Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

Simultaneous Multiple MS Binding Assays Targeting the Monoamine Transporters hDAT, hNET, and hSERT

Patrick Sebastian Neiens

aus

München, Deutschland

2017

Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Professor Dr. Klaus T. Wanner betreut.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, 30.11.2017

P. Neiens

Dissertation eingereicht am 30.11.2017

1. Gutachter: Professor Dr. Klaus T. Wanner

2. Gutachter: Professor Dr. Franz Bracher

Mündliche Prüfung am 20.12.2017

Die vorliegende Arbeit entstand in der Zeit von Januar 2013 bis November 2017
am Department Pharmazie – Zentrum für Pharmaforschung – der
Ludwig-Maximilians-Universität München auf Anregung und unter Leitung von

Herrn Professor Dr. Klaus T. Wanner

Für die hervorragende und sehr engagierte Betreuung und Förderung meiner
Arbeit sowie die ausgezeichneten Forschungsbedingungen danke ich Herrn
Professor Dr. Klaus T. Wanner sehr herzlich.

Herrn Professor Dr. Franz Bracher danke ich herzlich
für die Übernahme des Korreferats.

Danksagung

Mein Dank gilt allen aus meinem beruflichen und persönlichen Umfeld, die mich während meiner Promotion begleitet haben.

Besonderer Dank geht an Georg. Auf deine Unterstützung konnte ich mich in den letzten Jahren stets verlassen. In unseren vielen Gesprächen und – teils leidenschaftlichen – Diskussionen konnte ich mich auf dem Gebiet der Analytik, wissenschaftlich im Allgemeinen und auch persönlich weiterentwickeln.

Bei Steffi und Sebi möchte ich mich dafür bedanken, dass ihr mir in meiner Anfangszeit im Arbeitskreis (und auch bis heute noch) für all meinen Fragen zur Verfügung standet. Mit euch hatte ich eine wunderbare Zeit im Labor, ich hatte eine Menge Spaß und ich war noch nie so gut über gewöhnungsbedürftige Musik oder das Leben diverser „Promis“ informiert. Außerdem danke ich Gerd für seine Betreuung bei meinen Syntheseversuchen sowie der Aufreinigung meiner Substanzen, für die Versorgung mit Markern und internen Standards und für unseren Gesprächen über das Reisen.

Des Weiteren geht mein Dank an meine Post-Doktorandin, Bacheloranten und Praktikanten Angela, Anna, Judith, Jan und Markus dafür, dass ihr motiviert und engagiert mit mir zusammen im Labor gearbeitet haben und ich durch euch neue Sichtweisen auf dieses Projekt gewinnen konnte.

Ich danke meinen Freunden und Kollegen aus dem Arbeitskreis von Professor Dr. Wanner für die tolle Aufnahme in diese Gruppe, die ausgezeichnete Arbeitsatmosphäre, die gute Zusammenarbeit in der Praktikumsbetreuung und die vielen fröhlichen Stunden, die wir neben der Arbeit hatten. Insbesondere möchte ich mich bei Simone, Lars, Jörg und Thomas für die gemeinsame Zeit, die Diskussionen über Gott und die Welt und die Rätselei in den Mittagspausen, das sommerliche Eisessen-Gehen, die Fernsehabende nach der Arbeit, die Gespräche und die geteilte Begeisterung über die neusten technischen Spielzeuge aber auch für das Zuhören und eure Ratschläge, wenn es mal nicht so rund lief, bedanken.

Meinen Eltern danke ich dafür, dass ihr euer Möglichstes zu meinem bisherigen Werdegang beigetragen habt. Eure Unterstützung während der Schulzeit und des Studiums hat mir erst den Weg zu dieser Promotion ermöglicht. Der familiäre Zusammenhalt durch euch beide und dich, Silva, hat mir immer den Rücken freigehalten, so dass ich mich auf das Wesentliche konzentrieren konnte.

Zuletzt möchte ich mich noch von ganzem Herzen bei meiner Freundin Vanessa bedanken. Du warst immer für mich da, um mich nach Rückschlägen aufzubauen, aber auch um dich mit mir über Erfolge zu freuen. Ich freue mich auf alle weiteren Erfolge, die wir gemeinsam feiern werden.

This cumulative thesis is based on the following original publications

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Patrick Neiens, Georg Höfner, and Klaus T. Wanner; *Chirality* **2017**, 29:294 – 303.

"Determination of the enantiomeric purity of the selective dopamine transporter inhibitor (+)-*R*,*R*-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol"

Patrick Neiens, Angela De Simone, Anna Ramershoven, Georg Höfner, Lars Allmendinger, and Klaus T. Wanner; submitted to *Biomedical Chromatography*

"Development and validation of an LC-ESI-MS/MS method for the quantification of D-84, reboxetine, and citalopram for their use in MS Binding Assays addressing the monoamine transporters hDAT, hSERT, and hNET"

Patrick Neiens, Angela De Simone, Georg Höfner, and Klaus T. Wanner; submitted to *ChemMedChem*

"Simultaneous Multiple MS Binding Assays for the dopamine, the norepinephrine, and the serotonin transporter"

Parts of this thesis have also been presented at international conferences

Poster presentation at the “SFB35 Symposium” 2014, Vienna

Patrick Neiens and Klaus T. Wanner

“Determination of the chemical and enantiomeric purity of D-84”

Poster presentation at the “Annual Meeting of the German Pharmaceutical Society – DPhG” 2016, Munich

Patrick Neiens, Angela De Simone, Georg Höfner, and Klaus T. Wanner

“Simultaneous MS Binding Assays for Monoamine Transporters”

Short presentation at the “DPhG – International PhD Students Meeting” 2017, Frankfurt am Main

Patrick Neiens

“Multiple MS Binding Assays for Monoamine Transporters”

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1. Introduction

1.1. Screening Tools in Drug Discovery

For a long time, new drug substances were found by chance before they were used in the therapy of diseases. Important examples of such coincidentally found drug substances, which are still in use are salicylic acid (administered as acetylsalicylic acid), morphine, and penicillin, just to name a few. With the advance of medicinal sciences, target-oriented screening techniques were employed in modern drug discovery. Typically, the binding of a ligand towards a target molecule is observed. These screening techniques are important tools in the early to medium stages of the drug discovery process [1,2]. They can be divided into two groups based on what component of the ligand-target complex is investigated – either the change at the target, caused by the binding of the ligand, is measured, or the bound ligand is detected. By measuring the change at the target induced by ligand binding, information on the binding mechanism and the binding site can be gained, but such techniques can only be used with a single ligand and they have a limited throughput. With techniques that detect the bound compounds, ligands can be identified in mixtures of compounds with a high throughput, but no information on the binding interactions and binding mechanism can be obtained [3].

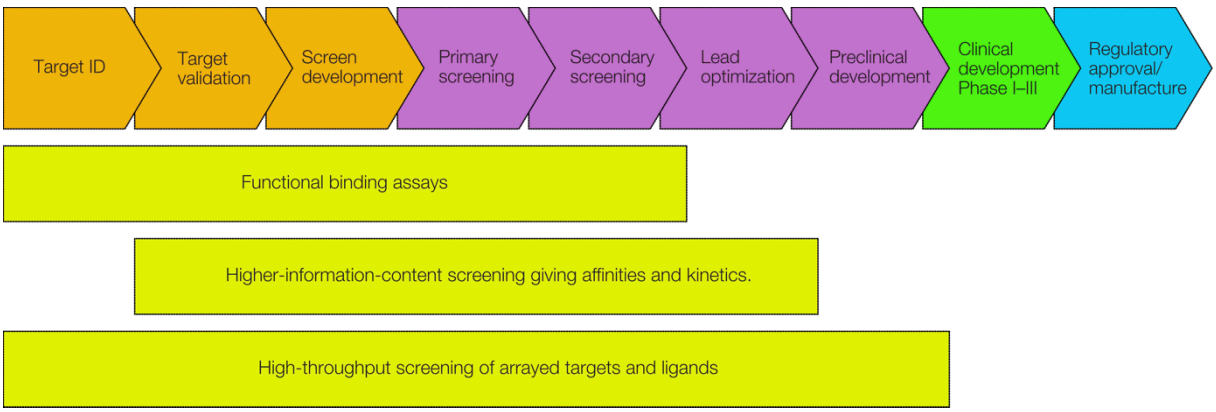

Figure 1: Simplified outline of the drug development process. Areas, in which binding assays can be applied are highlighted (adopted from Cooper [1]*).*

A schematic overview of the different stages in drug development is shown in figure 1. The first steps in contemporary drug discovery are the identification and validation of a target, that can be influenced in order to treat a disease. Afterwards, a strategy for the identification of ligands, which interact with the target, is established, since only such substances can influence the target and thus affect the progression of a disease. In multiple screening and optimization steps, mediators for the target are identified and developed, until a prospective new drug substance, which can then proceed to the clinical development, is found. Pharmacological assays are an essential tool in all stages of the drug development process up to the clinical development. In contrast to functional assays, monitoring a change in the biological response, induced by a test compound, binding assays are based on the binding of the test compound. In such binding assays, the bound ligand for a given target is quantified or identified. Various assays, based on the affinity of a ligand towards a target have been developed [3–8]. The readout of these assays can either be done by optical sensors or mass spectrometry (e.g. automated ligand identification system (ALIS), multitarget affinity/specificity screening (MASS)) [9–12]. A powerful and very versatile approach to quantify the bound ligand at a target, are radioligand binding assays [2,5,13,14].

1.1.1. Radioligand Binding Assays

Radioligand binding assays have been developed in the 1970s to analyse ligand-target binding [15]. They are based on a radioactively labelled reporter ligand, the so-called radioligand, which binds towards the target of interest and can be quantified by means of scintillation counting. Radioisotopes used for the synthesis of radioligands are commonly ^{3}H, ^{125}I, or ^{32}P. Compared to alternative labels, such as bulky fluorophores, necessary for fluorescence-based assays, the inclusion of radioisotopes only has negligible effect on the binding affinity of the radioligand towards its target. With different assay types (see 1.1.3 Ligand Binding Experiments) a variety of information about the target, the binding affinity and kinetics of the radioligand or the binding affinities of test substances can be obtained. Besides the above-mentioned benefit of only minor alteration of the reporter ligand by the insertion of the label, radioligand binding assays have further advantages. Commercial availability of a large number of highly affine and selective radioligands for various targets, enables the user to quickly set-up a radioligand binding assay. The readout by scintillation counting is selective for the

radioisotope and has a high sensitivity. Drawbacks are mainly associated with the use of radioactivity, which results in potential health hazards for the operators if the assays are not performed with care, expensive licences for the authorisation to work with radioactive materials, as well as high costs for the purchase of the radioligands and disposal of the radioactive contaminated waste.

To perform a radioligand binding assay, the target protein is incubated with the radioligand and – if needed for the experiment – an inhibitor. After the incubation, the unbound radioligand is separated from the radioligand-target complexes, which can be achieved by filtration or centrifugation (see figure 2). In radioligand binding assays, the bound reporter ligand is then quantified by scintillation counting.

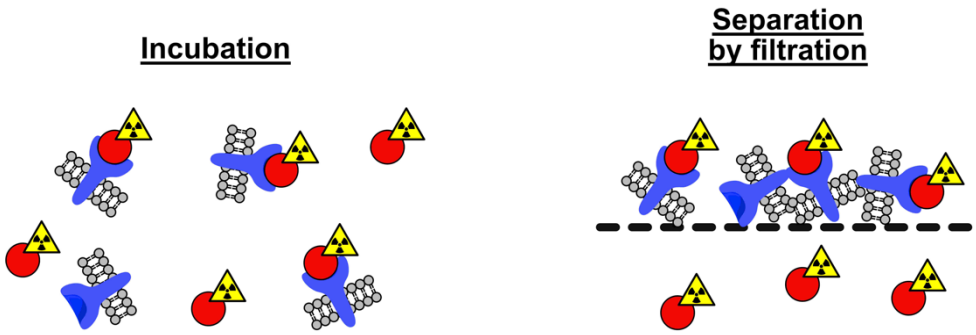

Figure 2: Experimental procedure of a radioligand binding assay. The target (blue) is incubated with the radioligand (red spheres). For the separation of the radioligand-target complexes from the unbound radioligand, the sample is filtrated (filter is represented by dotted line). Afterwards, the bound radioligand is quantified by scintillation counting of the filter with the radioligand-target complexes.

1.1.2. MS Binding Assays

As an alternative to radioligand binding assays, MS Binding Assays have been developed for a variety of different targets [16–22]. In contrast to radioligand binding assays, the quantification of the bound reporter ligand, which is called MS Marker or simply marker in MS Binding Assays, is done by liquid chromatography-mass spectrometry (LC-MS). Since the marker can be selectively detected by tandem mass spectrometry (MS/MS) based on the mass/charge-ratio (*m/z*) of its ionized species and specific fragment ions, no label is necessary.

Thus, the native compound can be used, which results in some advantages. Commercial availability of native compounds is much better, than for radiolabelled compounds. Additionally, the cost for the purchase of such native compounds is usually lower than for its radiolabelled counterparts. Due to the absence of a label, there is no effect on the binding affinity and kinetics, compared to (especially fluorophore-) labelled ligands. Further advantages originate from the avoidance of radioactivity in MS Binding Assays. The implementation of MS Binding Assays can be done in any suitably equipped chemical laboratory, without the need for special authorisations and risk of health hazards from radioligand binding assays. Arising waste can be treated like normal laboratory waste, without additional costs and ecological issues associated with the disposal of radioactively contaminated waste. The principle of MS Binding Assays is as simple, robust and flexible, as the one of radioligand binding assays.

For the use in MS Binding Assays, there are some requirements concerning the marker. First of all, like in radioligand binding assays, the reporter ligand should label the target of interest with a high affinity and selectivity. A low affinity is typically associated with a fast dissociation of the reporter ligand-target complexes, which results in problems during the separation of the reporter ligand-target complexes from the unbound reporter ligand [23]. Filtration and subsequent washing of the reporter ligand-target complexes takes a few seconds, in which the reporter ligand-target complexes should not dissociate. For reporter ligands with an affinity in the low nanomolar (nM) range, reporter ligand-target complexes are stable enough during the time required for the filtration and extensive washing. Contrary to this, too high affinity of the marker results in issues concerning the quantification of the bound marker in MS Binding Assays. Since free marker concentrations of 0.1 of the equilibrium dissociation constant (K_d) should be analysable and the target concentration should be not higher than 0.1 K_d (see 1.1.3 Ligand Binding Experiments), according to the Langmuir adsorption model (1, for further discussion of this formula, see 1.1.3 Ligand Binding Experiments)

$$[\mathrm{LT}] = \frac{[\mathrm{L}] \times B_{\max}}{[\mathrm{L}] + K_d} \quad (1)$$

marker concentrations of at least 0.0091 K_d have to be reliably quantified [19].

$$[\mathrm{LT}] = \frac{0.1\ K_\mathrm{d} \times 0.1\ K_\mathrm{d}}{0.1\ K_\mathrm{d} + K_\mathrm{d}} = 0.0091\ K_\mathrm{d}$$

So far, quantification limits in the low picomolar (pM) range were achieved in quantification methods of MS Binding Assays, allowing the quantification of markers with a low nanomolar affinity for the target.

An additional requirement is a high ionization efficiency of the marker. Chemical compounds can differ extremely in their ionization behaviour, which has a huge impact on the LC-MS quantification limits. The ionization efficiency of the marker can be influenced by the chosen LC-MS method parameters, but a low ionization efficiency can result in the unsuitability of the marker for an MS Binding Assay.

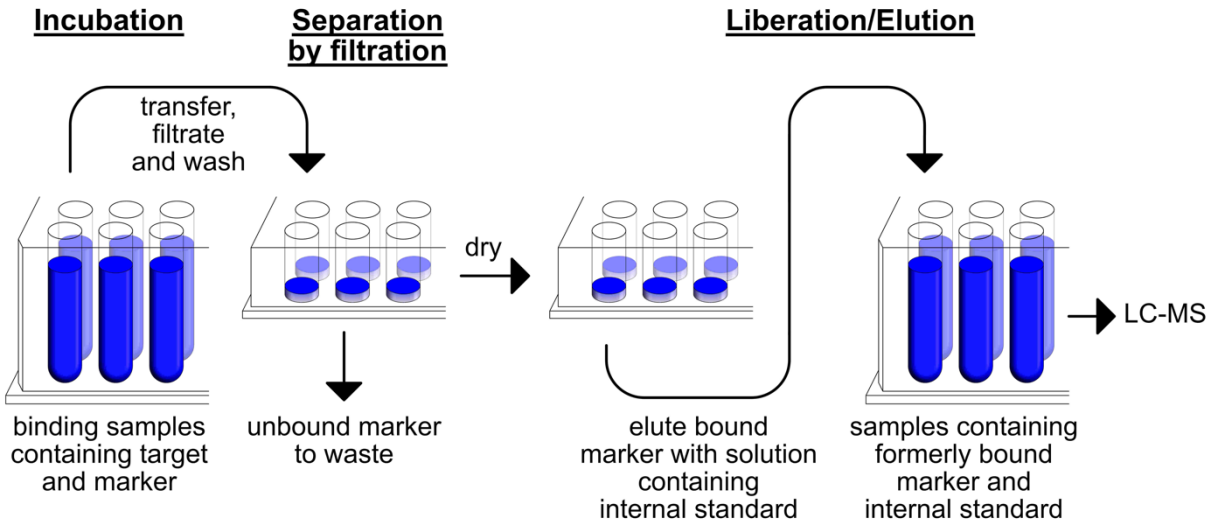

Figure 3: Experimental procedure of MS Binding Assays. The marker is incubated together with the target. After the incubation, the unbound marker is separated from the marker-target complexes by filtration. To denature the target proteins, the filters are dried prior to elution of the formerly bound marker.

MS Binding Assays are performed very similar to radioligand binding assays (see figure 3). Incubation of the marker with the target, as well as the separation of the marker-target complexes from the unbound marker is done analogous to radioligand binding assays. To quantify the bound marker in MS Binding Assays, the marker has to be liberated from the target protein. This can be achieved by denaturation of the target protein under the influence

of heat and an organic solvent. Finally, the organic solvent is used to elute the liberated marker from the filter. The obtained solution containing the formerly bound marker is then analysed by LC-MS.

1.1.3. Ligand Binding Experiments

With radioligand as well as MS Binding Assays, a variety of binding experiments can be performed. They can be divided into three subclasses, i.e. saturation, as well as competitive experiments and kinetic studies. All three assay types are based on the same experimental protocol, described above (see 1.1.1 Radioligand Binding Assays and 1.1.2 MS Binding Assays). Since the specific binding of the reporter ligand towards the target cannot be determined directly in binding experiments, the nonspecific binding (NSB) and the total binding (TB) have to be measured instead. Nonspecific binding is the amount of reporter ligand, that is bound to nonspecific binding sites in the binding sample, such as lipid membranes and non-target-proteins in the target preparation, or materials used to carry out the binding experiment, e.g. vessels used for the incubation or filter materials required for the separation of reporter ligand-target complexes and unbound reporter ligand. The sum of the nonspecifically and specifically bound reporter ligand is the total binding. Hence, the specific binding can be calculated as the difference between total binding and nonspecific binding (2).

$$\mathrm{SB} = \mathrm{TB} - \mathrm{NSB} \tag{2}$$

Since saturation and competition experiments were performed in this work, only these two types of binding experiments will be discussed in the following in detail.

Saturation Experiments

In saturation experiments, the affinity of the reporter ligand towards the target – the equilibrium dissociation constant (K_d) – can be determined. For this purpose, a defined target concentration [T] is incubated together with the reporter ligand [L], which is employed at different concentrations. The concentrations of the reporter ligand should cover a range of at

least 0.1 K_d to 10 K_d to ensure, that the resulting saturation isotherm is well defined by the obtained data points. Furthermore, to avoid reporter ligand depletion, the target concentration should not exceed 0.1 K_d [23]. Reporter ligand depletion is an effect, where a substantial fraction of the employed concentration of the reporter ligand is bound to the target and therefore not free in solution. For the calculation models, the concentration of free reporter ligand is required, but for reasons of simplicity, the nominal concentration can be used as long as the marker depletion is below 10 %. The incubation time should be chosen long enough to guarantee that the association of the reporter ligand-target complexes (k_{+1}) is in equilibrium with the dissociation of the reporter ligand-target complexes (k_{-1}).

Additionally to the samples for the determination of the total binding, a set of samples for the nonspecific binding are needed. In these samples, the target and the different reporter ligand concentrations are typically incubated in presence of a large excess of a highly potent inhibitor for the specific binding site, but also other options to avoid specific binding, such as denaturation of the target by heat shock are possible. Usually, the nonspecific binding can be described by a linear regression (see figure 4 a)). Based on the two data sets for the total and the nonspecific binding, the specific binding can be calculated according to the equation, mentioned above (see formula 2).

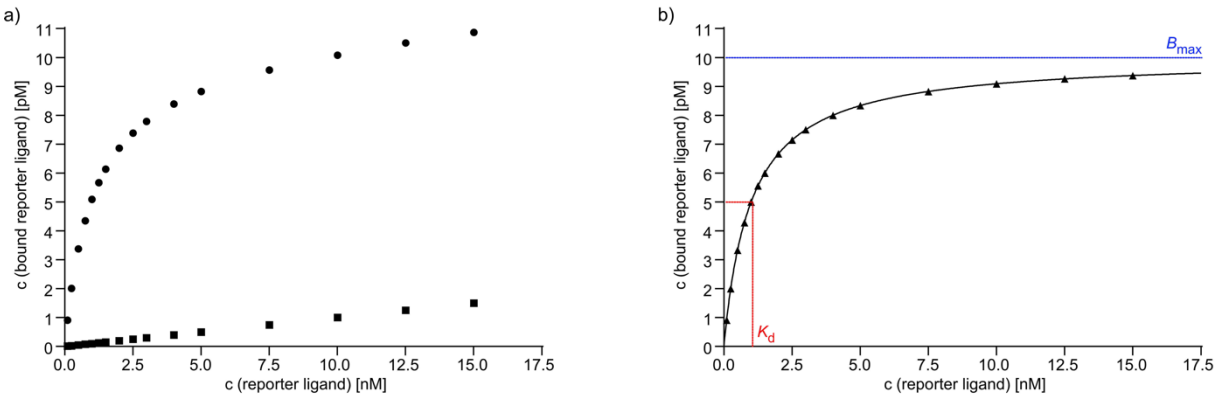

Figure 4: Simulated data for a saturation experiment. a) Concentration of bound reporter ligand is plotted against employed nominal concentration of the reporter ligand for nonspecific binding (■) and total binding (●). b) Saturation isotherm for specific binding resulting from data shown in a).

From the saturation isotherm, which is defined by the Langmuir adsorption model (equation 1), the affinity of the reporter ligand and the maximum amount of binding sites (B_{max}) can be derived. The B_{max} is represented by the plateau of the saturation isotherm. At this level, all specific binding sites are occupied by the reporter ligand. The equilibrium dissociation constant (K_d, see figure 4 b)) is defined by the reporter ligand concentration, which results in a target occupancy of 50 %.

Competition Experiments

To evaluate the binding affinities of large numbers of substances, competitive experiments are a powerful tool. In these experiments, the displacement of the reporter ligand from the target caused by the addition of a competing test substance, is analysed. To perform competitive experiments, different concentrations of the test substance [C] are incubated with the target in presence of a defined concentration of the reporter ligand (see figure 5). At the equilibrium state, both the reporter ligand and the test substance are in competition for the specific target binding sites, which can be expressed by equation 3.

$$[\mathrm{L}] + [\mathrm{C}] + [\mathrm{T}] \rightleftarrows [\mathrm{LT}] + [\mathrm{CT}] \tag{3}$$

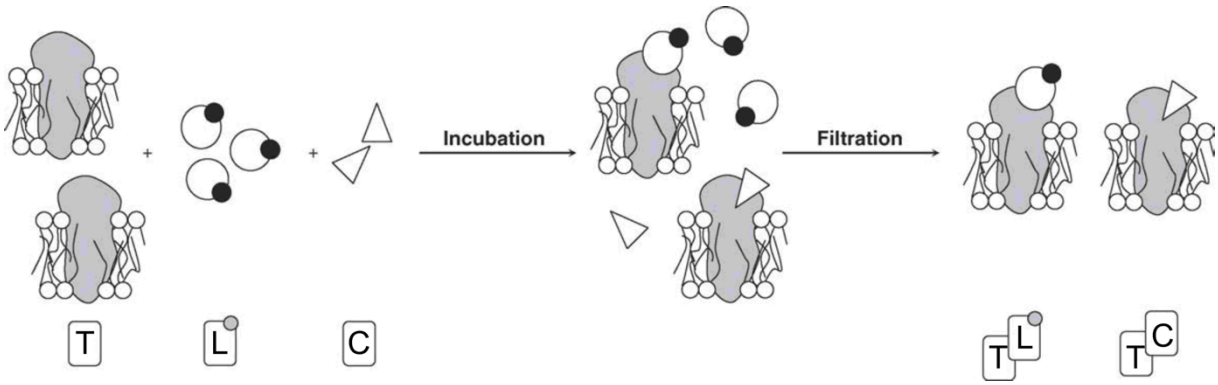

Figure 5: Schematic depiction of a competition experiment. The target (T) is incubated together with the reporter ligand L and a competing test substance (C). After the incubation, reporter ligand-target complexes and competitor-target complexes are separated from the unbound reporter ligand and competitor by a filtration step (adopted from de Jong [2]*).*

At increasing concentrations of the test substance, the reporter ligand will be gradually displaced from the complexes with the target and competitor-target complexes will be formed. In competition experiments, the affinity of the test substance is determined indirectly by quantification of the bound reporter ligand. An important benefit of this procedure is the circumstance, that it is not required to quantify the bound test substance. This means, that in radioligand binding experiments, unlabelled compounds can be used as test substances and in case of MS Binding Assays, only one quantification method for the marker is needed instead of quantification methods for each employed test compound.

After the incubation of the test compound with the target and the reporter ligand, the reporter ligand-target complexes are separated together with the competitor-target complexes from the unbound reporter ligand and competitor. The data from the quantification of the bound reporter ligand allows the construction of a competition curve as shown in figure 6. The left side of the competition curve is fixed to 100 % which represents the concentration of bound reporter ligand without the addition of the competitor. On the other side of the competition curve, the amount of nonspecific reporter ligand binding is defined as 0 % specifically bound reporter ligand. The inhibitory effect of the test substance is described by the IC_{50}-value, the concentration of the competitor, which reduces the specifically bound marker to a level of 50 % (see figure 6).

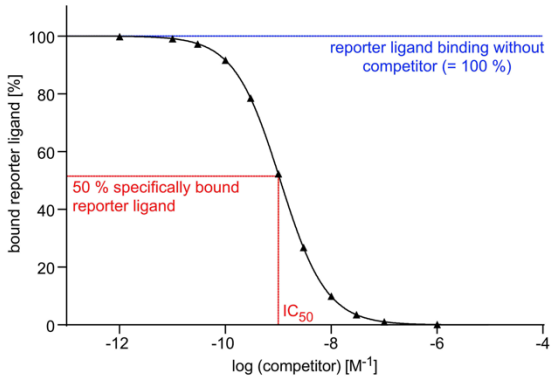

Figure 6: Simulated data for a competition experiment. Concentration of bound reporter ligand is plotted against employed concentration of a competitor.

To calculate the affinity of the test substance, the IC_{50}-value has to be transformed into the inhibition constant K_i with the Cheng-Prusoff equation (4) [24].

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}} \quad (4)$$

For this equation, the K_d of the reporter ligand has to be known. Similar to equation (3) for the saturation experiments, [L] represents the free concentration of the reporter ligand, which can be substituted by the nominal concentration of the reporter ligand, as long as the marker depletion is negligible. Since the inhibition constant K_i is independent from binding affinity of the used reporter ligand and its concentration in the binding experiment, it allows the comparison of binding affinities of different test substances, determined in different binding assays.

Saturation experiments are important to characterize the binding affinity of a reporter ligand towards a target. The results from these experiments can then be used to set up competition experiments for the screening of large numbers of test compounds. Hence, these two binding experiments are effective tools for the characterization of the binding affinities of test compounds in the modern drug development process.

1.2. The Neurotransmitters Dopamine, Norepinephrine, and Serotonin

The three biogenic monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT, see figure 7 for the structures of the three monoamines) act as neurotransmitters in the human brain and periphery.

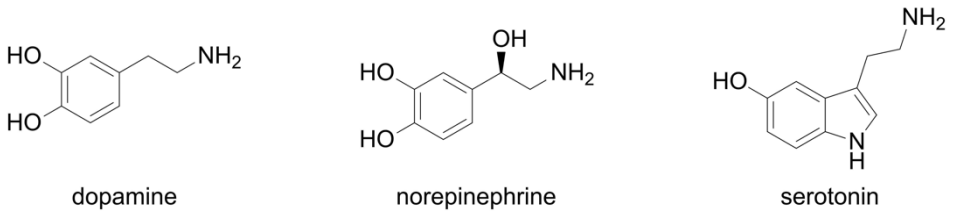

Figure 7: Structures of dopamine, norepinephrine, and serotonin

As neurotransmitters, these monoamines have distinct functions in different areas of the central nervous system (CNS).

1.2.1. Signal Transmission at Monoaminergic Neurons

The function of the mammalian CNS is based on neurons, which are able to receive and transmit the neuronal signals. This connection between neurons is usually achieved by neurotransmitters, like the above-mentioned monoamines. In the presynaptic neuron, the neurotransmitters are stored in vesicles, where they are protected from degradation (see figure 8 a) step 1). To transmit the neuronal signal from one neuron to another, the monoamines are released from vesicles in the presynaptic neuron into the synaptic cleft initiated by the influx of calcium ions as a response to an action potential arriving at the synapse (see figure 8 a) step 2) [25]. Due to the storage of the neurotransmitters in the vesicles, a fast release of high neurotransmitter concentrations can be achieved. In the synaptic cleft, the neurotransmitters can bind to receptors, located in the membrane of the postsynaptic neuron (see figure 8 a) step 3a). Most of the monoamine receptors are G-protein-coupled receptors, which are coupled to ion channels or enzymes. Only the serotonin receptor 3 (5-HT_3 receptor) is a ligand gated cation channel [26]. Activation of the receptors can lead to excitatory or inhibitory effects on the action potential of the postsynaptic neuron.

Furthermore, presynaptic G-protein-coupled monoamine receptors can reduce the release of the neurotransmitter, resulting in a negative feedback effect (see figure 8 a) step 3b). To terminate the signal transmission, there are two pathways for the removal of the released monoamines. They can either be degraded by the monoamine oxygenase (MAO) and the catechol-*O*-methyltransferase (COMT), or transported back into the presynaptic neuron by monoamine transporters (see figure 8 a) step 4). When transported back into the presynaptic neuron, the monoamines are taken up into secretory vesicles through vesicular transporters (see figure 8 a) step 5), where they are stored until the next release [26–28].

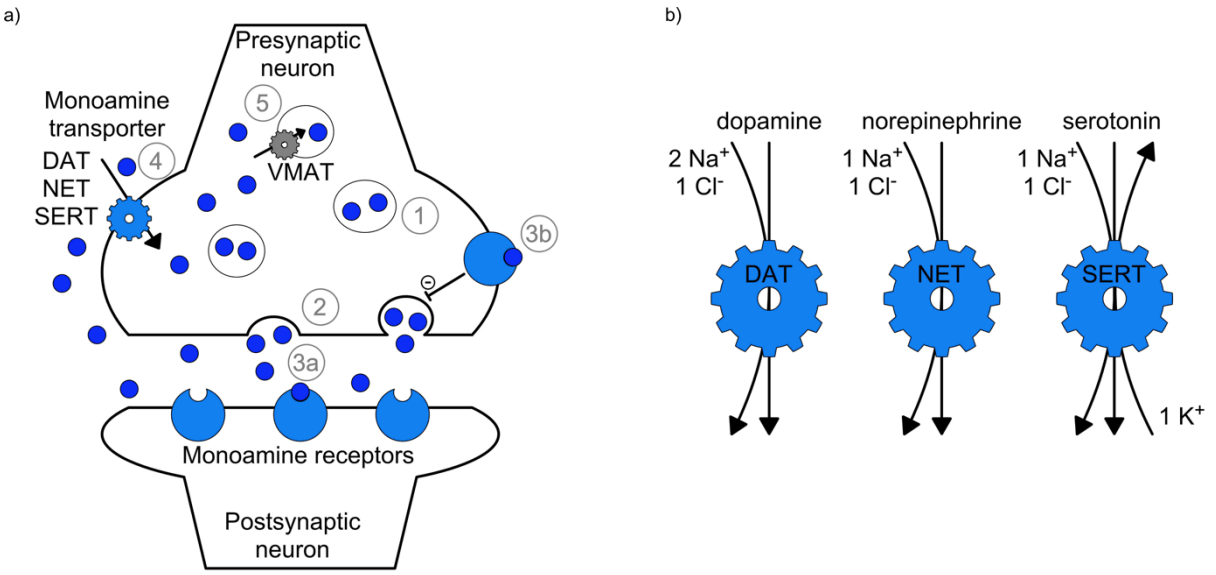

Figure 8: a) Schematic representation of the monoaminergic synapse. The monoamines (e.g. dopamine, norepinephrine, or serotonin) are stored in vesicles (1). Upon arrival of an action potential, the neurotransmitters are released into the synaptic cleft (2), where they bind to postsynaptic receptors (3a) or to presynaptic receptors, triggering a negative feedback effect (3b). To mediate neurotransmission, the monoamines are transported back into the presynaptic neuron by monoamine reuptake transporters (e.g. DAT, NET, or SERT) (4) and stored in vesicles again (5). b) Depiction of dopamine, norepinephrine, and serotonin transport, with stoichiometry of sodium and chloride symport, as well as potassium antiport. Figure adopted from Kristensen et al. [28].

1.3. Monoamine Transporters

The monoamine transporters are part of the solute carrier 6 (SLC6) family of transporters [29]. Besides the transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), the

SLC6 transporter family consists of 20 different transporters, among those are transporters for the neurotransmitters γ-aminobutyric acid (GABA) and glycine, as well as amino acids, betaine, taurine and creatine [30,31]. SLC6 transporters can be divided into subgroups, depending on the transported substrate. The neurotransmitter transporters (NTTs) consist of the three monoamine transporters, the GABA transporters, and glycine transporters. Since the transport of the neurotransmitters is based on a sodium gradient, the neurotransmitter transporters are also called neurotransmitter-sodium-symporters (NSS) in the "Transporter Classification System" from Milton Saier [31,32]. The cotransport of sodium ions enable the neurotransmitter transporters to transport the substrate against large concentration gradients [28]. In addition to the substrate and sodium, a chloride ion is transported into the cell by the monoamine transporters [33]. The stoichiometry of the transported substrate and ions differs for each of the three transporters. Generally, one substrate molecule is transported together with one sodium ion and one chloride ion. Differing from this, the DAT transports two sodium ions, while an additional potassium ion is transported from the SERT against the direction of the substrate (see table 1 and figure 8 b)) [33]. There is not much variance between the three monoamine transporters in the turnover rate, all three of them transport between 1 and 3 substrate molecules per second [28]. Affinities of the substrate towards the transporters and therefore the selectivity of the transporters for the substrate is different for each transporter. Only SERT is highly selective for its substrate serotonin. This can be explained by the structure of serotonin, which is very distinct, compared to dopamine and norepinephrine (see figure 7). The dopamine transporter has a slightly higher affinity for dopamine than for norepinephrine, while unexpectedly, the norepinephrine transporter also shows a lower affinity for norepinephrine than for dopamine (see table 1) [33].

Transporter	Substrate affinity K_i in µmol/L [a]			Turnover rate in molecules per s [b]	Substrate-ion stoichiometry (substrate : Na^+ : Cl^-) [b]
	DA	NE	5-HT		
DAT	6.40 ± 0.59	57.0 ± 13.0	549 ± 96	0.7 – 1.9	1 : 2 : 1
NET	28.0 ± 11.0	160 ± 33	360 ± 71	1.7 – 2.5	1 : 1 : 1
SERT	1110 ± 180	1470 ± 110	3.50 ± 1.20	1.0 – 3.1	1 : 1 : 1 (: 1 K^+ out)

Table 1: Characteristics of the monoamine transporters DAT, NET, and SERT. [a] Eshleman et al. [34]*. [b] Kristensen et al.* [28]*.*

1.3.1. Structure and Function of Monoamine Transporters

In recent years, X-ray structures of monoamine transporters were published. Until then, the crystal structure of the leucine transporter, expressed by the hyperthermophilic bacterium *Aquifex aeolicus* ($LeuT_{Aa}$), was used for studies on the structure and function of transporters from the SLC 6 family [28,35]. With a sequence identity of 20 – 25 % between the $LeuT_{Aa}$ and the monoamine transporters, the similarity of these two transporters seem to be quite low, but the homology at the substrate binding site is 76 % in the $LeuT_{Aa}$ and the human SERT (hSERT), which explains the good accordance of data, obtained from experiments with the monoamine transporter and from predictions based on the $LeuT_{Aa}$ crystal structure [28,36,37]. The elucidation of crystal structures for *Drosophila melanogaster* DAT (dDAT) and hSERT in the last years gave better insight into the structure and function of the monoamine transporters.

Crystal Structure of dDAT

In 2013, Penmatsa et al. published the crystal structure of dDAT (see figure 9) [38]. When extracted from the membrane with detergents, wild-type dDAT loses its ligand-binding activity and is therefore hard to crystallize. Crystallization was finally achieved by the insertion of five mutations, which resulted in a loss of dopamine-transport activity. The resolution of the X-ray structure was further enhanced by the creation of a complex of the transporter together with

an antibody fragment. In the crystal with nortriptyline, the transporter is in an outward-open conformation [38]. A later publication describes the crystallization of dDAT with nisoxetine and reboxetine bound to the transporter (see figure 10 a) and b)). Crystallization of the nisoxetine-dDAT was achieved with the previously published dDAT construct, containing five mutations (see figure 9 a)), while reboxetine-dDAT was crystallized with a dDAT construct with fewer mutations, which possessed a reduced dopamine-transport activity when compared to the wild-type [39]. In each case, the antidepressant is bound in the cavity halfway across the membrane bilayer, where it can only be accessed by solvent from the extracellular side of the transporter. The structure of the crystallized dDAT ($dDAT_{cryst}$) was in accordance with the predictions based on the crystal structure of the $LeuT_{Aa}$ ($LeuT_{cryst}$). While there are distinct differences in the periphery of structure of both transporters, the core of $dDAT_{cryst}$ closely resembles the one of $LeuT_{cryst}$ [38].

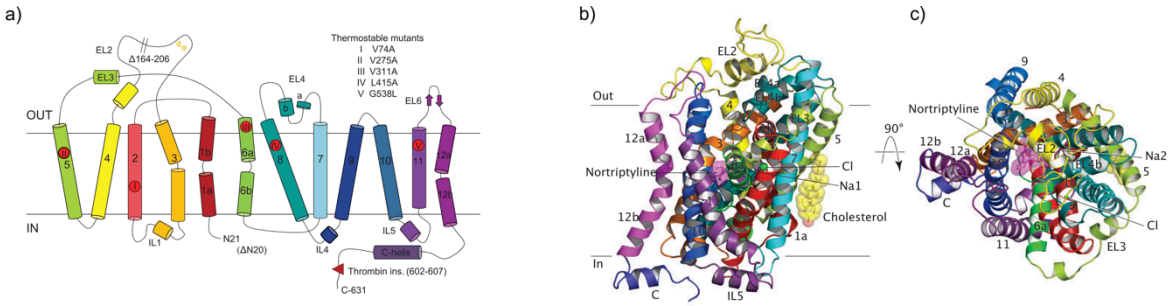

Figure 9: Crystal structure of dDAT as published by Penmatsa et al. [38]*. a) Schematic structure of dDAT. Mutations are labeled by red circles. b) Architecture of dDAT as viewed parallel to the membrane with bound nortriptyline, cholesterol, sodium and chloride ions. c) Architecture of dDAT as viewed from the extracellular face.*

Both transporters consist of twelve transmembrane helices (TMs) with intracellular N and C termini. TM 1, 3, 6, and 12 can be subdivided into two parts, connected by non-helical hinge-like regions (see figure 9). The arrangement of the TMs result in a C_2-pseudosymmetry regarding the helices 1 – 5 and 6 – 10, which is also referred to as the 5 + 5 inverted repeat fold [28,31,35,38,39]. As previously proclaimed in chimeric studies, the drug-binding site is localized between the helices 1, 3, 6, and 8, which corresponds to the position of the substrate-binding pocket in LeuT [40,41]. Close to the nortriptyline molecule in the crystal structure, three locations with high electron densities were identified in the non-helical

regions of TMs 1 and 6. Two of these locations are identical with the sodium ion-binding sites Na1 and Na2 of the LeuT-structure. The third location, proclaimed as the chloride ion-binding site is positioned between TMs 2, 6, and 7. Between TMs 5 and 7, a cholesterol molecule, modulating the movement of TM 1a, which takes place during the transport of the substrate, is positioned (see figure 9 b) and c)). In the crystal structure of dDAT, the ion and ligand binding sites are accessible to solvent from the direction of the extracellular side. To the intracellular side of the transporter, polar interactions are forming a barrier, so no solvent can reach the ligand binding site from this direction. The bound antidepressants in the crystal structures of dDAT sterically prevent a movement of TMs 1b and 6a into the direction of TMs 3 and 8, which is proclaimed to be necessary for closing of the extracellular gate of the transporter and thus the transport of the substrate (see figure 10 c)). This shows that in contrast to LeuT, where antidepressants bind to a noncompetitive binding site distant from the substrate binding site, the antidepressants stabilize the outward-open confirmation of the transporter in the DAT crystal structure, and thereby block it competitively [38,39].

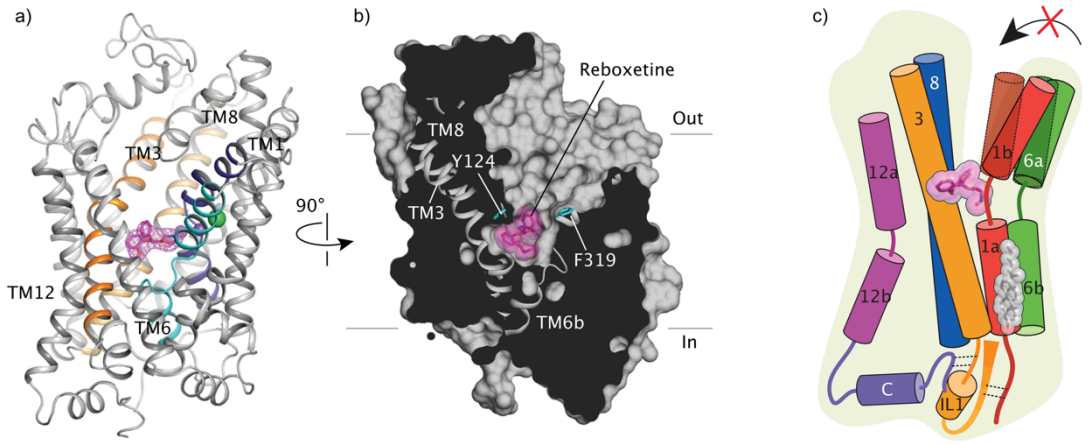

Figure 10: Inhibition of dDAT by antidepressants. a) Crystal structure of dDAT with bound reboxetine. b) Surface representation of dDAT with bound reboxetine (a) and b) adopted from Penmatsa et al. [39]*). c) Schematic representation of bound nortriptyline in the substrate binding site of dDAT and its mode of action. The bound antidepressant prevents movement of TMs 1b and 6a, which is necessary for substrate transport (adopted from Penmatsa et al.* [38]*).*

Crystal Structure of hSERT

The first crystal structures of a human serotonin transporter were published in 2016 by Coleman et al. (see figure 11) [42]. Together with a recombinant anti-SERT antibody fragment and two mutations in the hSERT, a crystal structure of the functional monoamine transporter with the bound antidepressant paroxetine was obtained. The introduction of an additional mutation resulted in a better resolution of crystal structures of the hSERT with bound paroxetine an (*S*)-citalopram, but also in a loss of transporter activity. According to the crystal structures of LeuT and dDAT, hSERT consists of twelve transmembrane helices, of which TMs 1 – 5 and 6 – 10 are arranged around a pseudo-two-fold axis (see figure 9 a)). A cavity between TMs 1, 3, 6, 8, and 10 represents the substrate binding site, in which the antidepressants are bound in the crystal structures. Additionally to the substrate, two binding sites for sodium ions (of which only one is transported) and one binding site for a chloride ion were identified in the same locations, as the ion binding sites in the dDAT. The two transmembrane helices 1 and 6 are interrupted by non-helical regions and contribute residues to coordinate a sodium and a chloride ion. As in the previously described dDAT crystal structure, the antidepressants lock the transporter in an outward-open configuration (see figure 10 c)) [42].

