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Drug lipophilicity and microsomal protein concentration as determinants in the prediction of the fraction unbound in microsomal incubations

Michael Gertz, Peter J. Kilford, J. Brian Houston and Aleksandra Galetin

School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, M13 9PT, United Kingdom

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Running title: Impact of drug lipophilicity and protein concentration on fu_{inc}

Corresponding author: Dr A. Galetin

School of Pharmacy and Pharmaceutical Sciences,

University of Manchester, Stopford Building

Oxford Road,

Manchester, M13 9PT, UK

Tel: (+) 44 161 275 6886

Fax: (+) 44 161 275 8349

Email: Aleksandra.Galetin@manchester.ac.uk

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Abbreviations used: fu_{inc}, fraction unbound in the incubation; pKa, acid ionisation constant; $logD_{7.4}$, distribution coefficient of all drug species between octanol and water at pH = 7.4; C, microsomal protein concentration; afe, average fold error; rmse, root mean squared error; logP, partition coefficient of unionised drug between octanol and water at pH that favours the unionized drug species; K_a, microsomal protein binding affinity; logP/D, descriptor for lipophilicity (logP for drugs where pKa > 7.4; logD for drugs where pKa < 7.4);

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Abstract

Two predictive tools have been proposed by Austin et al. (Drug Metab Dispos 2002, **30**: 1497-1503) and Hallifax and Houston (Drug Metab Dispos 2006, **34**:724-726) to estimate microsomal nonspecific binding (fu_{inc}). The current study was undertaken to elucidate the relative utility of these prediction tools over a range of drug lipophilicity and microsomal protein concentration. The fu_{inc} dataset (n=127) comprised of 35 drugs determined experimentally in this study and 92 collated from Austin and Hallifax data. The observed fu_{inc} values at three microsomal concentrations were compared to the estimates obtained using the Austin and Hallifax equations. In addition, the impact of variability in the logP on the fu_{inc} predictions was assessed. The current analysis highlights the importance of accurate estimation of lipophilicity for the prediction of the fu_{inc}, regardless of the prediction equation used. Both equations represent useful tools for estimation of fu_{inc} for low lipophilicity drugs (logP/D = 0 – 3), especially at low microsomal protein concentration. However, the accuracy of fu_{inc} predictions of highly lipophilic drugs was poor for both equations, implying that fu_{inc} should be experimentally confirmed for drugs with logP/D $\geq$ 3 unless the microsomal protein concentration is as low as 0.1 mg/mL in which case a cut-off of logP/D $\geq$ 5 can be applied. A significant difference in the predictions by the two proposed tools was observed in the area of intermediate lipophilicity (logP/D = 2.5 – 5) where the Hallifax equation provided more accurate fu_{inc} predictions on average and irrespective of the microsomal protein concentration investigated.

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The use of in vitro data to predict in vivo clearance or assess drug-drug interaction potential is well established (Bjornsson et al., 2003, Ito and Houston 2005, Galetin et al., 2006, Huang et al., 2007, Rostami and Tucker, 2007). Binding to microsomal protein and phospholipids has been recognized as an important parameter in the in vitro-in vivo extrapolation strategies (Obach et al., 1999, McLure et al., 2000, Tucker et al., 2001, Margolis and Obach 2003, Galetin et al., 2005, Ito and Houston, 2005, Brown et al., 2006). Nonspecific binding to microsomes may lead to under-estimation of in vivo clearance (Obach et al., 1996, 1999, Ito and Houston, 2005, Grime and Riley, 2006) or can result in significantly higher IC_{50} or K_i values in the assessment of inhibition interaction potential (Margolis and Obach 2003, Brown et al., 2006). Although generally accepted to improve the accuracy of in vitro-in vivo predictions (in conjunction with other in vitro parameters), the assessment of microsomal binding in the form of fu_{inc} is still challenging.

One way to avoid the complications of nonspecific binding is to use very low microsomal concentrations, a common practice in high-throughput screening, in particular with recombinant enzymes showing high enzyme activity (Obach et al., 2006). This is also consistent with recommendations that depletion incubations should be carried out at microsomal protein concentrations below 0.5 mg/mL (Jones and Houston, 2004). However, higher microsomal protein concentrations are required under certain conditions; for instance studying phase II metabolic reactions (Soars et al., 2002, Mohutsky et al., 2006) or intestinal metabolism (Galetin and Houston, 2006). In addition, most of the in vitro assessment of the time-dependent inhibition potential is based on the use of high protein concentrations (1 – 2 mg/mL) in order to allow adequate dilution in the two-step experimental procedure (Ghanbari et al., 2006). It should also be noted that highly lipophilic drugs might show significant nonspecific binding even at low microsomal protein concentration.

