Optimized experimental design for the estimation of enzyme kinetic parameters: an experimental evaluation

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Nonstandard abbreviations:

CYP, Cytochrome P450; $V_{\text{max}}$, maximum rate of the reaction; $K_m$, Michaelis constant; $\text{CL}_{\text{int}}$, metabolic clearance intrinsic; ODA, optimal design approach; MDCM, multiple depletion curves method; $k_e$, loss of enzyme activity constant; OFV, objective function value; $C_0$, starting concentration; $t_s$, time point of sampling
Abstract

A set of compounds (n=30) including traditional cytochrome P450 substrates and compounds from AstraZeneca’s compound library was used in an experimental evaluation of an optimal design approach (ODA) for the estimation of enzyme kinetic parameters (CL_{int}, V_{max} and K_{m}). A depletion method previously shown to provide reliable results, the multiple depletion curves method (MDCM), was used as reference. Experiments were conducted with human liver microsomes and samples were analyzed using LC-MSMS. CL_{int} estimated with the ODA were in >90% of the cases within a 2-fold difference compared to MDCM estimates. In addition, good agreement was generally seen for V_{max} and K_{m} estimates between the two methods as >80% of the estimates were within or almost within a 2-fold difference. The variability in V_{max} and K_{m} estimates were generally higher than for CL_{int} estimates. Also, decreased substrate turnover considerably increased the variability in V_{max} and K_{m} estimates whereas only a modest increase was observed for CL_{int} estimates. The experimental design of using multiple starting concentrations for the estimation of enzyme kinetics was shown to be appropriate even when there is a limitation to the number of samples. The method allowed for good estimates of CL_{int} and also for V_{max} and K_{m} in many cases. This approach is hence a good alternative for the estimation of enzyme kinetic parameters, especially if enzyme saturation and an assessment of a potential risk for nonlinear metabolism are of interest.
Introduction

The determination of metabolic intrinsic clearance \( (\text{CL}_{\text{int}}) \) has for many years been included in the characterization package of new molecular entities in drug discovery (Masimirembwa et al., 2001; Ito and Houston, 2005). Thoroughness and effort put in the determination is, depending on the stage in the discovery process, a balance of resources and expected outcome as well as intended usage of the result. Ranking and identification of certain metabolically unstable chemical structures may be the primary aim in an early discovery phase whereas the possibility and need for predictions of an in vivo situation becomes more relevant in later stages. Estimation of \( \text{CL}_{\text{int}} \) is commonly done with the well established in vitro half life method or a similar approach (Obach, 1999). Briefly, the disappearance of drug is followed over time at a single start concentration, usually 1 µM. Under the assumption that the overall metabolic process occurs under linear conditions a mono-exponential concentration-time relation is adopted where the rate constant is directly proportional to \( \text{CL}_{\text{int}} \). The convenient experimental setup and analysis are contributing factors to why this method has been widely accepted. However, a recent simulation study using the Optimal Design algorithm suggested that the conventional experimental setup might be sub optimal, especially if the assumption of linearity is violated or if risk assessment of non-linearity is of interest (Sjögren et al., 2011).

The Michaelis-Menten equation is central in traditional enzyme kinetic theory. The reaction rate is described by the maximum rate of the reaction \( (V_{\text{max}}) \) and the Michaelis constant \( (K_m) \) which represents the substrate concentration that yields a reaction rate equal to half \( V_{\text{max}} \). Estimates of \( V_{\text{max}} \) and \( K_m \) from a substrate disappearance experiment conducted in microsomal fractions (or any other mixed enzymatic systems) will reflect the combination of all present reactions of importance. With adequate interpretation, the \( K_m \) estimate could subsequently be used for an assessment of the general susceptibility for non-linear metabolism in vivo. The Optimal Design study indicated that multiple starting concentrations
(C₀) with late time points of sampling (tₛ) generally would be superior to the conventional method (Sjögren et al., 2011). The aim of this study was to experimentally evaluate the potential of this experimental approach with a limited number of samples and multiple starting concentrations for the estimation of enzyme kinetics. A depletion method previously shown to provide reliable estimates of enzyme kinetic parameters, the multiple depletion method (MDCM), was used as primary reference (Sjögren et al., 2009).
Material and Methods

Chemicals and reagents

Following compounds and reagents, supplier within brackets, were used in the study.