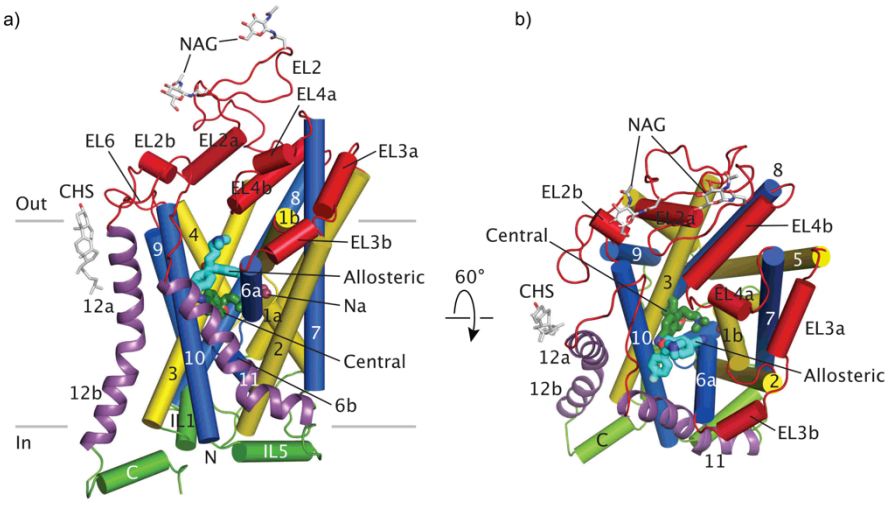

Figure 11: Architecture of hSERT with bound (S)-citalopram in central (dark green) and allosteric (cyan) binding site (NAG: N-acetylglucosamine, CHA: Cholesterol hemisuccinate). a) hSERT as viewed parallel to the membrane. b) hSERT as viewed from the extracellular face (adopted from Coleman et al. [42]*).*

In addition to the central ligand binding site, an allosteric binding site was found in the crystal structure of hSERT between TMs 1, 6, 8, 10 and extracellular loops (ELs) 4 and 6. A molecule of (*S*)-citalopram, bound to this allosteric binding site, slowed down the off-rate of the inhibitor, bound to the central binding site [42]. In summary, the crystal structure of hSERT confirms the findings of the crystal structure of dDAT. The allosteric binding site of hSERT, which was not identified in dDAT, can modulate the dissociation of the bound antidepressant in the central binding site.

Transport Mechanism of Monoamine Transporters

The mechanism of the monoamine transport is described by the alternating access model. This model states that the substrate binding site can be sealed off from the extracellular or intracellular side by the transporter. During the transport cycle, the transporter changes its confirmation, initiated by the binding of the substrate and the ions. The monoamine transporter can shuttle through at least three conformations, of which one is the outward-open conformation (captured in the above-mentioned crystal structures), the second is the occluded conformation in which the substrate binding side is not accessible from neither side of the transporter, and the third is the inward-open conformation, allowing the substrate to diffuse into the cell. The occluded conformation itself can be subdivided into an outward-facing occluded state, as well as an inward-facing occluded state (see figure 12). After the binding of the substrate and the ions in the central binding site, rearrangement of the TMs 1, 3, 6, 8, 2, and 10 results in the closing of the cavity to the extracellular face of the transporter. It has been proclaimed, that the non-helical regions in TMs 1 and 6 act as hinges, which enable the movement of the helical regions of these two TMs (see figure 10 c)). To release the substrate into the cell, TMs 1, 6, and 8 form a cavity to the intracellular side, through which the substrate can diffuse [28,31,43]. In an alternative model, TMs 1, 2, 6, and 7 form a bundle and the other 6 TMs a scaffold, in which the bundle can rock. This rocking movement is then responsible to close the transporter to each side of the membrane [31,44].

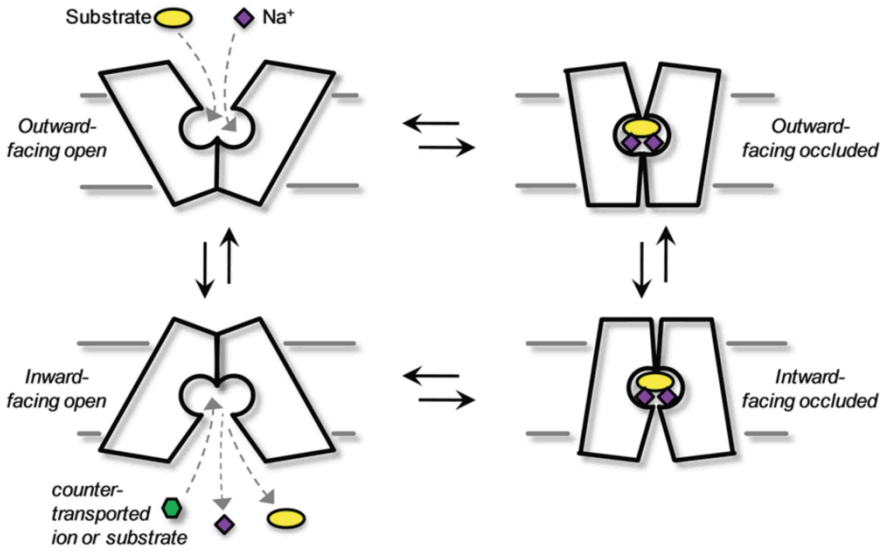

Figure 12: Schematic representation of the alternating access model for the transport of monoamines according to Kristensen et al. [28].

1.3.2. Localization, Physiological and Pathophysiological Implications

Expression of the monoamine transporters seems to be exclusive to their respective monoaminergic neurons. There they are present in dendrites and axons of the neurons, predominantly localized in the extrasynaptic regions [26,28,45–48]. Each of the monoamines and therefore also the monoamine transporter has distinctive effects on a variety of body functions, but they are all involved in the regulation of mood (see figure 13).

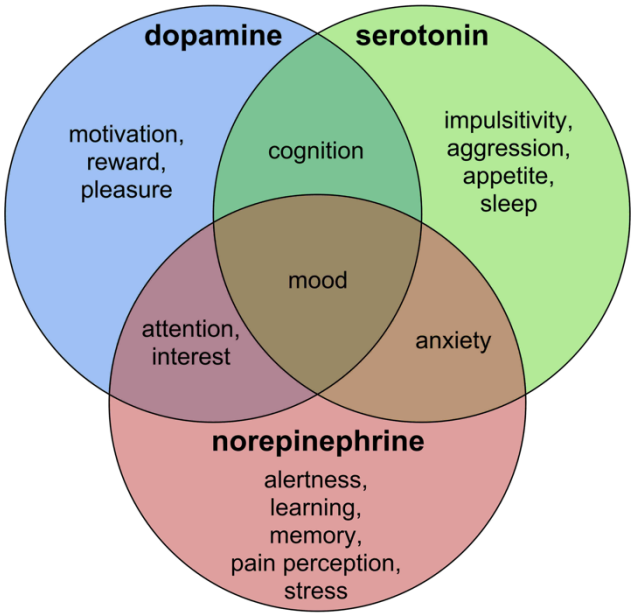

Figure 13: Areas of action for the monoamine neurotransmitters dopamine, norepinephrine and serotonin (adapted from Guiard [49]*).*

DAT

The dopamine transporter is mainly expressed in the substantia nigra of the brain, but it can also be found in the periphery, especially in the gut and the adrenal gland [33,45]. Movement, mood, pleasure, cognition, attention, and interest is mediated by the dopaminergic system [49,50]. In experiments with knockout mice, deficiency of functional dopamine transporters resulted in hyperactivity, cognitive deficits, disrupted sensorimotor gating and sleep dysregulation [51]. The lack of response to psychostimulants in these knockout mice supports the role of DAT in the mode of action of drugs as well as addiction behaviour [52,53]. This emphasizes the effect of the dopaminergic system on motivation and reward. Uptake of released dopamine is the major mechanism for the removal of this neurotransmitter from the synaptic cleft and therefore the termination of the neurotransmission [54].

NET

Norepinephrine transporters can be predominantly found in the central and peripheral nervous system, where they are confined to the membranes of axons, somata and dendrites of noradrenergic neurons. The norepinephrinergic system is responsible for learning and memory, as well as the regulation of mood, attention, interest, anxiety, alertness, stress, pain perception, and blood flow [33,48–50]. Knockout of the NET results in lower body weight and reduced locomotor response of test mice. Furthermore, heart failure can be associated with lower NET expression and reverse norepinephrine transport has been observed during cardiac ischemia [33].

SERT

The serotonin transporter is distributed in the brain as well as in peripheral tissue. SERT in the brain can be mainly found on extrasynaptic axonal membranes of dendrites [33]. Interestingly, SERT is located along the axonal membranes, distant to synaptic junctions [46]. In the periphery, SERT is expressed in epithelial cells and platelets [28,31,33]. Regulation of the serotoninergic system plays a role in mood, impulsivity, aggression, appetite, sleep, cognition and motor activity [49,50]. Anxiety, depression, suicide, schizophrenia, autism, substance abuse and gastrointestinal disorders can be associated with variations in SERT activity [33,49,50].

The monoaminergic neuronal systems play a role in a variety of diseases (see figure 14). They range from gastrointestinal disorders and cardiovascular diseases in the periphery of the body, to severe CNS diseases, like Parkinson’s disease and affective disorders. In affective disorders, especially in depression (also called major depressive disorder (MDD)), a dysfunction in the regulation of dopamine, norepinephrine, and serotonin is involved.

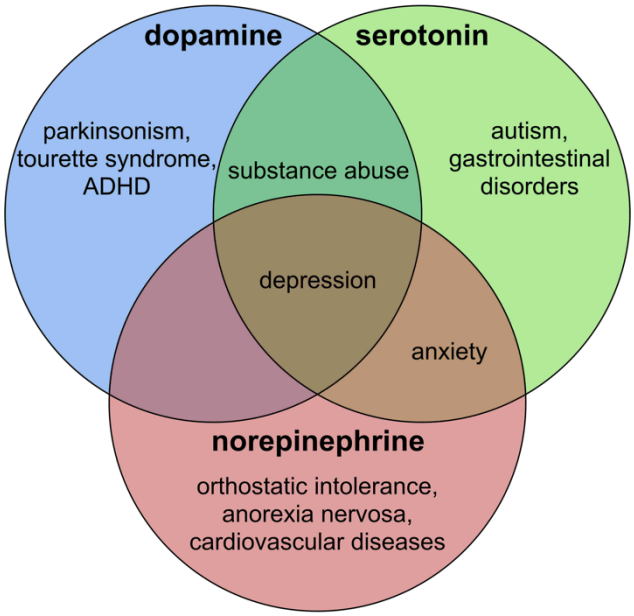

Figure 14: Diseases linked to malfunctions in the monoaminergic systems [33].

1.3.3. Pathophysiology and Therapy of Depression

Research on the therapy of depression started in the 1950s by the accidental discovery of the first antidepressive drugs. The discovery of these drugs resulted in insights into the genesis of major depressive disorders. Malfunctions in the regulation of both the serotonergic and norepinephrinergic neuronal system were identified as causes for the emergence of depression. This led to the formulation of the "catecholamine hypothesis of affective disorders" and the "serotonin hypothesis", which identify the lack of free norepinephrine and serotonin as responsible for the genesis of depression [55,56]. Later, also dopamine was considered to contribute to this disease.

Besides the minor role of electroconvulsive therapy in the treatment of depression, pharmacological therapy of depression is focussed on an increase of the monoamine concentration in the synaptic cleft. For this purpose, three targets have been identified, which can be affected by a variety of different drugs. Many of the symptoms, caused by malfunction of the monoaminergic neurotransmission, mentioned above (see 1.3.2 Localization, Physiological and Pathophysiological Implications, figure 13, and figure 14) can be recognized

in depressive patients and allow the allocation of side effects to the treatment with certain drugs.

MAO-Inhibitors

The first target for the treatment of major depressive disorder was found by accident. Inhibitors of the monoamine oxygenase (MAO) reduce the enzymatic degradation of the released monoamines. In this way, the monoamines stay in the extracellular matrix for a prolonged time, which results in a longer activation of the monoamine receptors. Due to severe side effects, such as an increased risk for hypertensive crisis and toxicity in overdose, today the role of MAO-inhibitors in the treatment of depression is only subordinate [50].

Monoamine Autoreceptor Antagonists

Another strategy for the increase of the extracellular monoamine concentration is an inhibition of the negative feedback effect, induced by stimulation of the presynaptic monoamine receptors (see figure 8 a) step 3b). The drugs trazodone, mianserin, and mirtazapine are antagonists for the norepinephrine and serotonin autoreceptors. Autoreceptors occupied by these drugs do not inhibit the exocytosis of vesicles containing the monoamines, thus the release of the neurotransmitter is not reduced by the released monoamines [50,57].

Monoamine Reuptake Inhibitors

Unlike the inhibition of the monoamine autoreceptors and in analogy with MAO-inhibitors, an inhibition of the monoamine reuptake transporters prolongs the residence time of the extracellular neurotransmitters. The first monoamine reuptake inhibitors were discovered during research on the therapy of schizophrenia. The tricyclic antidepressants (TCAs), such as imipramine, amitriptyline, and clomipramine possess antidepressant properties, but also severe side effects. These side effects are anticholinergic effects, toxicity and increased incidence of seizure in overdose. The broad variety of side effects can be explained by a lack

in selectivity of TCAs. Besides the SERT and NET, muscarinic, adrenergic, histaminic, noradrenergic and serotonergic receptors are also targeted by these drugs [50]. In order to reduce side effects, more selective drugs for the treatment of depression were developed as a new generation of antidepressants. In the 1980s selective serotonin reuptake inhibitors (SSRIs) were developed and introduced to the market. Fluoxetine, paroxetine, sertraline, and citalopram are members of this group of antidepressants, which are still widely in use [58]. These drugs selectively inhibit the SERT, thus they have considerably reduced side effects when compared to TCAs. Furthermore, their efficacy for anxiety disorders and depression is improved. To further enhance the efficacy of antidepressants, drugs targeting both the SERT and NET with higher selectivity than TCAs have been developed. These so-called serotonin-norepinephrine reuptake inhibitors (SNRIs), such as venlafaxine, milnacipran, and duloxetine have a mode of action and efficacy, comparable to TCAs, but they are better tolerated due to their much-improved side effect profile [50]. An additional benefit in the therapy of MDD is a faster onset of action, which is often a drawback of antidepressants. Reboxetine and nisoxetine have been developed as selective norepinephrine reuptake inhibitors (NRIs). These drugs target the NET selectively without an effect on the SERT. Since it mimics the adrenergic tricyclics to some extent, reboxetine has an activating effect, as is energizes the patients and improves their attention [50]. The norepinephrine-dopamine reuptake inhibitor (NDRI) bupropion is the only dual-action antidepressant with affinity for the DAT. Bupropion can be used to augment SSRIs in the therapy of MDD [59]. Today, inhibition of monoamine transporters represents the mainstay in the therapy of MDD [60].

2. Aims and Scope

For the identification of compounds, which bind towards a target, binding studies are an essential tool in drug discovery. The aim of this work was the development of a binding assay, suitable for the characterization of target-ligand interactions at the monoamine transporters hDAT, hNET, and hSERT. These newly developed binding assays should follow the procedure of MS Binding Assays, established in our group for various targets [16–22]. Furthermore, the MS Binding Assays should enable the use of a selective marker for each of the targets. The possibility to simultaneously quantify multiple markers in the same binding experiment is a feature, unique for MS Binding Assays. In radioligand binding studies, typically only one radioligand can be applied in the binding experiments, since the detection via scintillation counting has only limited selectivity. The high selectivity of mass spectrometry enables the performance of simultaneous MS Binding Assays, in which an individual marker for each of the employed targets can be used. To carry out simultaneous MS Binding Assays, two requirements have to be met. Additionally, to the use of a selective marker for each of the targets, a highly sensitive method for the quantification of all applied markers must be available.

Affective disorders have a high and even increasing importance in global economics. Major depression is already the main burden of disease in medium- to high-income countries and will be the primary burden of disease globally by 2030 [61]. Since only 60 – 70 % of patients treated with antidepressants respond to the therapy, the development of new drugs targeting the monoaminergic neurotransmission is still of great importance.

Recently, MS Binding Assays targeting the hDAT, hNET, and hSERT have been developed and published by Stefanie Grimm [19,62]. In these MS Binding Assays, (1*R*,3*S*)-indatraline is used as a marker for the three monoamine transporters. The advantage resulting from the use of only one marker for all three targets is the reduced work that has to be invested into the method development. Additionally, the performance of the binding experiments is very easy, since binding affinities of test compounds at different targets can be performed following the same procedure with different target materials.

The concept of Simultaneous Multiple MS Binding Assays provides some advantages in comparison to radioligand binding assays and classical MS Binding Assays (in which one marker for multiple targets is applied), as demonstrated recently by Schuller et al. [22]. The ability to characterize binding affinities at multiple targets in the same experiments means, that in Simultaneous Multiple MS Binding Assays only one binding experiment is necessary, whereas in radioligand binding assays and classical MS Binding Assays, one binding experiment for each target has to be performed individually. This results in a considerably lower number of samples, which have to be prepared and analysed in Simultaneous Multiple MS Binding Assays. So, regarding the effort of work and time required to perform binding studies, Simultaneous Multiple MS Binding Assays have a much higher efficiency than radioligand binding experiments and classical MS Binding Assays. The second advantage is concerning the purity of the used target material. In the above-mentioned (1*R*,3*S*)-indatraline MS Binding Assays, the selectivity is limited by the purity of the target material, so it is of fundamental importance to apply target material only containing one of the three targets. This can be achieved by the use of target material generated from cell lines stably expressing each of the three targets individually. In radioligand binding studies addressing the monoamine transporters, often native target material, containing the three monoamine transporters, is used together with [^{3}H]-WIN 35,428 (also known as [^{3}H]-β-CFT, [^{3}H]-(–)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane) or [^{125}I]-RTI-55 ([^{125}I]-β-CIT, [^{125}I]-(–)-2β-carbomethoxy-3β-(4-iodophenyl)tropane) as the radioligand. Both of these radioligands have low selectivities, which makes it necessary to use them together with selective inhibitors, blocking the other targets in the target preparation [63–67]. In the mentioned radioligand binding studies, neither the target material, nor the used radioligand offers the needed selectivity, so the selectivity can only be achieved by additional agents, blocking the targets which should not be addressed. As mentioned above, a major requirement for the feasibility of Simultaneous Multiple MS Binding Assays is the application of a selective marker for each of the targets. This provides the selectivity, which enables the use of native target material, containing multiple monoamine transporters, without the addition of blocking agents.

As a first step for the development of Simultaneous Multiple MS Binding Assays for hDAT, hNET, and hSERT, a suitable marker for each of the three targets had to be identified. The

markers should have a high affinity and only for one of the targets, which should be ideally in the low nanomolar range. Fulfilling these requirements, (*R,R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84) was chosen as marker for hDAT, (*S,S*)-reboxetine for hNET and (*S*)-citalopram for hSERT (see second publication "Development and validation of an LC-ESI-MS/MS method for the quantification of D-84, reboxetine, and citalopram for their use in MS Binding Assays addressing the monoamine transporters hDAT, hNET, and hSERT"). Since (*R,R*)-D-84 was not commercially available, a method for the semipreparative chiral separation of *rac*-trans-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol had to be developed and the resulting product had to be characterized concerning its enantiomeric and chemical purity (see first publication "Determination of the enantiomeric purity of the selective dopamine transporter inhibitor (+)-*R,R*-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol"). Additionally, *rac*-reboxetine was extracted from tablets and (*S,S*)-reboxetine was obtained after semipreparative resolution of the racemate (see supporting information in the second publication). Successive to the choice and production of the markers, a highly sensitive quantification method for the three markers had to be developed. In previously published MS Binding Assays, LC-MS proved to be suitable for the quantification up to low picomolar concentrations of markers in samples obtained from binding experiments [17,21,22,62,68]. During the LC-MS method development, MS-conditions had to be found, which allowed a sensitive detection of the markers and a HPLC method had to be developed, separating the markers from the matrix within a short chromatographic run time. To compensate for variations in the ionization efficiency and of matrix effects, a deuterated internal standard for each marker should be included in the MS Binding Assays. The binding samples should be prepared according to the previously published MS Binding Assays, applying a filtration step for the separation of the target-marker complexes and the unbound marker. To prove the reliability and robustness, the developed LC-MS quantification method should be validated according to the *CDER guideline for bioanalytical method validation* [69], concerning its selectivity, validity of the calibration curve, quantification range defined by the lower and upper limit of quantification (LLOQ and ULOQ), precision and accuracy.

In individual saturation experiments, performed with the newly established MS Binding Assays, the affinities and selectivities of the markers towards their targets should be

characterized. For these experiments, only one marker and one target should be used. After these individual saturation experiments, the feasibility of simultaneous saturation experiments, applying all three markers and targets in one binding experiment should be demonstrated. Finally, the binding affinities and selectivities of a variety of known monoamine inhibitors should be characterized in simultaneous competition experiments, again with all three markers and targets in a single experiment, carried out as simultaneous MS Binding Assays.

3. Results and Discussion

3.1. First Publication

Determination of the Enantiomeric Purity of the Selective Dopamine Transporter Inhibitor (+)-R,R-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol

3.1.1. Summary of the Results

(*R,R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84) is one of the most selective inhibitors for the DAT, known so far [19,70–72]. With an IC_{50} of 0.46 nM towards the DAT, an IC_{50} of 3600 nM towards the SERT and an IC_{50} of 1880 nM towards the NET, the (*R,R*)-enantiomer has a much higher affinity and selectivity for the DAT than the (*S,S*)-enantiomer (IC_{50} of 57 nM towards DAT, 1550 towards NET, and 1830 towards SERT) [70]. In an MS Binding Assay, (*R,R*)-D-84 showed the best selectivity for the hDAT in comparison to other DAT inhibitors [19]. The combination of high affinity as well as high selectivity for the hDAT implies that (*R,R*)-D-84 is a compound of great interest as a pharmacological tool for studies with the DAT. To ensure the quality of this compound prior to its use in pharmacological studies, the chemical and enantiomeric purity of (*R,R*)-D-84 has to be determined.

In the original literature, both enantiomers of trans-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol are derivatized with (S)-camphanic acid chloride to obtain diastereomers, which were then separated using a normal phase (NP) HPLC method. After the separation, the diastereomers were subjected to hydrolysis to liberate the enantiomers (*R,R*)-D-84 and (*S,S*)-D-83 [70]. Since this method is not suitable to determine the *ee* of the final, underivatized product, a method for the characterization of its enantiomeric purity had to be developed. A well-established method for the chromatographic separation of two enantiomers is the use of a chiral stationary phase (CSP). Therefore, we intended to develop and validate a CSP-HPLC method for the separation and quantification of (*R,R*)-D-84 and (*S,S*)-D-83. This method should further be used to separate both enantiomers on a semipreparative scale. As there is no reference standard for (*R,R*)-D-84 and (*S,S*)-D-83 with a certified purity available, the CSP-HPLC method cannot be used to determine the absolute

concentrations and chemical purities of the enantiomers. This requires an alternative method to assess the chemical purity of (*R,R*)-D-84 and (*S,S*)-D-83, for which quantitative ^{1}H NMR (qNMR) was selected.

During the method development, the influence of the mobile phase composition, the flow rate and the temperature on the chromatographic separation, retention and peak shapes of (*R,R*)-D-84 and (*S,S*)-D-83 was investigated. In the final analytical method, a Lux Celluose-1 column (250 x 4.6 mm, 5 µm, Phenomenex) was used in normal phase (NP) mode with a mobile phase consisting of *n*-hexane/propan-2-ol/ethanol/trifluoroacetic acid (TFA)/diethylamine (DEA) (85/13.5/1.5/0.5/0.1; v/v/v/v/v) at a flow rate of 2.0 mL/min. The temperature of the column was 40 °C, the injection volume 20 µL and 260 nm was used as the wavelength for the detection of the analytes. With these chromatographic parameters, a separation of both enantiomers within a run time of 7 min was achieved. The analytical method was then validated regarding its specificity, linearity, and range, resulting in a linear range from 25 µg/mL to 30 mg/mL. For the analysis of (*R,R*)-D-84 and (*S,S*)-D-83, samples with a concentration not lower than 25 mg/mL of the major enantiomer were analysed, thus the analytical CSP-HPLC method was able to determine an *ee* of up to 99.8 %. Based on the analytical CSP-HPLC method, a CSP-HPLC method which is suitable for the semipreparative separation of (*R,R*)-D-84 and (*S,S*)-D-83 was established. Since there were concerns about the stability of the enantiomers during the evaporation of the mobile phase, containing TFA as a strong acid, TFA was substituted with formic acid. Using a mobile phase consisting of *n*-hexane/propan-2-ol/ethanol/formic acid/DEA (85/13.5/1.5/1.0/0.1; v/v/v/v/v) at a flow rate of 2.0 mL/min in combination with the Lux Celluose-1 column (250 x 4.6 mm, 5 µm, Phenomenex) at a temperature of 5 °C, a good separation of large amounts of *rac*-trans-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol was achieved. With this method, 80 µL of a solution of 100 mg/mL of the racemic compound were separated per run, resulting in 137.3 mg (*R,R*)-D-84 and 148.8 mg (*S,S*)-D-83 after multiple chromatographic runs and a purification step, performed by column chromatography. The enantiomeric purity of (*R,R*)-D-84 was characterised with the analytical CSP-HPLC method as an *ee* > 99.8 %. Analysis by qNMR resulted in a chemical purity of 96.95 % for (*R,R*)-D-84. The high enantiomeric and chemical purity means, that the produced (*R,R*)-D-84 has a quality, which is sufficient for its use in pharmacological studies.

3.1.2. Declaration of Contributions

Racemic trans-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol was synthesized by Lars Allmendinger and Gerd Bauschke. The bachelor thesis "*Entwicklung einer Methode für die Analytik und semipräparative Trennung des Racemates D-83/D-84*" [73] by Jan Stöckl was prepared under my supervision. Results from this bachelor thesis were valuable as preliminary experiments for the method development performed by myself. I developed an HPLC method for the determination of the *ee* of (*R,R*)-D-84 as well as (*S,S*)-D-83, respectively. Furthermore the analytical HPLC method was modified to develop a semipreparative HPLC method for the chiral separation of large amounts of a racemic mixture of (*R,R*)-D-84 and (*S,S*)-D-83. This semipreparative HPLC method was used by Markus Stöckelhuber to obtain enantiomeric purified (*R,R*)-D-84 and (*S,S*)-D-83, which were subsequently subjected to additional purification by column chromatography. Validation of the analytical HPLC method as well as analysis of the purified enantiomers was performed by myself. Quantitative ^{1}H NMR experiments for the characterization of the chemical purities of (*R,R*)-D-84 and (*S,S*)-D-83 were performed by Lars Allmendinger and me. I wrote the manuscript and prepared the graphics and tables, assisted by Georg Höfner and Lars Allmendinger. Klaus T. Wanner corrected the manuscript.

Received: 11 November 2016 | Revised: 7 February 2017 | Accepted: 22 February 2017

DOI: 10.1002/chir.22688

REGULAR ARTICLE

WILEY

Determination of the enantiomeric purity of the selective dopamine transporter inhibitor (+)-*R*,*R*-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol

Patrick Neiens | Georg Höfner | Klaus T. Wanner

Department Pharmazie – Zentrum für Pharmaforschung, Ludwig-Maximilians-Universität München, Munich, Germany

Correspondence
Klaus T. Wanner, Department Pharmazie – Zentrum für Pharmaforschung, Ludwig-Maximilians-Universität München, Butenandtstr. 5–13, 81377 Munich, Germany.
Email: klaus.wanner@cup.uni-muenchen.de

Abstract

(+)-*R*,*R*-D-84 ((+)-*R*,*R*-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol) is a promising pharmacological tool for the dopamine transporter (DAT), due to its high affinity and selectivity for this target. In this study, an analytical method to ascertain the enantiomeric purity of this compound was established. For this purpose, a high-performance liquid chromatographic (HPLC) method, based on a cellulose derived chiral stationary phase (CSP) was developed. The method was characterized concerning its specificity, linearity, and range. It was shown that the method is suitable to determine an enantiomeric excess of up to 99.8%. With only a few adjustments, this analytical CSP-HPLC method is also well suited to separate (+)-*R*,*R*-D-84 from its enantiomer in a semipreparative scale.

KEYWORDS

cellulose tris-(3,5-dimethylphenylcarbamate), CSP-HPLC, enantiomeric excesss, selective DAT inhibitor, validation

1 | INTRODUCTION

Dopamine (DA) is an important neurotransmitter in the human brain and periphery, controlling physiological functions, such as voluntary movement, reward, hormonal regulation as well as hypertension.[1] After its release from the presynaptic neuron, DA can bind to dopamine receptors to transmit the signal to the postsynaptic neuron. To terminate the neurotransmission, dopamine in the synaptic cleft can be transported back into the presynaptic neuron by the dopamine transporter (DAT), where it can be enzymatically degraded or stored in vesicles. Dysregulation of the dopaminergic neurotransmission is associated with disorders like Parkinson's disease and schizophrenia.[1]

Besides therapy for the mentioned diseases, dopaminergic neurotransmission is considered a possible target to treat addiction, due to its regulation of the reward system. Since it is believed that the reinforcing effect of cocaine is mainly mediated by its binding to the DAT,[2,3] research was focused to develop ligands, binding selectively to this transporter.[4] In addition to their potential for therapy of cocaine addiction, such compounds are also of interest as pharmacological tools.

Many groups addressing the structure–activity relationships of DAT ligands have synthesized huge numbers of compounds with a broad variety of structural motifs,[5,6] such as tropane derivatives,[7,8] benztropine derivatives,[9,10] or piperazine derivatives, such as 1-[2-[bis(4-fluorophenyl) methoxy]ethyl]-4-(3-phenylpropyl)piperazin (GBR 12909) and 1-[2-(Diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine (GBR 12935)[11-13] and corresponding analogs derived from these selective DAT inhibitors, to name only a few.

Despite the enormous efforts in the search for DAT ligands, no highly potent and selective compounds that may serve as reporter ligands to label DAT binding sites are commercially available to date. There are, however, several

compounds described that are claimed to be highly selective for DAT in relation to the serotonin (SERT) and the norepinephrine transporter (NET), respectively.

Binding affinities are usually characterized by radioligand binding assays. As a powerful alternative to these radioligand binding assays, MS Binding Assays have been developed by our group.[14-18]

In DAT radioligand binding assays, typically [^{3}H]WIN 35,428 (also known as [^{3}H]CFT) or [^{125}I]RTI-55 are used as reporter ligands.[19-22] Studies on the selectivity of [^{3}H] WIN 35,428 show only a low selectivity for DAT against SERT, while [^{125}I]RTI-55 has even a slightly higher affinity towards SERT against DAT[23,24] and is therefore used in combination with agents blocking the binding to NET and SERT.[22] Also in MS Binding Assays, the reporter ligand represents an essential tool. As for radioligand binding assays, it should exhibit high affinity for the respective target. In addition, it should be highly selective for the individual target to avoid the need of blocking agents, in order to offer the perspective to perform simultaneous binding assays on multiple targets. Another important requirement of a compound to be used as a reporter ligand in MS Binding Assays is that it should be easily accessible by synthesis, including the option to synthesize a deuterated analog as internal standard for liquid chromatography / electrospray ionization / mass spectrometry (LC-ESI-MS) quantification.

Taking these criteria into account, 4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol, which was mentioned and characterized for the first time in 2003 by Dutta's group,[25] is a promising reporter ligand for DAT. Based on (GBR 12935), the introduction of a polar hydroxy group in the piperidine ring resulted in an elevated binding affinity towards DAT, especially for the (+)-*R*,*R* enantiomer of 4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol (*rac*-**1**), which was named (+)-*R*,*R*-D-84 in further publications (see Figure 1).[26,27] Both enantiomers differ substantially in their binding affinities towards the three monoamine transporters. With an IC_{50} of 0.46 nM measured at the DAT in rat striatum determined in radioligand binding assays, (+)-*R*,*R*-D-84, is 122-fold more potent than (−)-*S*,*S*-D-83 (IC_{50} 56.7 nM) towards this target. Furthermore, radioligand binding assays targeting SERT and NET in rat striatum showed better affinities for (−)-*S*,*S*-D-83, than for (+)-*R*,*R*-D-84.

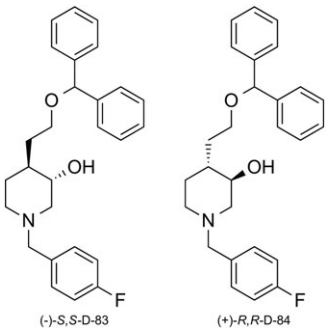

FIGURE 1 Chemical structures of (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84. The racemate of both enantiomers together correspond to *rac*-1

Overall, (+)-*R*,*R*-D-84 exhibits higher affinity for DAT as compared to (−)-*S*,*S*-D-84, and at the same time a higher subtype selectivity at this transporter with regard to SERT and NET.

Uptake assays for all three monoamine transporters confirmed the high potency and selectivity of (+)-*R*,*R*-D-84 towards DAT.[26] In an MS Binding Assay, which was established by our group, a set of DAT inhibitors containing commercially available, as well as compounds described in the literature of diverse structural classes, was pharmacologically characterized.[17] Here again, (+)-*R*,*R*-D-84 was shown to be the most potent and subtype-selective compound, concerning its affinity for the human DAT (hDAT) as compared to hSERT and hNET.

According to the data published for (+)-*R*,*R*-D-84, this compound offers high affinity (sub-nanomolar K_i) in combination with the highest selectivity towards DAT (vs. NET and SERT). In summary, these findings indicate that (+)-*R*,*R*-D-84 is a promising tool for pharmacological assays, targeting selectively DAT and thus as a reporter ligand in MS Binding Assays.

However, when used as a tool in pharmacological assays and also as a reporter ligand in MS Binding Assays, the corresponding compound samples of (+)-*R*,*R*-D-84 have to fulfil high demands with regard to chemical and enantiomeric purity. In the original publication, (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 were obtained by chiral resolution.[25] Derivatization with *S*-(−)-camphanic acid chloride resulted in a mixture of diastereomeric esters that were separated using a semipreparative normal phase high-performance liquid chromatographic (HPLC) method. Final hydrolysis of these esters led to the pure enantiomers (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84.[25] The obtained enantiomers have been characterized by their optical rotations, but their enantiomeric purity has not been finally determined. Since, for the MS Binding Assays to be developed, only the highly selective (+)-*R*,*R*-D-84 enantiomer should be used as a reporter ligand, it had to be assured that the respective samples of the compound are not contaminated by the (−)-*S*,*S*-D-83 distomer. So far, however, no method for the determination of the enantiopurity of (+)-*R*,*R*-D-84 has been published.

The aim of the present study was to develop an enantioselective UV-HPLC method with cellulose tris-(3,5-dimethylphenylcarbamate) as a chiral stationary phase (CSP) that allows the characterization of the enantiopurity of (+)-*R*,*R*-D-84 and (−)-*S*,*S*-D-83 at the stage of the final product without any need for derivatization.[28] To be able to rule out any substantial contamination with the minor enantiomer, the method should work for very high enantiomeric

excess (*ee*) values. To show its applicability for the determination of the enantiopurities of both enantiomers, the method should be validated with regard to specificity, linearity, and quantification range for both enantiomers. Furthermore, the method for the analytical separation should also be examined with regard to its suitability for semipreparative resolution of the enantiomers. A CSP-HPLC method, capable of separating reasonable amounts of *rac*-**1,** would facilitate the preparation of (+)-*R*,*R*-D-84, and thus be of high value. In addition, the characterization of samples of enantiopure (+)-*R*,*R*-D-84 by CSP-HPLC, their chemical purity should also be determined by quantitative ^{1}H nuclear magnetic resonance (qNMR) spectroscopy.[29,30] Both methods would be helpful tools to ascertain the suitability of samples of this compound as reporter ligand in future DAT MS Binding Assays and possibly also as a probe in pharmacological assays.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

HPLC-grade *n*-hexane (95%) and propan-2-ol were purchased from VWR International (Darmstadt, Germany), and ethanol (100%) was bought from Brüggemann (Heilbronn, Germany). Additives for the CSP-HPLC were diethylamine (DEA, >99.5%, Sigma-Aldrich, St. Louis, MO), trifluoroacetic acid (TFA, 99%, Acros Organics, Geel, Belgium), and formic acid (98–100%, Merck, Darmstadt, Germany). Ethyl acetate (pur.), heptane (puriss.) and diethyl ether (p.a.) were distilled prior to use. Sodium hydrogencarbonate was purchased from Fisher Scientific (Loughborough, UK) in analytical reagent grade. For ^{1}H qNMR, dimethyl sulfoxide-d_6 (d_6-DMSO; 99.9 atom %D), D_2O (99.9 atom %D), and maleic acid (99.94%, TraceCERT CRM for qNMR) were obtained from Sigma-Aldrich. DCl (99.5 atom %D, 36–38% in D_2O) was purchased from Deutero (Kastellaun, Germany). All mixtures of solvents used as mobile phases (including additives) or sample solvents are specified as volume fractions (*v*/*v*) if not indicated otherwise.

2.2 | Instrumentation

Chromatography was carried out with an Agilent 1100 HPLC system, consisting of a vacuum degasser, a binary pump with solvent selection valve, autosampler, column oven, and UV–Vis-detector (Agilent, Waldbronn, Germany) under control of EZChrom software (Agilent). As CSP, a Lux Cellulose-1 column (250 x 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) was used, which was protected with a SecurityGuard precolumn (Lux Cellulose-1, 4 x 3 mm, 5 µm, Phenomenex) and an inline filter (0.5 µm, stainless steel, IDEX, Lake Forrest, IL). For the determination of the calibration functions, GraphPad Prism 6.03 (GraphPad, La Jolla, CA) was employed. Calculations regarding validation parameters and enantiomeric purity were done in Microsoft Excel (Microsoft, Redmond, WA).

Chemical purity determination by NMR (qNMR) was performed with an Advance III HD 400 MHz NMR spectrometer equipped with a 5 mm PABBO broadband probe head with z-gradients (Bruker BioSpin, Billerica, MA).

2.3 | Solutions for analytical CSP-HPLC

For establishment of a calibration function, a stock solution of 60 mg/ml *rac*-**1** (purity: 97.6%, determined by ^{1}H–NMR) in propan-2-ol/DEA (9/1) was prepared resulting in a concentration of 30 mg/ml per enantiomer. Calibration standards in concentrations of 25 µg/ml to 20 mg/ml per enantiomer in propan-2-ol/DEA (9/1) were diluted from this stock solution.

The samples used for the determination of enantiomeric purity were prepared by dissolving the enantiomer to be analyzed in propan-2-ol/DEA (9/1) at a concentration of ~27.5 mg/ml. The resulting solutions were directly subjected to HPLC analysis.

2.4 | Analytical CSP-HPLC method

Enantioseparation of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 with the Lux Cellulose-1 column (250 x 4.6 mm, 5 µm) as stationary phase was performed with a mobile phase consisting of *n*-hexane/propan-2-ol/ethanol/TFA/DEA (85/13.5/1.5/0.5/0.1) at a flow rate of 2.0 ml/min at 40 °C. Twenty µl of the sample solutions were injected for analysis; for detection, a wavelength of 260 nm was used.

Quantification was carried out according to the external standard calibration method. Resulting peak areas (y) were investigated as a function of the analyte concentrations (x) of each enantiomer, separately. To obtain a calibration function, calibration standards in 11 concentrations reaching from 0.025 mg/ml up to 30 mg/ml of the free base of each enantiomer (employing the *rac*-**1**, see above, "solutions for analytical CSP-HPLC") were measured in triplicate. For calculation of the calibration function, linear regression was used. The standards were used to validate the method concerning its linearity and range. Furthermore, specificity (by comparison of the response of a solvent blank at the expected retention times with the standards) was investigated.

2.5 | Semipreparative enantioseparation of *trans*-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol

Resolution of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 with the Lux Cellulose-1 column (250 x 4.6 mm, 5 µm) as stationary phase

was carried out with a mobile phase consisting of *n*-hexane/propan-2-ol/ethanol/formic acid/DEA (85.0/12.4/1.5/1.0/0.1) at a flow rate of 2.0 ml/min at 5 °C using a detection wavelength of 260 nm. For semipreparative enantioseparation, 80 µl of a 100 mg/ml solution of the *rac*-**1** in propan-2-ol/DEA (9/1) were injected onto the column. All fractions collected during multiple chromatographic runs that corresponded to (−)-*S*,*S*-D-83 or (+)-*R*,*R*-D-84, respectively (for details see Supporting Information), were pooled. After evaporation of the solvent, both residues (containing (−)-*S*,*S*-D-83 or (+)-*R*,*R*-D-84, respectively) were subjected to flash chromatography, using silica gel (Merck 60, 40–63 µm) as stationary phase and ethyl acetate, heptane, and trimethylamine (49.5/49.5/1) as mobile phase. All fractions containing (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 were collected. After evaporation of the solvent, each enantiomer was dissolved in diethyl ether and washed with a saturated aqueous sodium hydrogencarbonate solution. The aqueous phase was reextracted 3 times with diethyl ether. Finally, all diethyl ether phases from one enantiomer were combined and dried ($MgSO_4$). Subsequent to filtration, the solvent was removed in vacuo.

2.6 | Quantitative ^{1}H NMR

(−)-*S*,*S*-D-83 or (+)-*R*,*R*-D-84 were weighed out separately to a mass of ~15 mg, then an exact amount (~5 mg) of maleic acid was added as an internal calibrant to each glass vial. The mixture of the respective analyte and the internal calibrant were dissolved in 600 µl d_6-DMSO and DCl (2 M in D_2O, 1.5 equivalents referred to the analyte) was added. This solution was quantitatively transferred into a 5-mm NMR-tube and directly subjected to qNMR measurement.

The measurement and data processing were performed according to the literature.[31] A standard pulse program ("zg" with 90° pulse) from the Bruker pulse program library, utilizing a single pulse without carbon decoupling, was used and the following settings were made: a relaxation delay (D1) of 60 sec, an acquisition time (AT) of 4 sec, and a spectral width (SW) of 30 ppm with a transmitter offset at 7.5 ppm. During acquisition the temperature was maintained at 25 °C. After the acquisition of four dummy scans (DS), the spectrum was acquired with 64 scans of 64 K data points. Prior to FT a line-broadening factor of 0.1 Hz and a zero-filling to 256 K data points were applied in MestReNova. After a manual phase correction followed by a 5th order polynomial fit as baseline correction, the signal of the internal calibrant (δ = 6.3 ppm) as well as chosen signals of the analyte [δ = 5.4 ($CH(Ph)_2$), 2.8 ($H\text{-}6_{eq}$), 2.1 ppm (CH_2CH_2O)] were manually integrated. Based on the absolute integrals, the chemical purity of the analyte was calculated with the Purity Calculator Tool of the MestReNova-Software.

3 | RESULTS AND DISCUSSION

3.1 | CSP-HPLC

The main goal for the method to be developed was at first to enable reliable enantiopurity determination of highly enantioenriched samples of (−)-*S,S*-D-83 and (+)-*R,R*-D-84. Accordingly, the method should allow the quantification of both enantiomers in a wide linear range, i.e., possess a lower limit of quantification (LLOQ) as low as possible for the minor enantiomer and an upper limit of quantification (ULOQ) as high as possible for the major enantiomer. Furthermore, the resolution of the two chromatographic peaks resulting from the enantiomers should be sufficient to guarantee a reliable separation of the small peak of the minor enantiomer from the large peak of the major enantiomer even when the major enantiomer elutes before the minor one. When having this feature, it should also be tested whether the method is suitable for a semipreparative resolution of (−)-*S,S*-D-83 and (+)-*R,R*-D-84. In that case, it should tolerate high loadings of the racemate onto the column, even in the mg range, distinctly surpassing the amounts typically applied to analytical columns. Favorably, the mobile phase should not contain any nonvolatile components to be able to easily isolate the enantiopure compounds by vaporization of the solvent. As the pure enantiomers (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 were not available for the development of the desired CSP-based separation method, the racemate synthesized according to the literature was employed.[25]

3.2 | Development of the analytical CSP-HPLC method

Experiments were started with a column containing a chiral stationary phase based on cellulose tris-(3,5-dimethylphenylcarbamate) (Lux Cellulose-1, 250 x 4.6 mm, 5 µm), as this CSP is known to enable enantiomeric resolution of a broad variety of different classes of chemical compounds.[32,33] Furthermore, this material proved to be successful in semipreparative applications. Therefore, it was assumed to be appropriate for our purpose, including the resolution of large amounts of *rac*-**1**.[33,34]

Development of the CSP-HPLC-method on an analytical scale was started—as mentioned—using the racemate. In preliminary experiments a resolution of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 could be achieved with a mobile phase consisting of *n*-hexane, propan-2-ol, ethanol, and TFA. Thereby, it was observed that addition of 0.1% DEA improved peak symmetry substantially. Therefore, a mobile phase, consisting of *n*-hexane/propan-2-ol/ethanol/TFA/DEA (80/14/6/0.5/0.1; all mobile phase compositions or sample solvent compositions are given as *v*/v/*v*/v/v; *n*-

TABLE 1 Effects of different mobile phase parameters (ratio of *n*-hexane/propan-2-ol/ethanol (*v*/v/v), flow rate, ratio propan-2-ol/ethanol and fraction of TFA)

No. (n[a])	Mobile phase: *n*-hexane/ propan-2-ol/ethanol/ TFA/DEA (*v*/v/*v*/v/v)	Flow rate (ml/min)	R_S	t_R (min)		A_S	
				(−)-*S,S*-D-83	(+)-*R,R*-D-84	(−)-*S,S*-D-83	(+)-*R,R*-D-84
Variation of the ratio of *n*-hexane and the sum of alcohols, ratio of propan-2-ol/ethanol kept constant at 7/3 (*v*/v), fraction of TFA kept constant at 0.5%. Flow rate kept constant at 1.0 ml/min.							
1a (3)	**80/14/6**/0.5/0.1	1.0	4.08	7.11	9.14	1.32	1.25
1b (4)	**85/10.5/4.5**/0.5/0.1	1.0	4.46	8.74	11.6	1.37	1.30
1c (2)	**90/7/3**/0.5/0.1	1.0	14.7	13.3	18.4	1.50	1.38
Variation of the flow rate. Mobile phase composition kept constant at *n*-hexane/propan-2-ol/ethanol/TEA/DEA 85/10.5/4.5/0.5/0.1							
2a (1) ≙ 1b[b]	85/10.5/4.5/0.5/0.1	**1.0**	4.48	8.62	11.4	1.30	1.29
2b (1)	85/10.5/4.5/0.5/0.1	**1.5**	3.89	5.72	7.54	1.25	1.24
2c (1)	85/10.5/4.5/0.5/0.1	**2.0**	3.55	4.21	5.53	1.24	1.17
Variation of the ratio of propan-2-ol and ethanol, ratio of *n*-hexane/alcohols kept constant at 85/15 (*v*/v), fraction of TFA kept constant at 0.5%, fraction of DEA kept constant at 0.1%. Flow rate kept constant at 2.0 ml/min.							
3a (1)	85/**7.5/7.5**/0.5/0.1	2.0	2.82	3.82	4.80	1.39	1.37
3b (4)	85/**9/6**/0.5/0.1	2.0	3.01	3.98	5.03	1.22	1.21
3c (3) ≙ 2c[b]	85/**10.5/4.5**/0.5/0.1	2.0	3.50	4.28	5.57	1.10	1.10
3d (3)	85/**12/3**/0.5/0.1	2.0	3.72	4.64	6.21	1.11	1.13
3e (3)	85/**13.5/1.5**/0.5/0.1	2.0	3.85	4.84	6.54	1.13	1.14
Variation of the fraction of TFA, composition of *n*-hexane/propan-2-ol/ethanol/DEA kept constant at 85/13.5/1.5/0.1. Flow rate kept constant at 2.0 ml/min.							
4a (3)	85/13.5/1.5/**0.1**/0.1	2.0	3.57	4.30	5.67	1.11	1.02
4b (3)	85/13.5/1.5/**0.2**/0.1	2.0	3.62	4.32	5.71	1.12	1.09
4c (3)	85/13.5/1.5/**0.3**/0.1	2.0	3.65	4.34	5.75	1.12	1.09
4d (3)	85/13.5/1.5/**0.4**/0.1	2.0	3.69	4.34	5.76	1.11	1.11
4e (3) ≙ 3e[b]	85/13.5/1.5/**0.5**/0.1	2.0	3.72	4.35	5.80	1.10	1.09

The varied parameters are indicated as bold characters. On resolution, retention times and peak symmetries of (−)-*S,S*-D-83 and (+)-*R,R*-D-84. All chromatograms were acquired, using a Lux Cellulose-1 (250 x 4.6 mm, 5 µm) column at 20 °C with a detection wavelength of 260 nm.

