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RESEARCH PAPER

Estimation of binding rate constants using a simultaneous mixed-effects method: application to monoamine transporter reuptake inhibitor reboxetine

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Background and purpose: Reboxetine is a clinically used antidepressant and is a racemic mixture of two enantiomers, SS- and RR-reboxetine. The aim of the work described in this manuscript was to determine the kinetics of binding of the RR- and SS-reboxetine to the human noradrenaline transporter (hNET).
Experimental approach: We have applied a simultaneous mixed-effects method to the analysis of the transient kinetics of binding of SS-, RR- and racemic reboxetine to hNET. This method allowed simultaneous modelling of multiple datasets, taking into account inter-experiment variability, thereby facilitating robust parameter estimation and minimizing the assumptions made.
Key results: The mixed-effects method proved simple and robust. SS-reboxetine bound to hNET according to a one-step binding model with the SS-enantiomer having 130-fold higher steady state affinity than the RR-enantiomer (K_d = 0.076 ± 0.009 nM vs. 9.7 ± 0.8 nM respectively). The k_{on} for SS-reboxetine was *c.* 1.4×10^5 $M^{-1} \cdot s^{-1}$ and k_{off} 1.05×10^{-5} s^{-1} ($t_{1/2}$ ~18 h). The k_{on} for RR-reboxetine was *c.* 4.3×10^5 $M^{-1} \cdot s^{-1}$ and k_{off} 4.2×10^{-3} s^{-1} ($t_{1/2}$ ~3 min). The racemate behaved as expected for an equimolar mixture of RR- and SS-reboxetine, assuming mutually exclusive binding.
Conclusions and implications: These data will be useful for the interpretation of the behaviour of reboxetine and its enantiomers in man and the method used could be applied to other candidate drugs.
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Keywords: association rate (k_{on}); competition binding; dissociation rate (k_{off}); duration of action; kinetics; mixed-effects; reboxetine

Abbreviations: B_{max}, maximum receptor concentration; D, drug; K_d, equilibrium dissociation constant of the drug; k_{offD}, dissociation rate constant of the drug; k_{offT}, dissociation rate constant of the tracer; k_{onD}, association rate constant of the drug; k_{onT}, association rate constant of the tracer; K_T, equilibrium dissociation constant of the tracer; MVOF, minimum value of the objective function; R, receptor/target; RD, receptor–drug complex; RT, receptor–tracer complex; T, tracer

Introduction

Sustained and controlled duration of action is typically a desirable drug characteristic. In addition, compliance is an important issue in determining the efficacy of drugs and dosing regimens need to be pragmatic, for example daily (Cramer *et al.*, 1989). Usually this is achieved by ensuring a drug has appropriate pharmacokinetics. However, selecting drugs with sustained residence time on the molecular target could also be an additional important selectable parameter. Tiotropium is an example of this effect, as its efficacy in the management of chronic obstructive pulmonary disease relies substantially on its very slow dissociation from M_3 muscarinic receptors (Disse *et al.*, 1999).

In the simplest case of single-step reversible binding, it is the ratio of the first-order dissociation rate constant over the second-order association rate constant for binding that equals

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the equilibrium dissociation constant. Hence, equal steady state target binding affinity could be achieved by an infinite number of ratios of rate constants that give the same equilibrium constant. However, in practice this is often thought to be limited; for example, with the on rate constants for binding of small molecules to proteins typically being restricted to less than *c.* 5×10^7 $M^{-1} \cdot s^{-1}$ (limited by diffusion rate and the probability of binding to a specific binding site) and generally no more than two to three orders of magnitude less than this upper limit (Gutfreund, 1987). Nevertheless, this leaves a range of several orders of magnitude and determining the binding rate constants for potential drugs may provide very useful information for the selection of candidate compounds and for understanding drug pharmacokinetics and pharmacodynamics (see Tummino and Copeland, 2008).

A commonly used approach for the determination of transient binding kinetic parameters has already been described (Motulsky and Mahan, 1984; Dowling and Charlton, 2006) and uses, most often, radiolabelled tracers. The equilibrium and pre-steady state kinetic behaviour of tracers can readily be determined and this data used in the analysis of competition experiments. Typically, a non-linear least-squares regression method is used for this (Bevington and Robinson, 1992) together with sequential estimation of the association and dissociation rate constants. However, simultaneous analysis of all association and dissociation data is superior to sequential linear and non-linear regression analysis with respect to the accuracy and precision of parameter estimates (Karlsson and Neil, 1989). In addition, the sequential method inevitably involves making certain assumptions about the error, both in tracer parameter values and inter-experimental variability. These assumptions can be minimized using a population modelling approach that has been applied with great success in pharmacokinetic and pharmacodynamic data analysis (Yuh *et al*., 1994; Beal and Sheiner, 1999). Such models require a structural component (e.g. describing the time course of drug binding) and two statistical components, one for the residual random error and the other the inter-experiment and/or inter-occasion variability. The residual error links the structural model and the observations and can be estimated using ordinary non-linear regression, resulting in intra-individual variability estimates for parameters and approximate standard errors indicating the precision of parameter estimates. The population or mixed-effects approach expands this single-experiment model, by simultaneously estimating the curves for all experiments. Population typical parameters can then be estimated together with the inter-individual variability (IIV). The power of the approach is to combine the information for all experiments while preserving the individuality (see Schoemaker and Cohen, 1996). In this manuscript we describe the application of such a population approach to the analysis of transient kinetic binding data. Specifically, we have applied this to datasets for the noradrenaline reuptake inhibitors SS-, RR- and racemic reboxetine. Reboxetine is used clinically as an antidepressant (Fleishaker, 2000; Kasper *et al*., 2000; Wong *et al*., 2000; Hajos *et al*., 2004), and its efficacy is considered to derive from its potent and selective inhibition of noradrenaline uptake (Ressler and Nemeroff, 1999). Reboxetine has also been investigated as a treatment for pain (Krell *et al*., 2005) and other indications (Preskorn, 2004). In all these cases it is racemic reboxetine that is the clinically used form; however, it has been reported that the SS-enantiomer is more potent than the RR-enantiomer against the rat receptor (Wong *et al*., 2001) and that the different enantiomers exhibit different clearances and plasma protein binding in man (Fleishaker *et al*., 1999).

