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RESEARCH PAPER

Methodological Comparison of *In Vitro* Binding Parameter Estimation: Sequential *vs.* Simultaneous Non-linear Regression

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ABSTRACT

Purpose Analysis of simulated data was compared using sequential (NLR) and simultaneous non-linear regression (SNLR) to evaluate precision and accuracy of ligand binding parameter estimation.

Methods Commonly encountered experimental error, specifically residual error of binding measurements (RE), experiment-to-experiment variability (BEV) and non-specific binding (B_{NS}), were examined for impact of parameter estimation using both methods. Data from equilibrium, dissociation, association and non-specific binding experiments were fit simultaneously (SNLR) using NONMEM VI compared to the common practice of analyzing data from each experiment separately and assigning these as exact values (NLR) for estimation of the subsequent parameters.

Results The greatest contributing factor to bias and variability in parameter estimation was RE of the measured concentrations of ligand bound; however, SNLR provided more accurate and less bias estimates. Subtraction of B_{NS} from total ligand binding data provided poor estimation of specific ligand binding parameters using both NLR and SNLR. Additional methods examined demonstrated that the use of SNLR provided better estimation of specific binding parameters, whereas there was considerable bias using NLR. NLR cannot account for BEV, whereas SNLR can provide approximate estimates of BEV.

Conclusion SNLR provided superior resolution of parameter estimation in both precision and accuracy compared to NLR.

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KEY WORDS BEV, between-experiment variability · B_{NS}, non-specific binding · NLR, sequential non-linear regression · SNLR, simultaneous non-linear regression · α, proportional constant relating non-specific binding to ligand concentration

INTRODUCTION

Estimation of ligand binding parameters to receptors *in vitro* constitutes a basic operation in many areas of scientific research. Estimation of these parameters provides crucial information to understanding agonist and antagonist selectivity for binding sites. Therefore, accurate determination of these parameters is important for evaluation of newly synthesized ligand potency, interactions with other molecules, and prediction of *in vivo* performance. Numerous investigations are devoted to the determination of ligand binding parameters. Most prevalent are equilibrium experiments where the primary aim is to provide information on the progressive saturation of receptors by the ligand of interest. These experiments predominantly are performed to provide estimates of maximum binding capacity and the affinity of the ligand to the binding site (1). Additionally, association and dissociation time-course experiments are used to optimize conditions for subsequent use in equilibrium experiments and demonstrate the reversibility of ligand-receptor interaction. Association time-course experiments allow the determination of rate of binding of ligand to receptor and the time at which equilibrium has been achieved. Data from dissociation time-courses are also used to calculate the dissociation rate constant(s) and to test whether the data from the dissociation kinetic experiment suggest heterogeneity of binding sites (2). These experiments are usually considered secondary but can sometimes provide superior resolving power, especially for multiple

binding sites, a situation difficult to accurately resolve with equilibrium experiments (3).

However, information obtained is not useful if the data are unduly influenced by procedural errors. Misleading results could occur due to experimental artifacts, such as radioactivity background, improper subtraction of non-specific binding, ligand heterogeneity, and errors occurring during separation of bound and free radioligand (4). These problems could result in misinterpretation of the equilibrium dissociation constant, number of binding sites, specific binding site concentration, and association or dissociation rate constants. Many studies have evaluated the data provided by these various experiments independently and estimated parameters separately either through linear regression on transformed data or non-linear regression (NLR) (5–8). These approaches require that parameter estimates from one experiment are indefectible when introduced for parameter estimation of subsequent experiments (a sequential approach). Thus, error in the determination of one parameter could be propagated unduly influencing subsequent parameter estimation.

An analysis of data from the combination of equilibrium and kinetic experiments performed simultaneously could overcome the limits and shortcomings of each specific technique. Previous published work has demonstrated that the use of a simultaneous analysis of data has had superior resolving ability to estimate parameters compared to NLR (9). The focus of this previous work was concerned with detailing the resolution of two binding sites, relative binding densities, receptor occupancy and number of data points. The focus of this study is to compare simultaneous non-linear regression (SNLR) to NLR in terms of bias and precision with factors commonly encountered during experimentation, namely, measurement error, between experiment variability and non-specific binding. Examination of how these factors influence parameter estimates based on both methods as well as techniques to resolve these issues will be explored by applying Monte Carlo simulation techniques.

MATERIALS AND METHODS

In this study, the performance of methods for modeling ligand binding data was explored using Monte Carlo simulation. Data simulation and analysis were performed using NONMEM version VI (ICON, Ellicott City, MD). Models were estimated using the first-order conditional estimation method (FOCE) with interaction (INTER) when needed (10).

Simulation of Ligand Binding Data

Ligand binding data were simulated for equilibrium, dissociation, association and non-specific binding experiments to compare the estimation of parameters with sequential non-linear regression *versus* simultaneous non-linear regression under various different experimental conditions. Data were simulated using Eqs. 1–4 (9):

Equilibrium:

$$B_{eq} = \sum_{r=1}^{n} \frac{B_{\max,r}{}^{*}L}{\frac{k_{-r}}{k_r} + L} + \alpha^{*}L \tag{1}$$

Dissociation:

$$B_{dis}(t) = \sum_{r=1}^{n} \frac{B_{\max,r}{}^{*}L}{\frac{k_{-r}}{k_r} + L} * \left[1 - e^{-(L^{*}k_r + k_{-r})^{*}t}\right] * e^{-k_{-r}(t-\tau)} + \alpha^{*}L \tag{2}$$

Association:

$$B_{ass}(t) = \sum_{r=1}^{n} \frac{B_{\max,r}{}^{*}L}{\frac{k_{-r}}{k_r} + L} * \left[1 - e^{-(L^{*}k_r + k_{-r})^{*}t}\right] + \alpha^{*}L \tag{3}$$

Non-Specific Binding:

$$B_{NS} = \alpha^{*}L \tag{4}$$

where r represents the binding site number, n represents the total number of binding sites, $B_{max,r}$ represents the total binding site concentration for receptor *r*, k_{-r} is the rate constant of dissociation for receptor *r*, k_r is the rate constant for association for receptor *r*, *L* is ligand concentration, τ represents the time at which association is stopped and dissociation is initiated, and α is the proportional constant relating non-specific binding to ligand concentration. It is assumed that $L >> B_{eq}$ for pseudo-first-order approximation, and equilibrium was attained before initiation of dissociation.

