Short Communication

Potential Application of D-Optimal Designs in the Efficient Investigation of Cytochrome P450 Inhibition Kinetic Models

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ABSTRACT:
Correctly chosen D-optimal designs provide efficient experimental schemes when the aim of the investigation is to obtain precise estimates of parameters. In the current work, estimates of parameters refer to the enzyme kinetic parameters $V_{\text{max}}$ and $K_m$, but they also refer to the inhibition constant $K_i$. In general, this experimental approach is performed on a grid of values of the design variables. However, this approach may not be very efficient, in the sense that the parameter estimates ($V_{\text{max}}$, $K_m$ and $K_i$) have unnecessarily high variances. For good estimates of parameters, the most efficient designs consist of clusters of replicates of a few sets of experimental conditions. The current study compares the application of such D-optimal designs with that of a conventional approach in assessing the competitive inhibitory potency of fluconazole and sertraline toward CYP2C9 and 2D6, respectively. In each instance, the parameter estimates, namely $V_{\text{max}}$, $K_m$, and $K_i$, were predicted well using the D-optimal design compared with those measured using the rich data sets, for both inhibitors. We show that D-optimality can provide more efficient designs for estimating the model parameters, including $K_i$. We also show that real cost savings can be made by carefully planning studies that use the theory of optimal experimental design.

Cytochrome P450 (P450) enzymes play a major role in drug metabolism, and CYP1A2, 2C9, 2C19, 2D6, and 3A4 are responsible for the majority of these reactions (Hasler et al., 1999). Altering the routes or rates of a metabolic reaction for a compound is particularly relevant with drugs that have a narrow therapeutic index, because small changes in the plasma concentration of the drug can potentially lead to an adverse effect. Such effects include reduction in efficacy or, even worse, toxicity. Inhibition of cytochromes P450 involved in the metabolic clearance of a drug, whether it is the drug itself or any coadministered drug, can potentially result in a metabolic drug-drug interaction (DDI). Inhibition of P450s results in the most frequently observed DDIs, and the associated mechanism of action can be classified as reversible, quasi-irreversible, or irreversible. Reversible inhibitors, the mechanisms of action of which can be further subdivide into competitive, noncompetitive, or uncompetitive inhibition (Houston et al., 2003), pertain to the current work. Mixed inhibition has also been reported but is less common (Houston et al., 2003).

In a typical cytochrome P450 kinetic reaction, the enzyme binds substrate and metabolizes it into associated products. The binding step is reversible, whereas the catalytic step irreversible and is written as the following chemical model:

$$E + S \leftrightarrow ES \rightarrow E + P$$

where $S$, $E$, and $P$ denote substrate, enzyme, and product, respectively. The reaction rate ($v$) is represented by the standard Michaelis-Menten model (eq. 1):

$$v = \frac{V_{\text{max}}[S]}{K_m + [S]}$$ (1)

where $V_{\text{max}}$ denotes the maximal velocity of the enzyme, $[S]$ denotes the concentration of the substrate, and $K_m$ denotes the Michaelis-Menten constant, which represents $[S]$ at which half of the maximal velocity is reached. The most prevalent mechanism of inhibition is competitive, where a compound binds reversibly to the enzyme and prevents the binding of substrate and vice versa. The competition between substrate and inhibitor for the enzyme is represented by the following equation:

$$E + S \leftrightarrow ES \rightarrow E + P$$

$$I \quad \downarrow K_i$$

where $K_i$ is the competitive inhibition constant. The initial rate of reaction $v$ follows the mechanistic model (eq. 2):

$$v = \frac{V_{\text{max}}[S]}{K_m\left(1 + [I]/K_i\right) + [S]}$$ (2)

where $[I]$ denotes the concentration of the inhibitor. For a fixed $[I]$, the limit of this function is $V_{\text{max}}$ when $[S]$ becomes infinitely large. This result means that the same maximal velocity is obtained irrespective of the concentration of the inhibitor. However, in its presence, higher concentrations of the substrate are needed to come equally close to the asymptote of $V_{\text{max}}$. Hence, the apparent $K_m$ increases with the concentration of the inhibitor, i.e., the reaction is slowed down.