The aim of the work described in this paper was to characterize the transient binding kinetics of SS-, RR- and racemic reboxetine to the human noradrenaline transporter (hNET), using a novel mixed-effects method. To our knowledge this is the first report of the kinetics of binding of these drugs and the successful implementation of a population mixed-effects modelling method to the analysis of this type of data. We report the parameters and show that the method used is both simple and robust. Our conclusions may be used to develop a better understanding of the pharmacokinetic and pharmacodynamic behaviour of these and other drugs and to aid the development of more efficient experimental protocols.

Methods

Membrane preparation

Membrane homogenates of HEK-293 cells expressing human recombinant NET (hNET) were prepared in our laboratories as described elsewhere (Fish and Wakenhut, 2007) and were stored at –80°C at a protein concentration of 5 $mg \cdot mL^{-1}$. The maximum receptor concentration (B_{max}) was approximately 40 $pmol \cdot mg^{-1}$. Prior to use, the vials were thawed rapidly, briefly homogenized using a PowerGen 35 homogenizer for 10 s and diluted with assay buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4 at room temperature)

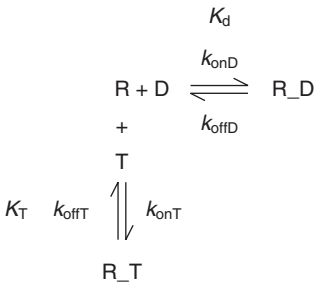
Transient binding kinetics of non-labelled reboxetine

All assays were carried out in 96-well polystyrene plates (Perkin-Elmer, Buckinghamshire, UK), typically with 2–4 replicates per plate. Non-specific binding was determined using 5 μM desmethylimipramine (DMI) or reboxetine. Compounds were dissolved in dimethyl sulphoxide (DMSO) to give stock solutions of 10 mM. These stock solutions were then diluted in assay buffer to give solutions 10 times the final assay concentration. Final DMSO concentration was *c.* 0.1% (v/v). Three sets of experiments were carried out in our laboratories to determine the transient binding kinetics of candidate drugs.

Experiment 1 was the estimation of the K_T of the tracer, [^{3}H]-DMI for hNET, by titration with increasing concentrations of [^{3}H]-DMI. Experiment 2 was the measurement of the rate of association upon mixing [^{3}H]-DMI with hNET. Experiment 3 was a competition experiment between SS-, RR- and racemic reboxetine and [^{3}H]-DMI, as tracer as shown in Scheme 1.

Experiment 1: binding of [^{3}H]-DMI to the recombinant hNET

Saturable binding was determined in quadruplicate at room temperature in a total assay volume of 200 μL, comprising 20 μL [[^{3}H]-DMI (30 pM–20 nM), 20 μL assay buffer or DMI (50 μM)] and 160 μL hNET (at 16 $\mu g \cdot mL^{-1}$ total protein to give ~0.5 nM hNET final). Following incubation for 90 min, reac-



Scheme 1 Schematic representation of the binding kinetics of a drug in presence of a radioactive labelled tracer. D, drug; K_d, dissociation constant of the drug for the receptor; k_{offD}, dissociation rate constant of the drug; k_{offT}, dissociation rate constant of the tracer; k_{onD}, association rate constant of the drug; k_{onT}, association rate constant of the tracer; K_T, dissociation constant of the tracer for the receptor; R, hNET (human noradrenaline transporter); R_D, receptor–drug complex; R_T, receptor–tracer complex; T, radioactive labelled tracer.

tions were filtered using a Brandel Cell Harvester through GF/B filter mats, which had previously been soaked in wash buffer (20 mM HEPES 120 mM NaCl, 5 mM KCl, pH 7.5 at 4°C) containing 0.5% (v/v) polyethylenimine (PEI) solution. Filtermats were washed three times with wash buffer and then punched into 7 mL vials and Starscint added. Following overnight incubation, vials containing filter mats were then counted using a scintillation counter (Tricarb 3100, Perkin Elmer, Buckinghamshire, UK) for 1 min per tube.

Experiment 2: association of [^{3}H]-DMI

Kinetic assays were performed essentially as for Experiment 1 in 1000 µL volume, to determine the association rate of [^{3}H]-DMI to the hNET. Total and non-specific binding were determined at time points ranging from 5 to 360 min for the association time course, using typically 1.5 nM of radioligand. Reactions were initiated at t = 0 by addition of the hNET at 16 µg·mL^{-1} to give *c*. 0.1 nM hNET and were terminated at appropriate times by filtration using a Unifilter Harvester (Perkin Elmer, Buckinghamshire, UK) and GF/B unifilters that had previously been soaked in wash buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.5 at 4°C) containing 0.5% (v/v) PEI solution. Unifilter plates were washed three times with wash buffer and dried overnight before adding 40 µL of Microscint 0. Plates were left for at least 30 min before counting (Packard Topcount NXT scintillation counter, Perkin-Elmer, Waltham, MA, USA).

Experiment 3: competition kinetics assay

All assays were carried out at room temperature in 1000 µL volume, comprising 100 µL [^{3}H]-DMI (to give final concentration *c*. 1 nM), 100 µL compound or 50 µM DMI (to estimate non-specific binding) and 800 µL hNET (at 3.2 µg·mL^{-1}, to give hNET *c*. 0.1 nM). Compounds were tested at three to four concentrations (quadruplicate wells per concentration), 0.1, 0.3 and 1 nM for reboxetine and SS-reboxetine, and 1, 3, 10 and 30 nM for RR-reboxetine. Each plate also contained 12 wells each to determine total and non-specific binding. Plates were incubated with shaking at room temperature until required for harvesting at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 90, 120 and 150 min and at 4, 6, 8, 20, 22 and 24 h. All competition kinetic assays were terminated by separation of bound and free radioligand by rapid vacuum filtration through glass fibre Packard Unifilters (Perkin-Elmer, Waltham, MA, USA) as described for Experiment 2.

Binding of [^{3}H]-SS-reboxetine to human recombinant norepinephrine transporter

Saturable binding of [^{3}H]-SS-reboxetine was determined as for Experiment 1 with [^{3}H]-DMI, but with free concentrations of [^{3}H]-SS-reboxetine between 0.004 and 16 nM and *c*. 0.12 nM hNET n = 3. Non-specific binding at hNET was defined at each concentration of radioligand using unlabelled DMI. Reactions were incubated for either 90 min or 24 h, prior to termination and counting as for the [^{3}H]-DMI saturation assays. The free concentration of [^{3}H]-SS-reboxetine was calculated from the difference between bound [^{3}H]-SS-reboxetine and the total added radioligand.

