Interpretation and Utilization of Effect and Concentration Data Collected in an *in Vivo* Pharmacokinetic and *in Vitro* Pharmacodynamic Study

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The effect (E) of some drugs may be monitored *in vitro* using the plasma drug concentration (C) following the *in vivo* dosing of drug. When using a specific analytical assay, counterclockwise hysteresis in the E vs C relationship can be explained only by the presence of an agonistic metabolite (M_A); the extent of hysteresis will depend upon the pharmacokinetics and relative "potency" of C and M_A. If a nonspecific assay is used, plots of E vs C may actually relate to E vs total agonist ($C + M_A$), and unusual hysteresis may be observed, e.g., clockwise hysteresis when C is more "potent" than M_A. Here, we simulate data for three models of relative C and M_A pharmacokinetics. The E vs C and E vs M_A data are simulated for both linear and noncompetitive agonist E_{max} models. When C and M_A are equally potent, hysteresis will not be observed in a plot of E vs $C + M_A$. However, when C and M_A are of differing "potencies," hysteresis will be observed (the direction of hysteresis is dependent on the relative potency of C and M_A). By appropriately "weighting" a respective agonist (C or M_A), hysteresis will "collapse" and the relative potencies of C and M_A can be estimated.

KEY WORDS: *in vitro;* pharmacokinetics-pharmacodynamics; hysteresis; metabolite(s); nonspecific analytical assay; drug discovery/development.

INTRODUCTION

Increasing attention is being given to pharmacokinetic-pharmacodynamic (PK-PD) modeling as part of the drug discovery/development program (1). A significant advance in pharmacodynamic modeling was achieved with the proposal of the "link model" (2), allowing estimation of the *in vivo* equilibrium relationship between effect (E) and plasma drug concentration (C) from non-steady-state "E vs time" and "C vs time" data. Justifiably, the conceptual emphasis has been placed upon interpretation of "apparent temporal displacement," i.e., hysteresis, in the relationship between C and *in vivo* measurements of E. However, it is not uncommon, especially for certain types of therapeutic groups such as immunosuppressants and antibiotics, to have a study design where the C vs time relationship is determined following *in vivo* non-steady-state dosing of parent drug and then the sampled plasma matrix is utilized in an *in vitro* pharmacodynamic study to estimate the E vs C relationship. The mixed lymphocyte reaction (MLR) following exposure to plasma samples containing the immunosuppressive agent cyclosporine (3) is an example of such an *in vitro* E vs C study. When E is estimated *in vitro* and E vs C plots generated, and connected in ascending order of time, potential for hysteresis in the relationship between E and C still exists, although its interpretation may be conceptually somewhat more straightforward than with the *in vivo* study design.

However, in the early stages of drug discovery/development, some additional factors need to be considered that may influence the interpretation and utilization of *in vitro* E vs C data: (a) the use of a nonspecific analytical assay that does not differentiate between parent compound and metabolite(s), or between enantiomers following racemate administration, and/or (b) the presence of a pharmacologically active metabolite(s) generated *in vivo*. The aims of this present simulation study are (i) to examine the possible causes for, and resultant direction of, hysteresis in *in vivo* pharmacokinetic–*in vitro* pharmacodynamic studies and (ii) to examine, when a specific analytical assay is utilized, an approach that collapses counterclockwise hysteresis in an *in vitro* E vs C relationship allowing estimation of the relative "potency" of parent compound and any *in vivo* generated agonistic metabolite(s). As a corollary, the *in vitro* pharmacodynamics of parent compound and metabolite will allow confirmation that all *in vivo* generated active metabolites have been identified. It should be noted that this technique is equally applicable to the evaluation of enantiomer PK-PD (4), and as such, in this report where parent compound metabolites are discussed, reference could also be made to R and S enantiomers.

