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Disparity in Holo/Apoprotein Ratios of Different Standards Used for Immunoquantification of Hepatic Cytochrome P450 Enzymes

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Running title: Holo/apoprotein ratios of standards used in CYP immunoquantification

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Non standard abbreviations: CYP, cytochrome P450; rhCYP, recombinantly expressed cytochrome P450; ELISA, enzyme linked immunosorbent assay; ISEF, inter system extrapolation factor; HLMSTD, human liver microsomal standard; WX, weighted mean; HMG, homogeneity number; VWX, variance of the weighted mean.

ABSTRACT

An analysis of reported hepatic abundances of CYP3A4 and 3A5 indicated that values determined by immunoquantification using commercially available, unpurified recombinant enzymes as standards are significantly lower than those determined using purified enzymes or human liver microsomes characterised using lysozomal peptides (CYP3A4: mean 45 vs 121 pmol/mg protein, $p < 0.01$; CYP3A5: mean 28 vs 83 pmol/mg protein, $p < 0.05$). When immunoquantifying CYPs it is assumed that the holo/apoprotein ratio is the same in the samples and the standard. Estimates of holo/apoprotein ratios from data reported for a range of CYPs purified from human liver and non-commercial recombinant systems indicated less than complete and variable haem coupling dependent on enzyme and system.

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The absolute abundances of cytochrome P450 (CYP) enzymes in human liver, expressed as amount of enzyme per mg microsomal protein, are required for scaling of *in vitro* data on drug metabolism by recombinant CYP systems (rhCYP) to *in vivo* hepatic clearance (Barter *et al.*, 2007). The use of inter system extrapolation factors (ISEFs) (Proctor *et* al., 2004) allows the differences in intrinsic activity (per unit CYP) between rhCYP and human liver enzymes to be accounted for. The corrected rhCYP *in vitro* data must then be combined with the abundance of the appropriate CYP enzyme in human liver as part of the scaling process. If such abundances are established for large numbers of individual human livers, it is possible to combine this information with activity per unit enzyme obtained with rhCYP systems to predict the distribution of drug clearance across populations, without the need to assess enzyme activity directly in large numbers of liver samples (Rostami-Hodjegan and Tucker, 2007). The accuracy of such *in vitro-in vivo* predictions will clearly depend on the fidelity of estimates of individual CYP abundances in human liver. The latter are usually determined by immunoblotting (Laemmli, 1970) or Enzyme Linked Immunosorbant Assay (ELISA). More recently, mass spectrometric methods have also been proposed for CYP quantification in human samples (Lane *et al*., 2004; Alterman, 2005; Jenkins *et al*., 2006). However, all of these methods measure apoprotein, which comprises active protein in which haem is incorporated (holoprotein) and that in which it is not. By contrast, only holoprotein is measured by carbon monoxide (Omura and Sato, 1964) or dithionite differerence spectroscopy (Matsubara *et al.*, 1976). A variety of protein standards have been used to immunoquantify CYPs in human liver microsomes, ranging from enzyme purified to electrophoretic homogeneity from either liver microsomes or rhCYPs (Guengerich and Turvy, 1991; Shimada *et al.*, 1994), human liver microsomes (HLMSTD; Westlind Johnsson *et al.*, 2003) characterised using lysozyme-peptide conjugates (Edwards *et al.*, 1998), to (more recently) commercially available (unpurified) rhCYP systems (Galetin *et al.*, 2003;

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Wang *et al.,* 2005; King *et al.,* 2003). An assumption in using any of these standards is that the holoprotein/apoprotein ratio is the same in the samples and the standard. To our knowledge the implications of this have never been assessed.

The aims of this study were two-fold. Firstly, to carry out a meta-analysis of CYP3A4 and 3A5 abundance values determined using different calibration standards, and secondly to assess holoprotein/apoprotein ratios from studies reporting the purification of CYPs from human liver and recombinant systems.