[a]Number of replicates, which were acquired for each No. The values for R_S, t_R and A_S represent the mean values.

[b]Data were acquired with the same chromatographic conditions in different experimental series.

hexane, propan-2-ol and ethanol were mixed to 100%, TFA and DEA were added to this mixture) at a flow rate of 1.0 ml/min was selected as the starting point for further method optimization.

In a first step, the fraction of *n*-hexane vs. the fixed combination of propan-2-ol and ethanol (7/3) was varied (see Table 1, no. 1a to 1c). It was observed that a higher proportion of *n*-hexane in the mobile phase results in better resolution and longer run times. The mixture of *n*-hexane/propan-2-ol/ethanol/TFA/DEA (85/10.5/4.5/0.5/0.1) was found to be the best compromise concerning a sufficient resolution and a tolerable run time. Keeping this mobile phase composition constant, next the flow rate was raised from 1.0 ml/min to 2.0 ml/min (see Table 1, no. 2a to 2c). This resulted in shorter run times, with a minimum of 4.21 and 5.53 min, respectively, and an increased back pressure of up to 180 bar. At the same time, the resolution declined from 4.48 to 3.55 (Table 1, no. 2a and 2c). Using the conditions given in Table 1, no. 1b [mobile phase composition: *n*-hexane/propan-2-ol/ethanol/TFA/DEA (85/10.5/4.5/0.5/0.1)], the flow rate of 2.0 ml/min, which had delivered considerably shorter run times with only a minor loss in resolution, the influence of the ratio of propan-2-ol vs. ethanol in the mobile phase on the chromatography results was investigated. To this end, the respective ratios were set to 7.5/7.5 (Table 1, no. 3a), 9/6 (Table 1, no. 3b), 10.5/4.5 (Table 1, no. 3c), 12/3 (Table 1, no. 3d) and 13.5/1.5 (see Table 1, no. 3e). With increased propan-2-ol and reduced ethanol contents, retention times became longer and resolution increased. The mobile phase composition of *n*-hexane/

propan-2-ol/ethanol/TFA/DEA amounting to 85/13.5/1.5/0.5/0.1 (Table 1, no. 3e) was considered the most suitable for the intended purpose, as it resulted in chromatograms with the highest resolution in this test series. Finally, the effect of the fraction of TFA in the eluent on the chromatography results was studied. Varying its percentage from 0.1–0.5% had only negligible effects on resolution and retention (Table 1, no. 4a to 4e). Due to a slight tendency towards better resolution at higher TFA contents in the eluent, the mobile phase with 0.5% of TFA was chosen for further experiments. Because of the high back pressure in the chromatographic system, the effects of temperature on the chromatography results and on the back pressure were also studied. When varying temperature from 5 °C to 50 °C, in line with expectations, a higher temperature resulted in a lower system back pressure, but also in reduced retention times and peak widths. Resolution had a maximum at 30 °C and declined slightly at lower as well as at higher temperatures. This might be explained by the decrease of retention time and peak width at higher temperatures. From 5 °C to 30 °C, the reduction of peak width results in an increasing resolution, although the retention times for both peaks are decreasing. After having reached optimum resolution at 30 °C, the decrease of retention times seems to become more dominant than the reduction in peak width, leading to a lower resolution (Figure 2). For the final conditions, a temperature of 40 °C was chosen, as it appears to provide a good compromise between a reasonable resolution and a not too high system back pressure (~160 bar).

Accordingly, the parameters for the final chromatography method at the chosen stationary phase (Phenomenex Lux Cellulose-1, 250 x 4.6 mm, 5 µm) were as follows: The mobile phase consisted of a mixture of *n*-hexane/propan-2-ol/ethanol/TFA/DEA in a ratio of 85/13.5/1.5/0.5/0.1. The flow rate was set to 2.0 ml/min, the column compartment to 40 °C, and the injection volume to 20 µl. For detection, a wavelength of 260 nm was chosen (for details, see Supporting Information).

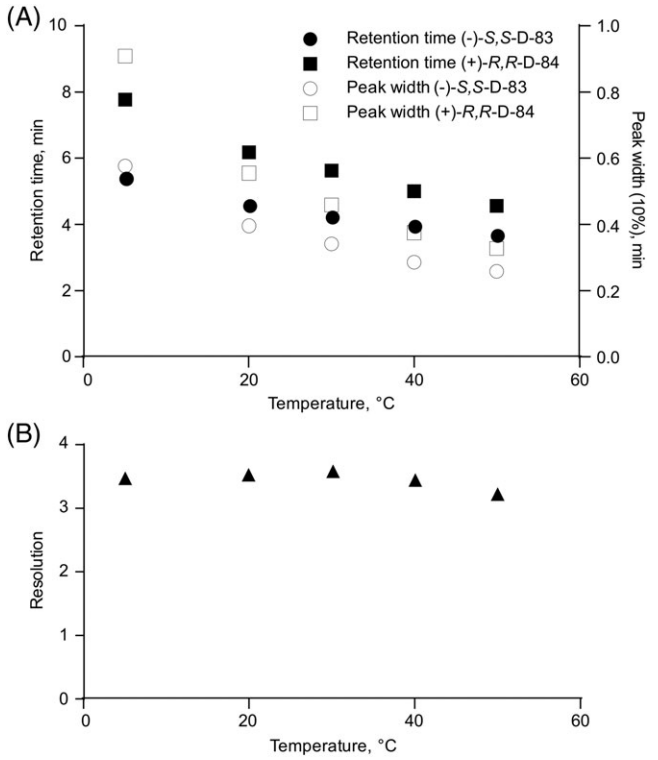

FIGURE 2 Effect of the temperature on the chromatography of (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 in the method development of the analytical CSP-HPLC method. **A**, Effect on the retention times and the peak widths (10 %) of (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84. **B**, Effect of the temperature on the resolution of (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84

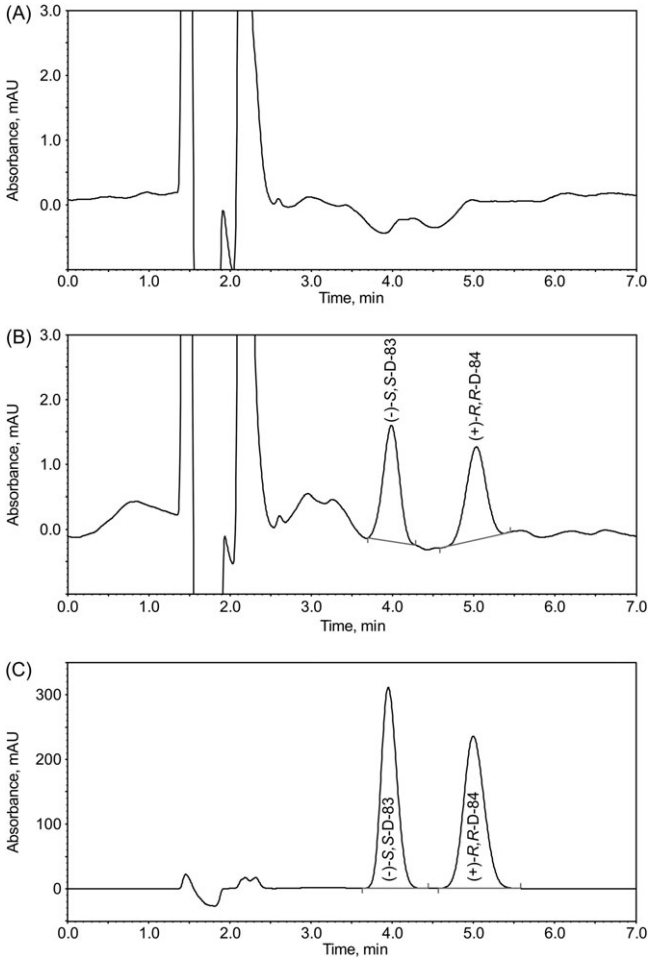

FIGURE 3 Chromatograms from the analytical CSP-HPLC method, using a Lux Cellulose-1 (250 x 4.6 mm, 5 µm) column as stationary phase in combination with a mixture of n-hexane/propan-2-ol/ethanol/TFA/DEA (85/13.5/1.5/0.5/0.1; v/v/v/v/v) as mobile phase at a flow rate of 2.0 mL/min at 40 °C, an injection volume of 20 µl at a detection wavelength of 260 nm. **A**, solvent blank; **B**, 25 µg/ml of (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84; **C**, 5 mg/ml of (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84

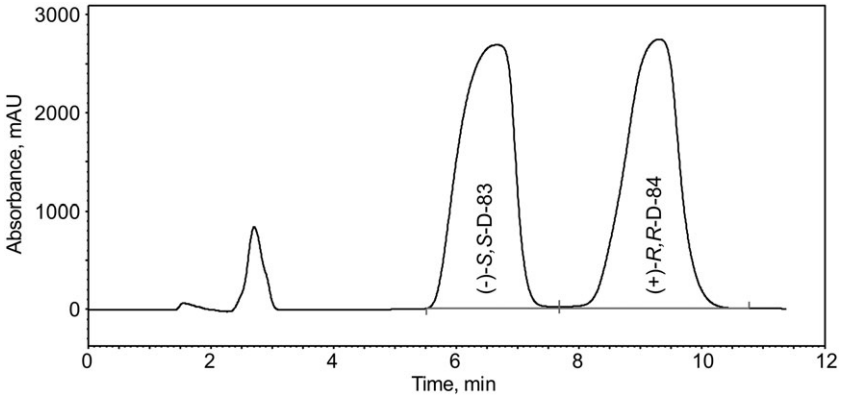

FIGURE 4 Chromatogram from the semipreparative CSP-HPLC method, using a Lux Cellulose-1 (250 x 4.6 mm, 5 µm) column as stationary phase in combination with a mixture of n-hexane/propan-2-ol/ethanol/formic acid/DEA (85/13.5/1.5/1.0/0.1; v/v/v/v/v) as mobile phase at a flow rate of 2.0 ml/min at 5 °C. For this chromatogram 80 µl of a 100 mg/ml solution of racemic (-)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 was injected and detected at a wavelength of 260 nm

These conditions resulted in retention times of 4.0 min for (−)-*S*,*S*-D-83 and 5.0 min for (+)-*R*,*R*-D-84* and a resolution of more than 2.0 (see Figure 3C for a chromatogram with a concentration of 5 mg/ml) even for the highest concentrations of 30 mg/ml per enantiomer and a run time of 7 min.

3.3 | Development and application of the semipreparative CSP-HPLC method

It was intended to adjust the analytical CSP-HPLC method described above in a way that it would allow the semipreparative resolution of *rac*-**1**.

As TFA is a very strong acid, it was worried that the samples of the enantiomers might be prone to degradation, e.g., by elimination of H_2O when during their isolation by evaporation of the solvent the TFA concentration increases. Therefore, TFA should be substituted by a weaker acid. Formic acid proved to be a good alternative to TFA, allowing a good separation of the enantiomers when present in a concentration of 1% in the mobile phase instead of TFA.

As expected, injection of large amounts of *rac*-**1** onto the column caused severe peak broadening. At high temperatures, the resolution tended to decrease; therefore the temperature for the semipreparative method was set to 5 °C. It may be assumed that this massive overloading results in a chromatographic behavior that does not follow the observations described for the analytical method. Nevertheless, these chromatographic conditions were suited for the semipreparative resolution of *rac*-**1** (at a still tolerable back pressure of around 180 bar). For these reasons, no further optimization of the semipreparative method was needed.

With a 100 mg/ml solution of *rac*-**1** in propan-2-ol/DEA (9/1), the effects of injection volumes in a range from 10–100 µl on the chromatography results were studied (see Figure 1 in the Supporting Information). Eighty µl was found to be the highest injection volume still allowing a sufficient separation of the two enantiomers of *rac*-**1** (resolution >1.5) (see Figure 4). Accordingly, 8 mg of *rac*-**1** could be resolved within a single chromatographic run.

With this modification of the analytical CSP-method, we separated ~300 mg racemic (−)-*S*,*S*-D-83/(+)-*R*,*R*-D-84 mixture. The eluents representing the peaks containing (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 were collected separately and concentrated in vacuo. ^{1}H–NMR spectroscopy revealed that there were still residual amounts of formic acid and DEA in the fractions of both enantiomers. Therefore, the fractions containing the individual enantiomers were further purified by column chromatography and thereafter also by base treatment ($NaHCO_3$) of diethylether solutions of the individual enantiomers. That way, 148.8 mg (−)-*S*,*S*-D-83 and 137.3 mg (+)-*R*,*R*-D-84 could be obtained as the final products. Their purities were determined later on by quantitative ^{1}H–NMR.

3.4 | Validation and quantification of the enantiomeric excess

The analytical CSP-HPLC method was validated concerning specificity, linearity, and range. For validation, blank samples and calibration samples at 11 concentration levels were prepared from *rac*-**1** (corresponding to concentrations of 25–30 mg/ml of each enantiomer) and analyzed in triplicate.

To ascertain the specificity of the method, blank samples (propan-2-ol/DEA; 9/1) were injected to the chromatographic system (see Figure 3A). Absence of any peaks at the expected retention times characteristic for (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 indicated that there are no compounds present that would interfere with the analytes and thus affect their quantification.

Based on the data obtained from calibration standards, calibration functions for both enantiomers were established by modelling the relation of the analyte peak areas (y) as a function of the concentrations (x) of corresponding calibration standards applying a linear regression (1/x weighting). Both calibration functions showed excellent linearity with r^2 of 1.000 in the investigated range. The LLOQ and ULOQ of the calibration curve were defined as the lowest and

*These retention times differ slightly from the ones observed during method development, as the column was employed in the meantime in another project with a different mobile phase.

TABLE 2 Results from the validation

Concentration (mg/ml per enantiomer)	(−)-*S*,*S*-D-83			(+)-*R*,*R*-D-84			Peak area (−)-*S*,*S*-D-83/ (+)-*R*,*R*-D-84
	Determined concentration (mg/ml)	Recovery (%)	RSD (%)	Determined concentration (mg/ml)	Recovery (%)	RSD (%)	
30	29.85	99.50	0.15	30.01	100.05	0.14	0.995
20	20.09	100.43	0.41	20.12	100.60	0.60	0.998
10	10.06	100.61	0.43	10.10	101.02	0.16	0.996
5	4.995	99.89	0.85	5.017	100.34	0.81	0.996
2.5	2.512	100.48	0.86	2.522	100.89	0.66	0.996
1	0.9940	99.40	0.21	0.9974	99.74	0.44	0.997
0.5	0.4969	99.39	0.30	0.5071	101.74	0.66	0.980
0.25	0.2490	99.61	0.41	0.2500	100.00	1.40	0.996
0.1	0.09912	99.12	0.76	0.1011	101.14	1.72	0.981
0.05	0.05060	101.20	1.37	0.05028	100.56	0.59	1.006
0.025	0.02507	100.27	0.75	0.02494	99.78	0.84	1.004

All samples were measured in triplicate and are indicated as means.

highest concentration of analyte, respectively, which can be quantified accurately and precisely. The criterion for accuracy defined as recovery was limited to a range from 98–102%, the criterion for precision defined as the relative standard deviation (RSD) of three injections was limited to ≤2%. For both enantiomers and all calibration levels, the recovery was between 99.12% and 101.74%. Precision is given as the RSD of the triplicates for each concentration level and did not exceed a value of 1.72% and 1.37% for (+)-*R*,*R*-D-84, and for (−)-*S*,*S*-D-83, respectively (see Table 2). In addition, based on the data obtained, a concentration of 25 µg/ml could be defined as the LLOQ of the calibration curve (see Figure 3B) and 30 mg/ml as the ULOQ.

Another parameter, investigated for the series of racemic calibration standards, was the ratio of the peak areas of both enantiomers. The peak area ratios of (−)-*S*,*S*-D-83 vs. (+)-*R*,*R*-D-84 for all concentration levels were between 1.006 and 0.980, thus indicating that both enantiomers show almost identical peak areas in the whole concentration range. This indicates that there are no chromatographic interferences affecting one of both enantiomers exclusively.

For the quantification of the *ee* of an analyte, first the concentration of both enantiomers in the sample has to be determined. Based on these concentrations, the *ee* can be calculated.

To allow *ee*-determinations of highly enantioenriched samples, the concentrations of the major enantiomer in the sample should be as high as possible with regard to the ULOQ of the method (30 mg/ml). Using, for example, a sample with an overall concentration of 25 mg/ml, in which the minor enantiomer is present at a concentration equal to the LLOQ, the area ratio of the minor enantiomer at the LLOQ (25 µg/ml) vs. the major enantiomer reaches almost 1:1000 (exactly 1:999). This means that the established method is capable for reliable determination of *ee* values up to 99.8% when the sample to be characterized is employed at concentrations between 25 and 30 mg/ml (overall concentration).

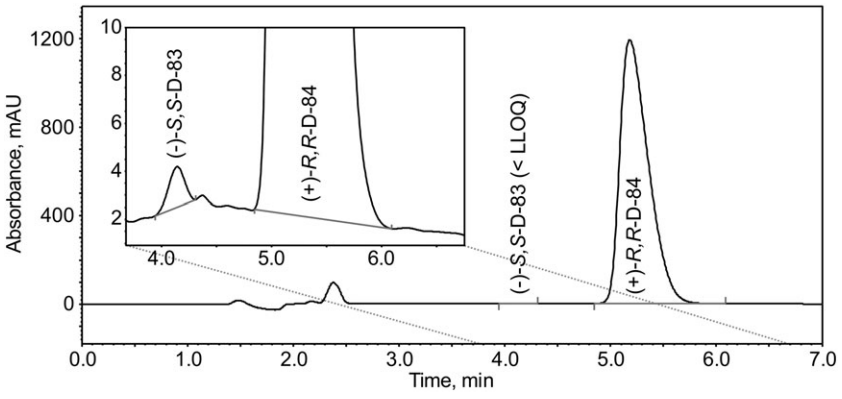

FIGURE 5 Chromatogram of 27.5 mg/ml (+)-*R*,*R*-D-84, using a Lux Cellulose-1 (250 x 4.6 mm, 5 µm) column as stationary phase in combination with a mixture of n-hexane/propan-2-ol/ethanol/TFA/DEA (85/13.5/1.5/0.5/0.1; v/v/v/v/v) as mobile phase at a flow rate of 2.0 ml/min at 40 °C, a detection wavelength of 260 nm and an injection volume of 20 µl

3.5 | Determination of the enantiomeric purity of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84

The enantiomeric purity of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84, obtained from the semipreparative separation, was determined with the established analytical CSP-HPLC method. For this, (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 were dissolved in propan-2-ol/DEA (9/1) to yield a final concentration of ~27.5 mg/ml. Both solutions were injected and analyzed three times. A representative chromatogram obtained for (+)-*R,R*-D-84 is depicted in Figure 5. The concentrations of the enantiomers were calculated from the calibration functions determined before (see above). For (−)-*S*,*S*-D-83 as well as for (+)-*R*,*R*-D-84, the signal intensities of the minor enantiomers were below the LLOQ and the concentrations of the major enantiomer were 28.8 mg/ml ((−)-*S*,*S*-D-83) and 26.4 mg/ml ((+)-*R*,*R*-D-84), the *ee*-values of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 (see Figure 5) being accordingly >99.8%.

3.6 | Determination of the chemical purity of (+)-*R*,*R*-D-84 and (−)-*S*,*S*-D-83

Due to the fact that neither a reference standard for (−)-*S*,*S*-D-83, (+)-*R*,*R*-D-84 nor the corresponding racemate was commercially available, the CSP-HPLC method was not useable for the determination of the chemical purity of the enantiomers. Therefore, to obtain the chemical purities of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84, quantitative ^{1}H–NMR experiments with maleic acid as internal calibrant were performed.[31] Either enantiomer (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 yielded only poorly resolved ^{1}H–NMR spectra signals being severely broadened (in d_6–DMSO). As this was assumed to be due to salt formation between the enantiomers and the internal standard used, maleic acid, ^{1}H–NMR probes were supplemented with DCl in D_2O (2 M, 1.5 equiv. of DCl with respect to the analyte). Thereupon, the resolution of the ^{1}H–NMR spectra was distinctly improved. The calculation of the chemical purity of the analytes was performed based on the integrals of the signals at δ = 6.3 ppm, (maleic acid) and at δ = 5.4 ppm, 2.8 ppm and 2.1 ppm (all from analytes). This led to a chemical purity of 91.18% for (−)-*S*,*S*-D-83 and 96.95% for (+)-*R*,*R*-D-84.

4 | CONCLUSION

In the present study, we developed an analytical CSP-HPLC method for the determination of the enantiomeric purity of (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84. For this method, a Phenomenex Lux Cellulose-1 column (250 x 4.6 mm, 5 μm) was used in combination with a mixture of *n*-hexane/propan-2-ol/ethanol/TFA/DEA (85/13.5/1.5/0.5/0.1) as mobile phase. The flow rate was set to 2.0 ml/min, the temperature in the column oven to 40 °C, and the injection volume was 20 μl, while the detection was done at a wavelength of 260 nm. Using these chromatographic parameters, we obtained chromatograms where the two enantiomers were sufficiently separated within a run time of 7 min. The method was successfully validated with regard to the parameters specificity, linearity, and range for both enantiomers. The achieved excellent range reached for both enantiomers from 25 μg/ml to 30 mg/ml enabled the determination of enantiopurities of up to 99.8% *ee* for each enantiomer. With minor changes, the method could also be applied to quantitative separation of up to 8 mg of racemate per run. Both, the analytical and the semipreparative CSP-HPLC method represent a substantial improvement in comparison to the published methods. Enantiomerically pure compounds have so far been accessible by intermediate formation of diasteromers. And an analytical CSP-HPLC method based on the determination of the enantiopurity of (−)-*S*,*S*-D-83 or (+)-*R*,*R*-D-84 has not been available at all. The highly enantioenriched sample of (+)-*R*,*R*-D-84 with an enantiopurity of >99.8% and a chemical purity of 96.95%, obtained in this study, will be of great value for the future development of MS Binding Assays for the dopamine transporter using this compound as reporter ligand.

ACKNOWLEDGMENTS

We thank Gerd Bauschke (LMU Munich, Department for Pharmacy) for the synthesis of the racemic mixture of (–)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84. We also thank Jan Stöckl for performing the preliminary experiments in the method development and Markus Stöckelhuber for the semipreparative resolution of (–)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Neiens P, Höfner G, Wanner KT. Determination of the enantiomeric purity of the selective dopamine transporter inhibitor (+)-*R,R*-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol. *Chirality.* 2017;29:294–303. https://doi.org/10.1002/chir.22688

Supporting Information

Determination of the enantiomeric purity of the selective dopamine transporter inhibitor (+)-*R,R*-4-(2-benzhydryl-oxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol

Patrick Neiens, Georg Höfner, and Klaus T. Wanner

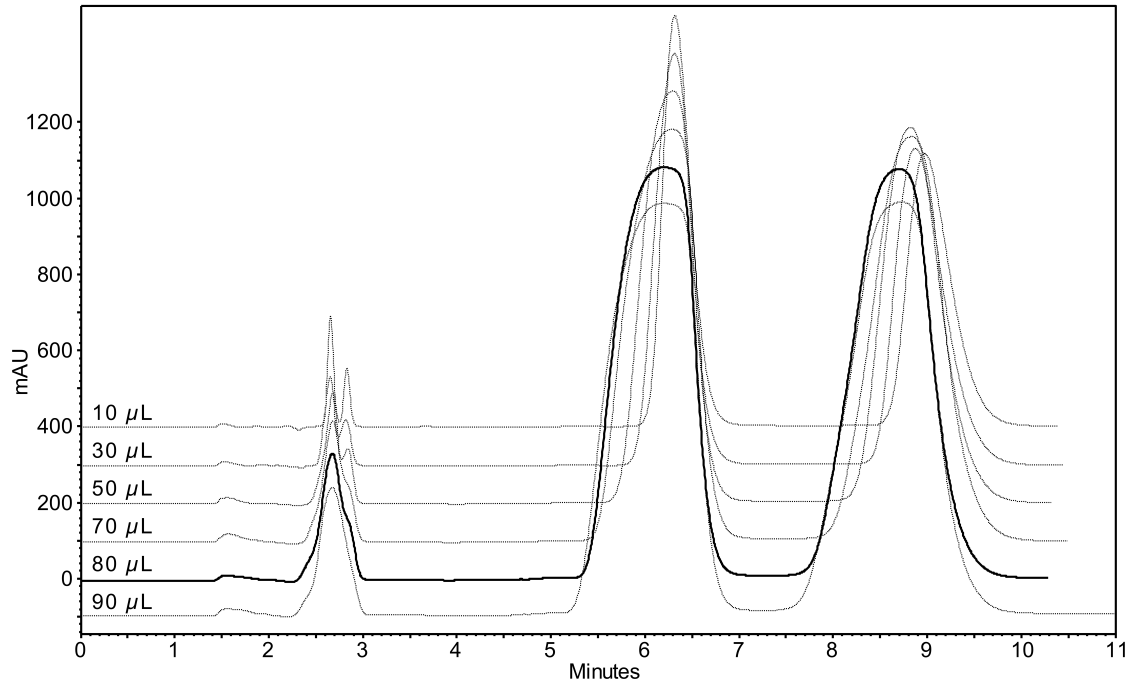

Figure 1: Comparison of chromatograms resulting from varying injection volumes of a 100 mg/mL solution of racemic (-)-*S,S*-D-83 and (+)-*R,R*-D-84, analyzed with the semipreparative CSP-HPLC method (Lux Cellulose-1 (250 x 4.6 mm, 5 µm) column as stationary phase in combination with a mixture of *n*-hexane/propan-2-ol/ethanol/formic acid/DEA (85/13.5/1.5/1.0/0.1; v/v/v/v/v) as mobile phase at a flow rate of 2.0 ml/min at 5 °C).

Choice of the detection wavelength

An UV-spectrum recorded for *rac*-**1** showed a shoulder at 224 nm and a local maximum at 260 nm. In preliminary experiments, we observed a better linear range due to the higher selectivity for 260 nm. Due to the higher selectivity expected for 260 nm, this wavelength was selected for analyte detection.

Collection of fractions in the semi-preparative HPLC method

Collection of the fractions for (-)-*S,S*-D-83 and (+)-*R,R*-D-84 was started as soon as the slope of the first peak was visible (at approximately 5.5 min) and the collection container was changed when the slope of the first peak changed from a negative to a positive slope (at approximately 7.6 min). The second fraction was collected for approximately 3 min.

3.2. Second Publication

Development and Validation of an LC-ESI-MS/MS Method for the Quantification of D-84, Reboxetine, and Citalopram for their Use in MS Binding Assays Addressing the Monoamine Transporters hDAT, hSERT, and hNET

3.2.1. Summary of the Results

In the last years, MS Binding Assays have been developed and established for a variety of targets. MS Binding Assays represent an alternative to radioligand binding assays and enable the implementation of experiments to determine reporter ligand-target interactions hardly feasible with radioligand binding assays. Due to the complex interactions regarding the action of antidepressant drugs with the three monoamine transporters DAT, NET, and SERT, it is important to characterize the binding of such drugs at all of these targets. The aim of this work was to develop an LC-ESI-MS/MS quantification method for the three monoamine reuptake transporter inhibitors (*R,R*)-D-84, (*S,S*)-reboxetine, and (*S*)-citalopram, which were chosen because of their high selectivity for each of the targets ((*R,R*)-D-84 for hDAT, (*S,S*)-reboxetine for hNET, and (*S*)-citalopram for hSERT). This method should be suitable for the quantification of the three markers down to low pM concentrations in samples generated from a biological matrix within a single and fast chromatographic run. The use of one method for the quantification of all three markers is an important requirement for the intended use in Simultaneous Multiple MS Binding Assays, which were to be established in a subsequent step. To demonstrate the selectivity, accuracy, precision, sensitivity, and linearity, the LC-ESI-MS/MS method should be validated according to the CDER guideline for bioanalytical method validation. The implementation of the method for saturation experiments of the markers towards their particular targets, should show the suitability of the method for MS Binding Assays.

For the chromatographic separations of the three markers from the matrix, a Luna pentafluorophenyl (PFP(2), 50 x 2.0 mm, 3 µm, Phenomenex) column was used in combination with a mobile phase consisting of acetonitrile/ammonium formate buffer (0.75 mg/mL; 85/15; v/v) at a flow rate of 800 µL/min. The column temperature was set to 20 °C and a volume of

30 µL of the sample was injected. For the detection of the analytes and their deuterated internal standards, an AB Sciex API 5000 mass spectrometer with an electrospray ionization (ESI) ion source was used in multiple reaction monitoring (MRM) mode, acquiring two MRM transitions for each analyte and one for every internal standard. During a chromatographic run time of 1.5 minutes, the three markers were separated and isolated from the matrix.

The procedure used for the binding experiments was adopted and adjusted from previously published MS Binding Assays. Binding samples containing the marker, target protein, and – if needed – an inhibitor to block specific binding sites in samples for the determination of nonspecific binding in an HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer, supplemented with NaCl and KCl, were incubated for 2 hours at 20 °C. As an inhibitor in the samples for the determination of the nonspecific binding, *rac*-[$^{2}H_3$]-indatraline was used, due to the ability of indatraline to efficiently block specific binding sites at all three targets. To avoid interferences with another MS Binding Assay based on non-deuterated indatraline, running on the same LC-MS system, the deuterated compound was used. After the incubation, the binding samples were filtrated through glass fiber filters and subsequently washed with ammonium formate buffer to separate the target-marker complexes from the unbound marker. The glass fiber filters were then dried before the markers were eluted from the target proteins with acetonitrile. Finally, the concentration of the marker in the acetonitrile eluates was determined with the developed LC-MS method.

During the method development and preliminary saturation experiments, an adsorption of D-84 to polypropylene reaction vessels and the glass fiber filters was observed. Addition of dimethylacetamide (DMA) to the aqueous solutions of D-84 and to the washing buffer was effective to prevent adsorption as well as to remove adsorbed D-84 from the glass fiber filters without affecting the formation and stability of the target-marker complexes.

To demonstrate that the markers are sufficiently separated from the matrix during the chromatographic step, post-column infusion experiments were performed with the final assay procedure. According to the results of these experiments the markers are not co-eluting with matrix components which appeared to cause multiple zones of signal suppression. Since one of these matrix suppression zones occurs after the elution of the last analyte peak, the effect

of the matrix on the following chromatograms was investigated. These experiments demonstrated, that no signal suppression is caused due to late-eluting matrix components in successive chromatograms. Therefore, the chosen chromatographic conditions warrant a good separation of the markers from all interfering matrix components.

The final LC-ESI-MS/MS quantification method was validated according to the CDER guideline for bioanalytical method validation in regard to selectivity, accuracy, precision, sensitivity, and linearity. For the validation, matrix samples from all three target proteins were prepared under the procedure of the binding assays. All requirements of the guideline were fulfilled for concentrations from 2.5 pM to 1 nM for (*R,R*)-D-84, from 1 pM to 2.5 nM for (*S,S*)-reboxetine, and from 2.5 pM to 2.5 nM for (*S*)-citalopram.

Saturation experiments performed under the established assay conditions utilizing the validated LC-ESI-MS/MS quantification method resulted in K_d–values of 3.66 $\pm$ 1.35 nM for (*R,R*)-D-84 towards hDAT, 3.06 $\pm$ 0.46 nM for (*S,S*)-reboxetine towards hNET, and 0.411 $\pm$ 0.003 nM for (*S*)-citalopram towards hSERT. These binding affinities are in good agreement with data published in literature, thus demonstrating the suitability of these MS Binding Assays for a reliable characterization of reporter ligand-target interactions.

3.2.2. Declaration of Contributions

[$^2H_4$]-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol and *rac*-[$^2H_3$]-indatraline were synthesized by Lars Allmendinger and Gerd Bauschke. Racemic reboxetine was extracted from tablets. The CSP-HPLC method for the semipreparative separation of *rac*-reboxetine was developed and applied by Anna Ramershoven to obtain (*S*)-reboxetine. Furthermore, Anna Ramershoven developed an analytical CSP-HPLC method for the determination of the enantiomeric purity of reboxetine and characterized the *ee* of the final products. The extraction, semipreparative enantioseparation and *ee*-determination is described in the bachelor thesis “Semipräparative Trennung des racemischen Antidepressivums Reboxetin sowie Entwicklung und Validierung einer HPLC-Methode zur Bestimmung der

Enantiomerenreinheit" by Anna Ramershoven, which was prepared under my supervision [74]. Characterization of MRM transitions for the markers and method development of the LC-ESI-MS/MS quantification method was done by myself. Experiments for the final method optimization, the validation, and the saturation experiments were performed by Angela De Simone and myself. I wrote the manuscript and prepared the graphics and tables, assisted by Georg Höfner, Lars Allmendinger, and Angela De Simone. Klaus T. Wanner corrected the manuscript.

Development and validation of an LC-ESI-MS/MS method for the quantification of D-84, reboxetine, and citalopram for their use in MS Binding Assays addressing the monoamine transporters hDAT, hSERT, and hNET.

Patrick Neiens,[a] Angela De Simone,[b] Anna Ramershoven,[a] Georg Höfner,[a] Lars Allmendinger,[a] Klaus T. Wanner[a*]

[a]Department Pharmazie – Zentrum für Pharmaforschung
Ludwig-Maximilians-Universität München
Butenandtstr. 7, 81377 Munich, Germany
Phone: +49 (0) 89 2180 77249
e-mail: klaus.wanner@cup.uni-muenchen.de
ORCID: 0000-0003-4399-1425

[b]Department for Life Quality Studies,
Alma Mater Studiorum – University of Bologna,
Corso D'Augusto 237, 47921 Rimini, Italy

Short title: LC-MS/MS quantification method for D-84, reboxetine, and citalopram
Keywords: MS Binding Assays, LC-MS/MS, monoamine transporters, method development, validation

Abstract

MS Binding Assays represent a label free alternative to radioligand binding assays. In this study, we present an LC-ESI-MS/MS method for the quantification of (*R,R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84, (*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) employed as highly selective nonlabelled reporter ligands in MS Binding Assays addressing the dopamine (DAT, (*R,R*)-D-84), the norepinephrine (NET, (*S,S*)-reboxetine), and the serotonin transporter (SERT, (*S*)-citalopram), respectively.

The developed LC-ESI-MS/MS method uses a pentafluorphenyl (PFP) stationary phase in combination with a mobile phase composed of acetonitrile and ammonium formate buffer for chromatography and a triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) mode for mass spectrometric detection. Quantification is based on deuterated derivatives of all three analytes serving as internal standards. The established LC-ESI-MS/MS method enables fast, robust, selective, and highly sensitive quantification of all three reporter ligands in a single chromatographic run. The method was validated according to the CDER guideline for bioanalytical method validation regarding selectivity, accuracy, precision, calibration curve, and sensitivity. Finally, filtration based MS Binding Assays were performed for all three monoamine transporters based on this LC-ESI-MS/MS quantification method as read out. The affinities determined in saturation experiments for (*R,R*)-D-84 towards hDAT, for (*S,S*)-reboxetine towards hNET, and for (*S*)-citalopram towards hSERT, respectively, were in good accordance with results from literature, clearly demonstrating that the established MS Binding Assays have the potential to be an efficient alternative to radioligand binding assays widely used for this purpose so far.

Introduction

According to the World Health Organization (WHO), an estimated 300 million people worldwide suffer from depression. This makes depression a leading cause of disability and can lead to suicide of the patient in the worst case (World Health Organization, 2017). The genesis of depressions is often explained with the catechol hypothesis (Lapin & Oxenkrug, 1969; Schildkraut & Kety, 1967), which links the dopaminergic, norepinephrinergic, and serotoninergic neuronal system to the emergence of this disease. In accord with this hypothesis, a lack of these monoamine neurotransmitters at corresponding synapses can be counteracted by the inhibition of the monoamine transporters, which terminate the neuronal signal transmission by removing the neurotransmitters out of the synaptic cleft. The inhibition of the monoamine transporters can be achieved by selective inhibitors of the serotonin transporter (SERT) or the norepinephrine transporter (NET) as well as by dual reuptake inhibitors targeting the SERT and NET or the NET and the dopamine transporter (DAT). These classes of monoamine transporter inhibitors are often used in the therapy of depression, but they inherit a few insufficiencies. It has to be noted that the therapy with these antidepressants has a considerable lag between the administration of the drug and the onset of

the therapeutic effect. A second shortcoming of these antidepressants is that only 60 – 70 % of the treated persons respond to the therapy (Ferrari & Villa, 2016). Thus, there is still a strong demand for the development of new drugs, targeting the monoamine transporters with an improved therapeutic profile.

To identify new potential drugs, screening methods are necessary for identification of compounds which can act at predefined targets. Such test systems can either detect a functional activity or affinity of a test compound at a target. Radioligand binding assays were performed for the first time in the 1970s (Lefkowitz, Roth, & Pastan, 1970) and have been extensively used to characterize binding affinities (R. Zhang & Xie, 2012). To apply this technique, a radiolabelled reporter ligand, which can be quantified by means of scintillation counting, is incubated together with the target. After the incubation, the target-radioligand complexes are separated from the unbound radioligand (e.g. by filtration) and the bound radioligand is quantified based on the radioactivity in the sample. Various types of binding experiments can be carried out following this strategy, such as the determination of the radioligand's equilibrium dissociation constant K_d towards the target in saturation assays or the determination of the inhibitory constant K_i of test compounds incubated together with the radioligand and the target in competition experiments. The challenge of quantification of low bound radioligand concentrations due to the low concentrations of the target is met by the high sensitivity offered by scintillation counting. But the radioactivity in radioligand binding assays also causes multiple problems associated with legal restrictions for the handling of the radioisotope labelled compounds as well as with complicated and expensive regulations regarding the disposal of the radioactive waste. To circumvent these issues, MS Binding Assays have been established and proven to be a powerful alternative to radioligand binding assays (Grimm, Höfner, & Wanner, 2015a; Hess, Höfner, & Wanner, 2011; Massink et al., 2015; Neiens, Höfner, & Wanner, 2015; Zepperitz, Höfner, & Wanner, 2006). MS Binding Assays follow the procedure of radioligand binding assays, but as a reporter ligand, a nonlabelled ligand, also referred to as MS marker, is used. The utilization of mass spectrometry (MS) as a detection principle allows the quantification of the reporter ligand with a sensitivity that can reach low picomolar concentrations (Grimm et al., 2015a; Hess et al., 2011; Neiens et al., 2015). As in radioligand binding assays, MS Binding Assays can use a filtration step for the separation of the formed target-marker complexes from the nonbound marker. After this separation, the target protein is denatured to liberate the formerly bound MS marker, which is then eluted to generate a sample that is subjected to marker quantification by LC-ESI-MS/MS. For the use in MS Binding Assays, the quantification

method has to fulfil an important criterion, regarding its sensitivity. To carry out saturation assays, samples with nominal marker concentrations ranging from 0.1 K_d up to 10 K_d should be analysed (Hulme, 1992). According to the calculation, described in a previously published work, the quantification method used in MS Binding Assays should be able to determine concentrations down to 0.0091 K_d (Grimm, Höfner, & Wanner, 2015b). Since the affinity of markers used in MS Binding Assays is in the low nM range, the quantification method to be developed should enable quantification of low picomolar concentrations of each marker in samples obtained from the binding experiment. As mentioned above, LC-ESI-MS/MS is a suitable analytical technique to quantify such low concentrations of nonlabelled substances, nevertheless, this can be still a very challenging task. Besides its high sensitivity, LC-MS/MS provides the possibility to analyse multiple analytes simultaneously, even if they are not chromatographically separated. In this study, we wanted to develop a single quantification method, which enables the quantification of the three selective markers (one for each target) under the same chromatographic conditions. This would allow us to develop the MS Binding Assays further to multiple simultaneous MS Binding assays, like the ones recently published for the dopamine D_1 and D_5 receptors (Schuller, Höfner, & Wanner, 2017). Due to the ability to perform binding experiments at multiple targets simultaneously, Multiple Simultaneous MS Binding Assays have huge advantages regarding their efficiency, compared to radioligand binding assays. Furthermore, it is worth to mention that the efforts made for LC-ESI-MS/MS method development can be considered as particularly efficient, as the established method can be applied for MS Binding Assays addressing three different targets in this case. In this study, we aimed at developing an LC-ESI-MS/MS quantification method for markers of the biogenic amine transporters hSERT, hDAT, hNET as the basis for the establishment of Multiple MS Binding Assays for the aforementioned targets.

The robustness of this LC-ESI-MS/MS quantification method and its suitability for the desired Multiple MS Binding Assays, we intended to develop, were considered to be best verified by its validation according to the CDER guideline for bioanalytical method validation (FDA, 2013). Finally, this established LC-ESI-MS/MS method should be applied for the quantification of all three markers in MS Binding Assays addressing hDAT, hNET, and hSERT, respectively. Accordingly, saturation experiments of each marker towards its specific target should be performed. For the control of the obtained binding affinities, the latter should be compared with published literature data. As in radioligand binding assays, and many related techniques also for MS Binding Assays the concentration of specifically bound marker in binding samples may be determined only indirectly. Two sets of experiments have to be

performed, one for the determination of the total binding (TB, entire bound marker in a binding sample), a second set of samples for the determination of nonspecific binding (NSB, marker bound to nonspecific binding sites), the difference of which provides the specific binding (SB) (SB = TB – NSB).

At first, markers suitable for the selective labelling of the monoamine transporters hDAT, hNET, and hSERT had to be chosen. In contrast to previously published MS Binding Assays using (1*R*,3*S*)-indatraline as a nonselective marker for all three targets (Grimm et al., 2015a), the MS Binding Assays to be developed in this study should be based on selective markers each selectively addressing DAT, NET, and SERT, respectively. As already indicated, this approach has the potential that the MS Binding Assays can determine the affinity of test compounds simultaneously at all three targets, a procedure which is hardly feasible with radioligand binding assays. Provided selective reporter ligands are employed such an approach would also allow the use of native target material in which the different monoamine transporters are present. An ideal marker for an MS Binding Assay should have a high affinity for its target, preferable a K_d in the low nM range. Furthermore, the marker should be commercially available in its native as well as its deuterated form or the latter should be at least synthetically easily accessible. Well characterized ligands such as radioligands used in radioligand binding assays, may also be a good choice for MS Binding Assays, which of course, were to be employed in their native, i.e. unlabelled form.

