Nonlinear mixed effects models applied to cumulative concentration–response curves

Chantal Thorin, Mohamed Yassine Mallem, Jacques Noireaud, Marc Gogny and Jean-Claude Desfontis

UPSP 5304 de Physiopathologie Animale et Pharmacologie Fonctionnelle, Ecole Nationale Vétérinaire, Agroalimentaire et de l'alimentation Nantes Atlantique, ONIRIS, France

Abstract

Objectives In experimental pharmacology, drug effect studies currently establish and analyse cumulative concentration–response curves (CCRC) under repeated measurements designs. Usually the CCRC parameters are estimated using the Hill's function in a nonlinear regression for independent data. The two-way analysis of variance is generally used to identify a statistical difference between the responses for two treatments but that analysis does not take into account the nonlinearity of the model and the heteroscedasticity (uneven distribution) of the data. We presently tested the possibility of finding a statistical solution for the nonlinear response in repeated measurements data using the nonlinear mixed effects (nlme) models.

Methods Experimental data sets, originating from studies on β-adrenoceptor-induced relaxation in rat thoracic aorta ring, were analysed using the nlme methods.

Key findings Comparison with classical methods showed the superiority of the nlme models approach. For each pharmacological parameter (E_m, n, pD_2), a point estimate, a standard error and a confidence interval are returned by the nlme procedures respecting the assumption of independency and normality of the residuals.

Conclusions Using the method presently described, it is now possible to detect significant differences for each pharmacological parameter estimated in different situations, even for designs with small samples size (i.e. at least six complete curves).

Keywords cumulative concentration–response curves; heteroscedasticity; nonlinear mixed effects model; statistic

Introduction

In experimental pharmacology, the drug–receptor interaction is commonly investigated at the molecular, cellular and tissue level using in-vitro approaches. When carrying out studies in isolated tissue, biological responses produced by drugs or agonists are often plotted as a concentration–response curve. The concentration of the drug is normally well controlled and the pharmacological response is measured for a range of cumulative concentrations of the potential agonist. Among a large choice of functions,[1] the Hill equation is the most commonly used to model the relationship between the pharmacological response and the agonist concentration:[2,3]

$$E = E_m \frac{[A]^n}{EC50^n + [A]^n} \tag{1}$$

with E_m as the maximum effect, EC50 as the agonist concentration ([A]) that produces a half-maximum effect and n as the Hill coefficient describing the cooperative binding of the agonist. Furthermore, when performing concentration–response curves, it is frequently convenient to plot the magnitude of effect versus the decimal logarithm ($\log_{10}$) of the concentration because a wide range of agonist concentrations are displayed and the parameters of different cumulative concentration–response curves (CCRCs) can be readily compared. A sigmoidal relationship between the $\log_{10}$ of the drug concentration and the response is classically observed and described by the Hill equation:

$$E = E_m \frac{1}{1 + 10^{n(pD_2 - pA)}} \tag{2}$$

where $pA = -\log_{10}[A]$ and $pD_2 = -\log_{10}(EC50)$

Correspondence: Mrs C. Thorin, UPSP 5304, ONIRIS, BP 40706, F-44307 Nantes, France. E-mail: chantal.thorin@oniris-nantes.fr

The demonstration of that function is shown in our website (http://www.oniris-nantes.fr/index.php?id=231). That function is clearly nonlinear and whatever the statistical analysis method employed the estimation of CCRC parameters (E_m, n and pD_2), from an experimental data set, has to be realised with a nonlinear regression analysis method. Moreover, experimental designs currently carried out are based on repeated measurements on the same individual and the statistical analysis must also take into account that dependency between data. Over the last few years, statisticians have developed a nonlinear mixed effect (nlme) model theory, which provides an excellent solution to that statistical problem.[4] To the best of our knowledge, only a small number of pharmacodynamic studies used such an alternative method to analyse their data (e.g. De Mayer et al.[5]) but without describing and detailing it. Being unfamiliar with, or uncomfortable in, using the nlme model, pharmacologists failed to adopt an appropriate approach to fit and compare CCRCs. The aim of this paper is to describe a valid and easy to use statistical method allowing the estimation and comparison of CCRC parameters taking into consideration the nonlinearity of the data and the repeated measurements designs. The modelling process was applied to a data set and results led to pharmacological interpretations.

