STATISTICS IN MEDICINE
Statist. Med. 2006; **25**:2932–2945
Published online 15 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/sim.2448

Estimating the parameters of the operational model of pharmacological agonism

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SUMMARY

The aim of this work is practical. We show that the parameters of the widely used operational model of pharmacological agonism are difficult to estimate from single dose–response curves. The parameters can be estimated using pairs of dose–response curves (usually treatment and control) sharing some parameters. Confidence bands for the estimators are developed. In the case of multiple dose–response curve pairs one can employ a non-linear mixed effects model to allow for inter-individual variation. The point estimates and the confidence intervals thus obtained are similar to the more naive construction based on mean and standard errors of parameter estimates. To test for difference of certain parameters between treatment and control we employ a permutation test and Wald's test. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: dose–response curves; concentration–response curves; operational model of pharmacological agonism; confidence interval; mixed effects

1. INTRODUCTION

Receptors are proteins interacting with extracellular physiological signals and converting them into intracellular effects. The receptor receives a signal and transduces the signal to an effector mechanism. Receptors can be divided into at least four groups: Receptors that act as enzymes, receptors that activate transmembrane ion channels, receptors that use as their transducer the G-protein and receptors located within the cell (transcription factors). Exposing the receptor to the endogenous ligand(s) normally found in the body gives rise to an agonist response—the normal positive effect of the endogenous ligand is duplicated. In fact, we can often identify substances not normally found in the body which have either a more specific effect on a particular receptor than does the normal ligand, a greater affinity for a particular receptor, or even a greater biological effect (greater intrinsic activity). In the absence of an agonist, a

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Received 11 May 2005
Accepted 28 September 2005

partial agonist may stimulate the receptor—however, if the receptor is already exposed to a full agonist, then the partial agonist will sit on the receptor and interfere, causing less agonist activity. This effect will be reversed by increasing the concentration of the agonist.

In 1983, Black and Leff introduced a model for agonist action [1]. This model, the operational model of pharmacological agonism, can be directly fitted to experimental agonist concentration-effect or dose-effect data in order to estimate affinities and relative efficacies of agonists. The model has been widely applied. Examples include α-adenoreceptor mediated vasoconstriction in rat aorta [2], 5-HT induced constriction in rabbit aorta [3], rabbit aorta contraction due to adenosine in the presence of methoxamine [4], adaptive changes in the pharmacodynamics of benzodiazepines upon chronic treatment in rats [5] and assessing the potency of a new δ-opioid receptor agonist [6]. There is however very scant information on the estimation procedure and the statistical analysis. The statistical properties of the estimates have only been studied in Reference [7]. In that study, the distribution of the estimators of the parameters for noisy measurements are studied for fixed parameter values, i.e. there is no provision for biological variation in the parameters themselves.

The aim of this paper is to show that the parameters are very difficult to estimate from single dose–response curves. Instead, the parameters can be estimated using pairs of dose–response curves (usually treatment and control) sharing some parameters. We also want to develop confidence intervals for the parameters and a test to detect changes in the differences in parameters between treatment and control.

In short we want to investigate the estimation procedure when applying the operational model of pharmacological agonism, as this potential problem has not been addressed properly before.

1.1. Dose–response curves

Dose–response curves express the relationship between the response or the effect, E and the dose or concentration of an agonist $[A]$. It is common to record the negative logarithm of the dose, which in analogy with the pH notation is denoted pA. Thus $pA = -\log_{10}([A])$. A wide range of pharmacological assays have sigmoidal dose–response curves, when E is plotted against pA. These curves are well described by logistic curves. Examples include the concentration–response curve that is seen when a contractile agonist, e.g. noradrenaline, is added to a blood vessel and the dose–response curve that is obtained when giving anesthetics, e.g. pentobarbitine, to elicit different degrees of drowsiness in patients.

The general form of a logistic dose–response curve with 0 response at 0 concentration is

$$E([A]) = \frac{E_m[A]^n}{EC_{50}^n + [A]^n} \tag{1}$$

E_m is the 'maximum' response, in the sense that $E(\infty) = E_m$. The EC_{50} is the agonist concentration at half the maximal effect. Thus, $E(EC_{50}) = E_m/2$. n is a factor that determines the steepness of the curve. The general logistic dose–response curve is thus a three parameter model.

1.2. Operational model of pharmacological agonism

The operational model of pharmacological agonism provides an explicit relation between agonist concentration and pharmacological effect using affinities and relative efficacies of

agonists [1, 3, 8, 9]. The agonist–receptor occupancy is linked in sequence to a transducer relation. Symbolically,

$$A + R \overset{K_A}{\longleftrightarrow} AR \overset{K_E}{\longleftrightarrow} E$$

This model assumes that the first step in the response produced by an agonist depends on a bimolecular interaction between the agonist and a receptor, where A denotes the agonist, R the receptor and AR the receptor–agonist complex, which is dependent on the dissociation constant of the receptor–agonist complex, K_A, the reciprocal of which defines agonist affinity. The effect E, is then produced in a second step, the transducer function. This step is dependent on the efficacy of the receptor, and is usually expressed as the number of occupied receptors when half the maximal effect is reached, K_E. Thus, K_E is a measure of the productivity of the AR, which depends both on the agonist and on the efficacy of the post-receptor biochemical events that link the AR to the effect E. K_E is thus both agonist and tissue dependent.