Recently, two algorithms have been introduced by Austin et al. (2002) and Hallifax and Houston (2006) for the prediction of fu_{inc}. Both predictive tools are based on the lipophilicity of the compounds investigated, as defined by either $logD_{7.4}$ (for acidic and neutral compounds) or logP for bases and the microsomal protein concentration (see

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Methods). Austin et al. (2002) compiled a dataset of 41 fu_{inc} values in rat liver microsomes and 15 fu_{inc} values in human liver microsomes at a concentration of 1 mg/mL. The dataset included compounds from different chemical classes and covered a wide range of logP/D values.

Hallifax and Houston (2006) extended the Austin et al. (2002) dataset by incorporating 36 additional drugs. The resulting dataset of 92 drugs covered a range of lipophilicity from logP/D = -2.11 - 7.2. The authors proposed that the relationship between logP/D and microsomal binding ($log((1-fu_{inc})/fu_{inc})$ was nonlinear, in contrast to the linear relation defined by Austin et al. (2002). Hallifax and Houston (2006) concluded that the latter empirical equation gave more unbiased predictions of fu_{inc} for drugs with low binding affinity ($fu_{inc} > 0.9$) when compared to Austin et al. (2002).

The aim of this study was to identify limitations within these empirical methods and differences between the respective predictions in order to asses their general applicability. A systematic comparison of predicted and observed fu_{inc} for a large dataset of compounds at different microsomal protein concentration is currently lacking in the literature. In addition, the source of logP/D (experimental or predicted value) and the impact of its variability on the prediction of fu_{inc} were also investigated.

The impact of lipophilicity and microsomal protein concentration was assessed using a dataset of 127 compounds (35 from our own investigation data and 92 from the dataset of Austin et al. (2002) and Hallifax and Houston (2006)).The fu_{inc} values were experimentally determined using high-throughput dialysis (Banker et al., 2003) at three protein concentrations (0.1, 0.5 and 1.0 mg/mL). The observed fu_{inc} values were compared to the values predicted by the Austin and Hallifax equations The impact of lipophilicity was examined at 0.1 - 1.0 mg/mL microsomal concentration, covering a representative range of logP/D values, from low (< 2.5), medium (2.5 – 5) to high (> 5). The implications of these findings on the prediction of fu_{inc}, in particular for medium and highly lipophilic compounds, are discussed.

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Materials and Methods

Sensitivity analysis. A simulated fu_{inc} dataset was generated using the Austin and Hallifax equations (equations 1 and 2, respectively) over a range of lipophilicity (logP/D = 0 to 8) and varying microsomal protein concentration (C) from 0.05 to 2 mg/mL. Comparison of the two equations was performed at four distinct areas of lipophilicity, namely logP/D = 0 – 2.5, 2.5 – 5, 5 – 7 and >7.

$$fu_{inc} = \frac{1}{1 + C \cdot 10^{0.56 \log P/D - 1.41}} \quad (1)$$

$$fu_{inc} = \frac{1}{1 + C \cdot 10^{0.072 \log P/D^2 + 0.067 \log P/D - 1.126}} \quad (2)$$

where $logD_{7.4}$ represents the logarithm of the ratio of the concentration of all drug species (ionized and unionized drug) distributed between octanol and water at pH = 7.4 (used for acidic and neutral drugs) and logP represents the logarithm of the ratio of the concentration of unionized drug partitioned between octanol and water (used for basic drugs).

Experimental fu_{inc} dataset. The fu_{inc} values were experimentally determined for 35 compounds at microsomal protein concentrations of 0.1, 0.5 and 1.0 mg/mL using the high-throughput dialysis method as described by Banker et al. (2003). All compounds were purchased from Sigma Chemicals Co. (Poole, Dorset, UK), apart from carvedilol, indinavir, ritonavir and zidovudine which were obtained from Sequoia Research Products (Pangbourne, West Berkshire, UK). Microsomal binding was determined using pooled human liver microsomes (n = 22) obtained from BD Gentest Co. (Woburn, MA, USA). Dialysis membranes had a 12 – 14 kDa molecular weight cut off and were purchased from HT Dialysis LLC (Gales Ferry, CT, USA). Phosphate buffer was added to the acceptor chamber with substrate (5 or 10 μM) and microsomes were added to the donor chamber at three protein concentrations, 0.1, 0.5 and 1.0 mg/mL. The dialysis plate was left to equilibrate for 20 hours on a plate shaker (450 rpm) at 37^0C. At the end of the experiment samples were transferred to a 96-well plate with acetonitrile containing the corresponding internal standard.

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Linearity. Peak areas for drugs with relatively low lipophilicity (logP/D < 2.5) were considered to be comparable in the donor and acceptor chamber. Additional, in house calibration data over the appropriate concentrations range was available for buspirone, warfarin, mycophenolic acid, codein, diclofenac, repaglinide, indinavir and nifedipine. In the range of logP/D 2.5 to 5, linearity data were available for 13 of the 17 drugs investigated; therefore, for the remaining drugs linearity, when comparing the area of the peaks, was assumed. For the compounds with high lipophilicity in house linearity was available for terfenadine, raloxifene and troglitazone, but not for mibefradil, ritonavir and tamoxifen.