Materials and Methods

Experimental design

In our laboratory, we currently carry out experiments on β-adrenoceptor activation, which mediates a concentration-dependent relaxation in rat, guinea-pig and equine isolated blood vessels.[6–9] To illustrate the use of nlme models, we chose the data obtained in a study of β-adrenoceptor-mediated effects in rat isolated thoracic aorta rings.[9] The experiments were performed in 12-week-old male Wistar Kyoto (WKY) rats. Animal procedures were approved by the Institutional Committee for Use and Care of Laboratory Animals at the National Veterinary School of Nantes and conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). The rats were housed under controlled conditions of temperature (22°C) and humidity (50%) and allowed free access to standard chow and drinking water. The preparation of aorta rings and the recording of data were the same for all experiments. Briefly, each aorta ring was suspended on stainless-steel wires in a 5-ml organ bath containing Krebs solution maintained at 37°C. Isometric tension was recorded by a force displacement transducer (Electromike; Electrocorporation, Sarasota, USA). Aorta rings were precontracted with 0.3 μM phenylephrine. Then, CCRCs to isoprenaline (nine concentrations ranging from 1 nM to 10 μM) were established at the steady-state plateau of phenylephrine contraction. In the first experimental series, relaxation to isoprenaline was studied in control intact aorta rings isolated from six WKY rats. In the second experimental series, carried out in a different group of six WKY rats, the involvement of β_1- and β_2-adrenoceptors in the isoprenaline-induced relaxation was determined in intact aorta rings incubated in the presence of 10 μM nadolol, a competitive β_1- and β_2-adrenoceptor antagonist.

Statistical analysis

As previously mentioned (see Introduction), the response to a given agonist concentration ([A]) was fitted by the Hill's equation, a nonlinear function with three parameters : E_m, n, pD_2. Respecting the experimental design with repeated measurements, where the aorta ring was the experimental individual while the statistical unit was the response measured for one concentration in one aorta ring, a nonlinear mixed effects model can be expressed as:

$$E_{ij} = E_{mi} \frac{1}{1 + 10^{n_i(pD_{2i} - pA_j)}} + \varepsilon_{ij} \text{ and}$$

$$\begin{cases} E_{mi} = E_m + \xi_i \\ n_i = n + \eta_i \\ pD_{2i} = pD_2 + \pi_i \end{cases} \quad (3)$$

where E_{ij} is the response of the individual i to the drug concentration j ($1 \le i \le m$ and $1 \le j \le k$ for m individuals included in the experiment and k levels of different drug concentrations). ε_{ij} is the residual or intra-individual random response under the assumption: ε_{ij} are independently and identically distributed as N(0; σ^2). E_m, n, pD_2 are the fixed effects terms and ξ_i, η_i, π_i are the random effects terms. This model has been used to establish the estimations of the pharmacological parameters.

Residuals and random effects terms have to be independent and normally distributed to deal with the accuracy of the different estimations. Data recorded in a repeated measurements design often show a great heterogeneity of variances. That heterogeneity of variances, so called heteroscedasticity, has two origins. Firstly, variances increase naturally with the amplitude of responses. Secondly, the correlation between responses measured on a same individual for two (or more) successive levels of the independent variable can be very strong and enhances the dispersion of measurements. The nlme modelling approach, using a pseudo-likelihood estimator, can accommodate this heteroscedasticity by giving an estimation of a power variance parameter (φ) and an estimation of a correlation parameter (α). Hence, the specificity of repeated measurements and their consequences are completely integrated in the fitting procedures by the mixed effects models theory.[4] A point estimate of each parameter value is given by the corresponding fixed effects value. Those fitting procedures are also particularly relevant to give confidence intervals for each parameter included in the model.[10] The assumptions of normal residuals distribution, independence of the residuals and independence of the parameters can be verified through graphical tools. R project (R Development Core Team (2009). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.) provides an efficient tool or script for computing all the estimations and graphs with its nlme library.[10] For a quite easy practice with R software, it is convenient to download the R commander package. The opened window of the package is divided into two parts. Part one is devoted to the scripts and the second part to the results. Scripts used in