For the hDAT, [^{3}H]-WIN 35,428 (also known as [^{3}H]-CFT) or [^{125}I]-RTI-55 are often applied in radioligand binding assays (Dersch et al., 1994; Gracz & Madras, 1995; Reith, de Costa, Rice, & Jacobson, 1992; Richard B Rothman et al., 2002). However, [^{3}H]-WIN 35,428 possesses only a low selectivity for DAT against NET and SERT, whilst the affinity of [^{125}I]-RTI-55 for DAT is even lower than for NET and SERT (Eshleman et al., 1999; R B Rothman et al., 1994), requiring additional ligands blocking the binding to NET and SERT when this ligand is used (Richard B Rothman et al., 2002). This apparent lack of selective DAT inhibitors that are expected to possess a high potential in the treatment of cocaine dependence has spurred an intensive search for these compounds (Ritz, Lamb, Goldberg, & Kuhar, 1987; Wilcox, Paul, & Woolverton, 1999). Thus, a wide variety of compounds from different structural classes (Gryzło, Zaręba, Malawska, Jakubowska, & Kulig, 2015; Singh, 2000), like tropane (F I Carroll et al., 1992; F Ivy Carroll et al., 1991), benztropine (Agoston et al., 1997; Newman, Allen, Izenwasser, & Katz, 1994), or piperazine derivatives such as 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazin (GBR 12909) and 1-[2-(Diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine (GBR 12935) (Hsin et al., 2002; R B

Rothman et al., 1993; Van der Zee, Koger, Gootjes, & Hespe, 1980) have been synthesized and tested for their selectivity as DAT inhibitors. This led to the identification of the (+)-*R*,*R* enantiomer of 4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84, (*R,R*)-**1**, see figure 1) as a highly selective and highly potent DAT inhibitor (Ghorai et al., 2003). In radioligand binding assays for DAT in crude rat striatum with [^{3}H]-WIN 35,428 as radioligand, for this compound an IC_{50} of 0.46 nM was found. For the two other monoamine transporters, an IC_{50} of 3,600 nM at SERT and an IC_{50} of 1,880 nM at NET were determined in studies with crude rat cerebral cortex with [^{3}H]-citalopram for SERT and [^{3}H]-nisoxetine for NET as radioligand. According to these results, (*R,R*)-D-84 ((*R,R*)-**1**) has a 7,800-fold lower affinity for the SERT compared to DAT and a 4,100-fold lower affinity for NET versus DAT (Ghorai et al., 2003). In MS Binding Assays for hSERT, hNET, and hDAT based on (1*R*,3*S*)-indatraline as MS marker, a series of commercially available and resynthesized DAT inhibitors selective for DAT from literature were analysed regarding their affinity and selectivity towards the human monoamine transporters. From all analysed compounds, (*R,R*)-D-84 ((*R,R*)-**1**) appeared to possess the highest selectivity for hDAT as compared to hSERT and hNET and a relatively low nM affinity thus allowing target concentrations in the low µg/mL-range in the planned MS Binding Assays (Grimm et al., 2015b). This and the easy synthetic accessibility of this compound allowing the synthesis of deuterated derivatives required as an internal standard made this compound appear to be well suited as an MS marker in multiple simultaneous MS Binding Assays for hSERT, hNET, and hDAT.

For NET, several selective and potent inhibitors have been published. Nisoxetine has been applied in radioligand binding assays (Ghorai et al., 2003; Meyers & Kritzer, 2009; Zavosh, Schaefer, Ferrel, & Figlewicz, 1999), while reboxetine was reported to be a potential PET radioligand (Zeng et al., 2008). In addition, talopram was found to have a high selectivity towards hNET (Andersen et al., 2011). Finally, according to data from the (1*R*,3*S*)-indatraline MS Binding Assays out of these three compounds *rac*-reboxetine has the highest selectivity for hNET (Grimm et al., 2015b). In addition, published data for (*S,S*)-reboxetine ((*S,S*)-**2**) state that this enantiomer has a binding affinity of 0.2 nM towards NET and one of 2,900 nM for SERT. For racemic reboxetine, a binding affinity of 1.1 nM towards NET and 129 nM towards SERT, respectively, has been published (Hajós, Fleishaker, Filipiak-Reisner, Brown, & Wong, 2004). Accordingly, (*S,S*)-reboxetine ((*S,S*)-**2**) can be considered well-suited for its use as a selective marker in MS Binding Assays which is even more true as deuterated form of reboxetine is commercially available that could serve as an internal standard.

For hSERT, a variety of drugs selectively targeting this protein are on the market commonly termed selective serotonin reuptake inhibitors (SSRIs). Examples are fluoxetine, paroxetine, sertraline, and citalopram, to name only a few. In radioligand binding assays (*S*)-citalopram ((*S*)-**3**) yielded K_i values of 0.28 nM towards SERT and 28,200 nM towards NET (Deupree, Montgomery, & Bylund, 2007), respectively, in one study, or K_i values of 1.1 nM towards hSERT, 27,410 nM towards hDAT, and 7,841 nM towards hNET, respectively, in another one (Owens, Knight, & Nemeroff, 2001). Thus, (*S*)-citalopram ((*S*)-**3**) displays the desired high selectivity for the hSERT in combination with an affinity in the low nM to high pM range. Moreover, as enantiopure (*S*)-citalopram ((*S*)-**3**) as well as deuterated citalopram are commercially available, this ligand seems to be an ideal marker for hSERT.

Based on the above given considerations, (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) were chosen as markers for the MS Binding Assays to be developed.

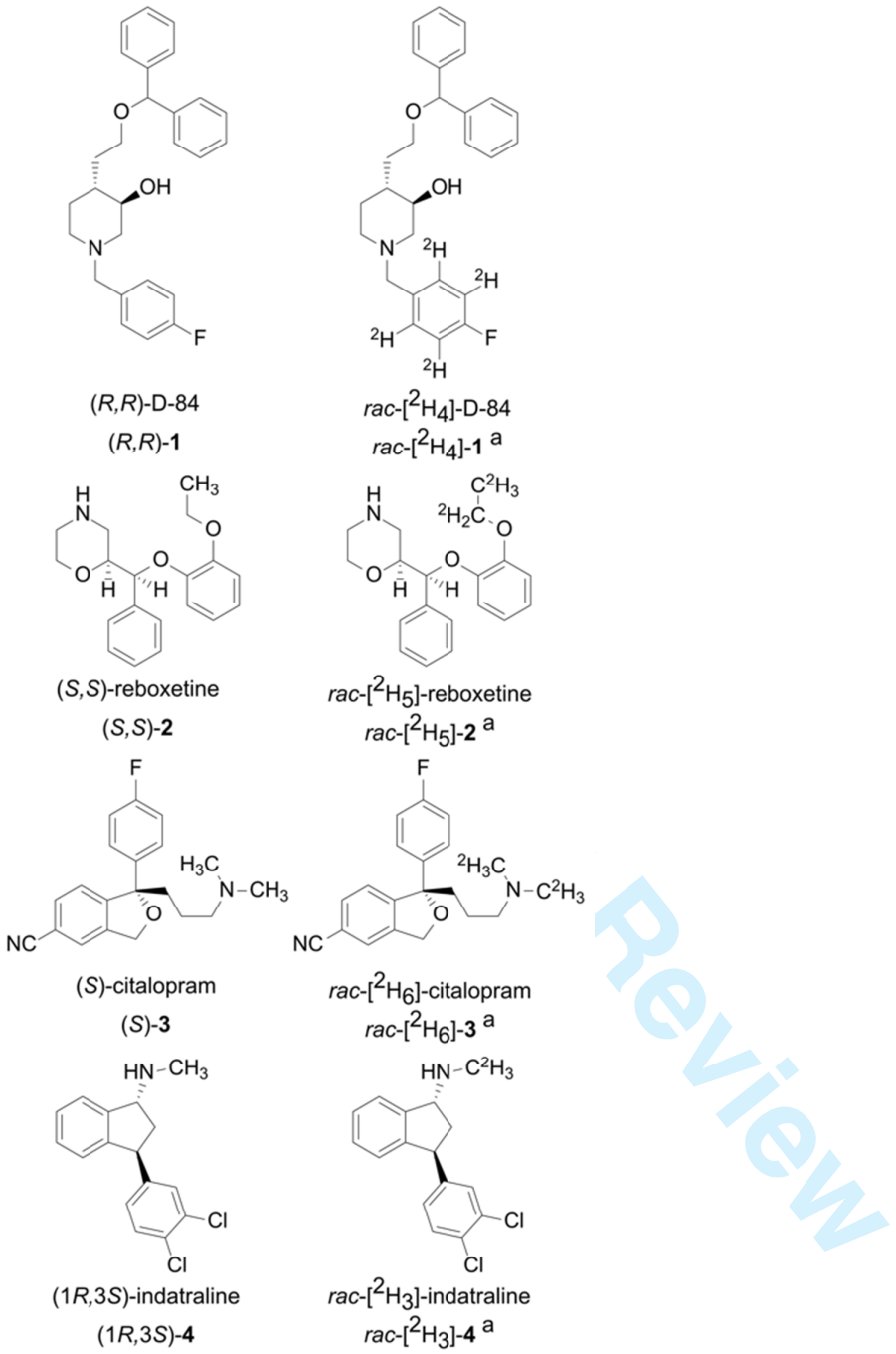

Figure 1: Structures of markers, internal standards and the inhibitor employed for determination of non-specific binding in MS Binding Assays. [a] Deuterated substances were used as racemates, but only one enantiomer is depicted in the figure. For the sake of simplicity, the racemic mixture of (*R,R*)-D-84 and (*S,S*)-D-83 (the enantiomer of (*R,R*)-D-84) is referred to as *rac*-D-84 and the racemic mixture of (*R,R*)-[$^2H_4$]-D-84 and (*S,S*)-[$^2H_4$]-D-83 is called *rac*-[$^2H_4$]-D-84 throughout this work.

Experimental

Chemicals

HPLC grade acetonitrile (for use at the API 3200) and LC-MS grade solvents (acetonitrile, methanol, water, for use at the API 5000) were purchased from VWR Prolabo (Darmstadt, Germany). HPLC grade methanol (for use at the API 3200) was bought from Fisher (Loughborough, UK). Water used for the preparation of mobile phase (for the API 3200), incubation and wash buffers had been purified by distillation of demineralized water (reverse osmosis) followed by filtration using a 0.45 µm filter. Additives for mobile phase buffers (ammonium formate, ammonium bicarbonate, formic acid, ammonium hydroxide solution ≥ 25 %; for mass spectrometry), were bought from Fluka (Teufelskirchen, Germany). *rac*-Citalopram (*rac*-**3**) HBr was purchased from Enzo Life Chemicals (Lörrach, Germany), (*S*)-citalopram oxalate ((*S*)-**3**) was bought from TCI (Eschborn, Germany), [$^2H_6$]-citalopram oxalate (*rac*-[$^2H_6$]-**3**) from Alsachim (Illkirch, France), and [$^2H_5$]-reboxetine mesylate (*rac*-[$^2H_5$]-**2**) was bought from TRC (North York, ON, Canada), all in the best commercially available quality.

4-(2-Benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol (*rac*-D-84, *rac*-**1**) was synthesized and purified according to Ghorai (Ghorai et al., 2003). For the sake of simplicity, the racemic mixture of (*R,R*)-D-84 and (*S,S*)-D-83 (the enantiomer of (*R,R*)-D-84) is referred to as *rac*-D-84 throughout this work. The enantiomer (*R,R*)-D-84 ((*R,R*)-**1**) was obtained after semipreparative enantioseparation of *rac*-D-84 (Neiens, Höfner, & Wanner, 2017). *rac*-Reboxetine (*rac*-**2**) was extracted from Edronax tablets (4 mg, Pfizer Europe, Kent, UK) as a racemate which was further used to perform a semipreparative CSP-HPLC to obtain (*S,S*)-reboxetine ((*S,S*)-**2**, see supporting information). The enantiopurities were determined by CSP-HPLC. Enantiomeric excesses amounted to ≥ 99.8 % for (*R,R*)-D-84 ((*R,R*)-**1**) and 99.3 % for (*S,S*)-reboxetine ((*S,S*)-**2**). [$^2H_4$]-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol (*rac*-[$^2H_4$]-D-84, *rac*-[$^2H_4$]-**1**) was synthesized utilizing an H/D exchange reaction (Allmendinger & Wanner, 2014) (total synthesis is to be published soon). *rac*-[$^2H_3$]-Indatraline (*rac*-[$^2H_3$]-**4**) HCl was synthesized according to Grimm et al. (for synthesis see supporting information) (Grimm, Allmendinger, Höfner, & Wanner, 2013).

Polyethylenimine (PEI) solution (50 % in H_2O) was bought from Fluka, HEPES and roti-quant (5 x concentrated) from Roth (Karlsruhe, Germany).
All percentages and ratios refer to v/v if not indicated otherwise.

Solutions for marker and internal standard

Marker and internal standard solutions for the performance of MS Binding Assays were prepared as follows. Stock solutions with a concentration of 1 mM of each marker and each internal standard in H_2O/dimethylacetamide (DMA; 80/20) were prepared separately and stored at -20 °C. For the binding samples, calibration standards and the quality control (QC) samples, solutions with the 100-fold concentration (regarding the concentration in the final sample) were prepared in H_2O/DMA (80/20) based on these stock solutions. For the binding samples, the 100-fold concentrated marker solutions in H_2O/DMA (80/20) were used to obtain the binding samples (see "MS Binding Assay"). A 10 nM solution with all three internal standards in H_2O/DMA (80/20) was diluted with acetonitrile to yield a 100 pM internal standards solution. Based on this internal standard solution, elution solutions for the validation and the MS Binding Assays were prepared. For the validation (see "Method validation"), 10-fold concentrated marker solutions (generated by dilution of a solution containing all three markers at 100-fold concentration with acetonitrile) were spiked to the 100 pM internal standard solution (1 part 10-fold concentrated marker solution + 9 parts 100 pM internal standard solution), resulting in a set of solutions containing the three marker at the final concentration for the calibration sample or QC sample and 90 pM of the three internal standards. To elute the bound marker in the MS Binding Assays (see "MS Binding Assay"), the 100 pM internal standard solution was diluted with acetonitrile (9 parts 100 pM internal standard solution + 1 part acetonitrile) to obtain a solution containing all three internal standards at a concentration of 90 pM (elution solution). All solutions were prepared in micro tubes (1.5 mL, polypropylene, Sarstedt, Nürmbrecht, Germany) and for the pipetting, non-coated pipet tips without filters (quality tips without filters, 20 µL neutral, 200 µL yellow 300 µL neutral, 1000 µL blue, and 1250 µL neutral, Sarstedt, Nürmbrecht, Germany) were used.

Instrumentation

HPLC method development was carried out on an API 3200 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with a Turbo V Ion Source, which was coupled with an Agilent 1200 HPLC system (vacuum degasser, binary pump, column oven; Agilent Waldbronn, Germany) and a Shimadzu SIL-HTA autosampler (Shimadzu, Duisburg, Germany). Mass spectrometric measurements for the optimization of the binding assay, validation, and the saturation experiments were performed on an API 5000 triple quadrupole mass spectrometer (AB Sciex) equipped with a pneumatically assisted Turbo V Ion Source, coupled to an Agilent 1200 HPLC system (vacuum degasser, binary pump, column oven) and a HTS-PAL autosampler (50 µL sample loop, 50 µL syringe; CTC Analytics, Zwingen, Switherland). For infusion experiments at the API 5000 a syringe pump (model 11 Plus MA 170-2208, Harvard Apparatus Inc., Holliston, MA, USA) in combination with a 1 mL syringe (Hamilton, Cinnamonson, NJ, USA) was used and connected to the ion source via a T-piece. In all mass spectrometric measurements, the resolution of Q1 and Q3 was set to unit resolution. The LC-MS systems were controlled with Analyst 1.6 software (AB Sciex).

As stationary phases a YMC Triart PFP column (50 x 2.0 mm, 3 µm, YMC Europe GmbH, Dinslaken, Germany) with a YMC Triart precolumn (10 x 2.0 mm, 3 µm) and a Luna PFP(2) column (50 x 2.0 mm, 3 µm, Phenomenex, Aschaffenburg, Germany), protected by a Luna PFP(2) security guard cartridge (4 x 2.0 mm, 3 µm, Phenomenex) were used. Furthermore, Amino, Diol NP, Polyamine, PVA SIL, and Silica (all 50 x 3.0 mm, 5 µm, YMC Europe GmbH, Dinslaken, Germany) were used as polar stationary phases. In all experiments, the stationary phase was additionally protected by two successive inline filters (IDEX, Wertheim-Monfeld, Germany) of 0.5 µm (stainless steel) and 0.2 µm (titanium) porosity.

Identification and tuning of MRM mass transitions

Identification of mass transitions and optimization of compound-dependent parameters for the operation in MRM mode was done with the Quantitative Optimization tool of the Analyst software. For this purpose, solutions with a concentration of 400 nM (for the API 3200) or 20 nM (for the API 5000) of each marker and internal standard separately dissolved in methanol/formic acid (0.1 %; 50/50) was infused (5 µL/min) directly into the ESI-source of the mass spectrometer (positive mode).

Development of the LC-MS/MS method

During the method development, the HPLC parameters were evaluated at the API 3200 with solvent samples containing *rac*-D-84 (*rac*-**1**), *rac*-reboxetine (*rac*-**2**), and *rac*-citalopram (*rac*-**3**) in acetonitrile. Detection was based on the mass transitions *m/z* 420.3/167.0 for *rac*-D-84 (*rac*-**1**), *m/z* 314.2/176.1 for *rac*-reboxetine (*rac*-**2**), and *m/z* 325.2/109.1 for *rac*-citalopram (*rac*-**3**).

Flow injection analysis

A solution of (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), (*S*)-citalopram ((*S*)-**3**), *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**), *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**), and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**), each in a concentration of 100 pM in acetonitrile containing the matrix (for preparation see "MS Binding Assay" below) was prepared for the flow injection analysis (FIA). For each run an injection volume of 10 µL of this solution was used and analysed with the FIA option in the Compound Optimization Tool of the Analyst software. The source-dependent parameters, such as ionization voltage, ion source temperature, and gas pressures were optimized in repeated chromatographic runs with the final HPLC method (see "LC-ESI-MS/MS quantification method).

Investigation of the matrix effect by post-column infusion experiments

Thirty µL of samples containing *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**), *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**), and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**) in a concentration of 100 pM, dissolved in blank matrix (preparation see "MS Binding Assay" below) were injected into the chromatographic system. The flow rate of the HPLC was set to 200 µL/min. The optimized source dependent parameters (determined with the FIA option of the Compound Optimization Tool of the Analyst software, see above) were as following: ion source temperature (TEM) 300 °C, ion spray voltage (IS) 5,000 V, nebulizing gas (GS1) 20 psi, and auxiliary gas (GS2) 50 psi. The acquisition time was set to 15 min. All other method parameters were identical to the final method, described in LC-ESI-MS/MS quantification method (see below). A solution of 300 pM (*R,R*)-D-84 ((*R,R*)-**1**), 2 nM (*S,S*)-reboxetine ((*S,S*)-**2**), and 600 pM (*S*)-citalopram ((*S*)-**3**) in mobile phase was infused at a flow rate of 100 µL/min into the eluent of the HPLC by means of a syringe pump with a Hamilton Gastight 5 mL syringe coupled to a 3-way-connector between the HPLC-column and the MS.

LC-ESI-MS/MS quantification method

For the quantification of the MS markers, a Luna PFP(2) column was used in combination with a mobile phase consisting of acetonitrile/ammonium formate buffer (0.75 mg/mL; 85/15) at a flow rate of 800 µL/min at a temperature of 20 °C. The injection volume was 30 µL. For the run time of the first 0.4 min the eluent was directed to waste. With positive polarity, the markers and internal standards were analysed under pneumatically assisted ESI conditions, using an ion source temperature of 650 °C, an ion spray voltage of 1,500 V, a curtain gas (CUR) pressure of 25 psi, a collision gas (CAD) of 5 psi, a nebulizing gas of 30 psi, and an auxiliary gas of 60 psi. For each marker two mass transitions were acquired (the first one with a dwell time of 50 ms the other one as a qualifier with a dwell time of 10 ms) in multiple reaction monitoring (MRM) mode. One mass transition with a dwell time of 50 ms was acquired for each internal standard. The mass transitions at the API 5000 were *m/z* 420.2/167.1 and *m/z* 420.2/109.1 for (*R,R*)-D-84 ((*R,R*)-**1**), *m/z* 314.1/176.2 and *m/z* 314.1/91.1 for (*S,S*)-reboxetine ((*S,S*)-**2**), *m/z* 325.2/109.2 and *m/z* 325.2/262.2 for (*S*)-citalopram ((*S*)-**3**), *m/z* 424.2/167.1 for *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**), *m/z* 319.2/176.2 for *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**) and *m/z* 331.2/109.0 for *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**).

Method validation

For validation six series, each containing blank samples, zero samples, calibration standards, and QC samples were investigated. In every validation series, matrix samples containing all three markers and internal standards were analysed. All standards (excluding the solvent blanks) were prepared similar to the binding samples ("MS Binding Assay", see below) but without markers during the incubation. Elution solutions for the validation samples ("Solutions for marker and internal standard", see above), which were transferred through the 96-well filter plate containing the membrane fractions, were spiked with the markers in varying concentrations and 90 pM of the internal standards.

For matrix blanks and zero samples (containing the matrix and the internal standards at 90 pM), six replicates were prepared and analysed.

The calibration curve was established with calibration standards in eleven concentration levels, ranging from 1 pM to 2.5 nM concentrations of each marker (the lower three concentrations in six replicates, all other concentrations in triplicates). The resulting area ratios (y, peak area MS marker/peak area internal standard) of each marker was plotted

against the concentration of the marker (x) and a linear regression with a weighting of 1/x was applied to obtain the calibration functions. To determine the intra- and inter-batch accuracy as well as the precision, QC samples at four concentration levels (2.5 pM, 10 pM, 100 pM, 1 nM) were prepared and analysed in six replicates for each level.

As a system suitability check, a 90 pM internal standard solution in acetonitrile was analysed prior to each series of measurements. The chromatogram obtained from this sample was evaluated in regard to the retention time (retention time of *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**): 1.15 – 1.25 min), the peak symmetry (no visible tailing or fronting for *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**)) and the intensity (given as peak heights, not less than 30,000 cps for *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**), not less than 10,000 cps for *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**), and not less than 20,000 cps for *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**)) of each internal standard.

MS Binding Assay

MS Binding Assays were carried out, using membrane preparations of HEK293 cells, stably expressing hDAT, hNET or hSERT, respectively (Grimm et al., 2015a). Aliquots of the membrane preparations, stored at -80 °C were thawed on the day of the assay. For saturation assays of (*R,R*)-D-84 ((*R,R*)-**1**) towards hDAT, 1 mL of the hDAT membrane preparation was diluted in 20 mL cold assay buffer (50 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4), centrifuged (20 min, 20,000 rpm, 4 °C, Sorvall Evolution, SS-34 rotor, Thermo Fisher Scientific, Dreieich, Germany) and the supernatant was discarded. The pellet was resuspended in 15 mL cold assay buffer and diluted with 10 mL assay buffer additionally to a final volume of 25 mL protein preparation. Membrane preparations for saturation experiments of the other two markers were prepared like described above, but with the following deviations: to perform saturation assays of (*S,S*)-reboxetine ((*S,S*)-**2**) towards hNET, 1 mL of the hNET membrane preparation was centrifuged in 20 mL assay buffer, the pellet was resuspended in 15 mL assay buffer and finally diluted with assay buffer to yield a volume of 50 mL protein preparation. For saturation assays of (*S*)-citalopram ((*S*)-**3**) towards hSERT, 2.5 mL of the hSERT membrane preparation was centrifuged in 20 mL assay buffer and the pellet was resuspended in 30 mL assay buffer without further dilution.

In a polypropylene 96-deep-well plate (1.2 mL, polypropylene, Sarstedt, Nürmbrecht, Germany), binding samples for the determination of the total binding were prepared in 32 marker concentration levels as triplicates for each concentration level in assay buffer to a volume of 200 µL. The incubation was started by addition of 50 µL membrane preparation to

give a final sample volume of 250 µL resulting in final marker concentrations of 25 pM to 500 nM and a protein content of 1.0 µg protein per well. The samples were then incubated at 20 °C in a shaking water bath for 2 h. To terminate the incubation, 200 µL of each sample was transferred to a 96-well glass fiber filter plate (AcroPrep Advance, glass fiber, 1.0 µm, 350 µL, Pall, Dreieich, Germany) which had been pretreated for 2 h with 150 µL 0.5 % (m/v) PEI solution per well at 4 °C. To transfer the samples row after row, a 12-channel pipette (25 µL - 300 µL, Eppendorf, Hamburg, Germany) was used. The filtration was carried out with a multi well plate vacuum manifold (Pall). After filtration of the samples row by row (i.e. 12 wells), the filters - with the membrane fragments containing the bound marker - were washed three times with 150 µL ice cold washing buffer (0.75 mg/mL ammonium formate, pH 7.4 + 10 % DMA) and finally one time with 150 µL ice cold washing buffer without DMA (0.75 mg/mL ammonium formate, pH 7.4) before the next row was transferred. Subsequent to the filtration of all samples, the 96-well filter plate was dried at 60 °C for 2 h. The bound marker was then liberated by the elution with 3 times 70 µL acetonitrile containing 90 pM of the three internal standards (*rac*-[$^2H_4$]-**1**, *rac*-[$^2H_5$]-**2**, and *rac*-[$^2H_6$]-**3**, elution solution, see "Solutions") into a 96-deep-well plate, leaving the elution solution each time on the filter for 20 s prior to aspiration under vacuum. The plates with the sample solutions were sealed with aluminium foil and centrifuged (10 min, 2000 rpm, 4 °C; Biofuge Stratos, Rotor: #3048, Heraeus, Germany) before they were analysed with the LC-MS/MS method.

Samples for the determination of the nonspecific binding were prepared by spiking of the binding samples with *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) prior to the addition of the target, to obtain a concentration of 10 µM of *rac*-[$^2H_3$]-indatraline in the final binding samples. Preparation of these samples was performed according to the procedure described above. All the other parameters (e.g. replicates, marker concentrations, incubation time) were kept the same.

Every day, when an MS Binding Assay was performed, at least one set of samples, containing all blanks, calibration standards, and QC samples described in the section "Validation", was prepared and analysed together with the binding samples. Based on this calibration, binding samples were quantified.

Matrix samples, used in the method development, for the determination of the matrix effect and for the validation were prepared analogously, in the same way as the incubation samples, except that no marker was added.

Data analysis

All chromatograms for method development, validation as well as marker quantification in saturation experiments were acquired with Analyst 1.6 software. The Analyst 1.6 software was further used to generate the calibration functions and the calculations for the accuracy of calibration and QC samples. In Prism 6 software (GraphPad Software, San Diego, CA, USA) the area ratios (peak area MS marker/peak area internal standard), obtained from the binding samples were transformed into the concentrations of bound markers. Only samples with a bound marker concentration within the quantification limits of the LC-MS/MS quantification method were used for the further data analysis. Specific binding was calculated as the difference between total binding and nonspecific binding and used for the construction of saturation isotherms. Nonspecific binding was analysed for all marker concentrations, but only marker concentrations ≥ LLOQ were used to calculate a line of best fit for the nonspecific binding, using a linear regression (forced through the origin). Based on the function of this line of best fit, the nonspecific binding for all marker concentrations (binding samples with a nonspecifically bound marker concentration < LLOQ as well as binding samples for which the nonspecifically bound marker concentration was ≥ LLOQ) was determined. The equilibrium dissociation constant (K_d) and the maximum number of binding sites (B_{max}) were obtained by nonlinear regression analysis ("one site – specific binding", Prism 6) of the data points for the specific binding.

Results

LC-MS/MS Method development

To quantify the amount of bound marker in an MS Binding Assay, an LC-ESI-MS/MS method had to be developed. LC-MS methods for the quantification of reboxetine and citalopram are already described in literature. Most of these methods are aimed at therapeutic drug monitoring of a variety of drugs, including either reboxetine (Zoerner et al., 2013) or citalopram (Amundsen, Øiestad, Ekeberg, & Kristoffersen, 2013; Licata et al., 2016) or both (Montenarh et al., 2014; Sempio, Morini, Vignali, & Groppi, 2014; Viette et al., 2011). Retention times in the published LC-MS methods are between 2.6 min (Zoerner et al., 2013) and 9.3 min (Viette et al., 2011) for reboxetine and range from 2.76 min (Amundsen et al.,

2013) to 9.2 min (Licata et al., 2016) for citalopram. All of the mentioned LC-MS methods have lower limits of quantification (LLOQs) not below 1 ng/mL (3.2 nM) for reboxetine (Zoerner et al., 2013) and not lower than 3.2 ng/mL (9.9 nM) for citalopram (Amundsen et al., 2013; Sempio et al., 2014). For D-84, no analytical method for the quantification is known at all. The method to be developed should enable us to quantify the markers with a sufficient sensitivity, so the LLOQ should be in the low pM range (see introduction), which is by a factor of approximately 1000 lower than the most sensitive quantification methods for reboxetine and citalopram known so far. Furthermore, the markers should be sufficiently separated from matrix components. Additionally, a separation of the three markers during the LC run appeared desirable as a mutual interaction of the coeluting compounds might be associated with signal suppression, thus falsifying the quantification which might be eventually true for a component present only in very low concentration coeluting with another compound present in very high concentration. Finally, the LC conditions should be optimized for short run times to obtain a method, capable of analysing samples with a high throughput. The published quantification methods for reboxetine and citalopram have retention times of at least 2.6 min for reboxetine and 2.8 min for citalopram, whereas the method to be developed was aimed to have a chromatographic run time of less than 2 min.

ESI-MS/MS

Since *rac*-D-84 [(*rac*-**1,** for the sake of simplicity, the racemic mixture of (*R,R*)-D-84 and (*S,S*)-D-83 (the enantiomer of (*R,R*)-D-84) is referred to as *rac*-D-84 throughout this work] was analysed by LC-ESI-MS/MS for the first time, mass transitions suitable for quantification were not known. Infusing a solution of *rac*-D-84 (*rac*-**1**) into the mass spectrometer (API 3200, ESI positive, for details the experimental section) the $[M+H]^+$ precursor ion for D-84 with *m/z* 420.3 could be observed as expected. Based on this precursor ion, the 5 most intensive fragment ions (*m/z* 167.0, 109.2, 152.2, 165.1, 121.2, see SI-figure 1) were identified and subsequently, the compound dependent parameters optimized with the Compound Optimization Tool of the Analyst software. Analogously, the mass transitions for *rac*-reboxetine (*rac*-**2**) *m/z* 314.2/176.1 and for *rac*-citalopram (*rac*-**3**) and *m/z* 325.2/109.1 were found to be in accordance with literature (Amundsen et al., 2013; Licata et al., 2016; Montenarh et al., 2014; Sempio et al., 2014; Zoerner et al., 2013) and used later on for method development at the API 3200.

For final method development with the API 5000 (API 5000, ESI positive, for details see experimental section) compound optimization was repeated with the markers as pure enantiomers and in each case the two most intensive mass transitions were selected for final method development: for (*R,R*)-D-84 ((*R,R*)-**1**) *m/z* 420.2/167.1 and *m/z* 420.2/109.1, for (*S,S*)-reboxetine ((*S,S*)-**2**) *m/z* 314.1/176.2 and *m/z* 314.1/91.1 as well as for (*S*)-citalopram ((*S*)-**3**) *m/z* 325.2/109.2 and *m/z* 325.2/262.2. In the same way, the mass transitions for the internal standards were analysed and optimized at the API 5000. For *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**, the $[M+H]^+$ precursor ion with *m/z* 424.2 could be observed as expected. Based on this precursor ion, again (as described above for the nondeuterated compound) the 5 most intensive fragment ions (*m/z* 176.1, 113.2, 168.2, 152.1, 165.2) were identified. Expected mass transitions for *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**) and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**) were found in agreement with published data (Licata et al., 2016; Sempio et al., 2014; Zoerner et al., 2013). For LC-MS/MS at the API 5000 *m/z* 424.2/167.1 for *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**), *m/z* 319.2/176.2 for *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**), and *m/z* 331.2/109.0 for *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**) were chosen.

LC

The aims of LC method development were to find chromatographic conditions that enable fast and robust quantification of all markers. Furthermore, a chromatographic separation of the markers from matrix components was intended in order to minimize matrix effects in the quantification of samples from MS Binding Assays via LC-ESI-MS/MS and to reach high ESI-MS/MS responses for all three analytes.

Under reversed-phase conditions in isocratic mode, we could not find conditions (even in the basic pH range up to 11) resulting in sufficient retention of the markers in a mobile phase consisting of at least 50 % acetonitrile (data not shown). An increase of the portion of the aqueous component in the mobile phase in order to enhance retention was not considered, due to the loss of sensitivity typically arising under LC-ESI-MS conditions from an enhanced water content (Naidong, 2003; Nguyen & Schug, 2008). Therefore, a set of distinctly more polar stationary phases (Amino, Diol NP, Polyamine, PVA SIL, Silica, all 50 x 3.0 mm, 5 µm, for detailed specification see experimental section) operated under HILIC conditions (Hemström & Irgum, 2006) as well as a pentafluorophenyl (PFP) stationary phase were investigated under various mobile phase compositions, with various additives and pH values in order to obtain an appropriate retention behaviour of the analytes. The PFP stationary phase

material has an enhanced selectivity in comparison to C8 and C18 stationary phases due to its dipole-dipole, π-π, charge transfer, and ion exchange retention mechanisms and turned out to be the most promising one for the intended purpose (Bell, Cramer, & Jones, 2005; Euerby, McKeown, & Petersson, 2003; Reta, Carr, Sadek, & Rutan, 1999). On a Triart PFP (50 x 2.0 mm, 3 µm, YMC), a method applying ammonium formate buffer (1.0 mg/mL) in combination with acetonitrile (15/85) at a flow rate of 800 µL/min resulted in a separation of all three markers. As an alternative to this stationary phase, chromatography on a Luna PFP(2) column (50 x 2.0 mm, 3 µm, Phenomenex) was also investigated. With the above mentioned mobile phase parameters, both stationary phases were compared concerning chromatographic parameters for the three markers (*rac*-**1**, *rac*-**2** and *rac*-**3**) by analysis of a solution containing each marker at a concentration of 1 nM (see table 1 and SI-figure 2). With the Luna PFP(2) column, reduced retention times in combination with lower peak widths were observed for all three markers in comparison to the chromatograms from the Triart PFP. This resulted in considerably higher peak intensities and signal-to-noise ratios (S/N). Furthermore, the peak asymmetry obtained with the Luna PFP(2) was closer to 1 for all three analytes. For this reason, the Luna PFP(2) column was chosen for the final LC-ESI-MS/MS method. Since the Luna PFP(2) showed a lower retention for all three markers than the Triart PFP, the mobile phase was slightly adjusted to an ammonium formate buffer concentration of 0.75 mg/mL, which was observed to prolong retention under these chromatographic conditions during the method development. All other method parameters were kept unchanged, resulting in retention times of 0.6 min for (*R,R*)-D-84 ((*R,R*)-**1**, k = 1.4), 0.78 min for (*S,S*)-reboxetine ((*S,S*)-**2**, k = 2.1), and 1.1 min for (*S*)-citalopram ((*S*)-**3**, k = 3.4, see figure 7d) while the results for peak height, S/N, peak width, and peak asymmetry were still better than for the Triart PFP column. It is also worth to mention that this LC-method is compatible with samples dissolved in pure acetonitrile, thus no addition of aqueous buffer is needed prior to analysis, which would dilute the samples resulting after elution of the bound marker from the filters (see also figure 3) and therefore reduce the sensitivity of the method.

For the developed LC method, the source dependent parameters of the mass spectrometer were optimized (for details see experimental section). The flow injection analysis (FIA) resulted in an optimal ion spray voltage (IS) of 1500 V, an ion source temperature (TEM) of 650 °C, a nebulizer gas (GS1) of 30 psi, and a heater gas (GS2) of 60 psi. In preliminary experiments, we found that a low pressure for the curtain gas (CUR) leads to higher

intensities, but since the CUR protects the mass spectrometer from contaminations, a low CUR also has huge disadvantages. As a compromise, the CUR was set to 25 psi.

Adsorption of the markers towards the container material

During LC-MS method development, it was observed that a freshly prepared aqueous solution of *rac*-D-84 (*rac*-**1**) resulted in considerably higher LC-ESI-MS/MS responses than a solution that was stored overnight. For a stock solution of *rac*-D-84 (*rac*-**1**) in water kept in a polypropylene tube, the peak area for the mass transition *m/z* 420.3/167.0 in LC-ESI-MS/MS analyses decreased by more than a half on the consecutive day when stored overnight (in each case prior to analysis the sample was subsequently diluted in acetonitrile. In contrast, when *rac*-D-84 (*rac*-**1**) was stored as a solution in acetonitrile, in a polypropylene tube such a loss of intensity was not apparent in corresponding MRM chromatograms. Nuclear magnetic resonance analysis of the *rac*-D-84 (*rac*-**1**) solution after varying storage durations showed no change in the ^{1}H-NMR spectrum (data not shown), indicating that the loss of compound is not due to degradation. Furthermore, storage of the aqueous *rac*-D-84 (*rac*-**1**) solution at different temperatures did not alter the loss of signal intensity, but it was noticed that this loss of signal intensity could be dependent on the materials, the container was made from. Therefore, a variety of containers from different manufacturers including coated and non-coated polypropylene tubes as well as glass vials were investigated. The adsorption and therefore loss of D-84 in aqueous solutions could not be sufficiently avoided with the tested materials. Hence, we did not pursue this strategy furthermore, as it seemed to be a hopeless endeavour to find suitable material (and corresponding containers) for all sample preparation steps of the MS Binding Assays (including e.g. pipetting, incubation, LC-MS analysis etc., see also figure 3). In this context, it is worth to mention that water as solvent is inevitable for incubation in the desired MS Binding Assays, so replacing water by an organic solvent was no alternative solution to overcome this problem.

The adsorption of analytes towards container material is an occasionally observed issue also described for analytical studies dealing with urine samples (Heinig, Wirz, Yuan, Tingler, & Mylott, 2011; Ji et al., 2010; Li, Luo, Smith, & Tse, 2010). Interestingly, in a publication by S. Sylvester and F. Zang (Silvester & Zang, 2012) adsorption of a test compound (AZD9164, (*R*)-1-(4-fluorophenethyl)-3-(((*S*)-2-phenyl-2-(piperidin-1-yl)propanoyl)oxy)quinuclidin-1-ium), which shares some structural features with D-84, towards plastic material of containers

in urine samples is reported. Several types of additives (such as proteins, detergents or cosolvents) are known to overcome this problem. Due to concerns that addition of proteins may lead to undesired interferences in the binding experiments and addition of detergents may cause severe matrix problems in LC-ESI-MS/MS based marker quantification, we decided to make use of dimethylacetamide (DMA), which has already been described to prevent adsorption of AZD9164 in urine samples in the above-mentioned study (Silvester & Zang, 2012). For investigation of the effect of DMA on the adsorption of *rac*-D-84 (*rac*-**1**) to the container, dilution series at six concentration levels between 500 pM and 25 nM were prepared in water without and with the addition of DMA. Each concentration level was prepared by dilution of the higher concentrated solution, thus every decrease in D-84 concentration was achieved by using a new container and pipet tip, on which adsorption could occur. These aqueous solutions were finally spiked with *rac*-reboxetine (*rac*-**2**) as internal standard (as the deuterated analogue of *rac*-**1** was not yet available at this time) and diluted tenfold in acetonitrile prior to analysis, to obtain solutions with 50 pM to 2.5 nM of *rac*-**1** with 2.5 nM *rac*-**2**. In the dilution series prepared in water without the addition of DMA, it could be seen that the relative response factors (RRF) decrease with each dilution step, illustrating a consecutive loss of analyte due to dilution in water (see figure 2). Preparation of the dilution series in water with 20 % DMA resulted generally in higher RFFs than for the dilution series in pure water and no decrease in RRFs during the course of the dilution series. This demonstrates that *rac*-D-84 (*rac*-**1**) stays at least largely dissolved in an aqueous solution with 20 % DMA. In this context, it is worth to mention that this conclusion is also supported by the excellent linear calibration functions obtained for (*R,R*)-**1** over a wide range of concentrations, which were prepared as a dilution series in DMA/water (20/80) prior to dilution in acetonitrile as described below (see "Validation"). It has to be noted that these adsorption-issues would also have been a major problem for the use of (*R,R*)-D-84 ((*R,R*)-**1**) as reporter ligand in radioligand binding assays. In the case of MS Binding Assays, these effects can be easily investigated and targeted during the method development in contrast to radioligand binding assays, where such problems may be overlooked.

In the MS Binding Assays to be developed, a high DMA content was expected to perturb a reliable affinity determination. Therefore, the DMA concentration in the binding samples had to be much lower. For this purpose, stock solutions were prepared in DMA/water (20/80) and diluted with the same solvent to obtain 100-fold concentrated marker solutions. The 100-fold concentrated marker solutions were diluted on the day of the assay with binding buffer to

generate the binding samples, resulting in the desired final marker concentrations. In this way, the DMA concentration in the final binding samples was reduced to 0.2 %.

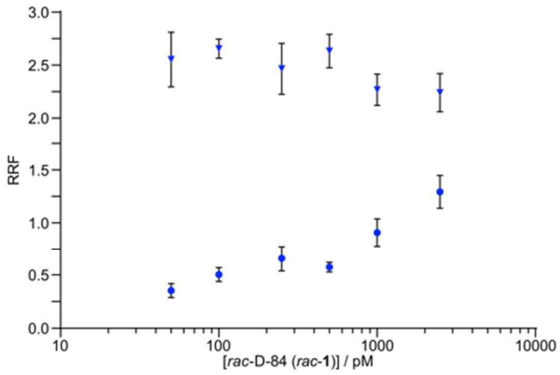

Figure 2: Relative response factors (RRF) for *rac*-D-84 (*rac*-**1**) calculated as, $\frac{\frac{\text{peak area } rac-\text{D}-84}{\text{concentration } rac-\text{D}-84}}{\frac{\text{peak area } rac-\text{reboxetine}}{\text{concentration } rac-\text{reboxetine}}}$, (mean ± SD, n = 3). Dilution series of *rac*-D-84 (*rac*-**1**, 500 pM – 25 nM) in pure water (circles) and 20 % DMA in water (triangles) were prepared, spiked with *rac*-reboxetine, subsequently diluted tenfold in acetonitrile and analysed. Stationary phase: Triart PFP (50 x 2.0 mm, 3 µm), mobile phase: acetonitrile / ammonium formate buffer (1.0 mg/mL, 85/15), 800 µL/min, detection at the API 3200: *rac*-D-84 (*rac*-**1**): *m/z* 420.3/167.0, *rac*-reboxetine (*rac*-**2**): *m/z* 314.2/176.1.

Binding assay

It was intended to perform MS Binding Assays according to the setup, previously published for targets like the γ-aminobutyric acid transporter GAT1 (Zepperitz et al., 2006), monoamine transporters (Grimm et al., 2015a; Hess et al., 2011), and dopamine receptors (Neiens et al., 2015) (see also figure 3 for the procedure of the established MS Binding Assays). For the formation of target-marker complexes, all of these MS Binding Assays consist of an incubation step under controlled conditions in a 96-well format. After an incubation time sufficient for equilibration, the target-marker complexes are then separated from the unbound marker by a vacuum filtration step employing a 96-well filterplate, followed by the washing of the filters with the entrapped membrane fragments to further reduce the presence of unbound and non-specifically bound marker. In successive steps, the target protein is denatured by the influence of heat and organic solvent, leading to a liberation of the bound

marker. The formerly bound marker - now dissolved in the organic solvent - can be eluted by filtration, resulting in a sample, which is finally analysed by LC-MS/MS.

Conditions for the incubation should be – as far as possible - chosen based on published binding assays for the corresponding targets and markers. Therefore, a HEPES buffer supplemented with NaCl and KCl (50 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4) was used for incubation as described by Grimm et al. (Grimm et al., 2015a) for MS Binding Assays addressing hDAT, hNET, and hSERT with (1*R*,3*S*)-indatraline as marker. With respect to incubation time and temperature, only information from radioligand binding experiments for *rac*-[^{3}H]-citalopram (*rac*-[^{3}H]-**2**) towards SERT was available. Chen et al. and Goulet et al. described incubation times from 1 h to 2 h at 4 °C for the mentioned radioligand binding experiments (Chen et al., 2005; Goulet et al., 2001). Since the affinities of (*R,R*)-D-84 ((*R,R*)-**1**) towards DAT and (*S,S*)-reboxetine ((*S,S*)-**2**) towards NET were expected to be in a similar range as the one of (*S*)-citalopram ((*S*)-**3**) towards hSERT, binding of all markers towards their targets was assumed to reach equilibrium within an incubation time of 2 h at 20 °C.

For the determination of the nonspecific binding, the triple reuptake inhibitor *rac*-indatraline (*rac*-**4**)) was intended to be used in a concentration of 10 µM in the binding assay to block specific binding sites of the markers at hDAT, hNET, and hSERT (Bøgesø, Vibeke Christensen, Hyttel, & Liljefors, 1985; Grimm et al., 2015b). Preliminary experiments using 10 µM *rac*-indatraline (*rac*-**4**) as an inhibitor enabled the reliable determination of the nonspecific binding. Since an MS Binding Assay using (1*R*,3*S*)-indatraline as a marker (Grimm et al., 2015a) was analysed on the same instrument, we wanted to avoid introducing high concentrations of indatraline into the MS, which could interfere with the (1*R*,3*S*)-indatraline MS Binding Assays. As an alternative to native indatraline, *rac*-[$^{2}H_3$]-indatraline (*rac*-[$^{2}H_3$]-**4**) was previously synthesized in our group (according to the procedure published in the supporting information of Grimm et al. (Grimm et al., 2013), synthesis is described in detail in the supporting information of this publication) for an earlier project. When used as inhibitor for the determination of the nonspecific binding in the newly developed MS Binding Assays, *rac*-[$^{2}H_3$]-indatraline (*rac*-[$^{2}H_3$]-**4**) did not interfere with the (1*R*,3*S*)-indatraline-MS Binding Assays, allowing its use in this project. It is, however, important to mention that the use of a deuterated indatraline species for this purpose is basically not imperative at all. There is a wealth of commercially available high affinity ligands for DAT, NET, and SERT that can be considered as alternatives as inhibitors for the determination of nonspecific binding in MS Binding Assays as well.