THEORY

Assuming the use of a specific analytical assay and that plasma concentration measurements of parent drug reflect unbound concentrations (i.e., assume either linear binding kinetics or measurement of unbound concentrations), then the presence of "apparent temporal displacement" resulting in counterclockwise hysteresis in the relationship between measured *in vivo* effect (E) and measured plasma parent drug concentrations (C) can reflect, as detailed below, nonequilibrium, nonstationarity (5,6), and/or the respective formation of antagonistic or agonistic metabolites (7).

Counterclockwise hysteresis can be explained by any of the following.

(a) "Nonequilibrium" results from any of the following: (i) instantaneous equilibrium between C and "effect-site" concentrations (Ce) is not achieved such that $Ce_{(t)}$ does not equal $C_{(t)}$, resulting in temporal displacement between Ce and C; (ii) the rate of change of Ce is much greater than the rates for pharmacological "receptor" deactivation/activation, such that the number of activated "receptors" (R^*) at time t is not reflective of Ce at time t, resulting in temporal displacement between R^* and Ce; (iii) the rate of signal transduction leading from R^* to measured E is much less than the rate of change of R^* such that $E_{(t)}$ does not equal $R^*_{(t)}$, resulting in temporal displacement between E and R^*.

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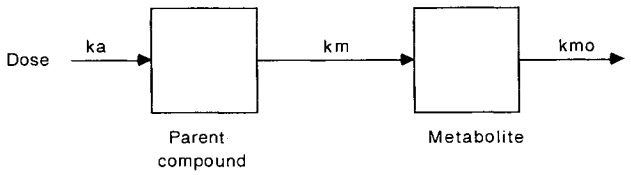

Fig. 1. Compartmental model of parent compound and metabolite pharmacokinetics used in simulations.

(b) A metabolite(s) with agonist actions (M_A) upon the same measured E utilized for the parent compound (PAR) is formed. The M_A may act either as a competitive agonist, i.e., the same receptor but with sufficient pharmacological activity such that competitive agonism does not occur [i.e., $E_{max(PAR)}$ is not much greater than $E_{max(MA)}$], or as a noncompetitive agonist, e.g., a different pharmacological receptor but the same E.

(c) Nonstationarity in the pharmacodynamics of the parent compound means that the pharmacodynamic relationship possesses a temporal component. For example, upregulation of pharmacologic response where, for example, EC_{50} may decrease with respect to time.

Similarly, clockwise hysteresis can be explained by any of the following.

(a) "Nonequilibrium" occurs when Ce precedes C in the time domain, e.g., the rate of equilibration between arterial plasma concentrations (compartment delivering drug to effect site) and venous plasma concentrations (sampling compartment for concentration analysis) is much less than the rate of equilibration between arterial concentrations and Ce.

(b) A metabolite(s) with antagonistic actions (M_T) is formed, acting either as a competitive antagonist, i.e., interacting at the same pharmacological receptor as the parent compound but essentially lacking any pharmacological activity [i.e., $E_{max(MT)}$ is much less than $E_{max(PAR)}$] such that competitive "receptor blockade" results, or as a noncompetitive antagonist, e.g., interacting with a pharmacological receptor different from that of the parent compound, and with effects opposing the measured E.

(c) There is nonstationarity in the pharmacodynamics of the parent compound, e.g., downregulation of the pharmacologic response, where, for example, EC_{50} may increase and/or E_{max} may decrease with respect to time.

However, when using an *in vivo* pharmacokinetic–pharmacodynamic study design, each separate *in vitro* incubate represents the measurement of a single E vs C observation conducted, for all incubates, at a single fixed time interval following plasma additions. Therefore each pair of E vs C observations will be subject to the same "prereceptor," "receptor," and "postreceptor" nonequilibrium phenomenon, i.e., the temporal component in $C_{(t)} \rightarrow Ce_{(t)} \rightarrow R^*_{(t)} \rightarrow E_{(t)}$ will be the same for each incubate, and hysteresis in the *in vitro* E vs C relationship resulting from nonequilibrium will not arise. In addition, the assumption of stationarity in parent compound pharmacodynamics should be maintained, as there will no previous exposure of the "pharmacological tissue" to the drug. However, if time-dependent changes in the pharmacodynamics were to occur in the *in vitro* system, e.g., in receptor number, affinity, or the signal transduction pathway, it would not lead to hysteresis in the E vs C relationship, as all incubates will have been exposed to the same experimental conditions. More importantly, these two assumptions can be validated by independent experiments using spiked plasma. Thus in an *in vitro* E vs C relationship the occurrence of counterclockwise or clockwise hysteresis can be explained only by the *in vivo* generation of a metabolite(s) (or endogenous compounds) that results in net agonistic or antagonistic E, respectively.

