Ignoring Pharmacokinetics May Lead to Isoboles Misinterpretation: Illustration with the Norfloxacin-Theophylline Convulsant Interaction in Rats

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Received October 2, 2001; accepted November 7, 2001

Purpose. To investigate the norfloxacin-theophylline convulsant interaction *in vivo*, with an experimental approach distinguishing between pharmacodynamics and pharmacokinetics contributions to the observed effect.

Methods. Male Sprague Dawley rats (n = 38) were infused each compound separately or in different combination ratios. Infusion was maintained until the onset of maximal seizures. Cerebrospinal fluid and plasma samples were collected for high performance liquid chromatography drug determination. The nature and intensity of the pharmacodynamics interaction between drugs was quantified with an isobolographic approach.

Results. Isobolograms suggested a relatively marked antagonism between norfloxacin and theophylline at the cerebrospinal fluid (previously shown to be part of the biophase) and dose levels, but not at the plasma (free and total concentrations) levels. These apparent discrepancies could be explained by nonlinear distribution or/and distribution desequilibrium phenomenon.

Conclusions. These findings showed that the quantitative isobolographic approach is appropriate to assess the nature and intensity of the pharmacodynamic interaction between two drugs when data are collected within the biophase, but that data interpretation outside the biophase can be risky due to further pharmacokinetic complexities, in particular slow or/and nonlinear diffusion into the biophase.

KEY WORDS: isoboles; interaction; fluoroquinolones; norfloxacin; theophylline; seizures; pharmacokinetic-pharmacodynamic modelling.

INTRODUCTION

Pharmacokinetics (PK) has been developing tremendously for several decades both in academia and industry, but pharmaceutical scientists have begun to integrate PK and pharmacodynamics (PD) only much more recently. Indeed PK determines the drug dosage regimen necessary to achieve a target concentration profile and PD determines the target concentration profile required to elicit the desired therapeutic effect (1). An important objective of pharmacokinetic-pharmacodynamic (PK-PD) modelling is to rationalise the frequently observed dissociation between the time courses of drug concentrations and biological effects, which may have several origins including slow distribution to the biophase. In such a case, drug concentrations measured outside the biophase, for example in plasma, at the onset of activity, vary with the input rate. This characteristic was first highlighted by Danhof & Levy (2) and then used on many occasions by this group to investigate the effect of diseases on the PD of drugs such as the convulsant activity of theophylline (3). The approach was subsequently adapted in our laboratory to assess the PK-PD contributions to the convulsant activity of fluoroquinolones (FQs) antibiotics in rats (4,5). However in these studies only one compound was responsible for the observed effect, whereas in clinical practice several drugs are frequently co-administered and interact with each other. Therefore distribution desequilibrium phenomenon, previously investigated following the administration of a single active compound (2–5), should also be considered when more than one compound contribute to the observed effect. We decided to address this question with special reference to the situation in which the interaction between two different active compounds is investigated through an isobolographic approach (6). Theophylline and norfloxacin as a representative FQ, were selected for this study.

MATERIALS AND METHODS

Animals

This work was done in accordance with the Principles of Laboratory Animal Care (NIH Publication #85-23, revised 1985), and the study protocol was approved by the local ethics committee. Male Sprague Dawley rats (n = 38) from Depres Breeding Laboratories (St Doulchard, France) with body weights at arrival ranging within the interval 220–240 g, were housed in the Animal Breeding Facilities of the Laboratory (authorisation No: 0028). The animals were placed in wire cages in a 12-h light/dark cycle for 5 days before the beginning of the experiment to accustom them to their new environment. During this period, they had free access to food (U.A.R. AO4, UAR Laboratories, France) and water.

Surgery

Surgery was carried out as previously described (7,8). Briefly, a polyethylene catheter (0.58 mm inside; 0.96 mm outside diameter, Harvard, France) was used when only one drug was infused (norfloxacin or theophylline), and two polyurethane catheters (0.51 mm inside; 0.71 mm outside diameter, Plastimed Laboratories, France) when the two compounds were infused simultaneously due to physical incompatibilities between the two drug solutions. In any case, catheters were implanted in the right jugular vein of the animals the day before the experiment under a 60 mg kg^{-1} sodium pentobarbital (Sanofi Laboratories, France) intraperitoneal anaesthesia. Following the surgery, the rats were kept under a heating lamp. After first signs of movement, the animals were placed into individual plastic cages. Food was withdrawn 12 h before the experiment, but the animals had free access to water until drug infusion.

