Dissertation zur Erlangung des Doktorgrades

der Fakultät für Chemie und Pharmazie

der Ludwig-Maximilians-Universität München

Development of MS Binding Assays Addressing the Human Dopamine, Norepinephrine, and Serotonin Transporter

Stefanie Heidrun Grimm

aus Buchen (Odw.)

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2015

Erklärung

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Stefanie Heidrun Grimm

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Für die hervorragende und sehr engagierte Betreuung und Förderung

meiner Arbeit sowie die ausgezeichneten Forschungsbedingungen danke ich

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“Development and validation of an LC-ESI-MS/MS method for triple reuptake inhibitor indatraline enabling its quantification in MS Binding Assays”

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2013:

Poster presentation at “LMU – AbbVie Joint Symposium, Munich”

Stefanie H. Grimm and Klaus T. Wanner

“Development of MS Binding Assays addressing hDAT, hNET, and hSERT”

Poster presentation at "6th SFB35 – Symposium 2013, Vienna"

Stefanie H. Grimm and Klaus T. Wanner

"Development of an LC-ESI-MS/MS method for quantification of Indatraline and its Application to MS Binding Assays addressing the human norepinephrine transporter"

Poster presentation at "Frontiers in Medicinal Chemistry, Munich"

Stefanie H. Grimm and Klaus T. Wanner

"Enantiopurity Determination of the Enantiomers of the Triple Reuptake Inhibitor Indatraline"

“ Give a girl the right shoes, and she can conquer the world. “

Marilyn Monroe

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Dedicated to my mother

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1 Introduction

1.1 Physiologic significance of Dopamine, Norepinephrine, and Serotonin

The biogenic monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT) are important neurotransmitters (Figure 1) which act in the human brain as well as in the periphery.

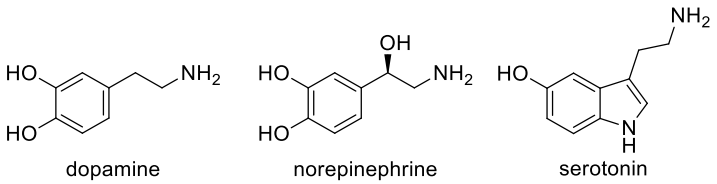


Figure 1: Structures of dopamine, norepinephrine, and serotonin.

However, this thesis will focus on the respective neural distribution and the functions of each of these monoamines (i.e. dopamine, norepinephrine, and serotonin):

- Dopaminergic neurons are most abundant in the corpus striatum, but high concentrations can also be found in frontal cortex, limbic system, and hypothalamus. Due to the distribution of the dopaminergic neurons in mammalian brain different functions arise from dopamine neurotransmission.[1] Dopamine not only influences coordination of movements, cognitive functions (i.e. attention, learning, work memory, decision making, etc.), sexual and eating behavior, it is also responsible for the human reward system and as a consequence for addictive behavior.[2]
- The highest concentration of norepinephrine neurons can be found in the locus coeruleus, which is located in the pons. It is also present in other parts of the brain, e.g. cortex, hippocampus, thalamus, hypothalamus, and cerebellum. The activation of the human fight-or-flight response induces a release of norepinephrine. It therefore increases vigilance, wakefulness, and attention. Next to these excitatory effects an activation of the respective adrenoreceptors, i.e. α_1 receptors, norepinephrine is thought to be involved in motor activity, cognition, and anxiety.[1]
- The serotonergic neurons are widespread in central nervous system (CNS), i.e. raphe nuclei, cortex, hippocampus, basal ganglia, limbic system, hypothalamus, cerebellum, medulla, and spinal cord.[1] Serotonin overlaps in its action with the previously described monoamines, e.g. regulation of eating and sexual behavior (DA), and anxiety (NE). Additionally, serotonin has specific functions, i.e. it controls aggression and impulse, sensory functions, and induces sleep.[1, 3]

Figure 2 depicts neuronal functions of the aforementioned monoaminergic systems and also illustrates that dopamine, norepinephrine, and serotonin neurotransmitter systems have a common influence on mood.

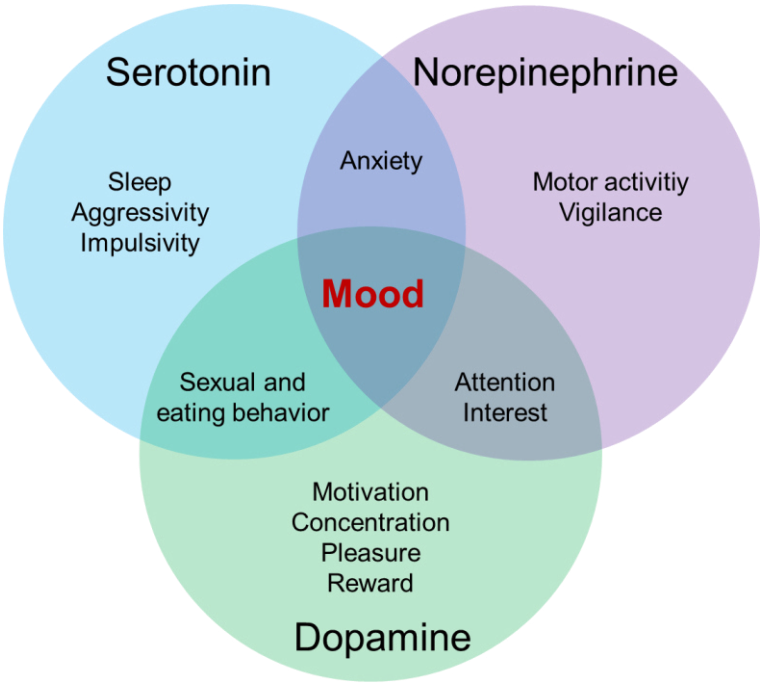


Figure 2: Neuronal actions of the dopaminergic, noradrenergic, and serotonergic neurotransmitter systems (adopted from Guiard [4]).

1.1.1 Neurotransmission

To fulfill the described functions in mammalian CNS, the neurons responsible receive and transmit signals via a process called neurotransmission, which will be briefly explained below.

After their biosynthesis, dopamine, norepinephrine, and serotonin are transported into vesicles via vesicular monoamine transporters (VMAT and VMAT2). The storage of the transmitters and the simultaneous increase in concentration has two reasons: first, the described monoamines are protected from degradation in cytosol, and secondly, a fast release of a high concentration is enabled (Figure 3, step 1).[5] The neurotransmitter release takes place in response of an arriving action potential in the nerve terminal and the associated influx of calcium ions at the terminal neuron. The resulting high intracellular concentration of calcium causes the vesicles to undergo an exocytosis with the plasma membrane releasing the stored monoamines (i.e. dopamine, norepinephrine, or serotonin) into the synaptic cleft (Figure 3, step 2). After their release into the synaptic cleft the neurotransmitters can bind to their respective receptors at the postsynaptic neuron which initiates a signal in this neuron (Figure 3, step 3a). Two different types of monoamine neurotransmitter (i.e. dopamine, norepinephrine, and serotonin) receptors can be distinguished:

- With one exception, all of these receptors belong to the family of G protein coupled receptors (GPCRs) which activate intracellular G proteins in response to ligand binding. These G proteins can then stimulate or inhibit two different intracellular signal pathways, i.e. the cyclic adenosine monophosphate (cAMP) and the

phosphatidylinositol signal pathway. Depending on the activated G protein, inhibitory as well as excitatory reactions in CNS are triggered.

- Only one serotonin receptor, i.e. 5-HT_3, is a ligand-gated sodium potassium cation channel, which upon activation leads to an excitatory response in neurons.

Next to these postsynaptic receptors, some presynaptic receptors also exist, called autoreceptors which reduce a further release of DA, NE, and 5-HT via a negative feedback mechanism (Figure 3, step 3b).[6-9] But even if the further release is inhibited, the neurotransmitters released in the synaptic cleft have to be removed from the synaptic cleft either by enzymatic degradation or by reuptake to terminate the neurotransmitter signal. For the three monoaminergic neurotransmitters discussed here presynaptic transporters (see below, *1.2 Monoamine Transporters*) which transport the monoamines back in the respective neurons (Figure 3, step 4) exist where they are stored again in the vesicles and can be reused later (Figure 3, step 5).

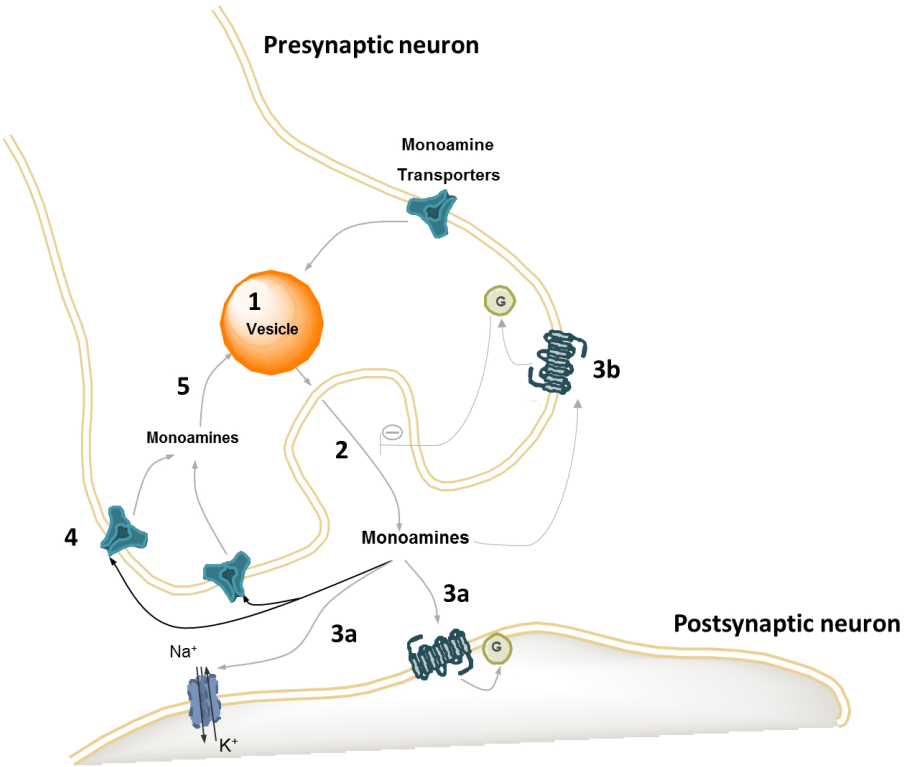


Figure 3: Monoaminergic neurotransmission. Monoamines (i.e. dopamine, norepinephrine, and serotonin) are stored in vesicles (1) and released in response to an action potential (2). In the synaptic cleft, the released monoamines can interact with postsynaptic receptors (3a) and therefore transmit the signal to the next neuron, or activate presynaptic autoreceptors (3b), which inhibit a further neurotransmitter release (2). Presynaptic transporters terminate signals via transporting the monoamines back into the neurons (4), where the transported monoamines can be stored again in the vesicles (5).

1.1.2 Pathology and Therapy

The physiological roles described above indicate that the monoamine neurotransmitter systems consisting of dopamine, norepinephrine, and serotonin (see *1.1 Physiological significance of Dopamine, Norepinephrine, and Serotonin*) are associated with a series of mental disorders, such as depression, schizophrenia, obsessive-compulsive disorders, etc.

This hypothesis was strengthened when in the 1950s and 1960s several links between clinical side effects of various drugs, namely an alleviation (e.g. iproniazid in treatment of tuberculosis) or induction (e.g. reserpine in treatment of hypertension) of symptoms of affective disorders, associated with the monoaminergic systems, were observed.[10] As a consequence, the so called 'catecholamine hypothesis of affective disorders',[11] and the 'serotonin hypothesis' [12] were formed explaining that depressions are a result of a functional deficit of norepinephrine and serotonin. Dopamine is today also assumed to be an important factor in the regulation of mood.[4]

An enhancement of the respective monoamine neurotransmitter systems is therefore an obvious therapeutic approach for the treatment of depressions. This enhancement can generally be realized following different strategies:

- Inhibition of enzymes catalyzing the degradation of monoamines:
  Inhibitors of catechol-*O*-methyltransferase (COMT) (e.g. entacapone and tolcapone) are only used in the therapy of Parkinson's disease, while inhibitors of the monoamine oxidase (MAO) (e.g. tranylcypromine, moclobemide, etc.) are used in treatment of both, Parkinson's disease and depressions. Since they cause adverse side effects and serious interactions MAO-inhibitors play a minor role as antidepressants today.[1]
- Regulation of respective autoreceptors:
  A different strategy is the direct inhibition of respective autoreceptors. By the inhibition of the negative feedback mechanism, a further monoamine release out of the neuron into the synaptic cleft is enabled. Next to the adrenergic autoreceptors other receptors can also be inhibited via antagonists (e.g. mirtazapine, mianserin).[1] This type of drug enhances mainly the noradrenergic and serotonergic neurotransmitter systems.
- Inhibition of reuptake:
  The mainstay in treatment of depression today is the inhibition of the responsible transporters, i.e. dopamine transporters (DAT), norepinephrine transporters (NET), and serotonin transporters (SERT), to effect a longer residence time of the monoamines in the synaptic cleft.[13]
  Diverse drug classes, namely tricyclic antidepressants (TCAs) as well as selective and dual reuptake inhibitors can realize an inhibition of the corresponding transporters. TCAs (e.g. clomipramine, desipramine, imipramine, etc.) were the first generation of

antidepressants, whose use started in the 1950s. The inhibition of NET and SERT is assumed to be the main mechanism of action. Unfortunately, TCAs are 'dirty drugs' since they do not possess a selective pharmacological profile. They not only inhibit reuptake, they also antagonize various serotonin, histamine, and muscarinic acetylcholine receptors, causing various side effects. The result of these adverse effects combined with the risk of an overdose brought about a replacement of TCAs in therapy by newer, safer antidepressants.[1, 10]

The next generation of drugs, the so called selective serotonin reuptake inhibitors (SSRIs) (e.g. citalopram, fluoxetine, sertraline, etc.), was introduced in the 1980s [14] and remains until now the most frequently prescribed class of antidepressants.[15] Due to their selective inhibition of SERT, they show an improved side effect profile combined with an efficiency comparable to that of the TCAs in the treatment of depression (except in the treatment of severe depression).[1] Nevertheless, selective norepinephrine reuptake inhibitors (NRI) (e.g. reboxetine, nisoxetine) were also developed. Their therapeutic efficiency showed a further improvement compared to the one of TCAs and SSRIs. For example, reboxetine has demonstrated its long-term safety and effectiveness in treatment of SSRI resistant melancholia and severe depressions.[16]

As a next step in the development of new antidepressants, the mode of action of SSRIs with the one of NRIs was combined. The result are dual reuptake inhibitors (SNRIs) (e.g. duloxetine, venlafaxine) enhancing both monoaminergic systems, i.e. noradrenergic and serotonergic. This new class is characterized by a safety profile comparable to that of the SSRIs and shows an improved clinical benefit comprising faster onset of action, a broader range of indications, and a higher efficacy.[15] It is worth to note that an authorized dual reuptake inhibitor inhibiting DAT and NET (NDRI), bupropion, also exists as an alternative in treatment of major depressions. Bupropion is the first antidepressant, which exhibits a dopaminergic component, [17, 18] and is also used for smoking cessation.

1.2 Monoamine Transporters

Thanks to their outstanding role in therapy of depressions, the monoamine transporters remain the most important target for the development of new antidepressants. Different aspects of the monoamine transporters have been investigated in the last decades. The knowledge discovered in these studies concerning function, regulation, and structure of these highly important targets is discussed in the following part.

The neurotransmitter transporters (NTTs) including monoamine transporters, γ-aminobutyric acid transporters, and glycine transporters, are part of the solute carrier 6 (SLC6) gene family. These NTTs are also called 'neurotransmitter-sodium-symporters' (NSSs) or Na^+/Cl^- dependent transporters.[19] As the names indicate, this group of transporters mainly works using a transmembrane sodium gradient for the transport of the respective substrates against the concentration gradient from synaptic cleft into the pre-synaptic neuron. The respective stoichiometry of the monoamine transport for each transporter as well as other characteristics, i.e. turnover rates of the different substrate molecules per second and their affinities, are shown in Table 1: At least one sodium and one chloride ion are co-transported with the respective substrate. In case of DAT, two sodium ions are involved in the transport process, whereas in case of SERT, in addition a counter transport of a potassium ion takes place.[20] Comparable results for all three monoamine transporters have been found regarding the velocity of the transport process, having a turnover rate of one to three substrate molecules per second. The affinities (shown as inhibition constant K_i) and substrate selectivities of the monoamines heavily depend on the targeted transporter. The only really selective transporter is SERT. This is not surprising considering the difference of the structure of the substrate serotonin, compared to the structures of dopamine and the related norepinephrine (Figure 1). However, it is noteworthy that dopamine shows a higher affinity towards NET than the native substrate norepinephrine (Table 1). This might be easily explained by the fact that NET is also responsible for dopamine clearance in the synaptic cleft, primarily in dopaminergic brain regions exhibiting only a minimal DAT expression for example in the frontal cortex.[21]

Table 1: Characteristics of the monoamine transporters DAT, NET, and SERT

transporter	substrate affinity K_i [µmol L^{-1}][a]			turnover rate [molecules s^{-1}][b]	substrate-ion stoichiometry (substrate : Na^+ : Cl^-)[b]
	DA	NE	5-HT		
DAT	6.40 ± 0.59	57.0 ± 13.0	549 ± 96.0	0.7 – 1.9	1 : 2 : 1
NET	28.0 ± 11.0	160 ± 33.0	360 ± 71.0	1.7 – 2.5	1 : 1 : 1
SERT	1,110 ± 180	1,470 ± 110	3.5 ± 1.2	1.0 – 3.1	1 : 1 : 1 : (1 K^+ out)

[a] Eshleman et al., 1999 [22]. [b] Kirstensen et al., 2011 [20]

1.2.1 Crystal Structures

Unfortunately, no crystal structures of the human monoamine transporters are available as the crystallization of membrane-associated proteins is very difficult. The analogous crystal structures of two homologues are however available: of a prokaryotic leucine transporter *LeuT* [23-28] and of a eukaryotic monoamine transporter of Drosophila melanogaster (*dDAT*).[29] They are discussed in the following section.

LeuT:

In 2005 Yamashita et al. published a crystal structure of the leucine transporter of the hyperthermophilic bacterium *Aquifex aeolicus* ($LeuT_{Aa}$, in the following denoted as LeuT), which is a prokaryotic homologue of the eukaryotic NSS.[23] This structure confirmed the earlier predicted 12 transmembrane segments (TMs) of the NSS members, but revealed an unprecedented C_2 pseudo-symmetry in the LeuT topology, where the subset of TM1-5 can be superimposed on TM6-10 by a rotation of 176.5° (Figure 4).

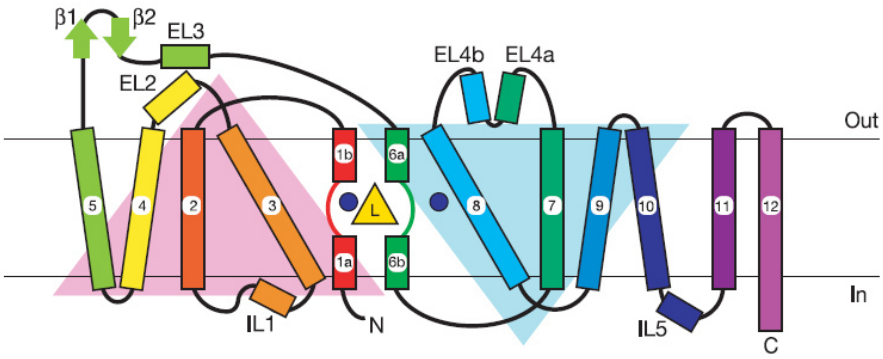


Figure 4: Topology of LeuT showing the 12 TMs, several loops, the N-, and C-terminus. Colored triangles (rose and light blue) illustrate the structural repetition of TM 1-5 and TM 6-10, which can be superimposed by a rotation of 176.5°. Bound substrate (yellow triangle) and two bound sodium ions (blue circles) are located in the core of the protein (adopted from Yamashita et al.[23]).

This X-ray analysis also showed that the co-crystallized substrate, *L*-leucine, is located in the core of LeuT in a cavity that is mainly formed by TM1, TM3, TM6, and TM8.[23] A later study examined additional binding of different TCAs (clomipramine, desipramine, and imipramine) in presence of the substrate in LeuT.[24] In these experiments, Singh et al. co-crystallized LeuT with its substrate, *L*-leucine, and different TCAs. The crystallographic analysis of the LeuT-leucine-TCA complexes obtained in this way revealed that the TCAs do not bind in the known substrate binding pocket in the core of the transporter (termed S1), which was still occupied by *L*-leucine, but directly above in a so called *extracellular vestibule* (later also denoted as S2) (see also Figure 5).[24] In line with that result, in competitive experiments was found that the TCA clomipramine could not significantly displace [^{3}H]leucine bound to the LeuT, indicating a noncompetitive inhibition of LeuT by clomipramine.[24]

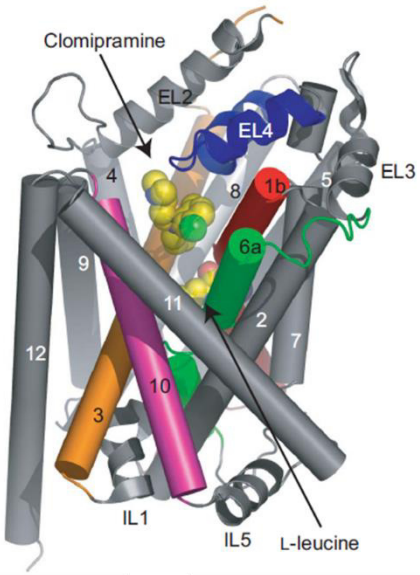


Figure 5: Binding site for clomipramine (yellow spheres in the upper part) and L-leucine (yellow spheres in the middle part of the structure) in LeuT (adopted from Singh et al.[24]).

The discovery of the existence of a second binding site S2 and in particular its potential function, is still controversially discussed by different groups. In 2008 Shi et al. postulated that not only noncompetitive inhibitors but also a second leucine molecule can bind in S2. It was proposed that this second substrate molecule in S2 allosterically triggers the release of the first bound leucine molecule bound in S1 into the cytoplasm.[26] This hypothesis of Shi et al. started a lively controversy about the postulated function of S2 for the transport process in LeuT.[26] In 2010 Piscitelli et al. examined the stoichiometry in substrate binding experiments revealing only one and not two high-affinity binding sites for *L*-leucine. It was suggested instead that S2 is a low-affinity binding site to which a second substrate can bind transiently. This conclusion contradicted the previously postulated transport mechanism of Shi et al..[28] The latest publication about this topic showed that conditions chosen (i.e. the employed concentration of the detergent) for the preparation of LeuT have a crucial influence on substrate binding in S2, which in the aforementioned study of Piscitelli et al. [28] falsified the results of the of S2 site.[27]

Even though the actual function of S2 is still unclear the results demonstrated that two binding sites (i.e. S1 and S2) exist in LeuT. If both can be occupied by substrate molecules or if S2 is only a binding site for inhibitors remains uncertain so far. The existence of this second binding site (i.e. S2) in LeuT might however serve as explanation for an allosteric modulation site in the mammalian monoamine transporters which was discovered in the late 1990s.[30] At this time it was discovered that the binding of various inhibitors can affect the dissociation kinetics of rDAT-, rNET-, and rSERT-marker complexes to higher but also to lower values. As a consequence the affinity of the employed marker substances towards the respective target can also be changed to higher or lower values (for further information regarding this kind of

experiment, see below *1.3.2.2 Kinetic Studies*).[30] According to this theory the employed inhibitor binds to a different binding site than the marker substance, i.e. S1, and this additional binding of the inhibitor affects the S1 binding site of the marker.[30] Plenge et al. called the addressed protein structure, which is responsible for this effect in the monoamine transporters, *affinity-modulating site*.[30] Different groups have confirmed the existence and relevance of this second binding site until now.[30-34] Nevertheless, as long as no crystal structure of a monoamine transporter with co-crystallized inhibitors occupying both binding sites is available, it will remain uncertain, whether this modulation site is equivalent to the S2 binding site in LeuT. However, it can be clearly concluded that monoamine transporters also have two binding sites, which can be addressed by different inhibitors.

dDAT:

Even though a large number of homology models based on LeuT have been developed for the monoamine transporters and have been found to provide data in good accord with experimental results,[35-37] many questions still remained unanswered. The limitation of these models are the result of the fact that with 20 – 24 % the sequence identity of LeuT and the monoamine transporters is quite low,[38] although the identity of the amino acids in the surrounding of the aforementioned S1 binding site is much higher, i.e. 76% comparing LeuT with hSERT.[39] Due to the differences and the fact that LeuT is a prokaryotic homologue we still lack many facts required for a complete understanding of the mammalian NSSs, i.e. occurrence of substrate selectivity, posttranslational modifications, the mechanism of inhibition by bound inhibitors, etc..[29] The results of Penmatsa et al. in 2013 were therefore a highlight in monoamine transporter research as they represent the first example of the crystal structure of an eukaryotic monoamine transporter, the dopamine transporter of *Drosophila melanogaster*, dDAT.[29] dDAT is a promising tool for further homology models for the mammalian NSSs, as it has a high sequence identity of 65 % compared to hDAT and hNET. Additionally, it is a pharmacologic hybrid of hDAT and hNET. That means that dDAT shows a substrate selectivity and transport kinetics more similar to hDAT than hNET in uptake experiments, but at the same time the rank order of potency for nonsubstrate inhibitors was found to be distinctly more similar with the one found for hNET than for hDAT.[40]

The currently unique crystal structure revealed some new aspects which improves our understanding of monoamine transporters:

First of all, the crystal structure of dDAT is in excellent accordance with the structure published for LeuT (see Figure 6a), exhibiting the same topology including C_2 pseudo-symmetry observed for the latter (see also Figure 4).[29]

The most important new finding regarding monoamine transporters derives however from the way the co-crystallized TCA molecule, namely nortriptyline, is bound in dDAT. This provides completely new insights into the mechanism of inhibition when compared to that of LeuT.[29] Unlike the structures of LeuT-inhibitor complexes (see above), nortriptyline is not bound in the extracellular vestibules (S2), but in a cavity equivalent to the substrate binding pocket S1 of LeuT mainly formed by TM1, TM3, TM6, and TM8 as shown in Figure 6b. It is probable that the binding of nortriptyline sterically prevents a spatial approach of TM1 and TM6 to TM3 and TM8, which is supposed to be crucial for the transport process. A stabilization of the transporter in a so-called *outwards-open state*, which prohibits the transport of substrate molecules, by TCAs is therefore assumed as mode of action of inhibitors for the monoamine transporters.[29]

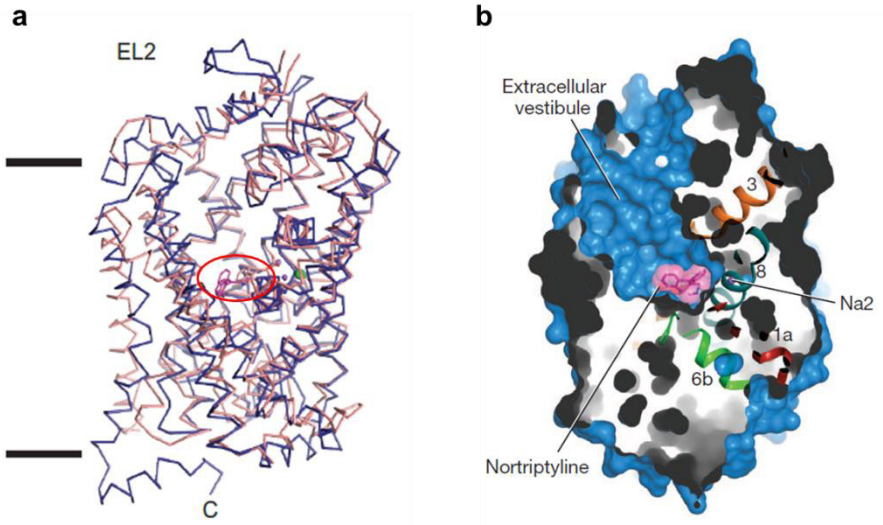


Figure 6: a) Superposition of dDAT (dark blue) with LeuT (salmon, bound to sodium, PDB code 3F3A) showing an excellent accordance between these two crystal structures. The bound TCA, nortriptyline, is labelled by a red circle. b) Surface representation of dDAT with a nortriptyline molecule bound in S1. TMs showing interactions with nortriptyline are colored as follows: TM1 (red), TM3 (orange), TM6 (green), and TM 8 (cyan). Nortriptyline is shown in magenta in the middle of the model (adopted from Penmatsa et al.[29]).

1.3 Screening Tools in Drug Discovery

The development of new, effective drug substances such as antibiotics, chemotherapeutics, etc., is a decisive factor in medicinal advance. While older substances, e.g. penicillin, were often found by chance, today the drug discovery process is well-organized and target-oriented. A representative scheme is shown in Figure 7.

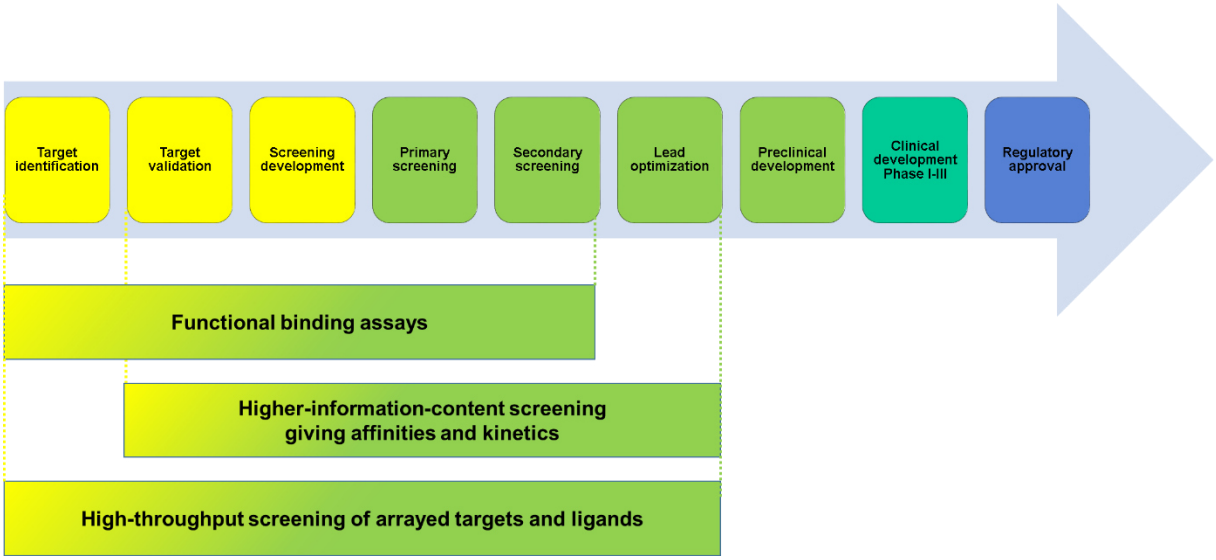


Figure 7: Simplified scheme of modern drug discovery process highlighting the application areas of binding assays below the respective stages (adopted from Cooper [41]).

After a target structure is identified and validated for inducing or affecting a pharmacologic effect, the next question is how we can influence, i.e. inhibit or activate, this target? To trigger such an effect, a mediator is necessary, e.g. small molecules or an increasing number of proteins are also used nowadays for this purpose. These mediators have to interact with the chosen target structure to affect them. The first and therefore most important step is to ensure that this substance binds to the target of interest.

Since the 1970s so called binding studies could be performed employing radioactively labelled ligands, also known as *radioligand binding assays* (for further information see below, *1.3.1 Radioligand Binding Assays vs MS Binding Assays*).[42] Even though radioligand binding assays were and still are a powerful tool in the drug discovery process, great efforts were made to develop other methods characterizing target-ligand interactions. Most research focused on finding ways to avoid the use of radioactivity in equivalent assays. This objective was achieved using optical sensors, measuring changes in the properties of light (e.g. due to absorption or fluorescence), as read-out, instead of radioactivity. Some of these approaches for the investigation of target-ligand interactions, e.g. *fluorescence resonance energy transfer* (FRET) and *fluorescence polarization* (FP), also require labelling either of the target structure and/or of the ligand.[43] One light based, nonlabelled method called *surface plasmon resonance* (SPR) was also developed.[41]

Though a large variety of techniques for the investigation of target-ligand binding exists, each has its disadvantages. Labelling, as required for radioligand binding and fluorescence assays (i.e. FRET and FP), is time consuming and expensive. Additionally, labelling can affect the affinity of the ligand towards the target, especially when sterically demanding fluorescence-labels are employed.[43] Even the nonlabelled SPR technique has disadvantages including high costs [43] and the employed targets have to be purified, a process that can be quite challenging in the case of membrane-bound proteins, and immobilized.[44]

For these reasons our group developed a new method termed MS Binding Assays which employs a nonlabelled ligand and allows the application of membranous targets, while, at the same time, allowing a reasonable throughput at low costs per sample.[45-47]

1.3.1 Radioligand Binding Assays *vs* MS Binding Assays

The following chapter focuses on MS Binding Assays comparing them with the long established radioligand binding assays. Similarities, differences, advantages, and disadvantages are also discussed.

As already described above, radioligand binding assays employ a radioactively labelled ligand, also called radioligand or simply marker. The labelling of the ligand is necessary to enable a reliable quantification even of very low concentrations of the formed target-marker complexes. Radioactivity can be quantified with high sensitivity via scintillation counting, which means that even targets being available in low concentrations, such as membrane associated proteins, can be investigated.

The general procedure for radioligand binding assays is depicted below (Figure 8):

A radioactively labelled substance, which is known to bind to the target, is incubated with some material containing the target for a defined period of time, the length of which is dependent on the aim of the experiment performed (for further information, see below *1.3.2 Types of Ligand Binding Assays*). Native material of the respective organs, whole cells, or membrane fragments as well as purified, soluble targets (if they can be immobilized) can be employed as source for the target. In order to be able to quantify the amount of radioligand bound to the target, the formed target-marker complexes have to be separated from the incubation mixture (i.e. free radioligand). This can be achieved via filtration or centrifugation. The last step of the assay is the quantification of the fraction of bound radioligand via scintillation counting.[48]

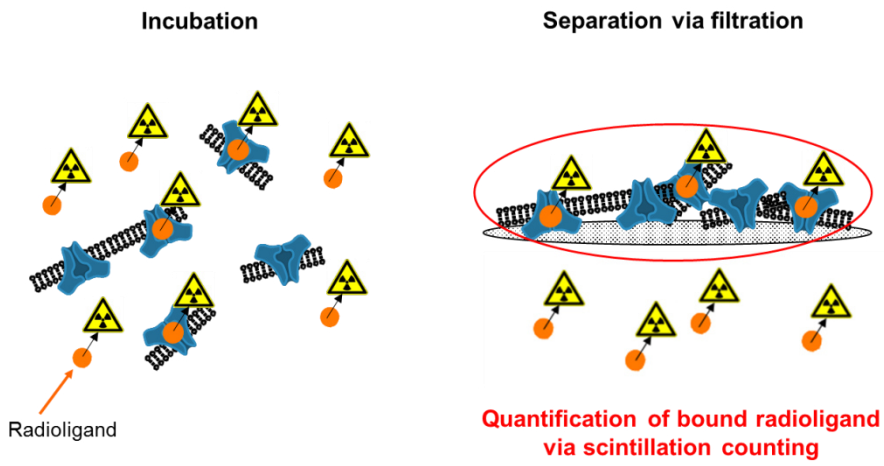


Figure 8: Performance of a radioligand binding assay. Radioligand (orange spheres with radioactivity sign) are incubated with a target (blue, in this case membrane associated proteins) and separated via filtration. Finally, bound radioligands are quantified via scintillation (red ellipse).

Unfortunately, this radioligand binding assays are accompanied by serious disadvantages as a consequence of the labelling. Radioactivity is dangerous both to the environment and humans, and strict legal regulations have to be followed when holding, working, and disposing radioactive substances and the resulting contaminated waste. MS Binding Assays offer a promising substitute for radioligand binding assays. This was made possible by the progress concerning throughput, selectivity, and sensitivity of modern mass spectrometry in the last decades.[49] Radioactive labelling of the employed marker has therefore become dispensable.

The main difference between MS Binding Assays and conventional radioligand binding assays is the principle employed for detection. Mass spectrometry is employed instead of scintillation measurements. Other parameters, such as incubation system, incubation time, temperature, material containing the target, etc., can be adopted from respective radioligand binding assays. Sometimes an additional sample preparation step can be necessary to remove, e.g. insoluble cell fragments, which can clog the employed *high-performance liquid chromatography* (HPLC) column or the *electrospray ionization* (ESI) needle, but would not affect scintillation counting. However, MS Binding Assays can substitute all types of experiments (see below, *1.3.2 Types of Ligand Binding Assays*), which have been realized employing radioligand binding assays to date, and offer some additional advantages:

First, due to the fact that no labelling of the marker is required for MS Binding Assays, every native compound is suitable as marker substance as long as it binds with an appropriate affinity towards the target. The number of appropriate, commercially available marker substances for the target of interest is therefore significantly higher than for radioligand binding assays.

Secondly, mass spectrometry allows the simultaneous detection and quantification of several compounds in one sample due to the differing monitored mass transitions (i.e. masses of the

respective $[M+H]^+$ parent ions and the resulting fragment ions). It is therefore possible to employ different selective ligands for several target structures in one pot and reliably quantify each individual marker substance in the presence of the other employed marker substances.

1.3.2 Types of Ligand Binding Assays

Binding studies can be subclassified in three different types of experiments, i.e. saturation, kinetic studies, and competitive experiments. Each delivers unique information about the binding behavior of a ligand towards a specific target.

The general workflow of all three types is as follows:

The ligand is incubated with the respective target. Depending on the information sought and as a consequence of the type of experiment to be performed, incubation is terminated after a defined time period via a separation step, which separates the bound from the free ligand (see also *1.3.1 Radioligand Binding Assay vs MS Binding Assay*). The amount of bound ligand is then quantified using the corresponding detection principle, i.e. scintillation counting or mass spectrometry.

The performance and the resulting information of the individual experiment types is presented in the following chapters.

1.3.2.1 Saturation Experiments

Saturation experiments are the most fundamental binding experiments. Here the binding of a ligand to a target is monitored by directly measuring the amount of ligand that binds to the target. From the data obtained a saturation curve can be established that allows the determination of the binding affinity of that ligand as well as the target concentration.

For this purpose, increasing concentrations of the ligand are incubated with a constant concentration of the target for a period sufficient to reach equilibrium state. The target-marker complexes are then separated by filtration or centrifugation and the amount of bound ligand is quantified. This quantified fraction represents the so-called *total binding* of the ligand, which is sum of the actual binding of the ligand to the target, called *specific binding*, and the binding of the ligand towards other binding sites, i.e. other protein structures or filter material, which is called *nonspecific binding*.[48, 50, 51] However, for the calculation of the affinity (equilibrium dissociation constant K_d) as well as the maximum amount of binding sites (B_{max}), the concentration of specifically bound marker has to be determined, which can only be calculated by subtracting the nonspecific binding from the total binding. Whereas total binding can be obtained as described above, nonspecific binding has to be determined in a separate experiment. The only difference between this experiment and the determination of total binding is the presence of another ligand addressing the target in a vast excess. This new ligand should occupy the same binding sites at the target as the marker does. The quantified, remaining

binding of the marker is therefore a result of nonspecific binding to other binding sites. In the next step, specific binding can be calculated and plotted *vs* the employed nominal marker concentration (Figure 9a and 9b). Based on the data points for specific binding a saturation isotherm can be created, which provides certain information: The plateau of the specific binding isotherm indicates the maximum number of binding sites, also called B_{max}, and the nominal concentration that produces an occupancy of 50 % of all relevant binding sites, is equal to the equilibrium dissociation constant, the K_d-value (Figure 9b).[50, 52]

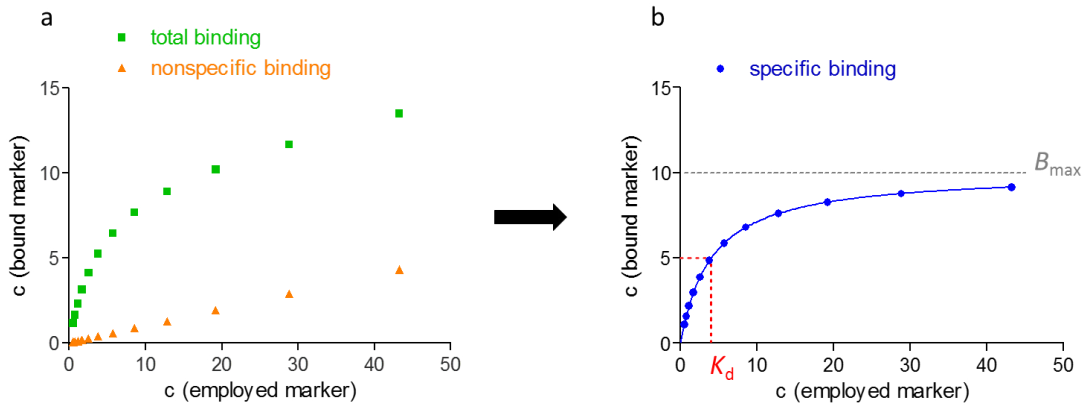


Figure 9: Typical saturation experiment. a) Quantified concentrations of total binding (green) and nonspecific binding (orange) are plotted vs the employed nominal concentration of the marker. b) Specific binding (blue) (difference of the shown total and nonspecific binding) is also plotted vs the employed nominal concentration of the marker, via nonlinear regression a saturation isotherm (blue line) can be created from which K_d (red) and B_{max} (grey) can be deduced.