METHODS

To evaluate the concept of "collapsing" counterclockwise hysteresis in an *in vitro* E vs C relationship, allowing estimation of the relative "potency" of parent compound and an *in vivo* generated agonistic metabolite (M_A), a series of "plasma concentration vs time" and "effect vs plasma concentration" data was generated. For parent compound and M_A, plasma unbound concentrations (C and C_{MA}, respectively) were simulated (PCSAS 6.04, SAS Institute Inc, NC) based upon the compartmental model in Fig. 1, assuming first-order kinetics for both parent compound and metabolite. Parent compound pharmacokinetics were described

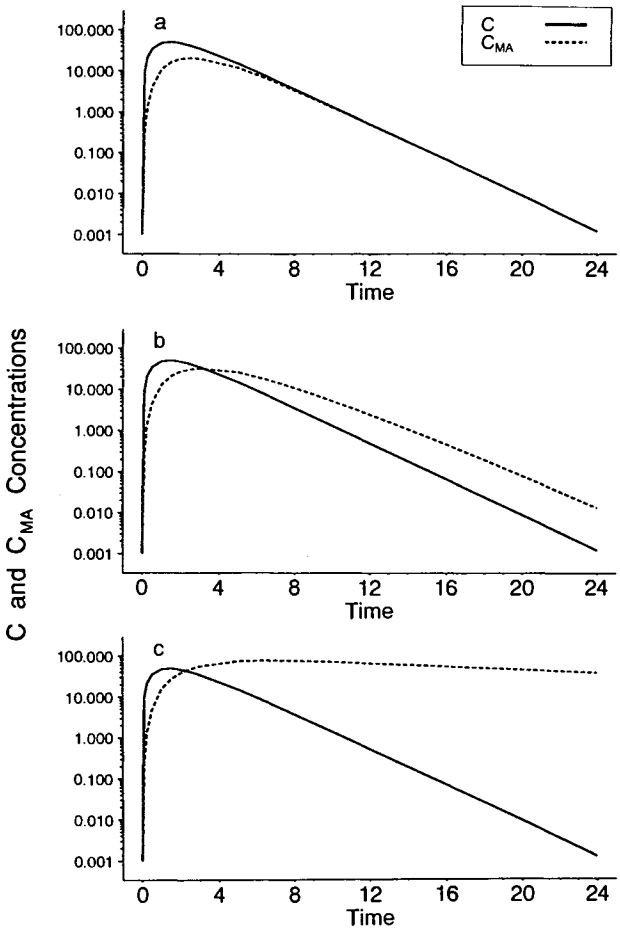

Fig. 2. *In vivo* pharmacokinetic plots of unbound plasma concentration for parent compound (C) (solid line) and agonistic metabolite (C_{MA}) (dashed line) vs time, where k_{mo} = 0.99 (a), k_{mo} = 0.49 (b), and k_{mo} = 0.05 (c).