For $[A] \approx 0$, we want the law of mass action to hold

$$[AR] = \frac{[R][A]}{K_A} \approx \frac{[R_0][A]}{K_A}$$

in which R_0 is the total functional receptor concentration. For $[A] > [R]$, we want $[AR] \approx [R_0]$. Therefore, at equilibrium, the concentration of occupied receptors, $[AR]$, may be modelled by

$$[AR] = \frac{[R_0][A]}{K_A + [A]} \tag{2}$$

In Reference [1] a transducer relation of the form

$$E([AR]) = \frac{E_m[AR]^n}{K_E^n + [AR]^n} \tag{3}$$

is suggested. Combining (2) and (3) and introducing $\tau = [R_0]/K_E$, we get

$$E([A]) = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \tag{4}$$

the operational model of pharmacological agonism, which is somewhat different from (1). Note that this four parameter model is logistic assuming a logarithmic scale on the dose. Again in analogy with the pH notation we introduce $pK_A = -\log_{10}(K_A)$. Using this, we may rewrite (4) as

$$E([pA]) = \frac{E_m}{1 + \tau^{-n}(10^{pK_A + pA} + 1)^n} \tag{5}$$

As pointed out before, dose–response curves are usually well modelled by a three parameter model, which raises concern regarding the possibility of fitting a four parameter curve. The maximum effect that can be generated in the system is $E_m\tau^n/(\tau^n + 1)$, which converges to E_m as $\tau \to \infty$. Further, n, is related to slope of the dose–response curve. It is a measure of the sensitivity with which a particular system transduces AR into E. In practice, a multitude of receptors and different second-messengers activated by an agonist will alter the slope of the curve. From (3) and the definition of τ, it follows that τ can be interpreted as the ratio of the total receptor concentration and the midpoint location of the transducer function. In theory,

it is possible to generate the same effect from a small amount of highly productive *AR* (low $[R_0]$, small K_E) as from a large amount of less productive *AR* (high $[R_0]$, large K_E). Therefore it is the ratio $[R_0]/K_E$ that defines how much effect an agonist elicits in a system. If the effect of the receptor stimulation is constant, τ will reflect the actual number of receptors. Further discussions on mathematical models of dose–response curves may be found in Reference [10].

2. SINGLE DOSE–RESPONSE CURVES

Let $\xi = (E_m, pK_A, n, \tau)^{\mathrm{T}}$ be the vector of parameters of (5), the pharmacological model of agonism. Suppose we have N noisy measurements

$$e_i = E_\xi[pA_i] + \varepsilon_i \quad i = 1, \ldots, N \tag{6}$$

of effects for the agonist concentrations $pA_1, \ldots, pA_N$. The dependence of E on ξ is indicated by E_ξ. We assume that $\{\varepsilon_i\}_{i=1}^N$ are independent and identically distributed error terms with mean zero and standard deviation σ. The homoscedasticity of the noise term is partly verified in Figure 2. Using vector notation, (6) can be written as

$$e = \eta(\xi) + \varepsilon \tag{7}$$

where $e = (e_1, \ldots, \ldots, e_N)^{\mathrm{T}}$, $\varepsilon = (\varepsilon_1, \ldots, \varepsilon_N)^{\mathrm{T}}$ and $\eta(\xi) = (E_\xi[pA_1], \ldots, E_\xi[pA_N])^{\mathrm{T}}$. Here $\varepsilon \in N(0, \sigma^2 I)$, where I is the identity matrix of dimension N.

In order to estimate ξ we define the maximum likelihood and non-linear least-squares estimator

$$\hat{\xi} = \arg\min_{\xi} \|e - \eta(\xi)\|^2 \tag{8}$$

where $\|e\|^2 = \sum_{i=1}^N e_i^2$ is the squared Euclidian norm. Then σ^2 can be estimated by

$$\hat{\sigma}^2 = \|e - \eta(\hat{\xi})\|^2/(N-4)$$

where $N-4$ is the number of degrees of freedom.

As N grows, $\hat{\xi}$ is an $\sqrt{N}$-consistent and asymptotically normal estimator of ξ under mild regularity conditions on the design points $\{pA_i\}_{i=1}^N$. A standard Gauss approximation argument (see Reference [11]) gives

$$\hat{\xi} \in AsN(\xi, (B_\xi^{\mathrm{T}} B_\xi)^{-1}\sigma^2) \tag{9}$$

where $B_\xi = (\partial\eta(\xi)/\partial E_m, \ldots, \partial\eta(\xi)/\partial\tau)$. The asymptotic covariance matrix for $\hat{\xi}$ can be estimated by $(B_{\hat{\xi}}^{\mathrm{T}} B_{\hat{\xi}})^{-1}\hat{\sigma}^2$. If the normal approximation is fair, then (9) gives an approximate confidence interval

$$(v^{\mathrm{T}}\hat{\xi} \pm \lambda_{\alpha/2}[v^{\mathrm{T}}(B_{\hat{\xi}}^{\mathrm{T}} B_{\hat{\xi}})^{-1} v]^{1/2}\hat{\sigma}) \tag{10}$$

for any linear combination $v^{\mathrm{T}}\xi$ of the elements of ξ with an approximate confidence level $1-\alpha$, if $\lambda_{\alpha/2}$ is the $(1-\alpha/2)$-quantile of the standard normal distribution.