Commonly, the separation of nonbound marker from the target-marker complexes can be easily achieved by rapid filtration of the binding samples through a glass fiber filter. Pretreatment of the filters with polyethyleniminie (PEI) is known to reduce filter binding in radioligand binding assays in many cases (Bruns, Lawson-Wendling, & Pugsley, 1983; Hampton, Medzihradsky, Woods, & Dahlstrom, 1982; Roche, Bergert, & Ryan, 1985) and was e.g. applied to [^{3}H]-citalopram binding assays (Chen et al., 2005; Goulet et al., 2001). After the filtration, the target material and the filters should be washed with ice cold ammonium formate washing buffer to remove remaining solution of the binding samples and loosely, nonspecifically bound marker from the target preparation and the filters. The filters with the target-marker complexes should be stored in the drying cabinet, before a solution of the internal standard in acetonitrile is applied to each filter and aspirated in the vacuum in order to elute the formerly bound marker. In this way, the formerly bound marker would be dissolved in the organic solvent of the mobile phase, which can then be used as the sample for LC-MS/MS analysis without any further sample preparation (e.g. solvent evaporation or addition of buffer).

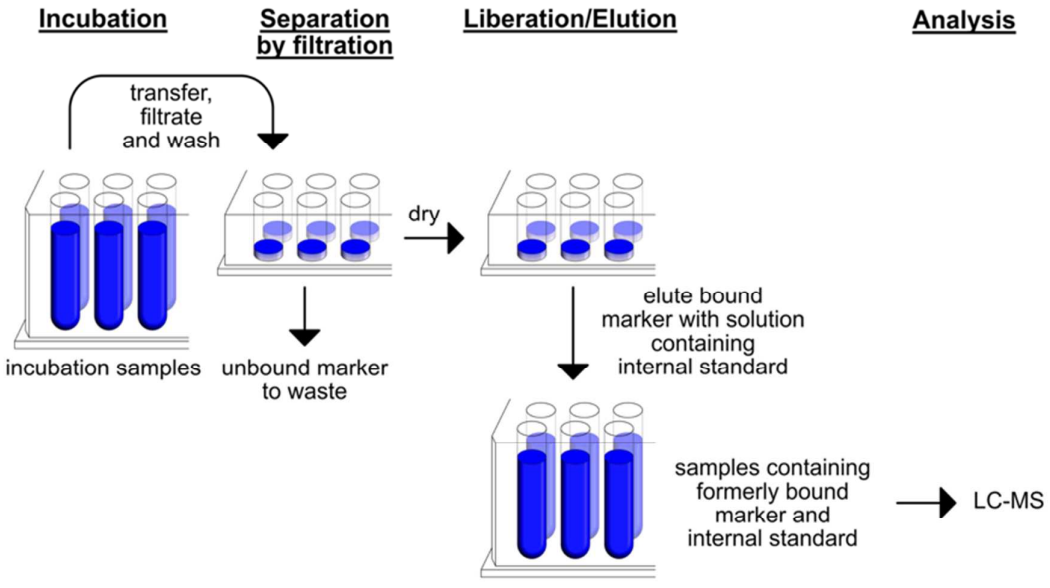

Figure 3: Workflow of the developed MS Binding Assays

Matrix effect

In LC-ESI-MS, the matrix of a sample can lead to signal suppression (Dams, Huestis, Lambert, & Murphy, 2003; Fu, Woolf, & Matuszewski, 1998; King, Bonfiglio, Fernandez-Metzler, Miller-Stein, & Olah, 2000; Matuszewski, Constanzer, & Chavez-Eng, 1998; Taylor, 2005). To compensate matrix effects, the use of stable isotopically labelled internal standards is typically advised and followed in LC-ESI-MS/MS quantification methods (Stokvis, Rosing, & Beijnen, 2005; Wang, Cyronak, & Yang, 2007; G. Zhang & Wujcik, 2009). Since we aimed for a high sensitivity, which is usually limited by the S/N ratio and therefore the intensity of the analyte signal in the chromatogram, we tried to reduce matrix suppressions that affect the performance of the method. To analyse the matrix effect, we tried to uncover possible matrix-effects that may be caused by the target material (i.e. hDAT, hNET, and hSERT membrane preparations), the buffers used for incubation and washing as well as by the glass fiber filter material (including its pretreatment with PEI) in post-column infusion experiments (Bonfiglio et al., 1999). At first, matrix from binding samples was generated according to the intended procedure for the binding experiments (for details see experimental section). After incubation of the target material (in absence of the markers) in HEPES buffer (50 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4) 200 µL of the binding samples were filtrated through PEI pretreated filter plates. Subsequently, the filters were washed with ammonium formate buffer (0.75 mg/mL, pH 7.4) and dried. Afterwards the filter plates were eluted with a solution of all three deuterated internal standards, each in a concentration of 100 pM, in acetonitrile. These matrices containing the internal standards were subjected to the developed LC-ESI-MS/MS method. In contrast to the standard conditions of the developed LC-MS/MS method, now a solution of 300 pM (*R,R*)-D-84 ((*R,R*)-**1**), 2 nM (*S,S*)-reboxetine ((*S,S*)-**2**), and 600 pM (*S*)-citalopram ((*S*)-**3**) in the mobile phase was infused into to the eluent leaving the column via a T piece before reaching the mass spectrometer by means of syringe pump at a flow rate of 100 µL/min. Furthermore, due to the pressure of the LC eluent directed into the syringe pump, the LC flow rate had to be reduced to 200 µL/min (and as a consequence, the source-dependent MS parameters were also adjusted for this purpose by FIA, for details see experimental section).

In the MRM chromatograms obtained in this way (see figure 5), the retention times of the markers under the altered flow rate were clearly indicated by the peaks of the internal standards (*rac*-[$^2H_4$]-**1**: *m/z* 424.2/167.1, 2.4 min, *rac*-[$^2H_5$]-**2**: *m/z* 319.2/176.2, 3.0 min, *rac*-[$^2H_6$]-**3**: *m/z* 331.2/109.0, 4.4 min). The void time is indicated by a peak in the traces of all

three markers ((*R,R*)-**1**: *m/z* 420.2/167.1, (*S,S*)-**2**: 314.1/176.2, (*S*)-**3**: *m/z* 325.2/109.2 at 1.1 min, which is assumed to be caused by higher acetonitrile content from the sample solvent eluting from the LC, leading to better volatility of the mobile phase and increased ionization efficiency. Furthermore, several suppression zones in the MRM traces of the markers could be recognized. The first one, directly after the void time and a second matrix suppression from 2.5 min to approximately 4.0 min can be clearly seen. After these suppression zones, which are located near or at the retention times of the markers, there was a third one after the peak of (*S*)-citalopram ((*S*)-**3**) visible in the chromatogram of the post-column infusion experiment (6.0 min – 8.0 min, see figure 5).

The third matrix suppression could be an issue, as it may affect successive chromatographic runs. An easy way to avoid this, would be a chromatographic run time, long enough to ensure that all the matrix is eluted from the column. Since short chromatographic run times were an important aim in our method development, we did not favour a solution in this way. Instead, we tried to determine, if there is indeed a significant suppression due to a matrix effect of the late-eluting matrix components onto successive chromatographic runs under the original conditions of the developed LC-ESI-MS/MS quantification method (with a flow rate of 800 µL/min). Therefore, a matrix standard with all three markers at a concentration of 100 pM was prepared according to the procedure of the MS Binding Assays (incubation, filtration of binding sample, washing with ammonium formate buffer, elution with solution containing 100 pM markers in acetonitrile, for details see experimental section) and analysed in a sequence together with a solvent standard containing the three markers ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) also in a concentration of 100 pM in acetonitrile (see figure 6). The sequence of the injections was chosen on the basis of the publication from S. Sylvester and F. Zang (Silvester & Zang, 2012), to determine signal suppressions on up to two solvent samples, which are analysed after a matrix sample, as well as an additional effect in a series of six matrix samples. In the first five injections of the solvent standard (see figure 5, injection no. 1 – 5), the peak intensities of the three markers ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) show a constant level for the peaks of the markers ((*R,R*)-**1**: *m/z* 420.2/167.1, ca. 800,000 cps (*S,S*)-**2**: 314.1/176.2, ca. 250,000 cps (*S*)-**3**: *m/z* 325.2/109.2), ca. 500,000 cps). In the six successive sequences of injections consisting of one matrix standard followed by two solvent standards (injection no. 6 – 23), no signs of suppression of the marker peaks due to preceding matrix samples could be observed. In the following series of six injections of the matrix standard (injection no. 24 – 29), the signal intensities of the peaks in all six chromatograms are at the same level, showing no effects of matrix accumulation over multiple injections caused by the matrix standards. A

lack of suppression on marker peaks caused by the matrix of preceding samples is further demonstrated by the successive solvent standards (injection no. 30 – 36), in which the peaks have the same level of intensity as in the solvent standards at the start of the sequence. As a result, this experiment shows, that under the standard method parameters (see experimental section "LC-ESI-MS/MS quantification method") the chromatographic cycle time including the time period necessary for the injection of the successive sample is long enough for the elution of the complete matrix before the next chromatographic run is started.

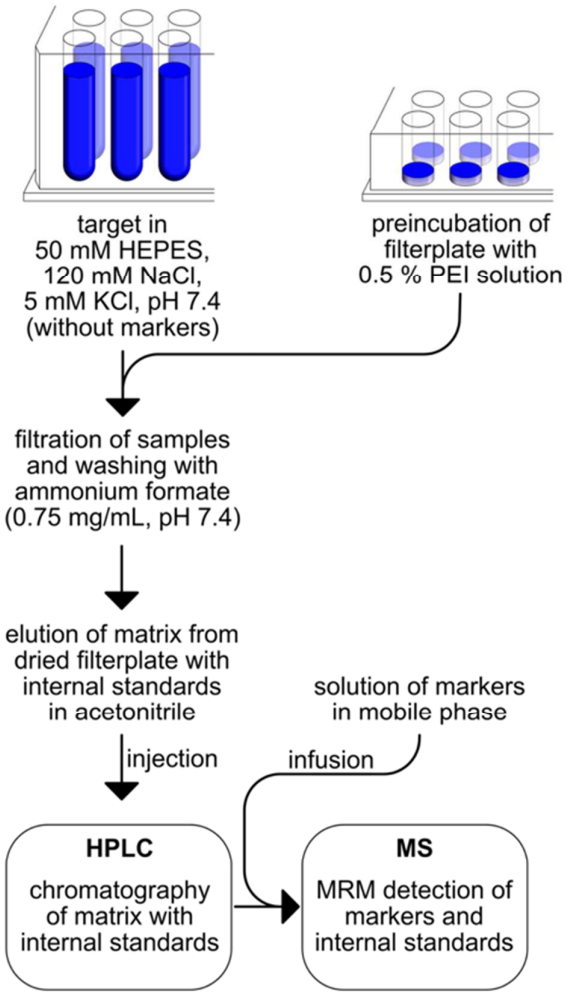

Figure 4: Schematic workflow of the post-column infusion experiment.

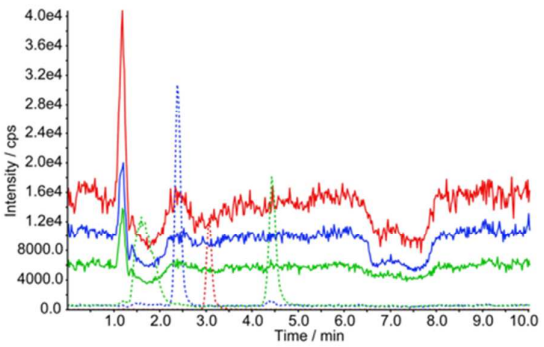

Figure 5: MRM Chromatogram from a post-column infusion experiment for the determination of matrix effects resulting from binding samples, as described in the experimental section ("Investigation of the matrix effect by post-column infusion experiments"). 300 pM (*R,R*)-D-84 ((*R,R*)-**1**, blue signal), 2 nM (*S,S*)-reboxetine ((*S,S*)-**2**, red signal), and 600 pM (*S*)-citalopram ((*S*)-**3**, green signal) were infused continuously, while blank matrix, containing 100 pM *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**, blue dotted peak), *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**, red dotted peak), and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**, green dotted peak at a retention time of 4.5 min; peak at 1.5 min is caused by matrix components, generating a signal with the same MRM transition as *rac*-[$^2H_6$]-**3**), which was subjected to the chromatography.

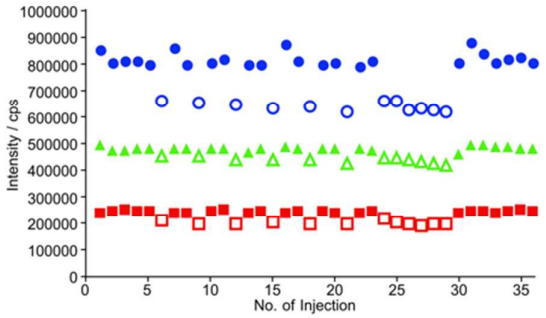

Figure 6: Plot of marker peak intensities for a series of injections of solvent standards (full symbols) and matrix standards (100 pM of each marker, empty symbols, obtained from a simulated binding experiment, for details on the sample preparation and analysis see experimental section); (*R,R*)-D-84 ((*R,R*)-**1**): blue dots, (*S,S*)-reboxetine ((*S,S*)-**2**): red squares, (*S*)-citalopram ((*S*)-**3**): green triangles.

Nonspecific binding of markers to glass fiber filters

In MS Binding Assays as well as in radioligand binding assays, separation of the target-marker complexes from nonbound marker is typically achieved by filtration (Chen et al., 2005; Grimm et al., 2015a; Hess et al., 2011; Neiens et al., 2015; Zepperitz et al., 2006; R. Zhang & Xie, 2012). In contrast to radioligand binding assays, where the whole filter with the remaining target material and the bound marker is subjected to measurement of radioactivity, in MS Binding Assays the target bound marker remaining on the filter has to be eluted by means of an organic solvent to enable its quantification by LC-MS (see also figure 3). Due to the adsorption problems observed for D-84 (see above), we found it worth to examine the adsorption of all three markers ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) when diluted in acetonitrile towards the glass fiber filters. Therefore, a 100 pM solution of all three markers ((*R,R*)-**1**, (*S,S*)-**2**, and (*S*)-**3**) in acetonitrile was filtered through pretreated glass fiber filters. The pretreatment of the glass fiber filters should be the same as in the final MS Binding Assays. Filters were incubated with 200 µL 0.5 % PEI solution or 200 µL water as a negative control for 1 h and afterwards washed three times with 150 µL ammonium formate buffer (0.75 mg/mL, pH 7.4). Additionally to the filtrated marker solutions, reference samples with unfiltrated marker solution (in the same nominal concentration) were prepared. Finally, all samples were spiked with a 100 pM solution of the three internal standards (*rac*-[$^2H_4$]-**1**, *rac*-[$^2H_5$]-**2**, *rac*-[$^2H_6$]-**3**) to compensate matrix effects. In comparison to the signals of the unfiltrated reference sample (100 % for each marker), the signals of the samples, which were passed through filters not pretreated with PEI are considerably decreased ((*R,R*)-**1**: 78.2 %, (*S,S*)-**2**: 35.0 %, and (*S*)-**3**: 9.6 %, see figure 7). Surprisingly, the adsorption of the markers towards the filter material is much higher for (*S,S*)-reboxetine and (*S*)-citalopram, than for (*R,R*)-D-84, when the markers are dissolved in acetonitrile. The comparison of the signals obtained from the samples filtrated through the PEI-coated filters (peak area ratios of (*R,R*)-**1**: 87.5 %, (*S,S*)-**2**: 91.1 %, and (*S*)-**3**: 89.6 %) shows that the pretreatment with PEI effectively reduces the loss of markers during liberation and elution (see figure 3) even though the filters are washed repeatedly after the pretreatment with PEI.

In preliminary saturation experiments performed for each of the three markers ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) in presence of their targets, an extraordinarily high nonspecific binding of (*R,R*)-D-84 was apparent. The proportion of the nonspecifically bound marker concentration for (*R,R*)-D-84 was between 1.4 and 2.8 % of the nominal marker concentration in the binding sample, whereas the nonspecifically bound marker concentrations of (*S,S*)-reboxetine and (*S*)-

citalopram were considerably lower with proportions between 0.025 and 0.030 % for (*S,S*)-**2** and between 0.054 and 0.074 % for (*S*)-**3** , respectively (see figure 8, filled symbols). It was assumed, that the high nonspecific binding of (*R,R*)-D-84 ((*R,R*)-**1**) may be mainly caused again by adsorption of the marker towards the filter material during the filtration step of the aqueous binding samples (see figure 3), which is not sufficiently reduced by the pretreatment with PEI.

Reliable determination of specific binding is typically hampered when the portion of nonspecific binding is high. Attempts to reduce the nonspecific binding of (*R,R*)-D-84 ((*R,R*)-**1**) by the use of other filter materials (hydrophilic polypropylene and polyethersulfone) resulted in even higher nonspecific filter binding when aqueous solutions of the markers (without target preparation) were filtrated (data not shown). Since the addition of DMA to the aqueous marker solutions proved to reduce the adsorption of D-84 towards the container surface, it was assumed that the nonspecific binding of (*R,R*)-D-84 ((*R,R*)-**1**) to the glass fiber filters could also be reduced by the addition of DMA to the washing buffer. Addition of DMA during incubation above 0.2% was avoided, as high concentrations of DMA were expected to affect the stability of the target-marker complexes. An alternative strategy was to remove nonspecifically bound D-84 from the filter material by the use of a washing buffer, which is supplemented with DMA. Therefore, 10 % DMA was added to the pure ammonium formate buffer (0.75 mg/mL, pH 7.4) This washing buffer was used for repeated washing of the filters (three times with 150 µL) after filtration of the binding samples. To reduce the DMA content in the filters prior to drying, a fourth washing step with pure ammonium formate buffer (0.75 mg/mL, pH 7.4) was implemented. In this way, the proportion of nonspecifically bound (*R,R*)-D-84 ((*R,R*)-**1**) in relation to the nominal marker concentration in the binding sample could be distinctly reduced to values between 0.31 and 0.40 % (see figure 7, blue empty symbols). This reduction of nonspecific binding enabled reliable determination of specific binding for (*R,R*)-D-84 ((*R,R*)-**1**) as shown below. In order to examine the suitability of these filtration and washing conditions, preliminary saturation experiments were exemplarily performed for (*S*)-citalopram ((*S*)-**3**) towards hSERT, as the results of these experiments could be easily validated by comparision with results published for [^{3}H]-(*S*)-citalopram binding experiments. The good agreement of the K_d values found for (*S*)-citalopram ((*S*)-**3**) with those obtained in radioligand binding assays (see supporting information) demonstrated that the established filtration, washing, and elution procedure enables a reliable determination of affinities.

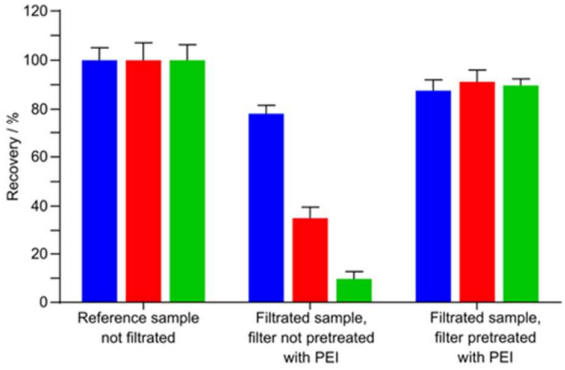

Figure 7: Recovery of markers ((*R,R*)-D-84 ((*R,R*)-**1**, blue), (*S,S*)-reboxtine ((*S,S*)-**2**, red), and (*S*)-citalopram ((*S*)-**3**, green, all at 100 pM) dissolved in acetonitrile after filtration through glass fiber filters. Reference sample was not filtrated, filtrated samples were filtrated through filters, pretreated with 200 µL water ("filter not pretreated with PEI") or 200 µL 0.5 % PEI solution ("filter pretreated with PEI") for 1 h and washed with 3 x 150 µL ammonium formate buffer (0.75 mg/mL, pH 7.4) prior to filtration. All non-filtrated reference and filtrated samples were spiked with internal standards (*rac*-[$^2H_4$]-**1**, *rac*-[$^2H_5$]-**2**, *rac*-[$^2H_6$]-**3**) at a concentration of 100 pM in acetonitrile prior to analysis. Bars represent means of recovery (% relative to reference sample) ± SD (n = 6).

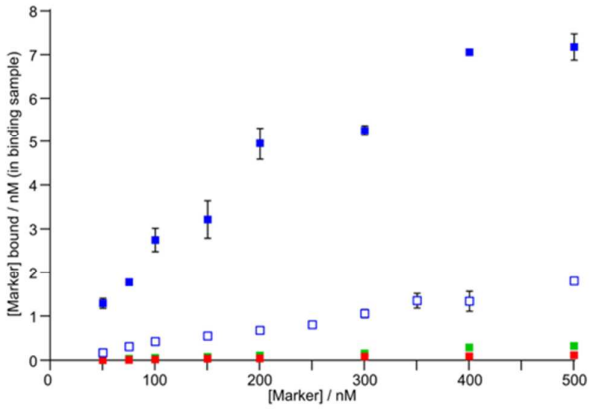

Figure 8: Nonspecific binding (mean ± SD, n = 3) derived from preliminary saturation experiments using different washing procedures. Binding samples were prepared according to the general procedure for the MS Binding Assays (see experimental section). After incubation, binding samples were filtrated over the glass fiber filters. Subsequently, the filters were washed with ammonium formate buffer (0.75 mg/mL, pH 7.4, filled symbols, (*R,R*)-**1**:

blue, (*S,S*)-**2**: red, (*S*)-**3**: green) or 10 % DMA in ammonium formate buffer (0.75 mg/mL, pH 7.4, only shown for (*R,R*)-**1**: empty blue symbols). Marker elution and analysis was carried out according to the procedure and with the LC-ESI-MS/MS quantification method, described in the experimental section.

Validation

The final LC-ESI-MS/MS quantification method was validated according to the CDER guideline for bioanalytical method validation (FDA, 2013) regarding selectivity, accuracy, precision, calibration curve, and sensitivity.

Calibration standards at eleven concentration levels in a range from 1 pM to 2.5 nM for each marker ((*R,R*)-**1**, (*S,S*)-**2**, and (*S*)-**3**) and quality control samples (QCs) at four concentration levels between 2.5 pM and 1 nM were prepared for the validation experiments. Matrix blanks, zero samples, calibration standards for the concentrations 1 pM, 2.5 pM, and 5 pM, as well as all concentration levels of the QC samples were prepared in six replicates for each concentration level. The other concentration levels for the calibration samples were prepared as triplicates. For all of these standards and samples, matrix blanks were prepared according to the procedure for the binding samples (but without the addition of marker and internal standard) and spiked with markers and internal standards at the elution step employing an acetonitrile solution containing the corresponding marker concentration and the internal standards each at a concentration of 90 pM. Sample series taken for the validation, were prepared at different days with membrane preparations for each of the three targets. In each series, the quantification for all three markers was validated.

To demonstrate the selectivity of the LC-MS/MS quantification method, matrix blanks, and zero samples were prepared with matrices of all three target materials. For each marker and internal standard, no interfering signals (i.e. no signals with an intensity of more than 20 % of the LLOQ) could be detected in the blanks at the estimated retention times of the analytes (see figure 9a). Furthermore, the absence of such peaks in the traces of the markers in the chromatogram of the zero samples shows, that there is no contamination of the markers originating from non-deuterated internal standards (data not shown).

To determine accuracy and precision of the method, QC samples were used. For the accuracy, the mean of the concentrations measured with the quantification method was compared to the actual concentration of the standard. The back-calculated concentration was between the

limits of the guideline (85 % and 115 % of the nominal concentration) for all three markers (see table 2 "Accuracy").

The precision is defined as the coefficient of variance or relative standard deviation (RSD) of multiple samples at each concentration level. It can be differentiated between intra-batch (within one validation run) or inter-batch (within all validation runs). In accordance to the guideline, the RSDs of the QCs were below 15 % for intra- as well as inter-batch precision (see table 2 "Precision").

For each of the three markers ((*R,R*)-**1**, (*S,S*)-**2**, and (*S*)-**3**), also calibration functions were established. The sensitivity defined by the lower limit of quantification (LLOQ) is the lowest analyte concentration, which can be determined accurately and precisely. For (*R,R*)-D-84 ((*R,R*)-**1**) and (*S*)-citalopram ((*S*)-**3**) the data for an analyte concentration of 2.5 pM (see figure 9c) met the criteria of the guideline in regard to signal-to-noise ratio (S/N ≥ 5), as well as precision (RSD ≤ 20 %) and accuracy (80 % - 120 %) of the calibration standards (see table 2 "LLOQ"). The LLOQ of (*S,S*)-reboxetine ((*S,S*)-**2**) was at a concentration of 1 pM (see figure 9b), where all criteria of the guideline were fulfilled.

The upper limit of quantification (ULOQ) is the highest concentration of the calibration function. For (*R,R*)-D-84 ((*R,R*)-**1**) the ULOQ was at 1 nM, for (*S,S*)-reboxetine ((*S,S*)-**2**) and (*S*)-citalopram ((*S*)-**3**) the ULOQ was at 2.5 nM. At these concentrations all requirements of the guideline regarding precision (RSD ≤ 15 %) and accuracy (85 % - 115 %) were met (see table 2 "ULOQ")).

The calibration functions were defined by a linear regression of peak area ratios (peak area marker/peak area internal standard) vs marker concentrations. A 1/x weighting of the calibration functions allowed for a reliable quantification of low as well as high concentrations. Based on the data obtained from the validation series, for (*R,R*)-D-84 ((*R,R*)-**1**) calibration functions from 2.5 pM to 1 nM with a coefficient of determination (r^2) of at least 0.9958 could be defined. For (*S,S*)-reboxetine ((*S,S*)-**2**), calibration functions ranging from 1 pM to 2.5 nM including eleven calibration levels were established ($r^2 \geq 0.9978$) and for (*S*)-citalopram ((*S*)-**3**), calibration functions with ten concentration levels between 2.5 pM and 2.5 nM were obtained ($r^2 \geq 0.9950$). The requirement that at least 75 % of all calibration standards should meet the acceptance criteria for accuracy was fulfilled for all three markers (see table 2 "Calibration").

Furthermore, the back-calculated concentration of at least 67 % of all QC samples and 50 % of the QC samples for each concentration level should deviate not more than 15 % from the

nominal concentration, which was fulfilled for all three markers (see table 2 "QCs"). This data supports the reliability of the established calibration functions.

The acquired data from the validation runs demonstrate the capability of the developed LC-MS/MS quantification method to deliver reliable results. This quantification method outperforms the published methods considering the sensitivities for (*S,S*)-reboxetine ((*S,S*)-**2**) and (*S*)-citalopram ((*S*)-**3**) by more than a factor of 1000 and has a much shorter run time of 1.5 min, which makes it highly suitable for the quantification of bound markers in a large number of samples. Furthermore, this is the first published quantification method of (*R,R*)-D-84 ((*R,R*)-**1**).

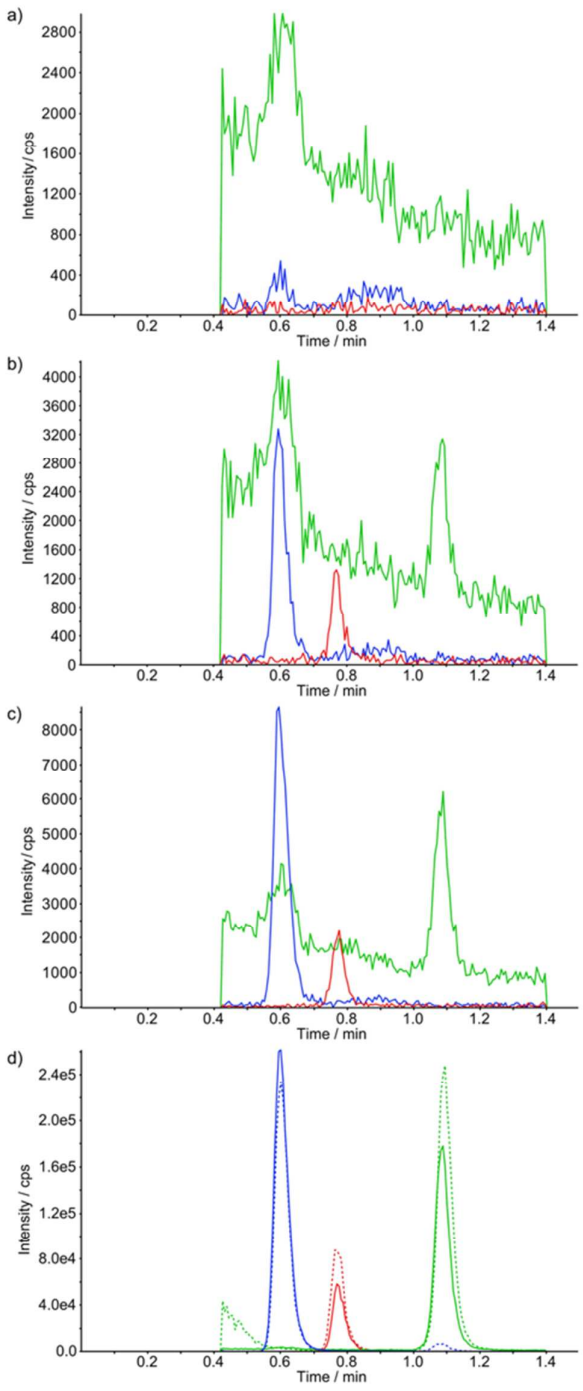

Figure 9: MRM chromatograms of (*R,R*)-D-84 ((*R,R*)-**1**, blue, *m/z* 420.2/167.1), (*S,S*)-reboxetine ((*S,S*)-**2**, red, *m/z* 314.1/176.2), and (*S*)-citalopram ((*S*)-**3**, green, *m/z* 325.2/109.1) obtained with the developed LC-ESI-MS/MS quantification method. a) Matrix blank, b) 1 pM, c) 2.5 pM, and d) 100 pM of each marker ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) with 90 pM *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**, blue dotted, *m/z* 424.2/176.1), *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**, red dotted, *m/z* 319.2/176.2), and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**, green dotted, *m/z*

331.2/109.0) as internal standards. All chromatograms were acquired, using a Luna PFP(2) column as the stationary phase and acetonitrile/ammonium formate buffer (0.75 mg/mL; 85/15) at 800 µL/min as mobile phase. The temperature was set at 20 °C and the injection volume was 30 µL. Only the eluent from 0.4 min to 1.4 min was sent to the API 5000.

Saturation assays

As a proof of concept, the validated LC-ESI-MS/MS quantification method was used to carry out saturation experiments in MS Binding Assays. To determine the total binding, samples containing 36 free marker concentrations from 25 pM to 500 nM were prepared (n = 3). The same marker concentrations were used to measure the nonspecific binding in presence of 10 µM *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**). Preparation of the binding samples, marker quantification and data analysis is described in detail in the experimental section. Based on the data of the total binding (TB) and the nonspecific binding (NSB, see figure 10 a, c, e), the SB were calculated for each nominal marker concentration. Therefrom saturation isotherms were generated (see figure 10 b, d, f).

Following this procedure, the affinities of the three markers towards their specific targets were calculated from the corresponding saturation isotherms. For (*R,R*)-D-84 ((*R,R*)-**1**) a K_d of 3.66 ± 1.35 nM together with a B_{max} of 57.91 ± 8.43 pmol/mg protein (mean ± SEM, n = 4) towards hDAT binding (see table 3 and figure 10 a, b) was determined, which is in accordance with published K_d values. In a published competitive radioligand binding experiment using [^{3}H]-WIN 35,428 as radioligand and rat striatum as target material, a K_d of 0.46 nM was found (Ghorai et al., 2003) and for a competition experiment using an MS Binding Assay with (1*R*,3*S*)-indatraline as a marker and hDAT expressed in HEK293 cells (same cell line as in the present study) a K_d of 35.5 nM (Grimm et al., 2015b). The K_d of (*S,S*)-reboxetine ((*S,S*)-**2**) towards hNET was determined as 3.06 ± 0.46 nM with a B_{max} of 31.59 ± 8.85 pmol/mg protein (mean ± SEM, n = 3, see table 3 and figure 10 c, d). In the literature, a K_d value of 0.2 nM was published for (*S,S*)-reboxetine ((*S,S*)-**2**). This result was obtained from a competition experiment with [^{3}H]-nisoxetine as a radioligand and hNET expressed in HEK293 cells as target material (Hajós et al., 2004; Tsuruda et al., 2010). K_d values published for *rac*-reboxetine (*rac*-**2**) range from 7.8 nM (determined in competition experiment with [^{3}H]-nisoxetine towards hNET in MDCK cells) (Millan et al., 2001) to 10.6 nM (determined in competition experiment with [^{125}I]-β-CIT towards hNET in COS-7 cells) (Andersen et al., 2011). With the above-mentioned MS Binding Assay employing

(1*R*,3*S*)-indatraline as marker a K_i of 3.6 nM for *rac*-reboxetine (*rac*-**2**) towards hNET (Grimm et al., 2015b) was found. The K_d values for *rac*-reboxetine (*rac*-**2**) are not directly comparable with the affinity for (*S,S*)-reboxetine ((*S,S*)-**2**), but in theory, the eutomer cannot show an affinity more than two times higher than the racemate. Thus, the affinities for (*S,S*)-reboxetine ((*S,S*)-**2**) could be between 1.8 nM and 5.3 nM with which our result of 3.06 nM corresponds quite well. For (*S*)-citalopram ((*S*)-**3**) a K_d of 411 ± 26 pM with a B_{max} of 10.11 ± 1.71 pmol/mg protein (mean ± SEM, n = 3) towards hSERT was obtained (see table 3 and figure 10 e, f). This result is in good agreement with published binding affinities for (*S*)-citalopram ((*S*)-**3**) towards SERT, determined in competition experiments with [^{3}H]-*rac*-citalopram and [^{3}H]-(*S*)-citalopram towards rat brain cerebellum and brain stem, as well as hSERT expressed in HEK293 and CHO cells, ranging from 0.28 nM to 1.1 nM (Deupree et al., 2007; Owens et al., 2001; Zhong et al., 2009). A published saturation experiment of [^{3}H]-(*S*)-citalopram towards hSERT expressed in COS-1 cells resulted in a K_d of 1.0 nM (Plenge & Wiborg, 2005), which is in the same range as the result from this MS Binding Assays, too.

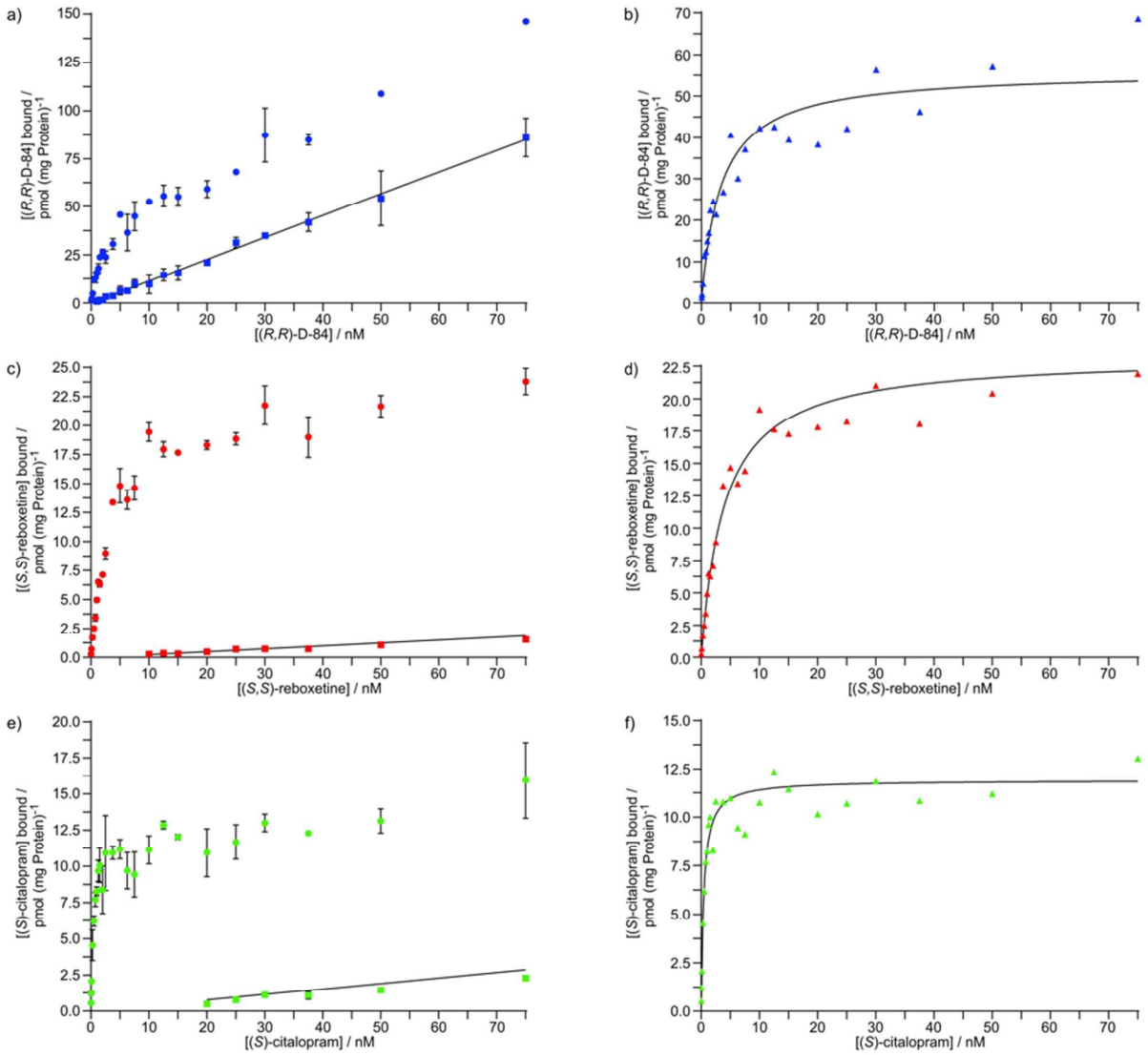

Figure 10: Saturation experiments performed with the established MS Binding Assays for (*R,R*)-D-84 ((*R,R*)-**1**) towards hDAT (blue symbols, a) and b)), (*S,S*)-reboxetine ((*S,S*)-**2**) towards hNET (red symbols, c), and d)), and (*S*)-citalopram ((*S*)-**3**) towards hSERT (green symbols, e) and f)). a), c), and e) Means ± SD (n = 3) of the total binding (circles) and the nonspecific binding in the presence of 10 µm *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**, squares, linear regression of the nonspecific binding is shown as a line). b), d), and f) Means of specific binding (triangles) and resulting saturation isotherms derived from the results shown in a), c), and e).

Conclusion

In summary, this study describes the successful development and validation of an LC-ESI-MS/MS method for the quantification of (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**) as well as (*S*)-citalopram ((*S*)-**3**) for their use as markers in MS Binding Assays addressing hDAT, hNET, and hSERT, respectively. This newly developed LC-ESI-MS/MS method enables the fast, robust, selective, and highly sensitive quantification of the three analytes within a chromatographic runtime of 1.5 min. A validation according to the CDER guideline for bioanalytical method validation demonstrates the selectivity, accuracy, precision, linearity, and sensitivity of the developed LC-MS/MS quantification method. The newly developed method allows for the simultaneous quantification of (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) in lowest pM to low nM concentrations under the same method parameters. This means that the effort to develop and validate the quantification method is considerably lower than for the development and validation of multiple quantification methods for MS Binding Assays selectively targeting DAT, NET, and SERT. The LC-ESI-MS/MS quantification method was applied to carry out saturation experiments using (*R,R*)-D-84 ((*R,R*)-**1**) as a marker for hDAT, (*S,S*)-reboxetine ((*S,S*)-**2**) as a marker for hNET, and (*S*)-citalopram ((*S*)-**3**) as a marker for hSERT. The procedure of the MS Binding Assays follows the well-established setup of previously published MS Binding Assays using a filtration with glass fiber filters for the separation of the target-marker complexes from the nonbound marker. This procedure allowed for the determination of total and nonspecifically bound marker in the binding samples and the calculation of specific binding as the difference of total and nonspecific binding. For the calculated specific binding, saturation isotherms for all three applied markers could be generated to determine their affinities (K_d values) towards their specific targets and the densities of binding sites (B_{max} values) in the corresponding target sources. The affinities of the markers towards their targets determined in this way were in good agreement with those published in literature, demonstrating that the established MS Binding Assays represent an efficient alternative to radioligand binding assays widely used for affinity characterisation at these targets so far. In this context, it is worth to mention that (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) can address their corresponding targets DAT, NET, and SERT, respectively, even in native target sources (e.g. brain homogenates), which may contain the desired target merely in the presence of many other targets. The use of (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) as highly selective markers for DAT, NET, and SERT, respectively, may further

enable the implementation of Multiple MS Binding Assays addressing these three targets simultaneously in a single binding experiment. Therefore, the described LC-ESI-MS/MS quantification method for (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) may have significant impact for mass spectrometry based affinity determination at DAT, NET, and SERT.

Acknowledgements

We thank Gerd Bauschke (LMU Munich, Department for Pharmacy) for the synthesis of D-84, [$^2H_4$]-D-84, and [$^2H_3$]-indatraline.

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Tables

	Luna PFP(2), 50 x 2 mm, 3 µm			YMC PFP, 50 x 2 mm, 3 µm		
	(*R,R*)-D-84 ((*R,R*)-**1**)	(*S,S*)-reboxetine ((*S,S*)-**2**)	(*S*)-citalopram ((*S*)-**3**)	(*R,R*)-D-84 ((*R,R*)-**1**)	(*S,S*)-reboxetine ((*S,S*)-**2**)	(*S*)-citalopram ((*S*)-**3**)
k	1.15	1.56	2.47	1.90	3.33	5.44
Peak Height / cps	1,510,000	397,000	1,390,000	1,010,000	197,000	681,000
S/N	20,850	15,350	6,885	10,050	5,167	2,785
Peak Width (50 %) / min	0.0469	0.0327	0.0401	0.0604	0.0634	0.0891
Peak Asymmetry	2.32	2.33	1.61	4.01	2.97	1.79

Table 1: Comparison of the chromatographic parameters obtained from chromatograms acquired with a Luna PFP(2) column and a Triart PFP column. Chromatography was performed with a mobile phase consisting of acetonitrile/ammonium formate buffer (1.0 mg/mL, 85/15) at a flow rate of 800 µL/min for both columns. The temperature was set to 20 °C and an injection volume of 30 µL of a 1 nM solution containing all 3 markers ((*R,R*)-**1**, (*S,S*)-**2**, and (*S*)-**3**) was used. The recorded mass transitions at the API 5000 were *m/z* 420.2/167.1 for (*R,R*)-D-84 ((*R,R*)-**1**), *m/z* 314.1/176.2 for (*S,S*)-reboxetine ((*S,S*)-**2**), and *m/z* 325.2/109.2 for (*S*)-citalopram ((*S*)-**3**). Results in the table represent means (n = 6).

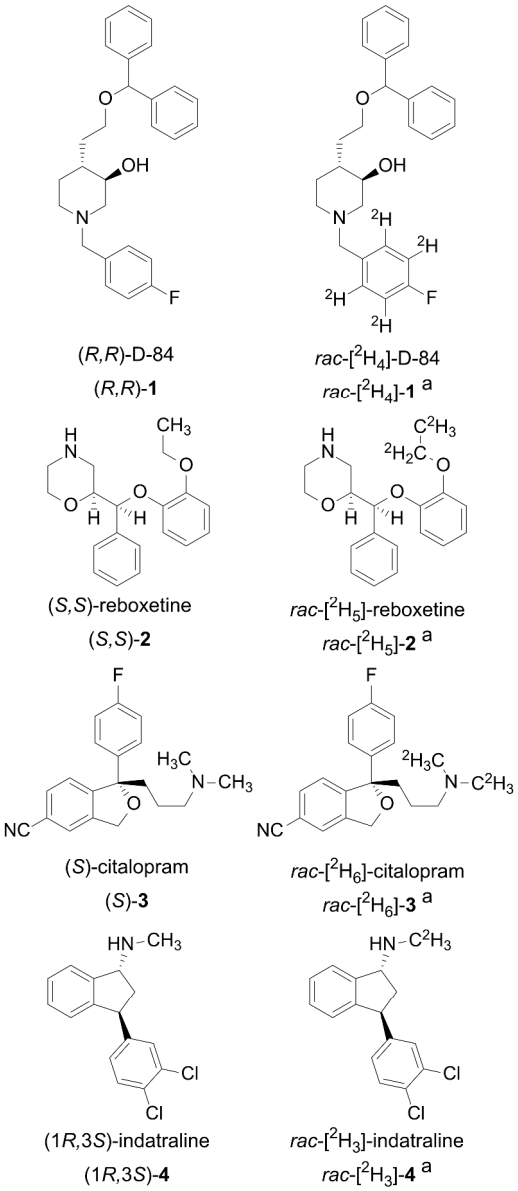
		(*R,R*)-D-84 ((*R,R*)-**1**)	(*S,S*)-reboxetine ((*S,S*)-**2**)	(*S*)-citalopram ((*S*)-**3**)
Accuracy	Accuracy in QCs	88.37 % - 112.73 %	96.08 % - 109.67 %	96.37 % - 110.33 %
Precision	Intra-batch precision in QCs	≤ 13.69 %	≤ 7.09 %	≤ 9.09 %
	Inter-batch precision in QCs	≤ 10.07 %	≤ 6.20 %	≤ 6.50 %
LLOQ	S/N at LLOQ	≥ 29	≥ 19	≥ 18
	Within-run precision at LLOQ (cal. std.)	≤ 9.75 %	≤ 9.01 %	≤ 6.24 %
	Between-run precision at LLOQ (cal. std.)	7.06 %	8.89 %	7.67 %
	Within-run accuracy at LLOQ (cal. std.)	95.87 % - 109.20 %	83.92 % - 99.55 %	88.80 % - 107.27 %
	Between-run accuracy at LLOQ (cal. std.)	101.60 %	93.65 %	97.04
ULOQ	Within-run precision at ULOQ (cal. std.)	≤ 1.37 %	≤ 4.87 %	≤ 2.37 %
	Between-run precision at ULOQ (cal. std.)	1.67 %	2.47 %	1.44 %
	Within-run accuracy at ULOQ (cal. std.)	94.10 % - 98.40 %	96.80 % - 99.60 %	94-00 % - 96.80 %
	Between-run accuracy at ULOQ (cal. std.)	96.93 %	98.33 %	95.82 %

Calibration	Range of calibration	2.5 pM – 1 nM	1 pM – 2.5 nM	2.5 pM – 2.5 nM
	r^2 of calibration function	≥ 0.9958	≥ 0.9978	≥ 0.9950
	Cal. stds. meeting criteria for accuracy	96.46 %	99.49 %	99.49 %
QCs	QCs meeting criteria for accuracy	99.30 %	99.30 %	100 %
	QCs per concentration level meeting criteria for accuracy	≥ 80.56 %	≥ 97.22 %	100 %

Table 2: Summary of results from the validation.

