Covariation of human microsomal protein per gram of liver with age: Absence of influence of operator and sample storage may justify inter laboratory data pooling.

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ABSTRACT

Scaling of metabolic clearance values from liver microsomal data or recombinantly expressed cytochrome P450 enzymes to predict human hepatic clearance requires knowledge of the amount of microsomal protein per gram of liver (MPPGL). Identification of physiological covariates of MPPGL requires analysis of values from large diverse populations which necessitates pooling of data from numerous sources. To ensure compatibility between results obtained within and between studies the impact of inter operator differences and sample storage on values of MPPGL were investigated. Using triplicate samples from one liver (HL86), no statistically significant difference was detected between values of MPPGL prepared from samples stored at -80°C (23.5±1.2 mg.g⁻¹) and those determined using fresh tissue (21.9±0.3 mg.g⁻¹). Whilst there was a significant difference in the yield of microsomal protein obtained from another liver sample (HL43) by 3 different operators (17±1, 19±2 and 24±1 mg.g⁻¹, p=0.004 ANOVA) no difference was observed in the estimated MPPGL after applying appropriate correction factors for each operator (28±1, 30±5 and 31±4 mg.g⁻¹). The result provided justification for pooling reported values of MPPGL for use in covariate analysis. Investigation of the relationship between age and MPPGL provided preliminary evidence that MPPGL values increase from birth to a maximum of 40 mg.g⁻¹ (95% CI mean<sub>geo</sub>: 37-43 mg.g⁻¹) around 28 years followed by a gradual decrease in older age (mean of 29 mg.g⁻¹ at 65 years; 95% CI mean<sub>geo</sub>: 27-32 mg.g⁻¹). Accordingly, appropriate age adjusted scaling factors should be used in extrapolating <i>in vitro</i> clearance values to clinical studies.
INTRODUCTION

The use of in vitro – in vivo extrapolation (IVIVE) of metabolic data in the prediction of population clearance has become an important tool in both discovery and preclinical phases of the drug development process (Rostami-Hodjegan and Tucker, 2007). Scaling of metabolic rate constants derived using microsomal protein (MSP) isolated from human livers, or those from recombinantly expressed cytochrome P450 (rhCYP) enzymes requires knowledge of the amount of microsomal protein per gram of liver (MPPGL) among other scaling factors (Barter et al., 2007). The most commonly used value of MPPGL in human IVIVE is that of 45 mg.g⁻¹ (for example, Obach, 1997; Soars et al., 2002; Andersson et al., 2004; Uchaipichat et al., 2006) reported in a review by Houston (Houston, 1994). However, this value is not obtained from human livers; instead it combines data generated using rat tissue from a number of sources (Joly et al., 1975; Lin et al., 1978; Baarnhielm et al., 1986; Chiba et al., 1990). Several values of MPPGL determined using human tissue have been reported in the literature and a detailed review of these studies has been the focus of a recent consensus paper on the most appropriate values of MPPGL for use in IVIVE alongside other scaling factors such as human hepatocellularity (Barter et al., 2007). Collation of values of MPPGL from 5 studies (114 observations; age range 11 – 80 years, median 48 years; 47F) has indicated a weak but statistically significant inverse relationship between MPPGL and donor age (Barter et al., 2007).

Barter et al., (2007) assumed that different experimental procedures and technical staff carrying out the exercise (“operators”) would not introduce bias into their analysis. However, these effects have not been assessed systematically. Assessing the “operator” effect also has implications for within laboratory pooling of data. The preparation and analysis of replicate samples, which are required to differentiate between methodological and true
biological variability in values of MPPGL, is time-consuming (2 days per liver). Although to increase output, processing of samples may be carried out by more than one individual (“operator”), this will require an indication of consistency between estimates of MPPGL by these “operators”.

In addition, determination of values of MPPGL from fresh tissue from large numbers of donors is hampered by the infrequent supply of human tissue and logistical problems associated with immediate analysis of the samples when they arrive at the laboratory. Theoretically, the use of tissue stored at -80°C solves this problem. However, the use of frozen samples assumes maintenance of protein structure and function through the freeze – thaw process. Thus, to ensure adequate compatibility between the results obtained within and between different studies (with the ultimate goal of combining data in large scale analysis) two major questions relating to experimental variables must be addressed:

- Does the “operator” involved in the preparation and analyses of samples have an impact on values of MPPGL derived from the experiments?
- Does the use of “frozen tissue” in the determination of values of MPPGL produce comparable results to those obtained from fresh tissue?