Amodiaquine, diltiazem, imipramine, dextromethorphan, phenacetin, diclofenac and dimethyl sulfoxide (DMSO) (Sigma Aldrich, Gillingham, Dorset, United Kingdom). Bufuralol (Toronto Research Chemicals Inc., Ontario, Canada). Midazolam (Lipomed, Arlesheim, Switzerland). 5, 5-diethyl 1, 3-diphenyl-2-imminobarbituric acid (used as internal standard) and in-house compounds (n=24) (AZ compound management). Human liver microsomes (batch 38289, 20 mg microsomal protein/mL, 150 donor pool) (BD Gentest, San Jose, CA, USA). Acetonitrile (ACN) 100% (Rathburn Chemicals, Walkerburn, Scotland). Formic acid (FA) 98-100% (Merck KGaA, Darmstadt, Germany). All compounds included in the enzyme kinetic investigation were stored as 100 mM DMSO stock solutions at 8 °C between experiments (except bufuralol that was stored at -20 °C).

Compound selection

The study was conducted using a set of traditional cytochrome P450 (CYP) substrates as well as a set of compounds from AstraZeneca’s compound library. Following well known CYP substrates, CYP enzyme(s) in brackets, were included in the study; amodiaquine (CYP2C8) (Li et al., 2002; Walsky and Obach, 2004), bufuralol (CYP2D6, CYP1A2) (Yamazaki et al., 1994; Ring et al., 1996; Youdim and Dodia, 2010), dextromethorphan (CYP2D6) (Walsky and Obach, 2004; Youdim and Dodia, 2010; Thörn et al., 2011), diclofenac (CYP2C9) (Leemann et al., 1993; Walsky and Obach, 2004; Youdim and Dodia, 2010; Thörn et al., 2011), diltiazem (CYP3A4) (Sutton et al., 1997; Jones et al., 1999; Zhao et al., 2007), imipramine (CYP2C19, CYP1A2, CYP2D6) (Lemoine et al., 1993; Koyama et al., 1997),
midazolam (CYP3A4) (Gorski et al., 1994; Ring et al., 1996; Walsky and Obach, 2004; Youdim and Dodia, 2010), phenacetin (CYP1A2) (Tassaneeyakul et al., 1993; Zhang et al., 2002; Walsky and Obach, 2004). Enzyme kinetic parameters for these compounds were collected from the literature to evaluate the overall result from this study. Selection of in house compounds were based on the main criteria of a CL_int between 10 and 300 µL/min/mg protein and a measured solubility at pH 7.4 higher than 100 µM. The existing CL_int estimate used in the selection had earlier been determined with a 4-time-points high throughput screening approach previously included in the discovery characterization routine at AstraZeneca. Compounds (n=22) were then randomly selected to obtain a representative set of compounds in respect of metabolic stability as well as some fundamental molecular properties, e.g., lipophilicity, molecular weight.

Experimental protocol
C₀ and tₛ for the evaluated optimal design approach (ODA) and the MDCM were selected on basis of previous reports as well as considering possible limitations in compound solubility and the LC-MSMS method. Also, considerations were made to create a straightforward experimental protocol. Under these considerations the protocol for the MDCM was set to include three C₀ values at 1, 10 and 100 µM conducted in triplicates with tₛ at 0, 10, 20, 30 and 40 min for each C₀ giving a total of 45 samples. The ODA protocol was limited to 15 samples and set to include following C₀’s (µM) with respective tₛ (min); 0.5:0, 0.5:40, 0.5:40, 2.5:5, 2.5:40, 15:5, 15:40, 30:5, 30:40, 60:40, 90:40, 90:40, 100:40, 100:40 and 100:40. The experimental protocol for ODA and the MDCM are summarized in Table 1. The theoretical optimality of the ODA, as objective function value (OFV), of this experimental setup using the same algorithms, data input and conditions as used in Sjögren et al., 2011, was calculated to OFV=13 (Sjögren et al., 2011). This should be compared to the highest theoretical
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optimality (maximum OFV=52), the OFV of the proposed experimental design in Sjögren et al., 2011, (OFV=17) and the conventional setup (OFV =0.011) (Sjögren et al., 2011). The theoretical loss of performance due to the modifications of the experimental protocol used in this study was hence assumed to be modest.