METHODS

Abundances of CYP3A4 and CYP3A5 in Human Liver. Values of human hepatic CYP3A4 and CYP3A5 abundance were collated from 2 electronic databases, "MEDLINE" and "Web of Knowledge", and personal files of the authors (1990-2006) containing references from "Current Contents" and "Reference Updates". The authors of the original articles were contacted directly when further information was required. Only data from adult Caucasians (> 16 years) were included, and sources were verified to exclude duplication of individual data in the analysis. Geometric mean values of abundance were used to represent central tendency as the frequency distributions of the data were not normal (Kolmogorov-Smirnov test ; SPSS v12, Chicago, USA). Overall weighted mean (WX) values of CYP3A4 and CYP3A5 abundance were calculated using equation 1:

$$W\overline{X} = \sum_{j=1}^{J} n_j . \overline{x}_j \Big/ \sum_{j=1}^{J} n_j \qquad \textit{Equation 1}$$

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Where there are "J" sources of data, n samples in each source and $\overline{X}$ is the mean value from each data source. The weighted geometric mean values (WX_{geo}) were calculated using equation 2:

$$W\overline{X}_{geo} = \text{Ln}\,(W\overline{X}) - 0.5 \times \sqrt{\ln\,(1+(CV))^2} \qquad \textit{Equation 2}$$

Where CV is the coefficient of variation (%).

Heterogeneity in the data was assessed from the homogeneity number (HMG), calculated using equations 3-5:

$$w_i = {}^{1}\!\Big/\!_{Variance_j} = {}^{1}\!\Big/\!_{(SD_j)^2} \qquad \textit{Equation 3}$$

$$VW\overline{X} = \sum_{j=1}^{J} w_j.\overline{X}_j \Big/ \sum_{j=1}^{J} w_j \qquad \textit{Equation 4}$$

$$\text{HMG} = \sum_{j=1}^{J}\left(w_j.(\overline{X}_j - \text{VWX})^2\right) = \sum_{j=1}^{J} w_j.\overline{X}_j^{\,2} - \frac{(\sum_{j=1}^{J} w_j.\overline{X}_j)^2}{\sum_{j=1}^{J} w_j} \qquad \textit{Equation 5}$$

Where w_j is the weight of each study based on the variance of the data and VWX is the variance of the weighted mean of all observations (1 to J).

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The significance of differences between CYP3A4 and CYP3A5 abundance values determined from studies using rhCYP standards and those using HLMSTD or purified enzyme were assessed by Student's *t*-test (Data Analysis Toolpack, Microsoft Office Excel 2003).

Determination of holoprotein/apoprotein ratios of purified CYPs. The "MEDLINE" database was searched for reports of CYP enzyme purification from both human liver microsomes and recombinant expression systems. The molecular weight (KDa) of each CYP (1A2 58.3; 2A6 56.5; 2B6 56.3; 2C8 55.8; 2C9 55.6; 2C19 56.0; 2D6 55.8; 2E1 56.9; 3A4 57.3; 3A5 57.1) was used to calculate the expected specific enzyme content, assuming 100% holoprotein. The actual percentage holoprotein content of each preparation was calculated using equation 6 by comparing the measured value of the specific CYP content per mg total protein determined by spectroscopy with the expected value

$$\%\,\text{Holoprotein} = \frac{[\text{Measured Specific CYP Content (nmol/mg)}] \times \left(\frac{10^{-9}\,(\text{mol})}{\text{nmol}} \times \frac{\text{mg}}{10^{-3}\,(\text{gram})} \right)}{[\text{Expected Specific CYP Content (1 (mol) / CYP MW (gram)}]} \times 100$$

Equation 6

Deviation of holoprotein protein contents from 100% were assessed for each CYP using the *z*-test. Differences in holoprotein content between CYPs were assessed by one-way analysis of variance (ANOVA) followed by Tukeys b *post hoc* test.