this work, explanations for the use of R and details for the organisation of the data set can be downloaded from our website.

Results

Data sets obtained with isoprenaline in the absence or in the presence of nadolol respectively were analysed with nlme models. Results are presented in Table 1.

Figure 1 shows an illustration of the nlme models built for data sets with isoprenaline in the absence and in the presence of nadolol respectively; for each aorta ring represented in a plot, two curves were drawn – one with dotted line for the individual model (individual fitted curve) and one with solid line for the population model (illustration of the fixed effects). The difference between the individual fitted curves illustrates the individual effect on the response particularly important with repeated measurement designs. The quantile–quantile plots (qqnorm plot) drawn for the isoprenaline fitted model and for the isoprenaline plus nadolol fitted model (Figure 2a, 3a), allow consideration of the accuracy of the normal residuals distribution assumption and then the validity of the fitted models. The scatter plots displaying standardised residuals versus fitted values for each fitted model (Figure 2b, 3b) show a symmetrical distribution of the residuals around 0, insuring their independence.

The fit of the two data sets with the nlme models were conducted separately, for one experimental condition at a time. Those two fits for the two experimental groups were completely independent. Thus, one way to compare the effects of different treatments on the response measured was to compare the parameter values obtained in each group and, more specifically, their confidence intervals. The point

estimate for E_m in the isoprenaline group (84.61) was not setting between the confidence limits of E_m for the isoprenaline plus nadolol group (36.82; 58.95) and, inversely, the point estimate for E_m in the isoprenaline plus nadolol group (47.89) was not setting between the 95% confidence limits of E_m in the isoprenaline group (80.03; 89.18), so there was a significant difference between E_m in the isoprenaline group and E_m in the isoprenaline plus nadolol group. For the pD_2 parameter, the situation was similar to the E_m estimation; there was a significant difference between pD_2 in the two groups. For the n parameter, the point estimate of n in the isoprenaline plus nadolol group (0.97) was included into the 95% confidence interval in the isoprenaline group (0.58; 0.98) but the point estimate of n in the isoprenaline group (0.77) was not included into the 95% confidence interval of n in the isoprenaline plus nadolol group (0.79; 1.16). It was impossible to conclude a significant difference of n in the two experimental situations.

Discussion

From a pharmacological standpoint, the results obtained in this study suggested a functional expression of β_1- and β_2-adrenoceptors in rat thoracic aorta.[9] The competitive nature of the nadolol antagonism was confirmed by the rightward shift of the isoprenaline CCRC and the lack of effect of nadolol on CCRC slopes. However, the decrease in the maximum response suggested that 10 μM nadolol induced an unsurmountable competitive antagonism. Ideally, several concentrations of nadolol should have been tested to rigorously define the quantitative relationship between such a displacement and the antagonist concentration. The concentration

Table 1 Results of nonlinear mixed effects procedures for CCRC to isoprenaline and for CCRC to isoprenaline in the presence of nadolol in rat isolated thoracic aorta rings