Data analysis

Parameter estimation method. A population modelling approach was applied to describe the relationship between the concentrations of one or more (labelled) compounds and receptor occupancy. A simple competitive binding model, shown in Scheme 1, was assumed for estimating the association and dissociation rate constants. To allow simultaneous analysis of data from the competition experiments, together with the kinetic experimental results using radioactive tracer, the following set of differential equations was used (Equations 1–4):

$$d[RT]/dt = k_{onT} * [R] * [T] - k_{offT} * [RT] \quad (1)$$

$$d[R]/dt = -(k_{onT} * [T] + k_{onD} * [D]) * [R] + k_{offT} * [RT] + k_{offD} * [RD] \quad (2)$$

$$d[RD]/dt = k_{onD} * [R] * [D] - k_{offD} * [RD] \quad (3)$$

$$d[T]/dt = -k_{onT} * [R] * [T] + k_{offT} * [RT] \quad (4)$$

$$B_{max} = [R] + [RT] + [RD] \quad (5)$$

in which R = receptor, T = radioactive labelled tracer, D = drug, RT = receptor–tracer complex, RD = receptor–drug complex, k_{onT} = association rate constant of the tracer, k_{offT} = dissociation rate constant of the tracer, k_{onD} = association rate constant of the drug, k_{offD} = dissociation rate constant of the drug. Depending on the experiment, the compartment corresponding to the differential equation of either [R] or [RT] was initialized with the total receptor concentration (B_{max}).

Binding isotherm data were described using Equation (6):

$$[RT] = \frac{B_{max} T}{K_T + T} \quad (6)$$

where B_{max} = total receptor concentration, T = free tracer concentration and K_T is the equilibrium dissociation constant for [^{3}H]-DMI binding to hNET.

The equilibrium dissociation constant for test drug was calculated according to Equation (7):

$$K_d = \frac{k_{offD}(s^{-1})}{k_{onD}(M^{-1}\cdot s^{-1})} \quad (7)$$

where K_d is the equilibrium dissociation constant for unlabelled drug.

Sequential analysis of the data was carried out as follows; first the K_T of the tracer was estimated from Experiment 1 data. Second, k_{offT} was estimated from Experiment 2 data while fixing K_T. Finally the data from the competition binding experiment were analysed to obtain equilibrium dissociation constant and dissociation rate of the drugs using the previously determined k_{offT} and K_T parameters.

Analysis of the binding curves for [^{3}H]-SS-reboxetine. The saturation curves were analysed using both a standard four-parameter logistic function that provided estimates of B_{max}, C_{50}, logC_{50} (the midpoint of the curve) and slope factor and by Equation (8) that takes into account of the slow kinetics of binding of the radioligand.

For 'saturation' curves measured at different times, t, for labelled drug concentrations [D], with log equilibrium dissociation constant, logK_d and with a slow dissociation rate constant, k_{off}, the concentration of bound receptor–drug complex [RD], is given by:

$$[RD] = \frac{B_{max} * (10^{\log[D]-\log K_d}) * \left(1 - e^{-k_{off}\cdot(1+10^{\log[D]-\log K_d})*t}\right)}{1+10^{\log[D]-\log K_d}} \quad (8)$$

where B_{max} is the receptor binding capacity.

Mutually exclusive binding of enantiomers. Assuming the racemate is a stable equimolar mixture of RR- and SS-reboxetine and that the enantiomers bind mutually exclusively, then the racemate will behave according to the assumptions depicted in Scheme 2. Steady state hNET occupancy was estimated using Equation (9);

$$\frac{R}{R_{TOT}} = \frac{1}{1+\frac{[SS\text{-}RBX]}{K_{dSS\text{-}RBX}}+\frac{[RR\text{-}RBX]}{K_{dRR\text{-}RBX}}} \quad (9)$$

where SS-RBX and RR-RBX are the free concentrations of drug. $K_{dSS\text{-}RBX}$ = equilibrium dissociation constant for SS-RBX and $K_{dRR\text{-}RBX}$ = equilibrium dissociation constant for RR-RBX. R = free hNET and R_{TOT} = total hNET = B_{max}.

To analyse or simulate the time dependence of the transient binding kinetics of racemic RBX-[^{3}H]-DMI association curve according to Scheme 2, the following set of differential equations was used;

$$d[RT]/dt = k_{onT} * [R] * [T] - k_{offT} * [RT] \quad (10)$$

$$d[R]/dt = -(k_{onT} * [T] + k_{onSS} * [SS] + k_{onRR} * [RR]) * [R] + k_{offT} * [RT] + k_{offSS} * [RSS] + k_{offRR} * [RRR] \quad (11)$$

$$d[RSS]/dt = k_{onSS} * [R] * [SS] - k_{offSS} * [RSS] \quad (12)$$

$$d[RRR]/dt = k_{onRR} * [R] * [RR] - k_{offSS} * [RRR] \quad (13)$$

$$d[T]/dt = -k_{onT} * [R] * [T] + k_{offT} * [RT] \quad (14)$$

$$B_{max} = [R] + [RT] + [RRR] + [RSS] \quad (15)$$

in which R = receptor, T = radioactive labelled tracer, SS = SS-reboxetine, RT = receptor–tracer complex, RSS = receptor–SS-reboxetine complex, RRR = receptor–RR-reboxetine complex, k_{onT} = association rate constant of the tracer, k_{offT} = dissociation rate constant of the tracer, k_{onSS} = association rate constant of SS-reboxetine, k_{onRR} = association rate constant of RR-reboxetine, k_{offSS} = dissociation rate constant of SS-reboxetine and k_{offRR} = dissociation rate constant of RR-reboxetine.

Computation. Data analysis or simulations were carried out using the non-linear mixed-effects modelling approach implemented in NONMEM software (version VI, release 2.0; Icon Development Solutions, Ellicott City, MD, USA). See online *Supporting information* for examples of the code used, or a copy can be provided on request. The models were compiled using Digital Fortran (version 6.6, Compaq Computer Corporation, Houston, TX, USA) and executed on a PC equipped with an AMD Athlon 64 processor 3200+ under Windows XP. The results were analysed using S-Plus for Windows (version 6.2 Professional, Insightful Corp., Seattle, WA, USA).