Microsomal incubations

Human liver microsomes from the same batch were used in all experiments. They were stored in a -80°C freezer and thawed at room temperature while kept on ice before each experiment. Dilutions from the compound stock solutions were made to obtain concentrations 100 times higher than the intended \( C_0 \) in the incubations. To ensure an equal concentration of organic solvent in all incubations, the ACN and DMSO content in each dilution were 50% and 10%, respectively. The incubations were performed by adding 5 µL of each compound dilution to 445 µL master mix (0.56 mg microsomal protein/mL, KPO4 (0.11 M, pH 7.4)). A volume of 18 µL of the master mix was transferred to the incubation plate and pre-incubated for 15 min. The reactions were then started by adding 2 µL of 10 mM NADPH solution to each well giving final concentrations of 1 mM NADPH, 0.5 mg microsomal protein/mL, 0.1 M KPO4, and intended \( C_0 \) of the investigated compound. The final concentration of organic solvents in the incubations was 0.5% ACN and 0.1% DMSO. Incubations were performed shaking (650 rpm) at 37 °C. At specific time points, according to the experimental protocol, the reactions were quenched through addition of 60 µL ice cold stop solution (ACN with 0.8% FA and 100 nM internal standard). The incubation plates were then centrifuged at 3220 g at 4 °C for 20 min. Aliquots of the supernatant (50 µL) were transferred to a new plate and diluted with an equal volume of purified water.

LC-MSMS analysis
The analysis equipment consisted of an Acquity UPLC sample and solvent manager, and an Acquity TQD triple quadrupole mass spectrometer (Waters Corp., MA, USA). Instrument control and data processing were performed using Waters MassLynx 4.1 software including QuanOptimise and QuanLynx. Prior to the experiments an automated optimization of a MSMS method for each compound was performed using Mass Lynx 4.1 (Waters Corp.). Samples (10 µL) were injected on an Acquity UPLC BEH C18 1.7 µm 2.1 x 30 mm column (Waters Corp.) and chromatography was performed at a total flow rate of 1 mL/min using the following gradient elution design: mobile phase A (0.1% (v/v) FA in water) and mobile phase B (0.1% (v/v) FA in ACN), t (min)=0: 4%B, t=0.7: 4%B, t=1.0: 95%B, t=1.01: 4%. Ionization occurred by means of electrospray using electrospray multiple reaction monitoring mode. The peaks were normalized to the internal standard. The lower limit of quantitation for each compound was set at a peak height of 5 times the background noise. Control samples verifying the chromatographic performance were injected before and after each analysis.

Data analysis

The disappearance rate of the compound in the incubation was described by Michaelis-Menten equation (eq. 1).

\[ v = \frac{-V_{max} \times C}{K_m + C} \]  

(1)

where \( C \) is the substrate concentration in the incubation.

It has previously been shown that correction for loss of the overall enzymatic activity during the incubation improved the results for specific reactions (Sjögren et al., 2009). Hence, the data from the MDCM experiments was also analyzed correcting for possible loss of enzyme
activity during the incubation period. This loss was modeled as a mono exponential decay giving equation 2.

\[ v = \frac{V_{\text{max}} \times C}{K_m + C} \times e^{-k_e t} \]  

(2)

where \( k_e \) is the loss of enzyme activity constant.

\( \text{CL}_{\text{int}} \) was calculated by dividing \( V_{\text{max}} \) by \( K_m \) (eq. 3).

\[ \text{CL}_{\text{int}} = \frac{V_{\text{max}}}{K_m} \]  

(3)

Substrate turnover represented as half-life (t\( \frac{1}{2} \)) was calculated as \( \ln(2)/\text{CL}_{\text{int}} \); were \( \text{CL}_{\text{int}} \) was compensated for the microsomal protein concentration in the incubation (0.5 mg/mL).