	Isoprenaline	Isoprenaline + nadolol
Em		
Estimation value	84.61	47.89
Standard error	2.33	5.65
95% Confidence interval	80.03; 89.18	36.83; 58.95
n		
Estimation value	0.78	0.97
Standard error	0.098	0.09
95% Confidence interval	0.58; 0.98	0.79; 1.16
pD_2		
Estimation value	7.28	6.49
Standard error	0.13	0.11
95% Confidence interval	7.55; 7.01	6.69; 6.28
EC50		
Estimation value	5.25×10^{-8}	3.23×10^{-7}
95% Confidence interval	2.82×10^{-8}; 9.77×10^{-8}	2.04×10^{-7}; 5.25×10^{-7}
Variance function (power)	0.20	0.56
95% confidence interval	0.005; 0.41[a]	0.4; 0.72[a]
Correlation parameter (φ)	0.63	0.64
95% confidence interval	0.42; 0.77[b]	0.43; 0.78[b]

[a]The power parameter was significantly different from 0 (0 not included in the confidence interval); [b]the correlation parameter, setting by definition between −1 and 1, was significantly different from 0 and from 1 in the two groups; thus it was then necessary to include them in the fitting model.
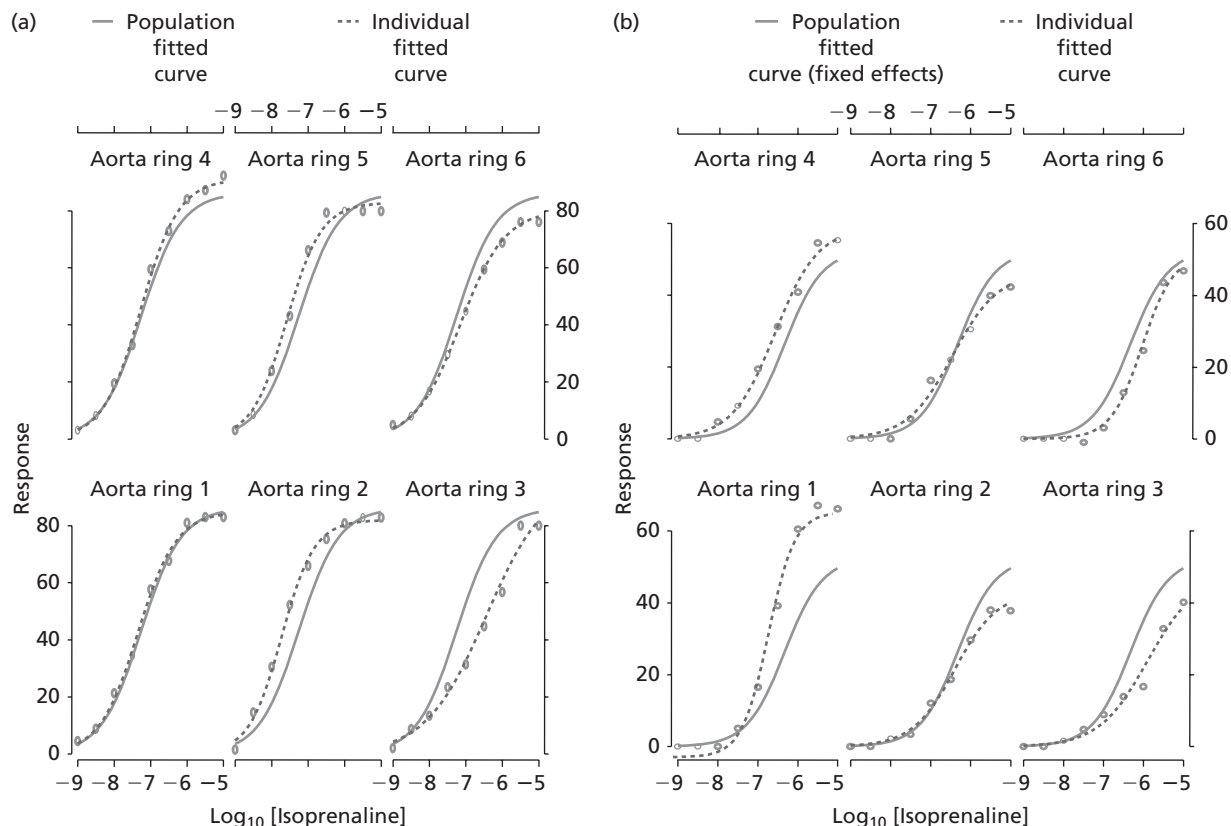
Figure 1 Population and individual fitted curves in data sets with isoprenaline in the absence (a) or in the presence (b) of nadolol. Two curves are drawn in each plot: the curve with dotted line for the fitted curve for one of six aorta rings (illustration of the individual effect) and the curve with solid line for the population fitted curve (fixed effects). The population fitted curve is the same in the six plots, individual fitted curves are different from one plot to the other; circles represent the observed responses