For each experimental condition (see below), 1,000 datasets were generated by simultaneously simulating data using Eqs. 1–4 assuming one binding site ($n=1$). Sampling, ligand concentrations and binding parameter values were based loosely on those previously reported (5). Each dataset consisted of duplicate samples for the equilibrium experiment and 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 min in the association phase at ligand concentrations of 20, 100, 200, 300, 400, 500 pM and duplicate samples at 90, 95, 100, 110, 120, 130, 140, 15, 160, 170, 180 min at a ligand concentrations of 200 pM in the dissociation phase. Non-specific binding was time-matched for samples simulated previously, and percent bound was based on highest ligand concentration at equilibrium. The binding parameters were as follows: $B_{max,1} = 3.7$ pM, $k_{-1} = 0.026$ min^{-1}, $k_1 = 0.000085$ min^{-1}pM^{-1}, and elimination half-life was equal to 26.7 min.

Experimental Conditions

Different experimental conditions were examined with the introduction of proportional residual error (RE), between-

experiment variability (BEV) using similar techniques as described previously as inter-occasion variability (11), and non-specific binding (B_{NS}) of ligand to sites other than receptor of interest. The specifics for each simulation/estimation for the experimental conditions described below are detailed in Table 1.

Residual Error (RE)

In all experimental conditions, the ligand binding data was simulated and analyzed with a proportional RE model (Eq. 5):

$$Y_{ij} = B_j^*\left(1 + \varepsilon_{ij}\right) \tag{5}$$

where Y_{ij} is the i^{th} sample of the j^{th} binding model, B_j is the j^{th} model predicted binding response (i.e. B_{eq}, B_{diss}, B_{ass}, B_{NS}), and ε_{ij} is the corresponding residual random variability. ε_{ij} is assumed to be normally distributed with zero mean and a variance of σ^2.

Between-Experiment Variability (BEV)

In some experimental conditions, the ligand binding data was simulated with BEV (Eq. 6) where the m^{th} parameter for experiment k, P_{mk} is defined as

$$P_{mk} = f(P_m, \kappa_{mk}) \tag{6}$$

where P_m is a typical value of parameter m in the population and κ_{mk} is assumed to be an independently and normally distributed parameter with mean zero and variance π_m^2. The κ_{mk} represents between experiment differences (11).

In experiments where BEV was included, the SNLR estimation method was analyzed both with and without this model component to investigate the effect of ignoring BEV in estimation. In the sequential NLR technique BEV cannot be estimated and was thus ignored in the estimation step.

Non-specific Binding (B_{NS})

In some experimental conditions, non-specific binding (B_{NS}) was assumed to be present in the experiments. For these conditions, the data was analyzed using two different methodologies. Method one involved subtraction of B_{NS} from total binding measurements (B_{eq}, B_{diss}, B_{ass}) at each sample measurement and subsequent parameter estimation, and method two involved estimation of the additional parameter α seen in Eqs. 1–4. For the second method, α was estimated both with and without inclusion of the simulated non-specific binding measurements (i.e. including Eq. 4 in the analysis or not) for SNLR. The non-specific binding estimates used for simulations based on highest ligand concentration at equilibrium were 0.00025, 0.00052, 0.00081, 0.00115 and 0.0015 for 5, 10, 15, 20 and 25% B_{NS}, respectively.

Sequential Non-linear Regression (NLR) and Simultaneous Non-linear Regression (SNLR)

NLR

In this work, parameter estimation based on sequential non-linear regression, the simulated non-specific binding data was analyzed first (if present) using the model in Eq. 4. The α parameter was then introduced as a constant for all subsequent analyses. Next the equilibrium data was analyzed using Eq. 7:

$$B_{eq} = \sum_{r=1}^{n} \frac{B_{\max,r}^* L}{K_{d,r} + L} + \alpha^* L \tag{7}$$

where $K_{d,i}$ represents the apparent dissociation constant (k_{-r}/k_r – 306 pM) and estimating both $B_{max,1}$ and $K_{d,1}$. $B_{max,1}$ and α were then introduced as constants for all subsequent

Table I Details of Experimental Conditions

Experimental condition	Simulation settings			Estimation Eqs. used		Comment
	RE	BEV	B_{NS}	NLR	SNLR	
1	0–25%	–	–	7,2,3,5	1,2,3,5	α set to zero
2.a	0–25%	0–25%	–	7,2,3,5	1,2,3,5,6	BEV on RE; α set to zero; SNLR estimated without BEV on RE
2.b	0–25%	0–25%	–	7,2,3,5	1,2,3,5	BEV on RE; α set to zero; SNLR estimated including BEV on RE
3.a	0–25%	0–25%	–	7,2,3,5	1,2,3,5,6	BEV on $B_{max,1}$; α set to zero; SNLR estimated without BEV on $B_{max,1}$
3.b	0–25%	0–25%	–	7,2,3,5	1,2,3,5	BEV on $B_{max,1}$; α set to zero; SNLR estimated including BEV on $B_{max,1}$
4.a	0–25%	–	0–25%	7,2,3,5	1,2,3,5	Subtraction of B_{NS} from total binding; α set to zero
4.b	0–25%	–	0–25%	7,2,3,5	1,2,3,5	Estimation of α without inclusion of B_{NS} data
4.c	0–25%	–	0–25%	4,7,2,3,5	1,2,3,4,5	Estimation of α with inclusion of B_{NS} data

analyses. Next the dissociation data was analyzed using Eq. 2, and both k_1 and k_{-1} were estimated. Subsequently, k_{-1}, $B_{max,1}$ and α estimates were then used as constants for estimation of k_1 using the association data and Eq. 3. One RE parameter was estimated per step.

SNLR

Simulated data from equilibrium, dissociation and association experiments were fitted simultaneously to Eqs. 1–3 to estimate binding parameters. In addition, Eq. 4 was introduced to the model to estimate B_{NS} when indicated. One RE parameter was estimated per model. When indicated, BEV was also assessed.

Bias and Imprecision in Parameter Estimates

Bias (%) and imprecision in parameter estimates using the root mean squared error (RMSE,%) were estimated (Eqs. 8 and 9):

$$\%Bias = \frac{100\%}{\theta_T * N} \sum_{s=1}^{N} (\theta_s - \theta_T) \tag{8}$$

$$\%RMSE = \frac{100\%}{\theta_T} \sqrt{\frac{1}{N} \sum_{s=1}^{N} (\theta_s - \theta_T)^2} \tag{9}$$

where s is the sth simulated data set (s = 1, 2, . . ., N), θ_s is the typical value of parameter estimates from the sth data set, and θ_T is the true parameter value used in simulation.