RESULTS

The analysis of CYP3A4 abundance values was based on 384 livers from 13 separate studies (Table 2). The overall weighted geometric mean value was 82 pmol per mg microsomal protein, and there was a 10-fold difference between mean estimates from

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different studies. The homogeneity test gave an HMG of 37 ($p < 0.001$), indicating that the reported average values of abundance in these studies did not conform to a unimodal distribution. Accordingly, the mean value of CYP3A4 abundance determined from studies using rhCYP systems as the calibration standard was significantly lower ($p < 0.01$) than the mean value from studies using characterised human liver microsomes or purified enzyme (45 *vs.* 121 pmol per mg microsomal protein). In all of the studies included in the meta-analysis rhCYP enzymes were obtained from commercial sources.

The analysis of CYP3A5 abundance values was based on 45 livers from 7 separate studies (Table 3). The overall weighted geometric mean value was 55 pmol per mg microsomal protein, and there was an 8-fold difference between mean estimates from different studies. An HMG value of 53 indicated significant ($p < 0.001$) heterogeneity in the results of the different studies. Accordingly, the mean value of CYP3A5 abundance determined from studies using rhCYP systems as the calibration standard was significantly lower ($p < 0.05$) than the mean value from studies using characterised human liver microsomes or purified enzyme (28 *vs*. 83 pmol per mg microsomal protein).

The percentage contributions of holoprotein to total CYP protein purified from human liver were found to be significantly less than 100% ($p < 0.01$ for CYP2C19 and $p < 0.001$ for CYPs 2C8, 2C9, 2D6 and 3A4) and the mean holoprotein/apoprotein ratio for CYP2D6 preparations was significantly ($p < 0.05$) less than that of the other CYP preparations (Figure 1A). There were also indications of inter-subject differences in holoprotein proportion, as exemplified by the analysis of data for CYP3A4 in preparations from 3 different livers purified in the same laboratory (Figure 1B). The percentage contributions of holoprotein to total CYP protein purified from rhCYP systems are shown in Figure 2. Unlike the rhCYP

preparations used in the meta-analysis of CYP3A4 and CYP3A5 abundance, rhCYPs used to obtain purified enzyme were from non-commercial sources.

DISCUSSION

The study of CYP3A4 and CYP3A5 abundances in human liver indicated that the use of different protein standards may result in different values, with commercial rhCYP standards providing generally lower estimates than characterised human liver microsomes and purified enzyme preparations (Tables 1 and 2). A possible explanation for the latter observation is that different standards have different holoprotein/apoprotein ratios. Thus, if the standard contains a lower proportion of holoprotein than that in the samples, the same immunoblot signal will indicate a lower amount of active enzyme, resulting in an underprediction of active CYP abundance in the samples.

Estimates of the holoprotein/apoprotein ratios of purified CYPs from human liver indicate incomplete heme coupling, and differences in this respect between specific CYPs and possibly between individual livers (Figure 1). While incomplete protein purification may explain the findings, it is unlikely that contamination would be more than 10% since loading of 5µg protein on a gel is usually sufficient to detect bands from other proteins running separately from the 'pure' enzyme. Thus, incomplete purification cannot account for the greater than 10% differences in holo/apoprotein ratios observed in many cases. If incomplete purification can be discounted, the observations are either due to incomplete haem incorporation *in vivo* or, experimental artefact (uncoupling of the haem from CYP protein during the purification process) or both. The latter assumes that the uncoupling happens to different extents in different purified systems. In any event, the findings have important

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implications for the immunoquantification of CYP abundances in human liver samples if there is a mismatch of the holoprotein/apoprotein ratio in standards and samples. Depending on the standard used, there could be either under- or over-prediction of CYP abundance.

The estimated holoprotein contents of purified rhCYPs also suggest incomplete haem coupling. The majority of purified rhCYP preparations have a similar holoprotein/apoprotein ratio to that seen in the human liver preparations, suggesting that these would be suitable to use as standards for immunoquantification (Figure 2). However, the CYP2D6 preparations had higher holoprotein contents than that seen in the human liver preparations. Therefore, if these were used as a standard, a significant overprediction in CYP2D6 abundance would result. It should be noted that these rhCYP standards have not been produced for commercial use. The low estimations of CYP abundance indicated in Table 1 and 2 suggest that commercially available rCYP systems would have a lower holoprotein content than those shown in Figure 2.