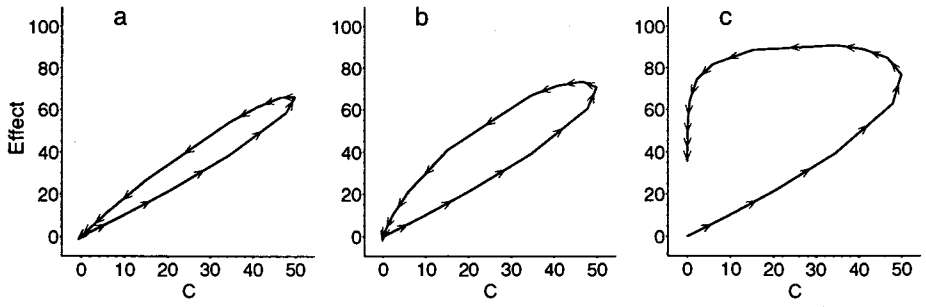

Fig. 3. *In vitro* pharmacodynamics: plots of observed effect (E) vs unbound plasma concentration for parent compound (C) showing counterclockwise hysteresis. The pharmacodynamics of parent compound and agonistic metabolite (M_A) are described by a linear model [Eq. (3)], with $P_{PAR} = P_{MA} = 1$ and $k_{mo} = 0.99$ (a), $k_{mo} = 0.49$ (b), and $k_{mo} = 0.05$ (c).

by Eq. (1), while Eq. (2) was utilized to describe the pharmacokinetics of M_A.

$$C(t) = \frac{\text{Dose } k_a}{V}\left[\frac{e^{-k_m t}}{(k_a - k_m)} + \frac{e^{-k_a t}}{(k_m - k_a)}\right] \quad (1)$$

$$C_{MA}(t) = \frac{\text{Dose } k_a k_m}{V_m}\left[\frac{e^{-k_{mo} t}}{(k_a - k_{mo})(k_m - k_{mo})} + \frac{e^{-k_m t}}{(k_a - k_m)(k_{mo} - k_m)} + \frac{e^{-k_a t}}{(k_m - k_a)(k_{mo} - k_a)}\right] \quad (2)$$

where Dose = 100 mass units; $V = 1$ and $V_m = 1$ volume units; $k_a = 1$ and $k_m = 0.5$ time unit^{-1}; and k_{mo} (time units^{-1}) was varied to allow three profiles for M_A pharmacokinetics, such as where M_A kinetics are (i) not formation rate limited, with $k_{mo} = 0.05$; (ii) in an intermediate situation, $k_{mo} = 0.49$; and (iii) formation rate limited, with $k_{mo} = 0.99$.

The *in vitro* effect vs plasma concentration data were generated using two pharmacodynamic models. First, the effect was considered as a linear function of the drug and metabolite concentrations, Eq. (3):

$$E = P_{PAR} \cdot C + P_{MA} \cdot C_{MA} \quad (3)$$

where the parent compound pharmacologic "potency" (P_{PAR}) was arbitrarily set to 1, and P_{MA} was varied to allow three pharmacological "potencies" for M_A, i.e., (i) where M_A is "equipotent" with the parent compound, with $P_{PAR} = P_{MA} = 1$; (ii) where the M_A "potency" is less than that of the parent compound, with $P_{MA} = 0.33$; and (iii) where the M_A potency is greater than that of the parent compound, with $P_{MA} = 3$.

In the second case, a competitive agonist E_{max} model was utilized, Eq. (4):

$$E = E_{max}\left(\frac{C}{EC_{50}}\frac{C_{MA}}{EC_{50_{MA}}}\right)\Big/\left(1 + \frac{C}{EC_{50}} + \frac{C_{MA}}{EC_{50_{MA}}}\right) \quad (4)$$

where E_{max} was set to 100 effect units, parent compound pharmacologic potency (EC_{50}) was set to 10 mass/volume units, and $EC_{50_{MA}}$ (mass/volume units) was varied to allow three pharmacological potencies for M_A, i.e., (i) where M_A is equipotent with the parent compound, with $EC_{50} = EC_{50_{MA}} = 10$; (ii) where the M_A potency is less than that of the parent compound, with $EC_{50_{MA}} = 30$; and (iii) where the M_A potency is greater than that of the parent compound, with $EC_{50_{MA}} = 3.33$.