Pharmacostatistical Analysis

NONMEM minimizes an objective function value (OFV), which is a global measure of the goodness of fit. The likelihood ratio test was used to test the effect of inclusion of BEV. In this test, the difference between OFV for a model containing n parameters and those containing n–1 parameters was assumed to approximate the Chi-square distribution with one degree of freedom. An *a priori* level of significance of $p<0.05$ was chosen which corresponds to a decrease in OFV of >3.841 when a single parameter is introduced. Beyond the decrease in OFV, evaluation of error of the estimate and impact on other parameter estimates was evaluated for inclusion into model (12,13).

RESULTS

Based on the ligand binding parameters used for simulations for the equilibrium, association and dissociation experiments, equilibrium was attained approximately 90 min after initiation of the association phase for 200 pM ligand concentration. Thus, 90 min was selected as "τ" at which dissociation was initiated and approximately 98% equilibrium was achieved.

In experimental setup 1, the effect of unexplained random variability, i.e. RE, in measurement of ligand binding data on parameter estimates using the NLR *versus* SNLR methods was examined. Across the span of RE used in these simulations, both methods typically provided unbiased model-based parameter estimates (<1%) that corresponded to the true estimates. However, as RE increased, there was slight increase in bias for the $B_{max,1}$ estimate up to 8% when the NLR was used. Overall, both methods provided precise estimates, but the accuracy was greater with the SNLR. The precision of parameter estimates using the SNLR was higher than those resulting from the use of NLR (Fig. 1). For example, at 25% RE, the RMSE was 30% for $B_{max,1}$ using the NLR and 8% for the SNLR. This relative difference in RMSE was observed across all three parameters and span of RE.

Parameter estimation can be significantly affected by methodological procedure or technique disparity between experiments. The effect of this BEV was examined in two different manners (experimental setup 2 and 3). Experimental setup 2.a included variability on the RE owing to unspecified differences in ligand binding measurement among experiments, while this variability was ignored in the estimation step. Parameter estimates obtained using both the NLR and SNLR provided reasonable estimates for the dissociation rate constant, k_{-1}, with a bias <1% and an RMSE of <8%. However, both methods demonstrated bias estimates for $B_{max,1}$. Using the NLR, bias increased from 0 to 8% as the RE increased but remained relatively constant as BEV increased. There was a 6–7% bias in the estimate of $B_{max,1}$ using the SNLR regardless of the extent of BEV and RE. Only the NLR showed bias in the association rate constant (10–11%) across the range of BEV. The variability of parameter estimates, RMSE, between experimental conditions was relatively unaffected. The RMSE estimates are similar to those obtained when only RE was included in the simulations. Thus, the parameter estimates using the SNLR were more precise than those using the NLR.

In experimental setup 2.b, attempts to account for the bias in the estimate of $B_{max,1}$ when using the SNLR were examined by implementing BEV on $B_{max,1}$. However, the parameter could not be estimated across the range of BEV and RE included in the simulations, and no further resolution of bias was attained.

In experimental setup 3.a, BEV was ascribed to the intrinsic quantity of receptors contained per experiment, $B_{max,1}$, but no BEV was assumed in the estimation step. Both NLR and SNLR demonstrate reasonably comparable increases in bias as both residual variability and BEV

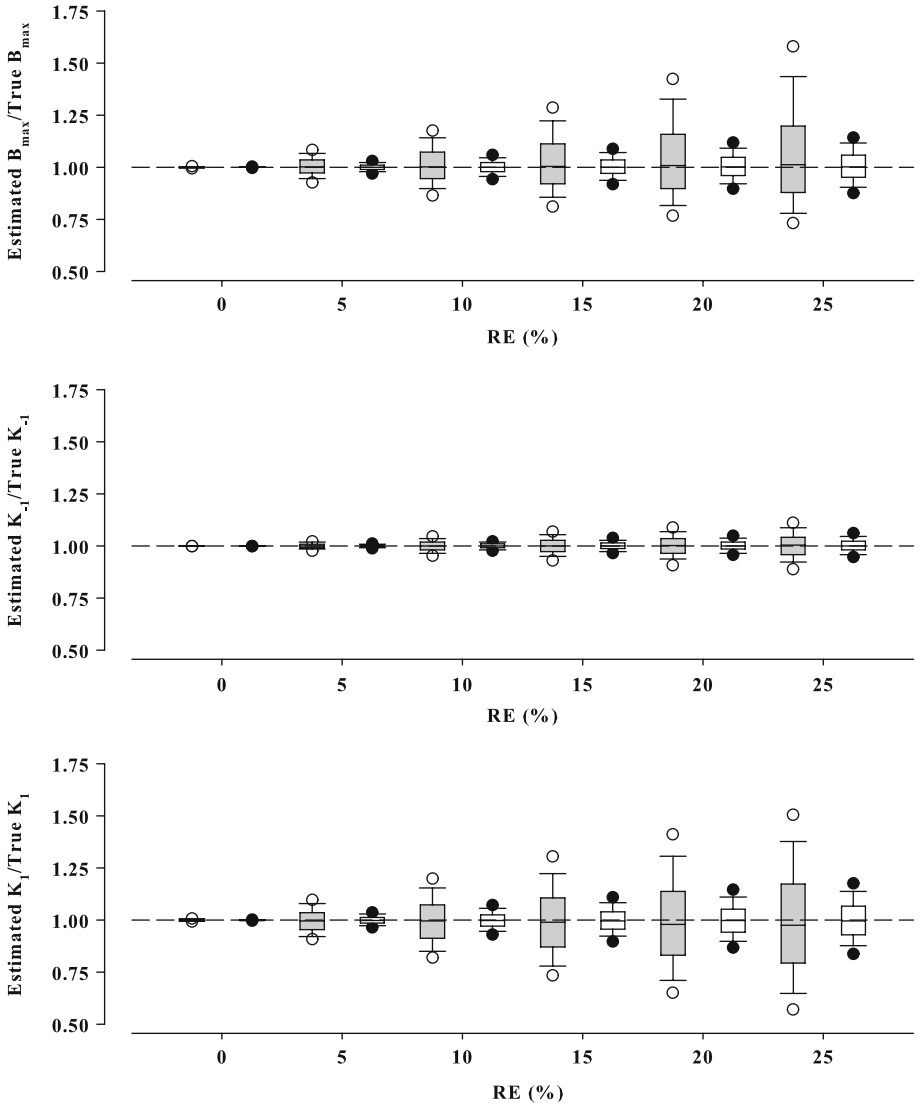

Fig. 1 Distribution of $B_{max,1}$, K_{-1} and K_1 estimates using the NLR (gray) and SLNR (white). Box plots graph data as a box representing statistical values describing the 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles.

increased for estimates of $B_{max,1}$ up to 10% (Fig. 2). However, the RMSE was less with SNLR compared to NLR and relatively consistent across increase in residual variability (Fig. 3). Estimation of k_{-1} was reasonably unbiased (<2%) using both NLR and SNLR, albeit higher with SNLR, and the RMSE was substantially higher with SNLR but consistent with changes in RE variability. The RMSE was consistent across increase in BEV for estimation of k_{-1} using NLR. Less than 1% bias was observed for estimation of k_1 using SNLR and RMSE less than 40%. However, there was an increase in bias up to 10% for k_1 estimation using NLR and higher RMSE up to 60% using NLR.