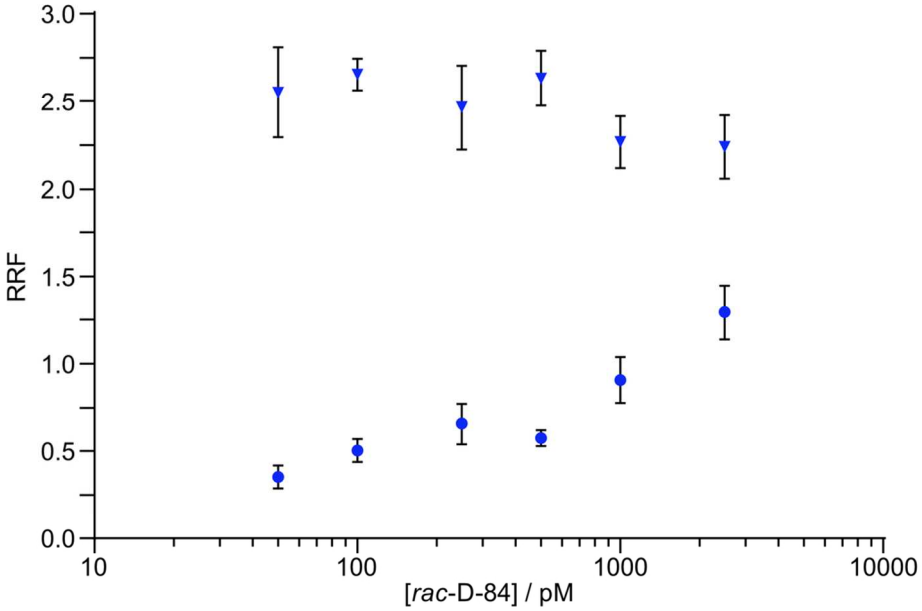
	(*R,R*)-D-84 ((*R,R*)-**1**) to hDAT (n = 4)	(*S,S*)-reboxetine ((*S,S*)-**2**) to hNET (n = 3)	(*S*)-citalopram ((*S*)-**3**) to hSERT (n = 3)
K_d in nM (mean ± SEM)	3.66 ± 1.35	3.06 ± 0.46	0.411 ± 0.0026
B_{max} in pmol/mg protein (mean ± SEM)	57.91 ± 8.43	31.59 ± 8.85	10.11 ± 1.71

Table 3: Results from the saturation experiments performed with the established MS Binding Assays.



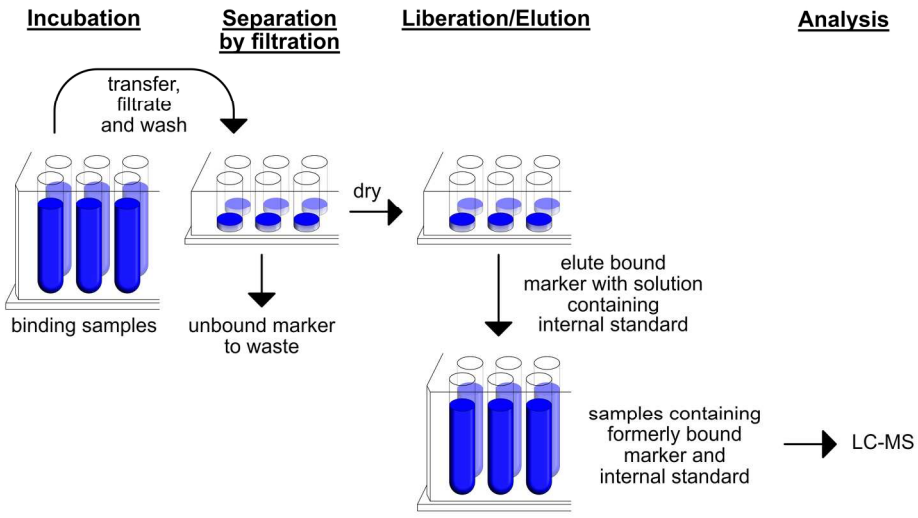
Structures of markers, internal standards and the inhibitor employed for determination of non-specific binding in MS Binding Assays. a Deuterated substances were used as racemates, but only one enantiomer is depicted in the figure. For the sake of simplicity, the racemic mixture of (*R*,*R*)-D-84 and (S,S)-D-83 (the enantiomer of (*R*,*R*)-D-84) is referred to as *rac*-D-84 and the racemic mixture of (*R*,*R*)-[^{2}H$_4$]-D-84 and (*S,S*)-[^{2}H$_4$]-D-83 is called rac-[^{2}H$_4$]-D-84 throughout this work.

193x438mm (600 x 600 DPI)



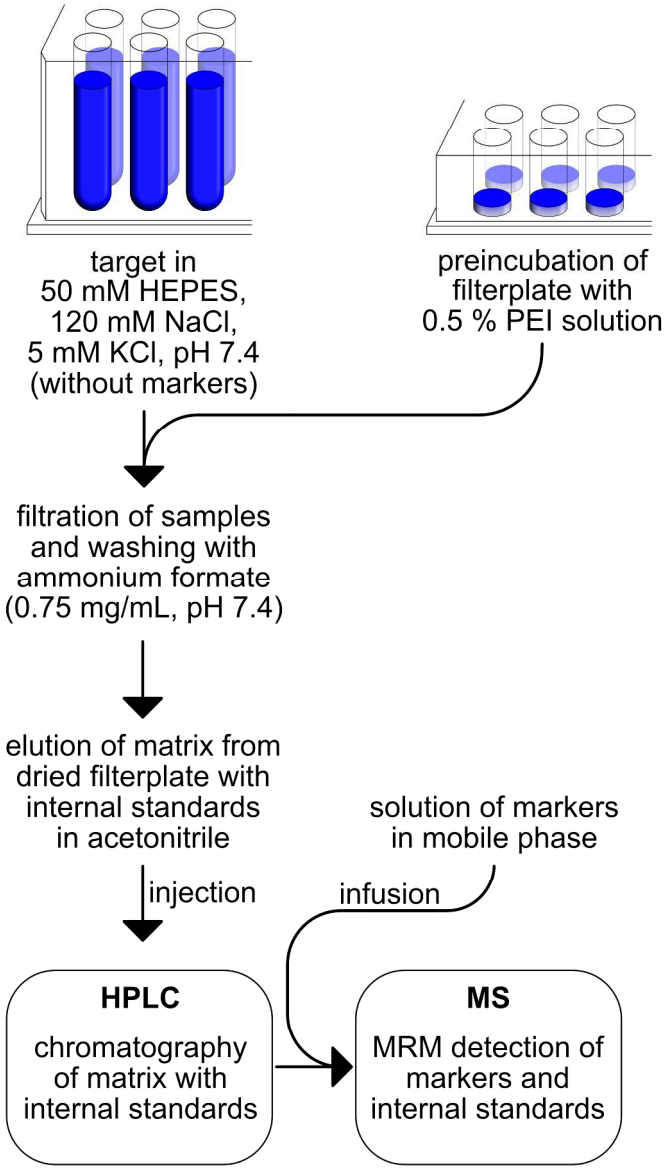
Relative response factors (RRF) for rac-D-84 (rac-1) calculated as, ((peak area *rac*-D-84)/(concentration *rac*-D-84))/((peak area rac-reboxetine)/(concentration rac-reboxetine)), (mean ±SD, n = 3). Dilution series of *rac*-D-84 (*rac*-**1**, 500 pM – 25 nM) in pure water (circles) and 20 % DMA in water (triangles) were prepared, spiked with rac-reboxetine, subsequently diluted tenfold in acetonitrile and analyzed. Stationary phase: Triart PFP (50 x 2.0 mm, 3 µm), mobile phase: acetonitrile / ammonium formate buffer (1.0 mg/mL, 85/15), 800 µL/min, detection at the API 3200: *rac*-D-84 (*rac*-**1**): m/z 420.3/167.0, *rac*-reboxetine (*rac*-**2**): m/z 314.2/176.1.

52x32mm (600 x 600 DPI)



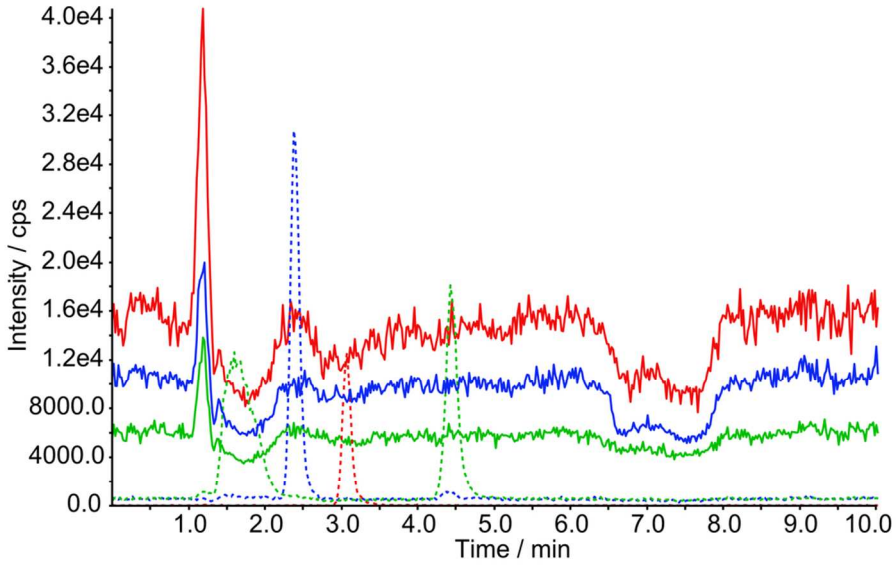
Workflow of the developed MS Binding Assays

92x48mm (600 x 600 DPI)



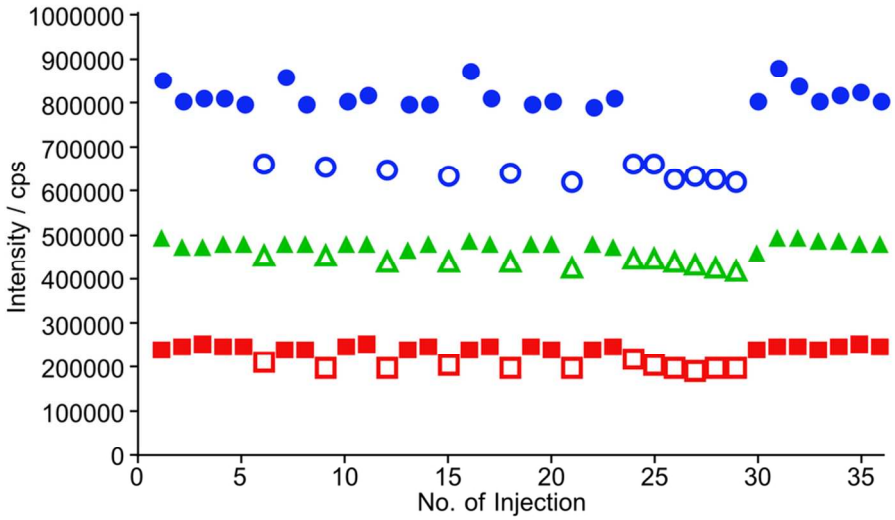
Schematic workflow of the post-column infusion experiment.

143x242mm (600 x 600 DPI)



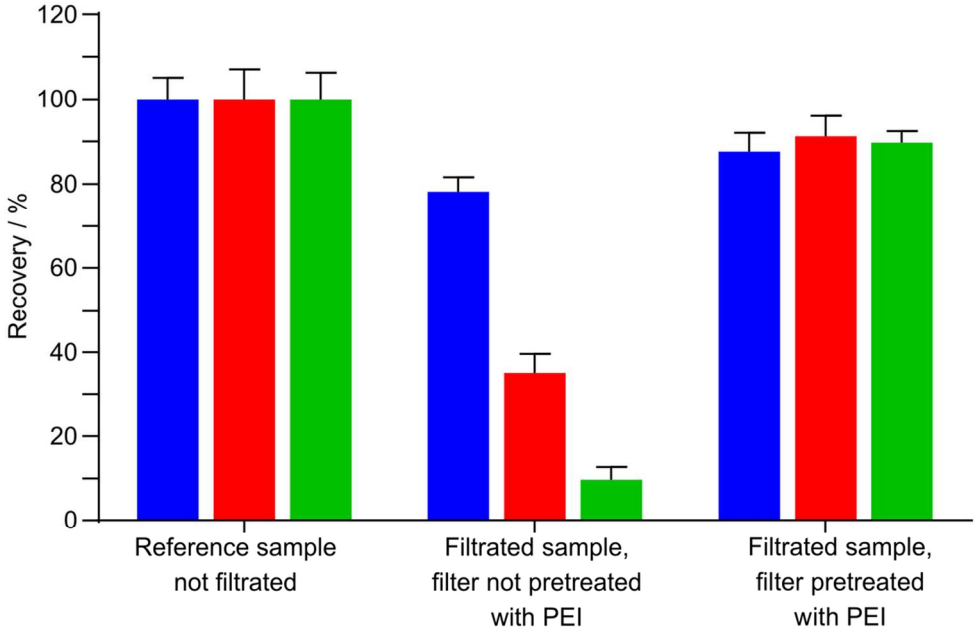
MRM Chromatogram from a post-column infusion experiment for the determination of matrix effects resulting from binding samples, as described in the experimental section ("Investigation of the matrix effect by post-column infusion experiments"). 300 pM (*R,R*)-D-84 ((*R,R*)-**1**, blue signal), 2 nM (*S,S*)-reboxetine ((*S,S*)-**2**, red signal), and 600 pM (*S*)-citalopram ((*S*)-**3**, green signal) were infused continuously, while blank matrix, containing 100 pM *rac*-[2H4]-D-84 (rac-[2H4]-1, blue dotted peak), *rac*-[$^2H_5$]-reboxetine (rac-[$^2H_5$]-**2**, red dotted peak), and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**, green dotted peak at a retention time of 4.5 min; peak at 1.5 min is caused by matrix components, generating a signal with the same MRM transition as *rac*-[$^2H_6$]-**3**), which was subjected to the chromatography.

51x31mm (600 x 600 DPI)



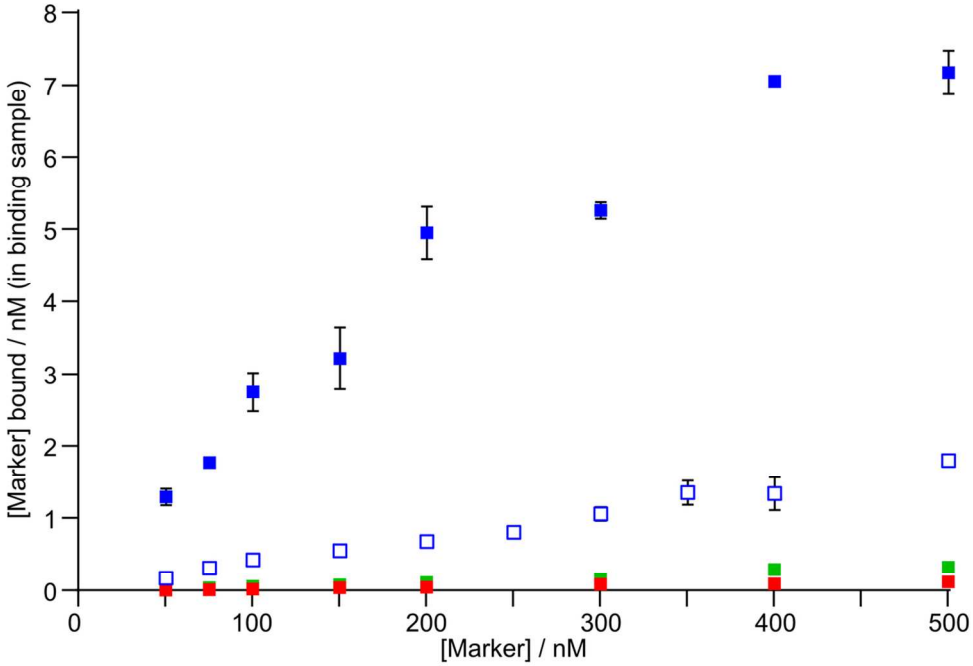
Plot of marker peak intensities for a series of injections of solvent standards (full symbols) and matrix standards (100 pM of each marker, empty symbols, obtained from a simulated binding experiment, for details on the sample preparation and analysis see experimental section); (*R,R*)-D-84 ((*R,R*)-**1**): blue dots, (*S,S*)-reboxetine ((*S,S*)-**2**): red squares, (*S*)-citalopram ((*S*)-**3)**: green triangles.

47x26mm (600 x 600 DPI)



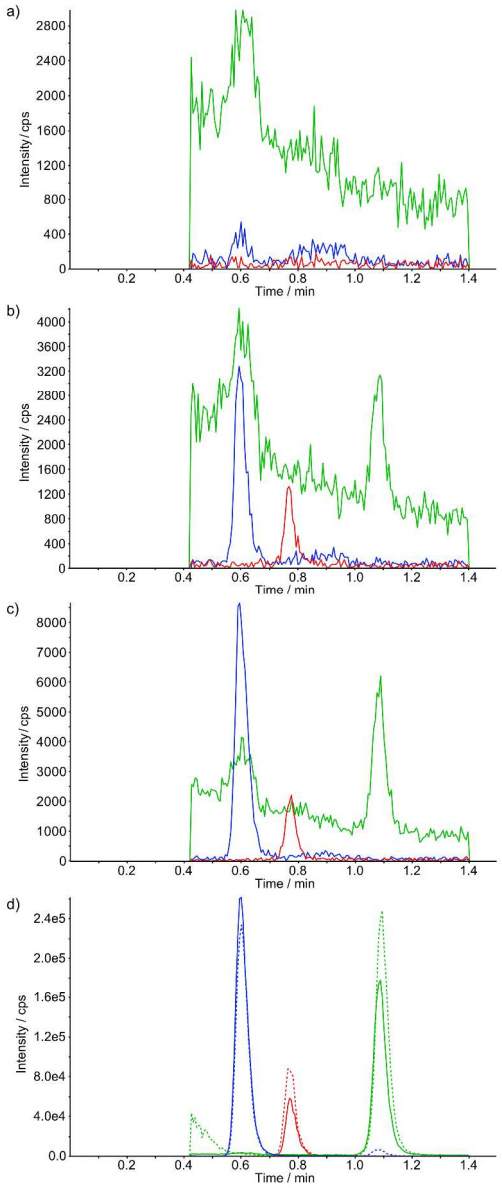
Recovery of markers ((*R,R*)-D-84 ((*R,R*)-**1**, blue), (*S,S*)-reboxtine ((*S,S*)-**2**, red), and (*S*)-citalopram ((*S*)-**3**, green, all at 100 pM) dissolved in acetonitrile after filtration through glass fiber filters. Reference sample was not filtrated, filtrated samples were filtrated through filters, pretreated with 200 µL water ("filter not pretreated with PEI") or 200 µL 0.5 % PEI solution ("filter pretreated with PEI") for 1 h and washed with 3 x 150 µL ammonium formate buffer (0.75 mg/mL, pH 7.4) prior to filtration. All non-filtrated reference and filtrated samples were spiked with internal standards (*rac*-[$^2H_4$]-**1**, *rac*-[$^2H_5$]-**2**, *rac*-[$^2H_6$]-**3**) at a concentration of 100 pM in acetonitrile prior to analysis. Bars represent means of recovery (% relative to reference sample) ± SD (n = 6).

54x34mm (600 x 600 DPI)



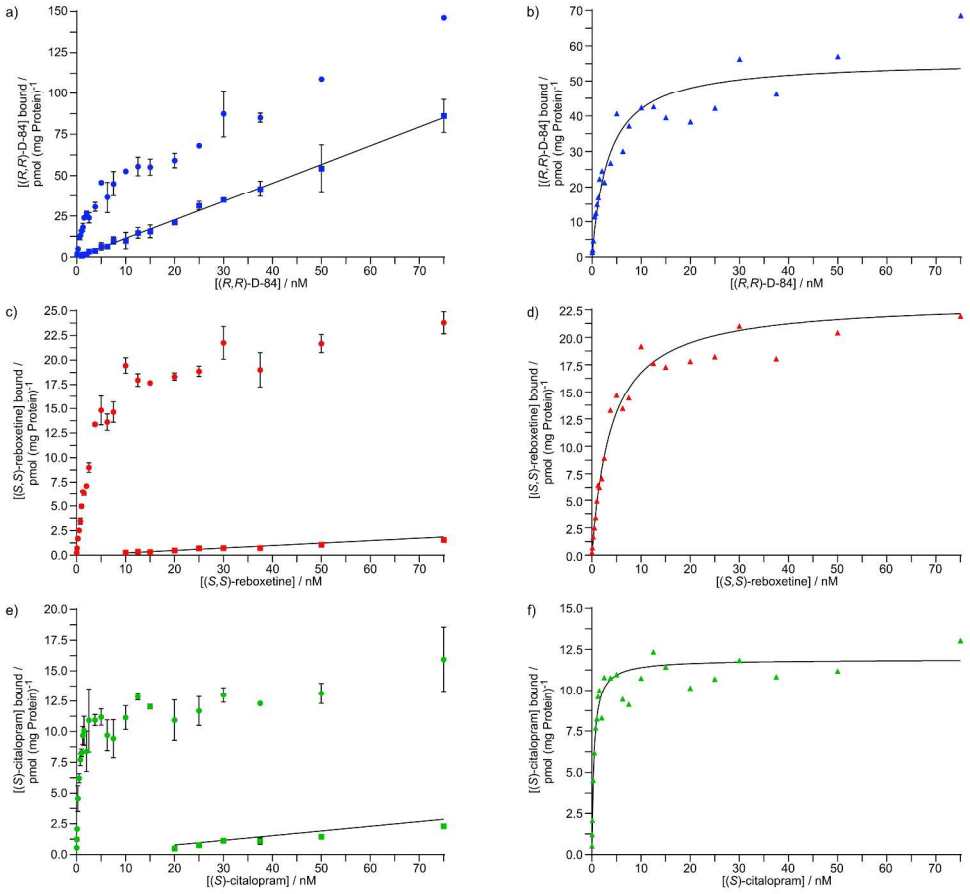
Nonspecific binding (mean ± SD, n = 3) derived from preliminary saturation experiments using different washing procedures. Binding samples were prepared according to the general procedure for the MS Binding Assays (see experimental section). After incubation, binding samples were filtrated over the glass fiber filters. Subsequently, the filters were washed with ammonium formate buffer (0.75 mg/mL, pH 7.4, filled symbols, (*R,R*)-**1**: blue, (*S,S*)-**2**: red, (*S*)-**3**: green) or 10 % DMA in ammonium formate buffer (0.75 mg/mL, pH 7.4, only shown for (*R,R*)-**1**: empty blue symbols). Marker elution and analysis was carried out according to the procedure and with the LC-ESI-MS/MS quantification method, described in the experimental section.

57x39mm (600 x 600 DPI)



MRM chromatograms of (*R,R*)-D-84 ((*R,R*)-**1**, blue, m/z 420.2/167.1), (*S,S*)-reboxetine ((*S,S*)-**2**, red, m/z 314.1/176.2), and (*S*)-citalopram ((*S*)-**3**, green, m/z 325.2/109.1) obtained with the developed LC-ESI-MS/MS quantification method. a) Matrix blank, b) 1 pM, c) 2.5 pM, and d) 100 pM of each marker ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) with 90 pM *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**, blue dotted, m/z 424.2/176.1), *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**, red dotted, m/z 319.2/176.2), and *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**, green dotted, m/z 331.2/109.0) as internal standards. All chromatograms were acquired, using a Luna PFP(2) column as the stationary phase and acetonitrile/ammonium formate buffer (0.75 mg/mL; 85/15) at 800 µL/min as mobile phase. The temperature was set at 20 °C and the injection volume was 30 µL. Only the eluent from 0.4 min to 1.4 min was sent to the API 5000.

197x458mm (600 x 600 DPI)



Saturation experiments performed with the established MS Binding Assays for (*R,R*)-D-84 ((*R,R*)-**1**) towards hDAT (blue symbols, a) and b)), (*S,S*)-reboxetine ((*S,S*)-**2**) towards hNET (red symbols, c), and d)), and (*S*)-citalopram ((*S*)-**3**) towards hSERT (green symbols, e) and f)). a), c), and e) Means ± SD (n = 3) of the total binding (circles) and the nonspecific binding in the presence of 10 µm *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4,** squares, linear regression of the nonspecific binding is shown as a line). b), d), and f) Means of specific binding (triangles) and resulting saturation isotherms derived from the results shown in a), c), and e).‼ ┼ ‼ ┼

157x141mm (600 x 600 DPI)

Supporting Information

Development and validation of an LC-ESI-MS/MS method for the quantification of D-84, reboxetine, and citalopram for their use in MS Binding Assays addressing the monoamine transporters hDAT, hSERT, and hNET

Patrick Neiens,[a] Angela De Simone,[b] Anna Ramershoven,[a] Georg Höfner,[a] Lars Allmendinger,[a] Klaus T. Wanner[a*]

[a]Department Pharmazie – Zentrum für Pharmaforschung
Ludwig-Maximilians-Universität München
Butenandtstr. 7, 81377 Munich, Germany
Phone: +49 (0) 89 2180 77249
e-mail: klaus.wanner@cup.uni-muenchen.de
ORCID: 0000-0003-4399-1425

[b]Department for Life Quality Studies,
Alma Mater Studiorum – University of Bologna,
Corso D'Augusto 237, 47921 Rimini, Italy

1. Enantioseparation and *ee*-determination of (*S,S*)-reboxetine ((*S,S*)-**2**)

1.1. Extraction of *rac*-reboxetine (rac-**2**) from Edronax tablettes

80 Edronax 4 mg tablettes were disintegrated in 50 mL 0.1 N HCl and filtrated. The filter was washed twice with 5 mL 0.1 N HCl. PH of the filtrate was adjusted to 9 with saturated $NaHCO_3$-solution and extracted with 5 x 40 mL diethyl ether. The combined ether phases were dried over $MgSO_4$, filtrated and the solvent was removed *in vacuo*. 290.9 mg (90.9 %) *rac*-reboxetine (*rac*-**2**) was obtained as yellow oil.

1.2. Semi-preparative enantioseparation of *rac*-reboxetine (*rac*-**2**)

Rac-reboxetine was dissolved in propan-2-ol/diethylamine (DEA, 9/1, v/v) to a concentration of 100 mg/mL. Enantioseparation was carried out on an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany, consisting of vacuum degasser, binary pump, autosampler, thermostated column compartment and UV-detector) with a Lux Cellulose-1 column (250 x 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany), protected by a security guard cartridge of the same stationary phase (4 x 3 mm, 5 µm, Phenomenex) and an in-line filter (0.5 µm, stainless steel,

IDEX, Lake forrest, IL, USA). As mobile phase, a mixture of propan-2-ol containing 2 % DEA and 3 % formic acid with *n*-hexane (20/80) was used at a flow rate of 1.5 mL/min. The column oven temperature was set to 20 °C and a volume of 60 µL of the solution described above was injected. For detection, a wavelength of 270 nm was used. Fractions, containing the peaks of each reboxetine enantiomer were collected, collection of the first fraction was started as soon, as the signal of the peak was visible and collection of this fraction was finished when the signal reached a minimum before the signal of the second peak started to increase. After the first fraction, the collection of the second fraction was started immediately and finished when the signal of the second peak reached the baseline-level. After multiple chromatographic runs, collected fractions of the same peaks were combined, the eluent was removed *in vacuo*, the obtained yellow oil was again dissolved in propan-2-ol/diethylamine (DEA, 9/1, v/v) to a concentration of 100 mg/mL. This solution was chromatographed a second time with the same procedure as described above, but this time an additional fraction between both peaks was collected and discarded. The eluent of each fraction was removed *in vacuo*, the residue was dissolved in a mixture of 1,4-dioxane and water and freeze dried. After the enantioseparation, 2 fractions each containing one enantiomer of 2-[(2-ethoxyphenoxy)phenylmethyl]morpholine-2-carbaldehyde as a yellow oil were obtained.

1.3. Hydrolysis of (*2R*)-2-[(*2R*)-(2-ethoxyphenoxy)phenylmethyl]morpholine-2-carbaldehyde and crystallization to (*S,S*)-reboxetine ((*S,S*)-**2**) mesylate

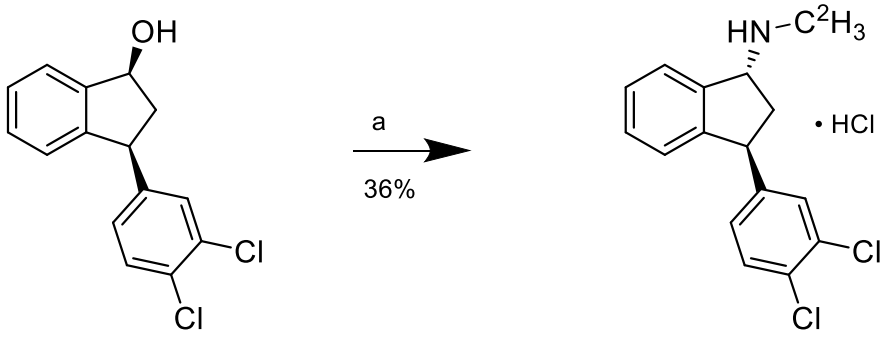
The fraction obtained from the second peak, containing (*2R)*-2-[(*R*)-(2-ethoxyphenoxy)phenylmethoy]morpholine was dissolved in water (2.5 mL) and dioxane (2.0 mL) and stirred at 50 °C over night after the addition of 53.8 mg KOH (85 %, 3.5 equiv.). The aqueous phase was extracted with diethyl ether (3 x 8 mL). The combined ether phases were dried ($MgSO_4$), filtered and the solvent was removed *in vacuo* to obtain 49.7 mg (*S,S*)-reboxetine ((*S,S*)-**2**) as yellow oil, which was then dissolved in diethyl ether, cooled to -77 °C and 11.2 µL methansulfonic acid was added. The solvent was removed *in vacuo*, the residue was dissolved in H_2O and freeze dried to obtain (*S,S*)-reboxetine ((*S,S*)-**2**) mesylate**.**

1.4. Determination of the enantiomeric excess of (*S,S*)-reboxetine ((*S,S*)-**2**)

Determination of the enantiomeric excess (*ee*} of (*S,S*)-reboxetine ((*S,S*)-**2**) was carried out on an Agilent 1100 series HPLC (consisting of vacuum degasser, binary pump, autosampler, thermostated column compartment and UV-detector) with a Chiralpak IB-3 column (250 x 4.6 mm, 3 µm, Daicel – Chiral Technologies, Illkirch Cedex, France), protected by an in-line filter (0.2 µm, titan, IDEX). Mobile phase A was ammonium bicarbonate buffer (20 mM, pH 9.0) with methanol (HPLC grade, VWR Prolabo, Darmstadt, Germany) in a ratio of 2/1, which was mixed with acetonitrile (HPLC grade, VWR Prolabo) as mobile phase B in a ratio of 7.5/92.5. The flow rate was 1.0 mL/min, the column oven was set to 5 °C and the detection wavelength was 270 nm. 5 µL of solution of 20 mM (*S,S*)-reboxetine ((*S,S*)-**2**) as the analyte in acetonitrile/H_2O (90/10) was injected. Based on calibration functions for both enantiomers in a range of 10 µM to 25 mM, the concentrations of both enantiomers in the sample were calculated and thus the *ee* was determined.

2. Synthesis of [$^2H_3$]-indatraline

2.1. Synthesis of indatraline hydrochloride ((1*R*,3*S*)-**4**)



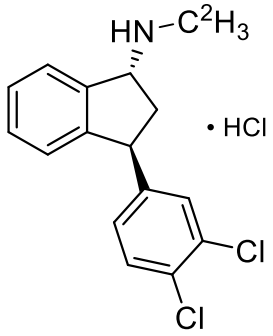
a) 1. methanesulfonyl chloride (2 equiv.), NEt_3 (4 equiv.), THF, -15 °C, 1 h; 2. $NH_2C^2H_3$, 0 °C to rt, 20 h, 3. aqueous HCl (1 M).

Depicted compounds are racemic although only one enantiomer is shown.

2.1. General Methods

THF and NEt_3 were distilled from sodium. Methanesulfonyl chloride (Fluka) was used without further purification. All other chemical reagents were used from bulk without further purification. Common solvents for recrystallization, column chromatography were distilled before use. TLC plates were made from silica gel 60 F254 on Glas plates (Merck). Compounds were first stained on silica then with 5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.2% $Ce(SO_4)_2\cdot 4H_2O$ and 5% conc. H_2SO_4. Merck silica gel (mesh 230–400) was used as stationary phase for flash chromatography (CC). Melting points: m.p. (uncorrected) were determined with an Electrothermal IA9100MK1 Melting Point apparatus. IR spectroscopy: FT-IR Spectrometer Paragon 1000 (Perkin Elmer), solid sample was measured as KBr pellet. HR Mass spectrometry: JEOL GC Mate II; NMR spectroscopy: NMR spectra were recorded on Bruker Avance III (Bruker BioSpin, 500 MHz) and integrated with the program of MestReNova version 11.0.4-18998.

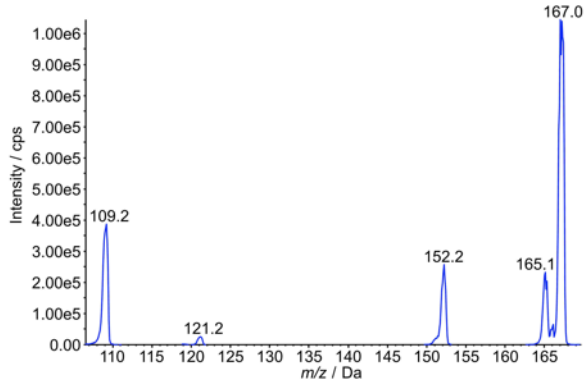
2.2. [$^2H_3$]-indatraline hydrochloride (*rac*-[$^2H_3$]-**4**) [42,43]



To a stirred solution of *cis*-(±)-3-(3,4-dichlorophenyl)indan-1-ol (0.500 g, 1.79 mmol) in THF (15 mL) was added NEt_3 (0.997 mL, 7.16 mmol) at -15 °C and methanesulfonyl chloride (208 µL, 2.69 mmol). After 1 h the reaction

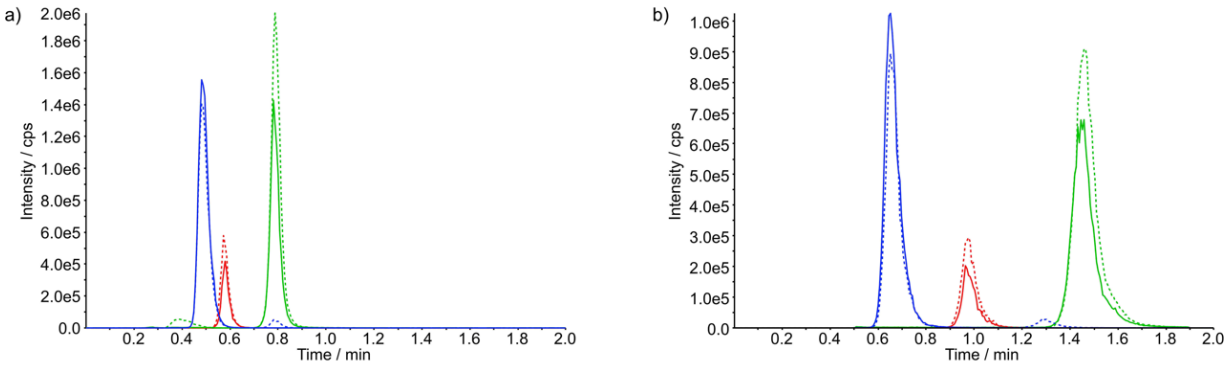
mixture was warmed to 0 °C and gaseous [$^2H_3$]methylamine was introduced for 15 min with a syringe from a second vessel containing a large excess of a stirred mixture (1:1) of KOH and [$^2H_3$]methylamine hydrochloride. The reaction mixture was allowed to warm slowly to r.t. over 10 h. The resulting orange solution was poured into saturated $NaHCO_3$ solution (25 mL) followed by extraction with EtOAc (6 x 20 mL). The combined organic layers were dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*. The resulting crude product was purified by flash chromatography (heptane/EtOAc/*i*-PrOH = 85:5:10 + 1% *N*,*N*-dimethylethylamine). The resulting colorless oil was dissolved in dioxane (0.2 mL), treated with aqueous HCl (1 M, 1 mL) and freeze dried. The resulting product was recrystallized from EtOAc/EtOH (92:8, 8 mL) to yield colorless crystals (188 mg, 36 %): m.p. 156-157 °C. ^{1}H NMR (500 MHz, D_2O): 2.28 (dt, J = 15.2/8.0 Hz, 1 H, CH_2), 2.66 (ddd, J = 15.2/7.6/2.1 Hz, 1 H, CH_2), 4.55 (t, J = 7.9 Hz, 1 H, CH_2CH), 4.82 (d, J = 7.8 Hz, 1 H, $CHNCH_3$), 6.84 (d, J = 7.6 Hz, 1 H, CH(OH)CCC*H*), 6.91 (dd, J = 8.3/2.1 Hz, 1 H, CC*H*CHCCl), 7.15 (d, J = 2.1 Hz, 1 H, CCHCCl), 7.32–7.18 (m, 3 H, $CH(NCH_3)CHCH$), $CH(NCH_3)CCCHCH$, $CCHCHCCl$), 7.53 (d, J = 7.6 Hz, 1 H, $CH(NCH_3)CCH$). ^{13}C NMR (125 MHz, D_2O): δ = 27.63 (quint., J = 23.5 Hz), 36.40, 45.98, 60.64, 123.77, 124.15, 125.94, 126.37, 127.87, 128.52, 128.81, 128.93, 130.26, 134.41, 142.27, 145.33. IR (KBr): $\tilde{\nu}$ = 3432 cm^{-1}, 2941, 2738, 22649, 2598, 1596, 1467, 766. HRMS (EI): *m*/*z* $[M\text{-}HCl]^+$ cacld. for $C_{16}H_{12}{}^2H_3N^{35}Cl_2$: 294.0770, found: 294.0778.

3. Identification and tuning of MRM transitions



SI-figure 1: Product ion scan of *m/z* 420.3 obtained in the Compound Optimization for *rac*-D-84 (*rac*-**1**).

4. Development of the LC-ESI-MS/MS method



SI-figure 2: Chromatograms of (*R,R*)-D-84 ((*R,R*)-**1**, blue), (*S,S*)-reboxetine ((*S,S*)-**2**, red) and (*S*)-citalopram ((*S*)-**3**, green). Mobile phase: acetonitrile/ammonium formate buffer (1.0 mg/mL, 85/15), flow rate: 800 µL/min, column oven temperature: 20 °C, injection volume: 30 µL. a) Stationary phase: Luna PFP(2) column (50 x 2.0 mm, 3 µm, Phenomenex), b) Stationary phase: Triart PFP (50 x 2.0 mm, 3 µm, YMC).

5. Preliminary saturation experiments

Preliminary saturation experiments to determine the influence of DMA in the washing buffer were carried out according to the final procedure for saturation experiments. For the preliminary experiments, no calibration was acquired, so the calculation for the K_d value was based on the area ratios (peak area marker / peak area internal standard). Experiments were carried out in duplicates, resulting in K_d values of 1.16 nM and 0.74 nM for the binding of (*S*)-citalopram ((*S*)-**3**) towards hSERT.

6. Validation

Validation (*R,R*)-D-84 ((*R,R*)-**1**)

	Intra-series																	Inter-series[b]		
	160223 hDAT			160226 hNET			160304 hDAT			160308 hSERT			160310 hNET			160311 hSERT					
c Marker [pM] (n)	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]
2.5 (6)	2.558	102.33	3.04	2.397	95.87	4.07	2.433	97.33	4.02	2.710	108.40	2.09	2.730	109.20	2.65	2.412	96.47	9.75	2.540	101.60	7.06
5 (6)	4.385	87.70	2.18	4.958	99.17	1.58	5.235	104.70	2.68	5.042	100.83	2.69	5.317	106.33	1.68	4.930	98.60	5.67	4.978	99.56	6.73
10 (3)	9.617	96.17	0.94	9.957	99.57	1.25	10.67	106.67	2.86	10.27	102.67	1.12	11.00	110.00	0.91	10.53	105.33	1.98	10.34	103.40	4.77
25 (3)	29.00	116.00	0.91	28.63	114.53	1.12	27.80	111.20	1.30	28.43	113.73	1.46	29.10	116.40	0.60	27.00	108.00	2.57	28.33	113.31	2.91
50 (3)	51.97	103.93	2.22	51.83	103.67	1.28	54.10	108.20	1.29	53.17	106.33	0.11	53.43	106.87	1.03	52.70	105.40	0.83	52.87	105.73	1.89
100 (3)	99.90	99.90	0.10	104.7	104.67	1.46	108.7	108.67	1.92	102.0	102.00	0.98	106.7	106.67	0.54	105.0	105.00	0.95	104.5	104.48	3.00
250 (3)	264.7	105.87	0.22	268.3	107.33	1.55	270.7	108.27	0.77	258.7	103.47	0.22	259.0	103.60	0.77	264.0	105.60	0.66	264.2	105.69	1.86
500 (3)	499.7	99.93	0.42	506.0	101.20	1.19	522.0	104.40	0.19	498.3	99.67	0.93	504.3	100.87	0.41	504.0	100.80	0.60	505.7	101.14	1.69
1000 (3)	981.7	98.17	1.00	965.7	96.57	0.36	941.0	94.10	0.32	984.0	98.40	0.88	971.3	97.13	1.37	972.0	97.20	0.80	969.3	96.93	1.67
QC 2.5 (6)	2.438	97.53	4.81	2.563	102.53	3.25	2.818	112.73	13.69	2.673	106.93	3.48	2.645	105.80	2.91	2.223	88.93	6.79	2.560	102.41	10.07
QC 10 (6)	9.283	92.83	1.89	10.01	100.07	1.60	10.98	109.83	1.95	10.78[a]	107.80	2.21	10.83	108.33	2.78	10.34	103.35	6.07	10.36	103.59	6.50
QC 100 (6)	99.13	99.13	0.64	102.6	102.62	2.00	109.8	109.83	1.86	104.7	104.67	4.59	108.0	108.00	2.19	106.3	106.33	0.97	105.1	105.10	4.06
QC 1000 (6)	889.8	88.98	0.85	939.8	93.98	1.28	951.2	95.12	1.03	960.5	96.05	1.05	947.5	94.75	0.89	982.7	98.27	0.88	945.3	94.53	3.17
Calibration function	y = 0.0111x + 0.013 (r^2 = 0.9988)			y = 0.011x + 0.00472 (r^2 = 0.9982)			y = 0.00968x + 0.0102 (r^2 = 0.9958)			y = 0.0116x + 0.00945 (r^2 = 0.9992)			y = 0.0104x + 0.00829 (r^2 = 0.9984)			y = 0.00964x + 0.00739 (r^2 = 0.9993)					

M: mean of calculated concentration, Acc: Accuracy, Prec: Precision, n: number of replicates, RSD: relative standard deviation, QC: quality control sample. [a] n = 5. [b] Number of samples (n) for 2.5 pM, 5 pM, QC 2.5 pM, QC 100 pM, QC 1000 pM: n = 36; 10 pM – 1000 pM: n = 18; QC 10 pM: n = 35.