Multicollinearity in arises in non-linear regression when the columns of $B_{\hat{\xi}}$ are almost linearly independent. As will be seen below, the single dose–response curve model exhibits

multicollinearity due to overparametrization. Following [12, p. 272–273], we may view $B_{\hat{\xi}}$ as the design matrix of a linear regression model and use collinearity diagnostics for linear regression in the non-linear setting as well. To quantify the amount of multicollinearity, we use κ, the condition number of the asymptotic correlation matrix of $\hat{\xi}$. See References [13, 14] for further details.

Example (Covariance for single dose–response curve model)
For $E_m = 25$, $pK_A = 8$ (i.e. $K_A = 10^{-8}$), $n = 2$, $\tau = 2$, $\sigma = 0.2$ and 17 equally spaced design points between 7 and 11, we obtained the asymptotic covariance matrix

$$\begin{pmatrix} 0.0273 & 0.0312 & 0.0103 & -0.7648 \\ 0.0312 & 0.0410 & 0.0148 & -1.0024 \\ 0.0103 & 0.0148 & 0.0060 & -0.3603 \\ -0.7648 & -1.0024 & -0.3603 & 24.5157 \end{pmatrix}$$

in (9). The κ condition number becomes 49 000.

We simulated a dose–response curve with the above parameters, and using (10) we obtained the poor 95 per cent confidence band for the parameters shown in Table I. By additionally simulating 500 dose–response curve pairs we estimated the actual confidence level. These values are also found in Table I. Thus, the asymptotic analysis reveals that the single dose–response curve estimation problem is ill conditioned.

An alternative method to generate confidence intervals is to use bootstrap methodology. This does not however remedy the fact that the parameters ξ are ill conditioned and difficult to estimate.

Alternatively, we may use a Bayesian approach with a multivariate normal prior on ξ. The resulting Bayes' estimator, which is then a non-linear analogue of the ridge regression estimator in linear regression, see e.g. Reference [15], is defined as in (8) by adding a penalty term $k\|\xi\|^2$ to the objective function on the right-hand side. We implemented this estimator and found that the MSE of $\hat{\xi}$ was only marginally improved.

Yet another possibility is to reparametrize ξ to have fewer parameters. The 'guided reformulation' of Reference [16] is one example, where a partial differential equation, corresponding to linear dependency among the original ξ-components, is solved to obtain a new approximate non-linear regression function with fewer parameters. However, we prefer to retain the biologically motivated model (5). Instead, we will solve the multicollinearity problem in the

Table I. 95 per cent confidence band and estimated actual confidence levels (with approximate 95 per cent confidence intervals estimated using the normal approximation of the binomial distribution) for the parameters of a single dose–response curve.

Parameter	Confidence band	Estimated actual confidence level
E_m	25.22 ± 0.32	$80 \pm 4\%$
pK_A	8.24 ± 0.39	$83 \pm 3\%$
n	2.05 ± 0.15	$82 \pm 3\%$
τ	11.57 ± 9.70	$76 \pm 4\%$

next section by simultaneously considering two or more dose–response curves that share some (but not all) parameters.

To allow for heteroscedasticity, one may consider non-constant variance functions $\sigma^2(pA) = \mathrm{Var}(\varepsilon_i | pA_i = pA)$. However, we do not think this will affect the ML-estimator $\hat{\xi}$ very much and in particular not make it less ill conditioned.

3. MULTIPLE DOSE–RESPONSE CURVES

Single dose–response curves contain too little information to give reliable estimates of the parameters. One solution is to use a multiple dose–response curve design. In such a design, it is assumed that E_m, pK_A and n remain constant after treatment [3]. Only τ is allowed to vary. If also K_E is assumed constant the variation in τ is an expression for changes in total receptor concentration $[R_0]$ after treatment.

In practice, the most common approach to the multiple curve design is to reduce the receptor number, usually with an irreversible alkylating agent, to such an extent that a full agonist can no longer produce the maximal response. The curve before alkylation is then compared to the curve after alkylation. In Reference [2] concentration–response curves for α-adenoreceptor-mediated vasoconstriction were registered before and after treatment with an alkylating agent. Another approach is to use a partial agonist. Instead, the dose–response curve of the partial agonist is compared to the dose–response curve of the full agonist. Experimental set-ups where multiple (often two) dose–response curves from the same sample or individual are registered are commonly used to reduce an observed variability in E_m between the samples (see Reference [17]). In summary, our analysis of receptor changes will rely on two crucial points:

(i) The operational model of pharmacological agonism (5) holds and
(ii) E_m, pK_A and n are unaltered by the treatment. Only τ is affected.

To formalize the multiple dose–response curve method, consider several dose–response curves simultaneously with the same E_m, pK_A and n but varying τ. More precisely we assume that

$$e_{ji} = E_{\xi_j}[pA_{ji}] + \varepsilon_{ji} \quad j = 1, \ldots, J \;\; i = 1, \ldots, N_j \tag{11}$$

where e_{ji} is the ith measured effect from the jth response curve, and $\xi_j = (E_m, pK_A, n, \tau_j)^{\mathrm{T}}$ is the parameter vector of the jth curve. Further $\{\varepsilon_{ji}\}$ are assumed to be i.i.d. with mean zero and standard deviation σ. The joint parameter vector is $\xi = (E_m, pK_A, n, \tau_1, \ldots, \tau_J)^{\mathrm{T}}$. Using vector notation, (11) can be written as