In experimental setup 3.b, estimation of BEV on $B_{max,1}$ was attempted with the SNLR method. The inclusion of BEV on $B_{max,1}$ provided substantially decreased bias estimates of $B_{max,1}$ and k_{-1} and resulted in significantly reduced RMSE for all three parameters (Figs. 2 and 3). When BEV was included on $B_{max,1}$, there was greater than 88% power to detect a significant change in OFV compared to the basic model without BEV for all but six of the experimental conditions explored (Table 2). However, when residual variability greatly exceeded the BEV, there was less power to detect BEV as a significant model parameter, and the precision of these BEV estimates was poor. Nevertheless, parameter estimates with rejection of inclusion of BEV on $B_{max,1}$ for these experimental conditions was not greatly biased or variable. The bias of BEV estimates is listed in Table 2. Generally, the BEV was estimated reasonably well, particularly when the BEV was greater or near the RE values. However, when RE greatly exceeded the BEV, the estimates approached the maximum of −34% bias. The same relationship was observed for RMSE and bias.

Ligand binding measurements usually are comprised of both specific and non-specific binding. Typically, the

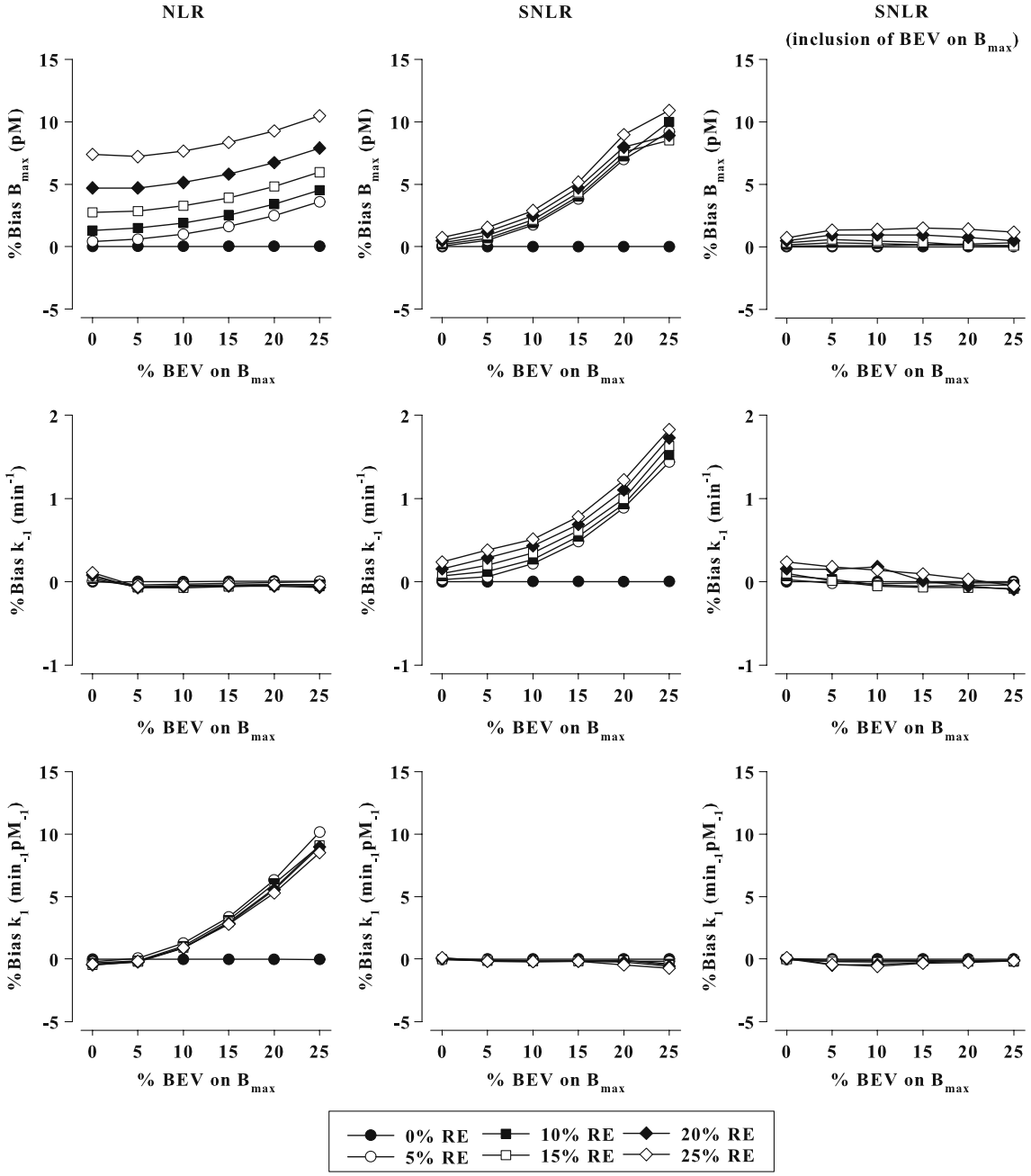

Fig. 2 Percent bias of $B_{max,1}$, K_{-1} and K_1 estimates using the NLR and SLNR with BEV on $B_{max,1}$.

amount of non-specific binding is subtracted from total binding on a time-matched basis for parameter estimation related to specific binding (experimental condition 4.a). This method was examined to assess the impact of non-specific binding on specific binding parameter estimation. The results demonstrate that this subtraction method of quantifying the specific binding parameters leads to significantly biased estimates of all parameters (Fig. 4) and increased the overall distribution of expected values for both NLR and SNLR (data not presented). For example, the maximum bias using the NLR method increased from 8% without B_{NS} to 17% with subtraction of B_{NS} for $B_{max,1}$ and less than 1% to 8% using SNLR. However, the SNLR still performed with better fidelity than the NLR, as the bias and RMSE were much smaller, although this method of estimation does not appear an appropriate method for specific ligand binding parameter estimation.