Literature fu_{inc} dataset. A dataset of fu_{inc} values for 92 compounds was collated from Austin et al. (2002) and Hallifax and Houston (2006). As the fu_{inc} values were determined at different microsomal protein concentrations, reported values were standardised to give fu_{inc} values at 0.1, 0.5 and 1.0 mg/mL using equations 3, where K_a is the microsomal protein binding affinity.

$$fu_{inc} = \frac{1}{1 + K_a \cdot C} \qquad (3)$$

LC-MS/MS. The LC-MS/MS system used consisted of a Waters 2790 with a Micromass Quattro Ultima triple quadruple mass spectrometer (Waters, Milford, MA). Samples were centrifuged at 2500 rpm for 10 min and an aliquot of 10 µL of both the dialysate and buffer was analysed by LC-MS/MS. Varying gradients of four mobile phases were used, the composition of which were; A – 90% water and 0.05% formic acid with 10% acetonitrile, B – 10% water and 0.05% formic acid with 90% acetonitrile, C – 90% water and 10mM ammonium acetate with 10% acetonitrile, D- 10% water and 10 mM ammonium acetate with 90% acetonitrile. For all compounds except carvedilol and buspirone a Luna C18 column (3µ, 50 x 4.6mm) was used for chromatographic separation of analytes. For carvedilol and buspirone a Luna phenyl-hexyl column (5µ, 30 x 4.6mm) was used for chromatographic separation of analytes. The flow rate was set at 1 mL/min and this was split to 0.25 mL/min before entering the mass spectrometer. Further analytical parameters are described in Table 1.

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Calculations. The fraction unbound was calculated using equation 4.

$$fu_{inc} = \frac{Peak\ area\ in\ buffer\ sample/\ Peak\ area\ internal\ standard}{Peak\ area\ in\ microsomal\ sample/\ Peak\ area\ internal\ standard} \quad (4)$$

The experimentally determined fu_{inc} values at microsomal protein concentrations ranging from 0.1 – 1.0 mg/mL for the current dataset (n = 35) were compared to the predicted values by both Austin and Hallifax equations. The experimental dataset in the current study was expanded by including the fu_{inc} values for further 92 drugs from previous publications (Austin et al. 2002, Hallifax and Houston, 2006), covering a lipophilicity range from -2.1 to 7.2. The bias of experimental fu_{inc} was assessed from the geometric mean of the ratio of predicted and actual value (average-fold error – *afe*, eq. 5). The root mean squared prediction error (*rmse*, eq. 6) provided a measure of precision for the predictions of the fu_{inc} values (Sheiner and Beal, 1981; Obach et al., 1997):

$$afe = 10^{\left|\frac{1}{n}\sum \log\frac{Predicted}{Observed}\right|} \quad (5)$$

$$rmse = \sqrt{\frac{1}{n}\sum (Predicted - Observed)^2} \quad (6)$$

Prediction of logP values. Experimental logP data were obtained from the online database ChemIDplus Advanced (2007) for 20 of the investigated 35 drugs. As experimental logP data were not available for all drugs investigated, five online predictive software packages, Drug Bank (2006), Sparc version 3.1 (2007), Syracus (2007), ACD logP (2007) and Interactive analysis logP (2007), were evaluated for their prediction accuracy. The evaluation was undertaken by comparing experimentally determined logP values for 49 drugs collated from the online database ChemIDplus Advanced (2007) to the predictions obtained by the aforementioned software packages. Consequently the mean of the Syracus, ACD and Interactive software packages was used to predict the values for the 15 drugs with unknown logP. The pKa values for the acids investigated were obtained from the online database ChemIDplus Advanced (2007) or from Sparc version 3.1. The $logD_{7.4}$ values were then calculated for the acidic drugs using equation 7:

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$$\log D_{7.4} = \log P - \log\left(1 + 10^{(7.4 - pKa)}\right) \quad (7)$$

The impact of variability in logP estimates on the fu_{inc} predictions by both predictive equations was assessed by propagating a 20% variation in the predictions of logP.

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Results

Sensitivity analysis. A simulated fu_{inc} dataset generated by both Austin and Hallifax equations was compared over a range of microsomal protein concentration (C = 0.05 – 2 mg/mL). At logP/D ≥ 0, lipophilicity had a greater influence on the fu_{inc} prediction than the microsomal protein concentration for both equations. This effect was more pronounced for the Hallifax equation. Both equations predicted drugs with very high lipophilicity (logP/D ≥ 7) to be bound > 90% to microsomal protein, even at C as low as 0.05 mg/mL (Figure 1).

