Twenty Year, Retrospective Analysis of CYP Activity Levels in Microsomes Isolated from Human Donor Livers

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Abstract

Human donor livers, originating from US-based Organ Procurement Organizations, have been the basis for the preparation of key reagents used for the characterization of drugs and drug candidates. Over time, the practices for liver transplantation have evolved which has indirectly affected the nature of organs which are unused for transplantation and are made available for research use. We have prepared microsomes from and characterized the enzyme activity levels of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A in over 300 donor livers, procured from 1985 to the present and conducted detailed statistical analyses of activity distributions and trends. We have found the following:

1. CYP2B6 and CYP2C19 show the highest variability among donors (CV 1.5-1.75) CYP2C9 (CV 0.47) shows the lowest variability with CYP1A2, CYP2D6 and CYP3A (CV 0.82-0.85) being intermediate. (Note: the CV values above are based on an assumption of Normality).
2. The age of donors did not change significantly with year of donation. The median age for females was 52 while males it was 50. The levels of CYP activity did not vary with donor age for all CYPs except CYP2C19 where a statistically significant decrease in activity with age was observed.
3. The gender distribution was 63% male and 37% female. The gender difference was observed for CYP3A. On average, females had 34% higher activity. While the average activity for CYP2C19 was 48% higher in females, this difference was not statistically significant.
4. No significant change in any enzyme activity as a function of year of donation. Therefore, liver samples obtained over a large period of time are suitable for creation and predicting the properties of pools.

Monte Carlo analyses for a 50 donor pool predicted an average CV for these 6 enzyme activities of 13%. The actual CV observed for 4 pools prepared in a manufacturing context was 12%. These observations indicate that in order to reduce the CV for all tested enzymes to 10% or less, donor number needs to be increased substantially. We have designed and developed a 150 donor pool.

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, CYP2C9, CYP2C19, CYP2D6 and CYP3A as those which are primarily responsible for human metabolism of small molecule (M.W. <1500) drugs and drug-like compounds[i]. 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[ii] [iii]. In contrast, inter individual CYP activities in animal model species is generally more consistent as these models are inbred and diet/environmental factors can be rigorously controlled.

Human liver microsomes (HLM) are commonly used as an *in vitro* reagent for the study of human CYP metabolism. These materials are derived from donor livers which may not represent the general population. A key to the successful use of HLM for quantitative studies of metabolism requires control of the inter individual variability to prepare a consistent reagent. This is typically performed by either precharacterizing 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. In this poster we present an analysis of CYP activity distributions for over 300 characterized HLM samples. These data were subsequently used to predict the variability in pool CYP activity as a function of donor number and select an appropriate donor number based on observed variability.

Materials and Methods

HLM 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 provide a rich source of CYP enzyme activities when fortified with NADPH or an NADPH generating system.

All CYP assays were conducted at 0.8mg/mL protein (except CYP3A which was at 0.5 mg/mL) with an 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 $MgCl_2$, and incubated for 20 minutes or 10 minutes (CYP2C9 and CYP3A). 0.1 M Potassium phosphate buffer (pH 7.4) was used for CYP1A2 (phenacetin O-deethylase), CYP2D6 (bufuralol 1'-hydroxylase) and CYP3A (testosterone 6β-hydroxylase). CYP2B6 ((S)-mephenytoin N-demethylase) and CYP2C19 ((S)-mephenytoin 4'-hydroxylase) assays used 0.05M potassium phosphate and CYP2C9 (diclofenac 4'-hydroxylase which used 0.1 M Tris (pH 7.5). Substrate concentrations were well above the apparent Km. Metabolite formation was quantified after HPLC separation using a standard curve of authentic metabolite. Activities expressed as pmole product per (mg protein x minute). Protein was assayed using the method of Lowry.

Results

We have characterized the CYP activities for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3Ain HLM 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 6β-hydroxylase were 640, 50, 2600, 70, 88 and 4800 pmole/(mgxmin), respectively. The median activities were 480, 24, 2500, 30, 80 and 3800 pmole/(mgxmin), respectively. The relative variability among the CYPs can be illustrated by the 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 two examples of the distributions (CYP2C9 and CYP2C19) and the skew to higher enzyme activities.