Another alternative approach for handling non-specific binding is to include an extra term, α, proportional to ligand concentration to the fitted kinetic and equilibrium equations that relate the non-specific binding to total binding (experimental condition 4.b). The NLR estimate

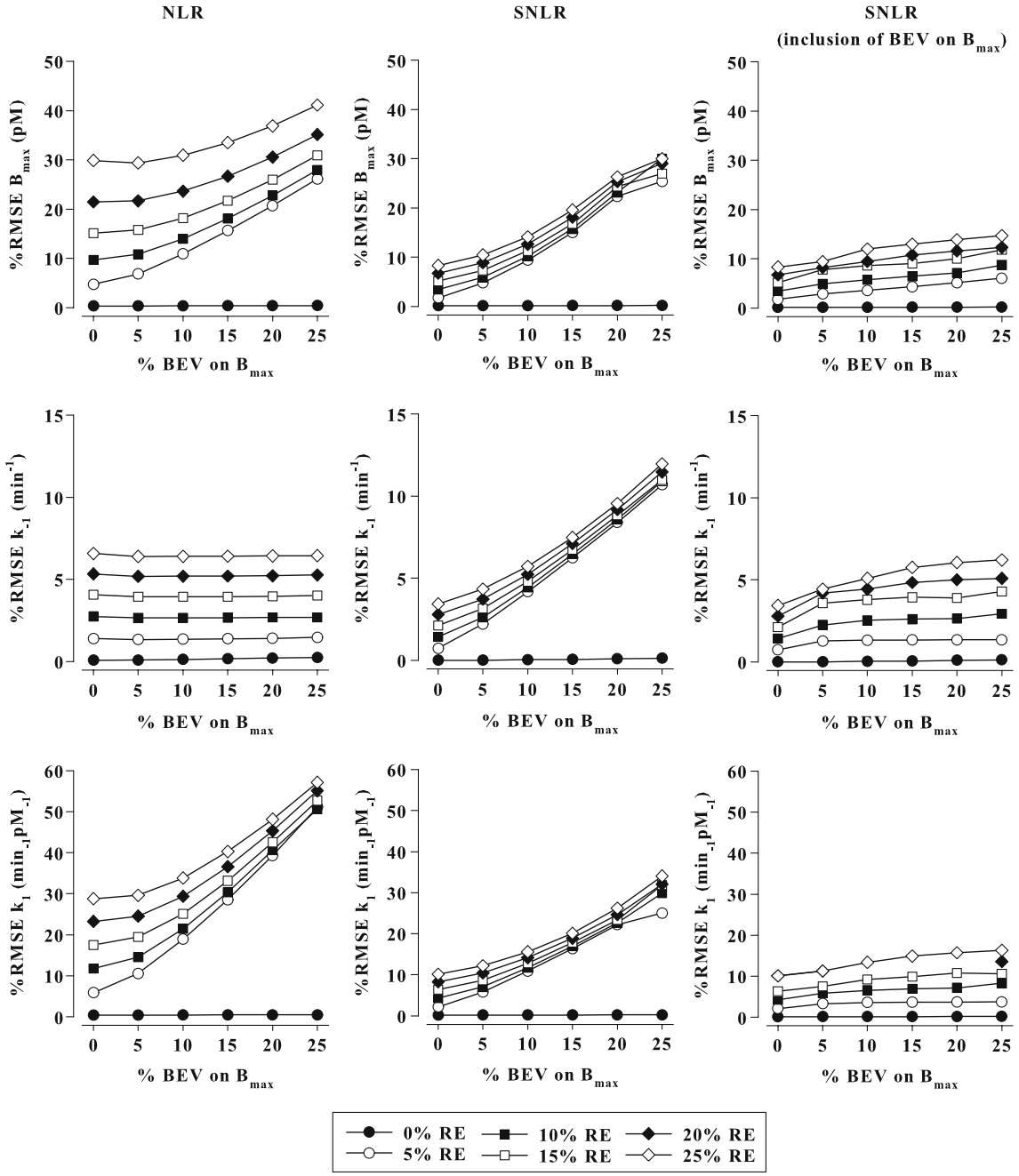

Fig. 3 Percent RMSE of $B_{max,1}$, K_{-1} and K_1 estimates using the NLR and SLNR with BEV on $B_{max,1}$.

Table 2 Percentage of Runs with BEV Included on $B_{max,1}$ (experimental condition 2.b) with Significant Change in OFV Relative to No Inclusion of BEV (experimental condition 2.a)

RE/BEV	Percent of Runs (% Bias of BEV Estimate)				
	5%	10%	15%	20%	25%
5%	99.9 (−7.31)	100 (−4.25)	100 (−3.87)	100 (−3.71)	100 (−3.61)
10%	77.0 (−16.1)	99.9 (−7.31)	100 (−4.95)	100 (−4.13)	100 (−3.81)
15%	35.6 (−25.4)	95.5 (−12.5)	100 (−7.52)	100 (−5.83)	100 (−4.77)
20%	15.0 (−31.0)	79.2 (−17.8)	97.3 (−12.0)	99.9 (−8.79)	100 (−6.27)
25%	8.00 (−34.1)	58.0 (−22.3)	91.7 (−15.0)	98.4 (−11.9)	99.8 (−8.98)