Four distinct areas of lipophilicity were investigated, namely low (logP/D = 0 – 2.5), intermediate (logP/D = 2.5 – 5), high (logP/D = 5 – 7) and very high lipophilicity (logP/D ≥ 7). No major difference between the two equations was observed for the areas of low and high lipophilicity. However, for logP/D values between 2.5 and 5 the Hallifax equation showed up to 3-fold higher predictions in comparison to Austin (Figure 1). At very high lipophilicity (logP/D ≥ 7), the largest discrepancy between the two equations was observed. Inversely to the situation at medium lipophilicity, predictions of fu_{inc} by Austin equation were higher than predictions by Hallifax equation and the ratio of the two equations tended towards 0 (Figure 1). Throughout the range of lipophilicity, minimal difference in the predicted fu_{inc} values was observed at low microsomal protein concentration.

Prediction of logP values. Due to the lack of availability of experimentally determined logP values for 15 of the 35 drugs investigated, a prediction based approach was used to obtain the unknown logP values. Out of the five software packages investigated, Syracus, ACD and Interactive showed the highest prediction accuracy and were ultimately chosen. Each of the individual predictive software tools gave logP predictions with low bias and high precision (data not shown). However, the mean of the three selected tools showed the highest prediction accuracy, as indicated by the correlation coefficient (r^2 = 0.957), bias (afe = 1.07) and precision (rmse = 0.52) (Figure 2). From the evaluation dataset, 78% of the predicted logP estimates were within 20% of the experimental values. Especially good

predictions were observed for drugs with log P/D ≥ 2, where 26/29 of the predictions were within 20% and 18/29 of the predictions were within 10% of the experimental values.

Impact of microsomal protein concentration on the predictions of fu_{inc}. In order to investigate the impact of microsomal protein concentration, the fu_{inc} values of 35 drugs were experimentally determined at three different microsomal protein concentrations, 0.1, 0.5 and 1.0 mg/mL. The experimental fu_{inc} values obtained were compared to the estimates predicted by both Austin and Hallifax equations, as shown in Table 2. In addition to these, fu_{inc} values for 92 drugs previously published by Austin et al. (2002) and Hallifax and Houston (2006) were included in the analysis, resulting in the dataset of 127 compounds. Overall, a decrease in prediction accuracy was observed with increasing microsomal protein concentration (Table 3). This effect was more noticeable for the equation of Austin, whereas the equation of Hallifax resulted in comparable bias in the predictions regardless of the microsomal protein concentration. Particularly high discrepancy between predicted and experimentally obtained values was observed for the fu_{inc} prediction of felodipine with the Hallifax equation, (6-fold over-prediction at C ≥ 0.5 mg/mL). Conversely, the fu_{inc} value of ritonavir was under-predicted by more than 1000% by both equations.

1. *Low microsomal protein concentration (0.1 mg/mL),* both equations gave highly accurate fu_{inc} predictions with very low bias. The success of prediction within 1.5-fold was comparable between the equations (81 and 85% for Austin and Hallifax, respectively). In addition, both equations resulted in a similar number of estimates outside 2-fold of the observed value (~ 8% for both equations).

2. *Medium microsomal protein concentration (0.5 mg/mL),* use of the Hallifax equation resulted in better predictions at this microsomal protein concentration, whereas the Austin equation on average under-predicted the fu_{inc} values by 22%. Overall the extent of predictions found within 1.5-fold decreased and a higher percentage of predictions was found outside 2-fold for both equations in comparison to 0.1 mg/mL (Table 3).

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3. *High microsomal protein concentration (1.0 mg/mL),* accuracy in the predictions further decreased for both equations. On average the Hallifax equation predicted fu_{inc} with higher accuracy; 69% of the fu_{inc} values were within 1.5-fold of the line of unity and 23% outside 2-fold, (Table 3).

Impact of lipophilicity on the prediction of fu_{inc}. In addition to microsomal protein concentration the impact of lipophilicity on the prediction of the fraction unbound was investigated. In the dataset of 127 compounds the lipophilicity ranged from -2.1 (cinoxacine) to 7.2 (amiodarone), providing a representative distribution across the range of lipophilicity of interest.

1. *Low lipophilicity (logP/D < 2.5).* Both equations gave highly accurate fu_{inc} predictions for 62 drugs with comparable low bias, as shown in Figure 3A and B. Zidovudine and nilvadipine were the only exceptions, where both equations over-predicted the fu_{inc} value by more than 50%.

2. *High lipophilicity (logP/D > 5).* Both equations gave equally inaccurate fu_{inc} predictions for 14 compounds investigated, with an average under-estimation at high microsomal protein concentration of 159 and 131% for Austin and Hallifax, respectively (Table 3, Figure 3).