There are several rules for the performance of such saturation experiments:

First, the employed nominal marker concentrations should cover a wide range around the assumed affinity (K_d-value), but at least from 0.1 K_d to 10 K_d. Secondly, the constant target amount for incubation should have a maximal concentration of 0.1 K_d.[52]

The latter is necessary to avoid an effect called marker depletion. This effect occurs when the concentration of the bound marker becomes a significant fraction of the employed nominal concentration. Since the applied calculation models are based on the free, unbound marker, the actual employed nominal concentration of the marker can only be used as substitute for the free marker concentration, if no marker depletion occurs. Otherwise the use of the calculation models leads to erroneous K_d- and B_{max}-values.[52]

1.3.2.2 Kinetic Studies

Kinetic studies provide information about the time course of formation of target-marker complexes and their dissociation. This information can e.g. be used to estimate the incubation time required to reach equilibrium state in saturation experiments.

Two types of kinetic experiments can be distinguished: association and dissociation experiments. Dissociation experiments are used for the determination of the dissociation rate constant (k_{off}) and half life of the target-marker complex ($t_{1/2}$). A prerequisite of this type of experiments is the existence of an appropriate number of complexes that enables a reliable quantification of the remaining, non-dissociated complexes during the experiment. In most cases this is ensured by a pre-incubation of a constant target concentration with a constant marker concentration. The time dependent dissociation of these complexes is then investigated. This dissociation can be initiated by two ways: Either hand, a second ligand is added in a vast excess (i.e. displacer), which occupies vacant binding sites of the target-marker complexes after dissociation and thus prevents re-association of the marker. Or the formed target-marker complexes can be diluted, at least by factor of 30.[34] This extensive dilution should ensure that during dissociation the concentration of the free marker is negligible for a re-association.[52] Whichever approach is used to initiate the dissociation process, the experiments are stopped after defined time intervals via separation. In this case commonly only filtration can be used, because a centrifugation step would require too much time and it would not be possible to realize short time increments. The remaining amount of target-marker complexes is then quantified and plotted *vs* the time (Figure 10a). The half life ($t_{1/2}$) and dissociation rate constant (k_{off}) of the target-marker complex can be calculated via nonlinear regression from the dissociation curve.

In contrast to k_{off} the corresponding association rate constant (k_{on}) cannot be determined directly, because the association does not proceed alone. There is always a parallel, inevitable dissociation of the formed target-marker complexes. It is therefore only possible to determine the so called *observed rate constant* (k_{obs}), which can be used for calculation of k_{on} according to the equation: $k_{obs} = k_{on} \times L + k_{off}$.[52] As this equation makes clear, k_{obs} is dependent on the concentration of the ligand (L). For the determination of very fast association rates (e.g. due to high incubation temperatures) a marker concentration as low as possible is therefore preferable, as this reduces k_{obs}. As in dissociation studies, a constant target concentration is incubated with a constant, preferably low concentration of the marker, but in this case incubation is terminated after defined time intervals via a separation step and the already formed amount of target-marker complexes is quantified and plotted *vs* the time (Figure 10b). Based on these data points, the employed marker concentration, and the previously determined dissociation rate constant (k_{off}), the desired value for k_{on} can be calculated according to the equation described above.

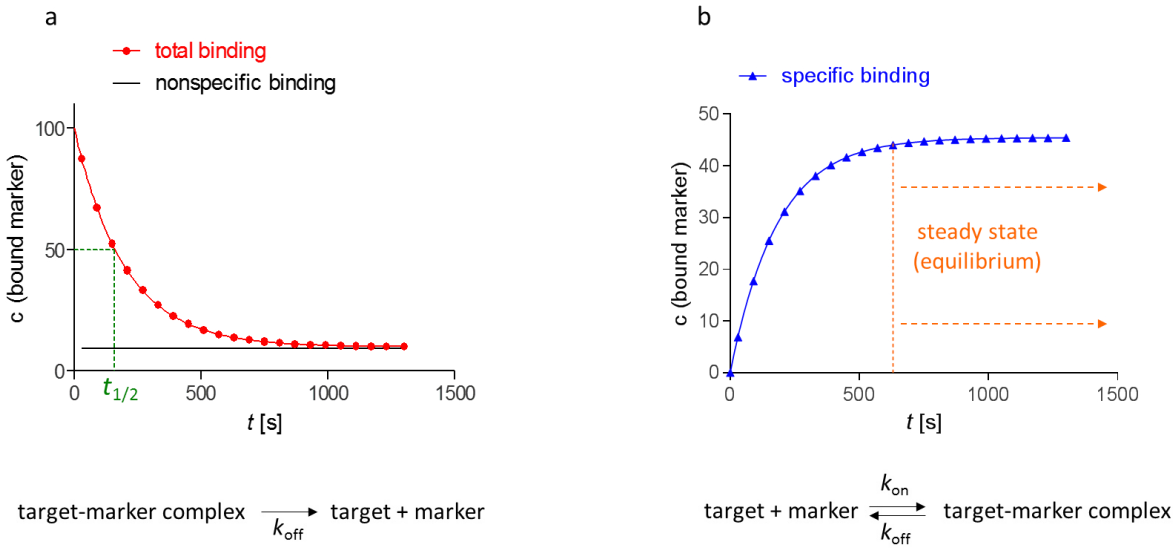


Figure 10: Typical kinetic studies. a) Dissociation experiment showing the time dependent decrease of total bound marker (red circles and dissociation curve) until the level of nonspecific binding (black line) is reached, and half life of the target-marker complex ($t_{1/2}$, green). b) Association experiment (blue triangles and association curve) showing the formation of the target-marker complex with parallel dissociation, steady state phase (orange) indicates equilibrium state of both process.

The results of such kinetic studies can be used to estimate a sufficient incubation time to reach equilibrium of the incubation system, which should be achieved after five times of the dissociation half life ($t_{1/2}$) of the target-marker complex. The studies also provide information about the affinity of the marker substance towards the target according to the equation: $K_d = k_{off} / k_{on}$.[52]

1.3.2.3 Competitive Experiments

Competitive studies are one of the most important applications of binding assays and are used for the determination of affinities of unknown substances, also called competitors. It should be noted that this is an indirect approach for affinity characterization since it is not the binding of the competitor that is monitored, but the influence of this competitor on the binding of the employed marker at the target. A prerequisite for the performance of such competitive binding experiments is therefore a previous characterization of the employed marker regarding its K_d-value at the respective target but also regarding its kinetics to determine an incubation time, which ensures an equilibrium state of the incubation system.

For competitive binding experiments increasing concentrations of a test compound are added to the incubation mixture (i.e. a constant target concentration and a constant marker concentration). The test compound and the marker are then competing for binding sites at the targets in the incubation mixture. After equilibrium of the system is reached, incubation is

stopped and the target protein including bound marker and bound competitor are separated from free marker/competitor, and the amount of bound marker is quantified. If the competitor addresses the same binding site as the marker, an increasing concentration of the competitor is accompanied by a reduction of the bound fraction of the marker. This is the result of the displacement of the marker at the binding sites by the competitor until the level of nonspecific binding of the marker substance is reached. As a last step, the specifically bound marker concentration, represented as a percentage of "total binding" (in this case termed as specific marker binding in absence of a competitor), is plotted *vs* the logarithmic concentration of the test compound for data analysis (Figure 11). The concentration of the competitor that reduces specifically bound marker to 50 % is called IC_{50}-value and provides information about the inhibitory effect of the test compound on the marker binding, but does not represent an independent physical variable.

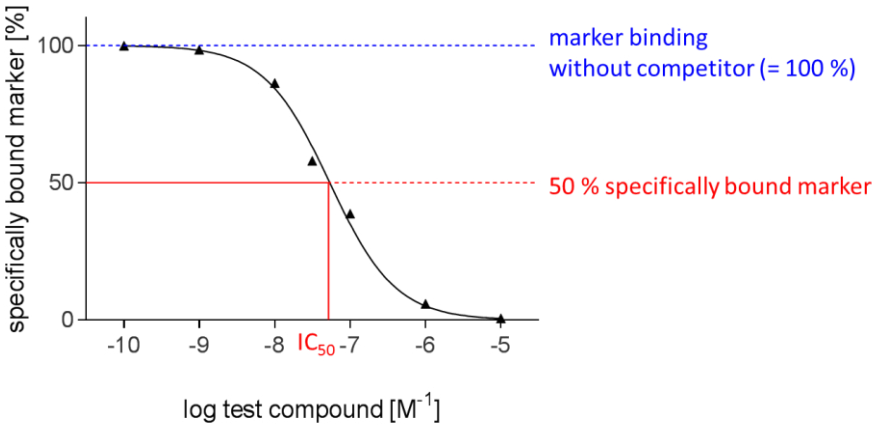


Figure 11: Typical competition curve. The increasing concentration of the test compound is accompanied by a reduced specific binding of the marker, in which 100 % represents the specific binding in absence of any inhibitor (blue dashed line) and IC_{50} shows the concentration of the competitor (red solid line), which reduces specific marker binding to 50 % (red dashed line).

But the IC_{50}-value can be used to calculate the affinity, i.e. the inhibition constant K_i, according to the equation of Cheng-Prusoff:[53]

$$K_i = \frac{IC_{50}}{1 + \frac{[M]}{K_d}}$$

In this equation K_i is the inhibition constant, which represents the affinity of the test compound according to the law of mass action, [M] represents the free concentration of the marker, and K_d the equilibrium dissociation constant of the marker. However, it should be noted that the calculation models for the data analysis of such competitive experiments are also based on the free, unbound marker concentration (see equation above, [M]). As long as the marker depletion is negligible, which is typically ensured by a employed target concentration ≤ 0.1 K_d,

the actual employed nominal marker concentration can be used as substitute for the free marker concentration [M] (for further information see also *1.3.2.1 Saturation Experiments*).

Competitive binding experiments have two main advantages: The affinity of an unknown substance towards a target can be determined without individual quantification methods for each substance and without time consuming characterization of their K_d-values in separate saturation experiments. Additionally, a substantially wider range of concentrations for the test compound can be employed since the concentrations of bound marker are used as surrogate therefore.

2 Aims and Scope

Even though a large number of different antidepressants (*1.1.2 Pathology and Therapy*) exists, only approximately 65 % of depressive patients show a sufficient therapeutic response to medical treatment with antidepressants.[54] There is therefore a substantial need for new compounds acting as antidepressants.

Binding studies are an indispensable tool for drug discovery (*1.3 Screening Tools in Drug Discovery*). Therefore, it was the aim of my research to develop a new binding assay which can be employed for binding studies addressing all three monoamine transporters (i.e. hDAT, hNET, and hSERT). The developed binding assay should serve as a screening tool for the identification of new inhibitors of one, two, or all three monoamine transporters, as their inhibition is considered today to represent the most promising approach in the treatment of depressions. The concept of MS Binding Assays recently used in our group for the development of binding assays for the murine γ-aminobutyric acid transporter subtype 1 (mGAT1)[47] and hSERT[45] was adapted for this purpose. MS Binding Assays have considerable advantages over the well-established radioligand binding assays, particularly with regard to safety of humans and the environment, since no radioactively labelled markers are needed. At the same time they offer all possibilities of radioligand binding assays with regard to the performance of the experiments and the resulting information. The general setup, i.e. incubation of target and marker, separation of formed target-marker complexes from the incubation system, and finally quantification of the bound marker, can be adopted from established radioligand binding assays. The main difference between these two types of binding assays is the method employed for quantification of the bound marker, which is mass spectrometry in MS Binding Assays but scintillation counting in radioligand binding assays (for further information see *1.3.1 Radioligand Binding Assays vs MS Binding Assays*). As very low amounts of bound marker have to be determined in these two types of assays, a highly sensitive quantification method is needed. As a consequence, such a quantification method based on mass spectrometry is an essential prerequisite for the performance of MS Binding Assays.

Two different strategies were conceivable for the intended MS Binding Assays addressing hDAT, hNET, and hSERT:

- Employing one marker substance addressing all three targets which is only applicable if the individual targets are available in separate form (e.g. by heterologous expression), or
- employing three different markers each with a selectivity for one target, which should allow an analysis of hDAT, hNET, and hSERT in one single experiment.

The application of one marker for all three targets can be considered as a more effective approach than the latter one, as the development of the above mentioned quantification method for the marker employed in MS Binding Assays can be considered as the most laborious and time consuming step when developing such assays.

We therefore decided to follow the first approach. An essential prerequisite was now a source providing the target proteins for hDAT, hNET, and hSERT in individual form. Expressing the individual targets in heterologous systems was considered the best choice as it has substantial advantages: The presence of targets (e.g. receptors, other transporters, etc.) in samples resulting from such cell systems potentially interfering with the desired target-ligand binding is reduced and a higher concentration of the demanded target in the cell preparation than in native brain preparations can be achieved. For HEK293 cells stably expressing hSERT such an expression system was already available in our group,[55] but equivalent HEK293 cell lines stably expressing the other targets demanded, i.e. hDAT and hNET, had still to be established.

Additionally, a native marker substance had to be found, which binds with a high affinity, ideally in the low nanomolar range, towards all three monoamine transporters (i.e. DAT, NET, and SERT). A highly sensitive MS based method for its quantification in biological matrices, as they result from respective binding samples, had to be developed as well. For quantitation a triple quadrupole mass spectrometer equipped with an ESI source for ionization was chosen as this system had already been successfully used for the marker quantification in formerly established MS Binding Assays.[45-47] However, ESI-MS/MS analysis is commonly prone to ion suppression, which is generally caused by components of the resulting biological matrix contained in the analytical sample. It was therefore intended to separate the matrix from the analyte (formerly bound marker) by *high performance liquid chromatography* (HPLC) connected upstream to the mass spectrometer. To compensate for uncontrolled sample loss or sample concentration and for any changes on the ionization efficiency an internal standard should be employed. To this end, a poly-deuterated analogue of the employed marker seemed most suitable. That way a reliable quantification even if the matrix might not be completely separated by HPLC should be amenable. The final method should be characterized by a short cycle time to enable a reasonable throughput of samples to remain competitive with comparable radioligand binding assays. To ensure the reliability and robustness of the method, it should be verified regarding its selectivity, the correctness of the calibration curve, *lower limit of quantification* (LLOQ), precision and accuracy according to the FDA guidance for bioanalytical method validation.[56]

With the MS Binding Assays established in this way, the affinity of the selected marker towards hDAT, hNET, and hSERT should be determined in saturation experiments as well as the

affinities and selectivities of various known monoamine transporter inhibitors in competitive MS Binding Assays.

3 Results and Discussion

3.1 Selection of the Native MS Marker–Indatraline

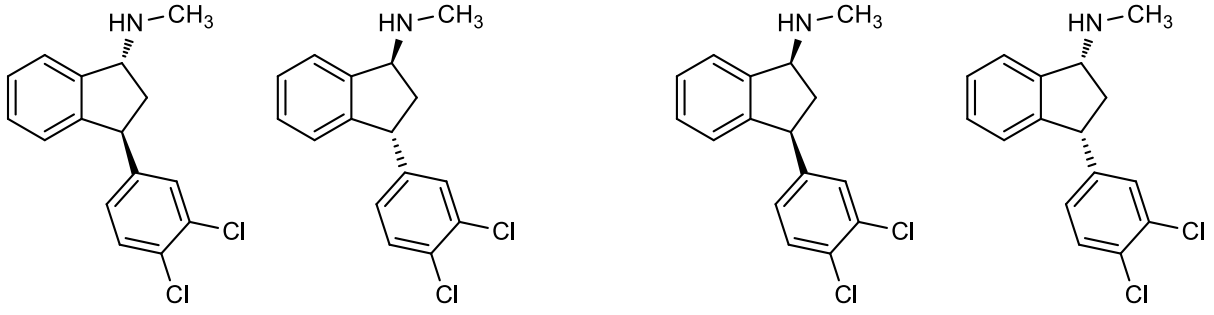
Next to the availability of the desired targets, the second basic requirement for the development of MS Binding Assays is an appropriate marker substance, which ideally should have the following characteristics:

First, it should exhibit an ideal level of affinity towards the relevant target. As a low k_{off} and a long $t_{1/2}$ are typically accompanied by a high affinity of marker substance towards the target the affinity should be high enough to enable filtration without affecting the fraction of bound marker due to uncontrolled dissociation of the target-marker complex (see 1.3.2.2 *Kinetic studies*). However, the affinity should not be too high to avoid that only very low target concentrations can be employed which might interfere with a reliable quantification of the bound marker (i.e. application of the target at a concentration ≤ 0.1 K_d and of the marker at 0.1 to 10 K_d, for further information see *1.3.2.1 Saturation Experiments*). The ideal affinity of a MS marker therefore lies in the low nanomolar range.

Secondly, since the readout of the assay should be accomplished by means of mass spectrometry, preferably by *electrospray ionization* (ESI), the marker substance should possess physicochemical properties allowing a good atmospheric ionization to enable its highly sensitive quantification.

Indatraline [*rac*-(1*R*,3*S*)-3-(3,4-dichlorophenyl)-1-methylamino-2,3-dihydro-1*H*-indene] has been identified as potential marker substance during extensive literature research, as it fulfills the first mentioned requirement, which means it is a highly affine triple reuptake inhibitor of DAT, NET, and SERT. Unfortunately, no data were available regarding indatraline's mass spectrometric properties, i.e. ionization, fragmentation, etc., and its suitability towards a highly sensitive quantification via MS was therefore far from certain. Furthermore, indatraline possesses two stereogenic centers giving rise to four stereoisomers, of which only the two enantiomers with *trans*-configuration inhibit nonselectively all three monoamine transporters and are called indatraline (see Figure 12). The racemic form of the *cis*-configured diastereomer was however reported to be selective for SERT. Bogeso et al. found for the two enantiomers of indatraline an eudismic ratio of five, nine, and 51 for SERT, NET, and DAT, respectively, with (1*R*,3*S*)-indatraline being the eutomer for all three monoamine transporters.[57] The different pharmacological profiles found for the two enantiomers of indatraline at the three monoamine transporters prompted me to characterize the binding affinities of both individual enantiomers in the intended MS Binding Assays. While the racemic indatraline is commercially available its single enantiomers are not. Moreover, for the preparation of the pure enantiomers only one method based on resolution of the racemate via crystallization[57] and several

asymmetric synthesis applying chiral catalysts[58-63] or chiral functional group transfer reagents[64] had been described when this study was started. In addition, no efficient analytical method for the determination of the enantiopurities of the two enantiomers, i.e. (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, was known then. The methods for the determination of the *enantiomeric excess* (*ee*) of (1*R*,3*S*)- and (1*S*,3*R*)-indatraline that had been published at that time, were based on polarimetry[57, 58] or ^{1}H NMR spectroscopy employing a chiral shift reagent which allowed a quantification of *ee* up to 95 % only.[57]



indatraline

Figure 12: The four stereoisomers of 3-(3,4-dichlorophenyl)-1-methylamino-2,3-dihydro-1H-indene.

It was therefore necessary to first develop an analytical method, which allows a reliable determination of the enantiopurity of (1*R*,3*S*)- and (1*S*,3*R*)-indatraline up to at least 99 % *ee*. Since I intended to gain access to the individual enantiomers by chiral resolution of the racemate via the crystallization of diastereomeric salts the analytical method for the determination of the enantiopurity was considered to be a useful tool to monitor the progress of the resolution process.

3.2 Summary of Published Results

3.2.1 First Publication:
Enantiopurity Determination of the Enantiomers of the Triple Reuptake Inhibitor Indatraline

Indatraline is a compound of great pharmacological interest due to its high potency as inhibitor of all three monoamine transporters.[57] On the basis of the results of Bogeso et al., who found an eudismic ratio of five, nine, and 51 for SERT, NET, and DAT, respectively, with (1*R*,3*S*)-indatraline representing the eutomer,[57] especially the enantiomers, (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, have been in the focus of research in medicinal chemistry in the last decade.[58-64] Unfortunately, no powerful and reliable analytical method existed then for the precise determination of the enantiopurity of the single enantiomers, which is essential if the pure enantiomers are to be characterized pharmacologically.

Such a method had therefore to be developed. For sertraline, an analogue of indatraline, a method for the determination of the *ee* based on ^{1}H NMR spectroscopy employing a chiral shift agent (CSA) is known from literature.[65] This approach served as a basis for the development of a method for the determination of the enantiopurity of the enantiomers of indatraline. To achieve this the influence of different CSAs and their stoichiometry ratios with regard to the analyte indatraline, the kind of solvent, and applied temperatures on the chemical shift difference between the signals of the diastereomeric indatraline-CSA complexes were investigated. The signal width, which also affects the signal resolution in the respective ^{1}H NMR spectra, was examined as well. According to these findings, the best conditions for the determination of the enantiopurity of (1*R*,3*S*)-indatraline in ^{1}H NMR are as follows: the use of the (*S*)-enantiomer of Mosher's acid [(*S*)-(-)-α-methoxy-α-trifluoromethyl-phenylacetic acid] as CSA in a molar ratio of 1:1 with regard to the analyte, i.e. (1*R*,3*S*)-indatraline, and chloroform-*d* as sample solvent, with the measurement being performed at 25 °C. Applying this ^{1}H NMR method to enantioenriched material, as it can be obtained from resolution via crystallization, allowed the determination of enantiopurities up to 98.9 % *ee*.

To determine even higher enantiomeric excesses for indatraline's enantiomers, a more sensitive method based on HPLC should be developed. The following aspects were investigated during the development of this method: the influence of buffer type, its concentration, the pH value of the aqueous fraction of the mobile phase, the kind and percentage of organic modifier, the temperature, the injection volume, and the sample milieu on chromatographic parameters (i.e. peak intensity, retention time, asymmetry factor, etc.) and the extent on the separation of the enantiomers (i.e. resolution). The final HPLC method consisted of a chiral stationary phase based on modified β-cyclodextrins, a mobile phase composed of triethylammonium acetate (TEAA) (0.1 %, v/v) pH 4.0 and acetonitrile (85:15,

v/v) with the analysis being performed at a flow rate of 1.0 mL min^{-1} and at a temperature of 20 °C. This method was successfully validated according to ICH guidance Q2(R1) regarding specificity, accuracy, precision, linearity, and quantitation limit. Not only is this the first HPLC quantitation method for indatraline described in literature so far, it also enabled a reliable determination of enantiopurities up to 99.75 % *ee* for (1*R*,3*S*)-indatraline and up to 99.67 % *ee* for (1*S*,3*R*)-indatraline.

Declaration of contributions:

Lars Allmendinger and Gerd Bauschke synthesized indatraline and performed together with me the resolution via crystallization yielding (1*R*,3*S*)-indatraline in an enantiomeric excess of 99.54 % *ee*. The bachelor thesis *"Entwicklung und Validierung einer HPLC-Methode zur Enantiomerentrennung von 1R,3S- und 1S,3R-Indatralin an einer dimethylierten β-Cyclodextrin Phase"*[66] by Sebastian Brodkorb was prepared under my supervision. Based on preliminary experiments regarding stationary and mobile phase performed by myself, Sebastian Brodkorb developed an HPLC method for the determination of the enantiopurity of (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, respectively. He employed this HPLC method to analyze samples obtained by resolution of racemic indatraline via crystallization to determine the enantiomeric excess (*ee*) of the desired enantiomer, (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, respectively. The validation according to the ICH guidance Q2(R1), numerous control experiments for HPLC results, as well as the ^{1}H NMR experiments were performed by myself. I also wrote the manuscript and generated all graphics and tables. Lars Allmendinger wrote the supporting information. Georg Höfner and Klaus T. Wanner corrected the manuscript.

Enantiopurity Determination of the Enantiomers of the Triple Reuptake Inhibitor Indatraline

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ABSTRACT The present study describes the development of two approaches for the determination of the enantiopurity of both enantiomers of indatraline. Initially, a method was developed using different chiral solvating agents (CSAs) for diastereomeric discrimination regarding signal separation in ^{1}H nuclear magnetic resonance (NMR) spectroscopy, revealing MTPA as a promising choice for the differentiation of the indatraline enantiomers. This CSA was also tested for its ideal molar ratio, temperature, and solvent. Optimized conditions could be achieved that made determination of enantiopurity for (1*R*,3*S*)-indatraline up to 98.9% enantiomeric excess (*ee*) possible. To quantify even higher enantiopurities, a high-performance liquid chromatography (HPLC) method based on a modified β-cyclodextrine phase was established. The influence of buffer type, concentration, pH value, percentage and kind of organic modifier, temperature, injection volume as well as sample solvent on chromatographic parameters was investigated. Afterwards, the reliability of the established HPLC method was demonstrated by validation according to the ICH guideline Q2(R1) regarding specificity, accuracy, precision, linearity, and quantitation limit. The developed method proved to be strictly linear within a concentration range of 1.25–1000 μM for the (1*R*,3*S*)-enantiomer and 1.25-750 μM for its mirror image that enables a reliable determination of enantiopurities up to 99.75% *ee* for the (1*R*,3*S*)-enantiomer and up to 99.67% *ee* for the (1*S*,3*R*)-enantiomer. *Chirality 25:923–933, 2013.*

KEY WORDS: ICH Q2(R1); validation; HPLC; NMR; CSA; cyclodextrine; enantiomeric excess

INTRODUCTION

Indatraline [*trans*-3-(3,4-dichlorophenyl)-*N*-methyl-indan-1-amine, *rac*-**1**], also known as Lu 19-005 (Fig. 1), was reported for the first time in 1982 in a study of K.P. Bøgesø[1] that aimed at the development of new psychopharmacologically active drugs for the treatment of psychic disorders such as depression. The *trans*-configured 3-phenyl-indan-1-amines with different halogen substitution patterns that in addition to indatraline (*rac*-**1**) had been studied as part of this project demonstrated nonselective inhibition of the neurotransmitter transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), proteins that are responsible for signal termination and recycling of the respective neurotransmitters, whereas most of the *cis*-configured 3-phenyl-indan-1-amines selectively inhibited SERT.[2]

Being *trans*-configured, indatraline (*rac*-**1**) also displayed similar and also very low IC_{50} values in uptake experiments for each of the three above-mentioned neurotransmitter transporters based on rat synaptosomes [dopamine (DA) = 0.99 nM, norepinephrine (NE) = 0.26 nM, serotonin (5-HT) = 0.48 nM]. Also for the single enantiomers of indatraline (*rac*-**1**), low to negligible selectivities as inhibitors of these transporters (DAT, NET, and SERT) were found, although one enantiomer, the (1*R*,3*S*)-stereoisomer, appeared to be distinctly more potent than its stereoisomeric counterpart. The eudismic ratio in favor of the (1*R*,3*S*)-enantiomer ranged from 5 (5-HT) to 51 (DAT).[2]

The neuronal systems of DA, NE, and 5-HT are involved in different mental disorders such as depression, Parkinson's disease, schizophrenia, and are also associated with drug abuse. Dual (NE and 5-HT, or NE and DA, respectively) as well as selective NE and selective 5-HT reuptake inhibitors [e.g., sertraline (**2**), Zoloft, a *cis*-configured structural analog of indatraline; see Fig. 1] are widely used in the treatment of depression, but about 35% of patients with depression do not or only partially respond to these agents.[3] That's why current efforts include the development of triple reuptake inhibitors, as so-called next-generation antidepressants, for a faster onset and better efficiency in therapy.[4] Due to its high and close affinities to DAT, NET, and SERT and its long-acting effect, indatraline (*rac*-**1**), and especially its (1*R*,3*S*)-enantiomer, (1*R*,3*S*)-**1**, could serve as a model substance to test the potential of this new class of agents,[5] e.g., for the treatment of depression[6] or even cocaine abuse.[7]

For the characterization of the pharmacological and pharmacokinetic properties of the enantiomers of a chiral compound, only highly enantioenriched samples should be used, as the wrong isomer will falsify the data the more it is present in the samples studied. In the study of the determination of the eudismic ratio of neurotransmitter transporter inhibition by the indatraline enantiomers, Bøgesø et al. used samples with enantiopurities of ≥ 95%.[2] The eudismic ratio for DAT inhibition of indatraline enantiomers was found to be 51 [(1*R*,3*S*)- as compared to the (1*S*,3*R*)-enantiomer]. But considering the enantiopurity of the samples used, which in the worst case could still contain 2.5% of the wrong enantiomer, the true eudismic ratio of the indatraline enantiomers as DAT inhibitors might possibly be distinctly higher than that. As can be seen from this

Additional Supporting Information may be found in the online version of this article.

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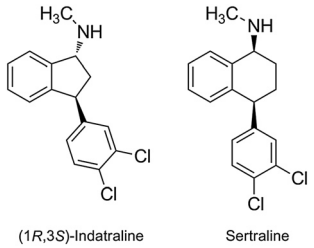

Fig. 1. (1*R*,3*S*)-indatraline [(1*R*,3*S*)-**1**] and sertraline (1*S*,4*S*)-4-(3,4-dichlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine) (**2**).

example, there is a substantial need for analytical methods that enable a reliable and precise determination of the enantiopurity even of highly enantioenriched indatraline samples. Of course, such methods will also be valuable tools for the preparation of the indatraline enantiomers, independent of whether this is accomplished by a resolution via crystallization (by formation of diastereomers) as described by Bøgesø et al.,[2] or by asymmetric synthesis applying chiral catalysts[8–13] or chiral functional group transfer reagents.[14]

So far, only two methods for the characterization of the enantiopurity of indatraline enantiomers have been described in the literature. One is based on polarimetry,[1,2,8] the other on ^{1}H nuclear magnetic resonance NMR measurements employing (*R*)-(-)-2,2,2-trifluoro-1-(9-anthryl)ethanol as shift reagent.[2] The latter represents the above-mentioned method Bøgesø et al. used for the determination of the enantiopurity of indatraline enantiomers which, however, allows the quantification of enantiomeric excesses (*ee*) of no higher than 95%.[2]

The aim of the present study was to develop a method enabling the determination of enantiomeric excesses up to at least 99% (*ee*) for the two indatraline enantiomers and to validate this method to demonstrate its reliability.

MATERIALS AND METHODS

Synthesis of Indatraline · HCl

The synthesis of indatraline · HCl (*rac*-**1** · HCl) was accomplished in five steps starting from commercially available 3,4-dichlorocinnamic acid (see also Supporting Information). The latter was reacted with benzene in the presence of $AlCl_3$ to give 3-(3,4-dichlorophenyl)-3-phenylpropanoic acid, which was transformed to the corresponding acid chloride utilizing thionyl chloride and subsequently subjected to an intramolecular Friedel Crafts acylation with $AlCl_3$ to yield 3-(3,4-dichlorophenyl)indan-1-one (93%). The following steps were performed according to Davies et al.[8]: reduction of the ketone with K-Selectride to afford the corresponding alcohol. In situ activation of the alcohol as mesilate followed by nucleophilic substitution with methylamine provided indatraline as free base, which was converted to its hydrochloride by treatment with aqueous HCl followed by freeze-drying. All procedures for the synthesis of *rac*-**1** as well as the analytical data are shown in the Supporting Information.

Analytical data for the free base (*rac*-**1**), which is the test material for all ^{1}H NMR experiments, is listed below.

^{1}H NMR for *rac*-**1** (chloroform-*d*, 500 MHz): δ = 2.24 (dt, J = 13.2/7.0 Hz, 1H, NCHC*H*$_2$), 2.44 (ddd, J = 13.2/7.8/3.3 Hz, 1H, NCHC*H*$_2$), 2.51 (s, 3H, NC*H*$_3$), 4.25 (dd, J = 6.8/3.2 Hz, 1H, CH$_2$C*H*), 4.50 (t, J = 7.6 Hz, 1H, NC*H*), 6.97 (m, 2H, H$_{indan}$), 7.22 (dd, J = 2.1 Hz, 1H, C*H*Cl),7.23-7.29 (m, 2H, H$_{indan}$), 7.35 (d, J = 8.2 Hz, 1H, CHC*H*Cl), 7.39 (dd, J = 8.2/2.1 Hz, 1H, C*H*CHCl). ^{13}C NMR (chloroform-*d*, 125 MHz): δ = 34.15, 43.21, 48.51, 63.64, 124.71, 125.25, 127.27, 127.45, 128.36, 129.88, 130.27, 130.44, 132.45, 144.74, 145.49, 145.53.

Infrared spectroscopy (IR) (film): $\tilde{\nu}$ = 3326 cm^{-1}, 3276, 3068, 3023, 2959, 2933, 2848,1469.

Mass spectrometry (MS): chemical ionization (CI, CH_5^+); *m/z* (%): 292 (100) $[M+H]^+$.

High-resolution mass spectrometry (HRMS): electron ionization (EI+): $M^{+\cdot}$ calcd. for $C_{16}H_{15}NCl_2$: 291.0576; found: 291.0571 (error 1.7 ppm).

Chemicals

L-(+)-tartaric acid p.a. for the racemate resolution was provided by Riedel de Haën.

HPLC-grade acetonitrile and methanol were purchased from VWR International (Darmstadt, Germany). Water was obtained by distillation and filtration (0.45 µm filter) of demineralized water, prepared by a reverse osmosis system.

Ammonium acetate (AAc) p.a. was purchased from Fluka (Taufkirchen, Germany), acetic acid p.a. from VWR International, and triethylamine (TEA) pure (≥ 99%), which was distilled prior to use, from AppliChem (Darmstadt, Germany).

(*S*)-(-)-1,1′-bi-naphthyl-2,2′-diol for synthesis 99.8% (99.7% *ee*) (Merck, Darmstadt, Germany), (*S*)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid 98%, (*S*)-(+)-*O*-acetylmandelic acid 99% (98% *ee*) (both Sigma-Aldrich, Steinheim, Germany), (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid 99% (Alfa Aesar, Karlsruhe, Germany), and (*S*)-(-)-α-methoxy-α-trifluoromethylphenylacetic acid p.a. 99% (97% *ee*) (Fluka) were used as purchased. The deuterated solvent benzene-d_6 (99.6% D) was purchased from Sigma-Aldrich, acetonitrile-d_3 (99.8% D) from euriso top (Saarbrücken, Germany), and chloroform-*d* (99.8% D) from Sigma-Aldrich, and euriso top.

Resolution of Indatraline Via Crystallization by Formation of Diastereomers

The resolution of *rac*-**1** was accomplished via crystallization according to Bøgesø et al.,[2] thereby the enantiopurity was monitored by the developed ^{1}H NMR or HPLC method, respectively. Contrary to Bøgesø et al.,[2] the tartaric acid salt was further transferred into the free base by an alkaline ether extraction, then finally treated with an excess of aqueous HCl (1 M) and subsequent freeze-drying of the resulting residue yielded (1*R*,3*S*)-indatraline · HCl [(1*R*,3*S*)-**1** · HCl], which was recrystallized from ethyl acetate to give colorless crystals of distinctly reduced hygroscopicity. The specific rotation of this product (melting point 172–173° C) amounted to $[\alpha]_D^{21}$ +16.4 (c 0.39, methanol) using a Perkin Elmer 241 C polarimeter (Perkin Elmer, Rodgau, Germany).

^{1}H NMR

Instrumentation. All ^{1}H NMR spectra were recorded on a JNM Eclipse +500 (500 MHz) NMR spectrometer from JEOL equipped with a direct 5 mm broadband probe operating at 500.16 MHz. The sample temperature was set to 25°C except for the investigations concerning the influence of variable temperature (VT) on the enantiomeric chemical shift differences (ΔΔδ). To achieve optimal results concerning peak shape, a gradient shim was applied using the gradient shim tool provided by the instrument operating software. The pulse angle was set to 45° and a relaxation delay of 1.3 s was used. In all, 64 transients over a frequency width of 7003 Hz were applied with 32 K data points, giving an overall digital resolution of 0.21 Hz per points. The spectra were processed using NMR software MestReNova v5.2.5-4119 provided by Mestrelab Research. No line broadening was applied prior to Fourier transformation (FT). The peak integration was performed manually with the integration tool included in the software. The comparison of the effect of the different solvents was made by measuring the chemical shift and the full width at half maximum (FWHM) of each *N*-methyl proton signal. The chemical shift was manually measured with MestReNova, and in contrast the FWHM values were measured with delta v4.3.3 software (JEOL). A 4 times zero-filling prior to FT was applied to obtain a reasonable number of data points suitable for the determination of FWHM. Signal-to-noise ratio (S/N) calculations were performed using the S/N tool in root mean square (RMS) mode provided by the delta v4.3.3 software (JEOL).

Preparation of the stock solutions for the ^{1}H NMR experiments and method development. All ^{1}H NMR experiments were measured using a total sample volume of 700 µl. To obtain stock solutions with a defined concentration, *rac*-**1** and the chiral solvating agents (CSAs) [(*S*)-(-)-1,1′-

binaphthyl-2,2′-diol (**3**), (*S*)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid (**4**), (*S*)-(+)-*O*-acetylmandelic acid (**5**), (*S*)-(-)-α-methoxy-α-trifluoromethylphenylacetic acid ((S)-**6**), and (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid ((*R*)-**6**)] (Fig. 2) were dissolved in an appropriate volume of the required deuterated solvent (chloroform-*d*, benzene-d_6, and acetonitrile-d_3, respectively) to yield final concentrations of 0.1 M. The samples were prepared by mixing a defined volume of a 0.1 M stock solution of *rac*-**1** (free base) and various volumes of 0.1 M stock solutions of the respective CSAs in a final volume (700 µl) of the respective deuterated solvent. When higher molar ratios (1:5 and 1:10, respectively) of the CSA (*S*)-(-)-1,1′-binaphthyl-2,2′-diol (**3**) were examined, no addition of the 0.1 M stock solution of **3** was possible without changing the final sample volume. Due to the low solubility of **3** in the chosen deuterated solvents (chloroform-*d*, benzene-d_6, and acetonitrile-d_3) the preparation of a higher concentrated stock solution, e.g., 1 M, was also impossible. Therefore, the required amount of **3** was weighed and directly dissolved in the final sample volume (700 µl) already containing the defined concentration of *rac*-**1**.

In the end, a 0.1 M stock solution of (1*R*,3*S*)-**1** in chloroform-*d* was made analogously to the *rac*-**1** sample and analyzed under the conditions established for the *rac*-**1**-CSA investigations.

UV-absorption measurements

The absorption spectrum of indatraline (*rac*-**1** · HCl) was measured in a 96-well quartz UV plate (Hellma, Müllheim, Germany) by means of a Spectra Max M2e plate reader (Molecular Devices, Ismaning, Germany). Spectra were corrected for solvent absorption by subtraction of the spectra of the pure solvents.

Chromatography

Instrumentation. An Agilent 1100 HPLC system consisting of an vacuum degasser, quaternary pump, column oven, diode array detector (Agilent, Waldbronn, Germany), and an SIL HTA Shimadzu autosampler (Shimadzu, Duisburg, Germany) in combination with an Astec Cyclobond I 2000 DM (25 cm x 4.6 mm; 5 µm) stationary phase (Sigma-Aldrich) was used. Data analysis was performed by means of Analyst 1.4.2 (Applied Biosystems, Darmstadt, Germany).