Parameters were estimated using the first-order conditional estimation method with interaction between the two levels of stochastic effects (FOCE interaction), as implemented in NONMEM (Beal and Sheiner, 1999). The IIV of appropriate parameters was modelled using multiplicative exponential random effects as described in Equation (16):

$$P_i = \theta * \exp(\eta_i) \quad (16)$$

Where θ = population typical value for the parameter, P_i the individual prediction and η_i the random deviation of P_i from θ. The values of η_i were assumed to be normally distributed with a mean of zero and a variance of ω^2.

Residual variability was described using a proportional error model;

$$Y_{ij} = F_{ij} * (1 + \varepsilon_{PROP,ij}) \quad (17)$$

where Y_{ij} denotes the observation for the ith individual at time t_j, F_{ij} denotes the corresponding prediction based on the model, and ε_{ij} denotes the residual random effect assumed to have zero mean and variance σ^2.

Goodness-of-fit was determined using the minimum value of the objective function defined as minus twice the log-likelihood and by visual inspection of the plots of individual predictions and the diagnostic plots of (weighted) residuals. For nested models, a decrease of 10.8 points in the objective function (MVOF) (corresponding to $P < 0.001$ in a chi-squared distribution) by adding an additional parameter was considered statistically significant.

For further details on the mixed-effects methods as implemented in NONMEM see Schoemaker and Cohen (1996).

Materials

All reagents were obtained from Sigma-Aldrich (Poole, UK) unless specified otherwise. Reboxetine, its enantiomers and

[^{3}H]-SS-reboxetine ([^{3}H]-SS-reboxetine) (specific activity 46 Ci·mmol^{-1}) were synthesized by Pfizer Global Research and Development laboratories. Starscint, Microscint O and [^{3}H]-desmethylimipramine [[^{3}H]-DMI (NET593) specific activity 90 Ci·mmol^{-1}] were purchased from Perkin Elmer (Buckinghamshire, UK). All drug and target nomenclature follow Alexander *et al*. (2009)

Results

Experiments 1 and 2 were carried out to obtain estimates of the equilibrium dissociation constant (K_T), the association rate constant (k_{onT}) and the dissociation rate constant (k_{offT}) for the radioactive tracer. The third experiment was a competition experiment to determine the transient kinetics of binding of the unlabelled compounds of interest (SS-, RR- and racemic reboxetine) and estimate k_{onD}, k_{offD} and K_d (Scheme 1). All data from Experiments 1–3 were analysed in a single step, affording parameter estimates derived from the total dataset consisting of 1500 data points from 53 experiments. Estimation of the inter-experiment variability for B_{max} was possible and inclusion of this alone improved the goodness of fit significantly as shown by a decrease in the MVOF of 58 points (<0.001). The estimated value of B_{max} was 72.4 ± 4.0 pM (or ~28 pmol·mg^{-1}). All other parameter estimates are summarized in Table 1. The parameters were estimated with good precision, with coefficients of variation (CV) ranging from 7% to 34%. We also analysed the data in a sequential manner as described in the *Methods* (Table 1). By comparing the simultaneous mixed and sequential fits to the total dataset, we found that the description of the data by the sequential analysis approach was significantly worse, as indicated by a difference in the MVOF of 297 points, versus a significance of 18.47. Interestingly, sequentially analysing the naïve pooled data from the different experiments resulted in estimates of K_d RR and and k_{off} DMI, which were not within the confidence intervals obtained from the simultaneous analysis (Table 1), but in general the parameter estimates were in close agreement, relative to typical variability in determining such parameters.

The tracer binding data were well described by a simple mass action isotherm with a K_T of 1.5 nM (logK_T = –8.82) for [^{3}H]-DMI. There was the expected tendency for higher variability in the data with increasing concentrations of radioligand (Figure 1A) because of the lower ratio of specific: non-specific binding.

Figure 1B shows the results of four independent experiments to measure the association of the tracer [^{3}H]-DMI. The individual component fits to the data are shown by the solid curves and the population typical theoretical curves are shown by the dotted curves. In general both the individual and population parameters fitted the data reasonably well (Figure 1B), with the exception of the time points around 24 h, where the observed bound radioligand was lower than that predicted by the model. The association rate constant of [^{3}H]-DMI was relatively fast (2×10^6 M^{-1}·s^{-1}), as was the dissociation rate constant of 2.9×10^{-3} s^{-1} (half time 4 min).

Figure 1C shows 15 illustrative transient binding time courses from five independent experiments where the time-dependent effects of different concentrations of SS-reboxetine (top two rows), RR-reboxetine (third and fourth rows) and the racemic reboxetine (bottom row) on the binding of [^{3}H]-DMI have been measured (Experiment 3 protocol). As found for the tracer saturation and association data, in general, the transient binding kinetics model (Equations 1–5) adequately described the kinetic data for SS-, RR- and racemic reboxetine. A rise and fall in the labelled species was observed for SS-reboxetine and the racemate. In the case of RR-reboxetine, equilibration was clearly more rapid, and the two phases were

Table 1 Summary of binding kinetic parameter estimates for tracer (DMI), SS-, RR- and racemic (rac) reboxetine