$$e = \eta(\xi) + \varepsilon \tag{12}$$

where $e = (e_{11}, \ldots, \ldots, e_{1N_1}, e_{21}, \ldots, e_{JN_J})^{\mathrm{T}}$, $\varepsilon_j = (\varepsilon_{11}, \ldots, \varepsilon_{1N_1}, \varepsilon_{21}, \ldots, \varepsilon_{JN_J})^{\mathrm{T}}$ and $\eta(\xi) = (E_{\xi_1}[pA_{11}], \ldots, E_{\xi_1}[pA_{1N_1}], E_{\xi_2}[pA_{21}], \ldots, E_{\xi_J}[pA_{JN_J}])^{\mathrm{T}}$. Here $\varepsilon_j \in N(0, \sigma^2 I)$, where I is the identity matrix of dimension $N = N_1 + \cdots + N_J$. σ^2 can now be estimated by

$$\hat{\sigma}^2 = \|e - \eta(\hat{\xi})\|^2 / [N - (J + 3)]$$

Formulas (9) and (10) still hold, with $B_\xi = (\partial\eta(\xi)/\partial E_m, \ldots, \partial\eta(\xi)/\partial\tau_J)$. Even though there are more parameters to estimate in (12) than in (7), the asymptotic covariance matrix is less ill conditioned.

Example (Covariance for multiple dose–response curve model)
Using 17 equally spaced design points between 7 and 11 and parameters $J=2$, $E_m=25$, $pK_A=8$, $n=2$, $\tau_1=2$, $\tau_2=20$ and $\sigma=0.2$ we obtained the following a symptotic covariance matrix

$$\begin{pmatrix} 0.0097 & -0.0002 & -0.0018 & -0.0004 & -0.0015 \\ -0.0002 & 0.0007 & 0.0010 & -0.0014 & -0.0316 \\ -0.0018 & 0.0010 & 0.0024 & -0.0019 & -0.0419 \\ -0.0004 & -0.0014 & -0.0019 & 0.0030 & 0.0615 \\ -0.0015 & -0.0316 & -0.0419 & 0.0615 & 1.4313 \end{pmatrix}$$

The problem is much better conditioned than in the single curve case as seen by the smaller variances and less collinear as indicated by the much smaller κ condition number of 170. We also simulated two dose–response curves using these parameter values which gave the accurate 95 per cent confidence band for the parameters shown in Table II. By additionally simulating 500 dose–response curve pairs we estimated the actual confidence levels. These values are also found in Table II.

In order to test the validity of (ii) we formulate it as a null hypothesis, which is tested against an alternative larger model with a total of $4J$ parameters $\xi_j = (E_{mj}, pK_{Aj}, n_j, \tau_j)$, $j=1\ldots,J$. Let $\hat{\hat{\xi}}_{\text{full}}$ be the ML estimator of the larger model. In order to test the null hypothesis (ii) we define the LR statistic

$$(\|e-\eta(\hat{\hat{\xi}})\|^2 - \|e-\eta(\hat{\hat{\xi}}_{\text{full}})\|^2)/\hat{\sigma}^2_{\text{full}}$$

with plug-in estimate $\hat{\sigma}^2_{\text{full}} = \|e-\eta(\hat{\hat{\xi}}_{\text{full}})\|^2/(N-4J)$ of variance. Asymptotically, it has a χ^2 distribution with $3(J-1)$ degrees of freedom under ii.

Table II. 95 per cent confidence band and the estimated actual confidence level (with approximate 95 per cent confidence intervals estimated using the normal approximation of the binomial distribution) for the parameters of a double dose–response curve.

Parameter	Confidence band	Estimated actual confidence level
E_m	25.03 ± 0.19	$95 \pm 2\%$
pA_A	8.01 ± 0.053	$94 \pm 2\%$
n	2.00 ± 0.096	$94 \pm 2\%$
τ_1	1.97 ± 0.11	$94 \pm 2\%$
τ_2	19.91 ± 2.34	$94 \pm 2\%$

Example (α_1-adrenoreceptor-mediated vasoconstriction)
In Reference [2], the role of endothelium on α_1-adrenoreceptor-mediated vasoconstriction in aorta from Wistar Kyoto (WKY) and spontaneously hypertensive rats was studied. The operational model of pharmacological agonism was used. In one set-up 12 WKY rat aortas with intact endothelium were subjected to varying doses of phenylepinephrine before and after treatment with the alkylating agent phenoxybenzamine. This causes a partial α_1-adrenoreceptor inactivation. We applied the multiple dose–response model with $J = 2$, $N_1 = N_2 = 8$ and $N = 16$ to each rat separately as well as calculating the LR-statistic and its pertaining p-value. The result of this analysis is presented in Figure 1 and Table III. Condition (ii) was not rejected for any of these 12 rats at the 90 per cent level. Also, as no trend is seen in the residuals in Figure 2, the homoscedasticity assumption of (12) is feasible, although the variance seems slightly larger for the control mice.