Validation (*S*,S)-reboxetine ((*S*,*S*)-**2**)

	Intra-series																		Inter-series[b]		
	160223 hDAT			160226 hNET			160304 hDAT			160308 hSERT			160310 hNET			160311 hSERT					
c Marker [pM] (n)	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]
1 (6)	0.947	94.67	8.15	0.996	99.55	6.18	0.988	98.83	4.86	0.891	89.07	8.83	0.839	83.92	9.01	0.959	95.88	5.30	0.937	93.65	8.89
2.5 (6)	2.487	99.47	4.06	2.473	98.93	5.62	2.543	101.73	4.17	2.582	103.27	3.19	2.698	107.93	4.19	2.445	97.80	4.89	2.538	101.52	5.29
5 (6)	4.792	95.83	5.48	4.983	99.67	2.36	4.965	99.30	1.78	5.082	101.63	5.50	4.970	99.40	3.08	50.20	100.40	3.77	4.969	99.37	4.06
10 (3)	9.770	97.70	1.43	9.587	95.87	3.41	10.08	100.77	7.08	9.867	98.67	0.51	10.20	102.00	0.98	9.880	98.80	2.18	9.897	98.97	3.55
25 (3)	27.57	110.27	4.24	25.57	102.27	2.22	25.23	100.93	3.20	26.10	104.40	2.65	27.27	109.07	0.92	26.17	104.67	4.30	26.32	105.27	4.23
50 (3)	51.60	103.20	1.03	49.40	98.80	1.61	49.17	98.33	2.27	51.50	103.00	1.03	51.00	102.00	1.87	51.07	102.13	2.08	50.62	101.24	2.44
100 (3)	99.97	99.97	2.70	100.3	100.33	0.58	99.30	99.30	0.70	100.2	100.17	1.62	103.0	103.00	0.00	101.7	101.67	1.14	100.7	100.74	1.73
250 (3)	263.3	105.33	2.16	262.0	104.80	0.76	252.7	101.07	4.34	255.0	102.00	1.04	251.7	100.67	0.83	258.0	103.20	1.78	257.1	102.84	2.55
500 (3)	507.7	101.53	3.20	504.3	100.87	4.37	497.7	99.53	0.46	508.7	101.73	2.02	493.0	98.60	0.88	510.7	102.13	1.11	503.7	100.73	2.43
1000 (3)	1057	105.67	2.73	1020	102.00	0.98	1008	100.80	2.96	1043	104.33	0.55	1037	103.67	1.47	1007	100.67	0.57	1029	102.86	2.40
2500 (3)	2420	96.80	2.71	2467	98.67	2.38	2490	99.60	2.24	2440	97.60	1.08	2467	98.67	4.87	2467	98.67	1.24	2458	98.33	2.47
QC 2.5 (6)	2.615	104.60	2.82	2.645	105.80	3.93	2.728	109.13	6.09	2.558	102.33	7.09	2.742	109.67	5.01	2.428	97.13	3.70	2.619	104.78	6.20
QC 10 (6)	9.860	98.60	1.86	10.20	102.00	6.44	10.30	102.95	2.37	10.58[a]	105.80	2.16	10.32	103.17	5.03	10.15	101.45	2.87	10.22	102.23	4.16
QC 100 (6)	102.3	102.33	1.92	99.28	99.28	1.16	100.8	100.80	2.16	102.3	102.28	3.94	104.2	104.17	1.65	102.2	102.17	1.14	101.8	101.84	2.54
QC 1000 (6)	960.8	96.08	2.56	983.2	98.32	2.05	998.7	99.87	2.88	999.3	99.93	2.14	973.8	97.38	0.93	1015	101.50	0.82	988.5	98.85	2.64
Calibration function	$y = 0.00608x + 0.0104$ ($r^2 = 0.9978$)			$y = 0.0065x + 0.00191$ ($r^2 = 0.9992$)			$y = 0.00603x + 0.000806$ ($r^2 = 0.9996$)			$y = 0.0071x + 0.00406$ ($r^2 = 0.9990$)			$y = 0.00638x + 0.00317$ ($r^2 = 0.9986$)			$y = 0.00589x + 0.0019$ ($r^2 = 0.9996$)					

M: mean of calculated concentration, Acc: Accuracy, Prec: Precision, n: number of replicates, RSD: relative standard deviation, QC: quality control sample. [a] n = 5. [b] Number of samples (n) for 1 pM, 2.5 pM, 5 pM, QC 2.5 pM, QC 100 pM, QC 1000 pM: n = 36; 10 pM – 2500 pM: n = 18; QC 10 pM: n = 35.

Validation (*S*)-citalopram ((*S*)-**3**)

	Intra-series																	Inter-series[b]		
	160223 hDAT			160226 hNET			160304 hDAT			160308 hSERT			160310 hNET			160311 hSERT					
c Marker [pM] (n)	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]
2.5 (6)	2.397	95.87	3.38	2.377	95.07	5.09	2.220	88.80	5.87	2.532	101.27	6.24	2.682	107.27	2.90	2.350	94.00	5.91	2.426	97.04	7.67
5 (6)	4.653	93.07	4.46	5.158	103.17	5.24	4.695	93.90	3.30	5.283	105.67	6.23	4.878	97.57	3.95	5.233	104.67	2.75	4.984	99.67	6.65
10 (3)	9.943	99.43	2.24	9.990	99.90	1.91	10.07	100.73	1.95	10.23	102.30	3.18	11.10	111.00	3.25	10.67	106.67	1.43	10.33	103.34	4.64
25 (3)	28.30	113.20	2.12	26.90	107.60	0.37	26.97	107.87	0.77	27.63	110.53	0.91	27.87	111.47	0.75	27.17	108.67	1.12	27.47	109.89	2.14
50 (3)	54.57	109.13	1.07	52.03	104.07	1.73	52.60	105.20	4.31	55.53	111.07	1.06	54.07	108.13	0.70	54.30	108.60	0.66	53.85	107.70	2.82
100 (3)	107.3	107.33	1.08	104.3	104.33	1.11	108.0	108.00	1.60	105.3	105.33	0.55	109.7	109.67	2.11	108.3	108.33	0.53	107.2	107.17	2.06
250 (3)	279.7	111.87	0.90	276.7	110.67	0.55	272.7	109.07	1.81	267.3	106.93	1.20	266.0	106.40	0.75	271.7	108.67	0.43	272.3	108.93	2.02
500 (3)	534.3	106.87	1.35	533.0	106.60	0.19	527.7	105.53	0.22	524.0	104.80	0.83	523.3	104.67	1.27	528.7	105.73	0.89	528.5	105.70	1.11
1000 (3)	1073	107.33	0.54	1037	103.67	0.56	1018	101.83	2.21	1043	104.33	0.55	1040	104.00	0.96	1027	102.67	0.56	1040	103.97	1.93
2500 (3)	2350	94.00	1.47	2397	95.87	0.24	2420	96.80	0.72	2397	95.87	0.87	2403	96.13	2.37	2407	96.27	1.05	2396	95.82	1.44
QC 2.5 (6)	2.672	106.87	3.12	2.562	102.47	9.09	2.435	97.40	5.78	2.585	103.40	7.23	2.567	102.67	4.66	2.490	99.60	6.01	2.552	102.07	6.50
QC 10 (6)	10.05	100.48	2.87	10.72	107.17	3.51	10.46	104.60	3.65	10.80[a]	108.00	3.00	10.65	106.50	1.54	10.63	106.33	2.57	10.54	105.44	3.61
QC 100 (6)	106.0	106.00	1.33	104.3	104.33	1.31	108.5	108.50	1.62	106.3	106.33	4.44	110.3	110.33	2.04	109.2	109.17	0.90	107.4	107.44	2.85
QC 1000 (6)	963.7	96.37	0.50	996.5	99.65	1.06	1048	104.83	0.94	1037	103.67	1.17	9950	99.50	0.48	1042	104.17	1.28	1014	101.36	3.20
Calibration function	$y = 0.0067x + 0.00556$ ($r^2 = 0.9950$)			$y = 0.00692x + 0.00479$ ($r^2 = 0.9974$)			$y = 0.0063x + 0.00665$ ($r^2 = 0.9982$)			$y = 0.00733x + 0.00723$ ($r^2 = 0.9976$)			$y = 0.00651x + 0.00608$ ($r^2 = 0.9976$)			$y = 0.00602x + 0.00386$ ($r^2 = 0.9978$)					

M: mean of calculated concentration, Acc: Accuracy, Prec: Precision, n: number of replicates, RSD: relative standard deviation, QC: quality control sample. [a] n = 5. [b] Number of samples (n) for 2.5 pM, 5 pM, QC 2.5 pM, QC 100 pM, QC 1000 pM: n = 36; 10 pM – 2500 pM: n = 18; QC 10 pM: n = 35.

3.3. Third Publication

Simultaneous Multiple MS Binding Assays for the Dopamine, the Norepinephrine, and the Serotonin Transporter

3.3.1. Summary of the Results

MS Binding Assays have been successfully established as an alternative for radioligand binding assays. In contrast to the latter, the use of mass spectrometry for the detection and quantification of unlabelled reporter ligands in MS Binding Assays result in various benefits. Besides the fact, that no radioactively labelled compounds are needed, meaning that MS Binding Assays can be performed without considerations about radiation protection regulations and problematic waste disposal, MS Binding Assays offer the possibility to monitor multiple reporter ligands simultaneously. Scintillation counting, used in radioligand binding assays, is hardly suited to distinguish between different radioactive labelled analytes, whereas LC-MS enables the quantification of large numbers of analytes in a single chromatographic run. To make use of this advantage, for each of the targets to be employed in the Simultaneous Multiple MS Binding Assays a selective marker has to be applied. Since this was already considered in the method development of an LC-ESI-MS/MS method for the quantification of D-84, reboxetine, and citalopram, this method can be used for Simultaneous Multiple MS Binding Assays. The aim of this work was to establish Simultaneous Multiple MS Binding Assays for the hDAT, hNET, and hSERT, which enable the performance of simultaneous saturation as well as simultaneous competition experiments.

Simultaneous saturation experiments were performed with a mixture of hDAT, hNET, and hSERT, which was incubated together with the three markers (*R,R*)-D-84, (*S,S*)-reboxetine, and (*S*)-citalopram according to the previously published conditions. As a result, saturation isotherms for each marker at its target could be generated in only one experiment. The affinities of the markers found in these experiments towards their targets (4.01 $\pm$ 0.21 nM for (*R,R*)-D-84 towards hDAT, 2.20 $\pm$ 0.29 nM for (*S,S*)-reboxetine towards hNET, and 0.440 $\pm$ 0.004 nM for (*S*)-citalopram towards hSERT) were in excellent agreement with results from individual saturation experiments, in which only one target and its selective marker were

applied. This demonstrates, that each marker selectively binds towards its target, even in the presence of the other targets and markers.

In simultaneous competition experiments, all three targets were incubated together with the three markers and a test compound. As test compounds, a set of seven monoamine reuptake inhibitors with different selectivity profiles were chosen. The test compound 3-α-bis-(4-fluorophenyl)methoxytropane is a selective dopamine reuptake inhibitor, *rac*-bupropione a selective dopamine and norepinephrine reuptake inhibitor, desipramine and *rac*-nisoxetine are selective norepinephrine reuptake inhibitors, (S)-fluoxetine and sertraline are selective serotonin reuptake inhibitors, while *rac*-indatraline (applied as *rac*-[$^2H_3$]-indatraline) is a non-selective triple reuptake inhibitor. In the simultaneous competition experiments, in which these test compounds were applied, three competition curves – one for each of the three target-marker systems – were obtained. The determined affinities of these test compounds resulting from these experiments were in good agreement with published data generated by an alternative MS Binding Assay and radioligand binding experiments.

The results of the simultaneous saturation as well as competition experiments demonstrate, that Simultaneous Multiple MS Binding Assays can be used to determine the affinity of multiple selective markers at their targets or the affinity of a test compound at several targets reliably in a single experiment. As a result, in comparison to radioligand binding assays and classical MS Binding Assays, Simultaneous Multiple MS Binding Assays offer a much-improved efficiency due to the possibility to characterize the affinity of a test compound at various targets with only one set of samples.

3.3.2. Declaration of Contributions

All binding experiments were performed and evaluated by Angela De Simone and myself. I wrote the manuscript and prepared the graphics and tables, assisted by Georg Höfner and Angela De Simone. Klaus T. Wanner corrected the manuscript.

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FULL PAPER

Simultaneous Multiple MS Binding Assays for the dopamine, the norepinephrine, and the serotonin transporter

Patrick Neiens,[a] Angela De Simone,[b] Georg Höfner,[a] Klaus T. Wanner*[a]

[a] Prof. Dr. K. T. Wanner, ORCID: 0000-0003-4399-1425, P. Neiens, Dr. G. Höfner
Department of Pharmacy – Center of Drug Research
Ludwig-Maximilians-Universität München
Butenandtstr. 5 – 13, 81377 Munich, Germany
E-mail: klaus.wanner@cup.uni-muenchen.de
[b] Dr. A. De Simone
Department for Life Quality Studies
Alma Mater Studiorum – University of Bologna
Corso D'Augusto 237, 47921 Rimini, Italy

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Abstract: In this work, we present label-free, mass-spectrometry based binding assays (MS Binding Assays), targeting the human dopamine, norepinephrine, and serotonin transporters (hDAT, hNET, and hSERT) in simultaneous binding experiments. Using a validated LC-ESI-MS/MS method for quantification of the selective dopamine transporter inhibitor (*R,R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84), the selective norepinephrine transporter inhibitor (*S,S*)-reboxetine, and the selective serotonin reuptake inhibitor (*S*)-citalopram, binding affinities at the three monoamine transporters could be characterized simultaneously in a single binding experiment. The performed simultaneous saturation and competition experiments yielded results that were in good accordance with those determined in MS Binding Assays addressing the monoamine transporters individually. The results obtained from this study underscore the potential of MS Binding Assays for simultaneous affinity determination at different targets which is hardly to accomplish with conventional radioligand binding assays.

Introduction

An important step in the development of new drugs is the identification of compounds interacting with a target of interest.[1,2] Various techniques directed towards determination of the functional activity of a test compound or its affinity at the desired target have been established serving this purpose.[3] As binding of a test compound towards a specific binding site at the target is the essential prerequisite for functional activity, characterization of binding affinity is a more direct approach in comparison to functional assays.[4] A broad spectrum of detection principles has been successfully exploited for affinity determination of potential ligands and deduction of corresponding structure-affinity relationships at the beginning of a drug development process.[5–11] Not all of these techniques, however, are equally well suited for each target. This is especially true for membrane-bound proteins, comprising G protein-coupled receptors (GPCRs), ion channels, and transport proteins for example, that belong to the most important drug targets addressed in current therapeutic approaches.[12,13] Many highly sophisticated detection principles are hardly applicable to these targets, as membrane-bound proteins are typically difficult to express in large scale and also difficult to purify without loss of function. For affinity determination of potential ligands at membrane proteins, radioligand binding assays have proven to be an efficient tool.[4,14] Although developed already in the 1970s, they are still the gold standard to characterize affinity of ligands to membrane–bound targets, such as GPCRs, ion channels or transport proteins.[6,15] To perform radioligand binding assays, a suitable target preparation (e.g. a membrane preparation containing the desired membrane protein) has just to be incubated with the radioligand (i.e. the radio-labeled reporter ligand). In the conventional setup, incubation is stopped by separation of the unbound radioligand from the bound radioligand (i.e. from the radioligand-target complexes), which is typically achieved by filtration or centrifugation.[4] After this, the radioactivity of the bound radioligand can be quantified with high sensitivity by radiometric methods. This experimental setup allows determination of the binding affinity of the radioligand towards the target in so-called saturation experiments and characterization of binding affinities of test compounds, which are competing with the radioligand for its specific binding site at the target, in so-called competition experiments and furthermore, kinetic experiments.[4] As an alternative to radioligand binding assays, MS Binding Assays have been developed that do, in contrast to radioligand binding assays, not require a radio-labeled reporter ligand. MS Binding Assays use instead of a radioligand a nonlabeled reporter ligand, which is often referred to as MS Marker or simply marker. MS Binding Assays follow for the most part the general setup of radioligand binding assays, using either a filtration or centrifugation step for separation of the marker-target complexes from the unbound marker. In contrast to the workflow common for radioligand binding assays, MS Binding Assays require an additional step, the liberation of the bound marker from the target prior to analysis by LC-MS (see figure 1 where the complete procedure of MS Binding Assays is explained in a scheme). This is typically achieved by denaturation of the target protein and subsequent elution of the formerly bound marker by an organic solvent. According to this strategy MS Binding Assays for several neurotransmitter receptors and transporters could be established in the last years.[16–20]

The use of mass spectrometry (MS) for the detection of the reporter ligand offers some important benefits in comparison to detection by radioactivity. Since a radioactive label is not necessary for the reporter ligands used in MS Binding Assays, binding experiments with native, i.e. nonlabeled compounds can

be performed. This means that issues inherently coupled with the use of radioactivity, such as obligatory safety precautions, strict regulations issued by authorities, or disposal of radioactive waste – to name just a few – become irrelevant when nonlabeled reporter ligands quantified by means of MS are used. An additional fundamental benefit of MS Binding Assays results from MS as the detection principle in comparison to measurement of radioactivity, the latter of which is typically accomplished by liquid scintillation counting (LSC). LSC provides high sensitivity, but no selectivity with respect to discrimination between different radioligands, labeled with the same radioisotope (that is typically tritium). To enable quantification of different radioligands present in one sample, different radioisotopes would have to be employed for labeling or alternatively, the different radioligands would have to be separated by an additional analytical technique (e.g. by chromatography) prior to quantification. As both approaches cause considerable additional expenses, it is not attractive to perform radioligand binding experiments targeting different targets simultaneously in a single binding experiment. In MS Binding Assays, LC-MS/MS is used for quantification of the reporter ligand that provides besides high sensitivity also excellent selectivity, due to its capability to differentiate all analytes that differ in their m/z ratios of precursor or fragment ions or in their chromatographic retention behavior. Therefore, LC-MS/MS allows quantification of an enormous number of different analytes simultaneously in the same sample in a single run. This means MS Binding Assays offer excellent conditions to perform multiple binding experiments in which binding affinities at different targets are characterized simultaneously in one and the same binding sample when different reporter ligands with sufficient selectivity for the individual targets are employed. This strategy termed “Simultaneous Multiple MS Binding Assays” has been recently introduced in a study by Schuller et al. where it was successfully applied to perform saturation as well as competition experiments simultaneously at D_1 and D_2 dopamine receptors.[21] Simultaneous Multiple MS Binding Assays are of particular value when it is necessary to characterize binding affinities of test compounds at various targets as it is the case, when for example several related targets contribute to the intended therapeutic effect or subtype selectivity has to be determined.

Such a scenario is given in the development of drug candidates addressing monoamine transporters, namely the dopamine (DAT), the norepinephrine (NET), and the serotonin transporter (SERT), which have an important function in the control of human mood and behavior and belong to the most relevant drug targets for many mental disorders. The relevance of the monoamine transporters for mental disorders finds expression in the catechol hypothesis.[22–24] This widely-accepted hypothesis implies, that an imbalance in the monoaminergic neurotransmitters dopamine (DA), norepinephrine (NE), and serotonin (5-HT) correlates with affective disorders. The importance of affective disorders is demonstrated in a study issued by the world health organization (WHO), which has identified mental disorders, especially depression, as a major burden of disease worldwide. Unipolar depressive disorders are the cause of the third highest healthcare expenditures of disease globally, primarily in middle- and high-income countries. Till 2030, unipolar depressive disorders may even surpass the abundance of the most prevalent diseases, e.g. lower respiratory infections and diarrhea.[25] Since only 60 – 70% of the patients treated against depression respond to the therapy, the search for new antidepressants is still a relevant topic in contemporary drug discovery.[26] The situation is even aggravated by the fact that, due to the complex impact of the different monoamines on the patient’s mood and behavior, the profile of binding affinities towards all three monoamine transporters is decisive for the intended therapeutic effects, but also for the side effects.

As Simultaneous Multiple MS Binding Assays can be a highly efficient tool to characterize binding affinities of test compounds at multiple targets simultaneously in a single binding experiment, the application of this strategy seems to be straightforward in this case. Such Simultaneous Multiple MS Binding Assays at DAT, NET, and SERT would clearly surpass the efficiency of conventional “individually performed” radioligand binding assays, which are commonly used for this purpose, by considerably reducing the number of binding samples required for affinity determination and thus - in total - also reduce expenditure.

Recently, an LC-ESI-MS/MS method for the quantification of (*R*,*R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R*,*R*)-D-84, (*R*,*R*)-**1**), (*S*,*S*)-reboxetine ((*S*,*S*)-**2**), (*S*)-citalopram ((*S*)-**3**) was developed and applied to establish MS Binding Assays individually addressing the human dopamine transporter (hDAT), the human norepinephrine transporter (hNET), and the human serotonin transporter (hSERT, see figure 1 and 2), respectively.[27] Sufficient selectivity of individual markers for the desired targets is a crucial requirement for the approach of Simultaneous Multiple MS Binding Assays, as it must be guaranteed that each of the different markers labels only one defined target in presence of other targets. As the above mentioned markers are claimed to be highly selective for their corresponding targets [18,28–30] and can, furthermore, be quantified with the sensitivity required in MS Binding Assays in a single LC-MS/MS run, the decisive prerequisites for Simultaneous Multiple MS Binding Assays addressing these targets appear to be fulfilled. It was thus the aim of the present study to establish Simultaneous Multiple MS Binding Assays for the three monoamine transporters based on the aforementioned LC-ESI-MS/MS quantification method for (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) previously published to prove their reliability and to demonstrate their high efficiency. Therefore, saturation and competition assays should be carried out with all three markers and the three targets (hDAT, hNET, and hSERT) in one binding experiment. To proof the reliability of Simultaneous Multiple MS Binding Assays, the results (i.e. the affinities) determined thereby should be compared with those obtained in individual MS Binding Assays and radioligand binding experiments.

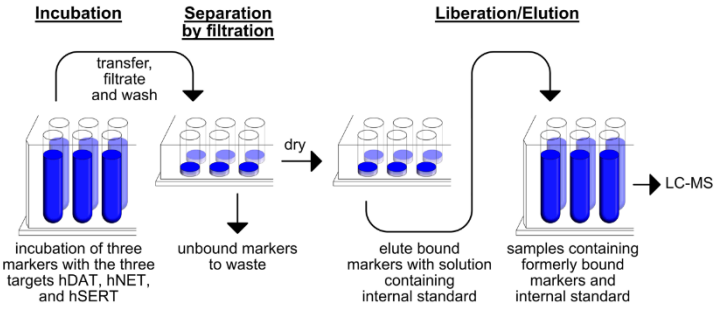

Figure 1. Workflow of the developed MS Binding Assays

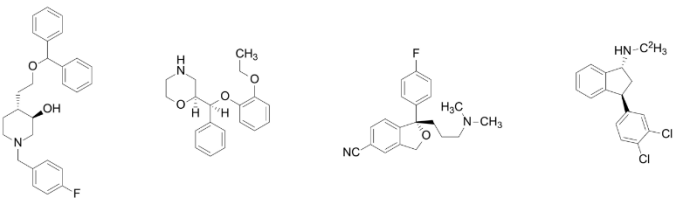

Figure 2. Structures of markers and the inhibitor employed for determination of nonspecific binding in MS Binding Assays, as published previously.[27]

[a] Deuterated indatraline was used as racemate, though only one enantiomer is depicted in the figure.

Results and Discussion

Calibration

In any case samples from binding experiments were analyzed together with a set of calibration standards and QC samples prepared at the day of the assay. A full validation of the LC-ESI MS/MS quantification method according to the CDER guideline for bioanalytical method validation[31] was performed and published previously.[27] Each subsequent calibration was evaluated pursuant to the CDER guideline to confirm the validation. For Simultaneous MS Binding Assays, the concentration of target material in the incubation samples had to be increased, but this change had no effect on linearity, accuracy, and precision of the LC-ESI-MS/MS quantification method. The results of all sets of calibration standards and QC samples complied with the limits set in the CDER guideline (see supporting information for results of the validation).

Simultaneous saturation assays

Binding affinities for (*R*,*R*)-D-84 ((*R*,*R*)-**1**) towards hDAT, (*S*,*S*)-reboxetine ((*S*,*S*)-**2**) towards hNET, and (*S*)-citalopram ((*S*)-**3**) towards hSERT have been characterized in individual saturation experiments published recently.[27] Simultaneous Multiple MS Binding Assays offer the possibility to determine the binding affinities of these three markers at their targets in a single experiment. We started to test the feasibility of this approach in the case of the three monoamine transporters with simultaneous saturation experiments. In these simultaneous saturation experiments, all three markers were incubated together with all three targets. Conditions of the simultaneous binding experiments were identical as for the individual experiments described previously.[27] In short, the three markers in 32 concentrations were incubated together with the three targets for 2 h at 20 °C before the target-marker complexes were separated from the nonbound markers by a filtration step. For simultaneous saturation experiments, the amount of each individual target was the same as in individual saturation experiments leading to higher total amount of membrane material in the binding samples in comparison with the individual binding experiments. To determine the nonspecific binding (NSB), the same set of samples was incubated together with *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) in excess to block specific binding sites as it was done recently (Neiens et al.[27]). After the elution of the bound markers and their quantification using LC-ESI-MS/MS, the specific binding (SB) was calculated by subtraction of the nonspecific binding from the total binding (TB, SB = TB – NSB). The simultaneous saturation experiments resulted in Kd values of 4.01 nM for the binding of (*R*,*R*)-D-84 ((*R*,*R*)-**1**) towards hDAT, 2.20 nM for (*S*,*S*)-reboxetine ((*S*,*S*)-**2**) towards hNET and 440 pM for (*S*)-citalopram ((*S*)-**3**) towards hSERT (see table 1 and figure 3). With the binding affinities for all three markers towards their individual targets agreeing excellently with those obtained from individual saturation experiments, it could be unambiguously demonstrated that simultaneous saturation experiments performed according the approach of Simultaneous Multiple MS Binding Assays lead to reliable results. Thus, each marker is able to label its target selectively, even in presence of the other markers and targets in the performed simultaneous saturation experiments. Since the membrane material employed for the simultaneous binding experiments was obtained by pooling of the individual membrane preparations prior to the protein determination, the Bmax values are given as bound marker in relation to the total amount of protein in the binding samples. According to the proportion the membrane preparation (hDAT, hNET, and hSERT) have been employed in the combined ("pooled") target preparation, the monoamine transporters hDAT, hNET, and hSERT were estimated to be present roughly in a ratio of about 1:1:2. To compare the Bmax values determined in the Simultaneous MS Binding Assays to the ones obtained in individual MS Binding Assays, it is necessary to calculate the Bmax values for each target transporter preparation individually instead of the Bmax values for the combined target preparation. Taking into account the proportion of the individual target membrane preparation in the binding sample, the Bmax for binding of (*R*,*R*)-D-84 ((*R*,*R*)-**1**) to hDAT in Simultaneous MS Binding Assays can be estimated approximately to 110 pmol/mg protein, being twice as high, as determined in individual MS Binding Assays. Bmax values obtained in Simultaneous MS Binding Assays for the binding of (*S*,*S*)-reboxetine ((*S*,*S*)-**2**) to hNET would be approximately 33 pmol/mg protein and for the binding of (*S*)-citalopram ((*S*)-**3**) to hSERT a Bmax value approximately 11 pmol/mg protein, both of which are in excellent agreement with results obtained from individual MS Binding Assays.

Table 1. Results from the saturation experiments performed simultaneously and those from individual MS Binding Assays for comparison purposes. B_{max} values in this table are related to the total protein content in the binding samples. As discussed above for comparison of the B_{max} values from this study with those in individual binding assays requires their calculation based on the individual target material employed in the Simultaneous Multiple MS Binding Assay. Results are given as mean ± SEM, n = 3, except for [a] n = 4 [27]

	Simultaneous Multiple MS Binding Assays		Individual MS Binding Assays	
	K_d in nM	B_{max} in pmol/mg protein	K_d in nM	B_{max} in pmol/mg protein
(*R*,*R*)-D-84 ((*R*,*R*)-**1**) to hDAT	4.01 ± 0.21	27.6 ± 7.7	3.66 ± 1.35[a]	57.9 ± 8.4[a]
(*S*,*S*)-reboxetine ((*S*,*S*)-**2**) to hNET	2.20 ± 0.29	8.22 ± 1.60	3.06 ± 0.46	31.6 ± 8.9
(*S*)-citalopram ((*S*)-**3**) to hSERT	0.440 ± 0.0038	5.26 ± 1.40	0.411 ± 0.0026	10.1 ± 1.7

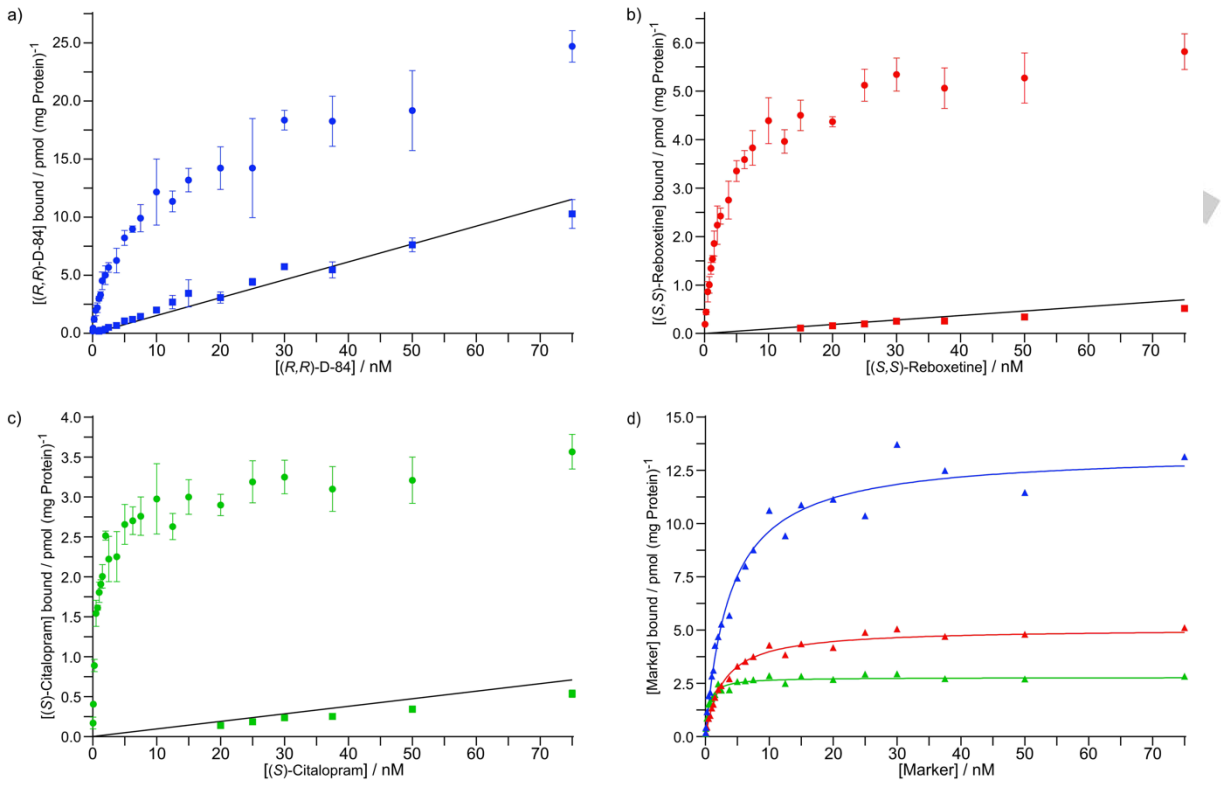

Figure 3. Simultaneous saturation experiments for (*R,R*)-D-84 ((*R,R*)-**1**) towards hDAT (blue symbols), (*S,S*)-reboxetine ((*S,S*)-**2**) towards hNET (red symbols) and (*S*)-citalopram ((*S*)-**3**) towards hSERT (green symbols). a), b), and c) Means ± SD (n = 3) of the total binding (circles) and the nonspecific binding in the presence of 10 µM *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) (squares, linear regression of the nonspecific binding is shown as a straight line). d) Means of specific binding (triangular symbols) and resulting saturation isotherms derived from the results shown in a), b), and c). Only data points up to a free marker concentration of 75 nM are shown. Data points of higher free marker concentrations do not change the plateau of the saturation isotherms.

Competition experiments

In competition experiments, the inhibitory constant of a test compound towards a target in presence of a marker can be determined. Simultaneous MS Binding Assays enable the determination of the affinity of one test compound at multiple target proteins in one experiment. To carry out simultaneous competition experiments, the procedure of the established simultaneous saturation experiments was adapted. For competition experiments, the markers are applied at a fixed concentration in all binding samples, whilst the competitor is added in multiple concentration levels. Commonly, the marker concentration is chosen near the K_d value of the marker.[32] In recently published MS Binding Assays for the three monoamine transporters, the selectivity of (*R,R*)-D-84 ((*R,R*)-**1**) was characterized as 1:9:84 (hDAT:hNET:hSERT).[18] Due to this low selectivity of (*R,R*)-D-84 ((*R,R*)-**1**) for hDAT, its concentration was chosen to be at one fourth of the K_d values, while concentrations of (*S,S*)-reboxetine ((*S,S*)-**2**) and (*S*)-citalopram ((*S*)-**3**) were chosen to be ten times the K_d values of these markers, thus 1 nM (*R,R*)-D-84 ((*R,R*)-**1**), 20 nM (*S,S*)-reboxetine ((*S,S*)-**2**), and 4 nM (*S*)-citalopram ((*S*)-**3**) were applied in the incubation samples. The use of these marker concentrations, the lower concentration for (*R,R*)-D-84 ((*R,R*)-**1**) and higher concentrations for (*S,S*)-reboxetine ((*S,S*)-**2**) and (*S*)-citalopram ((*S*)-**3**) were expected to ensure that hNET and hSERT are almost exclusively labeled by their respective markers, but not by (*R,R*)-D-84 ((*R,R*)-**1**).
Seven monoamine reuptake inhibitors with different selectivity profiles were chosen as test compounds for the competition experiments. The test compounds (see figure 1 and 4) were added to the binding samples in 15 concentration levels (20 concentration levels for sertraline (**10**), due to its large difference in affinity towards hNET and hSERT) in a range around published binding affinities, that was necessary to obtain competition curves for the marker displacement at all three targets (for concentrations of each compound, see “Competition assays” in the experimental section). All other conditions of the binding assay were maintained according to the procedure described above for the simultaneous saturation assays. As for the previously published saturation experiments, nonspecific binding was determined in samples where the specific binding sites were blocked by *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**), which was added in a large excess, for eight marker concentrations between 12.5 nM and 200 nM. Based on the concentrations of nonspecific binding for these marker concentrations, a linear regression was obtained, which was then used to calculate the nonspecific binding for marker concentrations used in the binding samples of the competition experiments.
After determination of the specific binding of three markers in the binding samples (for detail see “Data evaluation” in the experimental section), three competition curves – one for each target – were generated for a single simultaneous binding experiment for every test compound (see figure 5 for example). This reflects the major advantage of Simultaneous MS Binding Assays in comparison to radioligand binding assays and the previously published MS Binding Assays addressing DAT, NET, and SERT.[18] They allow to characterize binding affinities of test compounds at multiple targets in one experiment which results in a higher efficiency due to lower numbers of samples to be prepared and analyzed. The results for the investigated compounds are summarized in table 2 and discussed in detail in the following paragraph.
3-α-Bis-(4-fluorophenyl)methoxytropane (**5**), claimed as a selective dopamine reuptake inhibitor, was characterized in competitive Simultaneous MS Binding Assays with pK_i values of

7.43 at hDAT, 6.84 at hNET, and 5.73 at hSERT (see figure 5 a and table 2). These results can be best compared with published data, obtained from individual binding experiments with the previously established MS Binding Assay, which uses (1*R*,3*S*)-indatraline as a marker for all three targets and the same target material under assay conditions which differ only in the incubation temperature from the Simultaneous MS Binding Assays performed in the present study. In comparison to these (1*R*,3*S*)-indatraline MS Binding Assays, the results for the competition at hDAT (pK_i of 7.43 in Simultaneous MS Binding Assays vs. 7.52 in (1*R*,3*S*)-indatraline MS Binding Assays) and hSERT (pK_i of 5.73 in Simultaneous MS Binding Assays vs. 5.71 in (1*R*,3*S*)-indatraline MS Binding Assays) are in excellent agreement (see table 2). In the Simultaneous MS Binding Assays, the determined affinity towards hNET with a pK_i of 6.84 is slightly lower than the affinity (pK_i of 7.22) resulting from the (1*R*,3*S*)-indatraline MS Binding Assays.[18] In other references in literature, 3-α-Bis-(4-fluorophenyl)methoxytropane (**5**) is described with pK_i values of 7.93 at DAT, 5.07 at NET and 5.61 at SERT, determined by [^{3}H]-(–)-2-*β*-carbomethoxy-3-*β*-(4-fluorophenyl)tropane ([^{3}H]-WIN 35,428), [^{3}H]-desmethylimipramine and [^{3}H]-citalopram binding towards rat caudate putamen.[33,34] For the binding at DAT and SERT, the pK_i values determined in Simultaneous MS Binding Assays and radioligand binding experiments are in good agreement. The binding affinity of 3-α-bis-(4-fluorophenyl)methoxytropane (**5**) towards NET in Simultaneous MS Binding Assays was considerably higher than in the radioligand binding assays. This is likely to be due to the different incubation conditions and the different target materials used in both binding assays.

For *rac*-bupropione (*rac*-**6**), Simultaneous MS Binding Assays resulted in pK_i values of 5.83 at hDAT, 5.20 at hNET, and 5.15 at hSERT (see table 2). These results are in good accordance with pK_i values of 5.59 at hDAT, 5.08 at hNET and 4.83 at hSERT, determined with (1*R*,3*S*)-indatraline MS Binding Assays.[18] In radioligand binding experiments, slightly lower binding affinities with pK_i values of 5.58 at hDAT, 4.68 at hNET, and 4.46 at hSERT were determined showing a higher selectivity for hDAT, but the same rank order of potencies.[35]

Desipramine (**7**), a selective noradrenaline reuptake inhibitor, was characterized with pK_i values of 5.09 at hDAT, 8.92 at hNET, and 7.69 at hSERT in Simultaneous MS Binding Assays. Compared to the results from the (1*R*,3*S*)-indatraline MS Binding Assays (8.35 at hNET, and 6.80 in (1*R*,3*S*)-indatraline MS Binding Assays at hSERT), the pK_i values at hDAT and hNET are in good accordance whereas the pK_i value at hSERT is showing a lower affinity towards this target when determined in (1*R*,3*S*)-indatraline MS Binding Assays (see table 2).[18] Furthermore, the affinities determined in Simultaneous MS Binding Assays are in excellent agreement with pK_i values of 5.50 at hDAT, 9.08 at hNET and 7.75 at hSERT in radioligand binding assays using [^{3}H]-WIN 35,428, [^{3}H]-nisoxetine, and [^{3}H]-imipramine binding towards hDAT, hNET, and hSERT expressed in HEK293 cells reported in literature.[36]

(*S*)-fluoxetine ((*S*)-**8**) is the eutomer of the selective serotonin reuptake inhibitor fluoxetine. It was characterized in Simultaneous MS Binding Assays with pK_i values of 5.53 at hDAT, 6.10 at hNET, and 9.48 at hSERT (see figure 5 b and table 2). In (1*R*,3*S*)-indatraline MS Binding Assays pK_i values of 4.60 at hDAT, 5.99 at hNET, and 8.50 at hSERT were determined.[18] Though the pK_i values derived from Simultaneous MS Binding Assays are somewhat higher than those from (1*R*,3*S*)-indatraline MS Binding Assays, they are in good accord with the latter with regard to the subtype selectivity at the three transporters. Only a few other studies characterized the binding affinities of (*S*)-fluoxetine. One of these publications uses hNET and hSERT expressed in COS-7 cells to determine the binding affinities of (*S*)-fluoxetine which amounted to a pK_i value of 5.88 towards hNET and 8.52 towards hSERT in competition with [^{125}I]-(–)-2*β*-carbomethoxy-3*β*-(4-iodophenyl)tropane ([^{125}I]-*β*-CIT).[37] These affinities deviate only slightly from the ones determined by Simultaneous MS Binding Assays.

Since it had been used as a blocking reagent for the determination of the nonspecific binding in all Simultaneous MS Binding Assays, the affinities of *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) were also characterized in competition experiments. As the deuteration of indatraline is unlikely to lead to a considerable change of binding affinities, the results of the Simultaneous MS Binding Assays were compared to binding affinities obtained for nondeuterated, indatraline. In this context, it should be mentioned that only enantiopure indatraline had been used in the (1*R*,3*S*)-indatraline MS Binding Assays, this being the first time, that binding affinities of *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) towards monoamine transporters are characterized. So there are no reference values from MS Binding Assays (for deuterated as well as racemic indatraline) and from radioligand binding assays in general (for deuterated indatraline) for comparison. In Simultaneous MS Binding Assays, pK_i values of 8.34 at hDAT, 9.16 at hNET and 9.91 at hSERT were determined for *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**, see table 2). These results match well with pK_i values of 8.40 at hDAT, 8.70 at hNET and 9.22 at hSERT, found for the binding of racemic indatraline at rat brain homogenates as target material in radio ligand binding assays with [^{3}H]-WIN 35,428, [^{3}H]-nisoxetine, and [^{3}H]-citalopram as radioligands.[38] In other publications, a pK_i value of 8.52 for the binding at DAT in rat striatum, determined in competition with [^{3}H]-WIN 35,428, as well as a pK_i value of 9.89 at SERT in [^{3}H]-paroxetine binding at rat frontal cortical membranes and a pK_i value of 9.64 again at SERT in [^{3}H]-3-amino-4-(2-dimethylaminomethylphenylsulfanyl)-benzonitrile ([^{3}H]-DASB) binding at rat brain cortex material were determined, which are in good correspondence with the results obtained in this study in Simultaneous MS Binding Assays.[39–41]

In Simultaneous MS Binding Assays, the selective norepinephrine reuptake inhibitor *rac*-nisoxetine (*rac*-**9**) has been characterized with pK_i values of 5.49 at hDAT, 8.46 at hNET, and 7.37 at hSERT (see figure 5 c and table 2). These results are in good agreement with pK_i values of 5.67 at hDAT, 7.89 at hNET, and 6.55 at hSERT, determined in (1*R*,3*S*)-indatraline MS Binding Assays and pK_i values of 5.77 at hDAT, 7.49 at hNET, and 7.14 at hSERT, which were obtained in radioligand binding experiments.[18,35] Simultaneous MS Binding Assays show a slightly higher affinity of *rac*-nisoxetine (*rac*-**9**) towards hSERT than the (1*R*,3*S*)-indatraline MS Binding Assays, but are in good accordance to the results from radioligand binding assays.

A similar situation concerning the concordance of the characterized binding affinities could be observed for the selective SERT inhibitor sertraline (**10**) as a test compound in competition experiments. Simultaneous MS Binding Assays revealed pK_i values of 7.19 at hDAT, 6.66 at hNET, and 10.44 at hSERT, whereas (1*R*,3*S*)-indatraline MS Binding Assays had yielded the pK_i values of 6.61 at hDAT, 6.23 at hNET, and 8.72 at hSERT (see table 2).[18] Similarly, the binding affinities from radioligand binding experiments, using [^{3}H]-WIN 35,428, [^{3}H]-nisoxetine, and [^{3}H]-imipramine as radioligands in competition experiments towards hDAT, hNET, and hSERT expressed in HEK293 cells, yielding pK_i values of 7.60 towards hDAT and 6.38 at hNET are in good agreement with the results from the Simultaneous MS Binding Assay. The binding affinity towards hSERT has been characterized with a pK_i values of 9.54 in radioligand binding assays and is hence slightly lower than in Simultaneous MS Binding Assays.[36] Despite this slight disparity in binding affinity at hSERT, Simultaneous MS Binding Assays show the same selectivity of sertraline (**10**) for hSERT like all of the mentioned studies.

In summary, the pK_i values determined in Simultaneous MS Binding Assays for hDAT, hNET, and hSERT are for the most part in good correspondence with the results from the individual (1*R*,3*S*)-indatraline MS Binding Assays (see figure 6), indicating that Simultaneous MS Binding Assays are as reliable as individual MS Binding Assays for the characterization of binding affinities. A correlation of the pK_i values determined in Simultaneous (y-axes)

and with those from the (1*R*,3*S*)-indatraline MS Binding Assays (x-axes) all showing a linear relationship is illustrated in figure 6. This correlation was generated for the competition of the test compounds for each of the targets. The three graphs obtained are characterized by the following equations and coefficients of determination (R^2): y = 0.8414x + 1.164, R^2 = 0.8219 for the competition at hDAT, y = 1.106x - 0.4861, R^2 = 0.9421 for the competition at hNET and y = 1.331x - 1.474, R^2 = 0.9787 for the competition at hSERT. As apparent from these diagrams, Simultaneous MS Binding Assays tend to provide higher pK_i values than (1*R*,3*S*)-indatraline MS Binding Assays, especially for hSERT. A possible explanation for this finding could be that the binding sites labeled by (*R,R*)-D-84 ((*R,R*)-**1**) at hDAT, by (*S,S*)-reboxetine ((*S,S*)-**2**) at hNET and by (*S*)-citalopram ((*S*)-**3**) at hSERT might be slightly different to those addressed by (1*R*,3*S*)-indatraline at the corresponding targets.

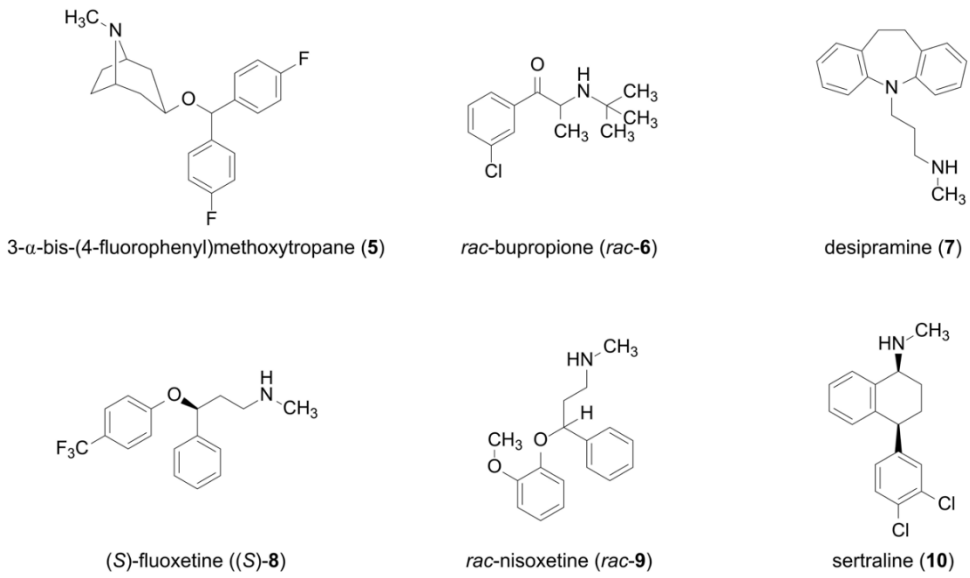

Figure 4. Structures of test compounds applied as competitors in simultaneous competition experiments

Table 2. Affinity (pK_i) and selectivity (K_i ratios hDAT:hNET:hSERT) values of inhibitors towards hDAT, hNET, and hSERT determined in competitive Simultaneous Multiple MS Binding Assays using (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) as markers with published data from (1*R*,3*S*)-indatraline MS Binding Assays for comparison. [18] [a] Simultaneous Multiple MS Binding Assays: Affinity values (pK_i; mean ± SEM, n = 4 – 5) determined in Simultaneous Multiple MS Binding Assays using (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) as markers. [b] (1*R*,3*S*)-indatraline MS Binding Assays: Affinity values (pK_i; mean ± SEM, n = 3 – 7) determined in MS Binding Assays using (1*R*,3*S*)-indatraline ((1*R*,3*S*)-**4**) as marker. [c] K_i ratios showing selectivity of the test compound.