ABBREVIATIONS: P450, cytochrome P450; DDI, drug-drug interaction.
The experimental procedure routinely used to determine these various parameters involves incubating the enzyme source (e.g., human liver microsomes) with a specific cytochrome P450 substrate over a range of different concentrations. The drug whose potency (i.e., $K_i$) is being determined is also studied at various concentrations under the aforementioned conditions, to see its effect on the amount of product being formed. From a pharmaceutical perspective, these P450 $K_i$ experiments are typically performed for compounds in the developmental phase and require the study to be done on multiple occasions. $K_i$ determinations are not limited to P450s, and there are a host of other screens (for example, target pharmacology potency screens) run within the pharmaceutical industry that generate $K_i$ values.

Traditionally, this experimental paradigm is performed on a grid of values of the design variables. However, this approach may not be very efficient and could potentially generate superfluous data. Hence, application of $D$-optimal designs allows for good estimates of parameters, where the most efficient designs consist of clusters of replicates of a few sets of experimental conditions. For linear models, the sets of conditions do not depend on unknown parameter values in the model. The design area is different for the model depicted by eq. 2, in which the parameters $K_m$ and $K_i$ enter nonlinearly. Then, the location of the clusters of design points depends on the $K_m$ and $K_i$ values, although not on the $V_{\text{max}}$ value, which enters the model linearly.

The current study investigated whether there was scope to optimize the traditional design (for estimating the inhibition constant $K_i$) through the application of $D$-optimal designs, and thus potentially affect efficiency by significantly reducing the sample numbers.

Materials and Methods

In Vitro Incubations. All in vitro incubations were carried out using a MicroLab-STAR Autoload with 8 channels and a 96-channel head (Hamilton Robotics, Bonaduz, Switzerland). The incubation mix consisted of the following (at their final concentrations): 50 mM KH$_2$PO$_4$ buffer (pH 7.4), 5 mM MgCl$_2$, human liver microsomes (BD Bioscience, Woburn, MA), and 1 mM NADPH. Incubation times and protein concentration were selected such that they provided a linear reaction velocity with respect to product formation (as identified in preliminary experiments; data not shown). Incubation mixes were then supplemented with relevant inhibitors [fluconazole (Diflucan; CYP2C9) and sertraline (Zoloft; CYP2D6); Pfizer in-house chemical store (Sandwich, England)] at up to eight concentrations. Reactions were subsequently initiated by the addition of specific P450 probes (Pfizer): dextromethorphan (CYP2D6) or diclofenac (Voltaren; CYP2C9) across 15 concentrations (0–50 μM), at each inhibitor concentration (0–60 μM). Reactions were terminated 1:2 in ice-cold acetone (v/v) containing isotopically labeled dextrophan or 4-hydroxy diclofenac metabolites (50 ng/ml). Terminated reaction mixtures were analyzed directly by high-performance liquid chromatography-mass spectrometry.

Samples were subsequently quantified using an Applied Biosystems/Sciex API 4000 QTRAP mass spectrometer (Applied Biosystems/MD Sciex, Foster City, CA) in the positive ionization mode. A Phenomenex Synergi Fusion high pressure-high performance liquid chromatography column, 2.0 × 20.0 mm, 2.5-μm particle size (Phenomenex, Torrance, CA) was used for chromatographic separation, at a flow rate 1 ml/min. A CTC auto sampler (CTC Analytics, AG, Zwingen, Switzerland) was used in conjunction with a Jasco XLC 3185PU high-pressure, low dead volume, binary gradient pump, Jasco XLC 3067CO column oven and Jasco XLC 3080DG degasser (Jasco, Tokyo, Japan) (Youdim et al., 2008).

Data Analysis. Conventional data were analyzed by nonlinear regression analysis using Grafit 4 (Eiritthus Software Ltd., Horley, Surrey, UK) and applying models for Michaelis-Menten kinetics with inhibition (eq. 2). The criteria used to select and check the most appropriate model included visual inspection of the residuals, together with tests of independence of the errors and the constancy of error variance, and $F$ tests for the values of parameters.