Parameter	*Population estimate – mixed-effects analysis (±SE)*	*CV (%)*	*95% confidence limits*	*Population estimate – naïve pool sequential analysis (±SE)*	*CV (%)*
k_{off} SS (s^{-1})	(1.05 ± 0.07) × 10^{-5}	7	(0.9–1.2) × 10^{-5}	(1.08 ± 0.13) × 10^{-5}	12
Half time SS (h)	18			18	
K_d SS (pM)	76 ± 9	12	57–94	67 ± 9	13
LogK_d	–10.12			–10.17	
k_{on} SS (M^{-1}·s^{-1})	1.4 × 10^5			1.6 × 10^5	
k_{off} RR (s^{-1})	(4.2 ± 0.8) × 10^{-3}	18	(2.7–5.7) × 10^{-3}	(4.7 ± 0.7) × 10^{-3}	16
Half time RR (h)	0.05			0.04	
K_d RR (nM)	9.8 ± 0.8	9	8.1–11.4	14 ± 2	17
LogK_d	–8.01			–7.84	
k_{on} RR (M^{-1}·s^{-1})	4.3 × 10^5			3.2 × 10^5	
$k_{off,obs}$ rac (s^{-1})	(0.6 ± 0.2) × 10^{-5}	34	(0.2–1.0) × 10^{-5}	(0.7 ± 0.4) × 10^{-5}	55
Half time rac (h)	32			27	
$K_{d,obs}$ rac (pM)	120 ± 30	27	55–180	109 ± 46	42
Log$K_{d,obs}$	–9.92			–9.96	
$k_{on,obs}$ rac (M^{-1}·s^{-1})	0.5 × 10^5			0.65 × 10^5	
k_{off} DMI (s^{-1})	(2.9 ± 0.2) × 10^{-3}	7	(2.5–3.3) × 10^{-3}	(3.4 ± 0.1) × 10^{-3}	4
Half time DMI (h)	0.07			0.058	
K_d DMI (nM)	1.5 ± 0.15	10	1.2–1.8	1.3 ± 0.15	11
LogK_d	–8.82			–8.88	
k_{on} DMI (M^{-1}·s^{-1})	20 × 10^5			25 × 10^5	

CV, coefficients of variation; DMI, desmethylimipramine.

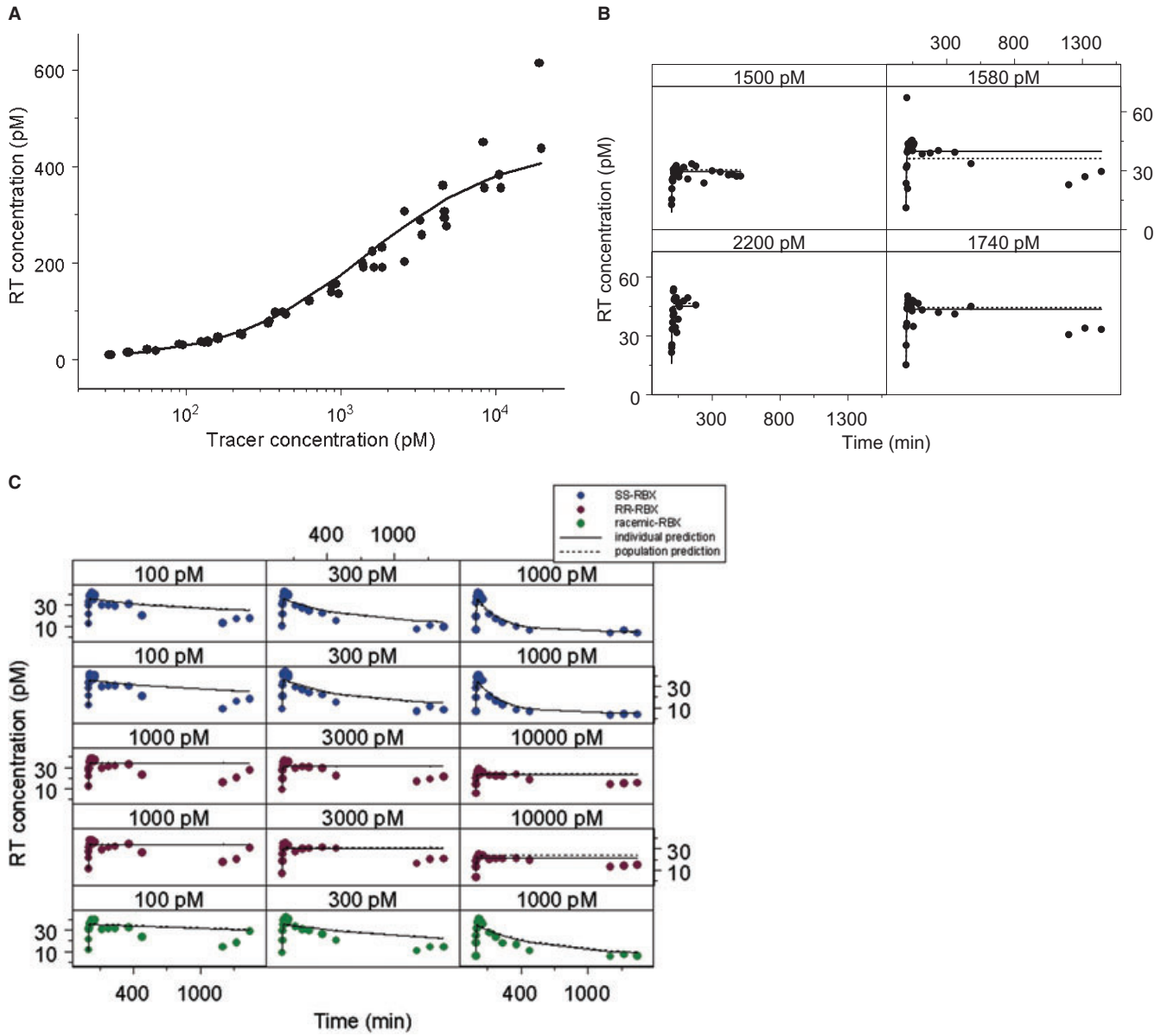


Figure 1 Model fits to the data from the three types of experiments carried out. (A) Experiment 1; the equilibrium binding of [^{3}H]-DMI to hNET. Model predictions are shown as a solid line and observed data shown as closed circles. (B) Experiment 2; time course of binding of [^{3}H]-DMI to hNET. Population or typical value predicted, individual predicted and observed data shown as dashed lines, solid lines and closed circles respectively. (C) Experiment 3; sample time course of $n = 5$ experiments showing binding of [^{3}H]-DMI to hNET in the presence of SS- (6 data sets), RR- (6 data sets) or racemic (3 data sets) reboxetine. Reactions were initiated at zero time with hNET, [^{3}H]-DMI and unlabelled competitor concentration as indicated. Population and individual predicted and observed data shown as dashed lines, solid lines and symbols respectively. DMI, desmethylimipramine; hNET, human noradrenaline transporter.

less distinct. In general both the individual and population parameters fitted the data well. The estimate of non-specific binding was equal to that found using unlabelled DMI.