Random error was incorporated into the concentration and effect variables according to Eq. (5), where Y_R is the observation incorporating the random error; Y_M is the model simulated "errorless" value; and ϵ represents the error term, with $\epsilon = 0.1$ (i.e., 10%) for concentration vs time data, and $\epsilon = 0.2$ (i.e., 20%) for the effect vs concentration data.

$$Y_R = Y_M (1 + \epsilon N) \quad (5)$$

N is a normally distributed random number (RANUNI func-

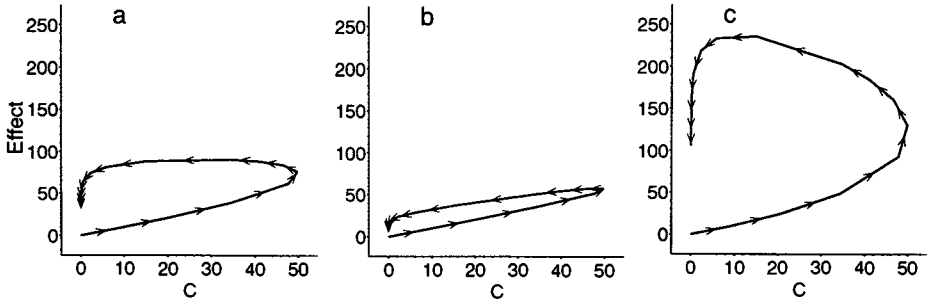

Fig. 4. *In vitro* pharmacodynamic plots of observed effect (E) vs unbound plasma concentration for parent compound (C) showing counterclockwise hysteresis. The pharmacodynamic parent compound and agonistic metabolite (M_A) are described by a linear model [Eq. (3)] and where, for M_A pharmacokinetics, $k_{mo} = 0.05$ and $P_{PAR} = P_{MA} = 1$ (a), $P_{PAR} = 1$ and $P_{MA} = 0.33$ (b), and $P_{PAR} = 1$ and $P_{MA} = 3$ (c).

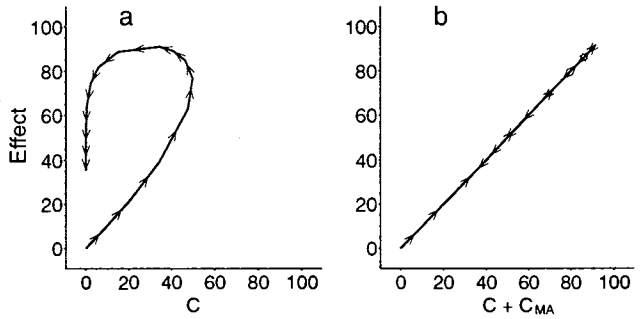

Fig. 5. (a) Plot of observed effect (E) vs unbound plasma concentration for parent compound (C) showing counterclockwise hysteresis. The pharmacodynamics of parent compound and agonistic metabolite (M_A) are described by a linear model [Eq. (3)], with P_{PAR} = P_{MA} = 1 and k_{mo} = 0.05. (b) Plot of observed effect (E) vs unbound plasma concentration (C + C_{MA}) for parent compound and M_A, showing "collapsed" hysteresis, where the pharmacokinetic-pharmacodynamic model is as in a.

tion, PCSAS) between −1 and 1, with a mean of 0. Fifty sets of concentration vs time and effect vs concentration data were generated for each combination of PK–PD model examined, each data set generated having 14 observations.

Analysis of the simulated concentration vs time and effect vs concentration data sets involved empirical fits of cubic spline functions to the C vs t and C_{MA} vs t profiles, while parametric models [Eqs. (3) and (4)] were utilized for the effect vs concentration data. As a further component of the analysis, plots of E vs C + C_{MA} were generated. In the plots of E vs C + C_{MA} no hysteresis was observed if the parent compound and M_A were equipotent, i.e., P_{PAR}/P_{MA} ratio = 1, or $EC_{50}/EC_{50_{MA}}$ ratio = 1. However, if the parent compound is more potent than the M_A, then clockwise hysteresis will be observed, while if the parent compound is less potent than the M_A, then counterclockwise hysteresis will be observed. Such hysteresis in the E vs C + C_{MA} plot was "minimized" by varying the P_{MA} parameter in the linear model, Eq. (3), or the $EC_{50_{MA}}$ parameter in the E_{max} model, Eq. (4), reflecting the relative potency of M_A vs parent compound.