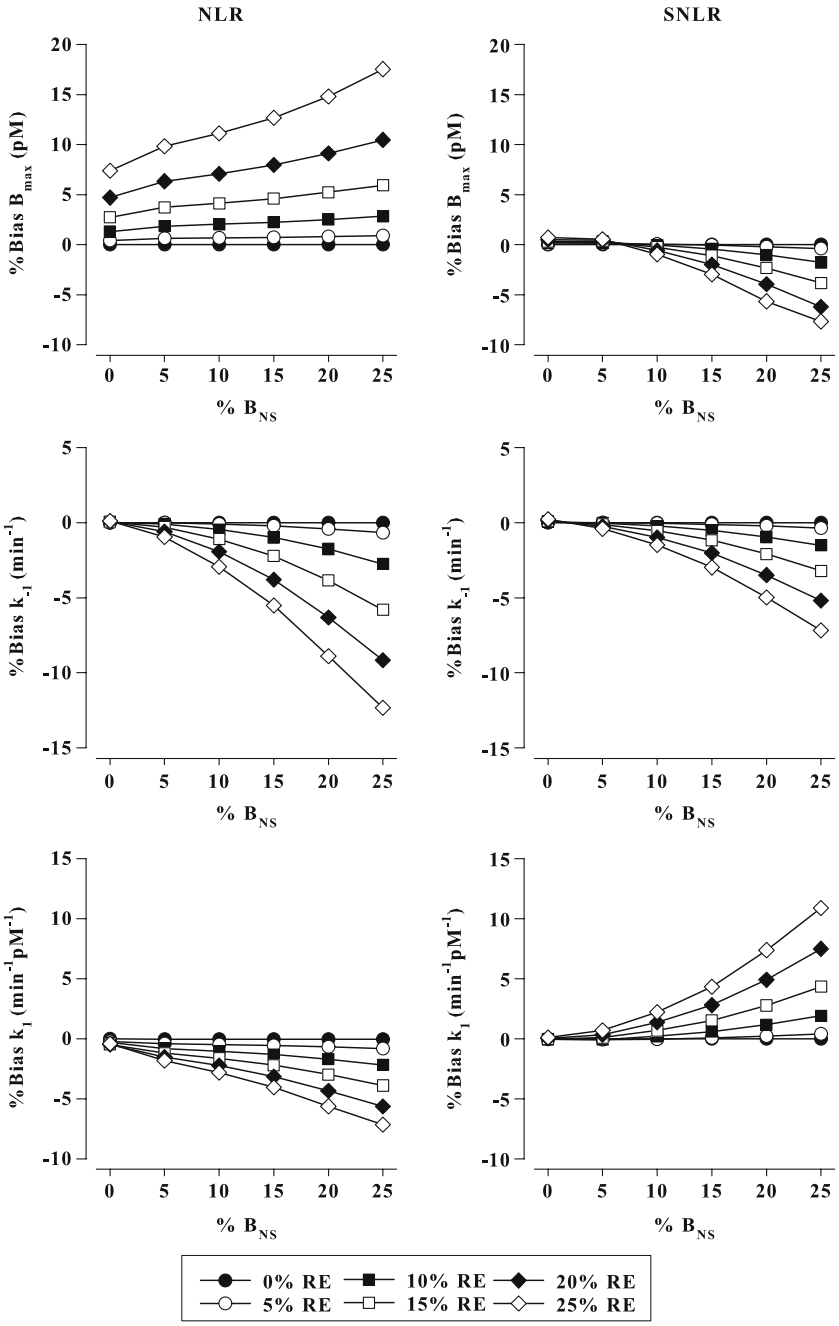

Fig. 4 Percent bias of $B_{max,1}$, K_{-1} and K_1 estimates using the NLR and SLNR with subtraction of B_{NS}.

of the proportional constant α relating B_{NS} to total binding were several fold higher than used during the simulations (data not shown). As a consequence of poor α estimation, $B_{max,1}$, k_{-1} and k_1 were significantly biased and imprecise by; several orders of magnitude.

An additional attempt to resolve the estimation of these parameters by NLR was examined by estimating α for each specific experiment and using those estimates in subsequent analyses to estimate specific binding parameters (experimental condition 4.c). Regardless of the degree of B_{NS}, the estimates of α were relatively consistent within each level of residual variability. However, there was a slight increase in bias and RMSE as residual variability increased—greatest for the equilibrium experiment. Overall, the estimates of α showed less than 1% bias, and the RMSE was less than 8%. Significant improvement of the estimation of the ligand binding parameters was observed compared to the previously mentioned method. The bias for k_{-1} and k_1 ranged from −2 to 1%, and the RMSE was less than 40%. However, the bias for the estimation of $B_{max,1}$ was up to 15%, and the RMSE was 50%. As increased RE would affect both the specific and B_{NS}, there was a slight increase in bias and RMSE using NLR as RE and B_{NS} increased (Figs. 5 and 6).

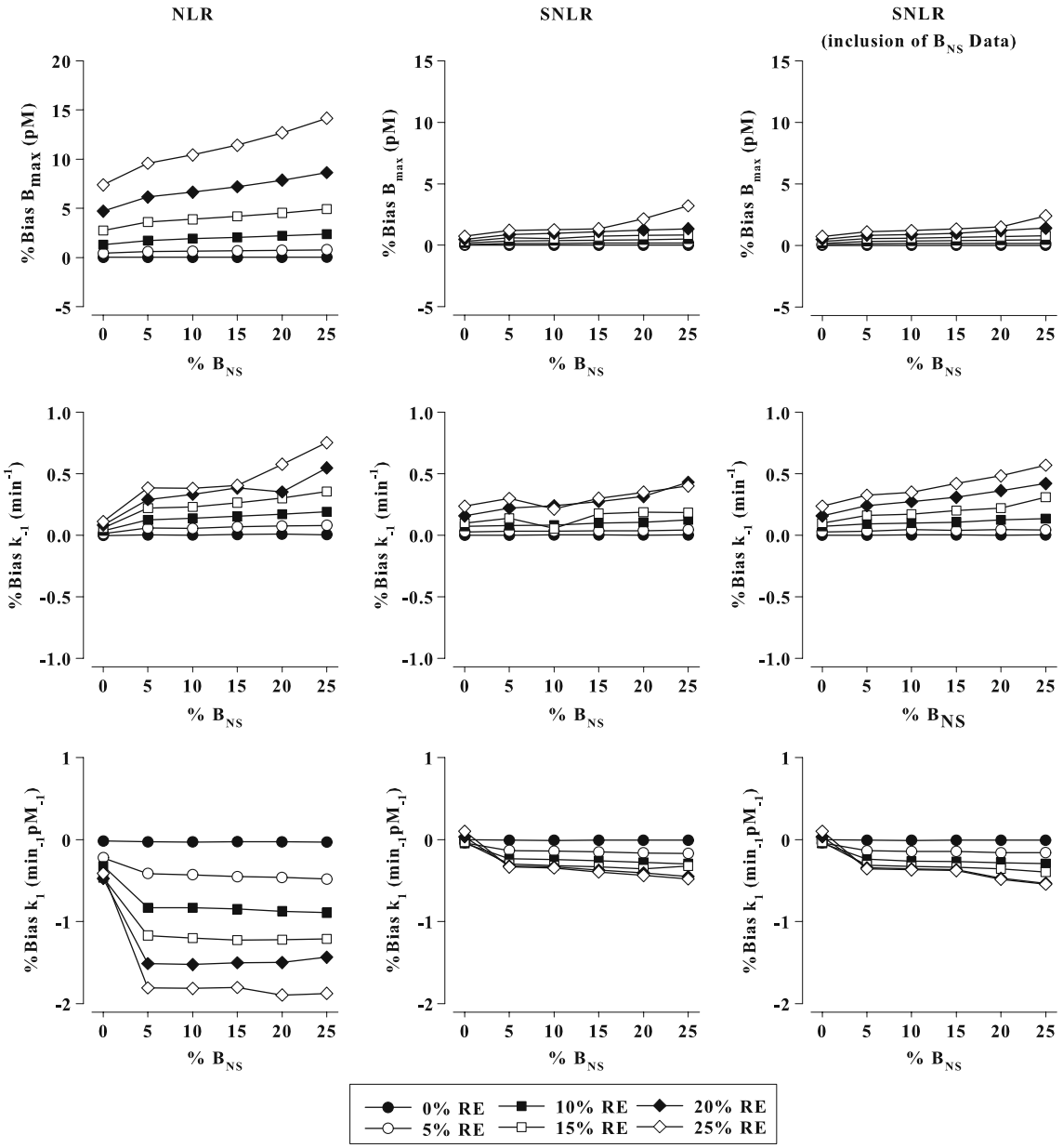

Fig. 5 Percent bias of $B_{max,1}$, K_{-1} and K_1 estimates using the NLR and SLNR with addition of α of B_{NS}.