(10 μM) of nadolol used in this study is well above the reported affinity of the drug for β_1- and β_2-adrenoceptors. A slow dissociation profile of nadolol from aortic β-adrenoceptors or a possible modification of the receptor signal transduction mechanism that may occur at higher concentration of nadolol may account for the unsurmountable competitive nature of antagonism observed. However, the possibility that the effect of isoprenaline in the presence of nadolol may be ascribed to non β_1- and β_2-adrenoceptor activation is not completely discarded. β_3- and low-affinity-state β_1-adrenoceptors co-exist with classical β_1- and β_2-adrenoceptors in rat thoracic aorta and could be involved in the isoprenaline-induced nadolol-insensitive effect. The hypothesis is supported by previous experiments showing, by using selective antagonists, that β_3- or low-affinity-state β_1-adrenoceptors (or both) can also play a role in the isoprenaline-induced relaxation in rat thoracic aorta.[9,11]

From a statistical point of view, the exploration of CCRC data by the nlme procedures appears to be an informative and more efficient tool than the method employed for CCRC analysis until now. CCRC comparisons were commonly performed with a two-way analysis of variance for repeated measurements, but the two-way analysis of variance always failed to give an accurate comparison of different treatments because (1) the nonlinearity of the data generated a large

effect of the concentration on the response and (2) a high level of interaction between concentration and treatment made hazardous the interpretation of the P-values of such an analysis. Moreover, analysis of variance has been designed for the analysis of linear data and is not accurate for nonlinear data. In our previous work, CCRC data analysis consisted of an estimation of the E_m, n, pD_2 values for each CCRC by nonlinear regressions according to Hill's equation using a least square estimator.[9] For each parameter and for each treatment group, the mean of the individual values previously computed was considered as an estimation of the population parameter value. A Student's t-test was then performed to compare each parameter value in the two groups (see Table 2). Although those estimation techniques are commonly used and are easy to execute and understand, they have to be criticised because a valid Student's t-test can only be computed if the values are sampled in two Gaussian populations with equal variances. Those conditions cannot be verified with