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ABBREVIATIONS: FQs, fluoroquinolones; CNS, central nervous system; PD, pharmacodynamics; PK, pharmacokinetics; CSF, cerebrospinal fluid; UF, ultrafiltrat; HPLC, high performance liquid chromatography.

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Solutions for Administration

Theophylline was administered as a 25 mg/ml solution of aminophylline (corresponding to 19.7 mg/ml or 109 mM of theophylline base) for intramuscular or intravenous administration (Assistance Publique des Hôpitaux de Paris, France). Norfloxacin (Sigma, Saint-Quentin Fallavier, France) was infused as a 76.6 mg/ml solution of norfloxacin hydrochloride (corresponding to 240 mM) dissolved in 5% glucose at pH 5.5 (4).

Drug Administration

The day after surgery, the jugular vein cannula was connected to a 2-way motor-driven syringe pump (SE400B, Vial Medical, France) equipped with two syringes containing norfloxacin for one and theophylline for the other one. Flow rates of each syringe were adjusted to achieve the desired rate of drug delivery (Table I). The total flow rate was equal to 4 ml/h. The rats were kept under a heating lamp to maintain body temperature. The infusion was stopped when the animals exhibited maximal seizures. Onset of maximal seizures was usually evidenced by tonic flexion of the forelimbs and tonic extension of the hind limbs. The total volume infused ranged between 0.90 and 4.25 ml. Drug administration was conducted between 2:00 p.m. and 7:00 p.m.

Samples Collection

Immediately after exhibiting maximal seizures, the rats were anaesthetised with an intramuscular injection of ketamin (KETALAR®, 50 mg/ml, Parke Davis Laboratories, France) plus xylazin hydrochloride (ROMPUN®, Bayer Laboratories, France), unless they had died following seizures. CSF was collected following the end of infusion, as previously described (4). Blood was subsequently withdrawn from the heart, collected in heparinised tubes (VACUTAINER®, Becton Dickinson, France) and immediately centrifuged at 3000 r.p.m. for 10 min at 37°C (CR 312 model, Jouan, France). Plasma was transferred into two separate tubes. One fraction was kept frozen at −20°C until HPLC drug determination. The other fraction was ultrafiltered by using a Centrifree system (CF50A model, Amicon, France) for determination of unbound concentrations.

Drug Analysis

Total (C_P) and unbound (C_{UF}) plasma concentrations, as well as cerebrospinal fluid (C_{CSF}) concentrations of norfloxacin and theophylline were determined simultaneously by HPLC as previously described (4,7).

Data Modelling

The nature of the convulsant interaction between norfloxacin and theophylline was analysed at the levels of Doses, plasma, UF, and CSF using a plausible model for the isobol (Eq. 1) (7):

$$\frac{C_{Theo}}{C_{0,\,Theo}} = \frac{1 - \dfrac{C_{Nor}}{C_{0,\,Nor}}}{1 + \alpha \times \dfrac{C_{Nor}}{C_{0,\,Nor}}} \quad (1)$$

where $C_{0,Nor}$ and $C_{0,Theo}$ are the geometric means of the concentrations (or doses) of norfloxacin and theophylline respectively, at the onset of activity when these drugs were administered alone, C_{Nor} and C_{Theo} are the concentrations (or Doses) of norfloxacin and theophylline respectively, at the onset of activity when these drugs were administered in combination, and α is the apparent interaction parameter according to Loewe, estimated from CSF concentrations (α_{CSF}), free (α_{UF}) and total (α_P) plasma concentrations, and Doses (α_{Dose}). Modelling was performed with WinNonlin, version 1.1 (SCI Software, Carry, NC, USA), with uniform weighting. Significance of α values was assessed by forming the 95% confidence interval of the estimates, α were considered significant if their confidence interval excluded zero (7).