RR-reboxetine had a relatively low affinity (K_d = 9.8 ± 0.8 nM, logK_d = –8.01) with 10-fold lower affinity than DMI. The association rate constant of RR-reboxetine of 4.3×10^5 $M^{-1}\cdot s^{-1}$ was *c*. fivefold slower than that of DMI whereas the dissociation rate constant of 4.2×10^{-3} s^{-1} ($t_{1/2}$ *c*. 3 min) was only slightly slower than that of DMI. SS-reboxetine was over 130-fold more potent than RR-reboxetine (K_d = 76 ± 9 pM, logK_d = –10.12) and exhibited 400-fold slower dissociation kinetics (1.1×10^{-5} s^{-1}, $t_{1/2}$ *c*. 18 h) but only a threefold slower association rate constant, relative to RR-reboxetine (Table 1).

To corroborate the results derived using the simultaneous analysis for SS-reboxetine, we carried out an experiment with [^{3}H]-labelled SS-reboxetine. hNET (*c*. 0.12 nM) was mixed with a range of [^{3}H]-SS-reboxetine concentrations as described in the *Methods*. Samples were then taken at 1.5 and 24 h after initiation of binding and the bound drug–receptor complex and free drug concentration were measured. The saturation curves illustrated in Figure 2 show a large (sevenfold) increase in binding potency of SS-reboxetine found at the longer incubation time, illustrating the fact that binding at 1.5 h is not in equilibrium. When a logistic function was used to fit the data, the mean values of parameters (logEC_{50}, slope factor) were (–8.97 ± 0.02, 1.44 ± 0.08) and (–9.79 ± 0.02, 1.18 ± 0.08) at

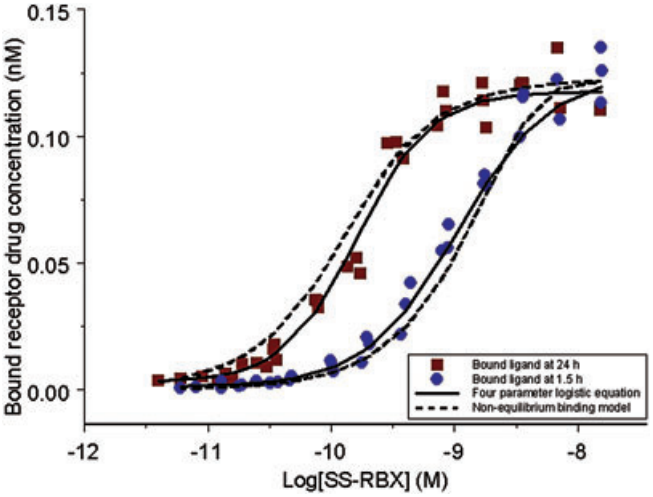
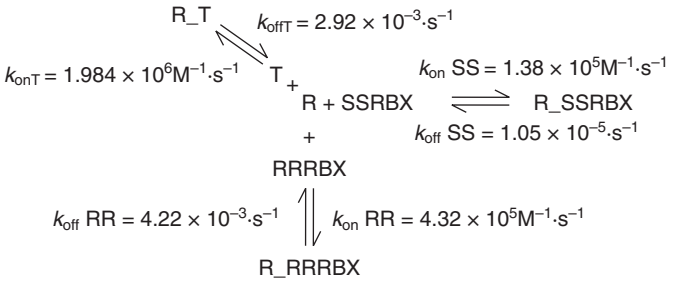


Figure 2 Binding isotherm data for [^{3}H]-SS-reboxetine (SS-RBX) binding to human noradrenaline transporter, showing shift in apparent K_d over a 24 h time course. Data are shown for bound ligand at 1.5 and 24 h. The solid lines represent the best fit curves to a four-parameter logistic equation. The dotted lines show the best fit curves to the non-equilibrium binding model (Equation 8). The data are from a single experiment repeated three times. The values of the fitted parameters are stated in the *Results* section.

1.5 and 24 h respectively ($n = 3$). The slope factor at 1.5 h was significantly greater than 1 ($P < 0.05$), as would be predicted if the binding of lower concentrations of [^{3}H]-SS-reboxetine were not in equilibrium at short incubation times. The binding data for [^{3}H]-SS-reboxetine were also analysed using Equation (8) described in *Methods*, which accounts for the effects of slow kinetics in a saturation experiment. The mean values of logK_d (–9.86 ± 0.08) and k_{off} (1.6 ± 0.3×10^5 s^{-1}) agree reasonably well with those values shown in Table 1. In this analysis the values of log$K_{SS\text{-}reboxetine}$ and k_{off} were quite strongly correlated with each other. If the value of k_{off} were constrained in the fitting procedure to the value found in the kinetic binding experiments (1.1 × 10^5 s^{-1}), the estimated value of log$K_{SS\text{-}reboxetine}$ was –9.99 ± 0.05 ($n = 3$) without the residual sum of squares being increased significantly. This latter value is in good agreement with the value estimated from the kinetic experiments shown in Figure 1.

The parameters obtained for racemic reboxetine (Table 1) have higher %CV values than those for the other ligands. This is not surprising given that data are fitted to a model assuming an interaction with a single non-radioactive ligand. The data clearly indicated a slow binding and potent component of the racemate (the SS-enantiomer) and the higher %CV reflects the fact that the faster binding and less potent RR-isomer also has an effect on the observed kinetics. These differences are manifested by the values of the $k_{off,obs}$ and $k_{on,obs}$ of the racemate being different from those of the SS- and RR-isomers and by the value of log$K_{d,obs}$ only being 50% higher than that of the SS-enantiomer.

The observed data for the racemate could be accurately simulated according to Scheme 2 in which two ligands, RR-reboxetine and SS-reboxetine are competing with [^{3}H]-DMI for binding to hNET and using the rate constants listed for these ligands in Table 1. This is illustrated in Figure 3.



Scheme 2 Proposed model for the binding of the racemate. Rate constant data from Table 1. R, hNET (human noradrenaline transporter); SS-reboxetine and RR-reboxetine, the RR- and SS-enantiomers of reboxetine, respectively; T, radioactive tracer ([^{3}H]-desmethylimipramine).

