

Optimizing Donor Number for Consistent Pooled Human Liver Microsomes

Application Note 465

CORNING

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Introduction

Oxidative drug metabolism via the cytochrome P450 (CYP) system is a principle means of drug clearance. Several decades of studies have pointed to five CYP forms—CYP1A2, 2C9, 2C19, 2D6, and 3A—as those which are primarily responsible for human metabolism of small molecule (MW <1500) drugs and drug-like compounds.¹ The absolute levels and CYP enzyme activities vary substantially among individual CYP forms and among individuals. This variability has been linked to genetic polymorphisms, disease and exposure to pollutants, drugs, herbal supplements, and other dietary materials which can either increase or decrease levels of individual or groups of CYPs.^{2,3} In contrast, interindividual CYP activities in animal model species is generally more consistent as these models are inbred and dietary/environmental factors can be rigorously controlled.

In vitro testing for the role of these CYPs in the metabolism of a drug candidate is standard practice in drug discovery and development. Regulatory guidance documents have been developed for this testing.⁴ In addition, the prediction of human pharmacokinetics (PK) typically requires testing of the rates of CYP metabolism with *in vitro* systems.

Human liver microsomes (HLMs) are commonly used as an *in vitro* reagent for the study of human CYP metabolism. A key to the successful use of HLMs for quantitative studies of metabolism requires control of the interindividual variability to prepare a consistent reagent. This is typically performed by either pre-characterizing the CYP activity levels for individual donors and then developing a formulation which yields specific, target CYP activity levels or by randomly pooling large numbers of individual donors. The lot-to-lot variability of the former approach and the relevance of the achieved activity levels to the population mean will be determined by the precision of the specific enzyme assays and the appropriateness of the target CYP activity levels, respectively. The precision of the latter approach is determined by the inherent variability of the activity of the CYP enzymes and the number of donors in the pool in accordance with the laws of statistics.

While the large pool, statistical approach has the potential to deliver a more consistent product, the current standard is to pool materials from 50 donors. We are unaware of any reports or analyses as to the expected variability in such a pool, whether 50 donors is the most appropriate number or whether some other number of donors would provide significantly improved product consistency and performance.

The purpose of this Application Note is to present an analysis of the CYP activity distributions for over 300 characterized HLM samples and to predict the variability in pooled CYP activity as a function of donor number and to select an optimal donor number based on observed variability.

Procedures

HLMs are typically prepared by differential centrifugation. Briefly, liver tissue is homogenized in KCl/Phosphate buffer and centrifuged at 9,000 x g. The supernatant, or S9, is then centrifuged at 100,000 x g to pellet the membrane fragments or microsomes. The initial pellet is typically resuspended in buffer and centrifuged again at 100,000 x g to remove residual cytosol. The final pellet is resuspended in sucrose buffer. These microsomes can be stored for years at -80°C and can provide a rich source of CYP enzyme activities when fortified with NADPH or a NADPH generating system.

All CYP assays were conducted at 0.8 mg/mL protein (except CYP3A4 which was at 0.5 mg/mL) with a NADPH generating system (1.3 mM NADP, 3.3 mM glucose 6-phosphate and 0.4 U/mL glucose 6-phosphate dehydrogenase), 3.3 mM MgCl₂, and incubated for 20 or 10 minutes (CYP2C9 and CYP3A4). 0.1 M Potassium phosphate buffer (pH 7.4) was used for CYP1A2 (phenacetin O-deethylase), CYP2D6 (bufuralol 1'-hydroxylase), and CYP3A4 (testosterone 6β-hydroxylase). CYP2B6 ((S)-mephenytoin N-demethylase) and CYP2C19 ((S)-mephenytoin 4'-hydroxylase) assays used 0.05 M potassium phosphate and CYP2C9 (diclofenac 4'-hydroxylase which used 0.1 M Tris (pH 7.5). Substrate concentrations were well above the apparent K_m. Metabolite formation was quantified after HPLC separation using a standard curve of authentic metabolite. Activities expressed as pmol product per (mg protein x minute). Protein was assayed using the method of Lowry.⁵