In the current report we provide evidence for consistency of MPPGL values obtained from different “operators” and fresh vs. frozen liver samples. In addition, we re-analyse the influence of age on MPPGL by incorporating neonatal and paediatric MPPGL values to the pool of the previously reported dataset (Barter et al., 2007).

**MATERIALS AND METHODS**

**Reagents**

All laboratory chemicals were purchased from either Sigma (Dorset, UK) or Bio-Rad (Herts, UK).
Source of Human Tissue

Samples of adult human liver were obtained from the collection of human liver samples held within the Academic Unit of Clinical Pharmacology at the University of Sheffield as described previously (Wilson et al., 2003). Samples of paediatric human liver were obtained from Vitron (Arizona, US) by the Environmental Protection Agency and shipped to the University of Sheffield for analysis of microsomal protein content.

Study 1: Fresh vs. frozen tissue

Values of MPPGL were determined in the “same liver” sample (HL84), immediately post surgical excision (“fresh”) and again from the same liver tissue, following storage for 1 week at -80°C (“frozen”). All samples were from the same lobe. Post extraction, liver samples were handled as is routine in our laboratory: placement on ice within 10 minutes of excision, dissection into approximately 3 g amounts followed either by immediate homogenisation (within a further hour; “fresh”) or flash freezing in liquid nitrogen and storage at -80°C (“frozen”). Homogenate and microsomes were prepared and CYP and total protein content measured as described previously (Wilson et al., 2003). The CYP content of samples were determined by Dithionite Difference Spectroscopy according to the method of Matsubara et al., (1976) and MSP was determined using the method of Bradford (1976). After 1 week, 3 g of tissue were thawed and homogenate and microsomal samples prepared and assayed for CYP and total protein content. Values of MPPGL corrected for the fraction of microsomal protein lost during centrifugation were calculated using paired homogenate and microsomal samples using the following equations:

\[
\text{Fractional loss of MSP} = 1 - \left( \frac{\text{CYP}_{\text{microsomal}}}{\text{CYP}_{\text{homogenate}}} \right) \left( \text{nmoles(nmoles)} \right)
\]

\[
\text{MPPGL (mg g}^{-1}) = \frac{\text{Yield of microsomal protein (mg g}^{-1})}{(1 - \text{fractional loss of MSP})}
\]
Levels of microsomal yield and values of MPPGL in the two samples were then compared using the unpaired t test (Data Analysis Toolpak, Microsoft Office Excel 2003). The significance of relationships was assessed by evaluation of p values.

Study 2: Inter operator variability

In order to investigate the effect of multiple operators on the determination of values of MPPGL, homogenate and microsomal samples were prepared in triplicate from tissue from a single donor (HL43) by 3 different individuals (“operators”) following the same protocol and using the same instrumentation (as described previously by Wilson et al., 2003) on the same day. Repeated measurements of the liver sample by each operator allowed the estimation of inter-operator variability in MPPGL. Differences in MPPGL between operators were assessed using One-way ANOVA (SPSS v12, Chicago, IL, USA).

Study 3: Assessing the effect of age on MPPGL from birth to old age

Homogenate and microsomes were prepared from liver tissue from 4 Caucasian paediatric donors (3F; 2, 4, 9 and 13 years). Evaluation of the literature identified one study reporting values of MPPGL (Pelkonen et al., 1973) from 11 foetal samples (age since conception / developmental age not provided). Values of MPPGL from the 4 paediatric samples determined in this study and the 11 foetal samples from Pelkonen et al., (1973) were incorporated into the meta-analysis carried out previously by Barter et al., (2007) (n=114 livers; 11-80 years of age; 43F).