small samples. At present, the mixed effects models fitting technique is the most efficient method to model correctly the dispersion of the data and to provide estimations of pharmacological parameters minimising the risk of mis-estimation and consequently minimising the risk of mis-interpretation. Moreover, the ability to separately assess the dose–response curve parameters in the same
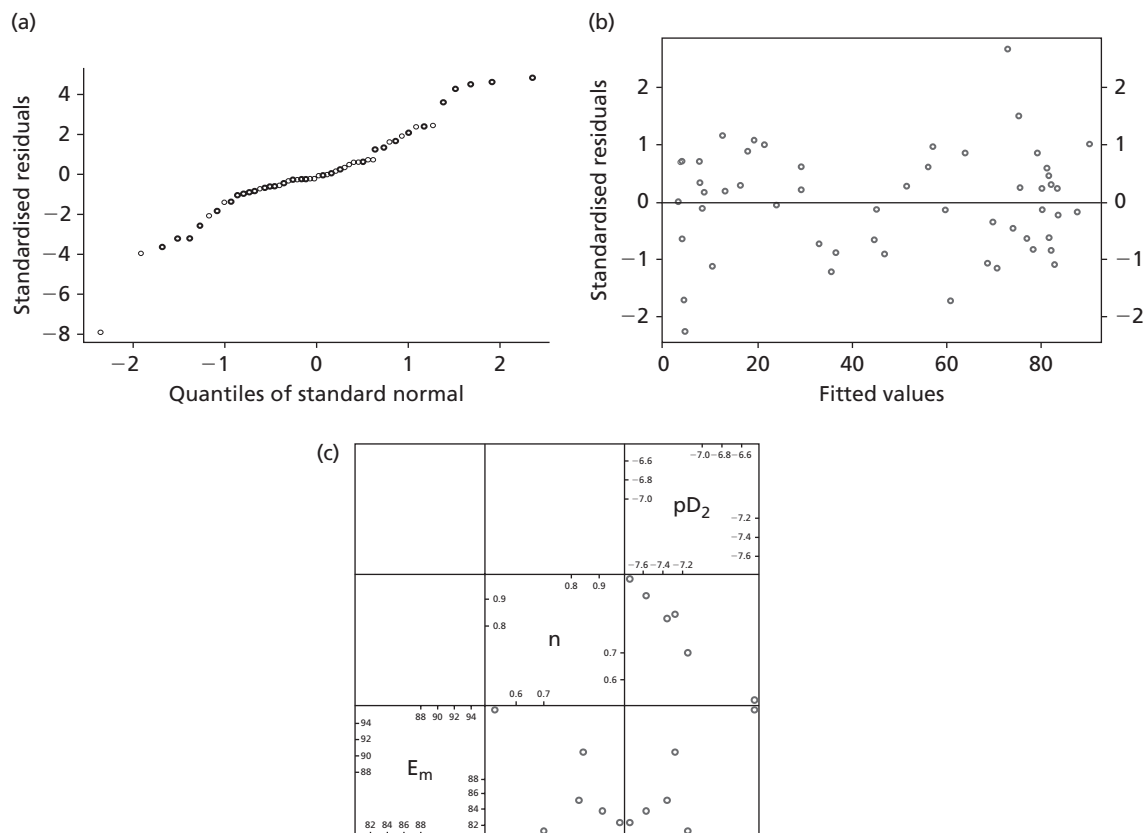
Figure 2 Adequacy for isoprenaline fitted model. (a) Accuracy of the normal distribution of the residuals; if the points in the graph are close to the diagonal line with the same scale on each axis then the normality of the residuals is considered to be achieved. (b) Accuracy of the independence of the residuals with a plot of the standardised residuals versus fitted responses; the residuals were distributed symmetrically around 0 with a constant variance. (c) Diagnostic for overparameterisation with a pairs plot; each scatter plot crossed the parameter values. In the scatter plot crossing n and pD_2 the points were close to a line so n and pD_2 seemed to be correlated; random effects have then been computed only for E_m and pD_2 parameters

package represents an advantage, particularly in the Hill coefficient estimation that reflected cooperativity in the stimulus–response chain. This is why it should be preferred to any other fitting technique.

Two more points of interest with the nlme procedure are that it is now possible (1) to compare each pharmacological parameter in different experimental situations and characterise the agonist effect and (2) to compute simulations particularly useful to study sample size considering all the variances sources in the same model.

However, despite their great interest, nlme methods present several disadvantages. First of all, missing values in the data are absolutely forbidden. The nlme procedures need complete curves, so any incomplete curve has to be removed from the data set. Secondly, for each experimental situation, six parameters have to be estimated (E_m, n, pD_2, σ^2, θ, φ) requiring quite big sample sizes. However, because of ethical and economical considerations, the number of animals included in pharmacological experiments is always small, the risk of overparameterisation is great and the number of necessary equations to estimate all the parameters may not be achieved. Using the fitting methods described in this paper, six complete curves are only, but absolutely, necessary to avoid trouble with overparameterisation.