From the last example, it is clear that there is a great deal of variation in the parameters between the subjects, and one may suspect that this variation must be in the parameters E_m and τ. Model (11) may not be an optimal description, since a separate model is needed for

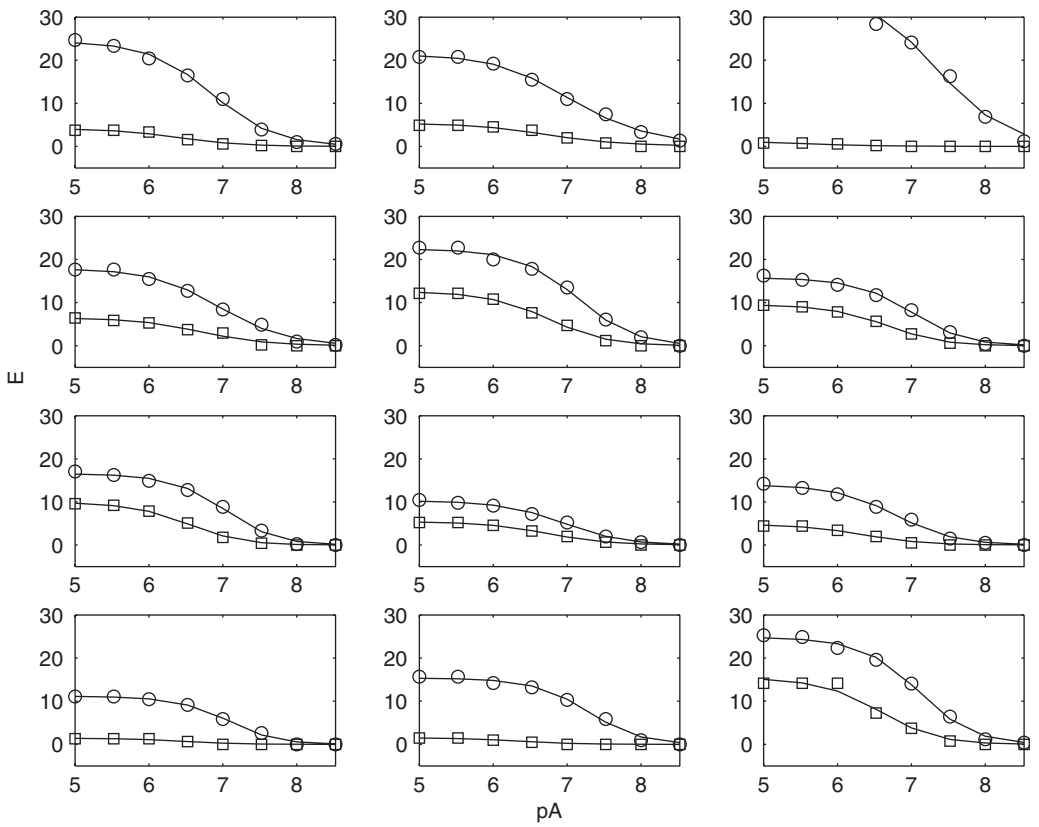

Figure 1. The contractile response of 12 rat aortas to phenylepinephrine before (circles) and after (boxes) treatment with phenoxybenzamine. The solid lines indicate the estimated curves.

Table III. Multiple dose–response curve estimates of ξ_k for 12 rat aortas.

k	1	2	3	4	5	6	7	8	9	10	11	12
$\hat{E}_m$	34.7	40.2	36.8	32.9	28.4	20.1	18.8	17.0	18.7	13.6	16.6	28.1
$\widehat{pK_A}$	6.34	6.42	5.57	6.56	6.60	6.54	6.39	6.62	6.29	6.64	6.45	6.36
$\hat{n}$	1.02	0.66	0.90	0.92	1.18	1.29	1.36	1.10	1.12	1.49	1.28	1.23
$\hat{\tau}_1$	2.29	1.17	56.62	1.19	3.03	2.69	4.31	1.44	2.58	2.80	6.74	5.12
$\hat{\tau}_2$	0.14	0.05	0.02	0.21	0.81	0.92	1.08	0.49	0.38	0.23	0.16	1.16
LR	0.95	4.81	0.12	1.27	1.18	2.86	5.36	0.54	2.15	3.28	4.26	3.81
p	0.81	0.18	0.98	0.73	0.75	0.41	0.14	0.90	0.54	0.34	0.23	0.28

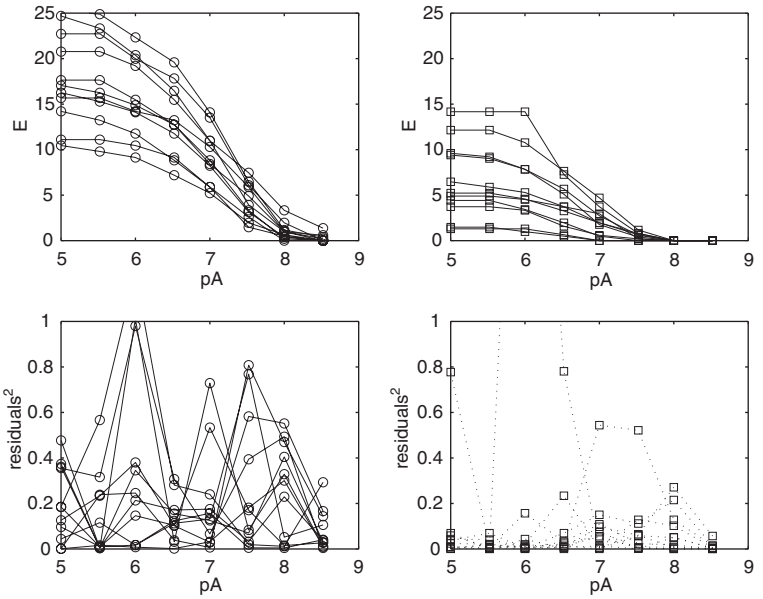

Figure 2. Dose–response curves and residual plots for control (left) and treatment (right) for the α_1-adrenoreceptor-mediated vasoconstriction example.

each rat. This observation motivates the use of one single model which takes into account the biological variation between the subjects.