Since the Kolmogorov Smirnov test showed that the data were compatible with a log normal distribution the assessment of model fit was performed on log transformed values of MPPGL. Following the incorporation of paediatric and foetal liver samples to the data set reported previously (Barter et al., 2007) five models, including the model described by Barter et al., (2007), were assessed (Figure 2) in order to best explain the relationship between donor age and MPPGL (Graphpad Prism 5, Graphpad Software Inc, San Diego, USA).
Model 1 was the model used in Barter et al., (2007). Models 2, 3 and 4 were extensions of Model 1 (i.e. nested in Model 1). However, Model 5 had a different structure. Therefore, the F Test was not appropriate for comparison of the models and hence AIC was used (Gabrielsson and Weiner, 2007).

RESULTS

Study 1: Fresh vs. frozen tissue

No significant difference (p > 0.05) was detected in either homogenate (Mean ± SD: fresh 11.5 ± 0.7; frozen 11.2 ± 0.6 nmol.g liver⁻¹) or microsomal (fresh 0.52 ± 0.03; frozen 0.48 ± 0.02 nmol.mg microsomal protein⁻¹) CYP content and hence values of MPPGL prepared from liver samples stored at -80°C (23.5 ± 1.2 mg.g⁻¹) and those determined using fresh tissue (21.9 ± 0.3 mg.g⁻¹).

Study 2: Inter operator variability

Despite using the same liver, same protocol and same instrumentation, significant differences in microsomal protein yield were found between operators (Mean ± SD: 17 ± 1, 19 ± 2 and 24 ± 1 mg.g⁻¹; p = 0.004 ANOVA; Figure 1A). Significant differences in the percentage of microsomal protein lost during the preparation process were also observed (38 ± 3, 37 ± 6 and 22 ± 5 %; p = 0.01 ANOVA; Figure 1B). However, no significant difference in estimated values of MPPGL corrected for losses in microsomal protein during preparation was observed; indicating that higher yields of microsomal protein were associated with lower losses of microsomal protein during preparation (28 ± 1, 30 ± 5 and 31 ± 4 mg.g⁻¹, p = 0.64 ANOVA; Figure 1C). Post hoc analysis of the data (Tukeys b) indicated microsomal losses determined by Operators 1 and 2 (38 and 37%) to be significantly different to that determined by Operator 3 (22%; p < 0.05). Thus, accounting for inter operator differences in yield by application of a recovery factor (1-fraction of microsomal protein lost), resulted in comparable values of MPPGL.
Study 3: Assessing the effect of age on MPPGL from birth to old age

The geometric mean value of MPPGL corrected for loss of protein incurred during preparation for the 4 paediatric livers was 28 mg.g⁻¹ (range 23 – 30 mg.g⁻¹). A foetal MPPGL value of 26 mg.g⁻¹ was calculated from data reported by Pelkonen et al., 1973.

The log linear model reported by Barter et al., (2007) is shown in Figure 2A and MPPGL values from the paediatric and foetal samples are overlaid. Visual inspection of the overlaid data indicates a systematic departure and hence inadequacy of the previous model to describe MPPGL values at lower ages than those examined in the earlier meta analysis. Graphical representation of the fit of the 5 models to the extended data set (n=129 livers) is provided in Figure 2B-F. Incorporation of the additional samples from younger donors into the data set indicated a non monotonic relationship between age and MPPGL with values increasing from birth to a maximum of 40 mg.g⁻¹ around 28 years (95% CI mean geo: 37-43 mg g⁻¹; 95% CI obs 19-85 mg g⁻¹) followed by a gradual decrease in older age (mean of 29 mg.g⁻¹ at 65 years; 95% CI mean geo: 27-32 mg g⁻¹; 95% CI obs: 14-63 mg g⁻¹). Having disregarded Model 1 as an appropriate model, a combination of comparison of AIC and visual inspection for the remaining models indicated Model 4 to provide the best (most parsimonious) fit. This was mainly due to the ability of the model to generate physiologically relevant values of MPPGL even at the extremes of the population. The probability of Model 4 being the correct model over models 2, 3 and 5 was 100%, 25% and 5% more likely respectively (differences in AIC: Model 2 vs. 4 17.0; Model 3 vs. 4 1.03; Model 4 vs. 5 0.21).