The nlme models, through the nlme library in R software, allow a lot of combinations of the different effects to solve troubles in the different procedures. If the lack of convergence resists those improvements in the fitting model, then a resampling method is necessary to give the required estimations.[12] Moreover the nlme library also allows one to integrate covariates in the model, which could be interesting to compare the effects in different experimental conditions but it is more difficult to update the model in that way than to compute confidence intervals; details on those computing procedures can be found on our website and questions can be sent to our e-mail address.

Conclusions

The nlme model method described in this paper is satisfactory to estimate curve parameters and to perform their comparison. Compared with analysis of variance and Student's *t*-test, the nlme model method is more valid in relation to statistical hypothesis and provides more accurate information on the pharmacological properties of an agonist. This study should help pharmacologists to overcome their uneasiness about using an nlme model approach to analyse their data when the need arises.
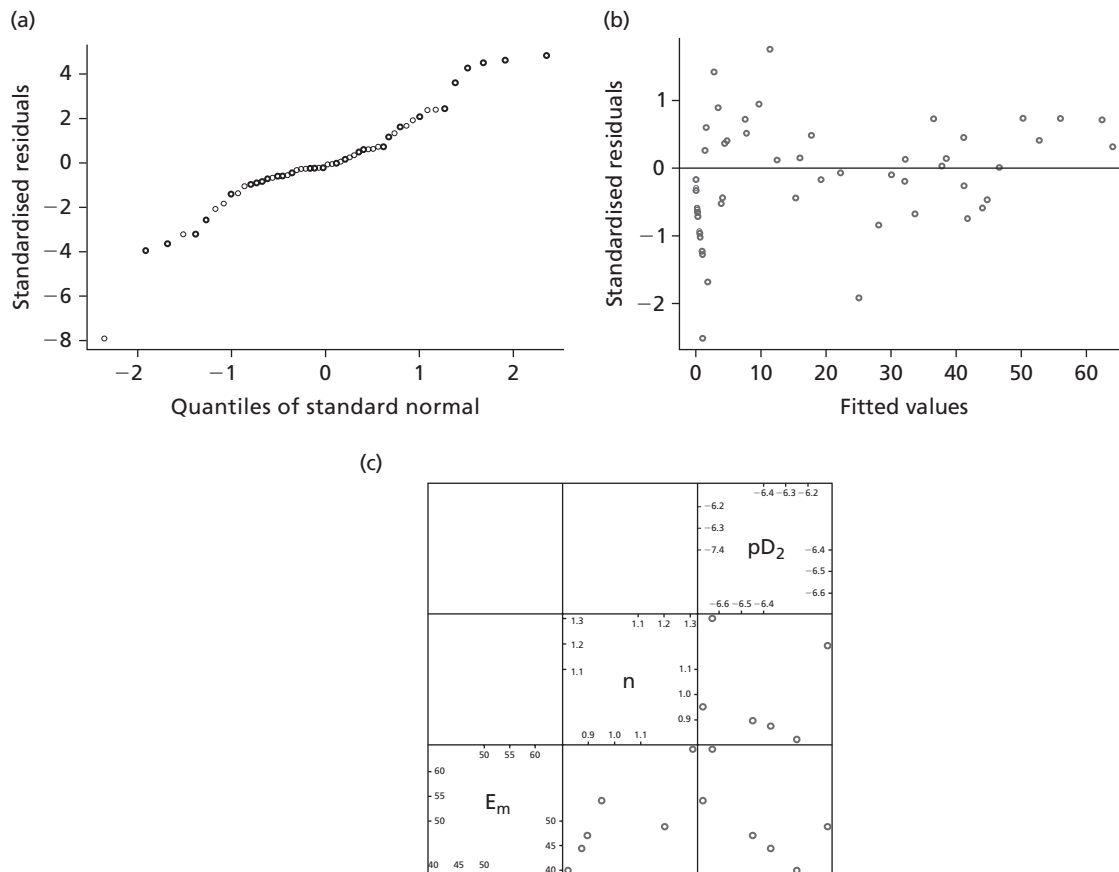
Figure 3 Adequacy for isoprenaline plus nadolol fitted model. (a) Accuracy of the normal distribution of the residuals; the points in the graph are close to the diagonal line with the same scale on each axis then the normality of the residuals is considered achieved. (b) Accuracy of the independence of the residuals with a plot of the standardised residuals versus fitted responses; the residuals were distributed symmetrically around 0 with a quite constant variance. (c) Diagnostic for overparameterisation with a pairs plot; each scatter plot crossed the parameter values and all the scatter plots were dispersed without any drawn direction, the parameters have then been considered as independent