Results and Discussion

Corning Life Sciences has characterized the CYP activities for CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 in HLMs from over 300 donors. The mean enzyme activities for phenacetin O-deethylase, (S)-mephenytoin N-demethylase, diclofenac 4'-hydroxylase, (S)-mephenytoin 4'-hydroxylase, bufuralol 1'-hydroxylase, and testosterone 6 β -hydroxylase were 640, 50, 2600, 70, 88, and 4800, respectively. The median activities were 480, 24, 2500, 30, 80, and 3800, respectively. The relative variability among the CYPs can be illustrated by the Coefficient of Variations (CVs) which were 0.8, 1.8, 0.5, 1.5, 0.8, and 0.9, respectively. However, the distributions were not Normal and this calculation can not be used for purposes beyond this illustration. All of the distributions were skewed with a tail out to higher CYP activities and did not fulfill the requirements for Normality. Figure 1 provides a distribution example of CYP3A4 and the skew to higher enzyme activities.

Summary for CYP3A4

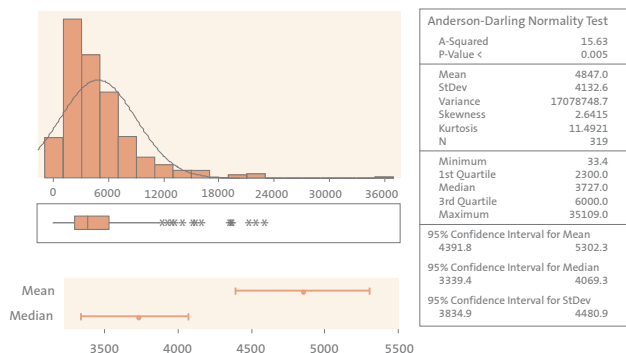


Figure 1. Distribution of CYP3A4-catalyzed testosterone 6 β -hydroxylase activities for HLM prepared from over 300 different donor livers.

Organ transplantation practices have evolved over time and Americans have become more obese over the past two decades. This has the potential to indirectly impact the properties of the available organs which are available for research use. In general, the livers available for research use have become less healthy with a notable trend towards more higher fat content livers. It is unknown whether this has affected CYP activity levels. We examined whether there were any statistically significant changes in median CYP activity based on year of donation. We found no statistically significant trends in the median CYP activities for any CYPs. Figure 2 provides an example of the data for median CYP1A2-catalyzed phenacetin O-deethylase activity plotted by year the liver was donated.

These observations indicate no significant impact of any potential changes in characteristics of livers available for research use on CYP activities. Therefore, the data set as a whole can be used to model pool performance.

Median of CYP1A2 vs. DOD

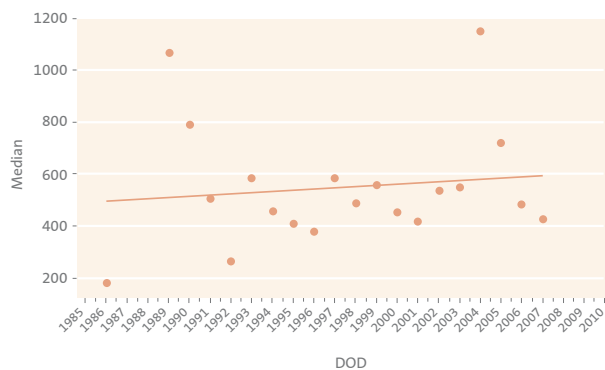


Figure 2. Graph of median CYP1A2 activity as a function of date of donation (DOD). Blue circle is the median activity for each year. The line represents the linear regression. No statistically significant change was observed ($P=0.61$).

In our experience, the ratio of males to females in the donor livers for research has been consistently 60:40 over the years of tissue collection. Activities of CYP1A2, 2B6, 2C9, 2C19, and 2D6 did not vary significantly between males and females. However, CYP3A activity was significantly higher in females relative to males (30-35%). Higher CYP3A activity in females has been reported by others.⁶ The higher proportion of males and the higher CYP3A activity in females results in about a 3% lower average CYP3A activity in this population relative to a 50:50 population of males and females.