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 shows the median CYP activity levels for all 6 enzymes as a function of the date of donation. No statistically significant trend was observed for any enzyme.

We have analyzed the effect of donor age on median CYP activity. Only with CYP2C19 did we see an effect. With this enzyme, activity decreased with age with highest levels in pediatric donors (Figure 3).

The table below illustrates the gender differences. The majority of donor livers were obtained from males (63%). CYP3A activity was statistically higher in females relative to males. The average increase was 34%. The observed difference with CYP2C19 was not statistically significant.

	Gender	Age	Number	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A
Average	Female	51	120	587	52	2662	83	83	5728
Std Dev	Female	14		453	59	1185	123	65	4236
Average	Male	47	192	656	49	2607	56	92	4282
Std Dev	Male	15		554	103	1264	74	78	4035

			CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A
Ratio	1.08	0.63	0.89	1.06	1.02	1.48	0.90	**1.34**
						Not Significant		Significant

	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A
Overall Mean	637	50	2617	70	88	4850
50% Male 50% Female	621	50	2635	69	87	5005
Ratio	1.03	1.00	0.99	1.01	1.01	0.97

Figure 1. CYP2C9 and CYP2C19 activity distributions.

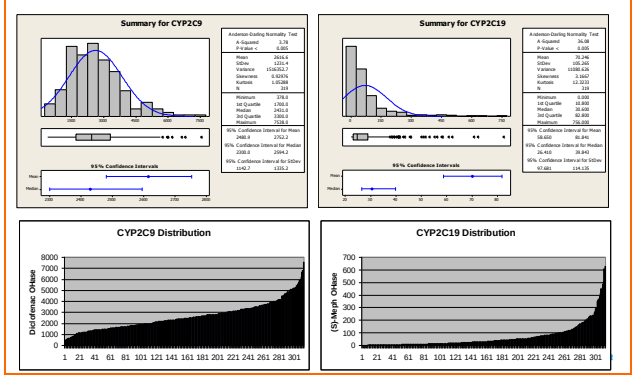


Figure 2. Median CYP activity level versus date of donation.

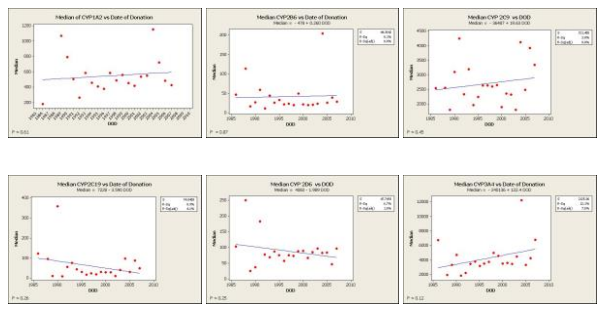


Figure 3. Median CYP Activity Versus Donor Age.
Note. Only three donors in the 80 year age group.

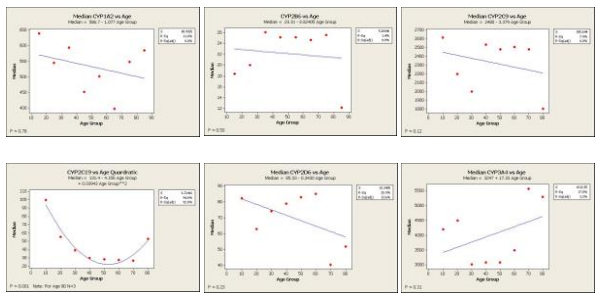


Figure 4. Monte Carlo Analysis – variability in CYP2C19 activity as a function of pool size (from 25 to 200 donors).

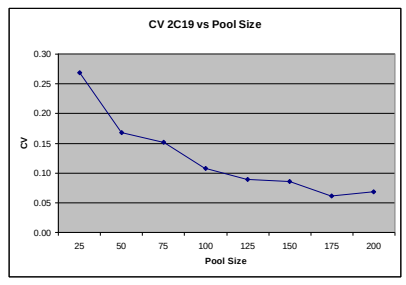


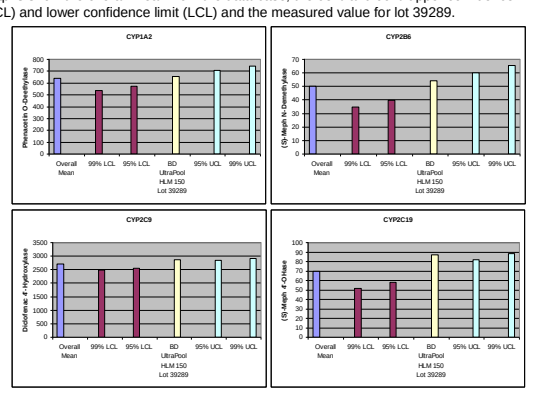
Figure 5. Actual and predicted variability in CYP activity levels for a 50 donor pool.