The relationship between CSF and unbound plasma concentrations for each drug was analysed by comparison of a linear (Eq. 2) and a non-linear (Eq. 3) model (9–11):

$$C_{CSF} = Kd \times C_{UF} \quad (2)$$

$$C_{CSF} = \frac{C_{CSF,\,max} \times C_{UF}}{C_{UF,50} + C_{UF}} \quad (3)$$

where Kd is the distribution coefficient of the unbound drug between CSF and unbound plasma, $C_{CSF,max}$ is a maximum drug concentration achievable into the CSF according to the model, and $C_{UF,50}$ is the unbound concentration corresponding to half $C_{CSF,max}$. Modelling was performed with WinNonlin, version 1.1 (SCI Software, Carry, N.C., USA) with uniform weighting. Discrimination between linear and non-linear models of CSF diffusion was assessed from different criteria, including visual inspection, residual analysis, sum of squared residuals, correlation coefficient between observed and predicted values, and Akaïke information criteria (10,11). Results are presented as mean ± s.d.

RESULTS

A total of 38 rats were used in this study. However, technical problems such as failure in determining the infusion time or in obtaining plasma concentrations as well as blood contamination of CSF samples, accounted for the loss of several values. Under these experimental conditions, maximal seizures occurred within 29.2 ± 6.1 min when norfloxacin was administered alone to 50.8 ± 6.0 min when theophylline was administered alone (Table I). The isobolograms related to Doses, C_P, C_{UF}, and C_{CSF} are displayed on Fig. 1 and suggest a relatively strong antagonism at the levels of Doses and CSF,

Table I. Summary of Experimental Conditions of the Interaction Study, Data Are Presented as Algebraic Mean ± s.d.

Drug combination				
Nor/Theo ratio[a]	Nor/Theo ratio[b]	Number of animals	Body weight (g)	Infusion time (min)
4/0	infinity	11	255 ± 24	29.2 ± 6.1
2/2	2.2	7	272 ± 24	58.0 ± 8.7
1.5/2.5	1.3	6	251 ± 6	55.3 ± 6.1
1/3	0.7	3	251 ± 1	58.8 ± 6.2
0/4	0	11	257 ± 15	50.8 ± 6.0

[a] Ratio of flow rates; the total flow rate was constant at 4.0 ml h^{-1}.
[b] Ratio of input rates in molar units.

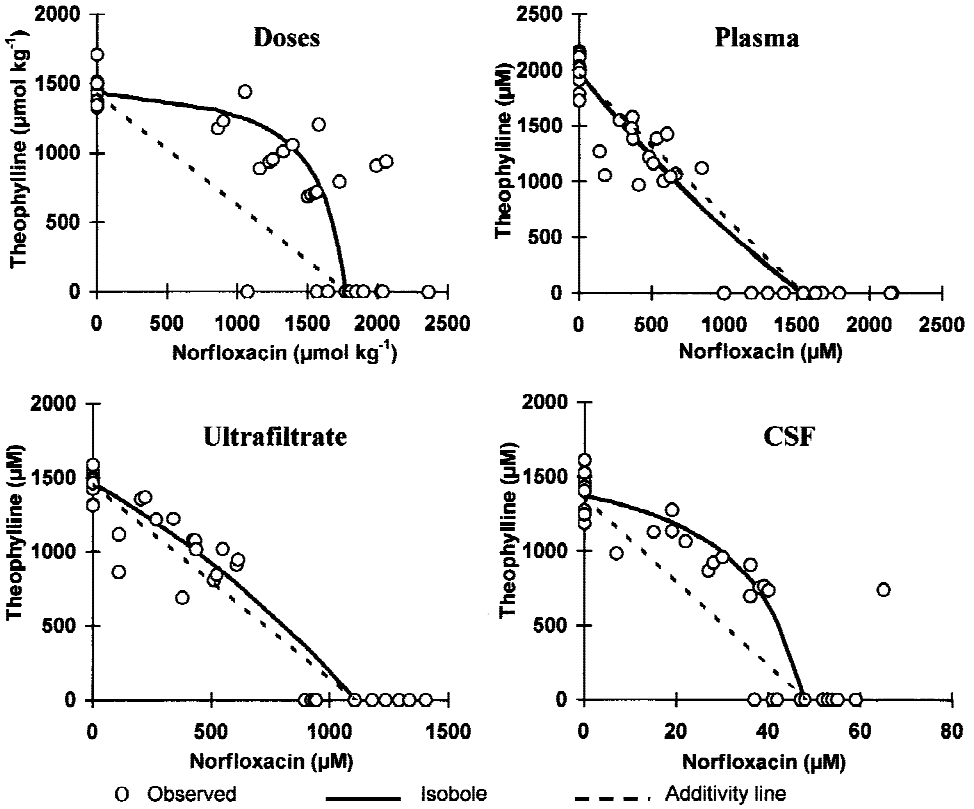

Fig. 1. Isobolographic representation of the convulsant interaction between theophylline and norfloxacin in rats at the level of doses, plasma, UF and CSF. Each point is the actual injected dose of norfloxacin and theophylline alone or in combination (n = 38), or the corresponding plasma (n = 38), UF (n = 37) and CSF (n = 34) concentrations. The solid line is the isobole simulated from Eq. 1. The dashed line is the theoretical isobole for additivity, which connects the mean dose or concentration of theophylline to that of norfloxacin when each drug is given alone.