4. PAIRWISE DOSE–RESPONSE CURVES

4.1. Mixed effects model

In a mixed effects model we will allow the parameter vector to be random and vary from subject to subject. Let $\xi_{kj} = (E_{mk}, K_{Ak}, n_k, \tau_{jk})^{\mathrm{T}}$ be the parameter vector for the kth control or treatment curve, and $\xi_k = (E_{mk}, K_{Ak}, n_k, \tau_{1k}, \tau_{2k})^{\mathrm{T}}$ be their joint parameter vector. Using vector

notation

$$e_k = \eta_k(\xi_k) + \varepsilon_k \quad k = 1, \ldots, K \tag{13}$$

where $e_k = (e_{k11}, \ldots, e_{k1N}, e_{k21}, \ldots, e_{k2N})^{\mathrm{T}}$, $\varepsilon_k = (\varepsilon_{k11}, \ldots, \varepsilon_{k1N}, \varepsilon_{k21}, \ldots, \varepsilon_{k2N})^{\mathrm{T}}$ and $\eta_k(\xi_k) = (E_{\xi_{k1}}[pA_{k11}], \ldots, E_{\xi_{k1}}[pA_{k1N}], E_{\xi_{k2}}[pA_{k21}], \ldots, E_{\xi_{k2}}[pA_{k2N}])^{\mathrm{T}}$. Here $\varepsilon_k \in N(0, \sigma^2 I)$, where I is the identity matrix of dimension $2N$. Let further

$$\xi_k = \mu + u_k$$

where μ is a 5×1 vector of fixed effects and

$$u_k \in N(0, \Sigma)$$

is a 5×1 vector of random effects. We will assume that Σ is a diagonal matrix, i.e. the parameters are uncorrelated. This mixture of fixed and random effects results in a non-linear *mixed effects model* with unknown parameters μ, Σ and σ^2. We wish to write all the K models simultaneously, and to this end we introduce $e = (e_1^{\mathrm{T}}, \ldots, e_K^{\mathrm{T}})^{\mathrm{T}}$, $\xi = (\xi_1^{\mathrm{T}}, \ldots, \xi_K^{\mathrm{T}})^{\mathrm{T}}$, $\eta(\xi) = (\eta_1(\xi_1)^{\mathrm{T}}, \ldots, \eta_K(\xi_K)^{\mathrm{T}})^{\mathrm{T}}$ and $\varepsilon = (\varepsilon_1^{\mathrm{T}}, \ldots, \varepsilon_K^{\mathrm{T}})^{\mathrm{T}}$. Using this notation, we may write

$$e = \eta(\xi) + \varepsilon \tag{14}$$

Define the objective function

$$g(\mu, \xi) = -\tfrac{1}{2}\,\sigma^{-2}\,(e - \eta(\xi))^{\mathrm{T}}(e - \eta(\xi)) - \tfrac{1}{2}\,(\xi - \mu)^{\mathrm{T}}\tilde{\Sigma}^{-1}(\xi - \mu) \tag{15}$$

where $\tilde{\Sigma} = \mathrm{diag}(\Sigma, \ldots, \Sigma)$.[‡] Estimates $\hat{\mu}$ and $\hat{\xi}$ are defined by jointly maximizing g with respect to μ and ξ, replacing Σ and σ^2 by plug-in estimates $\hat{\Sigma}$ and $\hat{\sigma}^2$ defined in the appendix. Following References [18, 19], $\hat{\mu}$ can be motivated as a maximum likelihood estimate of μ with an approximate marginal distribution assigned to the components of e. We are interested in finding confidence intervals for the components of μ. For this purpose, we introduce the design matrices $X_\xi = (B_{1\xi_1}^{\mathrm{T}}, \ldots, B_{K\xi_K}^{\mathrm{T}})^{\mathrm{T}}$ of dimension $2KN \times 5$ and $Z_\xi = \mathrm{diag}(B_{1\xi_1}, \ldots, B_{K\xi_K})$ of dimension $2KN \times 5N$, where $B_{k\xi_k} = (\partial\eta_k(\xi_k)/\partial E_{mk}, \ldots, \partial\eta_k(\xi_k)/\partial\tau_{2k})$. By Taylor expanding η and using the fact that asymptotically, $\hat{\mu}$ is equivalent to a generalized least-squares estimator, we obtain

$$\mathrm{Cov}(\hat{\mu}) \approx (X_\xi^{\mathrm{T}} V_\xi^{-1} X_\xi)^{-1} \tag{16}$$

where $V = \sigma^2 I + Z_\xi \tilde{\Sigma} Z_\xi^{\mathrm{T}}$ and I is the identity matrix of order $2KN \times 2KN$, cf. Reference [18]. Confidence intervals are constructed by assuming asymptotic normality of $\hat{\mu}$, and replacing σ^2, Σ, X_ξ, Z_ξ with $\hat{\sigma}^2$, $\hat{\Sigma}$, $X_{\hat{\xi}}$ and $Z_{\hat{\xi}}$ in the covariance matrix (16).

[‡]For a matrix A, $\mathrm{diag}(A)$ denotes the vector of the diagonal elements. For a vector v, $\mathrm{diag}(v)$ denotes the square matrix with diagonal elements v. For a collection of matrices $A_1, \ldots, A_n$, $\mathrm{diag}(A_1, \ldots, A_n)$ denotes the block matrix with matrices $A_1, \ldots, A_n$ on the diagonal and zero blocks outside the diagonal.