The data from the ODA was treated in following way: in each run the measured concentration for \( C_0=0.5 \) at \( t_0=0 \) was used as a reference point for all other \( C_0 \)'s, e.g., if the measured value was 10% higher than intended all other \( C_0 \)'s was assumed to be 10% higher. The measurements in the ODA protocol were then modeled as individual depletion curves with a \( C_0 \) equal to the corrected \( C_0 \). Two different approaches were used to evaluate how to handle these semi-predicted \( C_0 \) values. The analysis was either performed by 1) forcing the model fit through the corrected \( C_0 \)'s, i.e., no residuals in measured and predicted concentrations was allowed at \( t=0 \) min, or by 2) including the corrected \( C_0 \) values in the analysis as measurements.

Parameter estimations were in all cases performed by simultaneous fitting of respective equation and model approach to all available data in each run using non-linear regression. The regression was performed with Pharsight WinNonlin software version 5.2 (Pharsight, Mountain View, CA, USA) using a weighting scheme of \( 1/\hat{y}^2 \). Akaike information criterion
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(AIC), visual examination of data, residual plots and the precision of parameter estimates were used for evaluation and comparison of the goodness of fit. Parameter estimates are shown as mean values with coefficient of variation (CV%). Concentration measurements higher than respective C₀, below limit of quantitation and obvious outliers were excluded from the analyses.
Results

Enzyme kinetic parameters ($V_{\text{max}}$, $K_m$ and $CL_{\text{int}}$) determined with the ODA and the MDCM are summarized in Table 2. The estimated parameter values for $CL_{\text{int}}$ covered approximately two orders of magnitude (min: 11 µL/min/mg protein, max: 830 µL/min/mg protein). The parameter space covered by estimates of $V_{\text{max}}$ and $K_m$ was more narrow, $V_{\text{max}}$ (min: 147 pmol/min/mg protein, max: 2770 pmol/min/mg protein), $K_m$ (min: 1.2 µM, max: 25 µM). Compensation for loss of enzymatic activity improved the goodness of fit for the MDCM analysis for five compounds, amodiaquine ($k_e$=0.035), imipramine ($k_e$=0.042), diltiazem ($k_e$=0.035), midazolam ($k_e$=0.063) and AZ21 ($k_e$=0.024). A better fit (using the goodness of fit criteria stated) was obtained for the ODA in >95% of the cases when forcing the model fit through the $C_0$’s instead of including them as measurements in the analysis. This approach was consequently adopted for all ODA analyses and no other results are presented.

Comparative plots for parameter estimates are shown in Fig. 1. $CL_{\text{int}}$ estimated with the ODA were in >90% of the cases within a 2-fold difference compared to MDCM estimates. A tendency for underestimation of $CL_{\text{int}}$ for high clearance compounds was observed. However, the MDCM analyses for the two least metabolic stable compounds, amodiaquine and midazolam, were performed with compensation for loss of enzyme activity during the incubation. Equally high precision (always <50% in CV%) was overall obtained in $CL_{\text{int}}$ estimates for both methods. Good agreement was generally seen for $V_{\text{max}}$ and $K_m$ between the methods as >80% of the estimates were within or close to within a 2-fold difference. Only one compound (AZ20) distinctly deviated from the reference in both $V_{\text{max}}$ and $K_m$ estimates (>5-fold). This compound was also the most metabolic stable entity, associated with a $t_{1/2}$ equal to 125 min. The variability in $V_{\text{max}}$ and $K_m$ estimates was generally higher than for $CL_{\text{int}}$ estimates for both methods. For both model approaches the variability in $V_{\text{max}}$ and $K_m$ estimates increased markedly with decreased substrate turnover. In comparison the increase in
variability in $\text{CL}_{\text{int}}$ estimates were only moderate. The dependency of turnover for the variability in estimates is shown in Fig. 2. The compiled literature data for amodiaquine, bufuralol, dextromethorphan, diclofenac, diltiazem, imipramine, midazolam and phenacetin are shown in Table 3. Plots comparing the literature data to estimates acquired in this study are shown in Fig. 3.
Discussion

A generic experimental approach with a limited number of samples conducted at multiple 
C0’s, the ODA, was evaluated towards a more sample rich method, the MDCM, previously 
shown to produce reliable results (Sjögren et al., 2009). Estimates of $V_{\text{max}}$, $K_m$ and $CL_{\text{int}}$ from 
the ODA were altogether in good agreement with those obtained from the MDCM. This 
indicates that the ODA is a good alternative for enzyme kinetic investigations in drug 
discovery.