Designing the Experiments. From a pharmaceutical perspective, P450 $K_i$ experiments are typically performed for compounds in the development phase and consist of taking measurements across several different concentration combinations of the substrate and inhibitor, with each combination normally repeated in triplicate. However, application of $K_i$ studies for compounds during discovery, where numbers will exceed those in development, necessitates a reduced design. As such, single studies might be performed. However, a different set-up is required if we want to optimize the experiment for parameter estimation. Then, the design usually consists of far fewer combinations of the concentrations, but each one is replicated several times. We denote such designs by $\xi$ with a subscript $N$ to indicate the total number of observations to be taken (i.e., eq. 3):

$$\xi_N = \left\{ \frac{x_1, \ldots, x_n}{w_1, \ldots, w_n} \right\}$$

where $x_i$ denotes the design support points and

$$w_j = \frac{1}{N}$$

represents the proportion of experimental effort at $x_j$, $j = 1, \ldots, n$. Note the following:

$$\sum_{j=1}^{n} w_j = 1 \text{ as } \sum_{j=1}^{n} r_j = N$$

For the current study, the support points $x_j$ specify $n$ combinations of substrate and inhibitor concentrations that come from the design region $\Omega$, i.e., $\Omega = \{0, \ldots, \Omega \} 	imes \{0, \ldots, \Omega \}$, where $[S]\text{max}$ and $[I]\text{max}$ are the maximal allowable concentrations of the substrate and inhibitor, respectively. Although, in practice, setting such concentrations may be subject to error, in our experiments all $x_j$ are determined with good precision, because the experiments were performed using an automated procedure.

In designing the current experiments, we assume that the errors in observing the rate of reaction $r$ (eq. 2) are additive, independent, exhibit constant variance, and are approximately normally distributed. Analysis of conventional data supports these assumptions (data not shown). As such, the appropriate method of parameter estimation is nonlinear least-squares. If the model were linear, the confidence region for the parameter estimates would be elliptical, or ellipsoidal with three or more parameters. For $D$-optimal designs, we choose the $x$ and $r$ values to minimize this volume. If the parameter estimates are uncorrelated, this value is equivalent to finding a design that minimizes the variances of the estimates. However, the estimates are usually not independent, and $D$-optimality minimizes the generalized variance of the estimates defined as the determinant of the matrix of variances and covariances of the estimates. This determinant is proportional to the square root of the volume of the confidence ellipsoid. A succinct summary of the optimal experimental design theory is given by Fedorov and Hackl (1997). For $D$-optimal design for nonlinear models, see Atkinson et al., (2007).

Results and Discussion

The purpose of the current investigation was to compare a conventional approach with those that are $D$ optimum. Our conventional designs had $n$ (the number of support points) equal to 120, which consisted of a grid of 15 values of $[S]$ and 8 values of $[I]$ on a logarithmic scale, referred to as a “rich” data set. These rich data sets of substrate inhibitor pairings allowed us to estimate the parameters for both sertraline against CYP2D6 and fluconazole against CYP2C9 (Table 1). In each case, the inhibitory potencies obtained were consistent with those available in the literature for sertraline (Otton et al., 1993, 1996) and fluconazole (Youdim et al., 2008).

For many nonlinear models, the $D$-optimal designs have to be found by numerical maximization. However, in our case, we were able to obtain the following analytical expressions for a $D$-optimal design for the competitive model that has the form

$$\xi_N = \left\{ \begin{array}{ccc} ([S]\text{max}) & ([I]\text{max}) & (s, s, s) \\ 1 & 1 & 1 \\ 3 & 3 & 3 \end{array} \right\}$$

The model contains three parameters, and, in this case, the $D$-optimal design has an equal number of replicates at each of the three
Note also that in our model, estimates of the same accuracy. The diclofenac 30 observations evenly split over the three support points of the D-optimal efficiency for parameter estimation could be obtained with approximately these three support points with relevant replications of each. The show that the experimental analysis supports our theory.