Preparation of stock solutions for the HPLC experiments. All solutions and buffers were prepared in water unless stated otherwise. For method development and validation we used 10 mM *rac*-**1** · HCl stock solutions. These stock solutions were prepared by dissolving *rac*-**1** · HCl in the appropriate volume of water or 7.3 mM TEAA (triethylammonium acetate) pH 4.0, respectively. Analogously, a 10 mM stock solution of (1*R*,3*S*)-**1** · HCl in 7.3 mM TEAA pH 4.0 was prepared. All stock solutions were frozen at -20°C, thawed on the day of the experiment, and finally diluted in the corresponding sample solvent to obtain the samples for method development and validation (calibration standards, QCs, spiked samples).

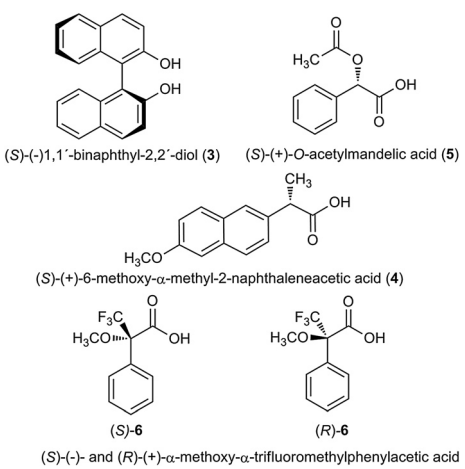

Fig. 2. Investigated CSAs for the ^{1}H NMR spectroscopic enantiomeric discrimination.

HPLC method development, data analysis, and validation. For each modification in the development of the HPLC method, the samples were measured in triplicates. Buffers (i.e., AAc and TEAA) were adjusted to the required pH value by addition of glacial acetic acid.

Peak intensity during method development was defined as peak height [mAU] of the (1*S*,3*R*)-**1** peak. Resolution (R_s) and asymmetry factors (A_s) were calculated according to Kuss et al.[15]

For quantification, peak areas (y) of both indatraline enantiomers as a function of their concentration (x) were investigated. Linearity was investigated by measurement of a series of calibration standards (13 concentration levels, i.e., 2.5, 5, 10, 25, 50, 75, 100, 250, 500, 750, 1000, 1500, 2000, and 3000 µM *rac*-**1** · HCl).

The final method based on a mobile phase composition of 7.3 mM TEAA pH 4.0 and acetonitrile (85:15, v/v), at a flow rate of 1.0 ml/min and a temperature of 20°C, an injection volume of 50 µl, a sample solvent of 7.3 mM TEAA pH 4.0 and a detection wavelength of 230 nm was validated for specificity (comparison of a sample solvent blank with a *rac*-**1** · HCl sample at the expected retention time), linearity (described above), accuracy (defined as recovery obtained for QCs in five concentrations each in five replicates), precision (calculated as RSD for the same QCs used for accuracy), and quantitation limit (QL) according to the ICH guidance Q2(R1).[16] Validation of accuracy and precision was extended to (1*R*,3*S*)-**1** · HCl samples of high enantiopurity that were spiked with defined amounts of *rac*-**1** · HCl as described above.

RESULTS AND DISCUSSION

^{1}H NMR Method Development and Optimization

In 1985 Bøgesø et al. published an ^{1}H NMR method based on the use of a shift reagent that enabled the determination of the enantiopurity of (1*R*,3*S*)- and (1*S*,3*R*)-**1** up to 95% *ee*.[2] Since this determination limit was not sufficient for our purpose, we intended to develop an alternative NMR method that allows the determination of enantiomeric excesses (*ee*) up to 99% and higher. To realize this method, we wanted to make use of a technique recently published by Claridge et al. which had distinctly improved the sensitivity for the quantification of diastereomeric excesses (*de*) by comparing the integrals of the signals of the minor diastereomer with the integral of ^{13}C satellites of a suitable ^{1}H NMR signal of the major diastereomer.[17] That way *de* of up to 99.8% could be successfully determined.[17] Our plan was to apply this technique to the determination of *ee* of indatraline samples. Actually, we considered this approach especially rewarding as an ^{1}H NMR-based method would, in addition, allow detection of sample impurities, such as solvent remnants, and recovery of the samples used.

Due to the fact that the *N*-methyl group gives rise to the most intense signal in the ^{1}H NMR spectrum of *rac*-**1**, it was selected as the reference signal for the development of the above-described ^{13}C satellite method. As this secondary methylamino moiety is directly attached to one of the two chiral centers of **1**, the chemical shift differentiation of this group was expected to be especially sensitive to the different nature of the diastereomeric ion pairs.

Various CSAs were tested to determine their effect on the chemical shift differences between the signals of the diastereomeric complexes of *rac*-**1** in the respective ^{1}H NMR spectra.

As such, (*S*)-(-)-1,1′-binaphthyl-2,2′-diol (**3**) was selected as it had been successfully applied to the determination of the enantiopurity of the structurally closely related sertraline (**2**), a method that had been established by Salsbury and Isbester[18] (Fig. 1). Also, the well-established CSAs (*S*)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid (**4**), (*S*)-(+)-*O*-acetylmandelic acid (**5**), (*S*)-(-)-α-methoxy-α-trifluoromethylphenylacetic acid

[(*S*)-**6**], and (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid [(*R*)-**6**] were studied. With these CSAs, a series of experiments was performed in which the molar ratios (CSA : *rac*-**1**, Table 1), solvents (Table 2), and temperature (Table 3) were varied to identify the best-suited reagent and optimal conditions for the determination of the enantiopurity of the enantiomers of **1**.

Regarding the molar ratios between the individual CSA and indatraline (**1**), the optimal value was defined as the ratio that led to the largest enantiomeric chemical shift difference (ΔΔδ) between the *N*-methyl signals of the diastereomeric complexes, together with low line broadening of the respective signals, which were measured as FWHM.

In brief, in chloroform-*d* at 25°C the best results were obtained using CSA (*S*)-**6** or (*R*)-**6**, at a molar ratio of 1:1 (Table 1 entries 14 and 20, respectively, 64 transients were taken) (Fig. 3). Under these conditions, not only ΔΔδ values amounting to 0.046 to 0.047 ppm were very high, but also the FWHM values in the range of 1.3–1.5 Hz were very low.

Due to the fact that the diastereomeric complex of **1** and (*S*)-**6** is the mirror image of **1** and (*R*)-**6**, the ΔΔδ and FWHM values should be the same; the slight difference of entry 14 compared to entry 20 (Table 1) can be explained by different enantiopurities of the purchased CSAs used and a determination imprecision originating from manual measurements of both parameters. Actually, for (*S*)-**6** and (*R*)-**6**, the ΔΔδ had reached their maximum when 1.0 equivalent of the CSA was employed and decreased when the molar ratio of the CSA was further increased (entries 15–17 and 21–13, Table 1), which at the same time was accompanied by a worsening of the FWHM values. In contrast, the ΔΔδ values either improved or reached a plateau for the CSA **3**-**5** (see entries 7 and 8, Table 1) when the molar ratios of the CSA as compared to *rac*-**1** were increased. Using (*S*)-**6** and (*R*)-**6** at a molar ratio of 1:1 (CSA : *rac*-**1**) gave the highest ΔΔδ values for all tested CSAs in the respective molar ratio range studied, except for CSA **3**. Also, **3** when employed in molar ratios of 2:1, 5:1, and 10:1, respectively (Table 1, entries 3-5) gave rise to ΔΔδ values reaching or even surpassing the optimal results observed for (*S*)-**6** and (*R*)-**6**, but this approach suffered from two major disadvantages. First, an overlap of the relevant *N*-methyl signals with a signal resulting from one of the methylene protons of *rac*-**1** occurred which complicated the analysis of these signals, but also the line broadening become distinctly worse. Thus, compound **3** appeared to be less suitable than (*S*)-**6** and (*R*)-**6** for use as a CSA for the analysis of the enantiopurity of the enantiomers of *rac*-**1**.

In further experiments the aprotic solvents acetonitrile-d_3 and benzene-d_6 were also tested for their suitability in chemical shift experiments with **3**, **5**, and (*S*)-**6** as CSA, but clearly turned out to be less appropriate than chloroform-*d* (Table 2). Finally, varying temperature experiments were performed for the CSA (*S*)-**6** in chloroform-*d*. According to the results of these experiments, a temperature of 25°C represents a good compromise, yielding a reasonably high ΔΔδ together with an adequately low FWHM value (entry 4, Table 3).

TABLE 1. Influence of different CSA and their molar ratio on enantiomeric chemical shift difference (ΔΔδ) and FWHM of *rac*-1

No.	CSA	Molar ratio[a]	ΔΔδ [ppm][b]	FWHM[c] [Hz]	
1	**3**	0.5 : 1	0.014	0.9	0.9
2		1 : 1	0.026	1.0	1.0
3		2 : 1	0.044	1.1	1.1
4		5 : 1	0.068	1.5	1.4
5		10 : 1	0.078	2.2	1.9
6	**4**	0.5 : 1	0.007	1.1	1.1
7		1 : 1	0.013	1.2	1.2
8		2 : 1	0.013	1.2	1.2
9	**5**	0.5 : 1	0.019	1.6	1.8
10		1 : 1	0.027	1.3	1.3
11		2 : 1	0.033	1.7	1.8
12	(*S*)-**6**	0.5 : 1	0.027	1.7	2.1
13		0.75 : 1	0.039	1.7	1.9
14		1 : 1	0.046	1.3	1.4
15		1.25 : 1	0.043	1.5	1.5
16		1.5 : 1	0.044	1.8	1.8
17		2 : 1	0.036	1.5	1.5
18	(*R*)-**6**	0.5 : 1	0.024	1.5	1.9
19		0.75 : 1	0.035	1.6	1.9
20		1 : 1	0.047	1.4	1.5
21		1.25 : 1	0.042	1.5	1.5
22		1.5 : 1	0.042	1.8	1.8
23		2 : 1	0.036	1.6	1.6

Standard conditions for these experiments were chloroform-*d*, at 25°C and 64 transients.
[a]Molar ratio CSA:*rac*-**1**.
[b]Chemical shift difference between the *N*-methyl signal of the diastereomeric enantiomer-CSA complexes.
[c]First value of the low frequency signal, second value of the high frequency signal.

TABLE 2. Influence of benzene-d_6 and acetonitrile-d_3 as solvent on enantiomeric chemical shift difference (ΔΔδ) and FWHM

No.	Solvent	CSA	Molar ratio[a]	ΔΔδ [ppm][b]	FWHM[c] [Hz]	
1	benzene-d_6	**3**	5 : 1	0.065	1.7	1.8
2		**5**	1 : 1	0.013	1.2	1.2
3		(*S*)-**6**	1 : 1	0.023	1.2	1.2
4	acetonitrile-d_3	**3**	5 : 1	0.005	1.7	1.7
5		**5**	1 : 1	0.006	1.0	1.0
6		(*S*)-**6**	1 : 1	0.024	0.9	0.9

Standard conditions for these experiments were 25°C and 64 transients.
[a]Molar ratio CSA: *rac*-**1**.
[b]Chemical shift difference between the *N*-methyl signal of the diastereomeric enantiomer-CSA complexes.
[c]First value of the low frequency signal, second value of the high frequency signal.

TABLE 3. Influence of the temperature on enantiomeric chemical shift difference (ΔΔδ) and FWHM using *rac*-1 and (*S*)-6 (1:1)

No.	*T* [°C]	ΔΔδ [ppm][a]	FWHM[b] [Hz]	
1	- 50	0.076	6.0	11.5
2	- 25	0.064	2.4	3.3
3	0	0.055	1.7	2.0
4	25	0.045	1.2	1.4
5	50	0.037	1.2	1.3

Standard conditions for these experiments were chloroform-*d* and 64 transients.
[a]Chemical shift difference between the *N*-methyl signal of the diastereomeric enantiomer-CSA complexes.
[b]First value of the low frequency signal, second value of the high frequency signal.

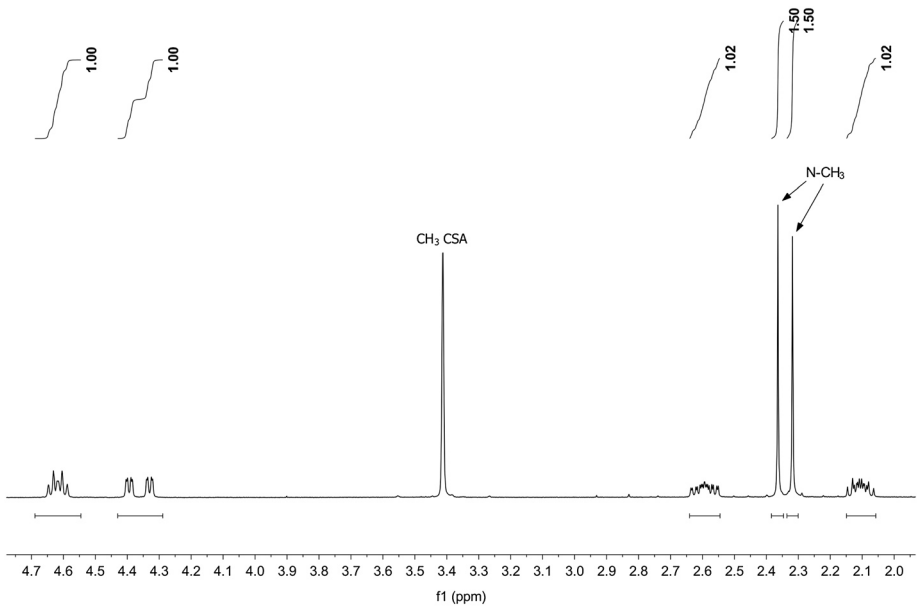

Fig. 3. Excerpt of the ^{1}H NMR spectrum of *rac*-**1** with (*S*)-**6** in a molar ratio of 1:1 measured in chloroform-*d* at 25°C with 64 transients. Separated *N*-methyl signals for the determination of ΔΔδ as well as FWHM values are labeled.

In the end, the optimized method was subjected to testing at a sample that had been obtained from multiple resolution via crystallization of the racemic compound *rac*-**1**, which was expected to be of exceptional high *ee*. Corresponding to Parker and Taylor,[19] the size of the ΔΔδ values in general varies depending on the enantiomeric composition of the analyte and the absolute configuration of the CSA employed. Hence, differing ΔΔδ values had to be expected for this highly enantioenriched sample depending on the enantiomer of the CSA used [(*S*)-**6** or (*R*)-**6**]. Therefore, the (1*R*,3*S*)-**1** obtained by resolution of *rac*-**1** via crystallization (see Materials and Methods) was analyzed by means of the optimized ^{1}H NMR method using (*S*)-**6** and (*R*)-**6** as CSA as well. The spectra resulting from these studies revealed that (*S*)-**6** is better suited for the analysis of the enantiopurity of (1*R*,3*S*)-**1**, as this CSA enantiomer gives higher values for ΔΔδ (0.080 ppm compared to 0.050 ppm for (*R*)-**6**). Moreover, when (*R*)-**6** was used as CSA the *N*-methyl signals of the two enantiomers of **1** were overlapping, severely impeding their separate integration. According to Claridge et al.,[17] theoretically diastereomeric excesses of up to 99.8% *de* can be determined provided an acceptable S/N ratio is reached. Unfortunately, we were not able to reliably quantitate the *N*-methyl signal of the (1*S*,3*R*)-enantiomer with 64 transients, as its integral was substantially lower than that of the ^{13}C satellite signal of the respective *N*-methyl signal of the major isomer the (1*R*,3*S*)-**1**. Although an S/N of 12:1 was found for one ^{13}C satellite which did fulfill our key criterion of S/N ≥ 10:1 for accurate quantification, the *N*-methyl signal of the minor isomer almost vanished in the signal noise for this real sample with high enantiopurity. Therefore, an S/N ratio optimization was performed by incrementally increasing the number of transients (128, 512, 2048, and 8192). But even with 8192 transients [S/N 66:1 for one ^{13}C satellite of the *N*-methyl group of (1*R*,3*S*)-**1**], the enantiopurity of this highly enantioenriched material of (1*R*,3*S*)-**1** could not be analyzed accurately, as slight changes of phase correction performance had significant effects on the integration values. To overcome this obstacle, more concentrated solutions could be used. But as we intended, for practical reasons, to employ only low amounts of enantioenriched samples obtained during the racemic resolution of *rac*-**1**, this opportunity was rejected. Nevertheless, as the integral of the *N*-methyl signal of the minor enantiomer was in any case unequivocally lower than the integral of one of the ^{13}C satellite signals of the *N*-methyl signal of the major enantiomer, it can be stated that the *ee* of the analyzed sample must have been higher than 98.9%. The *ee* being ≥ 98.9%, follows from the fact that the natural occurrence of ^{13}C amounts to 1.108%, and for this reason the ratio between the integrals of the ^{12}C-^{1}H NMR signal and a single ^{13}C satellite signal is 178.5:1,[17] which compares to an integral of 0.56% (= major signal, equivalent to 100%, divided by 178.5) for a ^{13}C satellite signal in relation to the parent signal. Accordingly, for theoretical reasons, the *ee* must amount to ≥ 98.9% if the integral of the ^{12}C-^{1}H NMR signal of the minor isomer is less than the integral of the ^{13}C satellite signal of the major isomer.

In any case, the above-described method represents a simple and quick tool for the analysis of the progress of racemic resolutions of indatraline (**1**) up to even very high enantiomeric excesses of *ee* ≤ 98.9%.

HPLC Method Development and Optimization

With the aim of developing an analytical method suitable for determination of *ee* ≥ 99% for both indatraline enantiomers, we tried next to benefit from the capabilities of a chiral stationary phase.

The method described by Rao et al.[20] for the separation and quantitation of stereoisomers and related enantiomeric impurities of sertraline (**2**) by means of a dimethylated β-cyclodextrine material as stationary phase was considered a suitable starting point. The column employed in this study, an Astec Cylcobond I 2000 DM, seemed to be promising for our purpose due to the structural analogy of indatraline (**1**) compared to sertraline (**2**). The described mobile phase, however, employing 0.4% (v/v) trifluoroacetic acid (TFA) at pH 3.0[20] can be considered relatively harsh and possibly destructive for the stationary phase as these conditions are at or even not within the manufacturer's advice

for handling of the Astec Cylcobond I 2000 DM. Additionally, for TFA a memory effect was found by Ye et al.[21] which was assumed to impair a rapid switching between different kinds of buffers. Hence, a gentle but powerful HPLC method avoiding TFA should be developed for this stationary phase. First, in order to select an appropriate wavelength for analyte detection, an absorption spectrum of *rac*-**1** · HCl was recorded. Figure 4 shows the absorption spectrum of a 25 μM aqueous solution of *rac*-**1** · HCl. It reveals a weak maximum at 265 nm and a steep increase of absorption at a wavelength <240 nm with a slight shoulder at about 230 nm.

Further spectra recorded in possible mobile phases such as 10 mM AAc pH 4.0, 7.3 mM TEAA pH 4.0, or 7.3 mM TEAA pH 4.0 and acetonitrile (85:15, v/v), employing different concentrations of *rac*-**1** · HCl and varying pH values of the buffers used as solvent (pH 3.0–7.0) covering the suggested operation range of the Astec Cyclobond I 2000 DM confirmed these absorption characteristics. These spectra were also similar to those of **2** reported by Mandrioli et al.,[22] who identified absorption maxima at 208 nm and 275 nm (conditions not specified), respectively. For the present study, we decided to use 230 nm as the detection wavelength for the HPLC analysis, just as Mandrioli et al.[22] had done in the case of **2**, as it provides high analytical sensitivity and at the same time is still sufficiently distant from the cutoff of the mobile phase.

To develop the required HPLC separation method for the enantiomers of indatraline (**1**) based on the selected Astec Cylcobond I 2000 DM column, we started with the chromatographic conditions suggested by the manufacturer for separation of racemic hydrobenzoine. When these were employed [i.e., 10 mM AAc buffer pH 4.0 and acetonitrile 90:10 (v/v)] injecting 10 μl of a 1 mM aqueous solution of *rac*-**1** · HCl, at a flow rate of 1.0 ml/min and a temperature of 20°C, a reasonable separation for the two enantiomers of indatraline (R_s = 3.95) was obtained (see Table 4a, entry 2). As discrimination of enantiomers by means of cyclodextrine phases is known to depend on the nature of the buffer, its concentration and pH value, on the organic modifier as well as on temperature,[23] we investigated the influence of these parameters on separation, run time, peak symmetry, and peak intensity [defined as peak height of (1*S*,3*R*)-**1**]. Regarding the latter, the peak resulting from the (1*S*,3*R*)-**1** enantiomer was considered to be crucial due to its longer retention time and broader peak width.

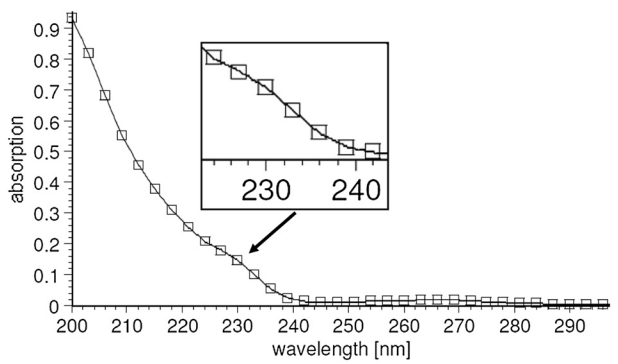

Fig. 4. Absorption spectrum, corrected by subtraction of the corresponding absorption spectrum of the sample solvent, of *rac*-**1** · HCl (25 μM in water) in the range from 200–300 nm with an enlargement of the slight shoulder at about 230 nm.

Several trends can be recognized from the results obtained when varying the chromatographic conditions, which are shown in Table 4a and 4b. First, the resolution of the enantiomers appeared to be sufficient for all conditions investigated ($R_s \geq 3$, all entries Table 4a and 4b). Second, for both buffers (AAc and TEAA) a decrease in buffer concentration led to shorter retention times (Table 4a and 4b, entries 1–3, respectively) associated with increased intensities (at least for AAc, Table 4a entries 1–3) and lower peak widths (data not shown). Third, the opposite effect (i.e., longer retention time associated with decreased intensities and broader peaks) was observed when the pH value was increased (Table 4a and 4b, entries 2 and 4–6, respectively). Fourth, enhanced amounts of acetonitrile resulted in shorter retention times, higher intensities, and improved peak shapes (i.e., lower A_s Table 4a and 4b, entries 2 and 7–8, respectively). Fifth, decreased temperatures caused a conspicuous peak broadening (data not shown) and a marked loss of intensity due to longer retention times (Table 4a and 4b, entries 2 and 9–11, respectively). Finally, it should be mentioned that substitution of acetonitrile for methanol led to markedly worse results regarding intensity, resolution, run time, and peak shape (data not shown).

Based on the conditions of entry 8 (Table 4b, i.e., 7.3 mM TEAA pH 4.0 with acetonitrile in a ratio of 85:15 (v/v), flow rate of 1.0 ml/min, temperature of 20°C) that were considered a good compromise regarding intensity (at the same noise level compared to AAc), resolution, and peak shape, the effect of enhanced injection volumes was investigated. Thus, to gain higher intensities, the original injection volume of 10 μl of the 1 mM sample solution of rac-**1** · HCl in water was raised to 50 μl.

The results shown in Table 5 document a proportional increase of intensities in relation to the injection volume at acceptable resolution and asymmetry factors for both enantiomers (Table 5, entries 1–3) the highest intensity thus being observed for the injection volume of 50 μl. But as peak splitting of the (1*S*,3*R*)-**1** peak of *rac*-**1** · HCl samples in water and in the mobile phase with concentrations > 1 mM occurred, additional experiments to optimize the sample solvent were performed. We found that by using 7.3 mM TEAA pH 4.0 as sample solvent the situation distinctly improved, giving higher intensities for (1*S*,3*R*)-**1** and better peak shape for (1*R*,3*S*)-**1** (Table 5, entry 4), with the remaining analytical characteristics unchanged. Thus, in this way, peak splitting of the (1*S*,3*R*)-enantiomer could be suppressed up to concentrations of 1.5 mM of *rac*-**1** · HCl. A representative chromatogram recorded after injection of 50 μl of a 1 mM rac-**1** · HCl sample in 7.3 mM TEAA pH 4.0 under these optimized conditions is shown in Figure 5a. The chromatographic parameters calculated for this sample are shown in Table 5.

These results were considered appropriate for a reliable quantitation of high enantiomeric excesses (i.e., >99% *ee*) for both enantiomers of indatraline. Therefore, we intended to verify the validity of the method used based on an Astec Cyclobond I 2000 DM as stationary phase (25 x 4.6 cm, 5 μm), a mobile phase composition of 7.3 mM TEAA pH 4.0, and acetonitrile (85:15, v/v) at a flow rate of 1.0 ml/min at 20°C and an injection volume of 50 μl of the samples dissolved in 7.3 mM TEAA pH 4.0 in the next step.

Validation

When a single enantiomer is selected for development as a new drug, the other enantiomer, i.e., the minor isomer, should be analyzed in the same manner as required for other

TABLE 4a. Effect of concentration and pH value of ammonium acetate buffer, organic modifier and temperature on intensity, resolution, retention times, and asymmetry factors of the indatraline enantiomers

				t_R [min]		A_s	
No.	Conditions[a]	Peak height[b] [mAU]	R_s	1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*S*,3*R*
	Buffer conc. [mM]						
1	5	135.00	3.51	7.64	9.55	2.43	2.40
2	***10**	**93.83**	**3.95**	**11.00**	**13.90**	**2.32**	**2.40**
3	15	78.10	4.24	13.33	16.97	2.22	2.72
	pH						
4	3.5	115.33	3.87	9.53	11.94	1.71	1.59
2	***4.0**	**93.83**	**3.95**	**11.00**	**13.90**	**2.32**	**2.40**
5	4.5	89.00	3.99	12.40	15.70	2.60	2.74
6	5.0	70.33	4.06	15.10	19.23	2.96	3.03
	A : B ratio[c]						
7	95:5	37.80	4.57	19.30	26.33	3.31	2.94
2	***90:10**	**93.83**	**3.95**	**11.00**	**13.90**	**2.32**	**2.40**
8	85:15	148.33	3.17	6.96	8.20	1.69	1.62
	T [°C]						
9	5	63.83	3.90	12.33	16.14	2.19	1.88
10	10	60.30	3.98	12.43	16.13	2.33	2.23
11	15	79.27	4.02	11.53	14.80	2.38	2.14
2	***20**	**93.83**	**3.95**	**11.00**	**13.90**	**2.32**	**2.40**

Highlighted entries represent starting conditions, during variation of each parameter (e.g., concentration, pH, A : B ratio, temperature) the three remaining parameters were kept constant.
[a]10 µl of an aqueous 1 mM *rac*-**1** · HCl solution injected on an Astec Cyclobond I 2000 DM (25 cm x 4.6 mm, 5 µm).
[b](1*S*,3*R*)-**1**.
[c]Ammonium acetate buffer (A):acetonitrile (B) (v/v).

defined impurities.[24] The ICH Q2(R1) guidance,[16] which addresses this topic, recommends quite generally "to demonstrate that it (i.e., the developed method) is suitable for its intended purpose." However, the ICH Q2(R1) guidance contains almost no exactly defined (i.e., quantitative) requirements regarding validation parameters for methods to determine enantiopurity. For the intended purpose of the described method it is mandatory for the accurate and precise

TABLE 4b. Effect of concentration and pH value of triethylammonium acetate buffer, organic modifier and temperature on intensity, resolution, retention times, and asymmetry factors of the indatraline enantiomers

				t_R [min]		A_s	
No.	Conditions[a]	Peak height[b] [mAU]	R_s	1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*S*,3*R*
	Buffer conc. [mM][d]						
1	3.65	133.00	3.28	6.94	8.65	2.20	2.16
2	***7.30**	**134.67**	**3.66**	**8.66**	**10.87**	**2.49**	**2.50**
3	14.60	105.33	4.08	12.63	16.2	2.63	2.63
	pH						
4	3.5	135.33	3.68	8.18	10.30	1.86	1.89
2	***4.0**	**134.67**	**3.66**	**8.66**	**10.87**	**2.49**	**2.50**
5	4.5	101.67	3.78	10.57	13.47	2.58	2.88
6	5.0	80.20	3.82	13.00	16.60	2.87	2.94
	A: B ratio[c]						
7	95:5	58.40	3.99	15.33	20.90	4.00	4.40
2	***90:10**	**134.67**	**3.66**	**8.66**	**10.87**	**2.49**	**2.50**
8	85:15	176.33	3.00	5.97	7.03	2.25	2.07
	T [°C]						
9	5	85.17	3.72	10.37	13.63	2.44	2.47
10	10	116.00	3.68	9.94	12.90	2.67	2.70
11	15	126.67	3.67	9.64	12.37	2.60	2.78
2	***20**	**134.67**	**3.66**	**8.66**	**10.87**	**2.49**	**2.50**

Highlighted entries represent starting conditions, during variation of each parameter (e.g., concentration, pH, A : B ratio, temperature) the three remaining parameters were kept constant.
[a]10 µl of an aqueous 1 mM *rac*-**1** · HCl solution injected on an Astec Cyclobond I 2000 DM (25 cm x 4.6 mm, 5 µm).
[b](1*S*,3*R*)-**1**.
[c]Triethylammonium acetate buffer (A):acetonitrile (B) (v/v).
[d]Preparation of triethylammonium acetate buffer by diluting triethylamine in water and pH adjustment using glacial acetic acid (7.3 mM are equivalent to 0.1% (v/v) triethylamine in water).

TABLE 5. Effect of the injection volume on intensity, resolution, retention time, and asymmetry factors of the indatraline enantiomers based on the chromatographic conditions of entry 8 table 4b*

No.	Sample solvent	Injection volume[a]	Peak height[b] [mAU]	R_s	t_R [min]		A_s	
					1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*S*,3*R*
1	water	10 µl	176.33	3.00	5.97	7.03	2.25	2.07
2	water	20 µl	338.67	2.95	5.95	6.99	2.75	2.09
3	water	50 µl	778.67	2.74	5.91	6.90	3.28	2.81
4	7.3 mM TEAA	50 µl	934.83	2.69	5.72	6.64	1.81	3.18

[a]1 mM *rac*-**1** · HCl injected.
[b](1*S*,3*R*)-**1**.
*7.3 mM triethylammonium acetate pH 4.0 and acetonitrile (85:15, v/v) as mobile phase, at a flow rate of 1.0 ml/min at 20°C using an Astec Cylclobond I 2000 DM (25 cm x 4.6 mm, 5 µm) as stationary phase.

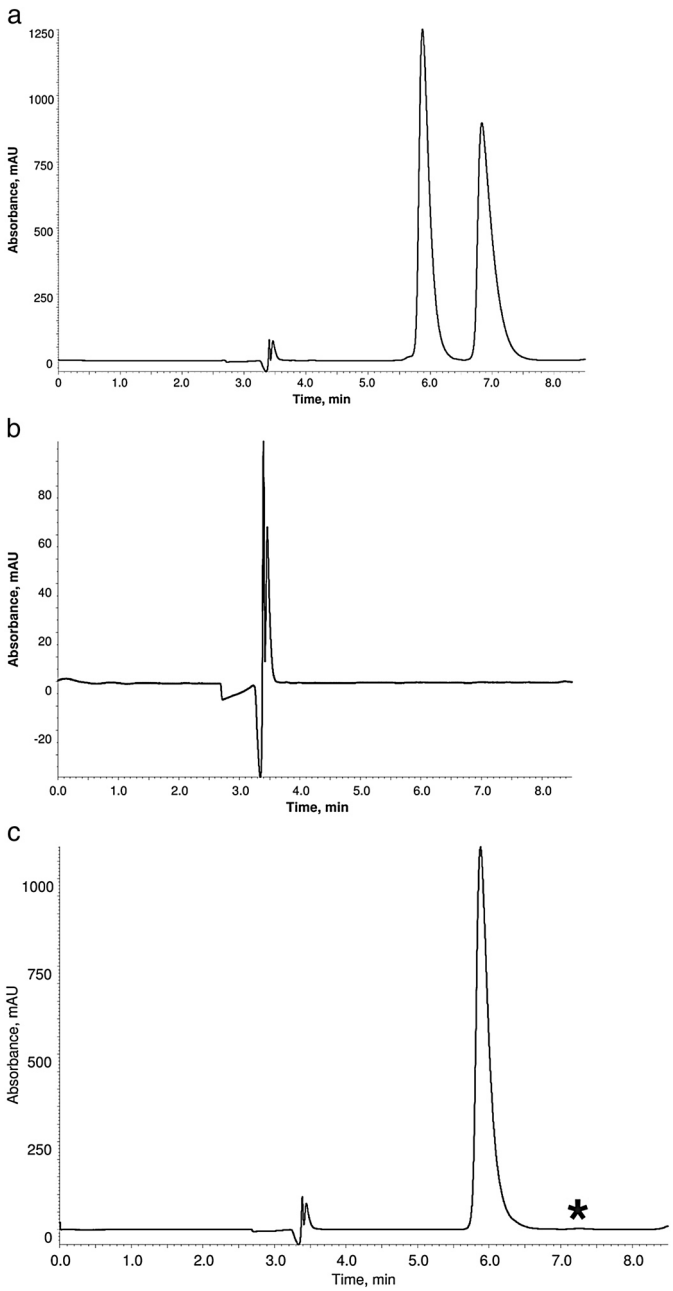

Fig. 5. Chromatograms of (**a**) 1 mM *rac*-**1** · HCl, (**b**) sample solvent blank (7.3 mM triethylammonium acetate pH 4.0, (**c**) 500 µM of (1*R*,3*S*)-**1** · HCl (obtained by racemate resolution, see Materials and Methods), an asterisk denotes t_R of the (1*S*,3*R*)-enantiomer. All chromatograms were recorded employing a mobile phase of 7.3 mM triethylammonium acetate pH 4.0 and acetonitrile (85:15, v/v), at a flow rate of 1.0 ml/min at 20°C on an Astec Cylclobond I 2000 DM (25 cm x 4.6 mm, 5 µm).

determination of both enantiomers – even if one is present in vast excess – that the range covered by the method is as large as possible. Following these considerations, we tried to demonstrate the performance of the established method for both indatraline enantiomers examining relevant validation parameters [i.e., range, quantitation limit (QL), linearity, precision, i.e., repeatability, accuracy, i.e., recovery, specificity] for both indatraline enantiomers by investigation of *rac*-**1** · HCl samples (due to the fact that no certified enantiopure material was available) over a broad concentration range.

Specificity. Injection of a blank sample (i.e., 7.3 mM TEAA pH 4.0) led to the chromatogram shown in Figure 5b. The absence of peaks at the retention times expected for the indatraline enantiomers indicates that there are no substances interfering with the indatraline enantiomers. Also, the resolution for the separation of the enantiomers ($R_s = 2.69$, see Fig. 5a) could be considered sufficient, thus demonstrating that this method is specific for both enantiomers.

Quantitation limit (QL). The S/N recorded for five 2.5 μM calibration standards of *rac*-**1** · HCl was calculated by means of Analyst 1.4.2 software. It was found to be ≥ 23 for (1*R*,3*S*)-**1** and ≥ 18 (1*S*,3*R*)-**1**,. This means that the ICH recommendation regarding QL (S/N >10) is fulfilled for both enantiomers at a concentration of 1.25 μM.

Linearity – range. From the resulting peak areas obtained for both enantiomers, calibration functions were generated by means of linear regression. Employing a $1/x^2$ weighting to the calibration functions gave best results regarding a concentration range as broad as possible. In this way, a calibration function of y = 29.55x - 1.731 was found for the (1*R*,3*S*)-**1** ($r^2 = 0.9995$ and a negligible y-intercept corresponding to 4.38% of the area obtained at QL comply with the requirements for linearity [25]) for a range of 1.25–1000 μM (i.e., 2.5–2000 μM *rac*-**1** · HCl). For (1*S*,3*R*)-**1**, this led to y = 29.63x – 1.623 ($r^2 = 0.9999$, y-intercept corresponds to 4.62% of the area obtained at QL) as calibration curve for a range of 1.25–750 μM (i.e., 2.5–1500 μM *rac*-**1** · HCl). Regarding the upper concentrations of the range, it should be recalled that for (1*S*,3*R*)-**1** a tendency to peak splitting had been observed when samples of *rac*-**1** · HCl with concentrations ≥ 2000 μM had been injected (see above). Furthermore, the injection of calibration standards containing 3000 μM *rac*-**1** · HCl (see above) appeared to overload the column, resulting in peak broadening and decreased resolution.

Accuracy – precision. Accuracy (i.e., recovery) and precision (i.e., repeatability) were examined for quality control samples (QCs) at five concentration levels (i.e., 2.5, 10, 50, 500, and 1500 μM) of *rac*-**1** · HCl, each in replicates of five. Table 6 shows the recovery (ratio of experimentally determined concentration to nominal concentration ± confidence interval, α = 0.05) and the repeatability (given as relative standard deviation, RSD) calculated from the experimentally obtained areas by means of the respective calibration functions. The developed method yielded accurate and precise results with recoveries between 98.6 ± 0.4% and 101.0 ± 1.0% and RSDs between 0.3% and 1.4% for (1*R*,3*S*)-**1**, and recoveries of 98.9 ± 0.3% to 100.5 ± 1.0% with RSDs of 0.2% to 1.1% for (1*S*,3*R*)-**1**. As all recoveries and RSDs are distinctly within the commonly accepted limits (recovery: 98.0%–102.0%, RSD ≤ 2.0%[25]), the established method is clearly in agreement with the ICH recommendations for accuracy (i.e., recovery) and precision (i.e., repeatability).

Considering the results of the validation based on *rac*-**1** · HCl samples, characterization of enantiopurities up to 99.75% *ee* [i.e., 1000 μM (1*R*,3*S*)-**1** in presence of 1.25 μM (1*S*,3*R*)-**1**] for (1*R*,3*S*)-**1** and 99.67% *ee* [i.e., 750 μM (1*S*,3*R*)-**1** in presence of 1.25 μM (1*R*,3*S*)-**1**] for (1*S*,3*R*)-**1** should be possible, when enantioenriched samples are analyzed at the upper concentration of the enantiomer representing the major isomer of the sample.

In the next step, we employed this method to study a sample of (1*R*,3*S*)-**1**, that was expected to possess an extremely high enantiopurity in the range of the analytical limit of the aforementioned method that had become available to us by resolution of *rac*-**1** via crystallization using L-(+)-tartaric acid as auxiliary reagent (see Materials and Methods) (chromatogram shown in Fig. 5c). As a (1*R*,3*S*)-**1** reference standard was still not available, the obtained (1*R*,3*S*)-**1** · HCl material was analyzed employing the calibration function established for *rac*-**1** · HCl.