The observation that estimates of CYP3A abundances are lower when using commercially available rhCYP systems as standards relative to human liver standards could reflect a lower holoprotein/apoprotein ratio in these systems. To establish whether this is the case it would be necessary to show that the commercial rhCYP systems give a greater immunoblot signal for the same level of spectrally determined holoprotein compared to purified liver enzyme. Preliminary studies have suggested that this is the case for CYP3A4 and CYP3A5 rhCYP systems (Wilson *et al.*, 2005; Perrett *et al.*, 2006). This work is ongoing and we hope that the outcome will enable determination of appropriate correction factors to apply when measuring enzyme abundance with rhCYP systems. The utility of such factors would also depend on the extent of variability in the holoprotein/apoprotein ratio between

individual liver samples. It is possible that the ratio of holoprotein to apoprotein might also be affected by genotype since single residue changes can markedly affect protein stability.

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Figure Legends

Figure 1: **(A)** Mean percentage contribution of CYP enzyme holoprotein to total CYP protein in preparations purified from human liver tissue. Standard deviations are shown by error bars where the number of livers was sufficient for calculation.

(B) Inter-individual variability in percentage contribution of holoprotein to total CYP3A4 protein purified from three individual livers (from Guengerich *et al*., 1986).

[References to the data for each of CYP in (A):

1A2 = Dislerath *et al*., 1985; 2A6, Yun *et al*., 1991; 2C8 = Lasker *et al*., 1998, Wang *et al*., 1980, Wrighton *et al*., 1987a; 2C9 = Lasker *et al*., 1998, Sandhu *et al*., 1993, Lasker *et al*., 1987, Shimada *et al*., 1986, Komori *et al*., 1988, Kawano *et al*., 1987; 2C19 = Lasket *et al*., 1998, Lasker *et al*., 1987; 2D6 = Dislerath *et al*., 1985, Gut *et al*., 1984, Gut *et al*., 1986; 2E1 = Wrighton *et al*., 1987, Lasker *et al*., 1987; 3A4 = Lin *et al*., 2002, Guengerich *et al*., 1986, Kawano *et al*., 1987, Watkins *et al*., 1985, Komori *et al*., 1988, Wang *et al*., 1983.]

Figure 2: Percentage holoprotein contribution in preparations purified from recombinant CYP expression systems. For comparison, the dotted lines indicate the mean percentage holoprotein contribution observed for each CYP enzyme when purified from human liver tissue.

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Table 1: Literature values of mean CYP3A4 abundance.

Purified enzyme (PUR), Baculovirus-insect cells (Supersomes–Gentest®) (SUP), human lymphoblastoid cells (Gentest®) (LYMPH), *Saccharomyces cerevisiae* (YEAST) and a sample of HLM quantified for total CYP3A4 protein (HLM-CYP3A4 Std) (Westlind-Johnsson *et al*, 2003). *Significant difference ($p < 0.01$)

Study	n	Mean CYP3A4 (pmol/mg)	Standard
Tateishi *et al.*, 1999	15	49	SUP
King *et al.*, 2002	22	33	SUP
Galetin *et al.*, 2004	12	73	SUP
Wang *et al.*, 2005	5	37	SUP
Wolbold *et al.*, 2003	39	56	LYMPH
Von Richter *et al.*, 2004	15	24	LYMPH
Mean		**45***	
Guengerich and Turvy, 1991	36	248	PUR
Shimada *et al.*, 1994	28	106	PUR
Wandel *et al.*, 1998	14	68	PUR
Lin *et al.*, 2002	60	81	PUR
Lamba *et al.*, 2002	53	80	PUR
Westlind-Johnsson *et al.*, 2003	32	171	HLMSTD
Barter *et al.*, (in preparation)	53	91	HLMSTD
Mean		**121***	
Total Weighted Mean		***82***	

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Table 2: Literature values of mean CYP3A5 abundance.