3. *Intermediate lipophilicity (logP/D = 2.5 – 5).* This range of lipophilicity was the main focus of the current study, as the equations displayed a pronounced difference in this area (Figure 1). Out of the 51 drugs within this range, fu_{inc} values for 35 were predicted closer to the line of unity by the equation of Hallifax (Figure 3). This equation estimated 53 – 82% of the fu_{inc} values within 1.5-fold of the predicted/observed ratio of 1, for 1.0 and 0.1 mg/mL, respectively. Especially high accuracy was observed in the fu_{inc} prediction for buspirone, quinidine, diazepam and buprenorphine. The Austin equation estimated 22 – 73% of the fu_{inc} values within 1.5 fold of the predicted/observed ratio of 1, for 1.0 and 0.1 mg/mL, respectively. Particularly high accuracy was observed in the prediction of the fu_{inc} values for carvedilol, simvastatin, propafenone and trimeprazine. For this range of lipophilicity, 9.8 – 55% of fu_{inc} estimates predicted by the Austin equation were outside the 2-fold of the

observed values, in contrast to 7.8 – 35% seen in case of estimates obtained using the Hallifax equation (Figure 3). Significant outliers were lorcainide and imipramine for the Austin equation, which were under-estimated by 600 and 700% respectively, and felodipine and nicardipine for the Hallifax equation, which were over-estimated by 500 and 1000%, respectively. Over the entire range of lipophilicity a lower bias was observed for the predictions obtained by the Hallifax equation compared to the Austin equation (Table 3).

Due to the differences seen between the predictive equations in the area of moderate lipophilicity, the impact of variability in logP estimates on fu_{inc} predictions was assessed (Figure 4). A propagated 20% variation in the predictions of logP resulted in 50 and 15% variation of fu_{inc} using the equations of Austin and Hallifax, respectively, at a logP/D of 2.5. However, at the higher end of lipophilicity (logP = 5), 20% variation on logP resulted in 5.4 and 6.7-fold difference in the fu_{inc} prediction obtained by the Austin and Hallifax equations, respectively at a microsomal protein concentration of 0.1 mg/mL, as illustrated in Figure 4. Fold difference in the fu_{inc} prediction is even more pronounced if higher microsomal concentrations (1.0 mg/mL) are used, ranging from 10-21 fold in the case of Austin and Hallifax equation, respectively, indicating caution in the use of predicted logP values.

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Discussion

It has become widely accepted that the fraction of drug unbound in an incubation needs to be incorporated into the in vitro determination of clearance and inhibition potential to correct for nonspecific binding (Obach et al., 1997, Tucker et al., 2001, Margolis and Obach 2003, Ito and Houston 2005, Riley et al., 2005, Brown et al., 2006). In recent years two prediction equations have been published to estimate fu_{inc} (Austin et al. 2002, Hallifax and Houston, 2006). This study was undertaken to identify the limitations within these empirical methods and to provide recommendation when the predictive equations can replace experimental fu_{inc} values with confidence. Consequently, the fu_{inc} values predicted by both equations were evaluated against experimentally determined fu_{inc} for a dataset of 127 drugs (35 from current study and 92 from the literature values) at three different microsomal protein concentrations and over a representative range of lipophilicity.

Sensitivity Analysis. In the area of low lipophilicity (logP/D ≤ 0) negligible interaction is expected with microsomal protein, resulting in fu_{inc} values of ~ 1. The assumption that highly hydrophilic drugs interact minimally with microsomal proteins or phospholipids is met by the Austin equation. Due to the nonlinear nature of the Hallifax equation, the use of this equation was inappropriate in this area. The large discrepancy between the two equations highlighted at high microsomal protein concentration and lipophilicity (Figure 1) indicated that reliance solely on the predicted fu_{inc} values is inadvisable in this particular area.

Impact of microsomal protein concentration on the prediction of fu_{inc}. The sensitivity analysis indicated that the microsomal protein concentration had less effect on the fu_{inc} predictions than the lipophilicity. This is particularly true at low microsomal protein concentrations. However, it still had a significant effect on the extent of binding observed; this effect increased at higher microsomal protein concentration. The Austin equation showed a tendency to under-predict fu_{inc} values at all microsomal protein concentrations investigated

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in the current study (Table 3, Figure 3). In contrast, the equation of Hallifax predicted the fu_{inc} values ≥ 0.4 with high accuracy but displayed particular problems to accurately predict very highly lipophilic drugs; where $fu_{inc} \leq 0.2$ (Figure 3). Comparison of prediction accuracy at different microsomal protein concentrations indicated that the use of the Hallifax equation for fu_{inc} predictions was advantageous, as shown in Figure 3 and Table 3. Especially fu_{inc} values at higher microsomal protein concentrations were better predicted using this equation.

Impact of lipophilicity on the prediction of fu_{inc}. The impact of lipophilicity was studied at microsomal protein concentrations from 0.1 to 1.0 mg/mL. This range of microsomal protein concentration is widely used for drug depletion profiles and inhibition studies (Obach, 1999, Jones and Houston 2004, Galetin et al. 2005, Rawden et al., 2005, Riley et al., 2005, Brown et al. 2006, Galetin and Houston 2006, Mohutsky et al., 2006, Obach et al. 2006).