The (1*R*,3*S*)-**1** · HCl samples were investigated in a nominal concentration of 1000 μM in five replicates. The concentrations of both enantiomers calculated by means of the aforementioned calibration functions were 979 μM for (1*R*,3*S*)-**1** and 2.28 μM for (1*S*,3*R*)-**1**, indicating an *ee* of 99.5%. The concentrations obtained in this experiment indicated a recovery of 98.1 ± 0.3% (mean ± confidence interval, α = 0.05) related to the sum of both enantiomers. The small but nevertheless distinctly lower recovery determined for the (1*R*,3*S*)-**1** · HCl sample in comparison to the total recoveries of *rac*-**1** · HCl samples [e.g., 98.8% for 1500 μM *rac*-**1** · HCl, i.e., 98.6% for 750 μM (1*R*,3*S*)-**1** and 98.9% 750 μM (1*S*,3*R*)-**1**, see Table 6] is presumably due to a small amount of ethyl acetate present

TABLE 6. Validation results (mean values, $n = 5$) regarding accuracy (recovery ± confidence interval, CI, α = 0.05) and precision (relative standard deviation, RSD) for five different QC levels of Indatraline

Concentration [μM]						
	Found		Recovery [%] ± CI		RSD [%]	
Nominal	1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*S*,3*R*
1.25	1.26	1.26	101.0 ± 1.0	100.5 ± 1.0	1.1	1.1
5	5.04	5.02	100.9 ± 1.3	100.3 ± 0.3	1.4	0.32
25	25.1	25.0	100.3 ± 0.3	100.0 ± 0.3	0.29	0.28
250	252	251	100.8 ± 0.2	100.5 ± 0.2	0.25	0.19
750	740	742	98.6 ± 0.4	98.9 ± 0.3	0.46	0.34

TABLE 7. Validation results (mean values, $n = 5$) regarding accuracy (recovery ± confidence interval, CI, $\alpha = 0.05$) and precision (relative standard deviation, RSD) after spiking a 1 mM solution of highly enantiomerically enriched (1*R*,3*S*)-1 · HCl (99.5% *ee*) with small volumes of a 1 mM *rac*-1 · HCl solution

	Concentration [μM]								*ee* [%]	
	Expected[c]		Found		Recovery [%] ± CI		RSD [%]			
Spiking	1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*R*,3*S*	1*R*,3*S*	1*S*,3*R*	1*R*,3*S*	1*S*,3*R*	expected[d]	found
1[a]	977	4.77	970	4.72	99.3 ± 0.3	99.0 ± 1.0	0.28	1.1	99.0	99.0
2[b]	975	6.26	967	6.21	99.2 ± 0.4	99.2 ± 0.9	0.41	1.0	98.7	98.7

[a]Sample 1: spiking 995 μL of a 981 μM (1*R*,3*S*)-**1** · HCl (99.5% *ee*) solution with 5 μL 1 mM *rac*-**1** · HCl (both in 7.3 mM TEAA pH 4.0).
[b]Sample 2: spiking 992 μL of a 981 μM (1*R*,3*S*)-**1** · HCl (99.5% *ee*) with 8 μL 1 mM *rac*-**1** · HCl to (both in 7.3 mM TEAA pH 4.0).
[c]Expected concentration according to the experimentally determined concentration of 981 μM for the enantiomenriched (1*R*,3*S*)-**1** · HCl sample (99.5% *ee*) and the added amount of *rac*-**1** · HCl.
[d]Expected enantiomeric excesses calculated from the expected concentrations.

in the respective (1*R*,3*S*)-**1** · HCl material. Unfortunately, this small amount of ethyl acetate — unambiguously identified by ^{1}H NMR (≤ 1.0%) in the respective (1*R*,3*S*)-**1** · HCl sample — could not be completely removed even after intensive high-vacuum treatment. That EtOAc has been found in this sample, of (1*R*,3*S*)-**1** · HCl, but not in those of *rac*-**1** · HCl is certainly a result of the different procedures used for the preparation of these compounds, (1*R*,3*S*)-**1** · HCl having been recrystallized from EtOAc, whereas *rac*-**1** · HCl has been isolated by freeze-drying of an aqueous solution in the final step (see Materials and Methods).

Since validation was initially based on *rac*-**1** · HCl samples, we additionally tried to validate accuracy (i.e., recovery) and precision (i.e., repeatability) of the method for their application to highly enantioenriched (1*R*,3*S*)-**1** · HCl samples by means of spiking experiments, which should ensure that even slight changes in the *ee* of highly enriched (1*R*,3*S*)-**1** · HCl samples can be reliably quantified. To this end, we spiked the above-mentioned 981 μM (1*R*,3*S*)-**1** · HCl sample of 99.5% *ee* [979 μM (1*R*,3*S*)-**1** and 2.28 μM (1*S*,3*R*)-**1**], with different volumes of a 1 mM *rac*-**1** · HCl solution in replicates of five and determined the resulting concentrations of (1*R*,3*S*)-**1** and (1*S*,3*R*)-**1**, by means of the calibration functions established for the racemic sample. The results from this experiment are shown in Table 7. For both of the spiked samples the expected concentrations calculated from the concentrations of the original solutions [977 μM and 975 μM for (1*R*,3*S*)-**1** and 4.77 μM and 6.26 μM for (1*S*,3*R*)-**1**] matched those found experimentally almost exactly [970 μM and 967 μM for (1*R*,3*S*)-**1** and 4.72 μM and 6.21 μM for (1*S*,3*R*)-**1**]. To this end, the found recoveries of ≥ 99.0% and RSDs ≤ 1.1% are also within the commonly accepted limits (recovery: 98.0%–102.0%, RSD ≤ 2.0%) [25]; additionally, a perfect agreement between expected and found enantiomeric excesses could be deduced from the performed spiking experiments. As consequence, the developed method can be considered reliable for the enantiopurity determination of the triple reuptake inhibitor (1*R*,3*S*)-indatraline.

CONCLUSION

Even though enantiodiscrimination by ^{1}H NMR spectroscopy is a well-established approach, this work demonstrates its limits for indatraline concerning the determination of low amounts of the minor enantiomer in samples containing the major enantiomer in large excess. Nevertheless, the established ^{1}H NMR method may serve as tool to monitor the progress of a resolution of indatraline enantiomers. For a more precise and sensitive analysis of highly enantioenriched samples, an HPLC method was developed that could be shown to enable accurate and precise quantitation of *ee* up to 99.75% for the (1*R*,3*S*)-enantiomer of indatraline (eutomer) and 99.67% for the (1*S*,3*R*)-enantiomer (distomer). Additionally, the results of HPLC method development show that ESI-MS detection employing a mobile phase based on ammonium acetate buffer is possible as well. The reliability of the established method was confirmed by validation according to the ICH guideline Q2(R1). As indatraline is a promising triple reuptake inhibitor, it is worth mentioning that the presented HPLC method represents a prerequisite for a correct pharmacological characterization of the pure enantiomers of indatraline and their possible use as pharmacological tools.

ACKNOWLEDGMENTS

We thank Gerd Bauschke for performing the synthesis and resolution of indatraline via crystallization (LMU München, Department of Pharmacy).

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Enantiomeric Purity Determination of the Enantiomers of the Triple Reuptake Inhibitor Indatraline

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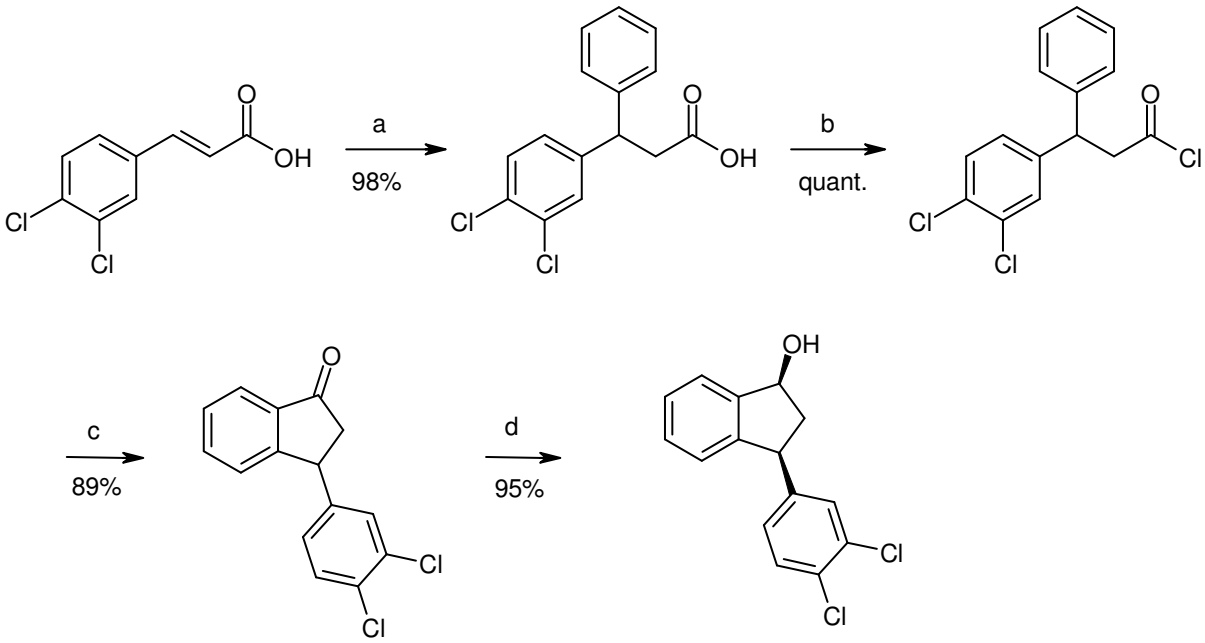
1. Synthesis of Indatraline hydrochloride (*rac*-**1**·HCl)



cis-(±)-3-(3,4-dichlorophenyl)indan-1-ol*

rac-**1** · HCl*

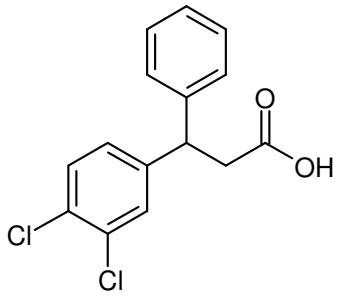
a) $AlCl_3$ (2 equiv.), benzene, rt., 20 h. b) $SOCl_2$ (3 equiv.), DMF (1.8 equiv.), DCM, rt., 45 h. c) $AlCl_3$ (3 equiv.), benzene, 90 °C, 3 h. d) K-Selectride® (1.1 equiv.), THF, -10 °C, 20 h. e) 1. methanesulfonyl chloride (2 equiv.), NEt_3 (4 equiv.), THF, -15 °C, 1 h; 2. NH_2CH_3 (2 M, 20 equiv.), 0 °C to rt, 20 h, 3. aqueous HCl (1 M).

*Depicted compounds are racemic although only one enantiomer is shown.

2. General Methods

Benzene, THF and NEt_3 were distilled from sodium and CH_2Cl_2 was distilled from CaH_2 under nitrogen. $SOCl_2$ (Fluka), DMF (Acros) and methanesulfonyl chloride Fluka) were 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 aluminium sheets (Merck). Compounds were first stained with I_2 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. If nothing else is stated, 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 1600 and Paragon 1000 (Perkin Elmer), oils were measured as film, solid samples as KBr pellets for measurements. Mass spectrometry: EI and CI, Mass Spectrometer 5989 A with 59980 B particle beam LC/MS interface (Hewlett Packard); ESI, API 2000 (Applied Biosystems). NMR spectroscopy: NMR spectra were recorded on JNMR-GX (Jeol, 500 MHz) with TMS as internal standard and integrated with the program of NMR-software Nuts (2D Version 5.097, Acorn NMR, 1995).

3. *3-(3,4-dichlorophenyl)-3-phenylpropanoic acid*[1]

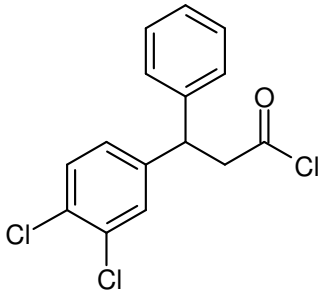


To a stirred solution of (*E*)-3-(3,4-dichlorophenyl)acrylic acid (2.06 g, 9.50 mmol) in benzene (40 mL) was added $AlCl_3$ (2.53 g, 19.0 mmol) at r.t. After stirring for 23 h the reaction mixture was poured into phosphate buffer (pH 5.5, 1 M, 70 mL) and the aqueous phase was extracted with EtOAc (5 x 80 mL). The combined organic layers were washed with saturated K-Na-tartrate solution (40 mL) then dried ($MgSO_4$) and filtered. The solvent was removed in vacuo. The resulting material was not subjected to a further purification step. Colorless oil (2.76 g, 98 %):

[1] Bøgesø KP, Christensen AV, Hyttel J, Liljefors T. *3-Phenyl-1-indanamines. Potential Antidepressant Activity and Potent Inhibition of Dopamine, Norepinephrine, and Serotonin Uptake.* J Med Chem 1985; 28: 1817-1828.

^{1}H NMR (500 MHz, CD_2Cl_2): δ = 3.06 (dd, *J* = 16.2/8.2 Hz, 1 H, CH_2), 3.10 (dd, *J* = 16.2/7.7 Hz, 1 H, CH_2), 4.47 (t, *J* = 7.9 Hz, 1 H CH_2CH), 7.11 (dd, *J* = 8.3/2.1 Hz, 1 H, CC*H*CHCCl), 7.18–7.23 (m, 3 H, H_{ar}), 7.27–7.33 (m, 2 H, H_{ar}), 7.34 (d, *J* = 2.1 Hz, 1 H, CCHCCl), 7.34 (d, *J* = 8.3 Hz, 1 H, CCHC*H*CCl). ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 39.9, 46.0, 127.2, 127.4, 127.6, 128.7, 129.0, 129.8, 130.7, 142.3, 144.0, 177.2. IR (film): $\tilde{\nu}$ = 3087 cm^{-1}, 3062, 3092, 2920, 2671, 1713, 1471. MS (CI, CH_5^+): *m*/*z* (%): 295 (4) $[M+H]^+$, 149 (100). HRMS (EI): *m*/*z* $[M]^+$ cacld. for $C_{15}H_{12}O_2{}^{35}Cl_2$: 294.0214, found: 294.0210.

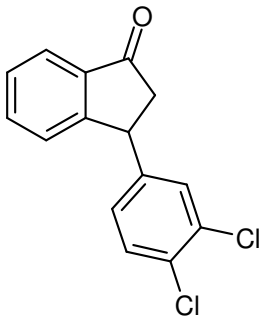
4. *3-(3,4-dichlorophenyl)-3-phenylpropanoyl chloride*



To a stirred solution of 3-(3,4-dichlorophenyl)-3-phenylpropanoic acid (11.4 g, 38.6 mmol) in CH_2Cl_2 (100 mL) was added $SOCl_2$ (8.41 mL, 116 mmol) and DMF (5.38 mL, 69.5 mmol) at 0 °C. The ice bath was removed and the reaction mixture was stirred at r.t. for 20 h. CCl_4 (200 mL) was added and the solvent was removed in vacuo. The resulting product was not subjected to a further purification step. Colorless oil (12.1 g, 100 %):

^{1}H NMR (500 MHz, CD_2Cl_2): δ = 3.62 (dd, *J* = 18.0/7.9 Hz, 1 H, CH_2), 3.66 (dd, *J* = 16.2/7.7 Hz, 1 H, CH_2), 4.55 (t, *J* = 7.7 Hz, 1 H CH_2CH), 7.11 (dd, *J* = 8.3/2.2 Hz, 1 H, CC*H*CHCCl), 7.18–7.23 (m, 2 H, H_{ar}), 7.23-7.38 (m, 1 H, H_{ar}), 7.31–7.36 (m, 3 H, 2 x $H_{ar,}$ CCHCCl), 7.61 (td, *J* = 7.5/1.3 Hz, 1 H, CCHC*H*), 7.77 (ddt, *J* = 7.7/1.2/0.6 Hz, 1 H,) ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 41.8, 47.8, 122.7, 123.0, 124.6, 125.2, 126.3, 126.5, 128.2, 136.4, 138.0, 144.0, 167.3. IR (film): $\tilde{\nu}$ = 3087 cm^{-1}, 3062, 3029, 2922, 1799, 1471. MS (EI): *m*/*z* (%): 312 (10) $[M]^+$, 235 (100). HRMS (EI): *m*/*z* $[M]^+$ cacld. for $C_{15}H_{11}O^{35}Cl_3$: 311.9876, found: 311.9882.

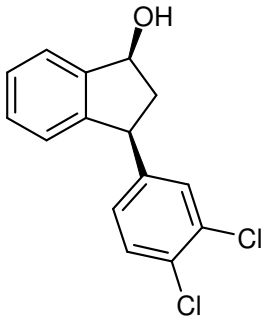
5. *3-(3,4-dichlorophenyl)indan-1-one*[1,2]



To a stirred solution of 3-(3,4-dichlorophenyl)-3-phenylpropanoyl chloride (2.94 g, 0.94 mmol) in benzene (36 mL) was added $AlCl_3$ (3.71g, 27.8 mmol) at r.t. and then refluxed for 3 h. After cooling to r.t. the reaction mixture was poured into H_2O (150 mL) followed by extraction with EtOAc (6 x 60 mL). The combined organic layers were washed with saturated K-Na-tartrate solution (40 mL) then dried ($MgSO_4$) and filtered. The solvent was removed in vacuo. The resulting crude product was purified by flash chromatography (heptane/EtOAc = 85:15). Colorless solid (2.25 g, 89%): m.p. 104-105 °C (Lit.[1] 113-15 °C). ^{1}H NMR (500 MHz, CD_2Cl_2): δ = 2.59 (dd, *J* = 19.1/3.9 Hz, 1 H, CH_2), 3.20 (dd, *J* = 19.1/8.2 Hz, 1 H, CH_2), 4.57 (dd, *J* = 8.2/3.9 Hz, 1 H, CH_2CH), 7.00 (dd, *J* = 8.3/2.2 Hz, 1 H, CC*H*CHCCl), 7.24 (d, *J* = 2.1 Hz, 1 H, CCHCCl), 7.27 (dq, *J* = 7.8/0.9 Hz, 1 H, C(=O)CCCH), 7.40 (d, *J* = 8.3 Hz, 1 H, CCHC*H*CCl), 7.46 (ddt, *J* = 8.0/7.5/0.9 Hz, 1 H, C(=O)CCHC*H*), 7.61 (td, *J* = 7.5/1.3 Hz, 1 H, C(=O)CCCHC*H*), , 7.77 (ddt, *J* = 7.5/1.2/0.6 Hz, 1 H, C(=O)CCH) . ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 43.6, 46.4, 123.4, 126.8, 127.4, 128.3, 129.7, 130.79, 130.83, 132.7, 135.3, 136.9, 144.5, 156.6, 204.6. IR (KBr): $\tilde{\nu}$ = 3050 cm^{-1}, 3014, 2947, 2914, 1699, 1471, 764. MS (CI, CH_5^+): *m*/*z* (%): 277 (100) $[M+H]^+$. HRMS (EI): *m*/*z* $[M]^+$ cacld. for $C_{15}H_{10}O^{35}Cl_2$: 276.0109, found: 276.0108.

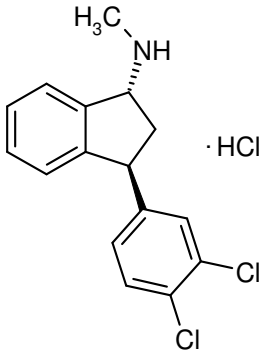
[2] Davies HML, Gregg TM. *Asymmetric synthesis of (+)-indatraline using rhodium-catalyzed C-H activation.* Tetrahedron Lett 2002; 43: 4951-4953..

6. *cis*-(±)-3-(3,4-dichlorophenyl)indan-1-ol[1,2]



To a stirred solution of 3-(3,4-dichlorophenyl)indan-1-one (1.18 g, 4.27 mmol) in THF (40 mL) was added a solution of K-Selectride® in THF (1 M, 4.70 mL, 4.70 mmol) at -10 °C. After stirring for 20 h the reaction mixture was poured into and then refluxed for 3 h. After cooling to r.t. the reaction mixture was poured into a saturated K-Na-tartrate solution (100 mL) followed by extraction with EtOAc (5 x 90 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 = 80:20). Colorless solid (1.15g, 97%): m.p. 73-74 °C (Lit.[1] 90-91 °C). ^{1}H NMR (500 MHz, CD_2Cl_2): 1.86 (ddd, *J* = 12.9/8.8/7.2 Hz, 1 H, CH_2), 2.98 (ddd, *J* = 12.9/7.8/7.0 Hz, 1 H, CH_2), 4.17 (t, *J* = 8.3 Hz, 1 H, CH_2CH), 5.27 (q, *J* = 6.5 Hz, 1 H, C*H*OH), 6.92 (dq, *J* = 7.7/1.0 Hz, 1 H, CH(OH)CCC*H*), 7.11 (dd, *J* = 8.3/2.1 Hz, 1 H, CC*H*CHCCl), 7.25 (tt, *J* = 7.4/0.9 Hz, 1 H, CH(OH)CHC*H*), 7.31 (tt, *J* = 7.5/1.1 Hz, 1 H, CH(OH)CCCHC*H*), 7.35 (d, *J* = 2.1 Hz, 1 H, CCHCCl) 7.40 (d, *J* = 8.3 Hz, 1 H, CCHC*H*CCl), 7.46 (dq, *J* = 7.5/0.9 Hz, 1 H, CH(OH)CC*H*). ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 46.7, 47.7, 74.9, 121.9, 122.8, 125.5, 125.9, 126.5, 128.20, 128.4, 128.5, 120.3, 142.6, 143.2, 143.5. IR (KBr): $\tilde{\nu}$ = 3334 cm^{-1}, 3070, 3026, 2964, 2927, 2862, 1469, 744. MS (EI) *m*/*z* (%): 278 (53) $[M]^+$, 105 (100). HRMS (EI): *m*/*z* $[M]^+$ cacld. for $C_{15}H_{12}O^{35}Cl_2$: 278.0265, found: 276.0281.

7. Indatraline hydrochloride (*rac*-**1**·HCl)[1,2]



To a stirred solution of *cis*-(±)-3-(3,4-dichlorophenyl)indan-1-ol (1.60 g, 5.73 mmol) in THF (40 mL) was added NEt_3 (3.19 mL, 22.9 mmol) at -15 °C and methanesulfonyl chloride (679 µL, 8.60 mmol). After 1 h the reaction mixture was warmed to 0 °C and gaseous 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 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 (50 mL) followed by extraction with EtOAc (6 x 70 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 = 85:15 + 1% *N*,*N*-dimethylethylamine). The resulting colorless oil was dissolved in dioxane (1 mL), treated with aqueous HCl (1 M, 10 mL) and freeze dried. The resulting product was recrystallized from EtOAc (15 mL) to yield colorless crystals (1.41 g, 75%): m.p. 120-123 °C (Lit.[1]: 183-185 °C). ^{1}H NMR (500 MHz, D_2O): 2.28 (dt, *J* = 15.2/8.0 Hz, 1 H, CH_2), 2.63 (s, 3 H, CH_3), 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 = 28.26, 36.4, 45.9, 60.7, 123.8, 124.0, 125.9, 126.2, 127.8, 128.3, 128.7, 128.9, 130.1, 134.5, 142.3, 145.3. IR (KBr): $\tilde{\nu}$ = 3406 cm^{-1}, 3963, 2712, 2479, 2444, 1590, 1486, 760. MS (CI, CH_5^+) *m*/*z* (%): 292 (100) $[M+H-HCl]^+$. HRMS (EI): *m*/*z* $[M-HCl]^+$ cacld. for $C_{16}H_{15}N^{35}Cl_2$: 291.0582, found: 291.0570.

3.2.2 Second Publication:
Development and validation of an LC-ESI-MS/MS method for triple reuptake inhibitor indatraline enabling its quantification in MS Binding Assays

Binding assays which characterize the affinity of a ligand to a defined target are an indispensable tool in the drug discovery process. Next to the well-established radioligand binding assays a concept called MS Binding Assays was recently introduced.[45-47] A basic prerequisite for both types of assays is a reliable and highly sensitive quantification of the employed marker substance in biological matrices. In radioligand binding assays quantitation is accomplished by the scintillation measurement, a highly sensitive method, of radioactively labelled markers, i.e. radioligands. MS Binding Assays are based on a quantification of a native, i.e. nonlabelled marker via LC-MS.

To develop MS Binding Assays addressing the human monoamine transporter (i.e. dopamine transporter, norepinephrine transporter, and serotonin transporter) an LC-MS quantification method for the chosen marker substance, i.e. the high affine triple reuptake inhibitor indatraline, had to be established. First of all, the previously unknown ESI-MS/MS mass transitions of indatraline and of the intended internal standard ($^2H_7$)-indatraline were investigated. Next, an LC method was developed which should allow a fast, reliable, and highly sensitive quantification of indatraline in binding samples. Different parameters concerning the LC such as composition of mobile phase, size of injection volume, composition of sample milieu, etc., but also concerning the generation of the matrix, i.e. filter material, incubation and washing buffer, as well as eluent for liberation of bound marker, were examined for their effect on signal intensity of the analyte as well as signal-to-noise ratio. The final HPLC method for quantification of indatraline [YMC Triart C_{18} column (50 mm x 2.0 mm, 3 µm) with a YMC Triart C_{18} precolumn (10 mm x 2.0 mm, 3 µm) as stationary phase, a mixture of acetonitrile and ammonium bicarbonate buffer (5 mmol L^{-1}, pH 10.0) in a ratio of 90:10 (v/v) as mobile phase, a mixture of acetonitrile and ammonium bicarbonate buffer (5 mmol L^{-1}, pH 10.0) in a ratio of 75:25 (v/v) as sample milieu, at a temperature of 20 °C, a flow rate of 600 µL min^{-1}, and an injection volume of 45 µL] was then validated according to the FDA guidance for bioanalytical method validation[56] regarding selectivity, calibration standard curve in a range from 5 pmol L^{-1} (LLOQ) to 5 nmol L^{-1}, accuracy, and precision. The developed LC-ESI-MS/MS method was used not only for the quantification of indatraline but also for its *cis*-configured diastereomer in biological matrices. It is also worth stressing that the developed method is the most sensitive quantification method for indatraline described in literature so far.

Finally, as proof of concept this method was applied to MS Binding Assays characterizing the affinity of (1*R*,3*S*)-indatraline towards hNET in saturation experiments and of desipramine in competitive experiments employing (1*R*,3*S*)-indatraline as marker. Both results, i.e. found

affinity of (1*R*,3*S*)-indatraline and desipramine towards hNET, demonstrated the efficiency of the developed MS Binding Assays and its suitability as substitute for conventional radioligand binding assays addressing NET.

Declaration of contributions:

Lars Allmendinger and Gerd Bauschke synthesized indatraline, its *cis*-configured diastereomer, and ($^{2}H_{7}$)-indatraline. The HEK cell line stably expressing hNET was generated (i.e. transfection and screening of clones) by myself. The whole cell culture, required for generating matrix samples, the development of the LC-MS method and its validation according to the guideline for bioanalytical method validation of the Center for Drug Evaluation and Research (CDER)[56] were done by myself. In the present case I also performed the resolution of indatraline via crystallization, the determination of the enantiopurity of the employed (1*R*,3*S*)-indatraline via HPLC, the saturation, and competitive experiments as well as the data analysis. Furthermore, I wrote the manuscript and generated all graphics and tables. Georg Höfner and Klaus T. Wanner corrected the manuscript.

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RESEARCH PAPER

Development and validation of an LC-ESI-MS/MS method for the triple reuptake inhibitor indatraline enabling its quantification in MS Binding Assays

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Abstract We herein present the first LC-MS/MS quantification method for indatraline, a highly potent nonselective inhibitor of the three monoamine transporters (for dopamine, DAT; norepinephrine, NET; serotonin, SERT), and its application to MS Binding Assays. For HPLC, an R18 column with a mobile phase composed of acetonitrile and ammonium bicarbonate buffer (5 mmol L^{-1}, pH 10.0) in a ratio of 90:10 (v/v) at a flow rate of 600 μL min^{-1} was used. Recording indatraline at *m/z* 292.2/261.0 and ($^2H^7$)-indatraline, employed as internal standard, at *m/z* 299.2/268.0 allowed reliable quantification from 5 pmol L^{-1} (LLOQ) to 5 nmol L^{-1} in biological matrices without additional sample preparation. Validation of the developed quantification method showed that selectivity, calibration standard curve, accuracy, as well as precision meet the criteria of the CDER guideline. Applying this method to mass spectrometry (MS) Binding Assays, a label-free MS-based alternative to conventional radioligand binding assays, binding of indatraline's eutomer, (1*R*,3*S*)-indatraline, towards NET could be characterized directly for the first time, revealing an equilibrium dissociation constant (K_d) of 805 pmol L^{-1}. Additionally, it could be shown that the established MS Binding Assays enable characterization of test compounds in competition experiments. As the established setup is based on a 96-well format and an LC MS/MS method with a short chromatographic cycle time (1.5 min), the developed MS Binding Assays enable considerable throughput and are therefore well suited as substitute for corresponding radioligand binding assays.

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Introduction

Since the catechol hypothesis was published in the 1960s as an explanation for the genesis of depressions [1, 2], the neuronal systems of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) are in focus for drug development. Inhibition of the corresponding transporters [dopamine transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT)], which terminate the signals that originate from these monoamines at their receptors by transporting them out of the synaptic cleft back into neurons, represent the mainstay for the treatment of major depression [3]. Nowadays, selective inhibitors for a single monoamine transporter type (SERT or NET) as well as dual reuptake inhibitors, which inhibit two of the monoamine transporters (SERT and NET or NET and DAT), are primarily employed to achieve this task. As currently only 65 % of the depressive patients treated with corresponding monoamine transporter inhibitors are showing a sufficient therapeutic response, there is still a substantial need for the development of new antidepressants with an improved therapeutic profile [4].

The identification of new drug candidates addressing monoamine transporters (or other targets aiming at the therapy of depression) requires suitable screening tools. Therefore, a broad spectrum of techniques providing information regarding affinity or functional activity is available. Competitive binding assays based on a reporter ligand, with high affinity for the target of interest, represent an efficient approach for affinity determination of test compounds. A prerequisite for this approach is a highly sensitive quantification of the reporter ligand. Commonly, this task is achieved by employing a ligand labelled with a radioisotope. Despite the extraordinarily

high sensitivity of this detection principle, the use of radioligands is associated with considerable disadvantages such as severe legal restrictions in the handling of these hazardous compounds and problems with the resulting radioactive waste. Mass spectrometry (MS) developed to be a promising detection technique to monitor binding of native ligands to defined targets in the last decades, either directly at the level of the target-ligand complexes or at the level of the ligands interacting with the targets. As it is beyond the scope of this introduction to give an overview over MS-based screening techniques, the interested reader is referred to literature [5–10]. A simple but very efficient strategy to avoid the drawbacks of radioligands in binding assays is the concept of MS Binding Assays recently developed in our group [11, 12]. It is based on nonlabelled reporter ligands, in this case termed MS marker, native marker, or simply marker, that can be quantified by means of mass spectrometry. MS Binding Assays follow the conventional setup of radioligand binding assays but offer all of their possibilities and are therefore a promising substitute for radioligand binding assays.

It was the aim of the present study to establish an LC-MS/MS method for an MS marker that can be employed to address DAT, NET, and even SERT in MS Binding Assays. As in this case the conditions of the intended binding experiments can be adopted from known radioligand binding and transport assays, the development of a highly sensitive, reliable, robust, and fast quantification method for the selected MS marker can be considered by far as the most time-consuming step in the process to set up corresponding MS Binding Assays. Therefore, selection of a marker, which can be used to address all of the three targets (DAT, NET, and SERT), can be considered as a particularly efficient approach, as the development of a suitable quantification method causes only a single effort. In this context, it should be mentioned that this approach is only feasible when the individual targets are individually accessible, as this is the case when heterologous expression systems are employed. According to this idea, we intended to benefit from indatraline [(1*RS*,3*SR*)-3-(3,4-dichlorophenyl)-1-methylamino-2,3-dihydro-1*H*-indene], also known as Lu 19-005 [13], that inhibits all three monoamine transporters (DAT, NET, SERT) with high potency and is therefore referred to as a triple reuptake inhibitor. For the MS Binding Assays to be established, we aimed to employ the eutomer, (1*R*,3*S*)-indatraline, as MS marker (Fig. 1).

So far, neither (1*R*,3*S*)-indatraline nor (1*S*,3*R*)-indatraline has been characterized in binding assays towards DAT, NET, or SERT. However, both enantiomers of indatraline have been investigated in uptake assays for their inhibitory potencies, revealing low nanomolar to subnanomolar IC_{50} values and a eudismic ratio of 5 (SERT) to 51 (DAT) in favor of (1*R*,3*S*)-indatraline [13]. Assessing the significance of these data regarding potency and selectivity of (1*R*,3*S*)-indatraline for our intended binding assays addressing DAT, NET, and SERT, we had to consider the conditions under which these uptake assays had been performed [13]. As Bogeso et al. employed indatraline enantiomers with a moderate enantiopurity (≥95 % *ee*) in the uptake experiments [13], the true potencies of the enantiomers may differ to some extent to those determined in this study. Furthermore, Bogeso et al. used crude animal (rat) brain preparations in their uptake experiments [13], which do not represent an appropriate target source for selective characterization of the individual monoamine transporters in uptake assays, as the substrates employed (in this case [^{3}H]DA, [^{3}H]NE, and [^{3}H]5-HT) could also interact with other targets present in the preparation. Thus, the data may be falsified to some extent. Distinctly more information is available from radioligand binding experiments at monoamine transporters regarding the racemic compound. Indatraline's affinity (i.e., inhibition constants, K_i) employing different radioligands such as [^{125}I]RTI-55 [14–16] or [^{3}H]WIN-35,428 [17–19] for DAT, [^{125}I]RTI-55 [16] and [^{3}H]nisoxetine [15, 20] for NET, and [^{125}I]RTI-55 [14, 16, 21], [^{3}H]citalopram [15], [^{3}H](*S*)-citalopram [22, 23], [^{3}H]DASB [23], [^{125}I]EINT [24], or [^{3}H]paroxetine [25] for SERT in binding assays (see Electronic Supplementary Material (ESM) Fig. S1) was found to be in the low nanomolar to subnanomolar concentration range. Again, most of the corresponding data were generated employing crude membrane preparations [except for [16, 22, 23] employing human embryonic kidney (HEK) membrane preparations]. Based on all the information available, (1*R*,3*S*)-indatraline's equilibrium dissociation constant (K_d) in binding experiments addressing DAT, NET, or SERT can only be assumed to be in the range from low nanomolar down to picomolar concentrations, but is not exactly known. Therefrom, the following consequences are arising.

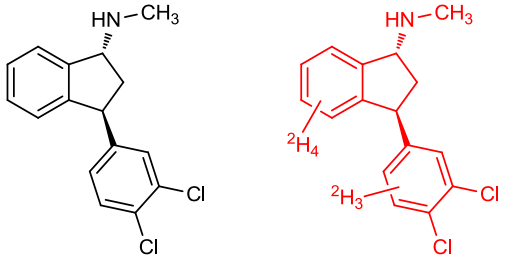


Fig. 1 (1*R*,3*S*)-indatraline [(1*R*,3*S*)-3-(3,4-dichlorophenyl)-1-methylamino-2,3-dihydro-1*H*-indene] (*black*) and ($^2H_7$)-indatraline [(1*RS*,3*SR*)-3-(3,4-dichloro(2,5,6-$^2H_3$)phenyl)-1-methylamino-2,3-dihydro(4,5,6,7-$^2H_4$)-1*H*-indene] (*red*)

At first and foremost, the quantification method for indatraline to be applied in the corresponding MS Binding Assays has to be extremely sensitive. An estimation for the required sensitivity can be derived from the expected K_d value of (1*R*,3*S*)-indatraline towards hDAT, hNET, or hSERT (low nanomolar down to picomolar range, see above) in combination with the rules generally accepted for saturation experiments in radioligand binding assays. These are nominal

marker concentrations in a range from at least 0.1 K_d to 10 K_d and target concentrations in a magnitude of up to 0.1 K_d that have to be employed to avoid a marker depletion [26]. It has to be mentioned that target concentrations distinctly below 0.1 K_d will further increase the challenge of marker quantification. For our intended hDAT, hNET, or hSERT saturation experiments following these recommendations, the concentration of the bound marker could be assumed to be in the low picomolar perhaps even in the femtomolar range for binding samples containing nominal (1*R*,3*S*)-indatraline concentrations in the low or middle picomolar range. As a consequence, our aspired lower limit of quantification (LLOQ) should be as low as possible but at least in the very low picomolar range. Such a highly sensitive method to quantify indatraline has—to the best of our knowledge—not been described so far. The only LC method known for indatraline employs UV detection [27]. Therefore, we started to establish a new LC-ESI-MS/MS quantification method employing indatraline in its racemic form, which is commercially available. Secondly, as ESI-MS/MS is prone to ion suppression that may in our case result from the matrix of the binding samples, it was our intention to make use of ($^2H_7$)-indatraline [(1*RS*,3*SR*)-3-(3,4-dichloro(2,5,6-$^2H_3$)phenyl)-1-methylamino-2,3-dihydro(4,5,6,7-$^2H_4$)-1*H*-indene] (Fig. 1) that was synthesized recently in our group to serve as internal standard [28]. Thirdly, to assure reliable results in MS Binding Assays, this LC-ESI-MS/MS quantification method should be validated according to generally accepted criteria for bioanalytical methods before its application. Furthermore, the LC-ESI-MS/MS method to be developed for quantification of indatraline should also be investigated for its capability to enable quantification of the *cis*-configured diastereomer of indatraline in MS Binding Assays, which is known as a SERT selective monoamine transport inhibitor [13, 16].

As an MS Binding Assay addressing hSERT [29] was already available in our group, the development of new MS Binding Assays employing (1*R*,3*S*)-indatraline as marker was focused on hDAT and hNET. Due to the fact that HEK cells stably expressing hNET were obtained first, we intended to set up saturation and competition experiments for this transporter as a proof of concept.

Materials and methods

Chemicals

All LC-MS grade solvents (acetonitrile, water) as well as HPLC grade methanol were purchased from VWR Prolabo (Darmstadt, Germany). Water for incubation and wash buffer was obtained in-house by distillation of demineralized water (prepared by reverse osmosis) and subsequent filtration using 0.45 μm filter material. Additives for LC-MS (ammonium bicarbonate, ammonium hydroxide solution ≥25 %, ammonium formate, and formic acid), all of LC-MS quality, were bought from Fluka (Taufkirchen, Germany). Ammonium acetate (HPLC grade) and HEPES (2-[4-(hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) were purchased from VWR Prolabo (Darmstadt, Germany).

Indatraline hydrochloride, ($^2H_7$)-indatraline deuterochloride, and the hydrochloride of the *cis*-configured diastereomer of indatraline were synthesized in-house. For (1*R*,3*S*)-indatraline hydrochloride employed in this study, an enantiopurity of >99.75 % *ee* was determined according to the method previously described [27]. [^{3}H]MPP$^+$ acetate (80–85 Ci mmol^{-1}) for uptake experiments was bought from BIOTREND (Cologne, Germany). Nonlabelled MPP$^+$ iodide and desipramine hydrochloride were from Sigma-Aldrich (Steinheim, Germany).

Dulbecco's modified Eagle's medium (DMEM) was bought from Sigma-Aldrich, and fetal bovine serum, penicillin, streptomycin, and geneticin were bought from PAA (Cölbe, Germany). HEK293 cells were purchased from American Type Culture Collection (LGC Standards GmbH, Wesel, Germany).

Unless otherwise indicated, all percentages and ratios are specified (*v*/*v*).

Preparation of standards and quality controls

For the preparation of stock solutions, the hydrochlorides of indatraline, (1*R*,3*S*)-indatraline, and the *cis*-configured diastereomer of indatraline as well as the deuterochloride of ($^2H_7$)-indatraline, respectively, were dissolved in an appropriate volume of water, resulting in a concentration of 10 mmol L^{-1} in each case. ($^2H_7$)-Indatraline stock solution was diluted with water, yielding a 1 μmol L^{-1} working solution, and indatraline, (1*R*,3*S*)-indatraline, and *cis*-configured diastereomer stock solutions were diluted in the same way, obtaining working solutions of 1μmol L^{-1}, 100, and 10 nmol L^{-1}. All solutions were stored at room temperature. On the day of the assay, respective working solutions were diluted with acetonitrile to yield the required concentrations for the preparation of matrix-based calibration standards, quality controls (QC), and zero samples. Generation of matrix samples was performed as described for binding samples ("MS Binding Assay—standard setup"; see below). After filtration and drying, the filter plates were eluted with 3× 75 μL of a solution, containing analyte and internal standard, or just the internal standard, in acetonitrile as required, to obtain calibration standards, QCs, and zero samples, or acetonitrile to obtain matrix blanks. Finally, 75 μL of a 5 mmol L^{-1} ammonium bicarbonate buffer (pH 10.0) was added to each well, obtaining the desired concentrations (see "Method validation") of ($^2H_7$)-indatraline and indatraline for calibration standards and QCs (the same procedure was

applied to obtain corresponding standards and QCs for the *cis*-configured diastereomer).