Table IV. Pairwise dose–response curve estimates of ξ_k for 11 rat aortas after exclusion of one outlier.

k	1	2	3	4	5	6	7	8	9	10	11	12
$\hat{E}_m$	33.3	32.8	—	27.3	28.4	20.8	19.9	15.9	20.0	13.7	17.9	28.9
$\widehat{pK_A}$	6.37	6.41	—	6.48	6.55	6.54	6.44	6.51	6.41	6.49	6.57	6.42
$\hat{n}$	1.08	0.75	—	0.95	1.14	1.29	1.30	1.03	1.18	1.27	1.22	1.24
$\hat{\tau}_1$	2.46	2.16	—	1.91	3.23	2.69	3.61	1.79	2.01	3.39	4.72	4.43
$\hat{\tau}_2$	0.16	0.11	—	0.30	0.82	0.92	0.97	0.54	0.36	0.19	0.14	1.08

Table V. μ^* and $\hat{\mu}$ and their pertaining approximate 95 per cent confidence bands. Subscripts oe and oi indicate exclusion, respectively, inclusion of outlier.

Parameter	Reported in Reference [2]	μ^*_{oe}	$\hat{\mu}_{\mathrm{oe}}$	μ^*_{oi}	$\hat{\mu}_{\mathrm{oi}}$
E_m	25.55 ± 5.15	24.51 ± 5.20	23.49 ± 5.70	25.54 ± 5.16	25.59 ± 5.77
pK_A	6.40 ± 0.16	6.47 ± 0.073	6.47 ± 0.12	6.40 ± 0.16	6.43 ± 0.20
n	1.13 ± 0.14	1.16 ± 0.13	1.12 ± 0.16	1.13 ± 0.13	1.08 ± 0.15
τ_1	3.39	3.03 ± 1.03	2.95 ± 1.24	7.50 ± 8.80	3.28 ± 9.21
τ_2		0.52 ± 0.24	0.50 ± 0.25	0.48 ± 0.23	0.44 ± 0.24

Example (α_1*-adrenoreceptor-mediated vasoconstriction*)
Returning to the real data example of α_1-adrenoreceptor mediated vasoconstriction in rat aorta, now using the pairwise dose–response curve model with $K = 12$, and excluding the obvious outlier, namely the third treatment/control pair (see right upper panel of Figure 1 and Table III) we obtain the new estimates $\hat{\xi}_k$ of ξ_k in Table IV for the remaining 11 rats. Notice that the parameter estimates in Table IV for the mixed model are slightly more shrunk towards their mean values than those of the multiple dose response curve model in Table III. The estimates of $\hat{\mu}$ for the mixed model are presented in Table V, together with the 95 per cent confidence bands based on the diagonal entries of (16) and normal approximation. We also computed the naive estimator $\mu^* = \sum_{k=1}^{K} \xi_k^*/K$ and the associated 95 per cent confidence bands. Here ξ_k^* is the preliminary estimate of ξ_k, see the appendix for details. To allow for a more direct comparison with the results in Reference [2] we also performed the calculations including the outlier. Looking at the estimates of τ_1, we note that $\hat{\mu}$ seems less sensitive to the outlier than μ^*. In fact they do not report confidence intervals in Reference [2] but the standard error of the mean (SEM) of E_m, K_A, n and $\log(\tau_1)$, i.e. the standard deviation divided by $\sqrt{K}$. For each parameter, an asymptotic 95 per cent confidence interval has the form mean $\pm\, \lambda_{0.025}$SEM.

4.2. Tests for treatment versus control

A common problem in the analysis of dose–response curves with the pharmacological model of agonism is to detect differences in τ before and after treatment, i.e. we want to test H_0 : $\tau_1 = \tau_2$. Given K pairs of treatment/control curves, there are 2^K possible permutations of the treatment/control curves under H_0.

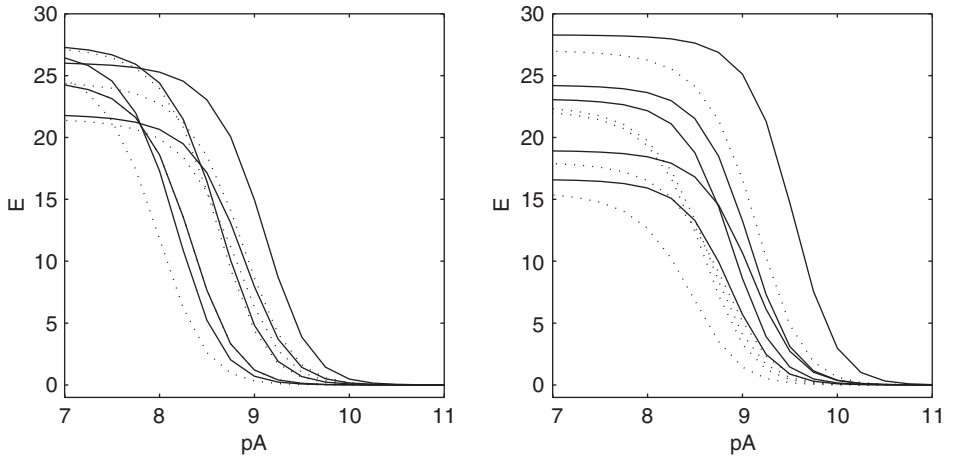

Figure 3. Estimated dose–response curves from a mixed effect model with $\tau_2 - \tau_1 = 1$ for the left panel and $\tau_2 - \tau_1 = 4$ for the right panel. The continuous curves correspond to τ_1 (control) while the dotted curves correspond to τ_2 (treatment).