but additivity of effect at the plasma (free and total) level. This finding was confirmed by the values of the corresponding apparent interaction parameters α (Table II). The CSF diffusion of theophylline was found linear with a parameter Kd calculated as 0.91 ± 0.02 whereas the CSF diffusion of norfloxacin was found non-linear, with $C_{CSF,max}$ and $C_{UF,50}$ estimated to 67.8 ± 10.1 µM and 443 ± 171 µM respectively (Fig. 2). Theophylline and norfloxacin were essentially unbound in plasma with free fractions respectively equal to 75±6 % and 74±11%.

DISCUSSION

The convulsant activities of both norfloxacin and theophylline administered in these conditions are related to their CSF concentrations, but not necessarily to their plasma concentrations, because the former but not the latter is part of the biophase (3,4). As a consequence, the PD interaction can only be investigated at the CSF level. Therefore only the α value estimated in CSF (α_{CSF}) allows characterisation of the pharmacodynamic interaction between the two compounds. Its negative value indicates an antagonistic interaction (7). Although this does not provide information about the interaction mechanism, it is interesting to note that antagonistic interactions have also been observed in previous experiments on the convulsant interactions between pefloxacin and theophylline (7) and between pefloxacin and norfloxacin (8), with α_{CSF} estimates respectively equal to –0.567 ± 0.079, and –0.766 ± 0.062, close to the value obtained in the present study (–0.775 ± 0.073). It could then be argued that the α_{CSF} values estimated in these various studies are close to each other.

Table II. Mean Values (Geometric Average) of Convulsant Doses (µmol.kg^{-1}) and Concentrations (µM) for Norfoxacin and Theophylline When Each Drug Was Given alone, and Apparent Interaction Parameter α Estimated at the Various Levels, followed by the 95% Confidence Interval into Brackets and the Number of Values Used for Calculation Into Parentheses

	Norfloxacin	Theophylline	α values
Dose	1774 [1577; 1996] (11)	1438 [1375; 1504] (11)	–0.897 ± 0.033 [–0.962; –0.832]
Plasma	1529 [1330; 1758] (11)	1974 [1892; 2058] (11)	0.274 ± 0.199 [–0.116; 0.664]
UF	1111 [999; 1236] (10)	1466 [1415; 1520] (11)	–0.283 ± 0.122 [–0.640; 0.076]
CSF	47.7 [43.7; 52.1] (11)	1370 [308; 6102] (9)	–0.775 ± 0.073 [–0.918; –0.632]