But for fluconazole rich 30, 0

For sertraline, the D efficiency of the rich design is even 2-fold (depending upon mechanism of inhibition), one could argue that there might be an advantage to having $K_i$ determined earlier during discovery to better guide predictions of DDIs Obach et al., 2005, 2006). However, a strategy to replace the conventional IC50 assay must balance the need for data with cost effectiveness. The conventional approach for measuring $K_i$ during early discovery clearly goes against this doctrine, as does the need to establish bespoke $D$-optimal designs for every compound. Hence, the next steps will be to establish optimal designs that are not governed by discrete point estimates of inhibitory potency (i.e., IC50) but rather designs that cover “regions” of potency, i.e., IC50 < 1 μM; 1 to 10 μM, >10 μM; a binning strategy often used by pharmaceutical companies as their first-tier approach to screen out compounds that pose a potential DDI risk. Such approaches are currently being investigated at the authors’ institutions, using D8 optimality, which is an extension of D optimality.

Here, “s” indicates that interest is in a subset of the parameters in the model, i.e, $K_i$. In the estimation of just a single parameter (s = 1), a design is found for which the estimate has minimal variance. These

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fluconazole–Diclofenac (CYP2C9)</th>
<th>Uniform (Rich Data Set)</th>
<th>n-Optimal Design</th>
<th>Sertaline–Dextromethorphan (CYP2D6)</th>
<th>Uniform (Rich Data Set)</th>
<th>n-Optimal Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (mmol/min/mg)</td>
<td>1.8 0.03</td>
<td>2.4 0.03</td>
<td></td>
<td>0.73 0.01</td>
<td>0.71 0.01</td>
<td></td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>5.6 0.30</td>
<td>8.9 0.39</td>
<td></td>
<td>4.4 0.23</td>
<td>4.2 0.23</td>
<td></td>
</tr>
<tr>
<td>$K_i$ (μM)</td>
<td>7.7 0.55</td>
<td>6.1 0.29</td>
<td></td>
<td>2.6 0.14</td>
<td>2.1 0.13</td>
<td></td>
</tr>
</tbody>
</table>

$\beta = \max \left\{ [S]_{\text{max}} K_m^0 + [I]_{\text{min}} \right\}$

$s_1 = \max \left\{ [S]_{\text{min}} \min \left\{ \frac{K_m^0 + [I]_{\text{min}}}{K_m^0} \right\} \right\}$

$s_2 = \max \left\{ [S]_{\text{max}} K_m^0 + [I]_{\text{min}} \right\}$

$s_3 = \max \left\{ [S]_{\text{min}} \min \left\{ \frac{K_m^0 + [I]_{\text{min}}}{K_m^0} \right\} \right\}$

$\xi_{S0} = \left\{ (50, 0), (4.6, 0), (49.1, 60) \right\}$

$\xi_{S1} = \left\{ (30, 0), (3.4, 0), (30, 20.2) \right\}$

In these displays, the pairs [such as (50, 0)] indicate the values of [S] and [I] to be applied to 10 or 7 samples, depending on the reaction being studied. As illustrated, all design support points were on the border of the design region for at least one of the substrate and inhibitor concentrations, and some were on the border for both concentrations. Given these D-optimal designs, D efficiencies can be calculated for any other design (Atkinson et al., 2007). These efficiencies are such that a design with an efficiency of 50% requires twice as many trials as the D-optimal design to provide parameter estimates of the same accuracy. The diclofenac–fluconazole rich design had an efficiency of 0.254 on a per observation basis; the same efficiency for parameter estimation could be obtained with approximately 30 observations evenly split over the three support points of the D-optimal design as that from the 120 observations from the rich design. For dextromethorphan–sertraline, the D efficiency of the rich design is even less, 0.182; a design with seven observations at each of the points of the D-optimal design provides greater efficiency than the rich design. The difference in the number of observations between the two studies arises from the dependence of the optimal design, as well as the efficiency of the design over a grid, on the prior values of the parameters. In theory, by running experiments that involve 30 and 21 observations, respectively, we should obtain parameter estimates that are as precise as those obtained from the 120 observations of the rich data sets. The results of Table 1 show that the experimental analysis supports our theory.