Discussion and conclusions

A key objective of this work was to define the transient binding kinetics of SS-, RR- and racemic reboxetine to hNET. To achieve this we have used a population mixed-effects modelling approach, on the basis that this minimizes the assumptions required. Typically analyses of kinetic data, such as those reported in this manuscript, are carried out in steps and by making assumptions about the inter-experiment and tracer parameter variability, for example fixing the latter at given values. In contrast, the mixed-effects method does not require this; first, simultaneous analysis of data from multiple experiments is enabled, which as discussed in the introduction has previously been found to be superior to sequential analysis. Second, the method allows the inclusion of inter-experiment variability. Defining each dataset as an individual experiment, we found that including inter-experimental variability on the B_{max} was a highly significant benefit. One possibility is that the variability in B_{max} occurs when experiments are repeated and that these can be accounted for using the exponential error model. Furthermore, comparing the sequential naïve pool fit with the mixed-effects fit to the same dataset suggested a highly significant increase in the quality of the fit using the mixed-effects method. Evaluating the parameters generated from the mixed-effects and naïve pool sequential fits showed that, in general, the estimates were within the typical variability for determining such parameters, although two of the parameter values determined via the sequential method were outside the 95% confidence intervals determined via the mixed-effects method. Together we interpret this as indicating that the mixed method is better, as it is simpler to implement and minimizes the assumptions required. Further investigation of the pros and cons of this approach to fitting transient kinetic data is warranted.

Apparent outlier points were observed at the highest concentrations of [^{3}H]-DMI in Experiment 1 (as expected) and for the data points around 24 h in Experiments 2 and 3. A likely explanation for this latter observation is degradation of the hNET in the assay. However, the use of the method described to estimate parameters gives confidence in relatively down-weighting the importance of these longer time point data. Using this approach we have shown that the enantiomers of reboxetine have very different transient and steady state kinetic properties. SS-reboxetine binds hNET with steady state

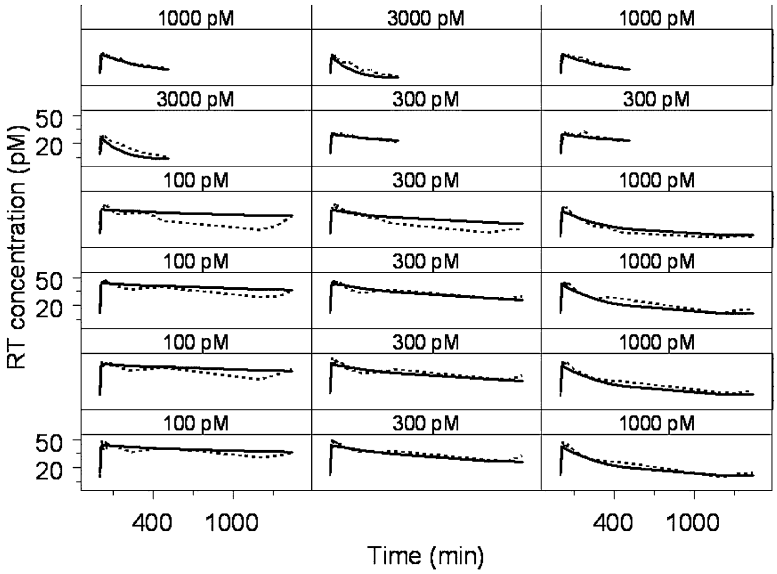


Figure 3 Observed (dashed) and simulated (solid line) behaviour of racemic reboxetine for 18 association curves using the SS- and RR-binding parameters from Table 1, assuming that the racemic reboxetine mixture contains 50% SS-reboxetine and RR-reboxetine, respectively, and according to Scheme 2. The tracer concentration varied between 1.5 and 1.7 nM between experiments. The concentration of cold ligand in each experiment is shown above each association curve.

K_d of approximately 80 pM, 130-fold more potently than RR-reboxetine. The analysis also provided an estimate of the K_T for the tracer [^{3}H]-DMI binding of *c*. 1.5 nM, compared with a reported K_i of 1.9 nM (Runkel *et al*., 2000). In principle, the steady state affinity of SS-reboxetine for hNET could also be derived from a standard binding isotherm experiment. However, the kinetic data indicated that the approach to equilibrium, at concentrations less than and around the K_d (80 pM), was likely to be slow, with equilibration taking more than 24 h. Wong *et al*. (2001) reported K_d values of 0.2 and 5.5 nM for SS- and RR-reboxetine, respectively, in rat cortical NET using a similar but not identical radioligand binding assay protocol. The lower apparent affinity and enantioselectivity of SS-reboxetine could be due to the relatively short duration incubation time of the binding assay (2 h).

To further test the prediction of non-equilibrium at short incubation times, we measured the binding of [^{3}H]-SS-reboxetine to hNET at 1.5 and 24 h. A considerable increase in potency was observed (of around sevenfold) over that time and that the magnitude of this increase correlated closely with that predicted from the kinetic binding data (Figure 2). As the estimated half time for dissociation of SS-reboxetine from Table 1 was *c*. 18 h it is not surprising that, even at 24 h, the [^{3}H]-SS-reboxetine saturation curve does not represent an equilibrium binding curve. For a simple binding curve, the rate constant describing the rate of approach to equilibrium, k_{obs}, at a given concentration of radioligand, [*T*], is given by the equation $k_{obs} = k_{-1}(1 + ([T]/K_T))$ where k_{-1} is the dissociation rate constant of *T* and K_T is its equilibrium dissociation binding constant. This equation predicts that, at concentrations lower than K_T, $k_{-1} \leq k_{obs}$ ($t_{1/2}$ 9–18 h). Even at 24 h, the binding of these concentrations of [^{3}H]-SS-reboxetine will not be in equilibrium whereas the higher concentrations will be. This results in the slope factors for the observed binding curve at both 1.5 h, and to a lesser extent at 24 h, being greater than 1. Notwithstanding this, the pre-equilibrium datasets can be fit to standard dose–response functions and yield apparently plausible estimates of K_d that over-predict the true K_d by *c*. 2–14-fold. This result highlights one of the benefits of measuring rate constants, relative to using inhibition or saturation binding curves. The latter makes an assumption of equilibrium that, in this case, would only be achieved at time points far greater than 24 h. In addition, in this particular case, defining the true affinity via an equilibrium experiment posed two technical challenges; first, reagent stability compromised the ability to obtain good data at time points greater than 24 h. Second, at the lower range of concentrations necessary to fully define the equilibrium dissociation constant, accurately measuring free drug concentration becomes technically more challenging. In contrast, we have found that the competitive pre-steady state experiment can be completed within an acceptable experimental time frame and yield accurate and precise estimates of both equilibrium and transient kinetic binding parameters, without the need for bespoke radiolabelled reagents. The accuracy of the estimates was confirmed by estimating equilibrium and the dissociation kinetic rate constants from the [^{3}H]-SS-reboxetine saturation experiment using Equation (8). We found that the parameters estimated were in good agreement with those predicted from the [^{3}H]-DMI competition experiments.

