. The final conditions used for LC–MS are reported in Section 2.

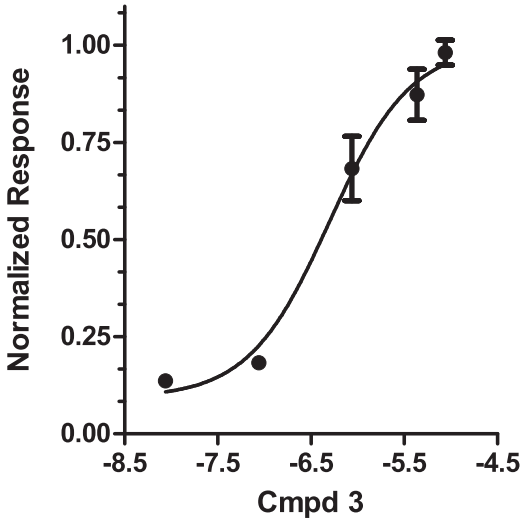

Fig. 5. Titration experiment for the binding reaction between AChBP and cmpd **3**.

Prevention of false negatives and false positives is one of the most challenging aspects of affinity selection methods [4,16]. False positives are related to non-specific binding of the ligand, while false negatives are due to either excessive washing steps, poor ligand detection limits, or ligand losses due to insufficient retention in the SPE step. These problems have been addressed by carefully optimizing the washing steps, by always using the competitive experiment, and by verifying the SPE retention for the tested compounds. The washing of the protein–ligand-bead complex trapped with the magnet is intended to remove non-specific binders from the receptor, PEEK tubing and beads, while binders should remain on the bead immobilized AChBP. The buffer used should be compatible with AChBP and able to efficiently wash away non-specific binders. In this respect, we selected NH_4HCO_3 (pH 7.4) with 10% of MeOH as washing buffer since it has previously proved to be efficient [4]. The amount of buffer needed to wash away the non-binders while maintaining most of the ligand on the receptor was optimized by measuring the retention of cmpd **1** in the absence of receptor and with various volumes of NH_4HCO_3 buffer. Under such conditions, the measured signal could only be attributed to non-specific retention on tubing and beads. The results obtained are presented in Fig. 3. Surprisingly, the non-specific retention of cmpd **1** on the beads was found to be around 25% with 400 μL of buffer, which is significantly higher than what was observed previously (<10% [4]). The non-specific binding to the beads was found to be compound dependent and is probably related to the chelating properties of the beads, especially with ionized basic compounds. The final conditions selected for this washing step were 800 μL of a 10 mM NH_4HCO_3 buffer at pH 7.4 containing 10% MeOH at a flow rate of 200 μL/min. Indeed, a higher volume would result in loss of bead immobilized AChBP due to the slow migration of trapped beads even at low flow rate [4].

Competitive experiments were performed to prevent false positives due to non-specific binding and were consequently carried out for every compound or mixture tested. In these experiments, AChBP was incubated with a mixture of a potential ligand (1 μM) and epibatidine, a strong AChBP binder ($pK_i \sim 9$), at a concentration of 10 μM. As a result of complete binding site competition, epibatidine fully displaces potential ligands from the receptor so the remaining ligand signals can be assigned to non-specific binding properties. As an example, the signals measured in the competitive