The determination of metabolic stability, i.e. $CL_{\text{int}}$, is a well established component of drug 
discovery for the prediction of human hepatic clearance. This is an important element in the 
selection process of optimal drug compounds. An assay based on very few samples has been 
shown to perform adequately well for preliminary assessment of the metabolic stability (Di et 
al., 2004). These kinds of assays are often well suited for an initial ranking of the vast number 
of new compounds generated in lead generation and optimization programs. Still, as 
compounds are excluded based on this information, it is important that the information is as 
accurate as possible and not misleading. The need for improved and more detailed 
information of the compounds enzyme kinetics also increases during the optimization process. 
Most conventional depletion methods only provide information of $CL_{\text{int}}$ while the ODA 
approach also facilitates the estimation of $K_m$ in many cases. This indicates that this approach 
would be a good complement to conventional metabolic stability assays for the assessment of 
enzyme saturation, i.e. risk of non-linear metabolism. There are numerous studies regarding 
optimization of experimental designs for the estimation of enzyme kinetics, inhibition and 
metabolic stability (Kakkar et al., 2000; Di et al., 2003; Saraswat et al., 2003; Di et al., 2004; 
Yang et al., 2007). Also, there are different approaches of depletion methods to the estimation 
of $CL_{\text{int}}$, $K_m$ and $V_{\text{max}}$ (Obach and Reed-Hagen, 2002; Sjögren et al., 2009). However, to our
knowledge this is the first study to experimentally evaluate a generic depletion approach for the assessment of non-linear metabolism suitable for a drug discovery setting, i.e., limited amount of samples and a suitable experimental protocol.

It has previously been shown that the quality of estimates from depletion based assays is highly dependent of substrate turnover (Sjögren et al., 2009). To perform a fair evaluation the intention was to include compounds covering a wide range of metabolic stability. However, in many cases the CL$_{int}$ estimates from the 4-time-points screening assay was underestimated (Fig. 4) and unfortunately only a few low-CL$_{int}$ compounds were therefore included in the evaluation. Consequently it is hard to draw any solid conclusions from this evaluation regarding low-CL$_{int}$ compounds. Still, the data indicates that high-quality estimates of CL$_{int}$ can be achieved for low-CL$_{int}$ compounds although they may suffer from poor accuracy in $V_{\text{max}}$ and $K_m$ estimates. This result is in agreement with previously performed simulations (Sjögren et al., 2011). Overall there was a clear trend of increased variability in $V_{\text{max}}$ and $K_m$ estimates with increased metabolic stability. As this was not observed for CL$_{int}$ estimates the conclusion is that this parameter is less sensitive to low turnover. No trend of higher precision in estimates for the MDCM was observed. This is not what could be expected since the MDCM includes 3-times as many samples and theoretically would be more robust. A possible trend of underestimation of CL$_{int}$ for high-CL$_{int}$ compounds was observed. However, the analysis of the two compounds with the highest MDCM estimates in CL$_{int}$, amodiaquine and midazolam, did benefit of compensation for loss of enzyme activity. The possibility for such compensation was not included in the ODA and therefore a lower estimate in CL$_{int}$ is expected in such cases. However, this discrepancy was not observed for the other three compounds, imipramine, dilatazam and AZ21, also analyzed with the MDCM compensating for enzyme activity loss. It should also be noted that the compensation of loss of enzyme activity was modeled towards a decrease in the overall metabolic rate. Hence, $k_e$ was not
estimated for individual CYP isoforms. Theoretically, this could have implications for individual results when several enzymes with significant differences in stability are of importance to the overall metabolism. The value of $k_e$ in itself should for this reason be regarded as a qualitative measurement of the stability in the specific assay and should not be interpreted or applied otherwise.