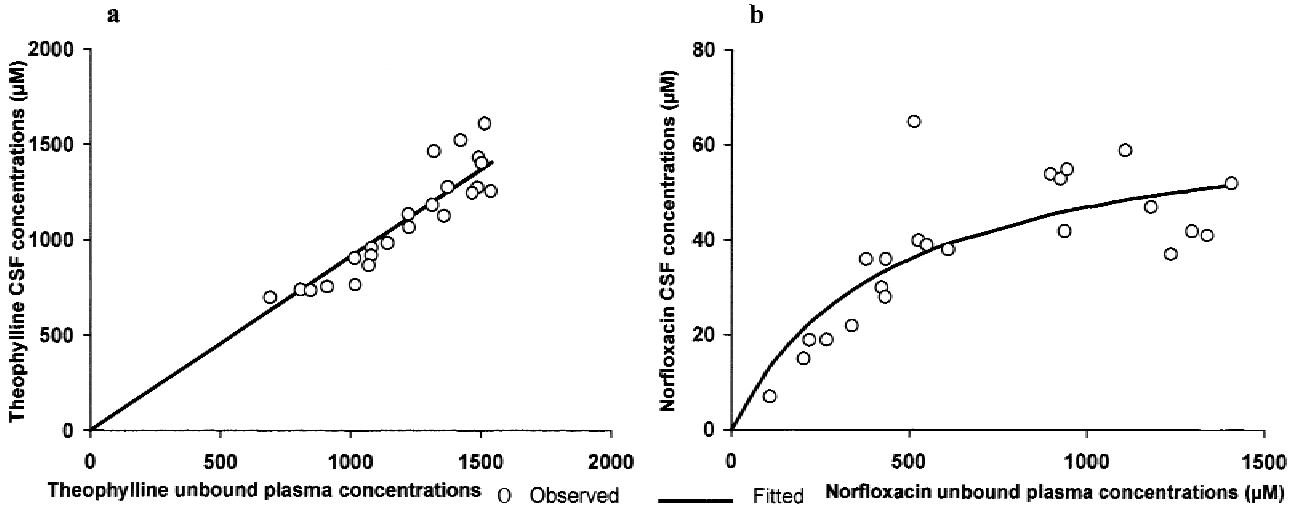

Fig. 2. Theophylline (Fig. 2a) and norfloxacin (Fig. 2b) CSF concentrations vs. UF concentrations. (Fig. 2a) pairs of data (n = 33) were fitted with a linear model (Eq. 2, solid line), with a parameter Kd calculated as 0.91 ± 0.02. (Fig. 2b) pairs of data (n = 33) were fitted with a non-linear model (Eq. 3, solid line), the two parameters CSF,max and $C_{UF,50}$ were estimated to 67.8 ± 10.1 µM and 443 ± 171 µM respectively.

However since α cannot take a value less than –1 in regards to the structure of the model (Eq. 1) (7), an α_{CSF} value of –0.775 corresponds to a really more antagonistic interaction than an α_{CSF} value of – 0.567, and so to a much greater observable curvature of the isobole.

Isobolograms analysis showed an apparent inconsistency between the value of the interaction parameter estimated in CSF and those obtained in plasma (total and unbound) (Table II). The relationships existing between the drugs concentrations in the various biological fluids are summarised on Fig. 3, adapted from a previous one (11) to a situation in which the two compounds exerted a similar type of PD effect. Figure 3 is the illustration that CSF data constitute the driving force of the system. The initial interaction model (Eq. 1) describing CSF data, may therefore apply to free plasma concentrations only if the C_{CSF}/C_{UF} ratios of the two drugs are constant throughout the experiment. This was not the case since the norfloxacin C_{CSF} vs. C_{UF} concentrations relationship was obviously non-linear (Fig. 2b). Therefore a new and indirect relationship had to be derived to