We have used a Monte Carlo analysis to predict the expected variability in random pools of differing sizes. For this exercise, HLMs are pooled based on an equal mg protein basis. Figure 3 provides an illustration of the result for CYP2C19 for pools ranging from 25 to 200 donors. The distribution of calculated pooled HLM activities was found to be Normal and CVs were calculated from the mean and standard deviations. The calculated CVs decreased from 0.27 to 0.07 and the donor number increased from 25 to 200. As is evident from the graph, once donor number increased significantly over 100 the further reduction in CV was relatively modest.

CV of CYP2C19 vs. Pool Size

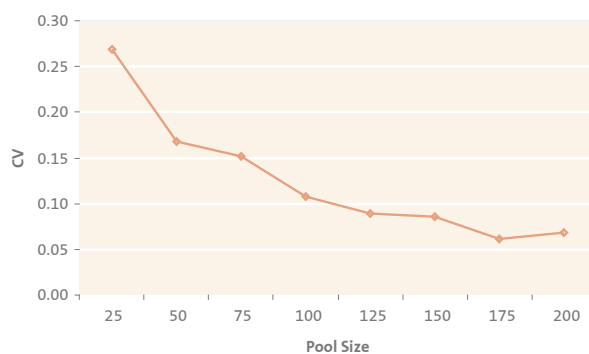


Figure 3. Monte Carlo analysis for random pools created from the 300+ donor database of CYP activities. Thirty simulations were run for each pool size, the mean and standard deviation were calculated. The CV was then calculated as the standard deviation divided by the mean. These mean values from these simulations were found to be Normally distributed and thus the calculated CV values could be used to design and model pool performance.

Table A below provides the calculated CVs for all six CYPs for pools of 50, 100, 150, and 200 donors. These were derived from 30 independent samplings from the database. At a pool size of 50 donors, 4 of the 6 CYPs have CVs greater than or equal to 0.10. At 100 donors, this drops to 2 of the 6 CYPs while at 150 donors CYP2B6 has the highest CV at 0.10. Therefore at the 150 donor pool size, all of the 5 major drug metabolizing CYPs demonstrate a CV of less than 0.10 while 4 of the 5 CYPs have CVs of 0.05 or less.

| | CYP1A2 | CYP2B6 | CYP2C9 | CYP2C19 | CYP2D6 | CYP3A4 |
|------------|--------|--------|--------|---------|--------|--------|
| 50 donors | 0.09 | 0.25 | 0.06 | .017 | 0.10 | 0.12 |
| 100 donors | 0.07 | 0.13 | 0.03 | 0.11 | 0.06 | 0.07 |
| 150 donors | 0.05 | 0.10 | 0.03 | 0.09 | 0.04 | 0.05 |
| 200 donors | 0.04 | 0.06 | 0.02 | 0.07 | 0.03 | 0.03 |

Table A. Calculated CVs from Monte Carlo analyses for six CYP enzymes and pools of 50, 100, 150, and 200 donors.

It is helpful to consider the calculated variability in the context of overall assay variability. Typically, *in vitro* ADME assays are validated to overall CVs of less than 0.20 with typical operational performance in the 0.05 to 0.10 range (keeping lots of reagent the same and reflecting principally analytical and volume transfer variability). Therefore, with HLM pools of 50 or fewer, variation in the HLM product between lots will be a significant contributor to overall assay variability. However, as the donor number increases to over 100, other sources of variability are more significant than that which would result from changing lots of pooled HLMs.

Another approach in considering the impact of variability is to convert CV values into expected ranges at a certain confidence limit. Of course, overall range is profoundly impacted by the number of observations as when the number of observations increases one is more likely to observe rare outliers. Table B below shows the expected range at 95% confidence level for a variety of CV values ranging from 0.05 to 0.30. We believe that the 95% confidence level is appropriate as typically about 20 CYP, UGT, and other enzyme activity assays are performed to characterize HLMs. Therefore, for each lot one would expect to see one assay to be at or near the 95% confidence level. The impact of the potential range of enzyme activities on specific applications is discussed below.

| Enzyme Activity CV | Enzyme Activity Range at 95% Confidence |
|--------------------|---|
| 0.05 | 1.2 |
| 0.10 | 1.5 |
| 0.15 | 1.9 |
| 0.20 | 2.3 |
| 0.25 | 3.0 |
| 0.30 | 4.0 |

Table B. Calculated range in enzyme activities at 95% confidence for various CV values. As the means were normally distributed, the 95% confidence level could be calculated as the mean plus or minus two standard deviations. The range was calculated and as the mean of "1" plus 2 x CV (the 95% upper confidence limit) divided by the mean of "1" minus 2 x CV (the 95% lower confidence limit).