As drug metabolism often occurs via several metabolic pathways, the parameters obtained with the suggested method are more accurately defined as total $V_{\text{max}}$ and apparent $K_m$. Nevertheless, the information about $K_m$ may still be very useful as it reflects the overall potential of saturation of the metabolism. Drugs with low $K_m$ values are at a higher risk of showing non-linear pharmacokinetics as well as supra-proportional dose-exposure. Also, an increased emphasis has during the last decade been put on predictive simulations of plasma exposure using physiologically based pharmacokinetic modelling (Rostami-Hodjegan and Tucker, 2007; Poirier et al., 2009; Zhao et al., 2011). As this approach is based on a high mechanistic level the demand on high quality and more profound information of involved processes is increased. Hence, for such exercises an estimate of $K_m$ would be of considerable benefit.

The comparison of the results acquired in this study towards literature values should be regarded as highly qualitative. In contrast to the methods used in this study a majority of the literature studies used as reference adopted methodologies for measurement of specific metabolic reactions. Even if these specific reactions contribute to a majority of the compounds metabolism at the studied concentrations, the possibility of contribution from other metabolic pathways and/or sequential metabolism cannot be excluded. The conclusion from this exercise is that the variability in literature data is very large, in some cases over a 10-fold difference between reported high-low measurements. However, the estimates from
this study were in general within the range of reported values indicating the aptness of the
adopted methods in this study.

One potential drawback for the approach suggested in this study is the requirement of
simultaneous model analysis of all data generated. This includes the use of sophisticated
software capable of non-linear regression and weighted residuals. However, it is possible to
automate such analysis and implement it in a standard analysis protocol. A more substantial
drawback with the ODA protocol presented is that it requires a solubility of 100 µM at pH
7.4. This is a relatively high concentration and many compounds are likely to suffer from a
lower solubility with a risk of precipitation. Although this study did not address this particular
aspect, a systematic approach needs to be developed and evaluated to handle this aspect if the
method would to be implemented in an industrial setting. The potential shortcoming of not
determining the actual concentration at t=0 min for each intended starting concentration could
by the presented result in this study be regarded as negligible. It was also shown that the
preferable way to handle the semi-predicted starting concentrations was to force the model
through these points, i.e., no residuals in measured and predicted concentrations were allowed
at t=0 min. This approach is already commonly used and is valid under the assumption that
the contribution from dilution and dispensing steps to the total variability are negligible.

Finally, the experimental design of using multiple starting concentrations for the estimation of
enzyme kinetics was shown to be appropriate even when there is a limitation to the number of
samples. The method generally allowed for good estimates of CL_{int} but also for V_{max} and K_{m}
in many cases. Hence, this approach is a good alternative for the estimation of enzyme kinetic
parameters, especially if an assessment of enzyme saturation and metabolic non-linearity are
of interest.
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Authorship Contributions:

Participated in research design: Sjögren, Svanberg, Kanebratt

Conducted experiments: Svanberg, Kanebratt

Contributed new reagents or analytic tools:

Performed data analysis: Sjögren

Wrote or contributed to the writing of the manuscript: Sjögren, Kanebratt, Svanberg
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Footnotes:

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**Fig. 1.** Comparative plots of enzyme kinetic parameter estimates using the optimal design approach (ODA) and the multiple depletion curves method (MDCM). MDCM analyses improved by compensation for loss of enzyme activity are represented by red squares. The green triangle represents AZ20, the compound in the data set associated with the lowest turnover. The solid and dashed lines represent the line of unity and a 2-fold range, respectively.

**Fig. 2.** Visualization of the correlation between variability (CV%) in parameter estimate to substrate turnover (t1/2=ln(2)/CL\textsubscript{int,MDCM}). Results from the multiple depletion curves method (MDCM) and the optimal design approach (ODA) are displayed in panel A and panel B, respectively. CL\textsubscript{int} : red circles, K\textsubscript{m} : blue triangles, V\textsubscript{max} : green squares.

**Fig. 3.** Comparison of estimated CL\textsubscript{int}, V\textsubscript{max} and K\textsubscript{m} values to literature data for amodiaquine, bufuralol, dextromethorphan, diclofenac, diltiazem, imipramine, midazolam and phenacetin. The ranges in the plots indicate the lowest and highest literature value divided by the estimate from the current study. The dotted line indicates the line of unity. CL\textsubscript{int}, V\textsubscript{max} and K\textsubscript{m} estimates determined with the multiple depletion curves method (MDCM) are compared in panel A, C and E, respectively. CL\textsubscript{int}, V\textsubscript{max} and K\textsubscript{m} estimates determined with the optimal design approach (ODA) are compared in panel B, D and F, respectively.