Highly accurate and similar fu_{inc} predictions were obtained by both equations for low lipophilicity drugs (logP/D $\leq$ 2.5), which is in accordance with the sensitivity analysis, indicating that both prediction equations can be used interchangeably in this area of lipophilicity. At the same time, both equations failed equally in predicting the fraction unbound of highly lipophilic (logP/D $\geq$ 5) drugs. The poor correlation observed between experimental and predicted fu_{inc} for some of the drugs in this lipophilicity area (e.g., ritonavir and mibefradil) may be a result of incomparability of peak areas in the analytical assay as no linearity study was performed. This study did not investigate the accuracy of fu_{inc} predictions for drugs exceeding logP/D of 7 due to the low availability of such lipophilic drugs. As the precision in fu_{inc} predictions decreased with increasing lipophilicity in this dataset, fu_{inc} predictions of very highly lipophilic drugs can be expected to be poor. Additionally, as even minor variation in logP predictions/determination resulted in a substantial variation in $fu_{inc,}$, the fraction unbound should be determined experimentally for drugs or new chemical entities with logP/D $\geq$ 5.

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Special consideration was given to the intermediate lipophilicity (logP/D = 2.5 – 5), as the sensitivity analysis showed a significant difference in the predictions between the two equations. As a large number of drugs and potentially new chemical entities can be expected to be found within this lipophilicity range, further emphasis was placed on experimental determination of fu_{inc} values for compounds in this particular logP/D range. Twenty-two drugs from our own experiments and 29 from the literature (n=51) were investigated in this lipophilicity range. On average the Austin equation resulted in an under-estimation of fu_{inc} (118 – 185%), whereas the equation of Hallifax generally over-estimated (113 – 121%) the extent of nonspecific binding (Table 3). The Hallifax equation predicted a larger number of drugs closer to the line of unity, as indicated by a larger proportion of predictions within 1.5-fold of the observed values (Figure 3).

The impact of using predicted (as opposed to the experimental) logP values, and of variability in those estimates, on the fu_{inc} predictions has also been investigated. Highly accurate logP predictions were obtained by three online software packages, Syracus, ACD and Interactive. The mean of these three software packages was found to be the most accurate to predict logP values for the dataset investigated. A very good agreement was observed in particular for drugs with logP $\geq$ 2 (Figure 2), whereas the prediction accuracy was lower at logP = 0 – 2. However, this was acceptable, as minimal interaction with microsomal protein is expected in this area of lipophilicity, as indicated by the sensitivity analysis. Still, even a relatively low bias in logP predictions may influence fu_{inc} predictions significantly, as lipophilicity was indicated as the most important parameter for fu_{inc} predictions. A propagation of a 20% variation in lipophilicity had a minor effect at low logP/D values (Figure 4). However, with increasing lipophilicity this effect became more pronounced, especially for the Hallifax equation, indicating caution in the use of predicted logP values in conjunction with fu_{inc} predictions. It is of concern that the inaccuracy in logP and consequently microsomal fu_{inc} prediction may be propagated further into hepatocytes studies, as it has been proposed that the extent of binding in hepatocytes incubations can be

extrapolated from microsomal fu_{inc} estimates, assuming a correlation between microsomal and hepatocyte binding at 1 mg/mL and 10^6 cells/mL, respectively (Austin et al., 2005).

In conclusion, the current analysis has highlighted the importance of drug lipophilicity as very sensitive parameter for the prediction of the $fu_{inc.}$ Both equations investigated showed very good agreement in the fu_{inc} estimates at low microsomal protein concentration, in particular for drugs with low lipophilicity. A significant difference in the fu_{inc} estimates was seen in the area of intermediate lipophilicity due to the nature of the prediction equations and their sensitivity on the variability in the logP estimates. On average, the Hallifax equation provided more accurate fu_{inc} predictions, in particular for lipophilic drugs (logP/D = 2.5 – 5) and at higher microsomal protein concentrations. The extent of nonspecific binding for highly lipophilic drugs was poorly predicted by both equations, suggesting that the fraction unbound should be determined experimentally for drugs with logP/D $\geq$ 5; this cut-off should be even lower (logP/D $\geq$ 3) if microsomal protein concentration above 0.1 mg/mL are used. As overall prediction accuracy was the highest at low microsomal protein concentration, it is prudent to perform kinetic and inhibition studies for new chemical entities at the lowest microsomal protein concentration possible.