Coefficient values for all of the models are provided in Figure 2. The correlation between predicted vs. observed values of MPPGL determined using Model 4 was statistically significant (p = 0.0004). The following equation was used to calculate age related values of MPPGL from birth to adult (Figure 3):

\[
\text{MPPGL (mg.g}^{-1}) = 10^{(1.407 + 0.0158 \times \text{Age} - 0.00038 \times \text{Age}^2 + 0.000024 \times \text{Age}^3)}
\]
DISCUSSION

A key point for consideration when determining values of hepatic scaling factors such as MPPGL is the quality of liver tissue used in their estimation. The CYP content of tissue left at 25°C for 6 hours has been shown to be significantly reduced compared to that maintained in a chilled environment (Yamazaki et al., 1997). Lengthy post-perfusion cold time of liver tissue may also reduce the CYP activity of prepared microsomes (Lipscomb and Garrett, 1998). Therefore, the length of time from tissue extraction to placement in chilled medium should be kept to a minimum. In the current study, the delay between extraction and transfer to ice was minimized to 10 minutes. The infrequent supply of fresh human liver tissue has led to the establishment of collections of frozen tissue samples in many institutions including that at which the present study was performed. Here, samples of human tissue or tissue fractions may be stored at -80°C for several years providing a constant supply of human tissue. However, when using frozen tissue in the determination of MPPGL the effect of storage on levels of CYP and total protein should be considered. Although the effect of sample storage on values of MPPGL has yet to be investigated, several studies have assessed the effect of storage on human liver microsomal CYP content (von Bahr et al., 1980; Powis et al., 1988; Pearce et al., 1996). Overall, very little difference in the microsomal protein content of fresh and frozen samples (10 mg g⁻¹ fresh vs. 12 mg g⁻¹ frozen; (von Bahr et al., 1980) was observed. Powis et al., (1988) found no change in the microsomal CYP content (nmol mg⁻¹) of liver samples stored either as microsomes, homogenate or liver pieces at -80°C for up to one year. However, studies carried out by Pearce et al., (1996) showed the CYP content of microsomes prepared from frozen liver to be around 30% less than that in microsomes prepared from fresh liver. No increase in levels of the degraded, inactive form of CYP, CYP420, were observed compared to those from fresh tissue, indicating that CYP degradation was not the cause of the reduction in CYP content. More likely the increase in
total MSP observed in microsomes prepared from frozen tissue was due to contamination by additional proteins such as haemoglobin, potentially resulting in the generation of artificially high values of MPPGL. Details on handling of the tissue post extraction, such as washing of samples to remove excess blood, to minimise haem contamination, were not provided by Pearce and co-workers. Although only investigated using samples (n=3) from a single donor, the current study, however, found no significant difference in either the homogenate (nmol.g liver\(^{-1}\)) or microsomal (nmol.mg\(^{-1}\)) CYP content of fresh and frozen tissue samples indicating that, provided care is taken prior to storage, samples stored at -80ºC are suitable for use in the determination of MPPGL. Additional support for the use of frozen tissue as a reliable source from which to generate values of MPPGL comes from the investigation of levels of CYP420 in microsomal samples prepared from frozen tissue. We have shown previously (Wilson et al., 2003) that levels of CYP420 in a subset of samples from 5 livers contributes less than 3% of the total CYP450, again indicating that, using the protocols described in the current study, tissue degradation is minimal.

A further experimental variable explored in the current study was the influence of different operators on the determination of MPPGL. Increasing the number of operators is advantageous in studies, such as the present one, where the nature of the analysis makes sample processing time consuming. As the aim of the study was to investigate inter-individual variability in MPPGL it was important to assess the impact, if any, of inter-operator variability on MPPGL determination. Differences in operator affected the yield of microsomal protein. However, measurement of microsomal yield does not account for the fraction of microsomal protein lost during the centrifugation process which can be substantial (Wilson et al., 2003). Determination of total microsomal protein per gram of liver (MPPGL) requires correction of microsomal yield \textit{via} application of a recovery factor (1 – fraction of microsomal protein lost; Figure 1). Application of recovery factors for each operator should
yield comparable values of MPPGL, as demonstrated in the present study. MPPGL values determined within the same laboratory by different individuals may, therefore, be compared. The effect of inter laboratory variables such as instrumentation and reagents on values of MPPGL has yet to be determined. Differences in instrumentation and reagents are also likely to produce different yields of microsomal protein. However, in theory, application of recovery factors specific to the conditions under which microsomal samples are prepared should produce similar corrected values of MPPGL across laboratories.