LC-ESI-MS/MS

LC-MS instrumentation

Preliminary experiments for HPLC method development and post column infusion experiments were performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent vacuum degasser, quaternary pump, column oven; Agilent, Waldbronn, Germany) and a Shimadzu SIL-10 autosampler (Shimadzu, Duisburg, Germany). For further method development, validation, and binding experiments, an API 5000 triple quadrupole mass spectrometer with a Turbo V ion source coupled to an Agilent 1200 HPLC system (Agilent vacuum degasser, binary pump, column oven) and an HTS-PAL autosampler (50 μL sample loop, 50 μL syringe, CTC Analytics, Zwingen, Switzerland) was used. Direct infusion experiments at the API 5000 were performed using a syringe pump (model 11 Plus MA 170-2208, Harvard Apparatus Inc., Holliston, MA, USA). For all MS-based investigations, Q1 and Q3 were operated with unit resolution.

For HPLC, a YMC Triart C18 (50×2.0 mm, 3 μm; YMC Europe GmbH, Dinslaken, Germany) with a YMC Triart C18 precolumn (10×2.0 mm, 3 μm) was employed. For routine MS Binding Assays, the column was additionally protected with two IDEX frits (Wertheim-Monfeld, Germany) of 0.5 and 0.2 μm porosity, respectively, and the first 0.6 min of the eluents was sent to waste. For all experiments, the column temperature was set to 20 °C.

Method development

Optimization of compound-dependent parameters for the precursor and the product ions of indatraline and ($^2H_7$)-indatraline was performed by direct infusion of a 1 μmol L^{-1} solution (API 2000) or a 20 nmol L^{-1} solution (API 5000), which were prepared in methanol/0.1 % formic acid at a ratio of 50:50, using the Analyst 1.4.2 Quantitative Optimization tool.

For the examination of the influence of the pH value on chromatography, matrix samples were generated by elution of hNET matrix ("MS Binding Assays—standard setup"; see below) with 3×100 μL methanol, drying overnight at 50 °C, and, finally, reconstitution of the dried sample residues in 300 μL of the respective mobile phase. The capacity factor (k) calculation was based on a total void volume comprised of the experimentally determined system void volume and the column void volume calculated to 131.95 mm^3 according to [30].

Using the flow injection analysis (FIA) option of the Analyst 1.4.2 Quantitative Optimization tool, 10 μL of a 500 pmol L^{-1} indatraline solution, dissolved in the mobile phase, was injected to determine the most appropriate source-dependent parameters for the final HPLC method.

Method validation

The validation was based on six different sample series (each including blank samples, zero samples, calibration standards, and QCs, all in the presence of the matrix obtained from hNET binding samples) investigated on different days using different batches of membrane preparation ("MS Binding Assays—standard setup"; see below).

Linearity was determined for calibration standards at ten different concentration levels (i.e., 5 pmol L^{-1}–5 nmol L^{-1}), which were prepared in triplicates, except for 5 pmol L^{-1} (LLOQ, six replicates). Obtained area ratios (y) of indatraline vs ($^2H_7$)-indatraline were plotted against the concentration of indatraline (x). A linear regression with a weighting of $1/x^2$ was applied to all calibration data sets. QC samples at five different concentration levels (i.e., 10, 25, 100, 500 pmol L^{-1} and 1 nmol L^{-1}), each consisting of six replicates, were used to evaluate intra- and inter-batch accuracy as well as precision. The LLOQ was defined as the lowest concentration of indatraline, which had at least a signal-to-noise ratio of 5:1, an accuracy of 80–120 %, and a precision of a relative standard deviation (RSD) ≤20 %. Acceptance limits for accuracy were 85–115 % (except LLOQ) and for precision an RSD ≤15 % (except LLOQ).

Stability of the aqueous stock solutions of indatraline was investigated by comparing 1 nmol L^{-1} solvent standards (supplemented with 1 nmol L^{-1} internal standard, n=6) prepared from a fresh indatraline stock solution or an 8-month-old indatraline stock solution stored at room temperature, respectively, employing in both cases an 8-month-old stock solution of ($^2H_7$)-indatraline.

Analogously, the *cis*-configured diastereomer of indatraline, also using ($^2H_7$)-indatraline as internal standard, was investigated (based on three different sample series prepared as those containing indatraline). Linearity was determined at six different concentration levels (i.e., 5 pmol L^{-1}–1 nmol L^{-1}, triplicates). Accuracy and precision were determined for QC samples at three different concentration levels (i.e., 10, 100 pmol L^{-1} and 1 nmol L^{-1}, hexaplicates).

Additionally, on each day an MS Binding Assay was performed, also individual matrix blanks, zero samples, and matrix standards were prepared and investigated to establish a calibration function in a range from 5 pmol L^{-1} to 1 nmol L^{-1} (six concentration levels in triplicates). Furthermore, accuracy and precision were examined by analysis of QCs (10, 100 pmol L^{-1} and 1 nmol L^{-1}, hexaplicates).

Cell culture and expression of hNET

The mammalian pRc/CMV expression vector containing the complementary DNA (cDNA) coding for hNET was kindly provided by Prof. Dr. Harald H. Sitte (Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna). HEK293 cells were cultured at 37 °C and 8 % CO_2 in DMEM containing 10 % (*m*/*v*) fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (culture medium). The plasmid linearized with *Kpn*I was employed for stable transfection. About 6×10^5 HEK293 cells per 21 cm^2 culture dish were plated in the culture medium 2 days before transfection. On the third day, a mixture of 15 μL FuGENE6 (Roche, Mannheim, Germany) and 40 μg linearized pRc/CMV hNET DNA was added to these cells. Two days later, the medium was exchanged using for further cultivation "selection medium" [DMEM supplemented with 10 % (*m*/*v*) fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 500 μg mL^{-1} geneticin]. Under these conditions, cells were cultured till an appropriate confluence was obtained in a 145-cm^2 culture dish. After that, these cells were detached (see below) and split in the selection medium for a dilution of ≤1 cell per 100 μL. Therefrom, aliquots of 100 μL were transferred in a 96-well culture dish. Single colonies of stably transfected cells were further cultivated in the selection medium and tested for their expression of hNET using a [^{3}H]MPP^+ uptake assay (for details, see supplementary material). The clone with the highest ratio of total uptake vs nonspecific uptake was used for hNET binding experiments.

hNET membrane preparation

HEK293 cells stably expressing hNET were cultivated in a selection medium, as described above. After detaching the cells by repeated aspiration and discharge of 10 mL phosphate-buffered saline (PBS, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 8 mmol L^{-1} Na_2HPO_4, 1.75 mmol L^{-1} KH_2PO_4, pH 7.4) per 145 cm^2 culture dish, the cell suspension was transferred to 50-mL tubes and centrifuged for 5 min at 1600 rpm at 4 °C (Biofuge Stratos, Heraeus, Hanau, Germany). The resulting pellet was washed twice with 10 mL PBS buffer per 145 cm^2 culture dish via resuspension and centrifugation as described above. Finally, the pellet was resuspended in 0.32 mol L^{-1} sucrose with a Polytron PT A7 (Kinematica, Littau-Luzern, Switzerland) and frozen in aliquots at -80 °C. On the day of the assay, an aliquot (1.0 mL) of the membrane preparation was thawed, diluted in 20 mL assay buffer (50 mmol L^{-1} HEPES, 120 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, pH 7.4), and centrifuged (20 min, 20,000 rpm, 4 °C, Sorvall Evolution, SS-34 rotor, Thermo Fisher Scientific, Dreieich, Germany). The resulting pellet was resuspended in ice-cold assay buffer to yield a final protein concentration of approximately 40–60 μg mL^{-1} (according to Bradford after incubation with 100 mmol L^{-1} sodium hydroxide and bovine serum albumin as standard [31]).

MS Binding Assays—standard setup

hNET membrane preparations and marker were incubated in assay buffer in polypropylene 96-deep-well plates at 37 °C in a shaking water bath. Incubation was terminated by filtration. Therefore, aliquots of the binding samples were transferred by means of a 12-channel pipette onto 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 μm, 350 μL, Multi Well Plate Vacuum Manifold, Pall, Dreieich, Germany), which had been pretreated for 1 h with 200 μL of a 0.5 % (*m*/*v*) polyethyleneimine solution. Membrane fragments with the bound marker remaining on the filter were washed five times with 150 μL ice-cold 150 mmol L^{-1} ammonium acetate buffer (pH 7.4). Afterwards, the filter plates were dried for 1 h at 50 °C and cooled down to room temperature. Subsequently, the bound marker was liberated via elution with acetonitrile containing 1.33 nmol L^{-1} ($^2H_7$)-indatraline (3 × 75 μL, 30 s for every aspiration step). After addition of 75 μL of ammonium bicarbonate buffer (5 mmol L^{-1}, pH 10.0) per well, the plates were centrifuged (10 min, 2500 rpm, 4 °C; Biofuge Stratos, Heraeus, Hanau, Germany), sealed with aluminum foil, and the samples analyzed according to the validated LC-MS/MS method.

Matrix samples for method development and validation were generated as hNET binding samples (protein according to Bradford in a range from 0.5 to 5 μg per well, total incubation volume 250 μL, aliquot transferred to the filter plate 200 μL) but in the absence of (1*R*,3*S*)-indatraline.

MS Binding Assays—saturation experiments

hNET membrane preparations (approximately 2.0 μg protein per well) and (1*R*,3*S*)-indatraline (15 concentration levels in a range from 50 pmol L^{-1} to 30 nmol L^{-1}, six replicates per concentration level) were incubated in polypropylene 96-well plates (2.2 mL per well; SARSTEDT, Nümbrecht, Germany; total incubation volume 2.0 mL) for 2 h. Incubation was basically terminated by filtration of the binding samples through a glass fiber filter plate as described for the standard setup. In contrast to the latter, however, two aliquots of 1800 μL per incubation replicate were each transferred to the same wells of the filter plate (i.e., two of the hexaplicate incubation samples were combined to obtain three replicates on the filter plate, each generated from 3600 μL of the respective binding samples per filter well). The remaining membrane

fragments retained on the filter were further treated as described for the standard setup.

Nonspecific binding was determined in the same way but in the presence of 10 μmol L^{-1} desipramine. Employing this modified setup, nonspecific binding could directly be determined at marker concentrations ≥500 pmol L^{-1}. Nonspecific binding for marker concentrations <500 pmol L^{-1} was extrapolated from the experimental data (as described below in "Data analysis").

MS Binding Assays—competition experiments

The described standard setup was also used for competitive experiments. The total incubation volume was 250 μL, and an aliquot of 200 μL therefrom was transferred to the filter plate. (1*R*,3*S*)-Indatraline was employed as marker in a concentration of 2.4 nmol L^{-1}. For affinity characterization, desipramine was incubated in the presence of the marker and the target (approximately 2.5 μg per well) at least at seven concentrations in a range from 50 pmol L^{-1} to 1 μmol L^{-1}.

Nonspecific binding was determined in an additional experiment in a range of 2.5 to 20 nmol L^{-1} of (1*R*,3*S*)-indatraline as described above (see saturation experiments). The corresponding nonspecific binding for the employed marker concentration, i.e., 2.4 nmol L^{-1}, was extrapolated from the respective experimental data (as described below in "Data analysis").

Data analysis

LC-ESI-MS/MS data for method development were obtained using Analyst 1.4.2, and data for validation as well as for binding experiments were obtained using Analyst 1.6.1. Calibration functions were generated by linear regression using Prism 5.0 (GraphPad Software, San Diego, CA, USA).

In saturation experiments, the equilibrium dissociation constant (K_d) and the maximum amount of binding sites (B_{max}) were calculated from specific binding using the nonlinear regression tool "one site – specific binding" of Prism 5.0. Specific binding was defined as the difference between total and nonspecific binding.

For determination of the inhibitory constants (K_i) of desipramine in competitive experiments, the nonlinear regression tool "one site – Fit Ki" of Prism 5.0 was used. Therefore, the corresponding specific binding, calculated as the difference of total binding and nonspecific binding, was plotted on a percentage basis of a respective binding sample in the absence of any inhibitor, which was set to 100 %, vs the employed log concentration of the test compound, whereby 0 % was equal to the nonspecific binding.

Nonspecific binding could be experimentally determined at a marker concentration ≥500 pmol L^{-1} (saturation experiments) and ≥2.5 nmol L^{-1} (competition experiments). At lower concentrations, nonspecific binding was calculated by extrapolation (linear regression) of the respective experimental data using Prism 5.0. Marker depletion was negligible in all experiments (≤10 %).

For statistical comparisons, data were examined by means of *F* test and *t* test (two sites, α=0.05 as far as not indicated otherwise in both cases).

Results and discussion

Method development

As the LC-MS method to be developed should be used for quantification of indatraline in MS Binding Assays for DAT, NET, and SERT, an LLOQ at least in the low picomolar range was a key requirement. To achieve this task, we tried to benefit from the high performance of an API 5000 triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source. To allow reliable and robust quantification of the analyte in the matrix resulting from the binding assays, ($^2H_7$)-indatraline was employed as internal standard. Furthermore, the method to be developed was expected to be fast enough to enable a considerable throughput and avoid any sample preparation.

ESI-MS/MS

As ESI-MS/MS mass transitions for indatraline and ($^2H_7$)-indatraline have not been described so far, the first aim was to find and optimize the potentials for the $[M+H]^+$ species of indatraline and ($^2H_7$)-indatraline in a Q1 scan. Afterwards, a product ion scan was performed to identify the most intensive product ions of the $[M+H]^+$ parent ions of indatraline (*m/z* 292.2) and ($^2H_7$)-indatraline (*m/z* 299.2), respectively. The five most intensive fragments observed were *m/z* 261.0, 225.9, 191.0, 189.2, and 115.1 for indatraline as well as *m/z* 268.0, 232.9, 198.2, 195.3, and 119.2 for ($^2H_7$)-indatraline, respectively (Fig. 2). For the most prominent mass transitions observed for indatraline (*m/z* 292.2/261.0) and for ($^2H_7$)-indatraline (*m/z* 299.2/268.0), which are presumably the result of a loss of a methylamine moiety, as it is described for other methylamine derivatives in literature [32] and also for sertraline, a tetrahydronaphthalene analogue of indatraline [33], an optimization of the mass spectrometer's compound-dependent parameters was performed and the optimized parameters resulting therefrom used for further method development. In detail, we found an optimal declustering potential (DP) of 61 V and entrance potential (EP) of 10 V for both compounds [indatraline and ($^2H_7$)-indatraline], a collision cell exit potential (CXP) of 32 V for indatraline and 30 V for ($^2H_7$)-indatraline, and a collision energy (CE) of 19 V for indatraline and 17 V for ($^2H_7$)-indatraline.

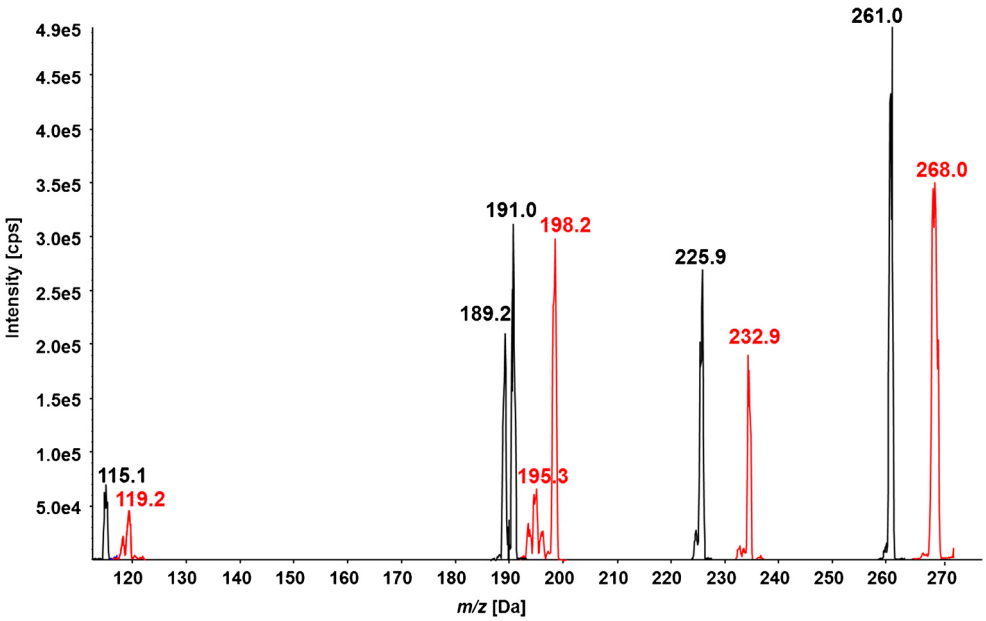


Fig. 2 Product ion scan with the five most prominent fragmentation products of the respective $[M+H]^+$ parent ions of indatraline (*m/z* 292.2; *black*) and ($^2H_7$)-indatraline (*m/z* 299.2; *red*)

LC

It was intended to establish a fast isocratic LC method based on an RP stationary phase with a mobile phase containing a high fraction of organic solvent, which should ensure high intensities and as a consequence high sensitivity under ESI conditions [34], but at the same time polar matrix components resulting from the binding samples should be separated by HPLC to avoid a respective suppression of the analyte signal.

In preliminary experiments with an RP18 stationary phase, the influence of the pH value (pH 3.0-11.0) of the aqueous component of the mobile phase on retention time and intensity of indatraline in LC-ESI-MS/MS chromatograms recording *m/z* 292.2/261.0 was studied. Furthermore, suppression of the analyte signal was investigated in post column infusion experiments using the same RP18 column and different acidic or basic mobile phases (5 mmol L^{-1} ammonium formate, pH 4.0, or 5 mmol L^{-1} ammonium bicarbonate, pH 10.0) in combination with acetonitrile at ratios of 20:80 and 10:90. The most important observations resulting from these experiments were the following. First, reasonable retention of indatraline (i.e., $k \geq 1$) employing such a high fraction of organic solvent in the mobile phase, in our case between 80 and 90 % acetonitrile, could only be obtained at pH values ≥8.5 of the aqueous component (see ESM Fig. S2). Secondly, intensities in the absence of the matrix were highest at the lower range of the investigated pH values, whereas intensities in the presence of the matrix were highest in the upper range of the investigated pH values. Thirdly, suppression of the analyte signal could be largely avoided at pH 10.0 when capacity factors (*k*) were >0.75 (see ESM Fig. S3).

These results prompted us to focus on a basic mobile phase in a pH range from 8.5 to 11.0 employing 5 mmol L^{-1} ammonium bicarbonate as additive for further method development. So, indatraline matrix standards (500 pmol L^{-1}) were employed to study this pH range at a mobile phase composition of 5 mmol L^{-1} ammonium bicarbonate and acetonitrile 10:90 at a flow rate of 600 μL min^{-1} using a 50×2.0 mm, 3 μm YMC Triart C18 column. The results from these experiments showed a slight decrease of *k* as a consequence of an increasing pH value of the aqueous mobile phase component (Fig. 3a). Additionally, it could be observed that signal intensity is hardly influenced by the presence of the matrix (Fig. 3b) but increases slightly with pH up to 10.0 and then drops markedly at a pH value above 10.5. Therefore, 5 mmol L^{-1} ammonium bicarbonate pH 10.0 was employed as aqueous component of the mobile phase (in combination with the other parameters mentioned above). This led to an acceptable intensity and was a good compromise regarding retention and chromatographic cycle time. Using the flow injection analysis (FIA) option of Analyst 1.4.2 Quantitative Optimization tool for these chromatographic parameters in the next step, the source-dependent parameters of the mass spectrometer were optimized. The most appropriate source-dependent parameters were as follows: a temperature (*T*) of 550 °C, an ion spray voltage (IS) of +3500 V, a curtain gas (CUR) of 20 psi, a nebulizing gas (GS1) of 60 psi, an auxiliary gas (GS2) of 60 psi, and a collision gas (CAD) of 5 psi for the mass transition *m/z* 292.2/261.0. In the end, these resulting optimal values were used for further method development.

Next, we tried to improve the signal intensity and also peak shape by investigating different sample solvents for the samples to be generated from the MS Binding Assays. In the setup

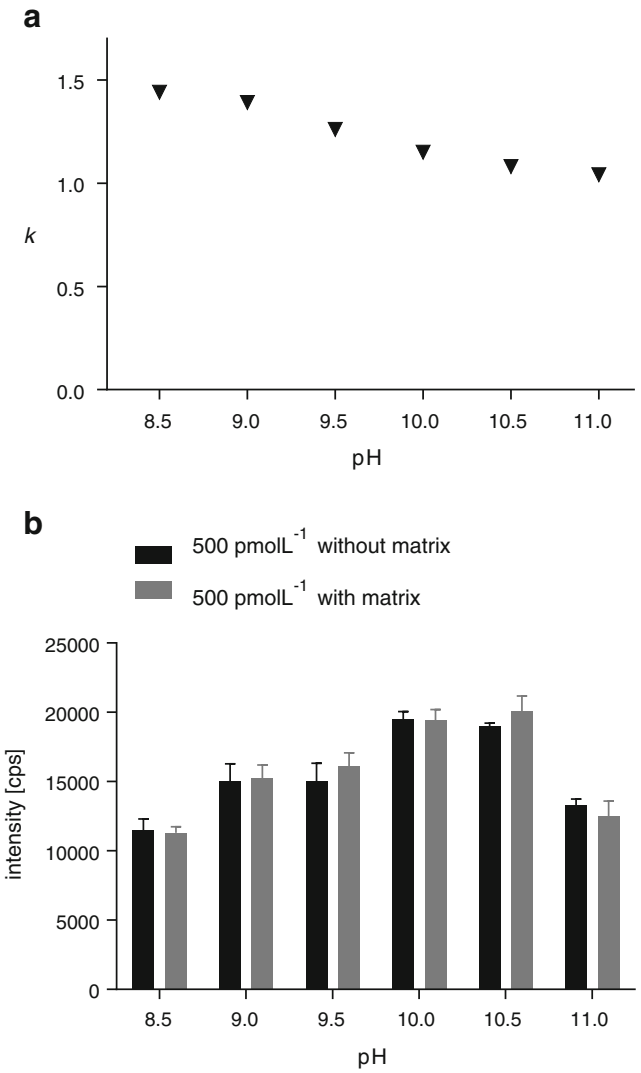


Fig. 3 Influence of aqueous phase pH on retention and intensity of indatraline. pH values in a range of 8.5 to 11.0 (*x*) are plotted against **a** *k* and **b** intensity, defined as peak height (*m/z* 292.2/261.0), of 500 pmol L^{-1} indatraline in absence (*black*) and presence of binding sample matrix (*grey*, means±SD, *n*=3). Chromatographic conditions: YMC Triart C18 (50×2.0 mm, 3 μm) with respective precolumn, 5 mmol L^{-1} ammonium bicarbonate (pH values as indicated), and acetonitrile (10:90, *v/v*) as a mobile phase at a flow rate of 600 μL min^{-1} at 20 °C and injection volume of 10 μL; for sample solvent, the mobile phase was used

of the MS Binding Assays established so far, the binding experiment is terminated by vacuum filtration over a 96-well filter plate. Subsequently, liberation and elution of the target bound marker from the membrane fragments remaining on the filter is achieved with methanol [11, 12]. Therefore, we considered a combination of methanol and 5 mmol L^{-1} ammonium bicarbonate, pH 10.0, as most convenient as sample milieu. Unfortunately, however, signals representing the mass transition of indatraline (*m/z* 292.2/261.0) as well as the mass transition resulting from the corresponding M+2 isotopologue of indatraline (*m/z* 294.2/263.0), at a retention time similar to the one of indatraline, were observed even in corresponding solvent blanks. These signals, which were small but nevertheless distinctly visible and clearly differing in their isotopic pattern from indatraline (data not shown), could be assigned to the use of methanol in combination with certain consumables, such as deep-well plates or reservoirs for multichannel pipettes, consisting of polypropylene (the longer the contact with these materials, the higher the signals).

Due to this problem, we tried to use acetonitrile instead of methanol as eluent, which is also known to denature proteins [35, 36], for liberation of the bound marker. Therefore, different ratios of the ammonium bicarbonate buffer and acetonitrile (i.e., 40:60, 30:70, 25:75, 20:80, 10:90 and pure acetonitrile) were investigated as possible sample milieu regarding their influence on peak intensity and peak shape. The results can be summarized as follows: first, rather broad peaks could be detected in pure acetonitrile; secondly, peak heights increased with increasing amount of ammonium bicarbonate buffer. As the marker is dissolved in the pure organic solvent (i.e., acetonitrile) after elution from the filter plate, addition of buffer leads to a dilution of the analyte in the sample. Therefore, we decided to use a ratio of 25 % ammonium bicarbonate buffer and 75 % acetonitrile as a compromise to gain intensity but to avoid a marked dilution of the eluted analyte.

Additionally, we examined the possibility to gain higher intensities as a consequence of higher sample injection volumes in a range from 10 to 100 μL. A linear increase of peak heights could be observed by raising the injection volume from 10 μL up to 60 μL, which was, however, accompanied by a rise of back pressure immediately after injection likely to be caused by the higher viscosity of the sample solvent in comparison to the mobile phase. To achieve a robust method suitable even for routine application with large sample numbers, we restricted the injection volume to 45 μL, in which case back pressure rose only by approximately 10 bar.

In the end, the developed method for quantification of indatraline is characterized as follows: a YMC Triart C18 column (50×2.0 mm, 3 μm) with a YMC Triart C18 precolumn (10×2.0 mm, 3 μm) as stationary phase, a composition of acetonitrile and ammonium bicarbonate buffer (5 mmol L^{-1}, pH 10.0) in a ratio of 90:10 as mobile phase, and a composition of acetonitrile and ammonium bicarbonate buffer (5 mmol L^{-1}, pH 10.0) in a ratio of 75:25 as sample milieu is used; temperature amounts to 20 °C, flow rate to 600 μL min^{-1}, and injection volume to 45 μL.

This resulting HPLC method was characterized by a chromatographic cycle time of only 1.5 min, whereby the eluent for the first 0.6 min was directed to waste to protect the mass spectrometer from the matrix. It is also noteworthy that we defined a system suitability parameter for the final HPLC method, in this case the retention time of the analyte, which had to be in a range of 0.82±0.08 min, as the pH value of the

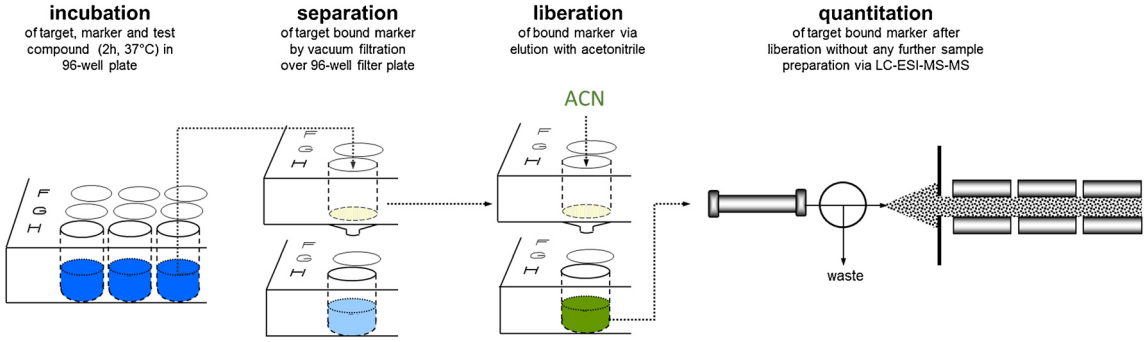


Fig. 4 Basic workflow for the developed MS Binding Assays consisting of incubation (*blue*), separation (*light blue*), liberation via elution (*green*), and quantification via LC-MS/MS

aqueous component of the mobile phase had an influence on the analytes *k*.

Binding assay development

For the development of hNET MS Binding Assays employing (1*R*,3*S*)-indatraline as marker, we basically followed the setup recently established for hSERT and mGAT1 MS Binding Assays [11, 12]; a basic scheme of the workflow is shown in Fig. 4. As several parameters, i.e., incubation and washing buffer, filter material, and employed inhibitor for nonspecific binding, affect the final matrix of the samples, we investigated these parameters for matrix samples generated in preliminary binding experiments with respect to signal intensity and signal-to-noise level observed in multiple reaction monitoring (MRM) chromatograms to optimize sensitivity of our quantification method (see supplementary material). The following setup could be shown to provide conditions suitable for the intended purpose and was selected for the assays.

For incubation, we employed a HEPES-based buffer (50 mmol L^{-1} HEPES, 120 mmol L^{-1} NaCl, and 5 mmol L^{-1} KCl, pH 7.4) instead of the commonly used Tris buffer [11] to avoid the potential reactive primary amino function of Tris in respect of further assay applications (such as combinatorial chemistry approaches employing aldehydes; see for example [37]). As washing with the HEPES buffer after termination of incubation by filtration caused an unfavorable signal-to-noise ratio and low peak height, we employed an isoosmotic 150 mmol L^{-1} ammonium acetate buffer (pH 7.4) for this purpose. Employing this buffer for washing instead of the incubation buffer did not change specific binding of indatraline towards hNET significantly.

For filtration, we employed glass fiber filter plates with a pore size of 1.0 μm, presoaked in 0.5 % (*m*/*v*) polyethyleneimine for 1 h, as they showed a low filter binding of the MS marker and almost no matrix effect and enabled a short filtration time.

Finally, desipramine, a highly potent norepinephrine reuptake inhibitor, was employed as competitor in a concentration of 10 μmol L^{-1} to determine nonspecific binding, as it neither affected indatraline's analyte peak area nor its peak shape.

LC-ESI-MS/MS method validation

The established method was validated in a range from 5 pmol L^{-1} to 5 nmol L^{-1} according to the FDA guidance for bioanalytical method validation [38] regarding selectivity, calibration standard curve, lower limit of quantification (LLOQ), intra- and inter-batch precision as well as accuracy, and stability.

A representative LC-ESI-MS/MS chromatogram of a matrix blank demonstrating selectivity for mass transitions of indatraline and the internal standard is shown in Fig. 4a. It exhibits no interfering signals at the mass transitions *m*/*z* 292.2/261.0 and *m*/*z* 299.2/268.0 at the retention time of analyte and internal standard. Linear calibration functions were obtained in a range from 5 pmol L^{-1} to 5 nmol L^{-1}, employing a $1/x^2$ weighting ($r^2 \geq 0.9912$, *y*-intercept ≤8.7 % of the LLOQ; see results in Table 1). For 5 pmol L^{-1} indatraline matrix standards (six series of hexaplicate samples), signal-to-noise ratios of ≥11.60, concentrations of 83.9–114.6 % of the nominal concentrations (calculated according to the corresponding calibration functions), and RSDs ≤8.7 % were found (see Table 1), showing that all these parameters are nicely in agreement with the recommendations of the Center for Drug Evaluation and Research (CDER) guidance for the LLOQ.

Corresponding chromatograms of a matrix blank, a 5 pmol L^{-1} matrix standard, and a 1 nmol L^{-1} matrix standard in the presence of 1 nmol L^{-1} internal standard are shown in Fig. 5a–c.

Also, the requirements for accuracy and precision were met perfectly as indicated by the results shown in Table 1. Thereby, the intra-batch accuracies and precision were 89.4–106.5 % (accuracies of all concentration levels of the respective series) and 1.7–8.9 % (RSD), respectively. Inter-batch accuracy was found to be 93.3–102.7 % (accuracies of all concentration levels) and precision, calculated as RSD, 2.9–6.1 %.

Due to the fact that there are no data published for the stability of aqueous solutions of indatraline, we compared a

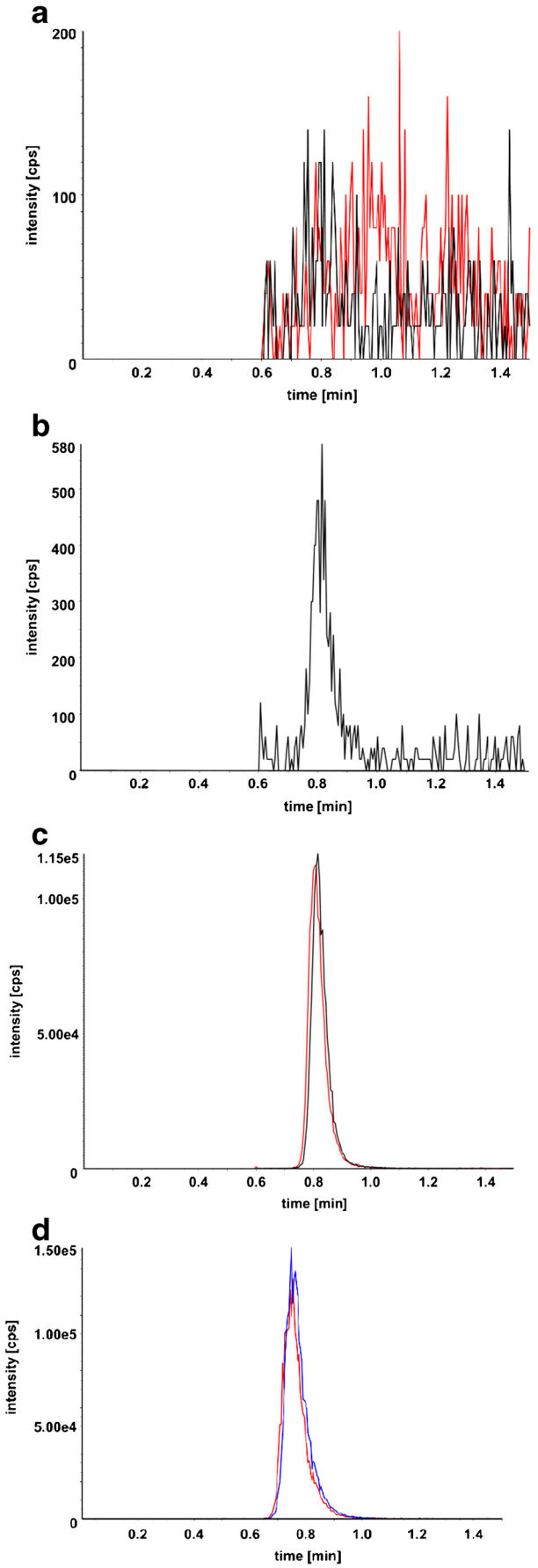
Table 1 Validation of indatraline quantification by LC-ESI-MS/MS with an API 5000 employing ($^2H_7$)-indatraline as internal standard

Sample (*n*)	Intra-series																		Inter-series		
	Series 1[b]			Series 2[b]			Series 3[b]			Series 4[b]			Series 5[b]			Series 6[b]					
	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD
5 pmol L^{-1} Cal (6)	4.876	97.8	7.7	4.824	96.5	7.7	4.985	99.7	8.6	4.771	95.4	5.5	4.911	98.2	8.7	5.017	100.3	7.4	4.872	97.4	7.4
10 pmol L^{-1} Cal (6)	10.70	107.0	4.3	10.89	108.9	5.1	10.29	102.9	3.7	11.15	111.5	2.7	10.58	105.7	4.9	10.12	101.2	7.5	10.61	106.1	5.6
25 pmol L^{-1} Cal (3)	23.35	93.4	0.2	23.39	93.5	7.3	22.70	90.8	4.2	23.69	94.8	10.5	22.33	89.3	1.7	23.27	93.1	5.2	23.12	92.5	5.5
50 pmol L^{-1} Cal (3)	47.56	95.1	3.6	48.51	96.9	1.3	47.30	94.6	5.0	45.87	91.8	1.3	47.60	95.2	3.9	47.63	95.2	4.6	47.40	94.8	3.5
100 pmol L^{-1} Cal (3)	101.2	101.2	4.7	97.38	97.4	1.2	99.86	99.9	2.0	101.3	101.3	2.5	96.80	96.8	2.2	102.5	102.5	2.8	99.84	99.8	3.2
250 pmol L^{-1} Cal (3)	235.4	94.2	2.1	236.4	94.6	3.4	241.0	96.4	1.7	234.9	94.0	2.3	242.8	97.1	0.9	237.0	94.8	1.2	237.9	95.2	2.2
500 pmol L^{-1} Cal (3)	481.2	96.3	3.0	471.7	94.3	1.9	491.0	98.2	3.1	479.2	95.8	1.7	489.3	97.9	1.4	487.5	97.5	5.4	483.3	96.7	3.0
1000 pmol L^{-1} Cal (3)	1019	101.9	2.3	1051	105.1	5.7	1039	103.9	1.9	1055	105.5	1.0	1037	103.7	1.3	1068	106.8	1.3	1045	104.5	2.8
2500 pmol L^{-1} Cal (3)	2452	98.1	1.8	2432	97.3	1.6	2509	100.4	4.8	2409	96.4	0.7	2532	101.3	0.5	2439	97.6	1.3	2462	98.5	2.7
5000 pmol L^{-1} Cal (3)	5463	109.3	2.5	5506	110.1	2.3	5522	110.5	0.9	5450	109.0	0.8	5511	110.2	0.4	5471	109.4	3.8	5487	109.7	1.9
10 pmol L^{-1} QC (6)	10.20	102.0	4.3	9.522	95.2	7.2	10.55	105.5	3.2	10.17	101.7	5.6	10.63	106.3	6.2	10.65	106.5	2.0	10.27	102.7	6.1
25 pmol L^{-1} QC (6)	23.34	93.4	2.8	23.40	93.6	5.4	22.82	91.3	4.2	23.75	95.0	7.8	23.69	94.8	6.1	22.89	91.6	5.1	23.31	93.3	5.3
100 pmol L^{-1} QC (6)	95.96	96.0	2.5	95.06	95.1	3.2	99.16	99.2	5.5	95.02	95.0	4.7	97.24	97.2	3.7	98.72	98.7	3.7	96.86	96.9	4.1
500 pmol L^{-1} QC (6)	469.3	93.9	3.1	447.1	89.4	2.8	473.6	94.7	2.0	457.1	91.4	4.5	476.2	95.3	4.6	487.0	97.4	8.9	468.4	93.7	5.4
1000 pmol L^{-1} QC (6)	987.0	98.7	2.8	989.0	98.9	3.7	1008	100.8	3.6	984.1	98.4	2.6	988.3	98.8	1.7	1010	101.0	2.3	994.4	99.4	2.9
1000 pmol L^{-1} SStd new[a] (6)	954.1	95.4	1.7	949.2	94.9	5.4	986.1	98.6	5.2	955.8	95.6	2.3	990.4	99.0	2.2	1003	100.3	3.5	973.7	97.3	4.5
1000 pmol L^{-1} SStd old[a] (6)	991.2	99.1	2.9	941.0	94.1	3.0	1000	100.0	1.6	1013	101.3	6.8	978.8	97.9	1.9	1020	102.0	2.5	990.6	99.1	4.3

M Mean of calculated concentrations (pmol L^{-1}), *Acc* accuracy (%), *RSD* relative standard deviation (%), *Cal* calibration standard, *QC* quality control sample, *SStd* solvent standard, *n* number of replicates

[a] Solvent standard of a freshly prepared stock solution (new) and an 8-month-old stock solution (old)

[b] Resulting calibration functions: series 1, $y=0.9410x-0.00004913$ ($r^2=0.9932$); series 2, $y=0.9503x+0.00043570$ ($r^2=0.9914$); series 3, $y=0.9420x-0.00027150$ ($r^2=0.9937$); series 4, $y=0.9846x-0.00036170$ ($r^2=0.9912$); series 5, $y=0.9792x-0.00006100$ ($r^2=0.9932$); series 6, $y=0.9804x-0.00027900$ ($r^2=0.9935$)



◀ **Fig. 5** MRM chromatograms of indatraline, ($^2H_7$)-indatraline, and *cis*-configured diastereomer of indatraline in presence of hNET binding sample matrix. For quantification, the mass transition *m/z* 292.2/261.0 for indatraline (*black*) and *cis*-configured diastereomer (*blue*), and *m/z* 299.2/268.0 for ($^2H_7$)-indatraline (*red*) were used. **a** Blank, **b** 5 pmol L^{-1} indatraline (LLOQ), **c** 1 nmol L^{-1} indatraline together with 1 nmol L^{-1} of ($^2H_7$)-indatraline, and **d** 1 nmol L^{-1} *cis*-configured diastereomer together with 1 nmol L^{-1} of ($^2H_7$)-indatraline. All chromatograms were recorded at an API 5000 employing a YMC Triart C18 (50×2 mm, 3 μm) and 5 mmol L^{-1} ammonium bicarbonate pH 10.0 with acetonitrile (10:90, *v/v*) as mobile phase at a flow rate of 600 μL min^{-1} at 20 °C. The injection volume was 45 μL; the eluents of the first 0.6 min were sent to waste to protect the mass spectrometer

10 mmol L^{-1} aqueous stock solution which was stored for 8 months at room temperature with a freshly prepared 10 mmol L^{-1} aqueous stock solution of indatraline. For this purpose, we prepared 1 nmol L^{-1} solvent standards of both stock solutions, both supplemented with 1 nmol L^{-1} ($^2H_7$)-indatraline (stock solution also stored at room temperature for 8 months), and analyzed them with the described LC-ESI-MS/MS method. The peak area ratios (i.e., area of the analyte/area of the internal standard) as well as the analyte peak areas found for both series, the samples prepared from the new and the old stock solution, showed no significant differences (n=36, α=0.01).