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FOOTNOTES

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Address correspondence to:

Dr A. Galetin, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Oxford Rd, Manchester, M13 9PT, UK

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Figure legends

Figure 1: Ratio of the fu_{inc} predicted by the Hallifax and Austin equations over a range of lipophilicity (logP/D = -8 – 8) and microsomal protein concentration (C = 0.05 – 2 mg/mL)

Figure 2: Comparison of predicted and observed logP values of 49 drugs using the mean of Syracus, ACD and Interactive software packages; (■) represents compounds chosen for evaluation; (···) line of best fit, (---) indicates 20% difference to the line of unity

Figure 3: Comparison between predicted/observed fu_{inc} ratio and lipophilicity (logP/D) for 127 drugs at C = 0.1 mg/mL (A) and C = 1.0 mg/mL (B) using (■) Hallifax and (□) Austin equations; (---) indicates 1.5 and (···) 2-fold of the line of unity.

Figure 4: Impact of 20% variation in logP predictions on the fu_{inc} estimates obtained by the Austin (---) and Hallifax (—) equations at microsomal protein concentration of 0.1 mg/mL

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TABLE 1

Experimental conditions for the selected compounds with details on the internal standards, mass transitions and retention times

Compound	**Internal Standard**	**Electrospray Ionisation**	**Transition**	**Retention Time (min)**
α-Napthoflavone	Triazolam	Positive	273.1 > 115.3	4.9
Buprenorphine	Mibefradil	Positive	468.5 > 396.3	2.7
Buspirone	Quinidine	Positive	386.4 > 122.2	3.6
Carvedilol	Buspirone	Positive	407.4 > 100.4	4.2
Codeine	Levallorphan	Positive	300.2 > 215.1	3.4
Desipramine	Zidovudine	Positive	267.0 > 72.30	3.7
Dextromethorphan	Qunidine	Positive	272.2 > 171.4	3.4
Diclofenac	Tolbutamide	Negative	293.9 > 250.1	4.6
Emodin	Diclofenac	Negative	269.3 > 225.3	3.0
Felodipine	Nifedipine	Positive	384.3 > 338.1	3.3
Gemfibrozil	Tolbutamide	Negative	249.2 > 121.2	4.5
Imipramine	Desipramine	Positive	281.2 > 86.50	3.7
Indinavir	Dextromethorphan	Positive	614.6 > 421.3	2.9
Levallorphan	Codeine	Positive	284.3 > 157.4	4.5
Mibefradil	Verapamil	Positive	496.3 > 202.2	3.8
Midazolam	Diazepam	Positive	326.0 > 291.3	4.4
Mycophenolic acid	Warfarin	Negative	319.4 > 191.2	4.0
Naloxone	Levallorphan	Positive	328.4 > 310.3	3.0
Nifedipine	Felodipine	Positive	347.3 > 315.4	2.7
Oxazepam	Midazolam	Positive	286.9 > 241.3	4.0
Quinidine	Dextromethorphan	Positive	325.1 > 307.3	3.0
Raloxifene	Terfenadine	Positive	474.4 > 112.3	3.3
Repaglinide	Indomethacin	Positive	453.3 > 230.1	4.3
Ritonavir	Dextromethorphan	Positive	743.4 > 573.2	2.6
Rosiglitazone	Buspirone	Positive	358.3 > 135.3	4.2
Saquinavir	Terfenadine	Positive	671.2 > 570.4	3.7
Simvastatin	Terfenadine	Positive	441.4 > 325.4	4.0
Tacrolimus	Verapamil	Positive	821.7 > 768.4	3.1

Tamoxifen	Verapamil	Positive	372.3 > 72.50	3.5
Terfenadine	Metoprolol	Positive	482.3 > 436.2	4.5
Triazolam	Diazepam	Positive	343.0 > 308.3	3.8
Troglitazone	Diltiazem	Positive	442.4 > 165.4	3.3
Verapamil	Mibefradil	Positive	455.4 > 165.4	3.3
Zidovudine	Desipramine	Positive	268.3 > 127.3	2.4

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TABLE 2

Predicted and experimentally determined fu_{inc} values at C = 0.1 and 1.0 mg/mL for 35 compounds investigated