- We have manufactured 4 lots of a 50 donor pool.
- The overall, observed variability for a 50 donor pool is the same as predicted by Monte Carlo simulations.

	4 Lots 50 Donor Pool	
CYP Form	Actual CV	Calc. CV
CYP1A2	0.15	0.09
CYP2B6	0.15	0.25
CYP2C9	0.13	0.06
CYP2C19	0.11	0.17
CYP2D6	0.14	0.10
CYP3A	0.06	0.12
	0.12	**0.13**

- With N of only 4, the individual CYP value comparisons are of limited value. Consider the overall mean N=24.
- This observation serves to validate the Monte Carlo approach.

Figure 6. Observed and expected CYP activity levels for 4 CYPs in BD UltraPool HLM 150. Graphs show the overall mean from the data base, the 95% and 99% upper confidence limit (UCL) and lower confidence limit (LCL) and the measured value for lot 39289.



Results (continued)

As discussed early, the distributions of CYP activities were skewed to higher activity levels and did not follow a normal distribution. We have used Monte Carlo analysis to predict the expected variability in random pools of differing sizes. Data are from 30 tirals. For this exercise, HLM are pooled based on an equal mg protein basis. Figure 4 provides as illustration of the result for CYP2C19 for pools ranging from 25 donors 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 CV 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.

The table below provides the calculated CVs for all six CYPs for pools of 50, 100, 150 and 200 donors. Again, Monte Carlo analysis was used. These were derived from 30 independent samplings from the data base. At a pool size of 50 donors, 4 of the 6 CYPs have CV 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. Given that in vitro ADME assays are typically validated to a CV of 0.2. At this performance level, the variability in activity for some CYPs is above 0.2 for a 50 donor pool. This creates the need to prequalify new lots of reagent for a 50 donor pool and potentially some adjustment in assay conditions.. However, at 150 donors, the reagent variability for all CYPs is substantially (2x) below the assay variability. Therefore, reagents with a design consisting of 150 donors should provide a much more consistent performance.

Figure 5 provides a comparison of the predicted and observed CV values for the six CYP and four lots of a 50 donor pool. Because N is only 4, the comparisons for the individual CYPs are of limited value. However, the average CV across the CYPs, which is derived from 24 values, is more robust. As reported in Figure 5, the actual CV, 0.12, is in very good agreement with the CV predicted from the Monte Carlo analysis, 0.13.

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

	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A4
50 Donors	**0.09**	**0.25**	**0.06**	**0.17**	**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**

Summary and Conclusions

1. We have analyzed CYP activity levels in a panel of over 300 human donor livers.
2. We observed the expected high level of variability in CYP activities with CYP2B6 and CYP2C19 being the most variable and CYP2C9 being the least variable.
3. There was an over representation of male donors relative to female donors.
4. The expression of CYP3A was found to be statistically higher in females relative to males.
5. The expression of CYP2C19 was found to be higher in young donors.
6. Monte Carlo analysis was used to predict the variability (CV) in HLM pools of different sizes. The validity of this approach was supported by a retrospective study with 50 donor pools.
7. The Monte Carlo analysis indicates that a donor number of 150 will substantially reduce variability in HLM pools relative to the 50 donor pools which are currently employed.
8. The totality of these analyses has lead BD Biosciences to design and manufacture BD UltraPool™ HLM 150. The design is: 150 donors with a 50:50 male/female split, equal mg microsomal protein from each donor and pediatric livers excluded. The results of 4 actual QC assays relative to the overall mean and predicted upper and lower confidence levels are presented in Figure 6. There is good agreement between observed and predicted values, especially given that product variability is expected to be less than assay variability.

References

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