**Fig. 4.** Comparative plot of CL\textsubscript{int} estimates obtained either with the 4-time-point screening assay (screen) or the multiple depletion curves method (MDCM) for the 22 included in-house compounds. The solid and dashed lines represent the line of unity and a 2-fold range, respectively.
Table 1. The experimental protocol, starting concentrations ($C_0$) and time points of sampling ($t_s$), for the optimal design approach (ODA) and the multiple depletion curves method (MDCM) adopted in the study. The ODA involved single measurements ($n_{samples} = 15$), and the MDCM involved measurements in triplicates ($n_{samples} = 45$).

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</table>
Table 2. Summary of enzyme kinetic estimates using the optimal design approach (ODA) and the multiple depletion curves method (MDCM). Values displayed as means with CV% in parentheses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CL_{int} (µL/min/mg protein)</th>
<th>V_{max} (pmol/min/mg protein)</th>
<th>K_{m} (µM)</th>
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<tbody>
<tr>
<td></td>
<td>MDCM</td>
<td>ODA</td>
<td>MDCM</td>
</tr>
<tr>
<td>Amodiaquine *</td>
<td>829 (5.1)</td>
<td>224 (6.7)</td>
<td>2770 (9.5)</td>
</tr>
<tr>
<td>Bufuralol</td>
<td>31.0 (26)</td>
<td>32.3 (30)</td>
<td>340 (99)</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>84.1 (18)</td>
<td>54.2 (12)</td>
<td>590 (46)</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>143 (4.2)</td>
<td>135 (11)</td>
<td>1680 (16)</td>
</tr>
<tr>
<td>Diltiazem *</td>
<td>47.6 (13)</td>
<td>75.4 (14)</td>
<td>495 (46)</td>
</tr>
<tr>
<td>Imipramine *</td>
<td>39.6 (16)</td>
<td>51.1 (27)</td>
<td>585 (42)</td>
</tr>
<tr>
<td>Midazolam *</td>
<td>717 (6.4)</td>
<td>220 (8.2)</td>
<td>880 (11)</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>39.5 (14)</td>
<td>50.4 (21)</td>
<td>794 (60)</td>
</tr>
<tr>
<td>AZ01</td>
<td>63.3 (12)</td>
<td>79.7 (6.2)</td>
<td>497 (39)</td>
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<td>AZ02</td>
<td>42.4 (21)</td>
<td>53.7 (16)</td>
<td>210 (68)</td>
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<td>AZ03</td>
<td>104 (9.1)</td>
<td>118 (6.4)</td>
<td>455 (30)</td>
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<td>AZ04</td>
<td>71.2 (8.4)</td>
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<td>1800 (35)</td>
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<td>AZ05</td>
<td>123 (5.7)</td>
<td>138 (12)</td>
<td>147 (36)</td>
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<td>AZ06</td>
<td>71.8 (20)</td>
<td>106 (14)</td>
<td>242 (72)</td>
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<tr>
<td>AZ07</td>
<td>85.8 (12)</td>
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<td>AZ08</td>
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<td>AZ10</td>
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<td>AZ11</td>
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<td>79.5 (14)</td>
<td>308 (64)</td>
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<td>AZ12</td>
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<td>370 (64)</td>
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<td>AZ13</td>
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<td>AZ14</td>
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<td>AZ15</td>
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<td>2300 (21)</td>
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<td>AZ16</td>
<td>156 (7.3)</td>
<td>172 (5.0)</td>
<td>632 (19)</td>
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<td>AZ17</td>
<td>42.8 (13)</td>
<td>36.3 (20)</td>
<td>314 (76)</td>
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<td>AZ18</td>
<td>188 (3.3)</td>
<td>187 (3.8)</td>
<td>784 (8.7)</td>
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<tr>
<td></td>
<td>AZ19</td>
<td>AZ20</td>
<td>AZ21*</td>
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<tr>
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<td>52.8 (13)</td>
<td>33.8 (8.9)</td>
<td>855 (45)</td>
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<tr>
<td>AZ20</td>
<td>11.1 (18)</td>
<td>166 (13)</td>
<td>274 (160)</td>
</tr>
<tr>
<td>AZ21*</td>
<td>87.1 (15)</td>
<td>76.8 (5.2)</td>
<td>914 (23)</td>
</tr>
<tr>
<td>AZ22</td>
<td>164 (5.3)</td>
<td>151 (4.3)</td>
<td>539 (16)</td>
</tr>
</tbody>
</table>