The conventional assay approach was subsequently repeated using these three support points with relevant replications of each. The estimates of the parameters determined from this experimental approach, using MATLAB (The MathWorks Ltd., Cambridge, UK) nonlinear least-squares procedure nlinfit, are shown in Table 1. The parameter estimates, namely $V_{max}$ and $K_m$, were predicted well using the D-optimal design compared with those measured using the rich data sets. Estimates of the inhibition constants were of particular importance. For sertraline, the $K_i$ toward CYP2D6 in the rich data set was estimated to be 2.6 μM, which agreed well with the value of 2.1 μM estimated using the D-optimal design. The $K_i$ for fluconazole toward CYP2C9 also compared well with an estimated value of 7.7 μM using the rich data set and 6.1 μM using the D-optimal design. More importantly, the similarities between the S.E.s for each parameter, given the significant reduction in sample numbers compared with the rich-data approach, demonstrate our cost saving implications using D-optimal designs.

The aforementioned D-optimal designs provide estimates of all three parameters with small variances. However, estimating the substrate/inhibitor pairings and relevant replicates requires retrospective analysis of the rich set. The need to have this information up front, together with the availability of high-throughput IC50 screens providing an estimate of the $K_i$ (Jones et al., 2009), limits the requirement to establish “bespoke optimized” $K_i$ experimental designs. Depending on the mechanism, the $K_i$ may reflect half the IC50 for competitive inhibitors or be equal to the IC50 for noncompetitive inhibitors. However, this approach may provide efficiency gains for compounds that progress during the developmental stage and at regulatory agencies that request more definitive measures of $K_i$. At this stage, there is likely to be sufficient information known about the parameter estimates, such as the $K_m$ and $V_{max}$ for substrates (probes) in the actual liver microsome matrix, against which the compound (inhibitor) is being tested against.

Given that $K_i$ estimates from IC50 data could vary by 2-fold (depending upon mechanism of inhibition), one could argue that there might be an advantage to having $K_i$ determined earlier during discovery to better guide predictions of DDIs Obach et al., 2005, 2006). However, a strategy to replace the conventional IC50 assay must balance the need for data with cost effectiveness. The conventional approach for measuring $K_i$ during early discovery clearly goes against this doctrine, as does the need to establish bespoke D-optimal designs for every compound. Hence, the next steps will be to establish optimal designs that are not governed by discrete point estimates of inhibitory potency (i.e., IC50) but rather designs that cover “regions” of potency, i.e., IC50 < 1 μM; 1 to 10 μM, >10 μM; a binning strategy often used by pharmaceutical companies as their first-tier approach to screen out compounds that pose a potential DDI risk. Such approaches are currently being investigated at the authors’ institutions, using D8 optimality, which is an extension of D optimality.

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TABLE 2

D-optimal and Ds-optimal designs and their efficiencies

The support points are the three pairs of values of ([S], [I]) for each experiment.

<table>
<thead>
<tr>
<th>Support Points</th>
<th>(30, 0)</th>
<th>(3.4, 0)</th>
<th>(30, 20.2)</th>
<th>Efficiency △νs</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Optimal design weights, r = 1</td>
<td>One third</td>
<td>One third</td>
<td>One third</td>
<td>100</td>
</tr>
<tr>
<td>Ds-Optimal design weights</td>
<td>r = 2</td>
<td>One fifth</td>
<td>Two fifths</td>
<td>Two fifths</td>
</tr>
<tr>
<td></td>
<td>r = 3</td>
<td>One seventh</td>
<td>Three sevenths</td>
<td>Three sevenths</td>
</tr>
<tr>
<td></td>
<td>r = 4</td>
<td>One ninth</td>
<td>Four ninths</td>
<td>Four ninths</td>
</tr>
<tr>
<td>Ds on D</td>
<td>1 × 10−3</td>
<td>Approximately one-half</td>
<td>Approximately one-half</td>
<td>4.07</td>
</tr>
</tbody>
</table>

TABLE 3

D-optimal and Ds-optimal designs and their efficiencies

The support points are the three pairs of values of ([S], [I]) for each experiment.