Collation of samples from donors aged 11 – 80 years (n = 113 adult; n=1 paediatric) previously suggested the relationship between human MPPGL and age to decrease monotonically with age (Barter et al., 2007). Incorporation of additional samples from younger donors (n=4 paediatric from the current study; n=11 foetal, Pelkonen et al., 1972) has indicated a non monotonic relationship between age and MPPGL with values increasing from birth to a maximum of 40 mg.g\(^{-1}\) around 28 years followed by a gradual decrease in older age. Values of MPPGL were around 36% and 31% lower in newborn and elderly (80 years) individuals than those in a 25 year old individual (typically used in clinical pharmacology studies). The use of a value of MPPGL of 40 mg.g\(^{-1}\), determined for a young adult, would be expected to result in an over prediction of clearance in very young or very old patients. Therefore, MPPGL values relevant to the age of the population in which predictions are being made should be used in IVIVE.

Although the correlation between predicted and observed values of MPPGL determined using Model 4 was statistically significant the relationship between MPPGL and age only explained 10% of the overall observed variation in the data. The remaining 90% cannot be attributed wholly to experimental variability as previous use of the repeat measures study design (Barter et al., 2007) has demonstrated experimental variability to contribute only 20% to the overall variability in MPPGL. All investigators working in the area are, therefore,
encouraged to record attributes of tissue samples in order to aid identification of these additional sources of variability.

In addition to further investigating the relationship between age and MPPGL, we have demonstrated that MPPGL values may be determined from frozen tissue by multiple operators. It is hoped that the provision of detailed protocols and validation studies such as those in the present study will serve to encourage wider investigation of MPPGL (including further exploration of the effect of tissue storage conditions, instrumentation and reagents on values of MPPGL). This will facilitate the identification of further covariates and examination of other factors such as ethnicity.
REFERENCES


Footnotes:

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Figure Legends

Figure 1 The effect of multiple operators on A) microsomal protein yield; B) microsomal loss during preparation and C) values of MPPGL corrected for loss. n = 3 determinations per operator ± standard deviation. ** p < 0.05 ANOVA

Figure 2 (A) Relationship between age and MPPGL adapted from Barter et al., (2007). Open circles represent the original data set of 114 livers reported by Barter et al., 2007. Closed circles represent the additional livers incorporated in the updated data set of 129 livers. Error bars represent the SD of the 11 foetal samples investigated by Pelkonen et al., 1973. (B) – (F) Relationship between age and MPPGL described using Models 1-5. Extended data set of 129 livers. Dotted lines indicate the 95% confidence interval of the mean. (B) Model 1: MPPGL = 10\(C_0 + C_1 \times \log\text{Age}\) ; C0 = 2.038, C1 = -0.3048 (C) Model 2: MPPGL = 10\(C_0 + C_1 \times \text{Age}\) ; C0 = 1.540, C1 = -0.00049 (D) Model 3 MPPGL = 10\(C_0 + C_1 \times \text{Age} + C_2 \times \text{Age}^2\) ; C0 = 1.434, C1 = 0.0080, C2 = -0.00011 (E) Model 4: MPPGL = 10\(C_0 + C_1 \times \text{Age} + C_2 \times \text{Age}^2 + C_3 \times \text{Age}^3\) ; C0 = 1.407, C1 = 0.0158, C2 = -0.0038, C3 = 0.00024 (F) Model 5: MPPGL = 10\(C_0(\exp(C_1 \times \text{Age}) + C_2(\exp(C_3 \times \text{Age}))\) ; C0 = 1.835, C1 = 0.0033, C2 = -0.4465, C3 = 0.0639

Figure 3 Examples of age specific values of MPPGL determined using Model 4.
Figure 1

A. Microsomal yield (mg.g⁻¹)

B. Recovery Factor

C. MPPGL (mg.g⁻¹)

**Statistical significance at p < 0.01**
Figure 2

A. Predicted MPPGL using linear relationship reported in Barter et al., 2007.