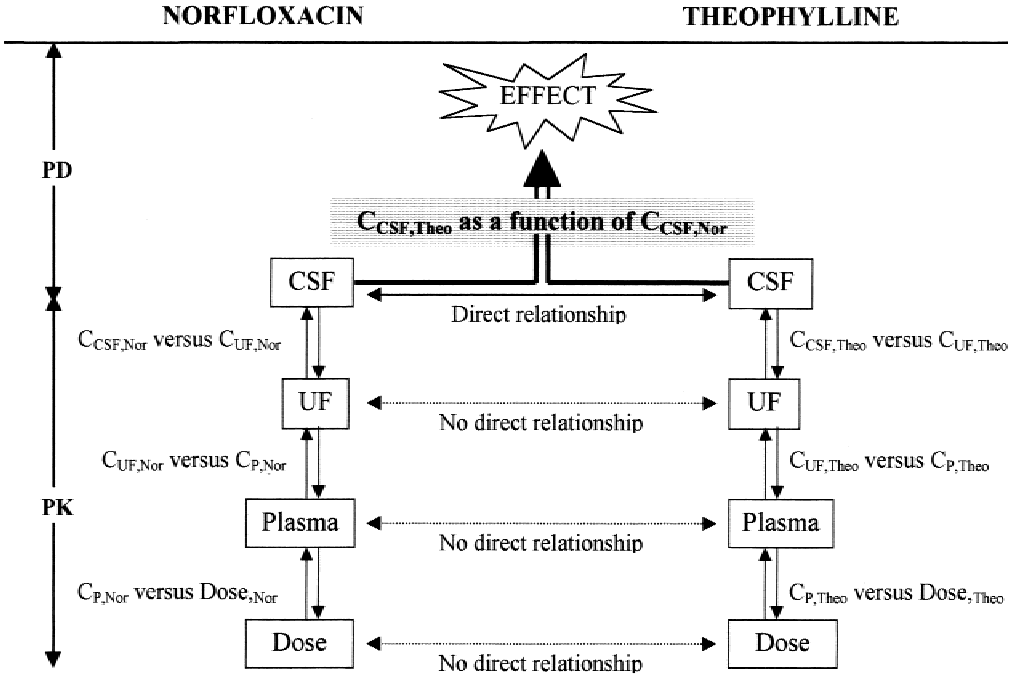

Fig. 3. Schematic representation of the convulsant interaction between norfloxacin and theophylline with distinction between the pharmacodynamic interaction of the two compounds in the CSF (biophase) and the pharmacokinetic relationships characteristic of the CSF diffusion and the plasma protein binding of each compound.

properly characterise the interaction at the unbound plasma concentrations level, taking into account: i) the PD interaction between norfloxacin and theophylline according to Loewe; and ii) the apparently non-linear CSF diffusion of norfloxacin.

It was previously shown using several infusion rates that the C_{CSF}/C_{UF} ratio of norfloxacin decreased from 0.063 ± 0.025 to 0.038 ± 0.008, without substantial change in C_{CSF}, but solely because the infusion duration decreased from 69.4 ± 8.9 to 12.9 ± 2.3 min (4). Therefore this apparent non-linearity could simply reflect a distribution desequilibrium since the C_{CSF}/C_{UF} ratio decreased when the proportion and therefore the concentrations of norfloxacin increased, and at the same time when the infusion duration decreased from 50.8 ± 6.0 to 29.2 ± 6.1 min (Table I). Empirical functions other that Eq. 3 could have been proposed to describe the apparently non-linear CSF diffusion of norfloxacin, in particular a power function. However only Eq. 3 permitted to reconcile data obtained in the various biological fluids, as illustrated on Fig. 4. The new relationship between $C_{UF,Theo}$ and $C_{UF,Nor}$ (Eq. 4) was obtained by integrating Eq. 2 and Eq. 3 into Eq. 1:

$$\frac{C_{UF,\,Theo}}{C_{UF0,\,Theo}} = \frac{1 - \dfrac{C_{UF,\,Nor}}{C_{UF0,\,Nor}}}{1 + \beta \times \dfrac{C_{UF,\,Nor}}{C_{UF0,\,Nor}}} \qquad (4)$$

with

$$\beta = \frac{C_{CSF0,\,Nor} \times (1 + \alpha_{CSF})}{C_{CSF,\,max} - C_{CSF0,\,Nor}} + \alpha_{CSF}$$

Complementary experiments are presently conducted in the laboratory to better investigate this apparent non-linearity, which has not only been previously observed with norfloxacin (11), but also with meropenem, another antibiotic with limited CSF diffusion (10).

As opposed to a previous situation in which the saturable protein binding of one of the two compounds had to be taken into consideration for the analysis of total plasma concentrations data (11), norfloxacin and theophylline are essentially unbound in plasma and therefore isoboles obtained from unbound and total plasma concentrations were virtually similar (Fig. 1). Corresponding α values were non significantly different from zero, suggesting an additive interaction (Fig. 1). However, differences appeared between the isobologram obtained from plasma concentrations and from Doses. This second apparent inconsistency was due to the fact that the norfloxacin C_P/Dose ratio was not constant as a consequence of a variation of the infusion time with the proportion of each drug infused (Table I). Variation of infusion duration had no effect on the theophylline C_P/Dose ratio because this drug distributes very rapidly following intravenous infusion in rats (12), but had an effect on the norfloxacin C_P/Dose ratio, because this compound distributes more slowly within the body, with a pharmacokinetic behaviour in rats characterised by a marked open two-compartment model (13). Simple rearrangement of the equation that predicts plasma concentration at the end of infusion for such a drug, describes how the Cp/Dose ratio varies with the infusion time (Eq. 5):

$$\frac{C_p}{Doses} = \frac{1}{V_c \times k_{10} \times t} \times \left(1 + \frac{\lambda_2 - k_{10}}{\lambda_1 - \lambda_2} \times e^{-\lambda_1 t} + \frac{k_{10} - \lambda_1}{\lambda_1 - \lambda_2} + e^{-\lambda_2 t}\right) \qquad (5)$$

where Vc is the volume of the central compartment, k_{10} the elimination rate constant from the central compartment, λ_1 and λ_2 the usual disposition rate constants, and t the duration of infusion.