* MDCM analysis performed compensating for enzyme activity loss: amodiaquine (k_e=0.035), imipramine (k_e=0.042), dilitiazem (k_e=0.035), midazolam (k_e=0.063) and AZ21 (k_e=0.024).
Table 3. Compilation of CL\textsubscript{int}, V\textsubscript{max} and K\textsubscript{m} values as well as the principal contributing Cytochrome P450 (CYP) enzyme(s) involved in the metabolism gathered from the literature for amodiaquine, bufuralol, dextromethorphan, diclofenac, diltiazem, imipramine, midazolam and phenacetin. Parameter values are shown as a low-high range.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CL\textsubscript{int}</th>
<th>V\textsubscript{max}</th>
<th>K\textsubscript{m}</th>
<th>CYP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µL/min/mg)</td>
<td>(pmol/min/mg)</td>
<td>(µM)</td>
<td></td>
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<tr>
<td>Amodiaquine(^a)</td>
<td>608 – 783</td>
<td>1460 – 1480</td>
<td>1.9 – 2.4</td>
<td>2C8</td>
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<tr>
<td>Bufuralol(^b)</td>
<td>3.0 – 39</td>
<td>70 – 320</td>
<td>8.2 – 23</td>
<td>2D6</td>
</tr>
<tr>
<td>Dextromethorphan(^c)</td>
<td>34 – 124</td>
<td>140 – 1200</td>
<td>1.4 – 11</td>
<td>2D6</td>
</tr>
<tr>
<td>Diclofenac(^d)</td>
<td>130 – 447</td>
<td>710 – 1670</td>
<td>1.7 – 8.9</td>
<td>2C9</td>
</tr>
<tr>
<td>Diltiazem(^e)</td>
<td>35 – 116</td>
<td>1880 – 2670</td>
<td>23 – 54</td>
<td>3A4</td>
</tr>
<tr>
<td>Imipramine(^f)</td>
<td>38 – 68</td>
<td>117 – 600</td>
<td>5.0 – 14.7</td>
<td>1A2, 3A4, 2C19</td>
</tr>
<tr>
<td>Midazolam(^g)</td>
<td>159 – 2058</td>
<td>636 – 4270</td>
<td>1.2 – 9.6</td>
<td>3A</td>
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<tr>
<td>Phenacetin(^h)</td>
<td>14.6 – 80</td>
<td>322 – 2090</td>
<td>9 – 47</td>
<td>1A2</td>
</tr>
</tbody>
</table>

\(^a\) (Li et al., 2002; Walsky and Obach, 2004), \(^b\) (Yamazaki et al., 1994; Ring et al., 1996; Youdim and Dodia, 2010), \(^c\) (Walsky and Obach, 2004; Youdim and Dodia, 2010; Thörn et al., 2011), \(^d\) (Leemann et al., 1993; Walsky and Obach, 2004; Youdim and Dodia, 2010; Thörn et al., 2011), \(^e\) (Sutton et al., 1997; Jones et al., 1999; Zhao et al., 2007), \(^f\) (Lemoine et al., 1993; Koyama et al., 1997), \(^g\) (Gorski et al., 1994; Ring et al., 1996; Walsky and Obach, 2004; Youdim and Dodia, 2010), \(^h\) (Tassaneeyakul et al., 1993; Zhang et al., 2002; Walsky and Obach, 2004)
Figure 4

The diagram displays a scatter plot with the x-axis labeled as \( CL_{\text{int.MDCM}} \) (\( \mu L/min/mg \) protein) and the y-axis labeled as \( CL_{\text{int.screen}} \) (\( \mu L/min/mg \) protein). The points are plotted on a logarithmic scale, with dashed lines indicating confidence intervals.