Purified enzyme (PUR), Baculovirus-insect cells (Supersomes–Gentest®) (SUP), human lymphoblastoid cells (Gentest®) (LYMPH), *E.Coli* (Bactosomes-Cypex®) (BAC) and a sample of HLM quantified for total CYP3A5 protein (HLMSTD) (Westlind-Johnsson *et al*, 2003). *Significant difference ($p < 0.05$)

Study	n	Mean CYP3A5 (pmol/mg)	Standard
Tateishi *et al.*, 1999	6	30	SUP
King *et al.*, 2002	5	42	SUP
Kamdem *et al.*, 2004	5	13	BAC
Mean		**28***	
Kuehl *et al.*, 2001	8	109	PUR
Lin *et al.*, 2002	13	78	PUR
Westlind-Johnsson *et al.*, 2003	3	44	HLMSTD
Barter *et al.*, (in preparation)	5	99	HLMSTD
Mean		**83***	
Total Weighted Mean		***55***	

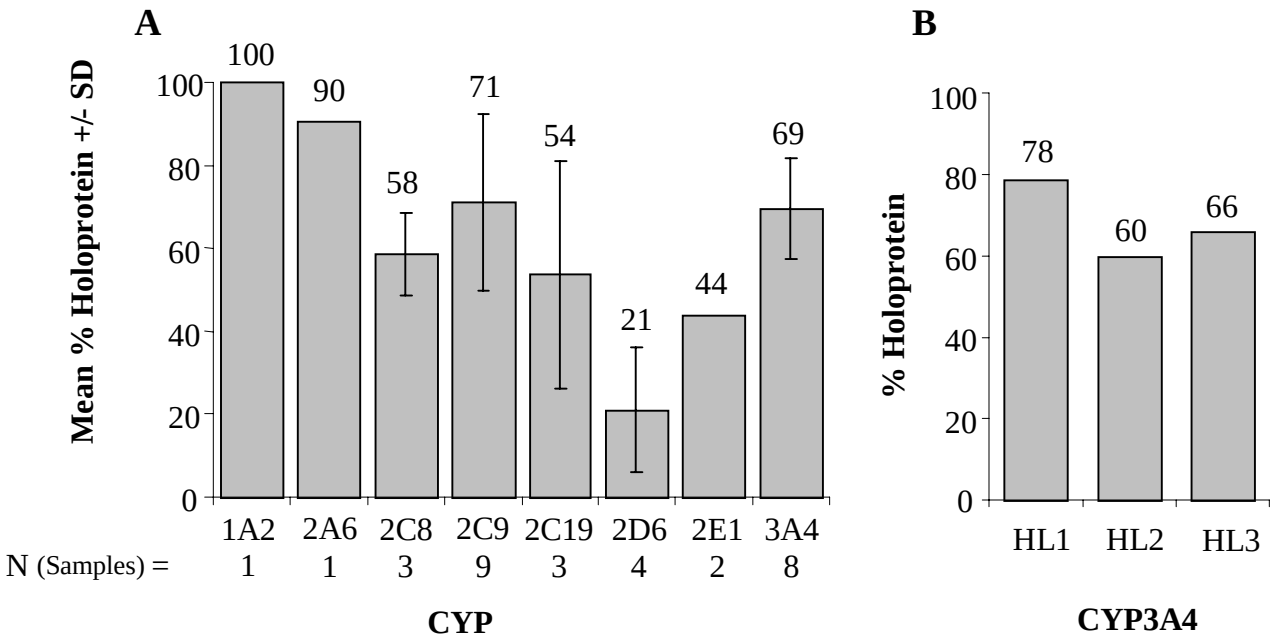

Figure 1

Figure 2