Data presented on Fig. 5 illustrate the good accordance between the C_P/Dose ratios determined experimentally and the corresponding values predicted by the model using mean values of parameters, coefficients and constants estimated from the literature (13). The apparent discrepancy observed between the interaction data at the C_P and Doses levels can

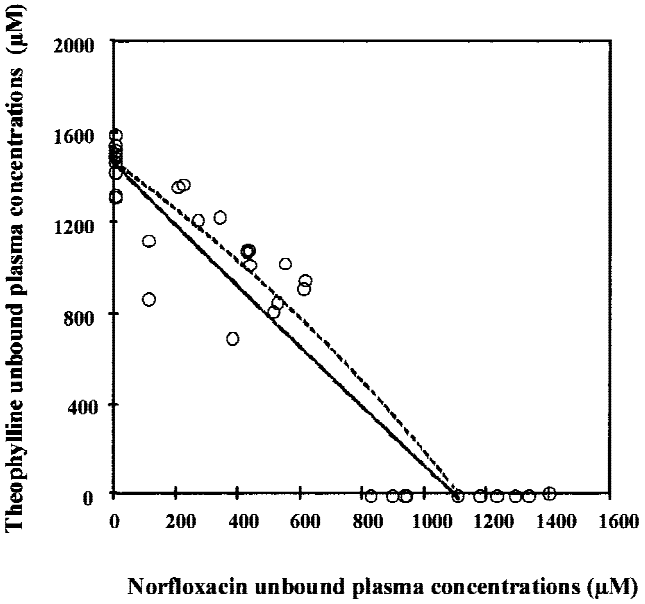

Fig. 4. Isobolographic representation of the convulsant interaction between theophylline and norfloxacin in rats at the level of unbound plasma concentrations. Data points correspond to measured free plasma concentrations. The dotted line simulated from Eq. 4, with previous estimates of α_{CSF} and $C_{CSF,max}$ (respectively –0.775 and 67.8 µM), was close to the solid line obtained by fitting Eq. 4 to the data, and would have been almost superimposed to the curve obtained by fitting Eq. 1 to unbound plasma concentrations data, presented on Fig. 1.

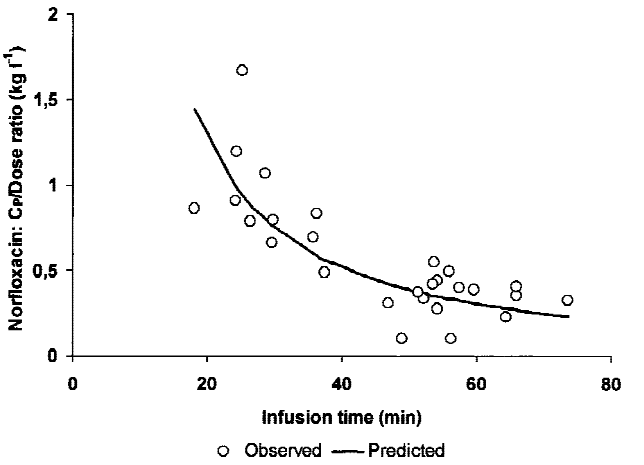

Fig. 5. Norfloxacin C_P/Dose ratio vs. infusion durations, the solid line was simulated from Eq. 5 and each point represents data from one rat (n = 27).

therefore be easily explained. Most often during pharmacology or toxicology studies drugs are administered by an extravascular route, such as the oral or intraperitoneal route, and it is then impossible to control the input rate of each compound. This is for exemple what happens when the proconvulsant effect of a new drug on a reference convulsant compound such as pentylenetetrazol is investigated. It must then be reminded that in such a case, not controlling the input rate of the compounds tested may introduce variability and even bias in the results, as shown in the present study.

In conclusion, this study has demonstrated that the PD interaction between the convulsant activities of norfloxacin and theophylline in rats is strongly antagonistic, and that this interaction can only be adequately investigated from data collected within the biophase. More generally this study has provided experimental evidence: i) that isobolographic interpretation from plasma concentrations may lead to false conclusions when the distribution of one compound within the biophase is delayed or/and saturable, and ii) that analysis of the isobolograms obtained from doses must be cautious because of the lack of control of the input rate of the compounds tested, which may also lead to false interpretation.

ACKNOWLEDGMENT

Isabelle Martineau is gratefully acknowledged for her excellent assistance in performing the experiments.

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