Additionally, we investigated the capability of our developed LC-ESI-MS/MS method for the quantification of the *cis*-configured diastereomer of indatraline in a range of 5 pmol L^{-1} to 1 nmol L^{-1} (again using ($^2H_7$)-indatraline as internal standard; see Fig. 5d). Calibration curves, LLOQ (again 5 pmol L^{-1}), and intra- and inter-batch accuracy and precision fulfilled again the requirements of the CDER guideline (for further information, see ESM Table S1). Accordingly, the developed LC-ESI-MS/MS method is suitable for quantification of all four stereoisomeric forms (enantiomers and diastereomers) of 3-(3,4-dichlorophenyl)-1-methylamino-2,3-dihydro-1*H*-indene as marker in MS Binding Assays.

Application of the LC-ESI-MS/MS method to saturation experiments

For the intended saturation experiments employing (1*R*,3*S*)-indatraline as marker for hNET, the following already mentioned aspects had to be considered:

It was reasonable to assume that the concentrations of (1*R*,3*S*)-indatraline bound to hNET are even below the LLOQ of the established quantification method (i.e., 5 pmol L^{-1}) in binding samples, especially when very low marker concentrations (i.e., about 0.1 K_d) combined with very low target concentrations (i.e., of approximately 0.1 K_d) are applied, the latter of which is necessary when the actual K_d value for (1*R*,3*S*)-indatraline binding towards hNET is very low, i.e., in the picomolar range (see also "Introduction"). Therefore, we decided to modify our standard setup for the intended

saturation experiments to ensure a reliable quantification of the bound marker even below our LLOQ of 5 pmol L^{-1}. A rather simple but efficient way to enable this task in MS Binding Assays is an increase of the incubation volume, while the volume of the final sample, containing the bound and subsequently liberated marker, is kept as low as possible, the approval of which has already been taken for the performance of MS Binding Assays addressing hSERT [11]. Increasing the ratio of incubation volume to final sample volume to a factor of 12 (3.6 mL binding sample to 300 μL final sample) enabled us to quantify even 400 fmol L^{-1} bound (1*R*,3*S*)-indatraline (1/12 of the LLOQ) in the binding samples.

Following this modified procedure, we characterized the binding of (1*R*,3*S*)-indatraline towards hNET in saturation experiments employing a wide concentration range of the marker, namely from 50 pmol L^{-1} to 30 nmol L^{-1}. Varying the employed marker concentrations to this large extent was considered to be sufficient to allow an accurate assessment of the K_d value of (1*R*,3*S*)-indatraline towards hNET, independent of where in the broad range it had to be expected, i.e., from middle picomolar to low nanomolar concentrations, would actually be. In Fig. 6a, total as well as nonspecific binding of (1*R*,3*S*)-indatraline towards hNET of a representative saturation experiment is shown. It is noteworthy that this modified setup enabled also the quantification of nonspecifically bound (1*R*,3*S*)-indatraline down to nominal marker concentrations of 500 pmol L^{-1}. At even lower concentrations, we extrapolated the respective data points for nonspecific binding based on the experimentally determined nonspecifically bound marker, which is possible due to an assumed linear correlation of nonspecific binding with nominal marker concentration [39, 40]. From these data for total and nonspecific binding, specifically bound (1*R*,3*S*)-indatraline at hNET (defined as the difference of total and nonspecific binding) was calculated and plotted vs the employed nominal marker concentration. Finally, these plots were analyzed by means of nonlinear regression (i.e., "one site – specific binding") to obtain a saturation isotherm, which gave information about the respective affinity of (1*R*,3*S*)-indatraline towards hNET (K_d) and the maximum amount of binding sites (B_{max}); see for example Fig. 6b. From these saturation isotherms, a K_d value of 805±71.4 pmol L^{-1} and a B_{max} value of 82.82±12.31 pmol mg^{-1} protein (mean±SEM, n=5; 82.8±34.2 pmol mg^{-1} protein, CL 95 %) were calculated, being in good agreement with the already published results, i.e., a K_i value of 2 nmol L^{-1} for indatraline obtained in [^{3}H]nisoxetine binding experiments addressing rNET [15, 13].

Finally, it is worth mentioning that also the results obtained for the individually prepared matrix blanks, zero samples, calibration standards, and QCs of this modified setup, i.e., starting with an incubation volume of 4.0 mL and using 3.6 mL of the binding sample to generate a final sample volume of 300 μL, were in agreement with recommendations

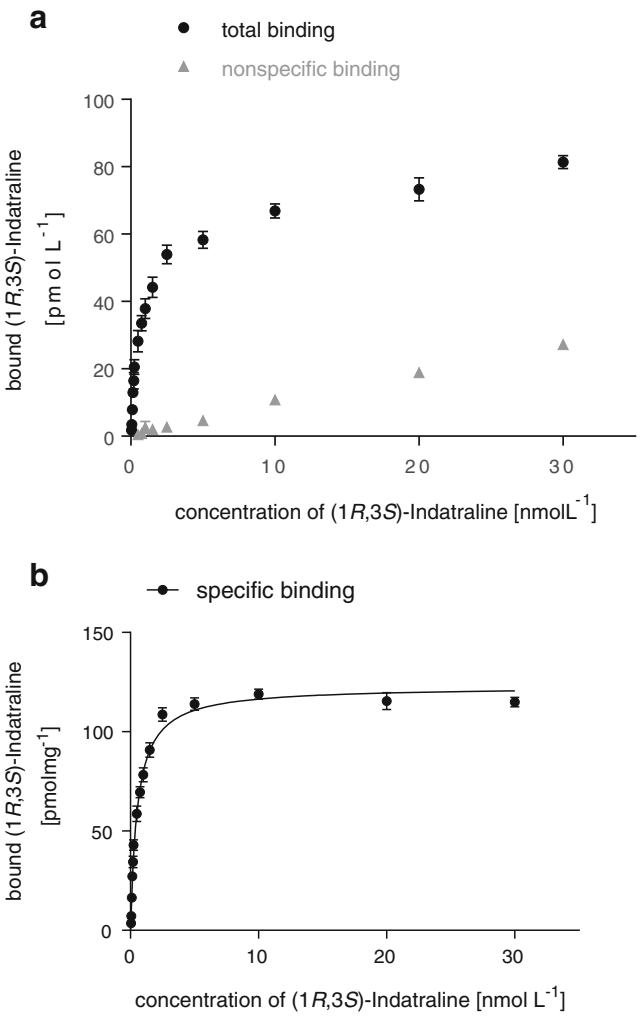


Fig. 6 Saturation experiment of (1*R*,3*S*)-indatraline binding towards hNET. **a** Means±SD (n=3) of total binding of (1*R*,3*S*)-indatraline towards hNET at nominal marker concentrations from 50 pmol L^{-1} to 30 nmol L^{-1} (*black spheres*) and nonspecific binding in presence of 10 μmol L^{-1} desipramine in a range of 0.5–30 nmol L^{-1} (*grey triangles*) of a representative saturation experiment (performed as described in the experimental section). **b** Specific binding and saturation isotherm (*black*) derived from the experiment shown in **a**. Data points for nonspecific binding below nominal marker concentrations of 0.5 nmol L^{-1} were extrapolated as described in the experimental section

for "application of validated method to routine drug analysis" of the FDA guidance for bioanalytical method validation [38] regarding selectivity, linearity, LLOQ, accuracy (85–115 %), and precision (RSD ≤15 %) (see also "Method validation"). This demonstrated that the modified setup employed in saturation experiments had no influence on the analytical reliability of our developed quantification method.

Competitive experiments

In the next step, competition experiments as the main application of reporter ligand-based binding assays should be

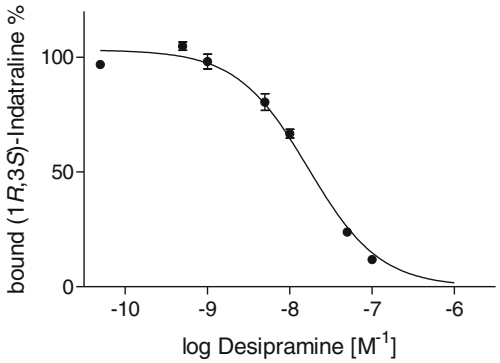


Fig. 7 Competition experiment targeting hNET. Competition curve for desipramine obtained in a representative MS binding experiment. *Data points* represent specific binding of (1*R*,3*S*)-indatraline in presence of different concentrations of desipramine (mean±SD, n=3). One hundred percent binding was equivalent to specific binding of (1*R*,3*S*)-indatraline without any inhibitor and 0 % to extrapolated nonspecific binding

implemented. To this end, we used our standard setup (i.e., 250 μL total incubation volume per well) that has been shown to allow a reasonable throughput as it is required for characterization of test compounds. In this case, the marker substance, (1*R*,3*S*)-indatraline, was employed at a nominal concentration of 2.4 nmol L^{-1} to ensure that even low percentages of specifically bound marker, which will occur at higher competitor concentrations, can be quantified reliably in the corresponding competition samples. As nonspecific binding at 2.4 nmol L^{-1} (1*R*,3*S*)-indatraline was typically below our LLOQ of 5 pmol L^{-1}, we pursued the same approach as already described in "saturation experiments." That means nonspecific binding was experimentally determined in a range from 2.5 to 20 nmol L^{-1} (1*R*,3*S*)-indatraline in the presence of 10 μmol L^{-1} desipramine and the corresponding value for 2.4 nmol L^{-1} (employed marker concentration in competition experiments) was extrapolated. As an example, we investigated the well-known NET inhibitor desipramine. For the determination of its inhibitory constant (K_i) concentrations in a range from 50 pmol L^{-1} to 1 μmol L^{-1}, desipramine was incubated in the presence of target and marker. After incubation, separation of nonbound from bound marker, and, finally, after elution of the target bound marker, the concentration of (1*R*,3*S*)-indatraline in the corresponding samples was quantified employing the established LC-ESI-MS/MS method. An inhibition curve for desipramine generated from a representative experiment is shown in Fig. 7. The affinity calculated for desipramine at hNET was 4.45±0.32 nmol L^{-1} (mean±SEM, n=3) being in excellent accordance with literature, in which affinities for desipramine towards NET of 1.6±0.2 nmol L^{-1} (rNET) [20] and 4.1±1.2 and 13.9±1.5 nmol L^{-1} (hNET) [41] based on results from radioligand binding assays are reported.

Conclusion

In summary, the present study describes the first highly sensitive method for quantification of the triple reuptake inhibitor indatraline so far. The LC-ESI-MS/MS method developed to quantify indatraline in MS Binding Assays is selective, fast (chromatographic cycle time of 1.5 min), and robust and avoids any sample preparation. According to the CDER guidance of the FDA, it could be demonstrated that reliable results regarding calibration standard curve and intra- and inter-batch accuracy as well as precision in a range from 5 pmol L^{-1} (LLOQ) to 5 nmol L^{-1} are obtained. The established method could also be applied to quantify the *cis*-configured diastereomer of indatraline with similar results for the investigated validation parameters.

The developed LC-ESI-MS/MS was successfully used in MS-based binding assays employing (1*R*,3*S*)-indatraline as a nonlabelled marker for hNET. With the established MS Binding Assays, the binding of (1*R*,3*S*)-indatraline towards hNET was characterized for the first time. The established setup (based on a 96-well microtiter plate format) of the MS Binding Assays in combination with the developed LC-ESI-MS/MS method proved to be sensitive enough to characterize even the affinity of (1*R*,3*S*)-indatraline towards hNET (K_d of 805 pmol L^{-1}) in saturation experiments under the conditions common to radioligand binding assays (marker concentration from 0.1 K_d to 10 K_d, target concentration ≤0.1 K_d), though it was very high. Besides this, the established MS Binding Assays were also demonstrated to be well suited to investigate test compounds for their affinity towards NET in competition experiments and represent therefore a promising substitute for the widespread [^{3}H]nisoxetine radioligand binding assays. It should be emphasized, however, that the scope of the approach presented in this study is distinctly more far reaching as it may additionally be applied to the other two monoamine transporters DAT and SERT without any further analytical efforts and will, besides (1*R*,3*S*)-indatraline, also allow to employ the other indatraline stereoisomers as nonlabelled markers.

Acknowledgments We thank Prof. Harald H. Sitte for providing the pRc/CMV vector containing the cDNA coding for hNET. For synthesizing indatraline and ($^2H_7$)-indatraline, we are very thankful to Lars Allmendinger and Gerd Bauschke (LMU München, Department of Pharmacy).

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Development and validation of an LC-ESI-MS/MS method for the triple reuptake inhibitor indatraline enabling its quantification in MS Binding Assays

Stefanie H. Grimm, Georg Höfner, Klaus T. Wanner

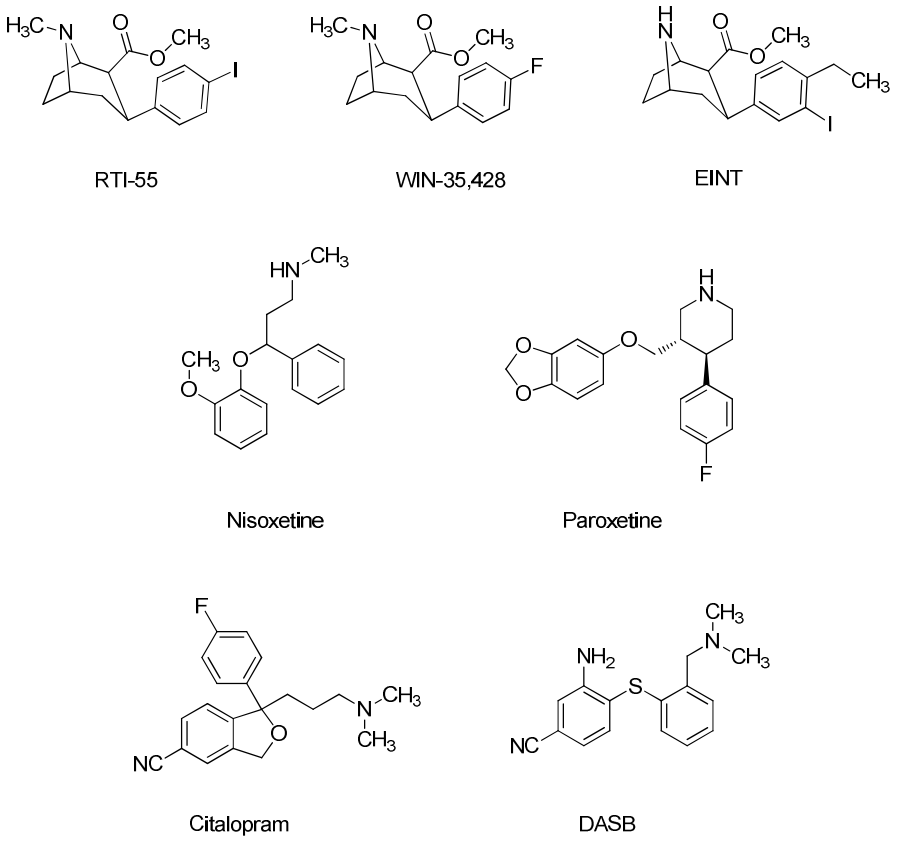

Fig. S1 Structures of respective nonlabelled species radioligands employed in literature for the affinity characterization (K_i-value) of Indatraline

[^{3}H]MPP$^+$ Uptake experiments

HEK293 cells stably transfected with hNET as described in "Cell culture and expression of hNET" were cultivated as described in "hNET membrane preparation". After washing with PBS for the second time, the resulting pellet was resuspended carefully in uptake buffer (10 mmol L^{-1} HEPES, 120 mmol L^{-1} NaCl, 3 mmol L^{-1} KCl, 2 mmol L^{-1} $CaCl_2$, 2 mmol L^{-1} $MgCl_2$ and 20 mmol L^{-1} glucose, pH 7.3) resulting in 6 x 10^6 cells mL^{-1}.

3 x 10^5 cells were added to uptake buffer which contained [^{3}H]MPP$^+$ (80-85 Ci $mmol^{-1}$) and MPP$^+$ in a ratio of 1:100, the sample for nonspecific uptake also included 100 nmol L^{-1} Indatraline, both series were prepared in triplicates, the resulting samples had a final concentration of 1nmol L^{-1} labelled MPP^{-+} per sample and a sample volume of 250 µL. After 10 min of incubation at 22 °C uptake was terminated via filtration and subsequently washing five times with 5 mL of ice cold incubation buffer, for that we used a Brandel M-24 harvester and Whatman GF/C filters to collect the cells after uptake. Glass fiber pieces with the cells containing labelled MPP$^+$ were transferred to scintillation vials and cells lysed by adding 3 mL of the scintillation cocktail. Each sample was measured for 4 min with a Packard TriCarb 2300.

Counts for total and nonspecific uptake of the respective hNET clone were compared to each other and the clones having at least a ratio of one to ten (nonspecific uptake to total uptake) were further tested using this [^{3}H]MPP$^+$ uptake experiment (n = 3). Finally, the clone having the highest ratio of total uptake *vs* nonspecific uptake in three individual experiments, was used for hNET binding experiments.

Preliminary LC-ESI-MS/MS experiments

pH dependent retention profile

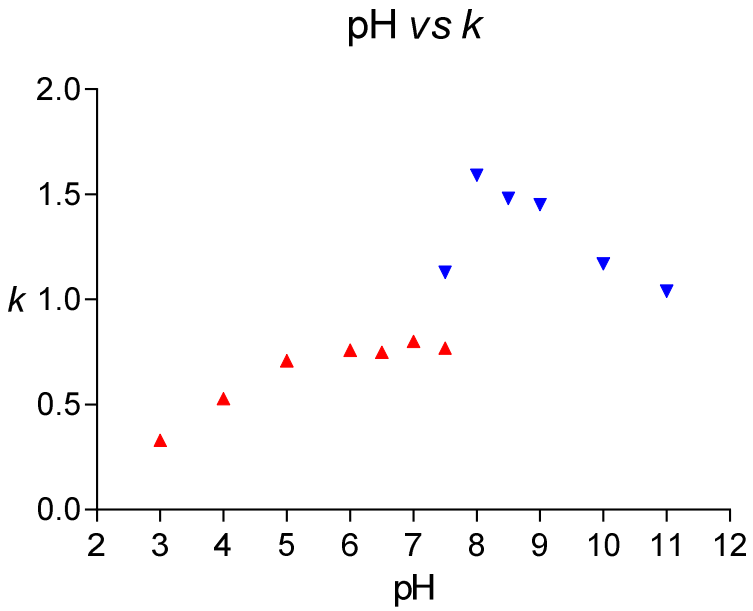

Fig. S2 pH-dependence profile of Indatraline retention. pH of the aqueous phase (x) is plotted against the *k* factor (y) using a YMC Triart C_{18} (50 x 2.0 mm, 3 µm) with respective pre-column and a mobile phase composition of a 5 mmol L^{-1} ammonium formate buffer (pH 3.0 – 7.5, red triangles) or 5 mmol L^{-1} ammonium bicarbonate (pH 7.5 – 11.0, blue triangles) and acetonitrile (10:90, v/v) at a flow rate of 600 µL min^{-1} (temperature was set to 20 °C and injection volume to 10 µL), for sample solvent the mobile phase was used

Post-column infusion experiments

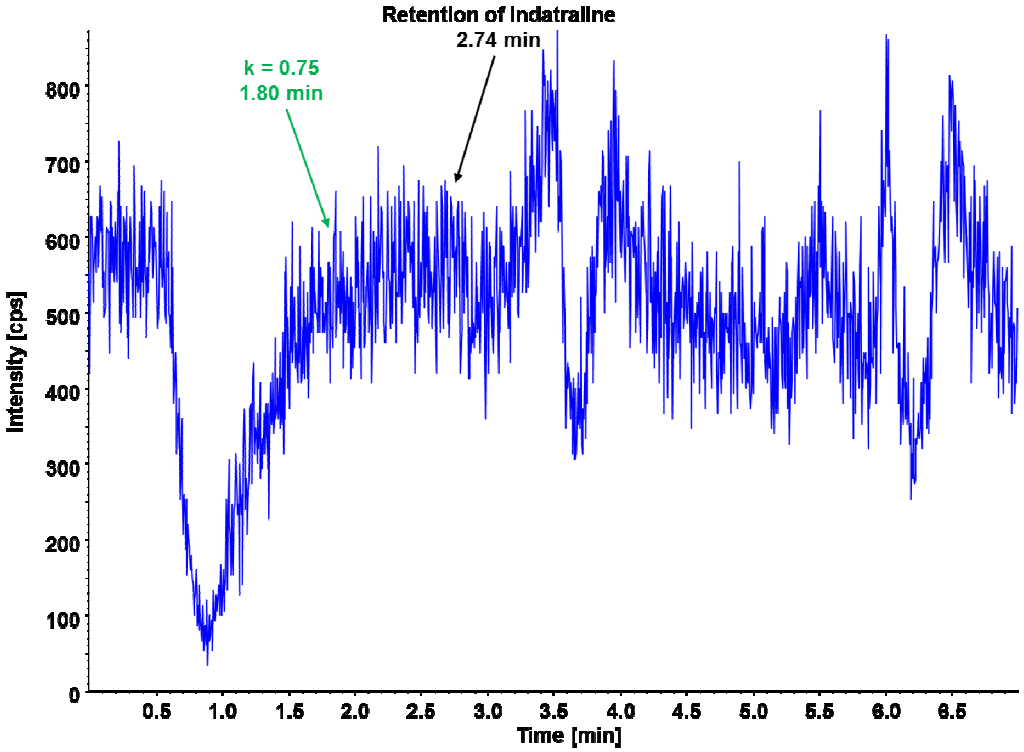

Fig. S3 Chromatogram of post-column infusion experiment, 10 nmol L^{-1} of Indatraline were infused (5 µL min^{-1}) after the column, 10 µL of a matrix blank were injected to the HPLC system. Chromatographic conditions were a mobile phase of 5 mmol L^{-1} ammonium bicarbonate pH 10.0 and acetonitrile (10:90, v/v), at a flow rate of 200 µL min^{-1} at 20 °C on an RP18 column (50 x 2 mm, 3 µm). Chromatogram was recorded using an API 2000 in the positive MRM mode for the mass transition (*m/z*) 292.2/261.0 of Indatraline

Table S1 Validation parameters for *cis*-configured isomers using ($^2H_7$)-Indatraline as internal standard

Sample (n[a])	Intra-series									Inter-series		
	Series 1[b]			Series 2[c]			Series 3[d]					
	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD	M	Acc	RSD
5 pmol L^{-1} Cal *cis* (3)	4.475	89.5	0.6	4.553	91.1	4.5	4.619	92.4	7.4	4.549	91.0	4.6
10 pmol L^{-1} Cal *cis* (3)	10.15	101.5	5.3	10.11	101.1	8.6	10.70	107.0	3.8	10.32	103.2	6.0
50 pmol L^{-1} Cal *cis* (3)	44.81	89.6	3.2	46.92	93.8	6.5	51.85	103.7	7.1	47.86	95.7	8.4
100 pmol L^{-1} Cal *cis* (3)	105.0	105.0	2.5	101.6	101.6	1.9	96.24	96.2	4.2	101.0	101.0	4.6
500 pmol L^{-1} Cal *cis* (3)	522.6	104.5	0.6	511.4	102.3	2.6	466.4	93.3	2.0	501.5	100.0	5.4
1000 pmol L^{-1} Cal *cis* (3)	993.9	99.4	1.8	1014	101.4	2.4	1102	110.2	1.2	1037	103.7	5.1
10 pmol L^{-1} QC *cis* (6)	9.658	96.6	4.5	10.67	106.7	7.6	9.968	99.7	9.5	10.10	101.0	7.6
100 pmol L^{-1} QC *cis* (6)	966.8	96.7.	4.8	99.17	99.2	4.5	99.97	100.0	6.7	98.60	98.6	5.8
1000 pmol L^{-1} QC *cis* (6)	1029	102.9	2.6	980.1	98.0	3.4	1018	101.8	9.2	1009	100.9	6.0

M Mean of calculated concentrations (pmol L^{-1}), *Acc* accuracy (%), RSD (%), *Cal* calibration standard, *QC* quality control sample

[a] *n* number of replicates. [b–d] resulting calibration functions:

series 1: y=1.016x+0.0001106 (r^2=0.9906)

series 2: y=1.064x+0.0003057 (r^2=0.9934)

series 3: y=0.984x–0.0000546 (r^2=0.9944)

MS Binding Assay development

The standard of our MS Binding Assays has workflow as follows: incubation, separation of target-bound from free marker via filtration employing a vacuum manifold in combination with 96-well filter plates and liberation via protein denaturation [references 5 and 6 of main paper]. As all mentioned parameters, i.e. incubation buffer, filtration system, eluent for protein denaturation, etc., affect the matrix of the binding samples, we investigated these regarding signal intensity of the analyte peak and signal-to-noise ratio, to increase the sensitivity of the developed quantification method.

Instead of the commonly used Tris based buffer which was used successfully used as incubation buffer in different radioligand binding studies and also in MS Binding Assays [references 11, 15, 19, 24 and 25 of main paper] we employed an analogous HEPES based buffer (50 mmol L^{-1} HEPES, 120 mmol L^{-1} NaCl and 5 mmol L^{-1} KCl, pH 7.4) to be more flexible for further assay applications.

In the next step, different kinds of 96-well filter plates (AcroPrep Advance, 350 µL, Pall, Dreireich, Germany) for separation of bound from nonbound marker were examined. Various filter materials, i.e. glass fiber, polytetrafluorethylene, hydrophilic polypropylene, and polyethersulfone, with different pore sizes in a range of 0.2 to 1.0 µm were tested. Unfortunately, hydrophilic polypropylene and polytetrafluorethylene as well as smaller pore sizes were not appropriate for our purpose due to a long filtration time and a higher hold up volume of organic solvents. Those based on polyethersulfone and glass fiber showed a markedly high nonspecific binding of Indatraline (i.e. approximately 10 %), therefore we investigated different pre-treatments (i.e. incubation buffer, water, 0.1 – 0.5 % (m/v) polyethyleneimine) to reduce this undesired nonspecific binding. In the case of glass fiber filter plates nonspecific binding could be reduced significantly (i.e. far less than 1 %) employing 0.5 % (m/v) polyethylene imine, but for polyethersulfone based filters nonspecific binding could only be reduced to approximately 5 %.

Additionally, we investigated the effect of different washing buffers, such as assay buffer, a 0.9 % (m/v) NaCl solution, 25 mmol L^{-1} ammonium acetate, 120 mmol L^{-1} NaCl and 5 mmol L^{-1} KCl buffer adjusted to pH 7.4, as well as a 150 mmol L^{-1} ammonium acetate buffer adjusted to pH 7.4, respectively, on intensity (peak height), signal-to-noise ratio of the analyte signal in corresponding MRM chromatograms and specific binding of the marker (nominal concentrations in a range from 2.5 nmol L^{-1} to 25 nmol L^{-1}). Using the incubation buffer also as washing buffer has the advantage that the binding properties are not influenced

during wash process, but in our case it also revealed two disadvantages, i.e. a rather high noise level and an intensity lower as that obtained using the other investigated washing buffers. The 0.9 % NaCl solution and the isoosmotic replacement of Tris by ammonium acetate led to an improved signal-to-noise ratio and also signal intensity, but best results concerning a more sensitive quantification method, i.e. a noise level as low as possible next to a peak height as high as possible, were found using the 150 mmol L^{-1} ammonium acetate buffer, pH 7.4. Also, it can be stated that all tested washing buffers showed no significant difference compared to the incubation buffer in specific binding, thus we can assume that there is no influence on affinity of Indatraline towards hNET resulting from the wash process presumed that the washing buffer is ice cold and the filtration process takes only a few seconds.

Finally, the chromatographic influence of high concentrations of Desipramine, as it was intended to use for the determination of nonspecific binding and as displacer in kinetic studies, was investigated. For this reason we prepared hNET binding sample matrix (like it is described for validation matrix samples) in absence and presence of 10 µmol L^{-1} Desipramine, these samples were treated as described in the standard setup and eluted with acetonitrile containing Indatraline and ($^{2}H_{7}$)-Indatraline. Comparing the resulting analyte areas and as a consequence the calculated concentrations, there was no significant influence of Desipramine on our quantification (samples without Desipramine, $n = 36$; samples with Desipramine, $n = 18$, $\alpha = 0.05$), and also the peak shape was not influenced. Thus, it can be stated that the application of high concentrations of Desipramine in our binding assay for the determination of nonspecific can be made.

3.2.3 Third Publication:
MS Binding Assays for the Three Monoamine Transporters Using the Triple Reuptake Inhibitor (1R,3S)-Indatraline as Native Marker

The human monoamine transporters hDAT, hNET, and hSERT are the most promising targets for the treatment of depressions. Additional screening tools addressing these targets for the identification of new drugs curing the above mentioned diseases are therefore required. Due to their efficiency and sample throughput MS Binding Assays are well suited to fulfill this task.

The previously developed highly sensitive LC-ESI-MS/MS method for the quantification of the triple reuptake inhibitor indatraline in biological matrices had already enabled its application to MS Binding Assays addressing hNET in saturation and competitive experiments. As a next step the developed MS Binding Assays were applied to different targets (i.e. hDAT, hNET, and hSERT) and also performed with additional markers (i.e. all stereoisomers of indatraline). First, the affinities of both enantiomers of indatraline, i.e. (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, as well as of the racemic *cis*-configured diastereomer towards hDAT, hNET, and hSERT were investigated, confirming (1*R*,3*S*)-indatraline to be the eutomer for all three monoamine transporters (i.e. hDAT, hNET, and hSERT) and the racemic *cis*-configured diastereomer to be highly selective for hSERT. A saturation isotherm and consequently a determination of the distomers, (1*S*,3*R*)-indatraline, K_d-value towards all three targets as well as of the *cis*-configured diastereomer towards hDAT was unfortunately not possible. Kinetic studies employing the eutomer, (1*R*,3*S*)-indatraline, were performed next and the dissociation rate constant (k_{off}) as well as the dissociation half life ($t_{1/2}$) could be successfully determined. It is worth noting, that within the performed dissociation experiments an allosteric effect of clomipramine on the dissociation of the hSERT-(1*R*,3*S*)-indatraline complex could also be identified. A reliable determination of the association rate constant (k_{on}) for the formation of target-(1*R*,3*S*)-indatraline complexes was for neither of the employed target, i.e. hDAT, hNET, or hSERT, possible due to its extreme fast progress.

Finally, the characterization of (1*R*,3*S*)-indatraline binding properties, i.e. affinities and dissociation kinetics, allowed its application as marker in competitive MS binding experiments addressing hDAT, hNET, and hSERT. In these experiments almost 40 known inhibitors including transporter substrates and narcotics were investigated concerning their affinities and also their selectivities for the three monoamine transporters. The results obtained by the employed MS Binding Assays were verified by comparison with those obtained in established radioligand binding assays employing [^{125}I]RTI-55 as marker, revealing an excellent correlation between our and literature data for all three monoamine transporters which proves the validity of the developed MS Binding Assays as a screening tool for hDAT, hNET, and hSERT.

Declaration of contributions:

Patrick Neiens synthesized the test compounds LR1111 oxalate and *rac*-(3*R*,4*R*)-4-(2-benzhydryloxy)ethyl)-1-(4-fluorobenzyl)piperidine-3-ol oxalate. He also provided the two enantiomers of the latter and determined the respective enantiopurities. The HEK cell line stably expressing hDAT (i.e. transfection and screening of clones) was generated by myself. Additionally, I performed the described experiments (i.e. saturation, kinetic studies, and competitions), and the data analysis self-reliant. During his volunteer internship, Martin Schröder supported me when I performed the first kinetic and competitive experiments. Giulia Cattaneo performed the competitive experiments employing (*S*)-citalopram, (*R*)-fluoxetine, and (*S*)-fluoxetine under my supervision. The manuscript, all graphics, and tables were generated by myself, and were corrected by Georg Höfner and Klaus T. Wanner.

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MS Binding Assays for the Three Monoamine Transporters Using the Triple Reuptake Inhibitor (1*R*,3*S*)-Indatraline as Native Marker

Stefanie H. Grimm, Georg Höfner, and Klaus T. Wanner*[a]

Dedicated to Prof. Dr. Theodor Severin on the occasion of his 85th birthday.

We herein present label-free, mass-spectrometry-based binding assays (MS Binding Assays) for the human dopamine, norepinephrine, and serotonin transporters (hDAT, hNET, and hSERT). Using this approach both enantiomers of the triple reuptake inhibitor indatraline as well as its *cis*-configured diastereomer were investigated toward hDAT, hNET, and hSERT in saturation experiments. The dissociation rate constants for (1*R*,3*S*)-indatraline binding at hDAT, hNET, and hSERT were determined in kinetic studies. These experiments revealed an allosteric effect of clomipramine on the dissociation of (1*R*,3*S*)-indatraline from hSERT. Finally, a comprehensive set of known monoamine transport inhibitors and substrates was studied in competition experiments at hDAT, hNET, and hSERT, using (1*R*,3*S*)-indatraline as nonlabeled marker. The results are in excellent agreement with those reported for radioligand binding assays. Therefore, the established MS Binding Assays are a promising alternative to the latter for the characterization of new monoamine reuptake inhibitors at DAT, NET, and SERT.

Introduction

Mental disorders have become a global problem, decreasing the quality of life for millions of people; they are expected to create a significant economic burden on the order of several trillion dollars over the next 20 years.[1] In 2010, approximately 40% of all mental disorders were diagnosed as depression.[1b] Unfortunately, a sufficient therapeutic response can only be realized in two out of three cases with currently available drugs.[2] It is currently believed that the main mechanism behind the genesis of emotional disorders is an imbalance in the monoaminergic neurotransmitter systems consisting of dopamine (DA), norepinephrine (NE), and serotonin (5-HT).[3] Therefore, these systems are the focus for the development of suitable medications. Most of the drugs in current use for the treatment of depression amplify the effect of DA, NE, and 5-HT. In general, this can be achieved by inhibiting enzymes that catalyze the degradation of monoamines, e.g., monoamine oxidase inhibitors (MAOIs), or by inhibiting monoamine transporters, i.e., the dopamine transporter (DAT), the norepinephrine transporter (NET), and the serotonin transporter (SERT), resulting in an increased residence time of the monoamines in the synaptic cleft. Monoamine transporter inhibitors, especially those that selectively inhibit one transporter type (i.e., mainly selective SERT inhibitors), are preferred in therapy because of their improved side effect profile relative to MAOIs; they are therefore the current mainstay in the treatment of depression.[4] Nevertheless, results of the *Global Burden of Diseases, Injuries, and Risk Factors Study 2010* (GBD 2010) clearly demonstrate a substantial remaining need for new antidepressants.[1b]

A fundamental task in the early stage of the development of new antidepressive drugs is to characterize the affinity of test compounds for targets such as hDAT, hNET, and hSERT. A common method for this involves competitive radioligand binding assays, which use a ligand labeled with a radioisotope (usually tritium) that has high affinity for the desired target. Despite all the advantages in simplicity, robustness, and sensitivity of radioligand binding assays, this technique also has some substantial drawbacks associated with the use of radioactivity: security issues, legal restrictions, problems with waste management, and expenses in ligand radiolabeling.

To overcome these obstacles, our research group recently introduced *MS Binding Assays*, which follow the conventional setup of radioligand binding assays, but use a native (i.e., nonlabeled) marker instead of a radioligand that is quantified by mass spectrometry.[5] Two basic requirements for the use of a marker in MS Binding Assays are the following: First, the marker should possess physicochemical properties that allow good atmospheric ionization, thereby enabling its highly sensitive quantification by LC–ESI-MS/MS. Second, the marker should exhibit suitable affinity (K_d) for its target. This means that the stability of the target–marker complex formed [defined by a sufficiently low dissociation rate constant of the complex (k_{off})] should be high enough to avoid substantial loss of specifically bound marker during separation by filtration and subsequent washing steps. For this purpose the k_{off} value of the target–marker complex should not exceed $10^{-2}\,s^{-1}$ if

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the separation step is to be performed by filtration.[6] According to Equation (1):

$$K_d = k_{off}/k_{on} \quad (1)$$

the required K_d value, which is associated with a sufficiently low k_{off}, should be in the high picomolar to low nanomolar range (provided that the association rate constant, k_{on}, is independent of the target used, but collision-limited, as can be assumed).[6] However, with increasing marker affinity, the analytical demand on the sensitivity of the LC–MS quantification method also increases. An estimate of the required sensitivity of the quantification method can be derived from the K_d value of the marker according to the rules generally accepted for radioligand binding assays (i.e., investigating the marker at nominal concentrations in a range from at least 0.1 K_d to 10 K_d, at a target concentration up to 0.1 K_d).[6,7] From this, a quantification of at least 0.0091 K_d bound marker, which results from binding samples employing a marker as well as target concentration of or slightly below 0.1 K_d (see Supporting Information), should be feasible employing the quantification method.

Our previously developed LC–ESI-MS/MS method for the quantification of indatraline and its *cis*-configured diastereomer (Figure 1) reaches a lower limit of quantification (LLOQ) of 5 pM in the final sample.[5b] However, even if the achieved sensitivity is very high, it could still be insufficient for saturation experiments designed to characterize highly affine markers with very low K_d values, as the resulting concentration of bound marker in binding samples with very low target concentrations (i.e., up to 0.1 K_d) combined with low marker concentrations (i.e., 0.1 K_d) might be still below the above-mentioned LLOQ. To overcome this obstacle, we modified our setup by increasing the incubation volume to 4.0 mL instead of the standard 250 μL setup. With the target concentration kept constant, this results in a 16-fold higher target amount in the 4.0 mL binding sample than in the 250 μL binding sample.[5b] Consequently, the amount of bound marker is also increased in the 4.0 mL setup. To quantify the resulting bound marker concentration in the 4.0 mL binding sample, we transferred 3.6 mL of the binding sample for filtration (for the standard setup, 200 μL of the 250 μL binding sample were subjected to filtration), while the final sample volume, resulting from liberation by elution of the bound marker, was kept constant for both setups at 300 μL (i.e., the modified setup having a 4.0 mL incubation volume, and the standard setup having a 250 μL incubation volume).[5b] The resulting twelvefold higher concentration of bound marker in the final sample (i.e., 3.6 mL binding sample resulting in a final sample volume of 300 μL) enabled us to quantify 400 fM bound marker in the corresponding binding samples (i.e., one twelfth the LLOQ) with this modified setup.[5b] Summarizing these considerations, K_d values down to at least 100 pM can be assumed to be reliably determinable by following the above-mentioned setup, as the minimum of quantifiable marker in binding samples (400 fM) is distinctly below 0.0091 K_d.

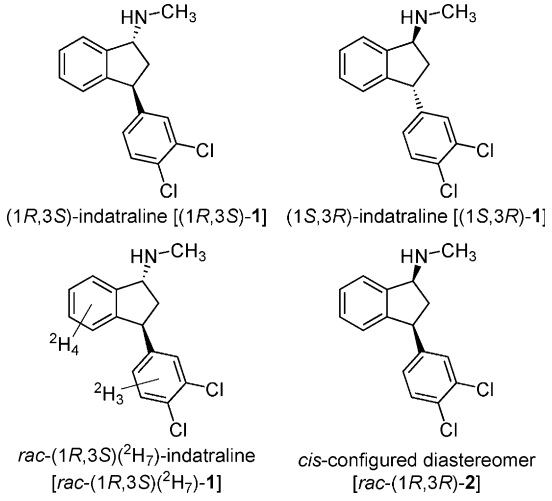

Figure 1. Native markers used in saturation experiments: (1*R*,3*S*)-**1**, (1*S*,3*R*)-**1**, the *cis*-configured diastereomer *rac*-(1*R*,3*R*)-**2**, and the internal standard used for LC–MS quantification, *rac*-(1*R*,3*S*)($^2H_7$)-**1**.