RESULTS AND DISCUSSION

Figure 2 shows the simulated plasma concentration vs time plots for parent compound (C) and metabolite (C_{MA}), with M_A accumulating significantly when k_{mo} = 0.05. When the analytical assay is specific, Fig. 3 shows that, for a given set of pharmacodynamic parameters, e.g., P_{PAR}/P_{MA} = 1, the degree of counterclockwise hysteresis in the *in vitro* E vs C plot is reflective of the pharmacokinetics of the parent compound and M_A. As expected, the degree of hysteresis increases as M_A accumulates, i.e., as k_{mo} decreases. Similarly, Fig. 4 shows that, for a given set of pharmacokinetic parameters, e.g., k_{mo} = 0.05, the degree of counterclockwise hysteresis in the *in vitro* E vs C plot is reflective of the relative "potency" of the parent compound and M_A; as the ratio of P_{PAR}/P_{MA} decreases in magnitude, i.e., as the potency of M_A increases, the degree of hysteresis increases. These observations are not unexpected and are conceptually predictable but clearly show both the kinetic and the dynamic contribution to the *in vitro* hysteresis relationships. When *in vivo* pharmacokinetic–*in vitro* pharmacodynamic methods are utilized together with a specific analytical assay, counterclockwise hysteresis in the E vs C plot is reflective of the *in vivo* formation of M_A(s) (Fig. 5a), and similarly clockwise hysteresis is indicative of the *in vivo* generation of M_T(s). However, if a plot of E vs C + C_{MA} is generated, hysteresis will be absent when the parent compound and M_A are "equipotent," e.g., when the P_{PAR}/P_{MA} ratio = 1 (Fig. 5b). Figure 6 exemplifies this point and shows no hysteresis in the E vs C + C_{MA} plot when the P_{PAR}/P_{MA} ratio = 1 (Fig. 6a), clockwise hysteresis when the P_{PAR}/P_{MA} ratio >1 (Fig. 6b), and counterclockwise hysteresis when the P_{PAR}/P_{MA} ratio <1 (Fig. 6c). Once again, this observation is predictable, since the relative contribution of M_A concentrations to a given C + C_{MA} concentration will be less at early time points following parent compound dosing, i.e., before peak C + C_{MA}, compared to later time points, i.e., after peak C + C_{MA}. Thus for E vs C + C_{MA} data plotted in time order, the E for a given C + C_{MA} concentration is (i) greater at later time points when M_A potency > parent compound potency and (ii) less at later time points when M_A potency < parent compound potency. These observations are equally applicable to the E_{max} pharmacodynamic model, that is, Eq. (4) (Fig. 7), which introduces nonlinearity into the E vs C and E vs C_{MA} relationships. It therefore follows that if the "full response" is known, i.e., combined E resulting from parent compound and M_A, and if C and C_{MA} are known, then regression analysis of the data to "collapse" the hysteresis will

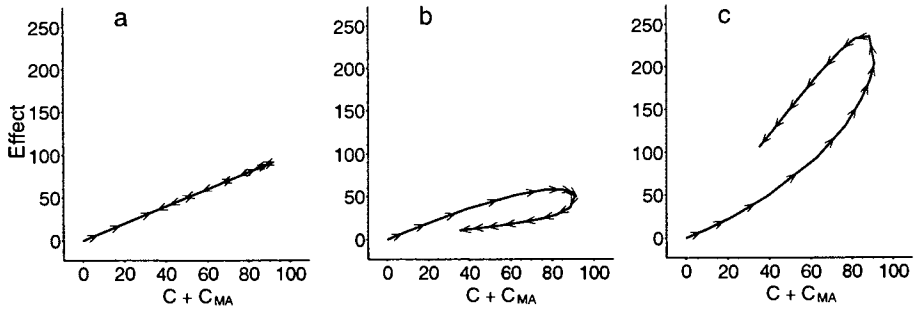

Fig. 6. Plot of observed effect (E) vs unbound plasma concentration (C + C_{MA}) for parent compound and agonistic metabolite (M_A). The pharmacodynamics of parent compound and M_A are described by a linear model [Eq. (3)] and where, for M_A pharmacokinetics, k_{mo} = 0.05 and P_{PAR} = 1: "collapsed" hysteresis with P_{MA} = 1 (a), clockwise hysteresis with P_{MA} = 0.33 (b), and counterclockwise hysteresis with P_{MA} = 3 (c).