In contrast, estimation of the specific ligand binding parameters utilizing SNLR were less than 5% biased, and RMSE did not exceed 20% with inclusion of the proportional constant α relating B_{NS} to total binding when simultaneously analyzed (experimental condition 4.b). The estimates of α were relatively consistent within each level of residual variability. The estimates of α showed less than 1% bias, and the RMSE was less than 8% when RE and B_{NS} were less than 15% and 20%, respectively. Above 15% RE, bias increased to 5% and RMSE 28%. As RE and B_{NS} increased, the bias and RMSE using SNLR remained relatively consistent to estimates obtained without B_{NS}, except when residual variability was above 15%, there was a slight increase (Figs. 5 and 6).

Whether the addition of the data from the non-specific binding to the dataset and including an additional equation to the model would provide any additional benefit of parameter estimates using SNLR (experimental condition 4.c) was also examined. The results demonstrated small improvement of the bias and RMSE for parameter estimates but were approximately equivalent to not including the data (Figs. 5 and 6). However, the estimation of α was significantly improved where the bias was less than 0.05% and RMSE was less than 2%. Overall, estimation of

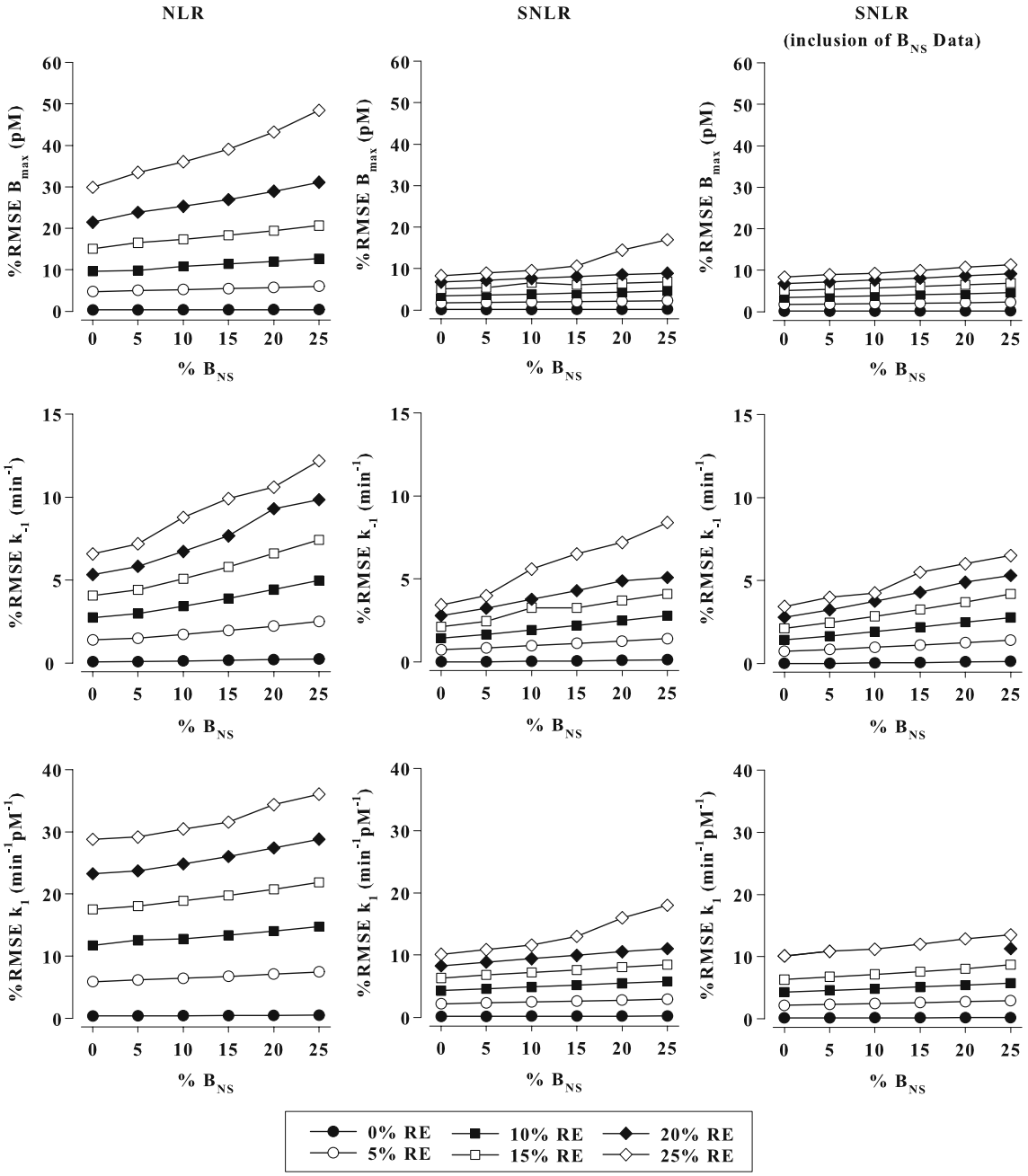

Fig. 6 Percent RMSE of $B_{max,1}$, K_{-1} and K_1 estimates using the NLR and SLNR with addition of α of B_{NS}.

the specific ligand binding parameter when B_{NS} is present was significantly improved using SNLR with less bias and RMSE compared to NLR whether subtracting the B_{NS} or inclusion of an additional term.