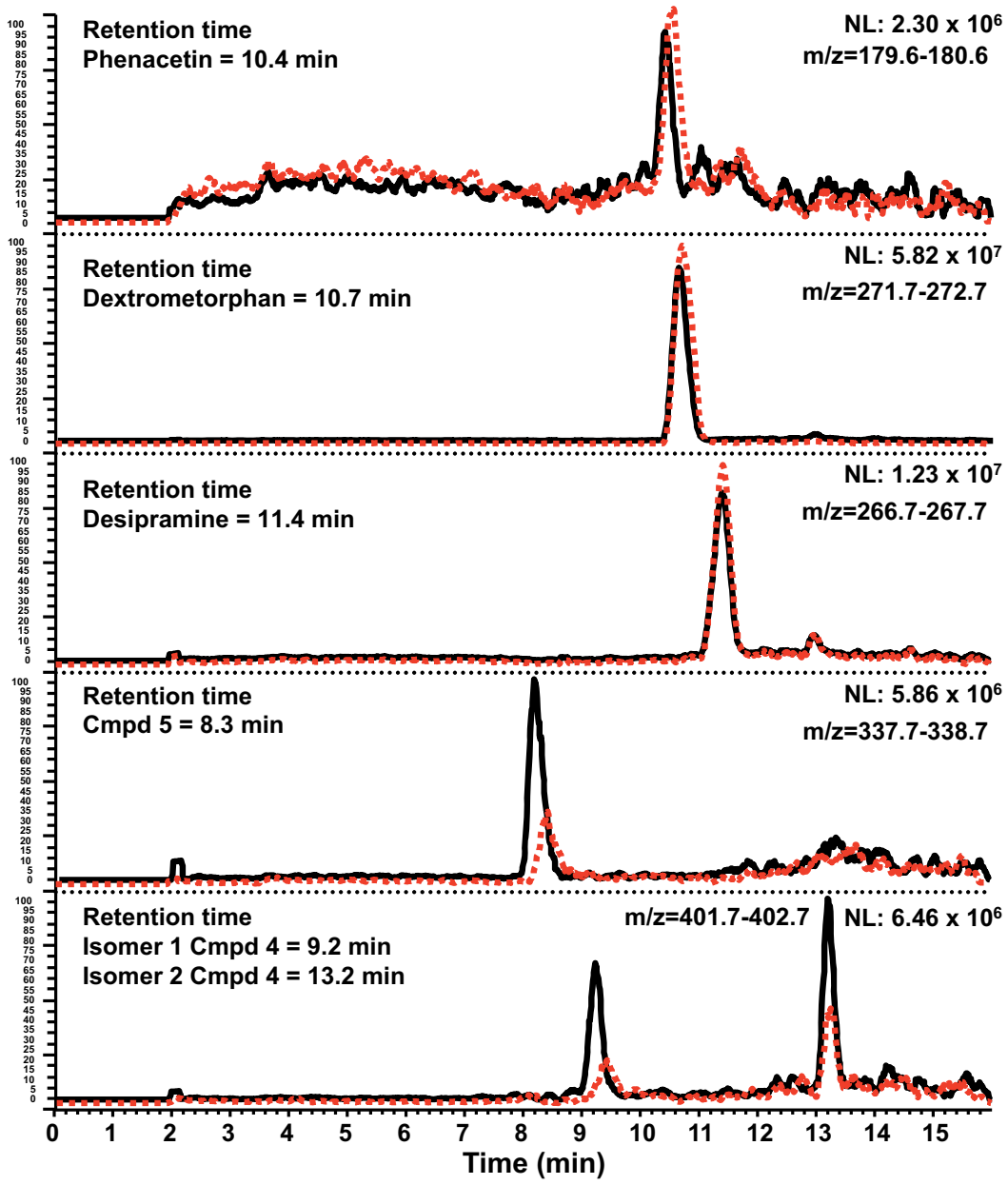

Fig. 6. Overlaid EICs of the binding and corresponding epibatidine displacement experiments of all compounds (binders and non-binders) present in the mixture (plain black curve) is without epibatidine and (red dotted line) is with epibatidine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

experiments for cmpd **2** (pK_i 7.38) and the non-binder cetirizine were 21 ± 4% and 94 ± 2%, respectively, compared to the actual binding experiments. These results demonstrated the feasibility of detecting only ligands as binders and allowed us to proceed to the actual screening of several ligands and non-binders.

3.3. Screening experiments towards the AChBP

3.3.1. Pure compounds

For demonstration of screening experiments, seven AChBP ligands (cmpd **1**–**7**) were selected from our in house library with pK_i values ranging from 6.26 to 8.46. Additionally, 4 non binders were incorporated in the analyses (dextromethorphan, desipramine, cetirizine and diclofenac). First, all compounds were analyzed with standard SPE LC–MS experiments under the same conditions as used during the actual dynamic protein affinity selection procedures to verify retention and assess sensitivity. All compounds were successfully retained on SPE under the conditions used and subsequently detected by LC–MS with sufficient sensitivity. Then, we moved to the actual screening experiments. Each experiment was performed in triplicate and both in the absence (binding experiments) or in the presence (competitive experiments) of 10 μM epibatidine. As typical example of a real binding experiment with the ligand cmpd **3**, Fig. 4A displays the two chromatograms obtained. The dotted line extracted ion chromatogram (EIC) represents the non-specific binding of cmpd **3**. The drawn line EIC represents the total binding of cmpd **3**. In the competitive experiment, the signal of cmpd **3** is clearly lower in the presence of epibatidine. From the peak areas, the relative non-specific binding can be calculated, which was 6 ± 8%. In Fig. 4B, similar EICs are shown for the non-binder desipramine. In this case, no diminution of the peak area is seen with the non-binder desipramine in presence of epibatidine, thus demonstrating that this compound is a non-binder. The results for all compounds tested are presented in