Three of the principle applications of pooled HLMs are:

1. Metabolic clearance measurement using a metabolic stability assay and measuring loss of parent compound.
2. CYP inhibition assays (IC_{50} or K_i) using CYP-selective probe substrates.
3. Reaction phenotyping (or enzyme mapping) which is the determination of the identity of the enzymes responsible for metabolic clearance and the percentage of total metabolism by each enzyme.

Variation in pooled HLMs can have differing impact on each of these applications. The potential impacts are discussed below in the context of a 50 donor pool (average CV of 0.13/range of 1.7-fold and highest CV (CYP2B6) of 0.25/range of 3-fold) and a 150 donor pool (average CV of 0.06/range of 1.27-fold and highest CV (CYP2B6) of 0.10/range of 1.5-fold).

Metabolic Stability Assays

The rates of compound metabolism in metabolic stability assays is principally determined by the activities of the enzyme or enzymes which carry out its metabolism. In general, there is less variability in metabolism when multiple enzymes are involved. However, there is a trend for many lipophilic discovery compounds to be principally metabolized by CYP3A. If we consider the expected range (at 95% confidence) for CYP3A, we expect the activity in a 50 donor pool to vary 1.6-fold while for a 150 donor pool it will vary only 1.2-fold. Clearly, a shift of 1.6-fold in rates of metabolism is undesirable. The use of a 150 donor pool provides a clear advantage.

CYP Inhibition Assays

Unlike metabolic stability assays, the output of CYP inhibition assays, IC_{50} or K_i values, are not substantially impacted by the activity levels of the individual CYPs. What can be affected is the amount of microsomal protein and the incubation time needed to maintain initial rate conditions for the assay—in particular ensuring that substrate depletion does not exceed 20% at low substrate concentrations and low inhibitor concentrations. For example, if an assay is designed and established to achieve 15% metabolism, the 1.7-fold range which is present on average for a new 50 donor pool could raise the extent of metabolism to over 25%, an unacceptable result. If the particular CYP was highly variable like CYP2B6, the extent of metabolism could reach 45%. However, if a 150 donor pool was used, the expected 1.27-fold range would result in conditions where the extent of metabolism is still less than 20%. Any adjustment in assay conditions would only need to be applied to the most variable enzymes (like CYP2B6) and the adjustment would only need to be about 10%. Once again, a 150 donor pool offers clear advantage over a 50 donor pool.

Reaction Phenotyping Assays

While much of the discussion in this Application Note has focused on the variation in individual enzymes, it is important to note that the variation in the ratio of two enzymes varies to a greater degree than the individual enzymes. Figure 4 illustrates the CV values for the individual enzymes (CYP2D6 and CYP3A4) and the ratio of 3A4 to 2D6 for both 50 and 150 donor pools. The impact of this variability is illustrated in the scenario below:

A compound is found to be cleared by only CYP3A4 and CYP2D6. The relative contribution of the two enzymes is 3:1 or 75% CYP3A4 and 25% CYP2D6. If CYP3A4 is completely inhibited in a drug-drug interaction, one would expect the systemic exposure to increase 4-fold. However, if this assay was conducted with a 50 donor pool, the range in activity ratios (at 95% confidence) means that the CYP2D6 contribution could vary from 17% to 33% and the increase in systemic exposure could vary from 3.1-fold to 5.7-fold. However, if the assay was conducted with a 150 donor