The aim of the present study was to establish MS Binding Assays for all human monoamine transporters: hDAT, hNET, and hSERT. For this purpose, saturation experiments were performed to investigate the affinities of the stereoisomers of indatraline [(1*R*,3*S*)-indatraline, (1*S*,3*R*)-indatraline, and the *cis*-configured diastereomer; Figure 1] toward their respective targets. Based on the results of these saturation experiments, the stereoisomer with the most appropriate affinities toward hDAT, hNET, and hSERT, being in the range from 100 pM (see discussion above concerning sensitivity) to low nanomolar (see discussion above concerning k_{off}), was selected as marker and further investigated in kinetic studies (i.e., dissociation and association experiments). Finally, competitive MS binding experiments based on the chosen marker were performed for hDAT, hNET, and hSERT to demonstrate the efficiency of MS Binding Assays as a promising substitute for corresponding competitive radioligand binding assays commonly used to characterize the affinities of test compounds for these targets.

Results and Discussion

Available information regarding indatraline's affinity and potency at monoamine transporters

Until now, no studies characterizing the binding affinities of the pure indatraline enantiomers toward monoamine transporters have been reported (except for our previous study regarding the binding of (1*R*,3*S*)-indatraline [(1*R*,3*S*)-**1**] at hNET),[5b] whereas the racemate has been characterized in competitive radioligand binding assays, showing affinities in the high picomolar to low nanomolar range.[8] There is one study in which the potencies of both indatraline enantiomers were individually investigated in uptake experiments.[9] In this study an appreciable difference in potency was noted between the enantiomers, (1*R*,3*S*)-**1** and (1*S*,3*R*)-**1**, showing (1*R*,3*S*)-**1** to be the eutomer, with inhibitory potencies (IC_{50} values) of 0.17 nM

for [^{3}H]DA uptake inhibition, 0.60 nM for [^{3}H]NE uptake inhibition, and 1.0 nM for [^{3}H]5-HT uptake inhibition. The distomer (1*S*,3*R*)-**1** exhibited IC_{50} values of 8.6 nM for [^{3}H]DA uptake inhibition, 5.3 nM for [^{3}H]NE uptake inhibition, and 5.2 nM for [^{3}H]5-HT uptake inhibition.[9] Based on these data, eudismic ratios of 5 ([^{3}H]5-HT uptake inhibition), 9 ([^{3}H]NE uptake inhibition), and 51 ([^{3}H]DA uptake inhibition) were calculated.[9] The latter results, however, must be critically scrutinized given the low enantiomeric purity ($\geq$95% *ee*) and the use of native rat brain preparations, which are considered to be inhomogeneous, as in addition to the required monoamine transporter type, they also contain other monoamine transporter types; that these assays may therefore lack the necessary selectivity.[9]

In contrast to indatraline, its *cis*-configured diastereomer *rac*-(1*R*,3*R*)-**2** was reported to be SERT-selective in uptake experiments[9] as well as in radioligand binding experiments using [^{125}I]RTI-55.[8l] The results of these two studies are, however, distinctly different concerning the degree of affinity and inhibitory potency of *rac*-(1*R*,3*R*)-**2**. Bogeso et al. found IC_{50} values for [^{3}H]5-HT uptake inhibition in the picomolar range, and for [^{3}H]DA and [^{3}H]NE uptake inhibition in the low nanomolar range,[9] whereas the radioligand binding experiments of Froimowitz et al. revealed affinities in the low nanomolar range for SERT, and in the high nanomolar range for DAT and NET.[8l]

In summary, the few reliable and to some extent contradictory data in the literature concerning the binding properties of indatraline's stereoisomers at the monoamine transporters do not allow a substantiated selection of the stereoisomer most suitable as a marker in MS Binding Assays for the biogenic amine neurotransmitter transporters DAT, NET, and SERT (see the Introduction).

Saturation experiments

We intended to initially characterize the affinities of the available indatraline stereoisomers for hDAT, hNET, and hSERT (except for (1*R*,3*S*)-**1** toward hNET, which was previously published)[5b] in saturation experiments using the MS Binding Assays recently published (Figure 2).[5b] From the aforementioned study, samples of the enantiomers of indatraline with high enantiomeric purities of >99.75% *ee* [(1*R*,3*S*)-**1**] and >99.67% *ee* [(1*S*,3*R*)-**1**] (determined according to the published HPLC method)[10] were available to us. Unfortunately, only the racemate of the *cis*-configured indatraline diastereomer was available, as attempts to separate the compound by crystallization after the formation of diastereomers using various acids (e.g., tartaric acid, mandelic acid, and dibenzoyl tartaric acid), an approach recently applied to indatraline, have so far been unsuccessful.

Due to the assumed high affinity of the eutomer (1*R*,3*S*)-**1** for all three monoamine transporters, and the *cis*-configured diastereomer *rac*-(1*R*,3*R*)-**2** toward hSERT, the same approach was used as previously applied to characterize the binding of (1*R*,3*S*)-**1** at hNET in saturation experiments.[5b] As discussed above, an increased incubation volume of 4.0 mL was used instead of 250 µL, of which 3.6 mL (instead of 200 µL) were subjected to filtration, which, in relation to an unchanged final sample volume of 300 µL, resulted in a twelvefold higher marker concentration in the final sample, thus enabling quantification of even 400 fM bound marker (one twelfth the LLOQ) in the binding samples.

Using this setup, increasing concentrations of (1*R*,3*S*)-**1** (for binding to hDAT and hSERT) and of *rac*-(1*R*,3*R*)-**2** (for binding to hSERT) from 50 pM to 30 nM were incubated with the corresponding targets as previously described. Nonspecific binding was determined directly at nominal marker concentrations $\geq$500 pM in the presence of 100 µM 1-[1-(benzo[*b*]thiophen-2-yl)cyclohexyl]piperidine (BTCP, **6**) for hDAT or 10 µM clomipramine (**10**) for hSERT, whereas nonspecific binding for the lower nominal marker concentrations was calculated by extrapolation after linear regression of the experimental data obtained for higher nominal marker concentrations ($\geq$500 pM). In the end, specific binding (defined as the difference between total binding and nonspecific binding) was plotted versus the nominal marker concentration. The resulting saturation isotherms were analyzed by nonlinear regression, revealing the K_d and B_{max} values listed in Table 1. We could confirm high-affinity binding of (1*R*,3*S*)-**1** toward all monoamine transporters, characterized by K_d values of 1.7 nM at hDAT, 0.81 nM at hNET,[5b] and 0.41 nM at hSERT, as well as high affinity of *rac*-(1*R*,3*R*)-**2** for hSERT, with a K_d value of 0.42 nM. Representative saturation isotherms showing binding of (1*R*,3*S*)-**1** at hDAT, hNET,[5b] and hSERT are shown in Figure 3.

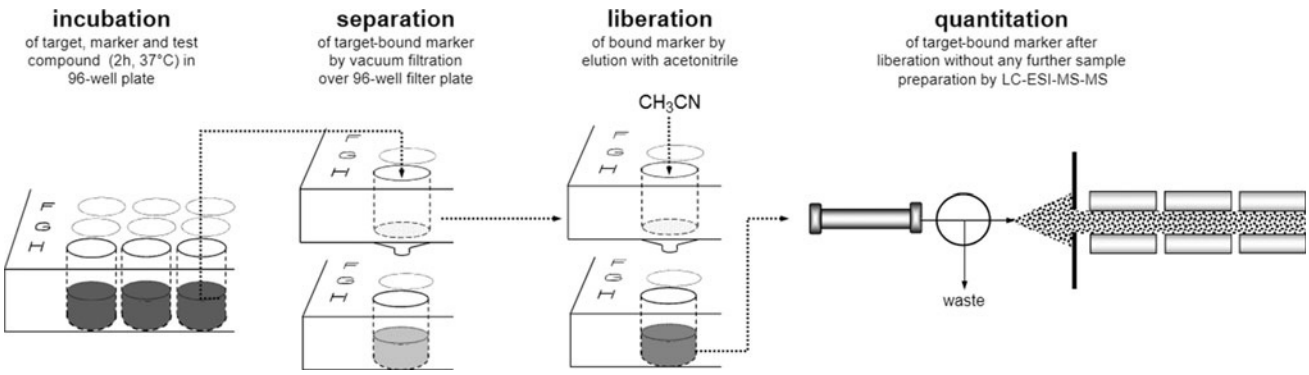

Figure 2. Basic workflow for MS Binding Assays: incubation, separation, and liberation steps followed by LC–MS/MS quantification (reproduced from ref. [5b] with kind permission. Copyright 2015, Springer-Verlag Berlin Heidelberg).[5b]

Table 1. K_d and B_{max} values for stereoisomers of indatraline obtained in saturation experiments toward hDAT, hNET, and hSERT.

Compound		K_d [nM] (B_{max} [pmol mg^{-1}])[a]			K_d ratio[b]
		hDAT	hNET	hSERT	hDAT/hNET/hSERT
(1*R*,3*S*)-**1**	(1*R*,3*S*)-indatraline	1.7 ± 0.1 (101 ± 32)	0.81 ± 0.07[5b] (83 ± 12)	0.41 ± 0.04[c] (33 ± 3)	4:2:1
(1*S*,3*R*)-**1**	(1*S*,3*R*)-indatraline	ND	ND	ND	ND
rac-(1*R*,3*R*)-**2**	*cis*-configured diastereomer of indatraline	ND	49 ± 6 (90 ± 18)	0.42 ± 0.07 (42 ± 8)	ND:117:1

[a] K_d and B_{max} values were determined in three individual experiments, unless otherwise indicated, by MS Binding Assays (means ± SEM, $n = 3$). ND: no data could be obtained in a concentration range from 100 pM to 500 nM [*rac*-(1*R*,3*R*)-**2**] and 500 pM to 500 nM [(1*S*,3*R*)-**1**], precluding the calculation of a saturation isotherm. [b] Ratios were calculated using mean K_d values. [c] K_d and B_{max} values of four individual experiments (mean ± SEM, $n = 4$).

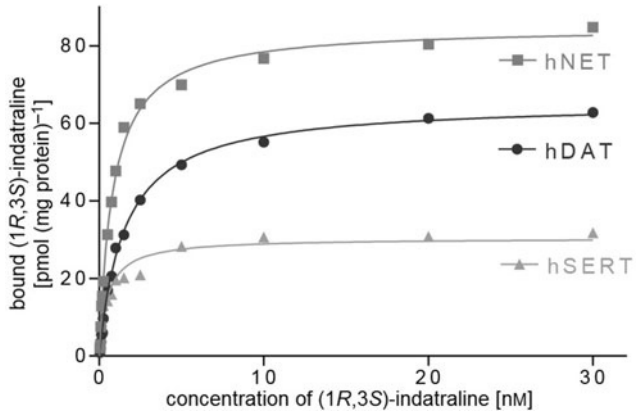

Figure 3. Specific binding of (1*R*,3*S*)-**1** at hDAT, hNET, and hSERT at nominal marker concentrations of 50 pM to 30 nM. Specific binding was determined as the difference between total binding and nonspecific binding (from experimental data in the range of 0.5–30 nM and from extrapolated data for nominal marker concentrations < 0.5 nM). Data points represent means of triplicates of an individual saturation experiment (performed as described in the Experimental Section).

We also examined binding of the indatraline stereoisomers at the transporters for which a lower affinity in saturation experiments was assumed, namely binding of the distomer (1*S*,3*R*)-**1** toward all three monoamine transporters, as well as binding of *rac*-(1*R*,3*R*)-**2** toward hDAT and hNET. Due to the expected markedly higher K_d values in these cases, the target concentration could also be distinctly enhanced in the corresponding saturation experiments, while still maintaining a target concentration at a magnitude up to 0.1 K_d (see above). As the amount of bound marker resulting under these conditions (i.e., higher target concentration; see discussion in the Introduction concerning target concentrations in various setups) was assumed to be sufficiently high for quantification with the established LC–MS method with an LLOQ of 5 pM in the final sample, we decided to revert to our standard setup for binding experiments at an incubation volume of 250 μL instead of 4.0 mL for these investigations (see above modified setup for saturation experiments for markers with high-affinity values). However, when marker binding was studied in the concentration range from 100 pM to 500 nM for *rac*-(1*R*,3*R*)-**2** and from 500 pM to 500 nM for (1*S*,3*R*)-**1**, no data were obtained to allow the calculation of a saturation isotherm for (1*S*,3*R*)-**1** binding to any of the monoamine transporters, or for *rac*-(1*R*,3*R*)-**2** to hDAT. We did not further enhance the concentrations of (1*S*,3*R*)-**1** or *rac*-(1*R*,3*R*)-**2** in saturation experiments, as in general, binding characterized by K_d values in the higher nanomolar range cannot be reliably determined in filtration-based binding experiments (see Introduction).[6] At least the experiments concerning the binding of *rac*-(1*R*,3*R*)-**2** at hNET yielded a satisfying saturation isotherm characterized by a K_d value of 49 nM (Table 1).

Taken together, the obtained K_d values confirm (1*R*,3*S*)-**1** to be a nonselective high-affinity ligand for the three monoamine transporters hDAT, hNET, and hSERT, with K_d values in the high picomolar to low nanomolar range and in a ratio of 4:2:1 (hDAT/hNET/hSERT). They also confirm *rac*-(1*R*,3*R*)-**2** as a highly selective high-affinity ligand for hSERT (Table 1). As (1*R*,3*S*)-**1** proved to be the only stereoisomer with an affinity for DAT and NET sufficiently high to be suitable as a marker in filtration-based binding assays, we decided to use this compound as a marker in competitive experiments for these targets. For competitive experiments with hSERT (1*R*,3*S*)-**1** also seemed better suited, as it was available as a pure enantiomer, in contrast to *rac*-(1*R*,3*R*)-**2**, which, although possessing high affinity for this transporter as well, was only available as the racemate. Therefore, only the kinetics of (1*R*,3*S*)-**1** binding to hDAT, hNET, and hSERT was investigated in the next step.

Dissociation studies

At first, the dissociation rate constant (k_{off}) and the corresponding half-life of the target–marker complex ($t_{1/2}$) were determined by the displacer technique by following our standard setup (250 μL incubation volume). A prerequisite for dissociation experiments in general is a sufficient amount of target–marker complexes formed at the beginning of the dissociation experiments (i.e., appropriate occupancy of the target by the marker), leading to concentrations of the remaining non-dissociated target–marker complexes after the various time steps that are sufficiently high for reliable quantification. Therefore, we allowed unhindered binding of (1*R*,3*S*)-**1** to hDAT, hNET, and hSERT for 2 h at 37 °C at concentrations of (1*R*,3*S*)-**1** (17.5 nM for hDAT, 25 nM for hNET, and 7.5 nM for hSERT) leading to nearly complete saturation of the target. After equilibrium was reached, the displacer [100 μM BTCP (**6**) for hDAT, 10 μM desipramine (**12**) for hNET, and 10 μM clomipramine (**10**) for hSERT] was added to initiate dissociation, which was terminated by filtration after various time increments ranging

from 30 s to 2 h. After further processing according to our standard setup, the concentration of remaining bound marker was determined in the final sample using the developed LC–ESI-MS/MS method. Due to the fact that nonspecific binding of (1*R*,3*S*)-**1** was found to be constant over the course of the dissociation experiment, total binding was used for data analysis. Based on these data, corresponding dissociation curves were generated, and k_{off} and $t_{1/2}$ values were calculated by nonlinear regression. Thereby dissociation rate constants (k_{off}) of $4.8\times10^{-3}\,s^{-1}$ for the hDAT–(1*R*,3*S*)-**1** complex, $1.4\times10^{-3}\,s^{-1}$ for the hNET–(1*R*,3*S*)-**1** complex, and $5.4\times10^{-3}\,s^{-1}$ for the hSERT–(1*R*,3*S*)-**1** complex were obtained (Table 2).

Table 2. Kinetic rate constants and dissociation half-life of (1*R*,3*S*)-indatraline [(1*R*,3*S*)-**1**] binding at hDAT, hNET, and hSERT.[a]

	hDAT	hNET	hSERT
k_{off} [s^{-1}][b]	$4.8\pm0.1\times10^{-3}$	$1.4\pm0.1\times10^{-3}$	$5.4\pm0.2\times10^{-3}$
$t_{1/2}$ [s][b]	145 ± 3	528 ± 38	129 ± 4
k_{off} [s^{-1}][c]	$4.2\pm0.5\times10^{-3}$	$1.8\pm0.2\times10^{-3}$	$0.88\pm0.17\times10^{-3}$
$t_{1/2}$ [s][c]	173 ± 25	364 ± 57	861 ± 179
k_{on} [$M^{-1}\,s^{-1}$][d]	$2.3\pm0.3\times10^{7}$	$2.4\pm0.5\times10^{7}$	$2.1\pm0.4\times10^{7}$

[a] Results are the mean ± SEM of three individual experiments unless otherwise indicated. [b] Dissociation experiments performed by displacer approach as described in the Experimental Section (hNET: $n=4$). [c] Dissociation experiments performed by dilution approach as described in the Experimental Section. [d] Association experiments performed as described in the Experimental Section (hDAT: $n=6$, hNET: $n=7$).

Surprisingly, the rank order of the calculated k_{off} values was not in agreement with expectations, as they did not parallel the K_d values obtained in saturation experiments. This is generally the case, as K_d and k_{off} values are related by Equation (1), $K_d = k_{off}/k_{on}$,[6] in which k_{on} is mostly collision-limited and thus independent of the target used.[6] For (1*R*,3*S*)-**1** we had found the affinity to be highest for hSERT over hDAT and hNET in saturation experiments (see *Saturation experiments* above). As a consequence, the k_{off} value should have been the lowest and not the highest for this transporter, as was found (see Table 2, k_{off} for displacer approach). One explanation for this discrepancy might be positive or negative allosteric (i.e., modulatory) effect mediated by one or more of the chosen displacers, on marker dissociation. This phenomenon was reported for some monoamine transporter radioligand binding assays (i.e., with [^{3}H]GBR 12935, [^{3}H]nisoxetine, or [^{3}H]citalopram).[11] Unfortunately, these allosteric effects cannot be predicted based on published data, as they depend on numerous factors such as the nature of the target, the marker used, as well as the employed displacer.[11b] To rule out that modulatory effects of the chosen displacers might affect the k_{off} value of (1*R*,3*S*)-**1** in the dissociation experiments, we followed an alternative approach in which dissociation is induced by diluting the equilibrated binding samples. Accordingly, dissociation was initiated under conditions assuring an almost 1:1000 dilution of the remaining unbound (1*R*,3*S*)-**1** and corresponding target–marker complexes (see the Experimental Section for details). Following this dilution approach, dissociation rate constants of $4.2\times10^{-3}\,s^{-1}$ for hDAT, $1.8\times10^{-3}\,s^{-1}$ for hNET, and $0.88\times10^{-3}\,s^{-1}$ for hSERT were observed (see Table 2, k_{off} for dilution approach, and Figure 4).

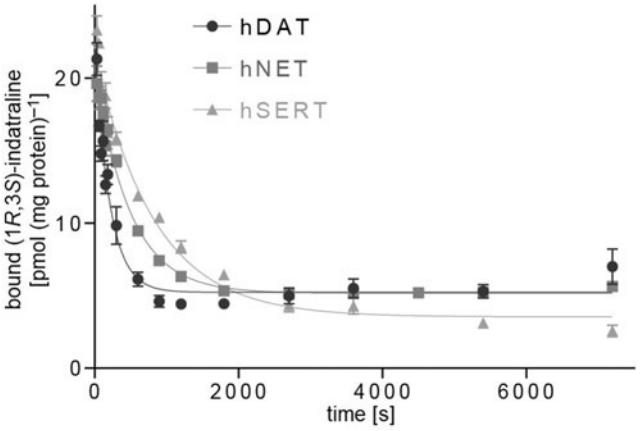

Figure 4. Dissociation kinetics of complexes between (1*R*,3*S*)-**1** and hDAT, hNET, and hSERT targets at a nominal marker concentration of 5 nM at 37 °C. Before dissociation experiments were started, unhindered binding of 5 nM (1*R*,3*S*)-**1** was allowed for 2 h at 37 °C to guarantee an equilibrium state. After separation by centrifugation, dissociation was initiated by dilution (at least 1:1000) of the remaining unbound (1*R*,3*S*)-**1** and corresponding target–marker complexes ($t=0$ s). Data points represent total binding (means ± SD, $n=3$) of an individual experiment (performed as described in the Experimental Section).

The results for hDAT and hNET were not significantly different from those obtained with the displacer method; the k_{off} value for hSERT, however, obtained with the dilution approach was significantly lower than that obtained with the displacer approach (*t*-test, $\alpha=0.05$, see Figure 5). As the rank order of k_{off} values observed in the dilution approach is in agreement with that of K_d values determined in saturation experiments, and the k_{off} values obtained for hDAT and hNET do not significantly differ between both approaches, a negative allosteric effect of clomipramine (**10**) enhancing the dissociation of the hSERT–(1*R*,3*S*)-**1** complex can be assumed.

Association studies

Subsequently, we also intended to get an estimate of the association rate constants (k_{on}) for (1*R*,3*S*)-**1** toward the monoamine transporters, to which end k_{obs} should be determined in association experiments which is related to k_{on} according to Equation (2) (with *L* as the marker concentration):[6]

$$k_{obs} = k_{on}\cdot L + k_{off} \quad (2)$$

As previous MS Binding Assays investigating the association of the highly affine and selective serotonin reuptake inhibitor (*S*)-fluoxetine [(*S*)-**15**] to hSERT showed that equilibrium was nearly reached within a few minutes, we used a nominal (1*R*,3*S*)-**1** concentration as low as possible for the association experiments with the monoamine transporters in order to keep k_{obs} as small as possible as a consequence of the above-mentioned Equation (2). Therefore, we chose a nominal marker

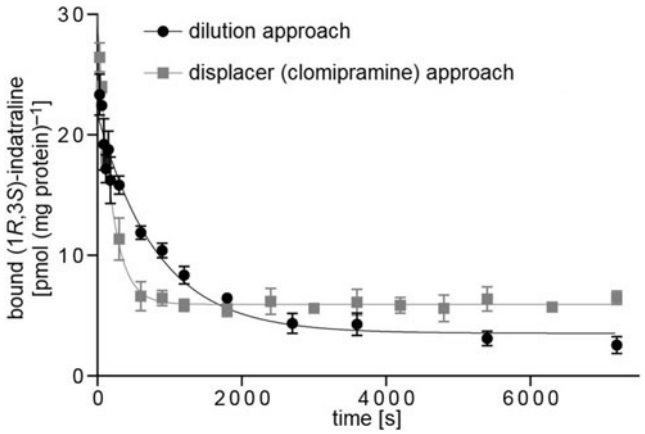

Figure 5. Dissociation kinetics of hSERT–(1*R*,3*S*)-**1** complexes at 37 °C at a nominal marker concentration of 7.5 nM (by displacer approach with clomipramine (**10**)) or 5 nM (by dilution approach). The incubation system was allowed to reach equilibrium [pre-incubation of (1*R*,3*S*)-**1** at 7.5 nM (displacer approach) and 5 nM (dilution approach) for 2 h at 37 °C] before the respective dissociation experiment was started. Dissociation was initiated by adding **10** (grey squares), resulting in a final concentration of 10 μM in the binding sample, or by dilution (at least 1:1000) of the remaining unbound (1*R*,3*S*)-**1** and corresponding target–marker complexes obtained after separation by centrifugation (black circles) ($t = 0$ s). Data points represent total binding (means ± SD, $n = 3$) of an individual experiment (performed as described in the Experimental Section).

concentration of 500 pM, resulting in concentrations of bound marker that could just be quantified reliably under conditions of our standard setup by means of the established LC–MS method (LLOQ 5 pM), even after the shortest incubation period of 20 s. The results of representative association experiments are shown in Figure 6. It is clearly visible that the observed association is almost complete within two minutes. Thus, association is indeed too rapid under the conditions chosen to provide exact data in conventional filtration-based binding assays. Nevertheless, k_{on} values of 2.3×10^7 M^{-1}s^{-1} (hDAT), 2.4×10^7 M^{-1}s^{-1} (hNET), and 2.1×10^7 M^{-1}s^{-1} (hSERT)

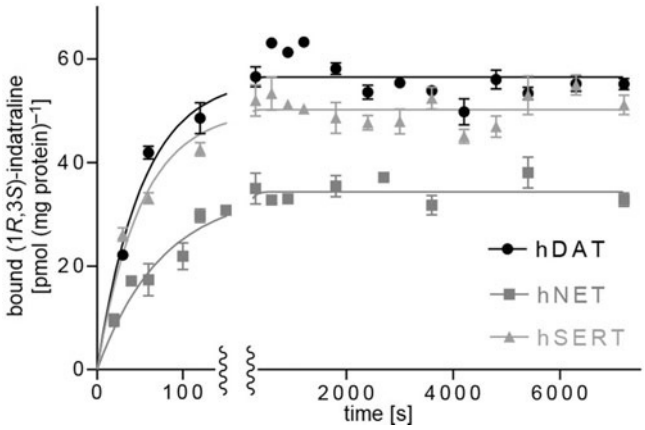

Figure 6. Association kinetics showing the formation of the various target–(1*R*,3*S*)-**1** complexes (i.e., hDAT, hNET, and hSERT) at a nominal marker concentration of 500 pM. Data points represent total binding (means ± SD, $n = 3$) of an individual experiment (performed as described in the Experimental Section).

(Table 2) were calculated from the determined k_{off} and k_{obs} values and the marker concentrations used. Although these association rate constants are only rough estimates, their magnitude is in agreement with association being collision-limited which has typically a k_{on} value of 10^7 M^{-1}s^{-1}.[6]

Summary of kinetic studies

Several conclusions can be drawn from the kinetic experiments performed. First, by using the dilution-based dissociation technique, the dissociation rate constants of the individual target–(1*R*,3*S*)-**1** complexes could be reliably determined, with a rank order of k_{off} values being in accord with the rank order of K_d values observed in saturation experiments. Second, the obtained dissociation rate constants for (1*R*,3*S*)-**1** binding to hDAT, hNET, and hSERT are clearly low enough to permit the use of filtration as a separation technique without significant loss of specific marker binding. Third, an allosteric effect of clomipramine (**10**) causing accelerated dissociation of the hSERT–(1*R*,3*S*)-**1** complex was identified. Fourth, association experiments revealed that association under the chosen conditions was too rapid for a reliable and exact determination of k_{on} in a filtration-based MS Binding Assays. Notably, however, the same applies to conventional radioligand binding assays.

Competitive experiments

(1*R*,3*S*)-Indatraline [(1*R*,3*S*)-**1**] proved to be the only stereoisomer with high affinity for hDAT and hNET. Thus, it was chosen to serve as a marker in competitive experiments for these targets. Compound (1*R*,3*S*)-**1** was also selected as a marker for competitive binding experiments with hSERT, as it could be used as a pure enantiomer, whereas the *cis*-configured diastereomer *rac*-(1*R*,3*R*)-**2**, with high affinity for this transporter as well, was only available as a racemate. Competitive MS Binding Assays with fixed (1*R*,3*S*)-**1** concentrations and varying concentrations of test compounds were performed, again following the standard setup (250 μL incubation volume) by which a reasonable throughput was warranted. This setup was used to study a series of known monoamine transporter inhibitors and substrates covering a wide range of affinities and selectivities (Figure 7). In each case, these compounds were used at a minimum of seven concentrations in a range spanning at least three orders of magnitude. Unfortunately, nonspecific binding resulting from the marker concentrations used in competitive binding experiments was typically below the LLOQ. Hence, an additional experiment was performed in which nonspecific binding of (1*R*,3*S*)-**1** in a range from 2.5 to 20 nM was determined as described above (see *Kinetic studies* in the Experimental Section). Therefrom, the extent of nonspecific binding at nominal marker concentrations used in competitive MS Binding Assays was extrapolated.

Competition curves were then established by plotting the percentage value of the resulting specific binding (*y*-axis), defined as difference of total binding and extrapolated nonspecific binding, versus the logarithm of the test compound used (*x*-axis) and analyzed by nonlinear regression. Representative

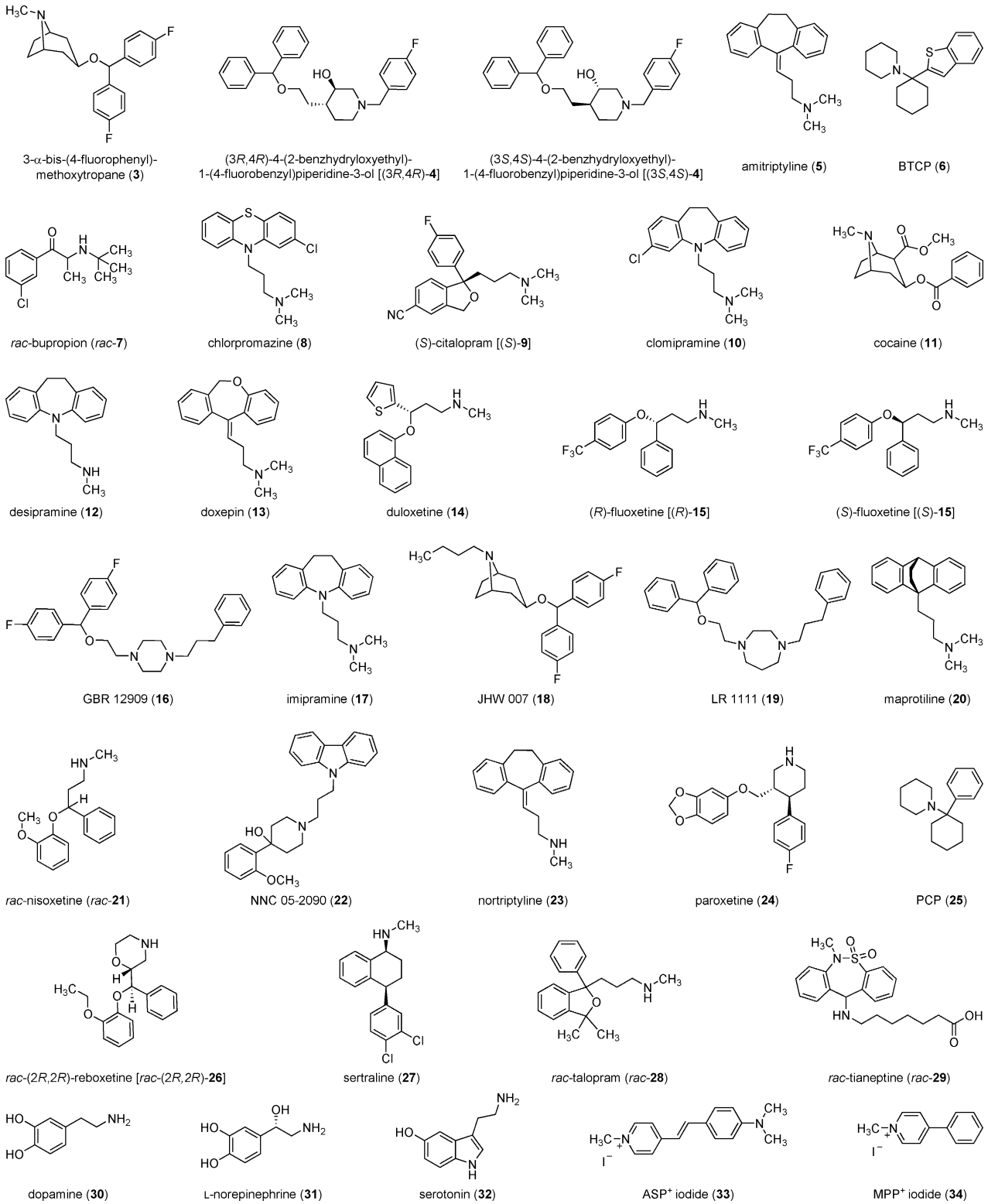

Figure 7. Structures of known inhibitors, including substrates **3**–**34** used in competitive experiments.

competition curves are shown for sertraline (**27**) in Figure 8. The competition curves yielded IC_{50} values from which the corresponding K_i values of the test compounds could be calculated. The pK_i values (means ± SEM, $n = 3$–7) derived from these MS Binding Assays for the three transporters hDAT, hNET, and hSERT using (1*R*,3*S*)-**1** as marker are summarized in Table 3 (see also Figure S1 in the Supporting Information for further details).

To verify the results obtained we compared them with data reported for corresponding radioligand binding assays. Un-

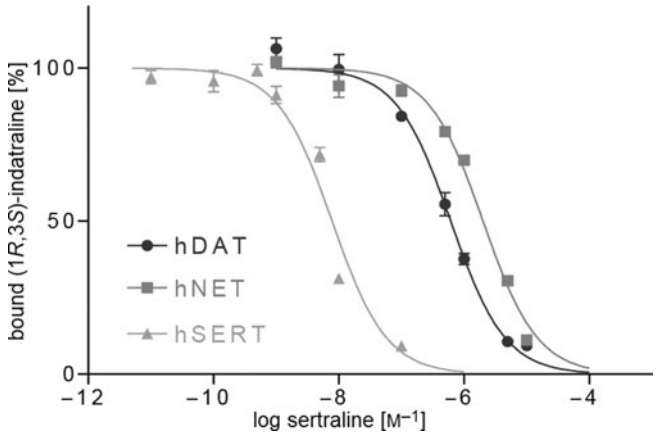

Figure 8. Representative competition curves for sertraline (**27**) obtained with hDAT, hNET, and hSERT resulting from individual competitive MS binding experiments (performed as described in the Experimental Section). Data points represent specific binding of (1*R*,3*S*)-**1** in the presence of sertraline at various concentrations in a single experiment (means ± SD, $n = 3$). The top level of the competition curves was constrained to 100%, which is equivalent to specific binding of (1*R*,3*S*)-**1** in the absence of inhibitor, and the bottom level to 0%, equivalent to nonspecific binding.

fortunately, there are only few studies that are based on consistent heterologous expression systems for all three monoamine transporters and that were carried out under consistent conditions. Most reports describe studies based on animal brain tissue preparations, which hardly allow a selective characterization of individual monoamine transporters (this is particularly true for DAT binding assays, lacking highly selective DAT ligands; see the discussion below). However, there is an [^{125}I]RTI-55 binding assay published by Eshleman et al. that also used membrane preparations of HEK293 cells expressing hDAT, hNET, and hSERT as respective target source and under consistent conditions. This offered the opportunity to compare the binding data of at least ten test compounds covering a broad affinity range and diverse selectivities with these from our study (see Table 3).[12]

Although even small differences in the performance of the binding assays (the nature of the chosen marker, the composition of the incubation buffer, temperature, etc.) may affect the resulting K_i values, an excellent correlation of our results with those of Eshleman et al.[12] was observed for all three transporters. In detail, the straight lines resulting from linear regression for the compared pK_i values determined by us with those reported by Eshleman et al.[12] (Figure 9) were characterized by the following equations and coefficients of determination (R^2): $y = 1.048x - 0.1569$, $R^2 = 0.8970$ (hDAT); $y = 1.099x - 1.028$, $R^2 = 0.9830$ (hNET); and $y = 1.131x - 0.6675$, $R^2 = 0.9726$ (hSERT). The observed agreement in pK_i values along with an almost equal rank order of potency verifies the reliability, and thus relevance, of our developed MS Binding Assays as a label-free alternative to radioligand binding assays addressing the studied monoamine transporters.

Furthermore, several important insights can be gained from these data. First, several DAT inhibitors that are claimed to be highly selective, i.e., 3-α-bis-(4-fluorophenyl)methoxytropane (**3**),[13] BTCP (**6**),[14] JHW007 (**18**),[15] and LR1111 (**19**)[16] did not show this selectivity in our MS Binding Assays (Table 3). This fact becomes most evident by the corresponding K_i ratios for

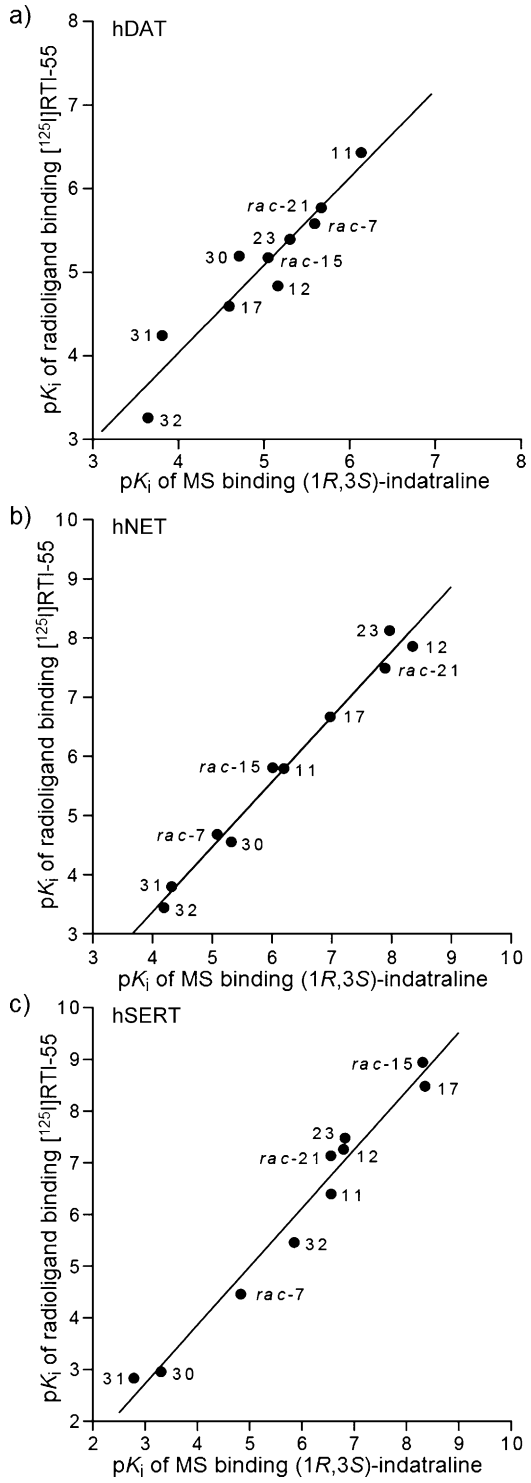

Figure 9. Graphical correlation of pK_i values for various competitors determined in MS Binding Assays (*x*-axes) and in [^{125}I]RTI-55 radioligand binding assays (*y*-axes).[12] Data points represent mean values from MS Binding Assays ($n = 3$–7) and the converted mean value of published K_i values ($n = 3$–8) of radioligand binding assays. Shown are the correlations of pK_i values for ten compounds at a) hDAT ($y = 1.048x - 0.1569$, $R^2 = 0.8970$), b) hNET ($y = 1.099x - 1.028$, $R^2 = 0.9830$), and c) hSERT ($y = 1.131x - 0.6679$, $R^2 = 0.9726$).