Example (Permutation test for treatment versus control)
We simulated $K = 5$ pairs of dose–response curves using the parameter distribution

$$N\left(\begin{pmatrix}25\\8\\2\\4\\5\end{pmatrix}, \begin{pmatrix}5 & 0 & 0 & 0 & 0\\0 & 0.4 & 0 & 0 & 0\\0 & 0 & 0.1 & 0 & 0\\0 & 0 & 0 & 0.5 & 0\\0 & 0 & 0 & 0 & 0.5\end{pmatrix}\right)$$

for ξ_k, $k = 1, \ldots, 5$ and $\sigma = 0.2$, as shown in Figure 3. Let $\tau_1 = \mu_4$ and $\tau_2 = \mu_5$ denote the average values of these two parameters in the whole population. Out of the $2^5 = 32$ possible permutations of the treatment/control curves, in three cases $|\hat{\tau}_2 - \hat{\tau}_1|$ was greater than or equal to $|\hat{\tau}_2 - \hat{\tau}_1|$ for the original unpermuted sample. This means we cannot reject H_0 at the 95 per cent level as the p-value is $3/32 \approx 0.094$. Repeating this procedure now with $\tau_2 = 8$ we get the p-value $1/32 \approx 0.031$. We may thus reject H_0 at the 95 per cent level.

Example (Wald's test for treatment versus control)
A more powerful alternative to the permutation test is to use Wald's test, i.e. the statistic $(\hat{\tau}_2 - \hat{\tau}_1)/s$, which asymptotically is standard normal. The standard error s is obtained through $s^2 = v^{\mathrm{T}}\mathrm{Cov}(\hat{\mu})v$, where $v = (0, 0, 0, -1, 1)$ and the estimated covariance matrix of (16) is used. As $\tau_2 > \tau_1$ in the permutation test example, we use a one-sided test. Already for $\tau_2 - \tau_1 = 1$ we get $2.24 > \lambda_{0.05} = 1.64$, i.e. rejection of the null hypothesis $\tau_1 = \tau_2$.

5. CONCLUSION

In this paper we have shown that the parameters of the widely used operational model of pharmacological agonism are difficult to estimate from single dose–response curves. The parameters can be estimated using pairs of dose–response curves (usually treatment and control) sharing some parameters. Standard errors and confidence intervals based on normal approximations are suggested for the estimators. In the case of multiple dose–response curve pairs one can employ a non-linear mixed effects model to allow for inter-individual variation. The point estimates and the confidence intervals thus obtained are similar to the more naive construction based on mean and standard errors of parameter estimates for different dose–response curve pairs. To test for difference in τ between treatment and control we have employed a permutation test and Wald's test.

APPENDIX

We will derive preliminary estimators of the parameters μ, Σ and σ^2 in Section 4.1. Let ξ_k^* be a non-linear least-squares estimator of ξ_k. A standard Gauss approximation argument gives that $\xi_k^* | \xi_k \in AsN(\xi_k, \Sigma_k)$, where $\Sigma_k = (B_{k\xi_k}^{\mathrm{T}} B_{k\xi_k})^{-1}\sigma^2$ and hence

$$\xi_k^* \in AsN(\mu, \Sigma + \Sigma_k)$$

μ can be estimated by $\mu^* = \sum_{k=1}^K \xi_k^*/K$, which asymptotically satisfies

$$\mu^* \in AsN\left(\mu, \frac{\Sigma}{K} + \frac{\sum_{k=1}^K \Sigma_k}{K^2}\right) \tag{A1}$$

Let

$$S = \sum_{k=1}^K (\xi_k^* - \mu^*)^{\mathrm{T}}(\xi_k^* - \mu^*)$$

By calculating the expected value of S,

$$E[S] = (K-1)\Sigma + (1 - 1/K)\sum_{k=1}^K \Sigma_k$$

we see that

$$\widehat{\mathrm{Cov}(\mu^*)} = S/(K(K-1))$$

is an unbiased estimator of the asymptotic covariance matrix in (A1). Moreover

$$\hat{\Sigma}' = \frac{S}{K-1} - \sum_{k=1}^K \hat{\Sigma}_k/K$$

is an unbiased estimate of Σ if $\hat{\Sigma}_k$ is an unbiased estimator of Σ_k. Our final estimate

$$\hat{\Sigma} = \mathrm{diag}(\mathrm{diag}(\hat{\Sigma}')) \tag{A2}$$

is defined by putting all non-diagonal entries of $\hat{\Sigma}'$ to zero. In practice it may happen that $\hat{\Sigma}'$ has negative diagonal elements. This can be remedied by using a flatter estimated prior for ξ_k, $\hat{\Sigma}'' = S/(K-1)$. In this case, we replace $\hat{\Sigma}'$ by $\hat{\Sigma}''$ in (A2). An asymptotically unbiased estimator of Σ_k is $\hat{\Sigma}_k = (B^{\mathrm{T}}_{k\xi^*_k} B_{k\xi^*_k})^{-1}\hat{\sigma}^2$, where

$$\hat{\sigma}^2 = \frac{\sum_{k=1}^{K} \|e_k - \eta_k(\xi^*_k)\|^2}{K(2N-5)}$$

ACKNOWLEDGEMENTS

The authors wish to thank Jesús Giraldo of Universitat Autònoma de Barcelona for the real data and two anonymous referees for useful suggestions on improving the paper.

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