<table>
<thead>
<tr>
<th>Support Points</th>
<th>(50, 0)</th>
<th>(4.6, 0)</th>
<th>(49.1, 60)</th>
<th>Efficiency △νs</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Optimal design weights, r = 1</td>
<td>One third</td>
<td>One third</td>
<td>One third</td>
<td>100</td>
</tr>
<tr>
<td>Ds-Optimal design weights</td>
<td>r = 2</td>
<td>One fifth</td>
<td>Two fifths</td>
<td>Two fifths</td>
</tr>
<tr>
<td></td>
<td>r = 3</td>
<td>One seventh</td>
<td>Three sevenths</td>
<td>Three sevenths</td>
</tr>
<tr>
<td></td>
<td>r = 4</td>
<td>One ninth</td>
<td>Four ninths</td>
<td>Four ninths</td>
</tr>
<tr>
<td>Ds on D</td>
<td>0.03</td>
<td>0.48</td>
<td>0.49</td>
<td>4.04</td>
</tr>
</tbody>
</table>

Designs differ from the D-optimal designs shown above. In particular, the weights on the design points are often far from equal. In some cases, the designs are even singular, putting weight (as in our example) on less than three support points. Although not immediately useful, because not all model parameters can be estimated, such designs provide a reference against which the Ds-efficiency of any other design can be calculated. Singular designs for nonlinear models have been reviewed previously (Atkinson et al., 2007). However, for illustrative purposes, the designs have been calculated for the current experimental data. Table 2 shows that the D-optimal design has a Ds-efficiency of 66.67% for dextromethorphan-sertraline. This measure of efficiency was found by comparison with the Ds-optimal design, which, for numerical reasons, was constrained to have a weight of at least 1 × 10−3 on the first support point. The rest of the weight is split equally between two points very close to those of the D-optimal design. The last line of Table 3 shows the efficiencies for a design on these support points, with weights found to minimize the variance of the estimates of Kt. This design has a Ds-efficiency of 100%, and a D-efficiency of only 4%. In between these extremes, a series of designs are presented in which the weights are in the ratio 1: r/r. The D-optimal design corresponds to r = 1. Designs for r = 2, 3, and 4 are also presented. As the weights become less equal, the D-efficiency decreases slowly and that for Ds increases. When r = 4 and the weights are one ninth, four ninths, and four ninths, the D and Ds efficiencies are 83.99 and 88.89. The results for diclofenac−fluconazole (Table 3) are similar, where, as a result of r increasing, the designs become less balanced, resulting in increased Ds efficiency and decreased D efficiency. Values of 3 or 4 for r give designs that are not highly unbalanced and that have good efficiencies on both measures. It is not even necessary for r to be an integer. For example, with 30 measurements, the numbers at the three design points could be 4, 13, and 13, giving a value of 3.25 for r and a design with good D and Ds efficiencies. We have found that the optimal designs depend both on the prior estimates of the parameters Kt and Kt (although not on Vmax) and on the assumed model. In practice, these parameter values will not be as well known as they are in our examples. However, if the value of the IC50 is known, only one design parameter remains unknown. Optimal designs and their efficiencies can then be calculated for a series of parameter values, and a design can be chosen with good efficiency over the range of values. If no such design can be found, D optimality can be extended by using the prior distribution of the parameters as weights in the calculation of a “Bayesian” design (Atkinson et al., 2007), which may require experiments at more than three combinations of concentrations. Likewise, compound optimality can be used (Atkinson et al., 2007) to find good designs when the mechanism of reaction is uncertain.

In conclusion, we have shown that D optimality can provide more efficient designs for estimating model parameters, including inhibitory Kt's. Such an approach may be of use for compounds that are in the later stage of drug development, where prior knowledge of potency can be used to guide these mathematical designs. Finally, because we have shown that D-optimal designs can be applied successfully, this approach can be extended to include Ds optimality, where there is less reliance of prior knowledge of parameter estimates.

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References


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