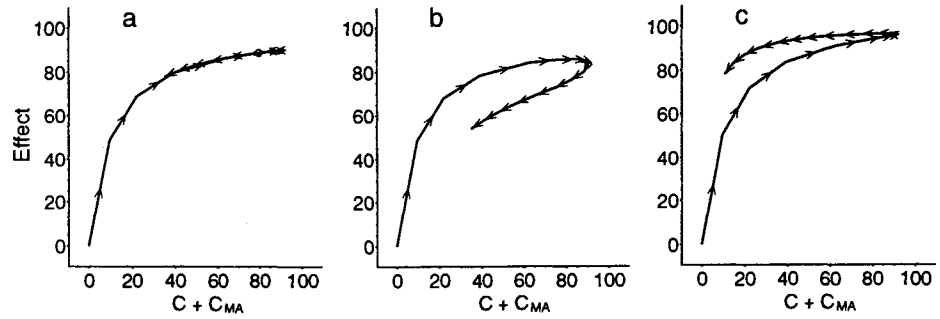

Fig. 7. Plot of observed effect (E) vs unbound plasma concentration ($C + C_{MA}$) for parent compound and agonistic metabolite (M_A). The pharmacodynamics of parent compound and M_A are described by an E_{max} model [Eq. (4)] and where, for M_A pharmacokinetics, $k_{mo} = 0.05$ and $EC_{50} = 10$: "collapsed" hysteresis with $EC_{50_{MA}} = 10$ (a), clockwise hysteresis with $EC_{50_{MA}} = 30$ (b), and counterclockwise hysteresis with $EC_{50_{MA}} = 3.33$ (c).

allow an estimate of the relative potency of the parent compound and M_A, i.e., estimates of P_{PAR}/P_{MA} and $EC_{50}/EC_{50_{MA}}$. As a corollary, a more readily applicable use of this concept may be envisaged, e.g., if P_{PAR} and P_{MA} are known through separate estimation in an *in vitro* study using "spiked" plasma, then analysis using plasma from an *in vivo* pharmacokinetic study would not generate hysteresis in the plot of E vs weighted total concentration, i.e., E vs $C/P_{PAR} + C_{MA}/P_{MA}$ only when all the pharmacologically active metabolite(s) has(have) been accounted for, and vice versa (the presence of hysteresis would indicate that active metabolites have yet to be identified).

Tables I and II show the results of the regression analysis of the simulated *in vivo* pharmacokinetic–*in vitro* pharmacodynamic data sets for the linear and E_{max} pharmacodynamic models, respectively. The analysis for the linear pharmacodynamic models estimated the mean potency of the parent compound and M_A to within 5% of error-free simulations [i.e., Y_M in Eq. (5)], with a %CV associated with estimates that was no greater than 17%. For the E_{max} pharmacodynamic models the mean potency of the parent compound and M_A was estimated to be within 28% of error-free simulations, with a %CV associated with estimates no greater than 47%. For the linear model the estimates are quite good and the CV is acceptable. For E_{max} models the mean parameter estimates were within an acceptable range but the CV was greater, as would be expected. If only the potency ratio were determined, less variability in the estimates would be observed. From our perspective it is important to estimate absolute values of both P_{PAR} and P_{MA}, since the variability in the estimates can be reduced by increasing the number of observations. To utilize this approach for estimating the comparative potency of the parent compound and M_A, the study assumes that (a) M_A may represent more than one metabolite, i.e., M_A's, but (i) all M_A's are agonists with respect to the same observed E as the parent drug, (ii) a specific analytical assay is utilized that can quantitate unbound concentrations of the parent drug and all M_A's; and (b) if M_T's are formed, then the M_T concentration and pharmacodynamics must be accounted for in the analysis. Though this concept is applicable to multiple agonists, the interpretation of hysteresis can be complicated depending upon the potencies and pharmacokinetic properties of the agonists. As indicated above, a readily applicable use for the approach would be where the *in vitro* pharmacodynamics of parent compound and an M_A are known, allowing confirmation that all *in vivo* generated active metabolites have been identified.