Table 3. Affinity (pK_i) and selectivity (K_i ratios hDAT/hNET/hSERT) values of various inhibitors toward hDAT, hNET, and hSERT obtained by competitive MS Binding Assays supplemented with published data from Eshleman et al.[12]

	Compound	pK_i hDAT		pK_i hNET		pK_i hSERT		K_i ratio
		MS[a]	RB[b]	MS[a]	RB[b]	MS[a]	RB[b]	MS[c]
3	3-α-bis-(4-fluorophenyl)methoxytropane	7.52 ± 0.04		7.22 ± 0.02		5.71 ± 0.05		1:2:65
rac-(3*R*,4*R*)-**4**	*rac*-(3*R*,4*R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol	7.43 ± 0.09		6.58 ± 0.03		5.86 ± 0.04		1:7:36
(3*R*,4*R*)-**4**	D-84	7.45 ± 0.08		6.52 ± 0.09		5.52 ± 0.04		1:9:84
(3*S*,4*S*)-**4**	D-83	6.97 ± 0.05		6.47 ± 0.08		5.96 ± 0.05		1:3:10
5	amitriptyline	5.02 ± 0.07		6.92 ± 0.07		7.59 ± 0.09		363:5:1
6	BTCP	7.49 ± 0.10		7.49 ± 0.08		6.71 ± 0.08		1:1:6
rac-**7**	*rac*-bupropion	5.59 ± 0.12	5.58	5.08 ± 0.09	4.68	4.83 ± 0.10	4.46	1:3:6
8	chlorpromazine	5.36 ± 0.07		7.49 ± 0.07		6.98 ± 0.14		123:1:3
rac-**9**	*rac*-citalopram	4.27 ± 0.03		5.45 ± 0.05		8.08 ± 0.05		6373:425:1
(*S*)-**9**	(*S*)-citalopram	4.27 ± 0.06		4.99 ± 0.08		8.32 ± 0.13		10484:2024:1
10	clomipramine	5.37 ± 0.05		7.02 ± 0.07		9.09 ± 0.13		5994:121:1
11	cocaine	6.13 ± 0.05	6.43	6.20 ± 0.05	5.80	6.56 ± 0.10	6.40	3:2:1
12	desipramine	5.16 ± 0.05	4.84	8.35 ± 0.03[5b]	7.86	6.80 ± 0.11	7.26	1760:1:44
13	doxepin	4.15 ± 0.07		6.99 ± 0.10		6.56 ± 0.03		684:1:3
14	duloxetine	6.01 ± 0.06		8.25 ± 0.08		9.41 ± 0.03		2520:15:1
rac-**15**	*rac*-fluoxetine	5.05 ± 0.04	5.18	6.01 ± 0.05	5.81	8.31 ± 0.07	8.94	1782:196:1
(*R*)-**15**	(*R*)-fluoxetine	4.88 ± 0.14		6.16 ± 0.06		8.30 ± 0.04		1048:141:1
(*S*)-**15**	(*S*)-fluoxetine	4.60 ± 0.08		5.99 ± 0.06		8.50 ± 0.01		2397:324:1
16	GBR 12909	7.43 ± 0.11		7.10 ± 0.04		6.40 ± 0.04		1:2:10
17	imipramine	4.59 ± 0.04	4.59	6.98 ± 0.05	6.67	8.35 ± 0.06	8.48	5656:24:1
18	JHW 007	7.19 ± 0.09		6.82 ± 0.05		6.10 ± 0.02		1:2:12
19	LR 1111	7.15 ± 0.02		7.22 ± 0.09		6.24 ± 0.09		1:1:10
20	maprotiline	6.12 ± 0.04		7.27 ± 0.09		4.98 ± 0.09		13:1:195
rac-**21**	*rac*-nisoxetine	5.67 ± 0.03	5.77	7.89 ± 0.06	7.49	6.55 ± 0.06	7.14	165:1:22
22	NNC 05-2090	5.92 ± 0.03		6.07 ± 0.00		6.45 ± 0.10		3:2:1
23	nortriptyline	5.30 ± 0.10	5.40	7.97 ± 0.12	8.13	6.83 ± 0.03	7.48	454:1:13
24	paroxetine	5.67 ± 0.06		6.47 ± 0.06		8.91 ± 0.03		1787:282:1
25	PCP	5.92 ± 0.07		6.01 ± 0.02		5.72 ± 0.09		1:1:2
rac-(2*R*,2*R*)-**26**	*rac*-(2*R*,2*R*)-reboxetine	4.79 ± 0.08		8.44 ± 0.15		6.53 ± 0.07		3928:1:71
27	sertraline	6.61 ± 0.05		6.23 ± 0.05		8.72 ± 0.08		128:303:1
rac-**28**	*rac*-talopram	4.49 ± 0.06		7.89 ± 0.07		6.13 ± 0.13		2570:1:75
rac-**29**	*rac*-tianeptine	< 4.00		< 4.00		< 4.00		ND
30	dopamine	4.71 ± 0.02	5.19	5.32 ± 0.08	4.55	3.31 ± 0.05	2.96	4:1:96
31	L-norepinephrine	3.81 ± 0.04	4.24	4.32 ± 0.06	3.80	2.79 ± 0.02	2.83	3:1:32
32	serotonin	3.64 ± 0.05	3.26	4.19 ± 0.11	3.44	5.85 ± 0.06	5.46	160:47:1
33	ASP^+ iodide	4.49 ± 0.05		5.47 ± 0.12		4.61 ± 0.15		9:1:8
34	MPP^+ iodide	3.83 ± 0.01		5.02 ± 0.14		4.23 ± 0.11		14:1:6

[a] MS binding: Affinity values (pK_i; mean ± SEM, $n = 3–7$) were determined in competitive MS Binding Assays using (1*R*,3*S*)-indatraline [(1*R*,3*S*)-**1**] as marker. [b] Radioligand binding: Affinity values of Eshleman et al. determined in [^{125}I]RTI-55 binding experiments ($n = 3–8$);[12] reported mean K_i values were converted into pK_i values. [c] K_i ratios showing selectivity were calculated with the mean K_i values of respective pK_i values determined in MS Binding Assays.

hDAT and hNET (Table 3), being 1:2 (**3**), 1:1 (**6**), 1:2 (**18**), and 1:1 (**19**). This discrepancy may be due to the fact that the radioligands used in the corresponding studies, i.e., [^{3}H]WIN 35428,[13,15] [^{3}H]BTCP,[14] and [^{3}H]JHW 007,[15] label binding sites at DAT slightly different from those addressed by (1*R*,3*S*)-**1**. Furthermore, the published data were obtained from radioligand binding and transport assays using brain tissue preparations, thus not ruling out that other targets apart from the desired one may also be addressed by the radioligand or the radiolabeled substrate to some extent. Second, *rac*-(3*R*,4*R*)-4-(2-benzhydryloxyethyl)-1-(4-fluorobenzyl)piperidin-3-ol [*rac*-(3*R*,4*R*)-**4**],[17] one of the most potent and selective DAT inhibitors described so far, could be confirmed as such, showing high affinity for hDAT (pK_i: 7.43) and the highest selectivity for this transporter characterized by a ratio of K_i values of 1:7:36 (hDAT/hNET/hSERT; Table 3). Determination of the affinity of both enantiomers, [(3*R*,4*R*)-**4**] and [(3*S*,4*S*)-**4**], in competitive MS binding assays confirmed that the eutomer, [(3*R*,4*R*)-**4**],[17] possesses even higher selectivity for hDAT (K_i ratios of 1:9:84, hDAT/hNET/hSERT; Table 3).

Third, investigating the γ-aminobutyric acid (GABA) transporter (GAT) inhibitor NNC 05-2090 (**22**), which, in contrast to most of the other known GAT inhibitors (GAT inhibitors are widely studied in our group), is devoid of a carboxylic acid function, affinities for this compound for hDAT (pK_i: 5.92), hNET (pK_i: 6.07), and hSERT (pK_i: 6.45; Table 3) were found to be in the range of the potencies observed at the GATs [i.e., pIC_{50} values of 4.7 (hGAT-1), 5.7 (BGT-1), 5.6 (hGAT-2), and 4.9 (hGAT-3)].[18] During the course of this project a study by Jinzenji et al. was published that also compared the uptake inhibition of compound **22** at monoamine and GATs. Also according to their results, **22** shows higher inhibitory potencies at DAT

(pIC_{50}: 5.4), NET (pIC_{50}: 5.1), and SERT (pIC_{50}: 5.3) in [^{3}H]DA, [^{3}H]NE, and [^{3}H]5-HT transport assays (based on CHO cells stably expressing the corresponding rat monoamine transporters) as compared with the inhibitory potencies at the four GAT subtypes in [^{3}H]GABA transport assays [based on murine transporters stably expressed in CHO cells, pIC_{50} values: 4.5 (GAT-1), 4.3 (GAT-2), 4.6 (GAT-3), and 5.0 (BGT-1)].[19]

Conclusions

The MS Binding Assays performed in this study allowed the first direct investigation of the binding of (1*R*,3*S*)-indatraline, (1*S*,3*R*)-indatraline, and the *cis*-configured diastereomer to hDAT, hNET, and hSERT. Saturation experiments confirmed (1*R*,3*S*)-indatraline to have high and nearly equal affinity for the three monoamine transporters, and the *cis*-configured diastereomer of indatraline to be highly SERT selective. Furthermore, it was demonstrated that the determination of K_d values down to 0.40 nM is feasible based on LC–MS quantification of a nonlabeled marker. After characterization of the binding kinetics of (1*R*,3*S*)-indatraline toward hDAT, hNET, and hSERT, this stereoisomer was used as a marker in competitive binding experiments. In this way, a comprehensive series (nearly 40 compounds) of selective and nonselective inhibitors as well as substrates were assayed for their affinities to all three targets. With respect to affinity, selectivity, and rank order of potency, the observed results were in excellent agreement with those obtained in [^{125}I]RTI-55 binding assays for all ten compounds also studied in the latter setup, clearly demonstrating the reliability of the data obtained in competitive experiments with our MS Binding Assays. After having processed almost 10000 samples during this study, it may be stated that with the developed setup, a throughput similar to that typical for conventional radioligand binding assays was reached, and that the established LC–MS-based quantification is characterized by remarkable robustness. Regarding LC–MS quantification it is worth mentioning that the MS Binding Assays described herein are based on a single method that enables quantification of the marker addressing three different targets (hDAT, hNET, and hSERT) and, furthermore, characterization of binding of all indatraline stereoisomers at these targets. The sensitivity of the developed MS-based quantification method (LLOQ of 5 pM) is close to that achieved by liquid scintillation counting in comparable radioligand binding assays (see the Supporting Information for a comparison). Thus, the MS Binding Assays described herein are a powerful substitute for radioligand binding assays addressing hDAT, hNET, and hSERT.

Experimental Section

Chemicals: The hydrochloride of NNC 05-2090 (**22**) was purchased from Abcam (Cambridge, UK). The hydrochloride of sertraline (**27**) was purchased from abcr GmbH (Karlsruhe, Germany), the hydrochlorides of *rac*-bupropion (*rac*-**7**), chlorpromazine (**8**), dopamine (**30**), and serotonin (**32**) were from Alfa Aesar (Karlsruhe, Germany). The sodium salt hydrate of *rac*-tianeptine (*rac*-**29**) was provided by AK Scientific, Inc. (Union City, USA). The hydrochlorides of *rac*-nisoxetine (*rac*-**21**) and *rac*-(2*R*,2*R*)-reboxetine [*rac*-(2*R*,2*R*)-**26**] were purchased from Biotrend (Cologne, Germany). The hydrobromide of *rac*-citalopram (*rac*-**9**), the hydrochloride of paroxetine (**24**), and U-0521 [1-(3,4-dihydroxyphenyl)-2-methylpropan-1-one] were obtained from Enzo Life Science (Lörrach, Germany). Ascorbic acid, the hydrochlorides of amitriptyline (**5**), doxepin (**13**), and imipramine (**17**) were purchased from Fagron (Barsbüttel, Germany). ASP^+ iodide (**33**), MPP^+ iodide (**34**), the hydrochlorides of BTCP (**6**), clomipramine (**10**), cocaine (**11**), desipramine (**12**), duloxetine (**14**), (*R*)-fluoxetine [(*R*)-**15**], (*S*)-fluoxetine [(*S*)-**15**], maprotiline (**20**), L-norepinephrine (**31**), and pargyline were provided from Sigma–Aldrich (Taufkirchen, Germany). The oxalate of (*S*)-citalopram [(*S*)-**9**] was obtained from TCI (Eschborn, Germany). The hydrochloride of 3-α-bis-(4-fluorophenyl)methoxytropane (**3**), the dihydrochloride of GBR 12909 (**16**), the hydrochlorides of JHW 007 (**18**) as well as *rac*-talopram (*rac*-**28**) were purchased from Tocris (Wiesbaden-Nordenstadt, Germany). The hydrochloride of *rac*-fluoxetine (*rac*-**15**) was bought from VWR (Darmstadt, Germany). Dr. Theo Rein (Max Planck Institute of Psychiatry, Munich, Germany) kindly provided the hydrochloride of nortriptyline (**23**). The oxalates of *rac*-(3*R*,4*R*)-4-(2-benzhydryloxy)ethyl)-1-(4-fluorobenzyl)piperidin-3-ol [*rac*-(3*R*,4*R*)-**4**] and LR 1111 (**19**) were synthesized in house. The oxalates of the enantiomers (3*R*,4*R*)-4-(2-benzhydryloxy)ethyl)-1-(4-fluorobenzyl)piperidin-3-ol, also known as D-84,[17] (>99.8% *ee*), [(3*R*,4*R*)-**4**], and (3*S*,4*S*)-4-(2-benzhydryloxy)ethyl)-1-(4-fluorobenzyl)piperidin-3-ol, also known as D-83,[17] (98.5% *ee*), [(3*S*,4*S*)-**4**] were separated and analyzed for enantiomeric purity in house. The hydrochloride of phencyclidine (PCP, **25**) was a donation of Emeritus Prof. Fritz Eiden (LMU München, Department of Pharmacy, Germany). All LC–MS-grade solvents (CH_3CN, H_2O) were purchased from VWR Prolabo (Darmstadt, Germany). Water for incubation and washing buffer was obtained in house by distillation of demineralized water (prepared by reverse osmosis) and subsequent filtration using 0.45 μm filter material. Additives for LC–MS ($(NH_4)HCO_3$, $(NH_4)OH$ solution ≥25%, all of LC–MS quality), were purchased from Fluka (Taufkirchen, Germany). HPLC-grade $(NH_4)OAc$ and HEPES were purchased from VWR Prolabo. The hydrochloride of indatraline [*rac*-(1*R*,3*S*)-**1**],[10] the deuterochloride of *rac*-(1*R*,3*S*)-($^2H_7$)-indatraline [*rac*-(1*R*,3*S*)-($^2H_7$)-**1**],[20] and the hydrochloride of the *cis*-configured diastereomer [*rac*-(1*R*,3*R*)-**2**][9] were synthesized in house according to published methods. The hydrochlorides of the pure enantiomers (1*R*,3*S*)-indatraline [(1*R*,3*S*)-**1**] and (1*S*,3*R*)-indatraline [(1*S*,3*R*)-**1**] possessed an enantiomeric purity of >99.75% *ee* and >99.67% *ee*, respectively, determined according to the method previously described.[10] The specific rotation of (1*S*,3*R*)-indatraline [(1*S*,3*R*)-**1**] hydrochloride (mp: 178–179 °C) amounted to $[\alpha]_{21}^{D} = -16.5$ ($c = 0.46$, CH_3OH, determined with a PerkinElmer 241 C polarimeter, PerkinElmer, Rodgau, Germany).

LC–ESI-MS/MS: LC–ESI-MS/MS was performed following our previously described method including all aspects of the validated method:[5b] YMC Triart C_{18} column (50 mm×2.0 mm, 3 μm) with a YMC Triart C_{18} pre-column (10 mm×2.1 mm, 3 μm) as stationary phase, CH_3CN and $(NH_4)HCO_3$ buffer (5 mM, pH 10.0) at a ratio of 90:10 (*v*/*v*) as mobile phase, flow rate of 600 μL min^{-1}, temperature of 20 °C for chromatography, CH_3CN and $(NH_4)HCO_3$ buffer (5 mM, pH 10.0) at a ratio of 75:25 (*v*/*v*) as sample solvent, injection volume 45 μL. Each day an assay was performed with individual matrix blanks, zero samples, matrix standards for generating a calibration function (six concentrations in a range of 5 pM to 1000 pM), and quality control samples (QCs) at three concentrations (10, 100, and 1000 pM) were prepared, each at least in triplicate, and analyzed with the markers (1*R*,3*S*)-(**1**), (1*S*,3*R*)-**1**, or *rac*-

(1*R*,3*R*)-**2**, respectively, in combination with *rac*-(1*R*,3*S*)($^2H_7$)-**1** as internal standard (see below).

Cell culture and expression of hDAT, hNET, and hSERT: The mammalian pRc/CMV expression vector containing the cDNA coding for hDAT was kindly provided by Prof. Dr. Harald H. Sitte (Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna). Stable transfection of HEK293 cells was performed as previously described using KpnI for linearization of the plasmid.[5b] Single colonies of stably transfected cells were further cultivated in selection medium containing geneticin and tested for their expression using a [^{3}H]MPP$^+$ uptake assay like described.[5b] HEK293 cell lines stably expressing hNET and hSERT (HEK-hNET and HEK-hSERT) were established as described recently.[5b,c]

hDAT, hNET, and hSERT membrane preparations: Membrane preparations of HEK293 cells stably expressing one of the monoamine transporters (i.e., hDAT, hNET, or hSERT) were prepared as described previously,[5b] with a final protein concentration of ~10–30 μg mL^{-1} (hDAT), 20–60 μg mL^{-1} (hNET), and 10–50 μg mL^{-1} (hSERT) determined according to the Bradford method after incubation with 100 mM NaOH and using bovine serum albumin as standard.[21]

General procedure for MS binding experiments: The standard setup for MS binding followed the procedure previously described for MS Binding Assays addressing hNET:[5b] membrane preparations and marker were incubated in triplicates in the assay buffer (50 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4) in polypropylene 96-well plates (1.2 mL well volume, total sample volume: 250 μL, Sarstedt, Nümbrecht, Germany) at 37 °C in a shaking water bath for 2 h. Incubation was terminated by filtration after transfer of the binding samples (aliquot of 200 μL per well) by means of a 12-channel pipette onto 96-well glass fiber filter plates (AcroPrep Advance, glass fiber, 1.0 μm, 350 μL, Multi-Well Plate Vacuum Manifold, Pall, Dreieich, Germany), which had been pretreated for 1 h with 200 μL of a 0.5% (*w*/*v*) polyethyleneimine (PEI) solution. Membrane fragments with the bound marker remaining on the filter were washed (5×150 μL) with ice-cold wash buffer (150 mM (NH_4)OAc buffer, pH 7.4). Afterward, the filter plates were dried for 1 h at 50 °C and cooled to room temperature before liberation of the bound marker by elution with CH_3CN containing 1.33 nM *rac*-(1*R*,3*S*)($^2H_7$)-**1** (3×75 μL, 30 s for every aspiration step). To obtain the final sample milieu, (NH_4)HCO_3 buffer (75 μL, 5 mM, pH 10.0) were added per well. Finally, the plates were centrifuged (10 min, 2500 rpm, 4 °C; Biofuge Stratos, Heraeus, Hanau, Germany), sealed with aluminum foil, and the samples were analyzed by the LC–ESI-MS/MS method described.[5b]

Saturation experiments: For saturation experiments two different setups were used. The standard setup was used for the characterization of the distomer, (1*S*,3*R*)-**1**, toward all three targets and *rac*-(1*R*,3*R*)-**2** toward hDAT and hNET (see *General procedure for MS binding experiments* above). For affinity determination of the eutomer (1*R*,3*S*)-**1** toward hDAT and hSERT, and *rac*-(1*R*,3*R*)-**2** toward hSERT, a modified setup, as described recently, was used:[5b] Membrane preparations and increasing concentrations of the marker (six replicates per concentration) were incubated in polypropylene 96-well plates (2.2 mL well volume, total sample volume 2.0 mL, Sarstedt, Nümbrecht, Germany) at 37 °C in a shaking water bath for 2 h. Incubation was terminated by filtration (glass fiber filter plate including pre-treatment as described above) after transfer of 1800 μL of two incubation replicates to the same wells of the filter plate (i.e., two of the six replicate incubation samples were combined to obtain three replicates on the filter plate, each generated from 3600 μL of the respective binding samples per filter well). The remaining membrane fragments with bound marker were treated exactly as described above (washing, drying, elution, centrifugation, and analysis), yielding samples in the same final volume (300 μL) as they were obtained following the *General procedure for MS binding experiments* (above). For determination of total binding in saturation experiments, the following concentration ranges of marker and amounts of target were used: (1*R*,3*S*)-**1** in a range from 50 pM to 30 nM, and the distomer (1*S*,3*R*)-**1** in a range from 500 pM to 500 nM in the presence of ~0.5–1.5 μg protein per well for hDAT, and 1.0–2.0 μg protein per well for hNET, and 1.5–2.5 μg protein per well for hSERT; *rac*-(1*R*,3*R*)-**2** toward hSERT in a range from 50 pM to 30 nM toward hDAT and hNET in a range from 100 pM to 500 nM in the presence of ~1.0–1.5 μg protein per well for hDAT, and 1.0–2.5 μg protein per well for hNET, and 1.5–2.5 μg protein per well for hSERT. Nonspecific binding was determined in the same way as total binding, but in the presence of a competitor in vast excess (100 μM BTCP (**6**) for hDAT, 10 μM desipramine (**12**) for hNET, and 10 μM clomipramine (**10**) for hSERT). Using the standard setup (250 μL incubation volume) nonspecific binding could be determined at nominal marker concentrations ≥2.5 nM. With the modified setup nonspecific binding could be determined at marker concentrations ≥500 pM. In both cases an extrapolation from the experimental data for nonspecific binding of lower marker concentrations based on linear regression was performed (see *Data analysis* below).

Kinetic studies: Dissociation experiments by the displacer approach were performed by pre-incubating (1*R*,3*S*)-**1** at a nominal concentration of 17.5 nM for hDAT, 25 nM for hNET, and 7.5 nM for hSERT with the respective target (~0.5–1.0 μg protein per well for hDAT, 0.5–3.0 μg protein per well for hNET, and 0.5–1.5 μg protein per well for hSERT) at 37 °C in a shaking water bath for 2 h (in a total incubation volume of 225 μL). After pre-equilibration, dissociation was initiated by adding 25 μL of a solution of the respective competitor to the incubation sample (i.e., 225 μL), to yield a final concentration of 100 μM BTCP (**6**) (hDAT), 10 μM desipramine (**12**) (hNET), and 10 μM clomipramine (**10**) (hSERT), and terminated after a defined time schedule (30 s–2 h, 16 dissociation time periods, samples for each time period were prepared in triplicate) by filtration as described above (see *General procedure for MS binding experiments*). For determination of the dissociation rate constant by dilution, unhindered binding of 5 nM (1*R*,3*S*)-**1** for 2 h at 37 °C in bulk was allowed to pre-equilibrate (incubation volume 10 mL and ~35–40 μg protein for hDAT, 100–120 μg protein for hNET, and 40–60 μg for hSERT). Pre-equilibration was terminated by centrifugation (20 min, 20000 rpm, 4 °C, Sorvall Evolution, SS34 rotor), and the resulting pellet (including residual solvent <200 mg) was resuspended in 20 mL assay buffer at 37 °C (providing a dilution of at least 1:1000). Again, dissociation was stopped by filtration after defined time intervals (30 s–2 h, 16 dissociation time periods, samples for each time period were prepared in triplicate) of the respective batch. To this end, 400 μL of the dilution experiment bulk sample were transferred to the filter plate and treated as described above under *General procedure for MS binding experiments*. Association experiments were performed by adding the target material (same protein amount as described for dissociation experiments by the displacer approach) to the incubation buffer containing 500 pM (1*R*,3*S*)-**1**. After a defined time schedule (20 s–2 h, 16 association time periods, samples for each time period were prepared in triplicate) association was stopped by filtration (transfer of a 200 μL aliquots of the binding samples making up a total volume of 250 μL, to the filter plate) as described above in *General procedure for MS binding experiments*. Additionally, nonspecific

binding was determined in dissociation experiments at the respective marker concentration used, i.e., 17.5 nM for hDAT, 25 nM for hNET, and 7.5 nM for hSERT for the displacer approach, and of 5 nM using the dilution approach in the presence of the displacer, i.e., 100 μM BTCP (**6**) (hDAT), 10 μM desipramine (**12**) (hNET), and 10 μM clomipramine (**10**) (hSERT) according to the *General procedure for MS binding experiments*. For association experiments nonspecific binding at a marker concentration of 500 pM was determined as follows: four different concentrations (i.e., 2.5, 5, 10, and 20 nM) of (1*R*,3*S*)-**1** were incubated in the presence of 100 μM BTCP (**6**) (hDAT), 10 μM desipramine (**12**) (hNET), and 10 μM clomipramine (**10**) (hSERT) according to the *General procedure for MS binding experiments*. The obtained experimental data were used for extrapolation of nonspecific binding at 500 pM (1*R*,3*S*)-**1**.

Competitive experiments: In all competitive studies the standard setup (see *General procedure for MS binding experiments*) was used, employing a nominal marker concentration of 1.75 nM (1*R*,3*S*)-**1** for hDAT, 2.4 nM for hNET, and 0.75 nM for hSERT. For affinity characterization of the test compounds, a minimum of seven concentrations covering a range of at least three orders of magnitude were incubated in the presence of the marker and the respective target (~0.5–1.0 μg protein per well for hDAT, 1.0–2.5 μg protein per well for hNET, 0.5–2.0 μg protein per well for hSERT). Due to their low stability, native substrates, i.e., dopamine (**30**) and L-norepinephrine (**31**), were incubated in an incubation buffer supplemented with 100 μM pargyline, 10 μM U-0521, and 10 μM ascorbic acid. Nonspecific binding was determined in an additional experiment in a range of 2.5 nM to 20 nM as described above for association experiments (see *Kinetic studies*).

Data analysis: LC–ESI-MS/MS data were obtained using Analyst 1.6.1. Calibration functions were generated by linear regression with a $1/x^2$ weighting with Prism 5.0 (GraphPad Software, San Diego, CA, USA). For saturation experiments, specific binding was defined as the difference between total and nonspecific binding, which was determined for lower nominal concentrations $\leq$2.5 nM (standard setup) and $\leq$500 pM (modified setup), respectively, by extrapolation (linear regression) of experimental data from nonspecific binding at a marker concentration $\geq$2.5 nM (standard setup) and $\geq$500 pM (modified setup) using Prism 5.0. Values for equilibrium dissociation constant (K_d) and maximum amount of binding sites (B_{max}) were calculated from specific binding isotherms using the nonlinear regression tool 'one site—specific binding' of Prism 5.0. Using the nonlinear regression tools of Prism 5.0, 'dissociation—one-phase exponential' and 'association kinetics—one conc. of hot', the corresponding rate constants for dissociation, half-life of the target–marker complex, and association were determined. For determination of the inhibition constant (K_i) the corresponding specific binding of the marker, calculated as the difference of total binding and nonspecific binding, was plotted on a percentage basis of a respective binding sample in the absence of inhibitor, which was set at 100%, versus the logarithm of the concentration of the test compound used, whereby 0% was set equal to the nonspecific binding. This plot was then analyzed with the nonlinear regression tool 'one site—Fit K_i' of Prism 5.0. Marker depletion was negligible in all experiments ($\leq$10%). For statistical comparisons data were examined by *F*-test and *t*-test (two sites, $\alpha = 0.05$ in both cases).

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Keywords: binding assays · mass spectrometry · monoamine transporters · neurotransmitters · triple reuptake inhibitors

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Supporting Information

MS Binding Assays for the Three Monoamine Transporters Using the Triple Reuptake Inhibitor (1*R*,3*S*)-Indatraline as Native Marker

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Supporting Information

Resulting bound marker in saturation experiments

According to the Langmuir adsorption model calculation of the isotherm resulting from saturation experiments is based on the following equation:

$$y = \frac{B_{max} * L}{K_d + L}$$

with B_{max} being the maximum amount of binding sites, L being the marker concentration, K_d being the affinity of the employed marker, and y being the formed target-marker complexes (which is equivalent to the bound marker). Therefore, the concentration of bound marker (y) for a nominal marker concentration (L) of 0.1 K_d at a target concentration (B_{max}) of 0.1 K_d can be calculated to:

$$y = \frac{0.1\ K_d * 0.1\ K_d}{K_d + 0.1\ K_d} = 0.0091 K_d$$

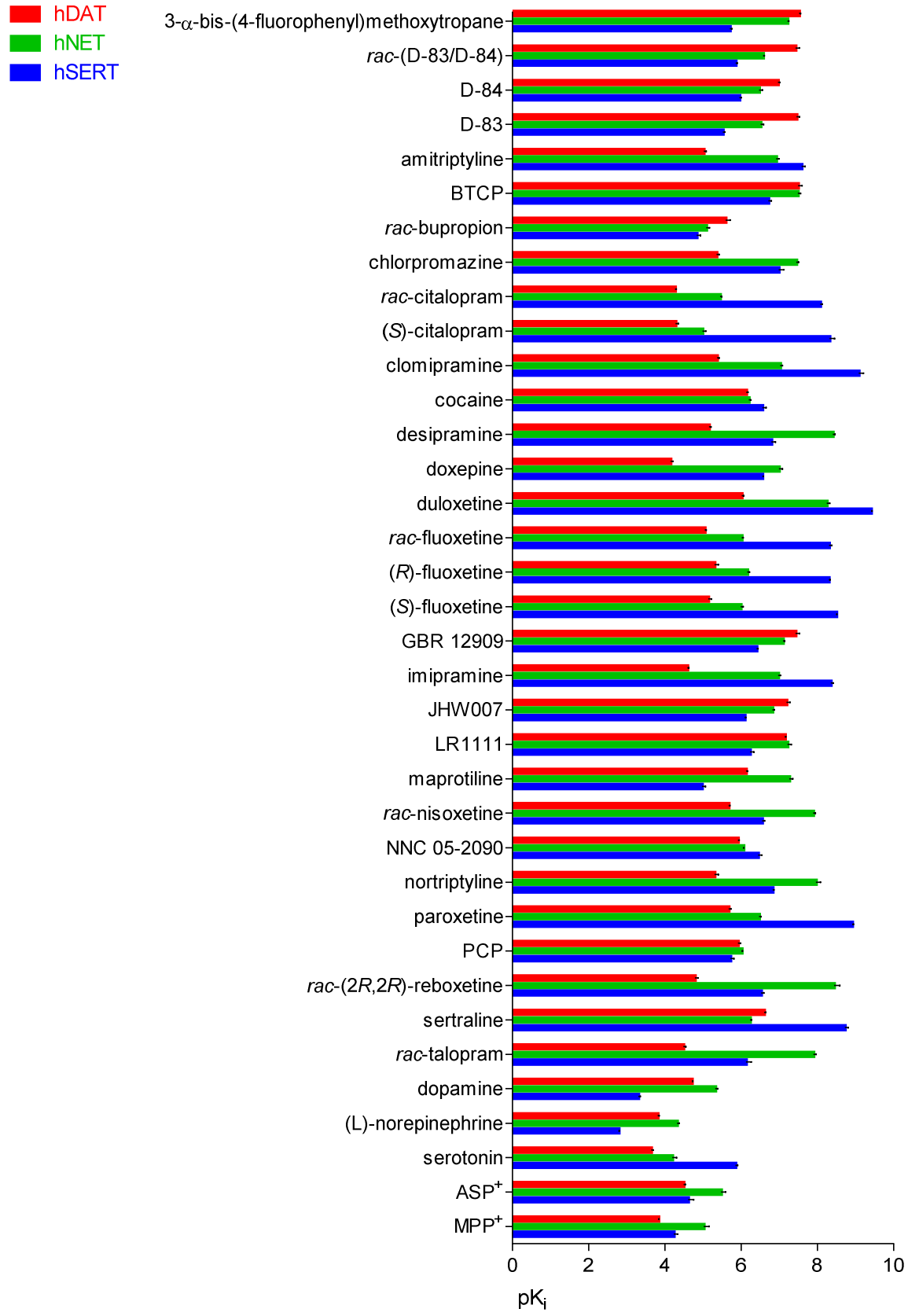
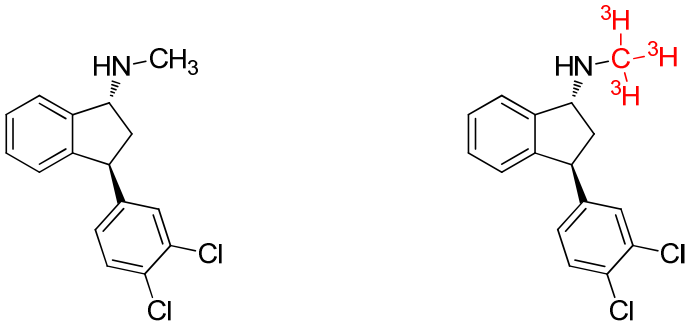


Figure S1. pK_i values for a series of known inhibitors and substrates for hDAT (red), hNET (green), and hSERT (blue) obtained in competitive experiments employing the developed MS Binding Assays utilizing (1*R*,3*S*)-indatraline as MS marker (mean ± SEM, n = 3-7).

Sensitivity of the developed LC-ESI-MS/MS method for the quantification of indatraline compared with the one expected in radioligand binding assays

The developed MS based quantification method for indatraline had an LLOQ of 5 pmol L^{-1} in the final sample having a volume of 300 µL. This corresponds to 1.5 fmol indatraline in the sample to be analyzed.

A corresponding radioligand of indatraline possessing a tritiated methylamine function (see figure S2) would have a specific activity of maximal 3.18 x 10^{15} Bq mol^{-1}. According to the assumed maximal specific activity of [^{3}H]indatraline, 1.5 fmol [^{3}H]indatraline would result in approximately 5 Bq, which is equivalent to 300 dpm (decays per min). Due to our experience concerning liquid scintillation counting a counting efficiency of approximately 25 % can be assumed for quantification of tritium in 96 well micro titer plates in conventional filtration based radioligand binding assays. As a consequence, for 1.5 fmol [^{3}H]indatraline (i.e. 300 dpm) in the sample to be analyzed, a signal of 75 cpm (counts per min) can be expected (based on our experience concerning such samples a noise level of approximately 10 cpm can be assumed).



MS marker
(1*R*,3*S*)-indatraline

Radioligand
(1*R*,3*S*)-[^{3}H]indatraline

Figure S2. One stereoisomer of the employed MS marker and a corresponding radioligand.

4 Further Applications

Within the scope of cooperation over 50 compounds have been subjected to the screening. During this screening new inhibitors for the monoamine transporters were identified. Their potencies as well as selectivities were subsequently determined in competitive binding experiments.

Based on these results the group intern division for routine analysis started an expanded screening investigating over 200 potential inhibitors.

The results (along with the corresponding structure formulas of the compounds) obtained by this screening are still confidential and are therefore not included in this study.

5 Summary of the Thesis

The biogenic monoamines, i.e. dopamine, norepinephrine, and serotonin, are important neurotransmitters, which act in the human brain as well as in the periphery. They control different functions, such as motor activity, attention, memory, sleep, but also mood. An imbalance of one of these neurotransmitter systems is considered to be associated with different mental disorders, e.g. depression. Even though there is a large number of different antidepressants, approximately 35 % of all depressive patient have no or no efficient response to their therapy. As a consequence there is a substantial need for new antidepressants. Nowadays, the human monoamine transporters, i.e. hDAT, hNET, and hSERT, are the most important targets for the treatment of depressions.

Binding studies are an indispensable tool in drug discovery process. They provide information regarding the affinity of a test compound towards a specific target. Up to now, radioligand binding assays are mainly employed to investigate binding affinities of potential inhibitors towards hDAT, hNET, and hSERT. Unfortunately, this technique has considerable disadvantages due to use of radioactivity.

It was the aim of the present thesis to develop MS Binding Assays addressing hDAT, hNET, and hSERT, which should serve as screening tool for the identification and characterization of potential monoamine transporter inhibitors utilizing one single marker for all three targets.

The marker to be selected should have high affinity, ideally in the low nanomolar range, towards each of the monoamine transporters, i.e. hDAT, hNET, and hSERT. This requirement is fulfilled by Indatraline [*rac*-(1*R*,3*S*)-3-(3,4-dichlorophenyl)-1-methylamino-2,3-dihydro-1*H*-indene], a known triple reuptake inhibitor, and was therefore chosen as marker for the intended purpose.

From uptake studies a significant difference of the inhibitory potency in regard to the employed enantiomer, i.e (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, is known for all three monoamine transporters, with (1*R*,3*S*)-indatraline being the eutomer. To be able to investigate the individual enantiomers in MS Binding Assays, the enantiopurity of the employed enantiomers had to be determined. Therefore, two analytical methods were developed. First, an ^{1}H NMR spectroscopy method employing a chiral shift agent was established. With this method a determination of enantiopurities up to 98.9 % *ee* for both enantiomers, i.e. (1*R*,3*S*)- and (1*S*,3*R*)-indatraline, was feasible. The second method was based on HPLC utilizing a cyclodextrine based chiral stationary phase. Thereby, even higher enantiopurities, i.e. for (1*R*,3*S*)-indatraline up to 99.75 % *ee* and for (1*S*,3*R*)-indatraline up to 99.67 % *ee*, could be determined. Additionally, the latter method was validated according to the ICH guidance Q2(R1) regarding specificity, accuracy, precision, linearity, and quantitation limit.

In parallel, MS Binding Assays were developed. A fundamental requirement therefore was a highly sensitive MS method that allowed the quantification of the chosen marker in biological samples. For this purpose, an API 5000 triple quadrupole mass spectrometer equipped with an ESI source for ionization was employed. As ESI ionization is prone to ion suppression caused by co-eluting matrix components, an LC method was developed that separates the analyte from the matrix by chromatography. Therefore, several parameters such as stationary and mobile phase, but also the injection volume and sample milieu were optimized. To allow a reliable quantification of the analyte, even in presence of traces of matrix, a poly-deuterated analogue of indatraline, ($^2H_7$)-indatraline, was employed as internal standard.

In the next step a suitable setup was developed for the intended MS based binding studies, where several parameters such as incubation buffer, filter materials, washing buffer, and elution solvent were investigated regarding their influence on the binding of the analyte to the respective target, but also on the signal intensity and the signal-to-noise ratio in the MRM chromatogram.

Afterwards, using matrix samples from MS Binding Assays, the developed LC-MS/MS method was validated according to the FDA guideline for bioanalytical methods regarding stability of the employed marker solutions, selectivity, calibration standard curve, the LLOQ, accuracy as well as precision. It could be demonstrated that the developed LC-MS/MS method allows a selective, accurate and precise quantification in a range of 5 pmol L^{-1} to 5 nmol L^{-1}.

Finally, using the established setup it was possible to characterize potential markers, i.e. the stereoisomers of indatraline, in MS Binding Assays. The respective results of saturation experiments confirm (1*R*,3*S*)-indatraline to be the almost equi-affine eutomer for all three transporters, i.e. hDAT, hNET, and hSERT, whereas the *cis*-configured diastereomer was found to be a highly selective SERT inhibitor. (1*R*,3*S*)-indatraline, the only stereoisomer having sufficient affinity towards all three targets, i.e. hDAT, hNET, and hSERT, was further characterized in kinetic studies.

The full characterization of the binding properties of (1*R*,3*S*)-indatraline allowed its application as marker in competitive MS binding experiments addressing hDAT, hNET, and hSERT. In these experiments the affinities and selectivities of about 90 potential inhibitors were determined.

For ten compounds the results could be compared with those obtained in radioligand binding assays under consistent conditions. This comparison demonstrated that the results from MS Binding Assays are in excellent agreement with those derived from radioligand binding assays. Therefore, the established MS Binding Assays represent a promising substitute for the so far

dominating radioligand binding assays widely employed to characterize affinity at monoamine transporters.

6 List of Abbreviations

5-HT	serotonin
cAMP	cyclic adenosine monophosphate
CDER	center for drug evaluation and research
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COMT	catechol-*O*-methyltransferase
CSA	chiral shift agent
DA	dopamine
DAT	dopamine transporter
DNA	deoxyribonucleic acid
ee	enantiomeric excess
EL	extracellular loop
ESI	electrospray ionization
FDA	food and drug administration
FRET	fluorescence resonance energy transfer
FP	fluorescence polarization
GPCR	G protein-coupled receptors
HEK	human embryonic kidney
HPLC	high-performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
ICH	international conference on harmonization
K_d	equilibrium dissociation constant
K_i	inhibition constant
k_{obs}	observed rate constant
k_{off}	dissociation rate constant

k_{on}	association rate constant
LeuT	leucine transporter
MAO	monoamine oxidase
mGAT1	murine γ-aminobutyric acid transporter subtype 1
mRNA	messenger ribonucleic acid
NE	norepinephrine
NET	norepinephrine transporter
NDRI	norepinephrine and dopamine reuptake inhibitor
NMR	nuclear magnetic resonance
NRI	selective norepinephrine reuptake inhibitor
NSS	neurotransmitter-sodium symporter
NTT	neurotransmitter transporter
SERT	serotonin transporter
SLC	solute carrier
SNRI	serotonin and norepinephrine reuptake inhibitor
SPR	surface plasmon resonance
SSRI	selective serotonin reuptake inhibitor
$t_{1/2}$	half life of the target-marker complex
TCA	tricyclic antidepressant
TEAA	triethylammonium acetate
TM	transmembrane helices/segments
VMAT	vesicular monoamine transporters

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8 Curriculum Vitae

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