As discussed earlier, the range of rate constants for binding that could plausibly be observed spans several orders of magnitude. The kinetic competition method has previously been shown to allow characterization of rapidly equilibrating compounds, for example with association rates of *c*. 5×10^7 $M^{-1}{\cdot}s^{-1}$ and dissociation rates of 2×10^{-2} s^{-1} (Motulsky and Mahan, 1984) and the slow rates for SS-reboxetine reported here ~1×10^5 $M^{-1}{\cdot}s^{-1}$ and ~1×10^{-5} s^{-1} respectively, showing that the range of rate constants that can be determined spans two to three orders of magnitude and hence the method

would appear to be applicable to most of the commonly observed drug binding kinetics.

The analysis of the kinetic data for the racemate yielded an apparent K_d of 120 pM. Assuming the racemate is a stable equimolar mixture of RR- and SS-reboxetine and that the enantiomers bind mutually exclusively, then the racemate will not behave according to the assumptions depicted in Scheme 1. Hence, the parameter estimates or the racemate should be regarded as observed and not as molecularly defined properties. However, bearing this caveat in mind, the data fitted well to the model for a single compound interacting with the radioligand, [^{3}H]-DMI, although the estimated off rate was apparently somewhat slower ($6 \pm 2)\times10^{-6}$ s^{-1} ($t_{1/2}$ ~30 h) than that found for SS-reboxetine. The K_d value of 120 ± 30 pM was approximately 1.5-fold that found for SS-reboxetine alone (76 ± 9 nM), which would be expected as the racemate contains only 50% SS-reboxetine; occupancy curves at hNET can be calculated according to Equation (9) using the K_d SS-reboxetine and K_d RR-reboxetine estimates as per Table 1, 50% occupancy of hNET would be observed at ~150 pM, which is within the 95% confidence interval of the experimental estimate of 120 pM. Furthermore, the analysis of the data for RR-reboxetine and SS-reboxetine and DMI alone resulted in population estimates that were less than 10% different from those reported in Table 1 (not shown). Taken together we interpret this as evidence for the hypothesis that RR- and SS-reboxetine bind hNET at the same site, but that SS-reboxetine is the predominant influence on the steady state pharmacological properties of reboxetine. To further explore the validity of this conclusion, we simulated the expected pre-steady state behaviour of racemate, given the data for RR- and SS-reboxetine (Scheme 2). The simulations were highly consistent with the observed data (Figure 3).

The nature of the transient kinetics of binding may influence outcome *in vivo*, for example as has been found with tiotropium. In the case of SS-reboxetine the data were particularly interesting in that the relatively slow on and off rates for binding may have a consequence for *in vivo* behaviour. For example, it is possible that the slow off rate could deliver a sustained effect independent of the plasma concentration of drug. This may have benefits relating to dose level, therapeutic index and impact of a missed dose. This effect of the observed *in vitro* binding kinetics in the *in vivo* context can be explored quantitatively. A typical dose for reboxetine is 4 mg, and at this dose a C_{max} for SS-reboxetine of approximately 33 $ng{\cdot}mL^{-1}$ and a free fraction of 2% has been reported (Hendershot *et al*., 2001). Given a molecular weight of 313 g, it can be calculated that the free C_{max} is ~2 nM. Under the assumption that free plasma and brain equilibrate rapidly, this would represent a concentration of about $20 \times K_i$ for the hNET at the site of action. With the slow offset of binding and the $t_{1/2}$ of *c*. 12 h (Fleishaker, 2000) this would imply high receptor occupancy will be achieved upon multiple dosing of reboxetine. One caveat is the temperature dependence of the rate constants. The experiments described herein were carried out at 25°C, whereas the *in vivo* temperature will be around 37°C. In general, reaction rates double with every 10°C increase in temperature (Arrhenius, 1889) and so a potentially significant difference may be observed *in vivo* relative to *in vitro*. In principle, it should be possible to further explore this by repeating the analysis of the rate constants at 37°C. Notwithstanding these caveats, predicting the impact of binding rate parameters on an outcome such as receptor occupancy is a complex question with the plasma protein binding, the pharmacokinetics of the drug, the brain permeability and the binding parameters all potentially contributing to the pharmacodynamic effect (Peletier *et al*., 2009). Evaluation of this complexity could be facilitated via incorporation of the binding kinetic model and parameters into pharmacokinetic and pharmacodynamic models (see Ploeger *et al*., 2009). In addition, such models could be applied to the optimal design of clinical investigations, for example PET studies (Zeng *et al*., 2008). Clearly although, given the differences in binding kinetics, pharmacokinetics and potentially plasma protein binding of SS- and RR-reboxetine, understanding the pharmacokinetic and pharmacodynamic behaviour of the racemate will be more challenging than that of the SS- or RR-enantiomers separately.

The simultaneous mixed-effects approach has the added benefit that it allows stochastic simulations to be carried out to explore optimal experimental design; using the parameter estimates in Table 1, our simulations indicated that the number of data points acquired could have been significantly decreased without compromising the accuracy and precision of the parameter estimates. Specifically, Experiment 2 could have been omitted and, by optimizing sample times in experiment three, the numbers of data points decreased by *c*. one-third (Benson *et al*., 2007). This approach can also be applied to other candidate drugs, although further data will be required before generic statements on optimal design can be made. Nevertheless, the use of a tool incorporating mixed-effects stochastic and structural elements should facilitate design on a case by case basis.

In summary, we have demonstrated a mixed-effects method can be used to robustly estimate parameters of binding of SS-, RR- and racemic reboxetine. We have shown that the enantiomers have very different kinetic and equilibrium binding properties. These parameters could be used to develop a better the understanding of the *in vivo* properties of these drugs. The mixed-effects method could be applied to other compounds with similar mechanism but is also flexible and could be adapted for other more complex *in vitro* transient binding kinetics and therefore will be of great utility in the further integration of target binding kinetic parameters in drug discovery programs.

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Conflicts of interest

N Benson, R Butt, C Napier and PH van der Graaf are employees of Pfizer.

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