Compound		pK_i						K_i-ratio determined in Simultaneous MS Binding Assays (hDAT:hNET:hSERT)[c]
No	Name	Simultaneous Multiple MS Binding Assays[a]			(1*R*,3*S*)-indatraline MS Binding Assays[b]			
		hDAT	hNET	hSERT	hDAT	hNET	hSERT	
5	3-α-bis-(4-fluorophenyl)methoxytropane	7.43 ± 0.10	6.84 ± 0.03	5.73 ± 0.04	7.52 ± 0.04	7.22 ± 0.02	5.71 ± 0.05	1:4:47
rac-**6**	*rac*-bupropione	5.83 ± 0.06	5.20 ± 0.05	5.15 ± 0.05	5.59 ± 0.12	5.08 ± 0.09	4.83 ± 0.10	1:4:5
7	desipramine	5.09 ± 0.15	8.92 ± 0.13	7.69 ± 0.15	5.16 ± 0.05	8.35 ± 0.03	6.80 ± 0.11	7347:1:18
(*S*)-**8**	(*S*)-fluoxetine	5.53 ± 0.09	6.10 ± 0.09	9.48 ± 0.11	4.60 ± 0.08	5.99 ± 0.06	8.50 ± 0.01	8651:2343:1
rac-[$^2H_3$]-**4**	*rac*-[$^2H_3$]-indatraline	8.34 ± 0.15	9.16 ± 0.09	9.91 ± 0.10				41:5:1
rac-**9**	*rac*-nisoxetine	5.49 ± 0.15	8.46 ± 0.02	7.37 ± 0.05	5.67 ± 0.03	7.89 ± 0.06	6.55 ± 0.06	1099:1:12
10	sertraline	7.19 ± 0.23	6.66 ± 0.04	10.44 ± 0.09	6.61 ± 0.05	6.23 ± 0.05	8.72 ± 0.08	2455:5756:1

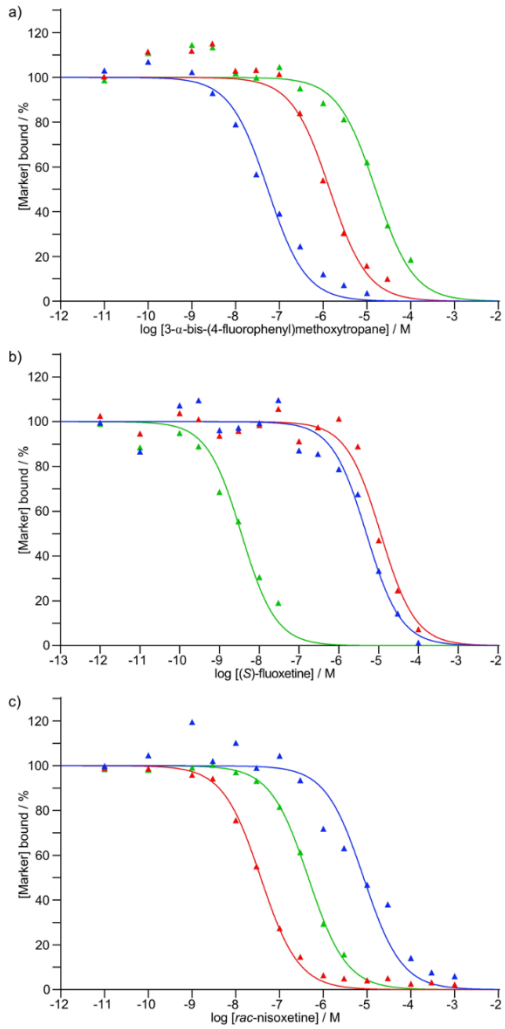

Figure 5. Results for three compounds investigated from simultaneous competition experiments. Diagrams show the specific binding given as means (n = 3) and the resulting competition curve of (*R,R*)-D-84 ((*R,R*)-1) towards hDAT (blue), (*S,S*)-reboxetine ((*S,S*)-2) towards hNET (red), and (*S*)-citalopram ((*S*)-3) towards hSERT (green) with a) 3-α-bis-(4-fluorophenyl)methoxytropane (*rac*-6), b) (*S*)-fluoxetine ((*S*)-8), and c) *rac*-nisoxetine (*rac*-9) as competitor.

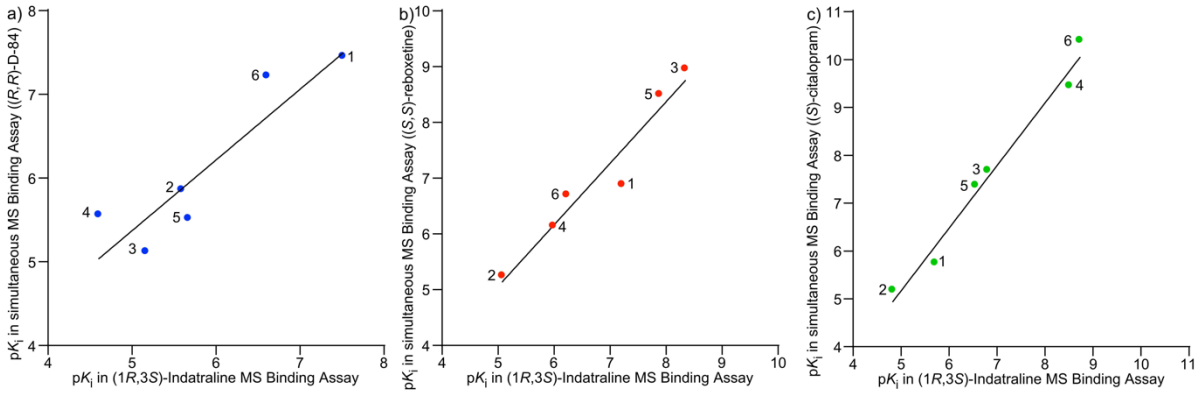

Figure 6. Correlations of pK_i values for six monoamine transporter inhibitors determined in Simultaneous Multiple MS Binding Assays using (*R,R*)-D-84 ((*R,R*)-**1**), (*S,S*)-reboxetine ((*S,S*)-**2**), and (*S*)-citalopram ((*S*)-**3**) as markers (y-axes) and those determined in MS Binding Assays using (1*R*,3*S*)-indatraline ((1*R*,3*S*)-**4**) as marker (x-axes) [18]. Shown are correlations of pK_i values at a) hDAT (y = 0.8414x + 1.164, R^2 = 0.8219), b) hNET (y = 1.106x - 0.4861, R^2 = 0.9421), and c) hSERT (y = 1.331x - 1.474, R^2 = 0.9787). Data points represent mean values as given in table 2.

Conclusions

In this work, we demonstrated that MS Binding Assays have the potential for the simultaneous characterization of binding affinities towards multiple targets, in this case, for the monoamine transporters DAT, NET, and SERT. With the previously established and validated LC-ESI-MS/MS quantification method, the bound concentrations of the three selective markers (*R,R*)-D-84 ((*R,R*)-**1**) addressing DAT, (*S,S*)-reboxetine ((*S,S*)-**2**) addressing NET, and (*S*)-citalopram ((*S*)-**3**) addressing SERT could be determined in a single LC-ESI-MS/MS run. Based on this quantification method Simultaneous Multiple MS Binding Assays were established in which all three markers were incubated together with all three targets in a single binding experiment. The simultaneous saturation experiments performed this way yielded the corresponding three saturation isotherms at once. The K_d values for each marker calculated therefrom were in good agreement with those obtained for the corresponding binding experiments addressing the targets individually. Additionally, we could show that this approach provides also the possibility for simultaneous affinity profiling of test compounds in competition experiments. The affinities exemplarily determined for a set of seven known monoamine reuptake inhibitors (consisting of selective as well as nonselective ones) were as well in a good accordance with published data.

Clearly, the established Simultaneous Multiple MS Binding Assays, addressing the monoamine transporters DAT, NET, and SERT, distinctly surpass the efficiency of radioligand binding assays predominantly used for this purpose up to now, though there is still some potential to improve the established setup especially by employing a more favored DAT inhibitor - especially with higher selectivity and less of the recently described analytical drawbacks.[27] Hence, the present study may be an important contribution for a more efficient affinity profiling at monoamine transporters that is still a highly important task in the development of drug candidates for mental disorders such as depression.

Experimental Section

Chemicals: LC-MS grade acetonitrile and water were obtained from VWR Prolabo (Darmstadt, Germany), water for incubation buffers and washing buffers was prepared by distillation of demineralized water (reverse osmosis) and filtration through a 0.45 µm filter. Ammonium formate was bought in mass spectrometry grade from Fluka (Teufelskirchen, Germany). (*S*)-citalopram ((*S*)-**3**) oxalate was bought from TCI (Eschborn, Germany), racemic [$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**) oxalate from Alsachim (Illkirch, France), racemic [$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**) mesylate was obtained from TRC (North York, ON, Canada), 3-α-bis-(4-fluorophenyl)methoxytropane hydrochloride from Tocris (Wiesbaden-Nordenstadt, Germany), hydrochlorides of desipramine and (*S*)-fluoxetine from Sigma-Aldrich (Taufkirchen, Germany), *rac*-bupropione from Alfa Aesar (Karlsruhe, Germany), *rac*-nisoxetine from Biotrend (Cologne, Germany), and sertraline hydrochloride from abcr GmbH (Karlsruhe, Germany), each in the highest commercially available quality. (*R,R*)-4-(2-Benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84, (*R,R*)-**1**) was synthesized and purified in-house [29,42] and racemic reboxetine (*rac*-**2**) was extracted from Edronax tablets (4 mg, Pfizer Europe, Kent, UK) which was further used to perform a semipreparative CSP-HPLC to obtain (*S,S*)-reboxetine ((*S,S*)-**2**).[27] Enantiopurities of ≥ 99.8% *ee* for (*R,R*)-D-84 ((*R,R*)-**1**) and 99.3% *ee* for (*S,S*)-reboxetine ((*S,S*)-**2**) were determined by CSP-HPLC.[27,42] Racemic [$^2H_4$]-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol (*rac*-[$^2H_4$]-D-84, *rac*-[$^2H_4$]-**1**, for the sake of simplicity, the racemic mixture of (*R,R*)-[$^2H_4$]-D-84 and (*S,S*)-[$^2H_4$]-D-83 ((*S*)-[$^2H_4$]-83 is the enantiomer of (*R,R*)-[$^2H_4$]-D-84 according to the denotation in literature[43,44]) is referred to as *rac*-[$^2H_4$]-D-84 throughout this work. synthesis of *rac*-[$^2H_4$]-D-84 is to be published soon) and racemic [$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) were synthesized in-house.[27] Polyethylenimine (PEI) solution (50% in H_2O) was obtained from Fluka, sodium chloride from Bernd Kraft (Duisburg, Germany), potassium chloride and dimethylacetamide (DMA) from Sigma-Aldrich (Taufkirchen, Germany), HEPES and roti-quant (5 x concentrated) for determination of total protein in membrane preparations from Roth (Karlsruhe, Germany). All percentages and ratios are specified as (v/v) if not indicated otherwise.

Instrumentation: MS Binding Assays were performed with an API 5000 triple quadrupole mass spectrometer, equipped with a TurboV ESI ion source (AB Sciex, Darmstadt, Germany). The mass spectrometer was coupled to an Agilent 1200 series HPLC system (Agilent, Waldbronn, Germany), consisting of a vacuum degasser, a binary pump and a column oven in combination with a HTS-Pal autosampler (50 µL syringe, 50 µL sample loop, CTC Analytics, Zwingen, Switzerland). As a stationary phase, a Luna PFP(2) column (50 mm x 2.0 mm, 3 µm, Phenomenex, Aschaffenburg, Germany) was used.

Solutions: Separately prepared stock solutions of the markers and internal standards with a concentration of 1 mM in H_2O/DMA (80/20) as well as stock solutions for the competitors with a concentration of 10 mM in H_2O were stored at -20 °C until the day of the experiment. For binding and calibration samples, equal volumes of marker stock solutions were mixed and diluted with H_2O/DMA (80/20) to working solutions containing all three markers with the 100-fold concentration of the intended final concentration in binding samples or calibration samples. In these working solutions, all three markers had equal concentrations for the use in simultaneous saturation experiments. For competition experiments a 100-fold concentrated working solution with 100 nM (*R,R*)-D-84 ((*R,R*)-**1**), 2 µM (*S,S*)-reboxetine ((*S,S*)-**2**), 400 nM (*S*)-citalopram ((*S*)-**3**) in H_2O/DMA (80/20) was prepared to obtain final concentrations of 1 nM (*R,R*)-D-84 ((*R,R*)-**1**), 20 nM (*S,S*)-reboxetine ((*S,S*)-**2**), and 4 nM (*S*)-citalopram ((*S*)-**3**) when diluted in the binding samples. Competitor stock solutions were diluted to a 100-fold concentrated working solution with respect to the intended final concentration in competition experiments. Equal volumes of internal standard stock solutions were mixed and diluted in acetonitrile to obtain a solution of all three internal standards at a concentration of 100 pM in acetonitrile/water/DMA (99/0.8/0.2). For the calibration, 100-fold concentrated working solutions of all three markers in H_2O/DMA (80/20) were diluted in acetonitrile to 10-fold concentrated solutions and finally diluted with the internal standard solution to obtain solutions containing 1 pM to 2.5 nM of all three markers and 90 pM of all three internal standards. All solutions were prepared in 1.5 mL polypropylene micro tubes (Sarstedt, Nürmbrecht, Germany). As pipet tips, non-coated tips without filter (quality tips without filters, 20 µL neutral, 200 µL yellow, 300 µL neutral, 1000 µL blue and 1250 µL neutral, Sarstedt) were used.

LC-ESI-MS/MS quantification method: Quantification of (*R,R*)-D-84, (*S,S*)-reboxetine and (*S*)-citalopram ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**) was performed with the LC-ESI-MS/MS method previously described.[27] In short, a Luna PFP(2) column was used in combination with acetonitrile/ammonium formate buffer (0.75 mg/mL; 85/15) at a flow rate of 800 µL/min as mobile phase. The column oven temperature was set to 20 °C, the injection volume was 30 µL and the eluate of the first 0.4 min of each chromatographic run was directed to waste. Using pneumatically assisted ESI in positive polarity, mass transitions for each marker (*m/z* 420.2/167.1 and *m/z* 420.2/109.1 for (*R,R*)-D-84 ((*R,R*)-**1**), *m/z* 314.1/176.2 and *m/z* 314.1/91.1 for (*S,S*)-reboxetine ((*S,S*)-**2**), *m/z* 325.2/109.2 and *m/z* 325.2/262.2 for (*S*)-citalopram ((*S*)-**3**)) and internal standard (*m/z* 424.2/167.1 for *rac*-[$^2H_4$]-D-84 (*rac*-[$^2H_4$]-**1**), *m/z* 319.2/176.2 for *rac*-[$^2H_5$]-reboxetine (*rac*-[$^2H_5$]-**2**) and *m/z* 331.2/109.0 for *rac*-[$^2H_6$]-citalopram (*rac*-[$^2H_6$]-**3**)) were acquired in multiple reaction monitoring (MRM) mode.

General procedure for MS Binding Assays: MS Binding Assays were performed according to the procedure previously described[27] with membrane preparations of HEK293 cells, stably expressing hDAT, hNET or hSERT, respectively.[45] For 20 mL of the target preparation containing hDAT, hNET, and hSERT to be used in Simultaneous MS Binding Assays, hDAT, hNET, and hSERT membrane preparation (stored at -80 °C and

thawed at the day of the experiment) were pooled (1 part hDAT, 1 part hNET and 2 parts hSERT) and diluted in 20 mL cold assay buffer (50 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4). The suspension was centrifuged (20 min, 20000 rpm, 4 °C, Sorvall Evolution, SS-34 rotor, Thermo Fisher Scientific, Dreieich, Germany), the supernatant was discarded and the remaining pellet resuspended in 20 mL cold assay buffer. Binding samples for determination of total binding were prepared as triplicates in a polypropylene 96-deep-well plate (1.2 mL, polypropylene, Sarstedt, Nürmbrecht, Germany) by addition of the 100-fold concentrated marker working solution ((*R,R*)-**1**, (*S,S*)-**2**, (*S*)-**3**, see "Solutions") and if needed the 100-fold concentrated competitor working solution (see "Solutions") to assay buffer (reaching a volume of 200 µL per well). Incubation was started by the addition of 50 µL target preparation to each well and carried out for 2 h at 20 °C in a shaking water bath. The incubation was terminated by filtration. For the pipetting, a 12-channel pipette (25 µL - 300 µL, Eppendorf, Hamburg, Germany) was used and the filtration as well as washing was carried out row after row. 200 µL of each binding sample were transferred from the 96-deep-well plate to a 96-well glass fiber filter plate (AcroPrep Advance, glass fiber, 1.0 µm, 350 µL, Pall, Dreieich, Germany) which had been pretreated for 2 h with 150 µL 0.5% (m/v) PEI solution per well at 4 °C. The binding samples were rapidly filtrated through the filter with a multi well plate vacuum manifold (Pall). Directly after the filtration of one row of binding samples, the same row on the filter plate was washed three times with 150 µL ice cold wash buffer 1 (0.75 mg/mL ammonium formate, pH 7.4 + 10% DMA) and once with 150 µL ice cold wash buffer 2 (0.75 mg/mL ammonium formate, pH 7.4) before the next row was filtrated and washed. After filtration of all binding samples, the filter plate was dried for 2 h at 60 °C. For elution of the binding samples, 70 µL acetonitrile containing all three internal standards (*rac*-[$^2H_4$]-**1**, *rac*-[$^2H_5$]-**2**, *rac*-[$^2H_6$]-**3**), each at concentration of 90 pM, were transferred into each well of the filter plate (containing the remaining marker-target complexes). After a residence time of 20 s the elution solution was aspirated into a 96-deep-well plate under vacuum. This step was repeated two more times resulting in 210 µL eluate per binding sample. The 96-deep-well plate was sealed with aluminum foil and centrifuged (10 min, 2000 rpm, 4 °C; Biofuge Stratos, Rotor: #3048, Heraeus, Germany) prior to analysis by LC-MS/MS. Nonspecific binding was determined under the same conditions as total binding, but in presence of 10 µM *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**). In all binding samples, the marker depletion was not higher than 10%. Matrix samples, used for the calibration (see "Calibration") were prepared equally but without the addition of marker or competitor to the binding samples. Every day an MS Binding Assay was performed, a set of samples, containing blanks, calibration standards (in a range of 1 pM – 2.5 nM for all three markers) and QC samples (2.5 pM, 10 pM, 100 pM, and 1 nM of all three markers in hexaplicates) was prepared and analyzed together with the binding samples, as described previously.[27] All binding samples were quantified based on this calibration.

Simultaneous saturation assays: Simultaneous saturation assays were performed according to the "General procedure for MS Binding Assays" with all three markers and a target preparation containing all three monoamine transporters. Binding samples with all three markers in 32 concentration levels ranging from 25 pM to 500 nM were prepared in triplicates. For the determination of nonspecific binding, samples for all 32 marker concentration levels were prepared.

Competition assays: The competition assays were performed according to the "General procedure for MS Binding Assays" with all three markers and a target preparation containing all three monoamine transporters. Binding samples were prepared with 1 nM (*R,R*)-D-84 ((*R,R*)-**1**), 20 nM (*S,S*)-reboxetine ((*S,S*)-**2**), 4 nM (*S*)-citalopram ((*S*)-**3**) and the competitor in 15 concentration levels (20 concentration levels for sertraline) ranging from 10 pM to 1 mM for 3-α-bis-(4-fluorophenyl)methoxytropane, *rac*-bupropione, desipramine and *rac*-nisoxetine, respectively from 1 pM to 100 µM for (*S*)-fluoxetine as well as *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**) and from 300 fM to 1 mM for sertraline. Each set of samples for a competitor consisted of the binding samples with varying concentrations of the competitor and one triplicate of samples without the addition of the competitor (100% level of the competition curve, see "Data evaluation"). To determine the nonspecific binding (which was set to be the 0% level of the competition curve, see "Data evaluation"), binding samples containing the three markers in eight concentration levels ranging from 12.5 nM to 200 nM, 10 µM *rac*-[$^2H_3$]-indatraline (*rac*-[$^2H_3$]-**4**), and the target preparation, were prepared and processed according to the procedure described under "General procedure for MS Binding Assays".

Data evaluation: Calibration functions for each marker were determined in the Analyst 1.6 software based on the calibration standards by plotting the obtained peak area ratios (y-axes, marker peak area / internal standard peak area) against the marker concentration (x-axes) followed by a linear regression with a 1/x weighting. Further data evaluation was carried out with Prism 6 software (GraphPad Software, San Diego, CA, USA). With the calibration functions, peak area ratios for each binding sample were transformed to bound marker concentrations. Samples with bound marker concentrations not covered by the linear range of the LC-ESI-MS/MS quantification method (linear ranges from validation: 2.5 pM – 1 nM for (*R,R*)-D-84 ((*R,R*)-**1**), 1 pM – 2.5 pM for (*S,S*)-reboxetine ((*S,S*)-**2**), and 2.5 pM – 2.5 nM for (*S*)-citalopram ((*S*)-**3**)) were not used for further calculations. For the determination of the nonspecific binding, a straight line was calculated for the data points of the nonspecific binding by a linear regression (forced through the origin). Concentrations of nonspecifically bound marker concentrations for each free marker concentration was calculated based on the resulting linear function. Subtraction of the calculated nonspecific binding (NSB) from the total binding (TB, mean concentration calculated from triplicate) resulted in specifically bound marker concentrations (SB = TB – NSB) for the binding samples at each nominal marker concentration level. In saturation assays, equilibrium dissociation constants (K_d) and the maximum amount of binding sites (B_{max}) were derived from the data of the specific binding by a nonlinear regression analysis ("one site – specific binding", Prism 6). For the evaluation of competition experiments, the specifically bound marker concentrations were transformed in % bound marker concentration where 0% bound marker represents the level of the nonspecific binding and 100% the level of specifically bound marker in binding samples without the addition of a competitor. The inhibitory constants (K_i) were then derived by processing this data with a "one site – Fit K_i" nonlinear regression. K_d values used for the calculation were as determined in simultaneous saturation experiments (4.01 nM for (*R,R*)-D-84 ((*R,R*)-**1**), 2.20 nM for (*S,S*)-reboxetine ((*S,S*)-**2**), and 0.440 nM for (*S*)-citalopram ((*S*)-**3**)).

Acknowledgements

We want to thank Gerd Bauschke and Lars Allmendinger (LMU München, Department Pharmazie – Zentrum für Pharmaforschung) for the synthesis of (*R,R*)-D-84, *rac*-[$^2H_4$]-D-84, and *rac*-[$^2H_3$]-indatraline. We also thank Katharina Heimberger for her help to bring the manuscript into the final layout.

Keywords: binding assays, liquid chromatography, mass spectrometry, monoamine transporters, neurotransmitters

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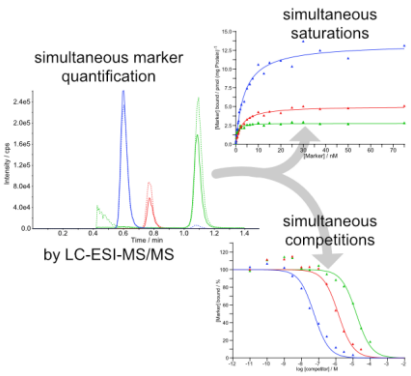

Simultaneous Multiple MS Binding Assays are a label-free, mass-spectrometry based technique for the concurrent determination of binding affinities at multiple targets. In this study, we realized this approach for the three monoamine transporters hDAT, hNET, and hSERT Using (*R*,*R*)-D-84, (*S*,*S*)-reboxetine and (*S*)-citalopram as selective markers profiling.

Supporting Information

Simultaneous Multiple MS Binding Assays for the dopamine, the norepinephrine, and the serotonin transporter

Patrick Neiens,[a] Angela De Simone,[b] Georg Höfner,[a] Klaus T. Wanner[a*]

[a]Department Pharmazie – Zentrum für Pharmaforschung
Ludwig-Maximilians-Universität München
Butenandtstr. 7, 81377 Munich, Germany
Phone: +49 (0) 89 2180 77249
e-mail: klaus.wanner@cup.uni-muenchen.de
ORCID: 0000-0003-4399-1425

[b]Department for Life Quality Studies,
Alma Mater Studiorum – University of Bologna,
Corso D'Augusto 237, 47921 Rimini, Italy

1. Validation

Validation (*R,R*)-D-84 ((*R,R*)-**1**)

	Intra-series																	Inter-series[b]		
	160315			160316			160318			160322			160323			160330					
c Marker [pM] (n)	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]
2.5 (6)	2.418	96,73	4,61	2.425	97.00	10.10	2.497	99.87	2.69	2.468	98.73	2.72	2.377	95.07	1.35	2.467	98.67	1.53	2.442	97.68	4.78
5 (6)	5,.08	102,17	2,28	5.228	104.57	9.84	5.093	101.87	1.30	4.957	99.13	0.69	4.995	99.90	1.88	5.043	100.87	2.47	5.071	101.42	4.49
10 (3)	10.47	104,67	0,55	10.67	106.67	7.16	10.47	104.67	2.21	10.53	105.33	1.45	10.87	108.67	1.41	10.57	105.67	1.45	10.59	105.94	3.05
25 (3)	27.13	108,53	1,13	26.17	104.67	0.22	27.17	108.67	1.39	26.80	107.20	0.37	27.23	108.93	0.56	27.23	108.93	1.29	26.96	107.82	1.67
50 (3)	52.77	105,53	1,45	54.53	109.07	5.93	52.67	105.33	0.58	51.73	103.47	0.95	52.93	105.87	0.79	54.13	108.27	1.23	53.13	106.26	2.88
100 (3)	107.7	107,67	0,54	103.9	103.90	8.81	105.7	105.67	1.09	105.0	105.00	0.95	107.3	107.33	0.54	109.0	109.00	1.59	106.4	106.43	3.48
250 (3)	266.3	106,53	0,22	267.7	107.07	2.49	256.3	102.53	0.60	263.7	105.47	0.22	265.0	106.00	0.00	265.7	106.27	1.15	264.1	105.64	1.74
500 (3)	507.0	101,40	0,86	506.7	101.33	2.96	514.3	102.87	1.27	510.3	102.07	0.23	506.0	101.20	0.86	496.7	99.33	1.11	506.8	101.37	1.65
1000 (3)	964.0	96,40	0,10	964.0	96.40	8.65	968.3	96.83	0.86	967.7	96.77	0.73	966.7	96.67	0.42	972.7	97.27	0.06	967.2	96.72	3.00
QC 2.5 (6)	3.152	126,07	2,95	2.320	92.80	6.44	2.517	100.67	1.79	2.502	100.07	1.59	2.398	95.93	2.71	2.982	119.27	1.31	2.645	105.80	12.20
QC 10 (6)	11.30	113,00	1,58	11.28	112.78	10.45	10.72	107.17	1.09	10.63	106.33	1.65	10.88	108.83	1.35	10.97	109.67	2.13	10.96	109.63	4.89
QC 100 (6)	112.1	112,17	1,31	10.93	111.62	9.79	106.7	106.67	0.97	106.0	106.00	0.84	104.8	104.83	1.27	106.0	106.00	0.60	107.9	107.88	4.78
QC 1000 (6)	983.7	98,37	0,78	985.7	98.57	5.95	1048.3	104.83	0.72	984.8	98.48	0.70	972.5	97.25	0.91	1002.3	100.23	0.61	996.2	99.62	3.43
Calibration function	y = 0.01x + 0.00478 (r^2 = 0.9991)			y = 0.0084x + 0.00628 (r^2 = 0.9975)			y = 0.01x + 0.00288 (r^2 = 0.9994)			y = 0.0103x + 0.00323 (r^2 = 0.9993)			y = 0.0103x + 0.00409 (r^2 = 0.9992)			y = 0.0108x + 0.00412 (r^2 = 0.9991)					

M: mean of calculated concentration, Acc: Accuracy, Prec: Precision, n: number of replicates, RSD: relative standard deviation, QC: quality control sample. [a] Number of samples (n) for 2.5 pM, 5 pM, QC 2.5 pM, QC 10 pM, QC 100 pM, QC 1000 pM: n = 36; 10 pM – 1000 pM: n = 18.

Validation (*S*,S)-reboxetine ((*S*,*S*)-**2**)

	Intra-series																		Inter-series[b]		
	160315			160316			160318			160322			160323			160330					
c Marker [pM] (n)	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]
1 (6)	0.872	87.22	4.22	0.921	92.10	14.49	0.907	90.65	4.75	0.946	94.63	9.12	0.930 [a]	93.04	15.28	0.924	92.43	10.50	0.916	91.64	10.07
2.5 (6)	2.490	99.60	4.36	2.387	95.47	6.32	2.532	101.27	3.54	2.390	95.60	5.47	2.307	92.27	13.87	2.547	101.87	2.06	2.442	97.68	7.25
5 (6)	5.088	101.77	1.68	5.223	104.47	11.35	5.088	101.77	3.51	4.938	98.77	3.58	5.037	100.73	9.57	4.825	96.50	4.57	5.033	100.67	6.79
10 (3)	10.050	100.50	3.26	10.277	102.77	4.24	10.05	100.53	3.89	10.237	102.37	7.31	10.47	104.67	2.21	10.163	101.63	3.05	10.208	102.08	3.90
25 (3)	25.93	103.73	1.74	24.63	98.53	0.23	25.70	102.80	1.95	25.87	103.47	1.98	26.47	105.87	2.08	25.90	103.60	2.91	25.75	103.00	2.78
50 (3)	52.60	105.20	1.06	53.90	107.80	4.65	51.97	103.93	1.28	51.77	103.53	2.30	53.70	107.40	4.21	52.33	104.67	2.97	52.71	105.42	3.05
100 (3)	104.00	104.00	0.96	100.8	100.83	7.39	102.67	102.67	1.12	103.8	103.77	4.20	101.8	101.77	2.32	104.0	104.00	2.88	102.8	102.84	3.41
250 (3)	265.0	106.00	1.00	262.3	104.93	2.76	248.7	99.47	0.61	264.7	105.87	0.79	259.7	103.87	3.70	256.7	102.67	2.25	259.5	103.80	2.89
500 (3)	523.0	104.60	0.88	507.3	101.47	4.28	513.3	102.67	2.10	520.0	104.00	1.68	515.7	103.13	0.73	501.3	100.27	1.00	513.4	102.69	2.32
1000 (3)	1013	101.33	1.51	1011	101.10	8.52	1020	102.00	0.98	1013	101.33	0.57	1002	100.17	1.68	1030	103.00	1.68	1015	101.49	3.21
2500 (3)	2440	97.60	1.08	2463	98.53	2.08	2463	98.53	1.30	2447	97.87	1.31	2467	98.67	1.30	2453	98.13	0.47	2456	98.22	1.20
QC 2.5 (6)	3.045	121.80	3.24	2.307	92.27	10.28	2.565	102.60	2.90	2.408	96.33	6.30	2.143	85.73	6.91	2.710	108.40	1.42	2.530	101.19	12.80
QC 10 (6)	10.967	109.67	2.92	10.75	107.47	11.55	10.58	105.83	2.27	10.32	103.22	2.82	10.24	102.43	4.30	10.39	103.85	4.69	10.54	105.41	5.87
QC 100 (6)	110.7	110.67	1.78	107.75	107.75	10.68	101.7	101.67	1.02	104.3	104.33	1.31	102.8	102.83	1.78	100.1	100.13	1.89	104.6	104.56	5.60
QC 1000 (6)	1045.0	104.50	1.17	1039.3	103.93	7.33	1101.7	110.17	0.37	1041.7	104.17	0.72	1013.5	101.35	1.31	1032	103.17	1.67	1045.5	104.55	3.93
Calibration function	y = 0.00589x + 0.00197 (r^2 = 0.9995)			y = 0.00601x + 0.00303 (r^2 = 0.9990)			y = 0.00625x + 0.0016 (r^2 = 0.9998)			y = 0.00598x + 0.00265 (r^2 = 0.9995)			y = 0.00583x + 0.0292 (r^2 = 0.9997)			y = 0.00504x + 0.00236 (r^2 = 0.9997)					

M: mean of calculated concentration, Acc: Accuracy, Prec: Precision, n: number of replicates, RSD: relative standard deviation, QC: quality control sample. [a] n = 5. [b] Number of samples (n) for 2.5 pM, 5 pM, QC 2.5 pM, QC 10 pM, QC 100 pM, QC 1000 pM: n = 36; 10 pM – 2500 pM: n = 18; 1 nM: n = 35.

Validation (*S*)-citalopram ((*S*)-**3**)

	Intra-series																		Inter-series[b]		
	160315			160316			160318			160322			160323			160330					
c Marker [pM] (n)	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]	M [pM]	Acc [%]	Prec [RSD in %]
2.5 (6)	2.347	93.87	6.68	2.265	90.60	12.09	0.846	97.20	1.56	2.368	94.73	2.45	2.308	92.33	2.41	2.383	95.33	3.25	2.350	94.01	5.88
5 (6)	5.120	102.40	3.38	5.385	107.70	9.00	2.430	99.67	1.58	5.015	100.30	2.15	5.012	100.23	2.83	5.017	100.33	1.65	5.089	101.77	4.98
10 (3)	10.60	106.00	0.94	10.500	105.00	5.79	4.983	103.33	0.56	10.70	107.00	0.00	10.80	108.00	3.34	10.67	106.67	1.95	10.60	106.00	2.82
25 (3)	27.10	108.40	1.69	25.80	103.20	2.71	10.33	107.73	0.43	26.97	107.87	3.00	27.57	110.27	1.47	27.57	110.27	0.91	26.99	107.96	2.77
50 (3)	54.53	109.07	2.09	55.13	110.27	5.75	26.93	106.87	0.47	54.20	108.40	0.80	55.00	110.00	0.18	54.33	108.67	1.18	54.44	108.88	2.43
100 (3)	108.7	108.67	1.06	105.1	105.13	8.37	53.43	108.00	0.93	108.3	108.33	0.53	109.3	109.33	1.90	108.3	108.33	1.41	108.0	107.97	3.22
250 (3)	269.3	107.73	0.57	267.3	106.93	1.77	108.0	104.27	1.23	272.3	108.93	0.56	273.3	109.33	1.28	267.7	107.07	0.22	268.4	107.38	1.82
500 (3)	535.7	107.13	0.88	527.7	105.53	3.02	260.7	106.93	0.92	538.7	107.73	1.24	530.3	106.07	0.71	514.3	102.87	1.70	530.2	106.04	2.05
1000 (3)	1043	104.33	0.55	1023	102.27	7.42	534.7	104.00	1.67	1040	104.00	0.96	1037	103.67	0.56	1033	103.33	0.56	1036	103.60	2.70
2500 (3)	2387	95.47	0.87	2420	96.80	0.83	1040	96.27	0.48	2387	95.47	0.24	2393	95.73	0.96	2417	96.67	0.86	2402	96.07	0.86
QC 2.5 (6)	2.958	118.33	2.65	2.382	95.27	10.01	2.482	99.27	2.88	2.365	94.60	4.46	2.405	96.20	1.59	2.667	106.67	2.14	2.543	101.72	9.47
QC 10 (6)	11.48	114.83	4.10	10.89	108.85	7.93	10.88	108.83	2.13	10.45	104.50	2.48	11.15	111.50	1.24	10.77	107.67	1.92	10.94	109.36	4.75
QC 100 (6)	113.0	113.00	0.79	112.7	112.67	10.66	109.5	109.50	1.26	111.0	111.00 [a]	0.90	108.5	108.50	1.27	108.2	108.17	1.08	110.5	110.46	4.62
QC 1000 (6)	1052	105.17	0.72	1054	105.35	6.31	1112	111.17	0.68	1055	105.50	0.79	1042	104.17	1.28	1062	106.17	1.10	1063	106.25	3.31
Calibration function	y = 0.00645x + 0.00405 (r^2 = 0.9986)			y = 0.00643x + 0.00402 (r^2 = 0.9986)			y = 0.00633x + 0.00365 (r^2 = 0.9989)			y = 0.00636x + 0.00459 (r^2 = 0.9985)			y = 0.0063x + 0.0039 (r^2 = 0.9986)			y = 0.00627x + 0.00205 (r^2 = 0.9992)					

M: mean of calculated concentration, Acc: Accuracy, Prec: Precision, n: number of replicates, RSD: relative standard deviation, QC: quality control sample. [a] n = 5. [b] Number of samples (n) for 2.5 pM, 5 pM, QC 2.5 pM, QC 10 pM, QC 1000 pM: n = 36; 10 pM – 2500 pM: n = 18; QC 100 pM: n = 35.

4. Summary of the Thesis

The monoamine transporters DAT, NET, and SERT are important targets for the treatment of affective disorders like major depressive disorders. Therapy of MDD is mostly based on nonselective or selective monoamine reuptake inhibitors, which interact with one or multiple monoamine transporters. Due to the different functions of the monoamines, the individual profile of binding activities at each monoamine transporter can help to explain intended effects – but also side effects – of antidepressants. Since MS Binding Assays, an alternative to conventional radioligand binding assays, have the potential to characterize binding affinities at multiple targets simultaneously, they are a promising technique to determine selectivities and activities of antidepressants at the monoamine transporters with high efficiency.

The aim of this work was to develop Simultaneous Multiple MS Binding Assays, which allow the characterization of binding affinities at hDAT, hNET, and hSERT in a single experiment, thus resulting in a much-improved efficiency in comparison to radioligand binding assays and the recently published (1*R*,3*S*)-indatraline MS Binding Assays.

As a requirement for Simultaneous Multiple MS Binding Assays, a selective marker for each of the three targets had to be found. These markers should have a high affinity, ideally in the low nanomolar range, just for one of the three monoamine transporters. (*R,R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol ((*R,R*)-D-84) is one of the most selective DAT inhibitors and was therefore chosen as a marker for this target. For hNET (*S,S*)-reboxetine was chosen and (*S*)-citalopram was used as marker for hSERT.

Since the enantiomerically pure compounds should be applied as markers and (*R,R*)-D-84 is not commercially available, a semipreparative method for the chiral separation of *rac*-D-84, which was synthesized in house according to the original publication, was developed [70]. This semipreparative HPLC method was based on a cellulose-based chiral stationary phase (CSP) and enabled the separation of up to 8 mg *rac*-D-84 per chromatographic run. For the successive determination of the enantiomeric purity of (*R,R*)-D-84, again a CSP-HPLC method with slightly modified conditions was used and validated. With the analytical CSP-HPLC

method it was possible to determine an *ee* of up to 99.8 %. The (*R,R*)-D-84 sample obtained from the semipreparative method was found to possess an *ee* > 99.8 %, thus its quality was adequate for its use in MS Binding Assays. (*S,S*)-reboxetine was extracted as a racemate from commercially available tablets and semipreparatively separated by CSP-HPLC. With a newly developed analytical CSP-HPLC method the *ee* of the obtained (*S,S*)-reboxetine was determined as 99.3 %.

For the quantification of (*R,R*)-D-84, (*S,S*)-reboxetine, and (*S*)-citalopram, an ESI-LC-MS/MS quantification method had to be developed, allowing the analysis of the three markers in a single chromatographic run. A HPLC method could be established that allowed the efficient separation of the markers from the matrix, hence minimizing matrix effects. During the method development, different stationary phase materials were investigated concerning their suitability for the chromatographic analysis of the three markers. Additionally, the influence of the mobile phase parameters, such as pH and concentration of the buffer, on the chromatography were assessed. The final chromatographic method was based on a LC-column with a pentafluorophenyl (PFP) phase, which was used in combination with a mobile phase consisting of acetonitrile and ammonium formate buffer. Under the chosen chromatographic conditions, all three markers were separated from each other and from interfering matrix components within a run time of only 1.5 min. For the detection, an API 5000 triple quadrupole mass spectrometer, equipped with a TurboV electrospray ionization (ESI) source was used. As internal standards, *rac*-[$^2H_4$]-D-84, *rac*-[$^2H_5$]-reboxetine, and *rac*-[$^2H_6$]-citalopram were employed to compensate for matrix effects and variations in the ionization efficiency.

The setup for the binding experiments was kept similar to that of the previously published MS Binding Assays. During the LC-MS method development it was observed, that (*R,R*)-D-84 undergoes extensive adsorption to the used containers, resulting in non-reproducible results. This issue was solved by the addition of DMA to the solutions containing this compound, preventing the adsorption of this marker in the working solutions. To separate the target-marker complexes from the unbound marker after the incubation, a filtration through glass fiber filters was used. Similarly to the adsorption of (*R,R*)-D-84 towards the containers, an extensive binding of (*R,R*)-D-84 towards the filter material was noticed. The filter binding of

(*R,R*)-D-84 could be dramatically reduced by the addition of DMA to the washing buffer. After the filtration and washing of the filters containing the target-marker complexes, the bound markers were liberated by exposing the protein samples to elevated temperatures and subsequently eluting them with an organic solvent. The resulting samples were then analysed with the developed LC-ESI-MS/MS quantification method.

To ensure the reliability and robustness, the LC-ESI-MS/MS quantification method was validated using matrix samples, obtained by performing the previously described procedure for the binding experiments. The validation was carried out according to the *CDER guideline for bioanalytical method validation*, regarding the selectivity, validity of the calibration curve, range, precision and accuracy. Based on the data from the validation, analytical quantification ranges from 2.5 pM to 1 nM for (*R,R*)-D-84, 1 pM to 2.5 nM for (*S,S*)-reboxetine, and 2.5 pM to 2.5 nM for (*S*)-citalopram could be defined. The developed LC-ESI-MS/MS quantification method is the first quantification method for D-84. In addition, it is by a factor of approximately 1000-fold more sensitive than other published LC-MS quantification methods reported for reboxetine and citalopram.

With the established MS Binding Assays, the binding affinities and selectivities of (*R,R*)-D-84, (*S,S*)-reboxetine, and (*S*)-citalopram towards their targets were determined in individual saturation experiments. Simultaneously performed saturation experiments, in which all three markers together with all three targets were employed, resulted in comparable binding affinities for the three markers towards their targets, thus demonstrating the feasibility of Simultaneous Multiple MS Binding Assays. Following the procedure of Simultaneous Multiple MS Binding Assays, the binding affinities and selectivities of a set of known monoamine transporter inhibitors were characterized in competition experiments. The results from the simultaneous competition experiments were in good accord with published data, which demonstrates the potency and reliability of Simultaneous Multiple MS Binding Assays as an alternative to radioligand binding studies.

5. List of Abbreviations

5-HT	serotonin
B_{max}	maximum amount of binding sites
[C]	competitor concentration
CDER	center for drug evaluation and research
CNS	central nervous system
COMT	catechol-*O*-methyltransferase
CSP	chiral stationary phase
DA	dopamine
DAT	dopamine transporter
dDAT	*Drosophila melanogaster* dopamine transporter
$dDAT_{cryst}$	crystal structure of *Drosophila melanogaster* dopamine transporter
DEA	diethylamine
DMA	dimethylacetamide
(*S,S*)-D-83	(*S,S*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol
(*R,R*)-D-84	(*R,R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol
ee	enantiomeric excess
EL	extracellular loop
ESI	electrospray ionization
GABA	γ-amonibutyric acid
hDAT	human dopamine transporter
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
hNET	human norepinephrine transporter
HPLC	high performance liquid chromatography
hSERT	human serotonin transporter
IC_{50}	half maximal inhibitory concentration
K_d	equilibrium dissociation constant
K_i	inhibitory constant
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LeuT	leucine transporter
$LeuT_{Aa}$	*Aquifex aeolicus* leucine transporter

$LeuT_{cryst}$	crystal structure of *Aquifex aeolicus* leucine transporter
[L]	reporter ligand concentration
[LT]	concentration of reporter ligand-target complexes
MAO	monoamine oxygenase
MDD	major depressive disorder
mGAT1	murine γ-amonibutyric acid transporter subtype 1
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass/charge-ratio
NDRI	norepinephrine-dopamine reuptake inhibitor
NE	norepinephrine
NET	norepinephrine transporter
NMR	nuclear magnetic resonance
NP	normal phase
NRI	selective norepinephrine reuptake inhibitor
NSB	nonspecific binding
NSS	neurotransmitter-sodium-symporter
NTT	neurotransmitter transporter
PFP	pentafluorophenyl
qNMR	quantitative nuclear magnetic resonance
SB	specific binding
SERT	serotonin transporter
SLC6	solute carrier 6
SNRI	serotonin-norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TB	total binding
TCA	tricyclic antidepressant
TFA	trifluoroacetic acid
TM	transmembrane helix
[T]	target concentration
X-ray	röntgen radiation
β-CIT	(–)-2β-carbomethoxy-3β-(4-iodophenyl)tropane
β-CFT	(–)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane

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