Table 2 Estimated values for E_m, n and pD_2 obtained by individual nonlinear regressions by the conventional method

	E_m	n	pD_2
Isoprenaline			
Mean	84.3	0.79	7.25
Standard error	1.9	0.07	0.15
Isoprenaline + nadolol			
Mean	55.33	0.88	6.25
Standard error	5	0.13	0.19
t-value	5.45	−0.54	4.31
P-value	0.0003	0.6	0.01

For each parameter, the mean displayed is the population estimation and its standard error (SE), *t*-value and *P*-value are the results of the Student's *t*-test performed.

The three parameters E_m, n and pD_2 derived from the Hill equation, an empirical model, can yield useful information for the understanding of drug activity or system sensitivity. However, recent developments in the receptor theory have encouraged improvement of the statistical analysis by leading to development of a new model as an instrument by which a biochemical interpretation can be attempted. The use of mechanistic models (especially for G protein-coupled receptors) would be preferable because the model formulation is then based on the correspondence between the equation parameters and the biochemical constants of the biological process. When based on valid assumptions, mathematical equation parameters can be considered as useful instruments for the understanding of biological function, for characterising ligands and receptors and for identifying more specific therapeutic drugs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

Acknowledgements

References

1. Bretz F *et al*. Combining multiple comparisons and modeling techniques in dose response studies. *Biometrics* 2005; 61: 738–748.

2. Hill AV. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J Physiol (London)* 1919; 40: iv–vii.

3. Kenakin T. Principles: receptor theory in pharmacology. *Trends Pharmacol Sci* 2004; 25: 186–192.

4. Davidian M, Giltinan DM. *Nonlinear Models for Repeated Measurement Data.* New York: Chapman & Hall-CRC, 1995.

5. De Mayer JH *et al*. Porcine left atrial and sinoatrial 5-HT4 receptor-induced responses: fading of the response and influence of development. *Br J Pharmacol* 2006; 147: 140–157.

6. Bidon J-C *et al*. Effect of guinea-pig purified immunoglobulin G1 on the responsiveness of tracheal, aortic, vas deferens and ileum smooth muscles. *Clin Exp Allergy* 1995; 25: 1246–1253.

7. Mallem MY *et al*. Evaluation of beta3-adrenoceptor-mediated relaxation in intact and endotoxin-treated equine digital veins. *Am J Vet Res* 2003; 64: 708–714.

8. Mallem MY *et al*. Impairment of the low-affinity state beta1-adrenoceptor-induced relaxation in spontaneously hypertensive rats. *Br J Pharmacol* 2004; 143: 599–605.

9. Mallem MY *et al*. Beta-adrenoceptor-mediated vascular relaxation in spontaneously hypertensive rats. *Auton Neurosci* 2005; 118: 61–67.

10. Pinheiro JC, Bates DM. *Mixed-Effects Models in S and S-Plus.* New York: Springer-Verlag, 2000.

11. Trochu JN *et al*. Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br J Pharmacol* 1999; 128: 69–76.

12. Huet S *et al*. *Statistical Tools for Nonlinear Regression: a practical guide with S-PLUS and R examples,* 2nd edn. New York: Springer-Verlag, 2004.