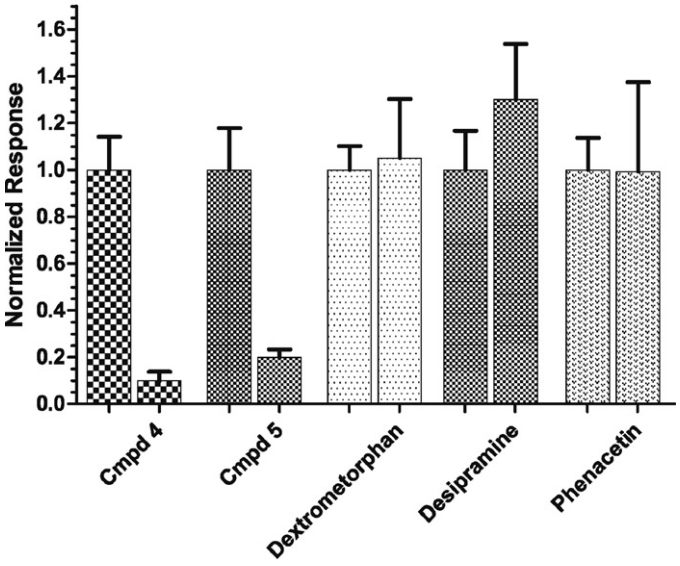

Fig. 7. Results of each compound for the screening experiments as a mixture represented as a bar graph. For each compound the left bar represents the response for the experiments without epibatidine normalized to the signal mean, while the right bar represents the normalized response of the competition experiments with epibatidine.

Fig. 4C; the peak areas from all the binding experiments were normalized. For this, the average signal of the binding experiments for each compound tested was set at 100%. The figure clearly shows that the average signal of all binders (cmpd **1**–**7**) is significantly lowered in the presence of epibatidine, whereas the average signal for all non-binders is not. This allows for a clear distinction between binders and non binders.

3.3.2. Concentration response dependency

To demonstrate concentration dependency of the binding of ligands, a titration experiment with cmpd **3** was performed at five (increasing) concentrations. In brief, the binding was performed by incubating cmpd **3** (10^{-5} to 10^{-8} M) with AChBP. The peak areas from EICs were measured and corrected for the non-specific binding effects using the competitive experiments with epibatidine (10^{-3} M). Fig. 5 gives the data obtained showing a characteristic concentration response curve, as expected.

3.3.3. Mixture analysis

The sample throughput of this screening method could be improved by testing mixtures of compounds rather than just one compound at a time. This is especially valid because the current methodology is well suited for mixture analysis of, for example, parallel synthesized compound mixture libraries from medicinal chemistry programs. In order to evaluate the potential of mixture screening with the system, a mixture containing two ligands (cmpd **4** and **5**) and four non-binders (dextromethorphan, desipramine, phenacetin and sulfamethoxypyridazine) was prepared at compound concentrations of 1 μM. The experiments were performed in the same way as discussed for the pure compounds. The results, normalized for each compound in the same manner as discussed for the pure compounds, are presented in Fig. 6. The figure shows the overlaid binding and epibatidine displacement EICs for each compound present in the mixture analyzed. Comparison between peak areas in the EIC for binding and displacement experiments with ligands showed significantly higher peak areas for binding experiments. Such differences in peak areas were not observed for the tested non-binders. This clearly demonstrates the capacity of this method to screen for ligands in mixtures of compounds. As can be seen in Fig. 6, cmpd **4** eluted as two isomers, which was also demonstrated by independent LC–MS and ^{1}H NMR analysis (data not shown). Finally, Fig. 7 summarizes the results for the analysis of the mixture in a bar graph. Again, the normalized results are shown for the binding and displacement experiment for every compound in the mixture; binding was only observed for the two binders in the mixture. As the methodology uses MS as a detector, the methodology could also be applied for analysis of mixtures of unknown compounds, such as metabolic mixtures or natural extracts, providing compound identification from MS data.

4. Conclusions

This work describes the development of an online magnetic bead based affinity selection methodology followed by SPE LC–MS analysis for ligand fishing in mixtures towards AChBP affinity. The sequence of operation is as follows: After in solution incubation of the His-tagged AChBP with (mixtures of) potential ligands, the AChBP ligand complexes are fished out of solution using IMAC based paramagnetic beads. The resulting suspension is injected into the online SPE LC–MS system. The bead complexes are trapped in the tubing by an adjustable magnet, while non-binders are sent to waste via a washing step. The protein–ligand complex is disrupted and the released ligands are sent to an SPE cartridge. Finally, the ligands are eluted from the SPE cartridge to LC–MS for analysis. The advantage of this affinity selection methodology is the capability of in solution incubation, while the separation step in which binders are separated from non-binders is performed in an efficient way by means of an immobilized target protein. Furthermore, magnetic beads are excellently suitable as mobile/online transportable affinity SPE material that can be used for selective ligand extraction in any type of sample container and from there be extracted/collected for further online analysis like in our current system. The results obtained with AChBP as target protein demonstrated reliable discrimination between binders with pK_i values ranging from 6.26 to 8.46 and non-binders. The sensitivity of this method, thus also enabling the detection of weak binders, could probably be improved by adjusting the washing volume used to remove the non-binders. This methodology proved to be effective for analyzing mixtures containing both binders and non-binders. This feature was evaluated for mixtures containing 6 compounds including two binders (pK_i 7.05 and 7.21). Since few hit compounds are present in larger libraries, it is expected that this methodology could be extended to screen such libraries by using more complex mixtures. This technique could also be used to fish and identify unknown structures as ligands by MS. This is particularly interesting for the screening of metabolic mixtures, natural extracts, or combinatorial libraries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.04.023.

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