DISCUSSION

The methods described in this article provide insight into the advantages and disadvantages of using sequential non-linear least squares curve fitting and simultaneous non-linear curve fitting in ligand binding parameter estimation. The use of sequential non-linear least squares curve fitting provides an advantage of the use of transformed ligand binding data used in linear regression for parameter estimation by computing variance of measured binding; however, interdependence of parameter estimation is not resolved via this method. Estimation of ligand binding parameter by simultaneously fitting all the data from these experiments can reveal this interdependence by the correlation matrix and can further identify variability across experiments. Additionally, SNLR provides a single residual

variability estimate that allows for statistical discrimination between nested models. Thus, these values describe the distribution of the unexplained random variability between the estimated parameters and noise in the measurements.

A variety of experimental conditions was applied to both NLR and SNLR. Overall, SNLR provided superior resolution of parameter estimates in terms of both precision and accuracy across all these conditions. The greatest contributing factor to bias and variability in parameter estimation was attributed to residual variability of the measured concentrations of total ligand bound. The use of NLR did not allow for the measurement error across experiments to be used in determination of these parameters and considered each parameter indefectible for the subsequent experiment. However, SNLR utilized data from all experiments and did not necessitate treating observations or estimated parameters as error free. This factor was observed more prevalently at higher levels of residual variability, but even at lower levels there was still better estimation using SNLR, especially for $B_{max,1}$ and k_1. The fitting sequence using NLR did not have an undue effect on parameter estimation (i.e. association experiment preceded equilibrium experiment or vice-versa) or increase in precision or variability of $B_{max,1}$ and k_1. Effectively, when dissociation data was fit without inclusion of a fixed value of $B_{max,1}$, the equation reduced to that in Eq. 10 and provided k_{-1} parameter estimated less than 0.1% different than when $B_{max,1}$ was assumed known.

$$B_{dis}(t) = \sum_{r=1}^{n} B_o * e^{-k_{-r}(t-\tau)} + \alpha * L \tag{10}$$

Using this value as known and not including $B_{max,1}$ provided k_1 estimates that were no more biased than initially evaluated, and subsequently the same was observed for the estimation of $B_{max,1}$. In this regard, NLR provided more bias and variable estimates of these parameters compared to SNLR. Although a constant coefficient of error proportional to the concentration of bound ligand was used for simulations and estimation of binding parameter values, additional RE models could be applied and expected to be better resolved using SNLR.

Another significant contributing factor to bias and variability in parameter estimation was non-specific binding and the method used to estimate the impact of this on specific binding parameter estimation. Overall, subtraction of B_{NS} from total ligand binding data provided poor estimation of specific ligand binding parameters using both NLR and SNLR. This method does not allow for the residual variability associated with measurement of B_{NS} to be separated from that of specific binding and effectively disseminates an increase in the variability of assumed specific binding measurements. However, this method is prevalently used in many ligand binding studies as a method to estimate specific binding parameters (5–8,14). In light of this shortcoming of computing B_{NS}, additional methods were examined and demonstrated for NLR; a better approach was to estimate α for each experiment. However, this method, while providing better parameter estimates, did not utilize the error associated to the measurement of B_{NS}. The use of SNLR provided better estimation of specific binding parameters regardless of the use of B_{NS} data; however, the estimates were significantly better when this data was included in the modeling. Although the experimental design was not optimal, including an additional ligand concentration (1,200 pM) to the experiment well above K_d still did not provide much improvement in the bias estimate of B_{max} using NLR (~140%) with RE and α at 25%. Thus, this information provides further evidence that this method of analysis has significant limitations. Additionally, a major assumption for B_{NS} was that it was constant over time and proportional to total ligand concentration. Another major advantage of using SNLR is if B_{NS} displays a different relationship, it can be modeled and incorporated into the equations, whereas it might not be able to be estimated for each experiment independently as used in NLR.

Finally, the impact of two sources of experiment-to-experiment variability was assessed on the impact of bias and variability on binding parameter estimation. The experiment-to-experiment differences associated with assay measurements led to cumulatively higher bias and imprecision than expected from residual variability alone. The most notable impact was on the estimation of k_1 for NLR and $B_{max,1}$ for SNLR. However, overall, SNLR performed better to approximate the true values. Variability associated with the intrinsic quantity of receptors contained per experiment, $B_{max,1}$ led to estimates approximately equivalent utilizing both methods when estimation of this variability was not accounted for using SNLR. With inclusion of estimates of BEV on $B_{max,1}$ for SNLR, this method proved superior in the estimation of the binding parameters. There was still some unresolved bias in the estimation of the BEV; however, this estimation became confounded with RE. These sources of variability in binding measurement can be attributed to many factors including binding affinity, uniformity of binding, stability on storage, working temperature and handling procedures (15), and without accounting for these in at least a portion leads to biased estimates.

Cumulatively, these factors mentioned above play an important role on the quantification of the specific ligand binding parameter estimation. Whereas for all cases, SNLR performed with better precision and accuracy in as much as if each of these factors were included into the simulation and estimation of these experiments, this method would provide better approximation to the true values. For

example, providing a modest range of variability of 10% as might be expected within each of these factors on BEV, B_{NS} and residual variability, the overall bias of $B_{max,1}$ and k_1 was ~5 times less using SNLR (accounting of BEV) compared to NLR and the RMSE was ~2.5 times less for $B_{max,1}$ and k_1. Additionally, the estimation of the dissociation binding constant, K_d, was significantly biased using NLR compared to SNLR. While specific values were used for these simulations, the relative merit of using SNLR over NLR is applicable to a range of parameter estimates and experimental conditions.

From the considerations mentioned above, it is clear that SNLR is the preferred method for ligand binding parameter estimation based on superior estimation properties, statistical advantages, and easy implementation. The use of SNLR required a reduced number of both model and data files and transfer of data. Additionally, utilizing SNLR data from multiple experiments can increase the power and precision of parameter estimates, and supplementary parameters can be added to account for different levels of variability. Furthermore, increased complexity of ligand-receptor interaction, increased binding sites, can be easily assessed based on changes of statistical inference, specifically the objective function value, whereas with NLR this comparison becomes increasingly more difficult.

CONCLUSION

As drug development becomes more costly and time consuming, accurate estimation and prediction of parameters related to physiological conditions becomes vital for the pursuit of better molecular entities in treating diseases. One method useful in drug development is the use of *in vitro–in vivo* correlation. Describing uncertainty related to the modeled parameter estimates and data can provide invaluable insight into the probability of predicting which molecules will perform with better reliability. The SNLR method described in this article can provide better approximation of the true values obtained from *in vitro* experiments for predicted performance *in vivo* leading to increase chance of choosing better molecules. This increase in probability of choosing better molecules can decrease the overall time and cost associated with drug development. Although the focus of this article is related to one binding site, these methods could be applied to multiple binding sites as previously demonstrated (16).

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