Table I. Estimates of the Mean "Potency" Based on the Linear Pharmacodynamic Model [Eq. (3)] from 50 Randomly Generated Data Sets, Each Comprising 14 Pairs of Data Observations and Incorporating 10% Error in the Concentration–Time Data and 20% Error in the Effect–Concentration Data

	P_{PAR}	P_{MA}	P_{PAR}	P_{MA}	P_{PAR}	P_{MA}
	1[a]	1	1	0.33	1	3
$k_{mo} = 0.99$						
Mean	1.01	0.98	0.98	0.31	0.97	2.98
CV%	8.60	6.98	9.83	6.52	11.7	6.82
$k_{mo} = 0.49$						
Mean	1.00	1.01	1.05	0.29	0.99	3.01
CV%	8.27	13.5	17.0	5.69	8.22	7.56
$k_{mo} = 0.05$						
Mean	1.01	1.00	0.97	0.31	0.97	3.02
CV%	9.20	6.13	5.52	6.13	10.6	5.87

[a] True values.

It is apparent that in using an "unsuspected" nonspecific analytical assay, i.e., where the investigator is unable to differentiate between parent compound and M_A (such as RIA), the E vs $C + M_A$ plots in Figs. 6 and 7 could represent plots of E vs apparent C, and that in such circumstances unusual relationships can arise, leading to misinterpretation of hysteresis. The latter point relating to hysteresis resulting from nonspecific analysis is equally applicable in the interpretation of *in vivo* E vs C relationships. Further, it is also applicable to the achiral analysis of racemate e.g., the use of an achiral analysis where both the PK and the PD of the enantiomers differ will result in the generation of hysteresis relationships in the E vs C plots despite all other causes of hysteresis being absent.

In conclusion, this report documents the potential causes of hysteresis in effect vs concentration data generated in an *in vivo* pharmacokinetic–*in vitro* pharmacodynamic study and describes an approach that allows estimation of the relative pharmacological potency of parent compound and agonistic metabolite. This approach can be

Table II. Estimates of the Mean "Potency," Based on the E_{max} Pharmacodynamic Model [Eq. (4)] from 50 Randomly Generated Data Sets, Each Comprising 14 Pairs of Data Observations and Incorporating 10% Error in the Concentration–Time Data and 20% Error in the Effect–Time Data

	EC_{50}	$EC_{50_{MA}}$	E_{max}	EC_{50}	$EC_{50_{MA}}$	E_{max}	EC_{50}	$EC_{50_{MA}}$	E_{max}
	10[a]	10	100	10	30	100	10	3.33	100
k_{mo} = 0.99									
Mean	9.64	11.4	102	10.2	33.3	102	10.6	3.25	103
CV%	7.81	15.7	2.99	6.31	20.6	3.87	38.9	11.1	6.95
k_{mo} = 0.49									
Mean	10.7	10.7	104	9.70	31.0	100	11.5	3.21	105
CV%	33.6	11.3	12.1	8.25	4.83	0.89	36.2	12.6	10.4
k_{mo} = 0.05									
Mean	12.8	11.8	106	13.3	36.3	111	12.3	3.59	105
CV%	29.2	19.1	8.13	46.4	18.6	13.2	35.9	21.8	6.33

[a] True values.

utilized in drug discovery/development to confirm that all active metabolites have been identified. Further, this report highlights some interpretational problems that can arise in using nonspecific analytical assays, e.g., RIA or achiral analytical methods in pharmacokinetic–pharmacodynamic studies.

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