Median of CYP1A2 vs. DOD

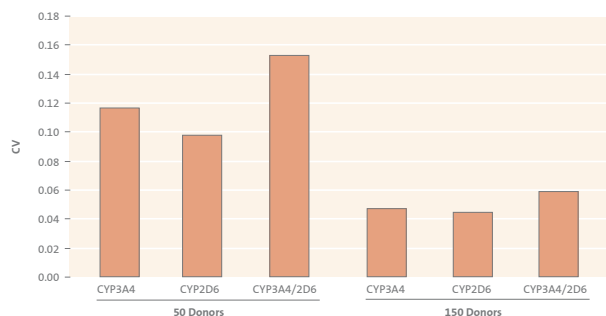


Figure 4. CVs calculated from the Monte Carlo analysis in Table A (30 trials) for CYP3A4, CYP2D6, and the ratio of 3A4 to 2D6 for 50 and 150 donor pools.

pool, the range in CYP2D6 contribution could vary from only 22% to 28% and the increase in systemic exposure could vary from 3.6- to 4.5-fold. Clearly, the use of a 150 donor pool reduces uncertainty in predicting these results. These results are illustrated in Figure 4.

Conclusions

The standards are increasing for reagents which are used in ADME screening and ADME development assays and reduction in the variability of these reagents and the degree to which they are representative of typical or average human metabolism are quite important. The current practice of pooling ~50 donors allows for considerable variability in the activity of key CYP enzymes. This has resulted in the need to perform laborious qualification assays and to adjust parameters in routine assays when lots of reagents change.

Based on a detailed statistical analysis of the variability in individual CYP activity levels for over 300 donors, we conclude that the number of donors in pooled HLMs should be increased more than 2-fold. A donor number of 150 appears to be a sweet spot providing low CVs for the major CYPs such that the variability in the HLM product is well below the overall assay variability. This conclusion has led to the decision to develop and commercialize Corning® UltraPool™ HLM 150, a pooled HLM product comprised of 75 male donor livers and 75 female donor livers. Figure 5 illustrates the observed CYP1A2 activity levels for the first lot of Corning UltraPool HLM 150 relative to 95% confidence limits calculated from the Monte Carlo simulation-derived CV values. The CYP1A2 levels for 4 lots of a 50 donor pool are provided for comparison purposes. This example illustrates the validity of the statistical model and underscores the reduction in variability relative to pools with fewer donors.

In addition, the design of the Corning UltraPool HLM 150 coupled with the amount of tissue which can be set aside from each donor for HLM preparation, has resulted in a consistent product

Corning acquired the UltraPool™ brand.

For additional Corning product, technical, or distributor information, please e-mail us at CLSTechServ@corning.com, visit our website www.corning.com/lifesciences or call 800.492.1110. Outside the United States, call 978.442.2200.

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Median of CYP1A2 vs. DOD

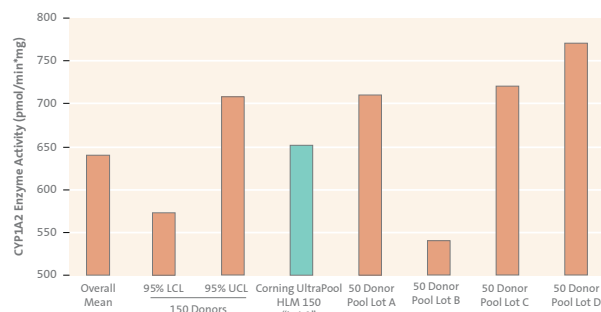


Figure 5. Illustration of the actual enzyme activity levels for CYP1A2 in the population of characterized HLM and various pools. The bars are (from left to right): the overall mean CYP1A2 (phenacetin O-deethylase) activity in the 300+ donors, the 95% lower confidence limit (LCL) calculated based on the CV from Monte Carlo simulations for a 150 donor pool, the 95% upper confidence limit (UCL) calculated based on the CV from Monte Carlo simulations for a 150 donor pool, the measured CYP1A2 activity in the first lot of Corning® UltraPool™ HLM 150, the measured CYP1A2 activity in four lots of a 50 donor pool.

with a large lot size ideally suited to support multiyear drug discovery programs and the follow on ADME assays in drug development.

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