	log P/D[a]	Fraction unbound (fu_{inc}) at 0.1 mg/mL			Fraction unbound (fu_{inc}) at 1.0 mg/mL		
		Observed[c]	Hallifax eq.	Austin eq.	Observed[c]	Hallifax eq.	Austin eq.
Buprenorphine	4.98	**0.47**	0.50	0.30	**0.10**	0.09	0.04
Buspirone	2.63	**0.94**	0.97	0.90	**0.85**	0.74	0.46
Carvedilol	4.19	**0.58**	0.79	0.54	**0.10**	0.28	0.10
Codein	1.19	**1.00**	0.99	0.98	**0.96**	0.90	0.85
Desipramine	4.90	**0.65**	0.54	0.32	**0.21**	0.11	0.04
Dextromethorphan[b]	4.19	**0.84**	0.79	0.54	**0.72**	0.28	0.10
Diclofenac	1.26	**1.00**	0.99	0.98	**0.87**	0.89	0.84
Emodin[b]	2.84	**0.46**	0.96	0.87	**0.19**	0.69	0.40
Felodipine	3.86	**0.40**	0.86	0.64	**0.06**	0.38	0.15
Gemfibrozil	1.80	**0.97**	0.98	0.96	**0.77**	0.86	0.72
Imipramine	4.80	**0.91**	0.58	0.35	**0.45**	0.12	0.05
Indinavir[b]	2.12	**1.00**	0.98	0.94	**0.88**	0.82	0.63
Levallorphan	3.48	**0.92**	0.91	0.74	**0.84**	0.51	0.22
Mibefradil[b]	6.23	**0.34**	0.075	0.077	**0.03**	0.008	0.008
Midazolam	3.80	**0.97**	0.87	0.66	**0.54**	0.40	0.16
Mycophenolic acid[b]	0.94	**0.95**	0.99	0.99	**0.79**	0.91	0.89
Naloxon	2.09	**0.87**	0.98	0.95	**0.87**	0.82	0.64
Nifedipine	2.20	**0.98**	0.98	0.94	**0.70**	0.81	0.60
Oxazepam	2.24	**0.83**	0.98	0.94	**0.72**	0.81	0.59
Quinidine	3.44	**0.86**	0.92	0.75	**0.56**	0.53	0.23
Raloxifene[b]	5.26	**0.51**	0.38	0.23	**0.08**	0.06	0.03
Repaglinide[b]	1.76	**0.96**	0.98	0.96	**0.73**	0.86	0.73
Ritonavir[b]	5.79	**0.87**[d]	0.18	0.13	**0.38**	0.02	0.02
Rosiglitazone[b]	2.62	**0.93**	0.97	0.90	**0.72**	0.74	0.47
Saquinavir[b]	3.09	**0.59**	0.94	0.83	**0.10**	0.63	0.32
Simvastatin	4.68	**0.39**	0.63	0.38	**0.06**	0.15	0.06
Tacrolimus[b]	2.97	**0.71**[d]	0.95	0.85	**0.18**	0.66	0.36
Tamoxifen[b]	6.44	**0.06**	0.049	0.060	**0.01**	0.005	0.006
Terfenadine[b]	6.60	**0.04**	0.034	0.049	**0.02**	0.003	0.005
Triazolam	2.42	**1.00**	0.97	0.92	**0.84**	0.78	0.53
Troglitazone[b]	5.02	**0.52**	0.49	0.29	**0.07**	0.09	0.04
Verapamil	3.79	**0.83**	0.87	0.66	**0.47**	0.41	0.16
Warfarin	0.28	**0.99**	0.99	0.99	**0.99**	0.93	0.95
Zidovudine	0.05	**0.75**	0.99	1.00	**0.60**	0.93	0.96
α-Naphthoflavone[b]	4.65	**0.20**	0.64	0.39	**0.07**	0.15	0.06

[a]Data represent either $logD_{7.4}$ (for acidic and neutral compounds) or logP for bases. [b]logP values were predicted using the mean of Syracus, ACD and Interactive software packages. [c]Data represent a mean of three replicates. [d]Obtained using average K_a value due to variability of data.

TABLE 3

Accuracy of fu_{inc} predictions using the Austin and Hallifax equations categorised into three different lipophilicity groups at different microsomal protein concentrations

	Austin eq.				Hallifax eq.			
log P/D	< 2.5	2.5 - 5	> 5	**all**	< 2.5	2.5 - 5	> 5	**all**
n	62	51	14	**127**	62	51	14	**127**
				C = 0.1 mg/mL				
Within 1.5-fold (%)	98.4	72.5	35.7	**81.1**	98.4	82.4	35.7	**85.0**
Outside 2-fold (%)	0	9.8	35.7	**7.9**	0	7.8	50	**8.7**
afe	1.01	1.18	1.26	**1.09**	1.02	1.13	1.13	**1.05**
rmse	0.06	0.23	0.26	**0.17**	0.06	0.20	0.25	**0.15**
				C = 0.5 mg/mL				
Within 1.5-fold (%)	98.4	39.2	21.4	**66.1**	96.8	58.8	21.4	**73.2**
Outside 2-fold (%)	2.0	37.3	42.9	**19.7**	2.0	23.5	57.1	**16.5**
afe	1.01	1.61	1.58	**1.28**	1.06	1.18	1.32	**1.07**
rmse	0.12	0.32	0.16	**0.23**	0.12	0.24	0.16	**0.18**
				C = 1.0 mg/mL				
Within 1.5-fold (%)	93.5	21.6	21.4	**59.1**	93.5	52.9	14.3	**68.5**
Outside 2-fold (%)	0	54.9	42.9	**26.8**	2.0	39.2	57.1	**22.8**
afe	1.03	1.85	1.59	**1.37**	1.07	1.21	1.31	**1.08**
rmse	0.17	0.31	0.10	**0.23**	0.15	0.23	0.10	**0.18**

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Figure 1

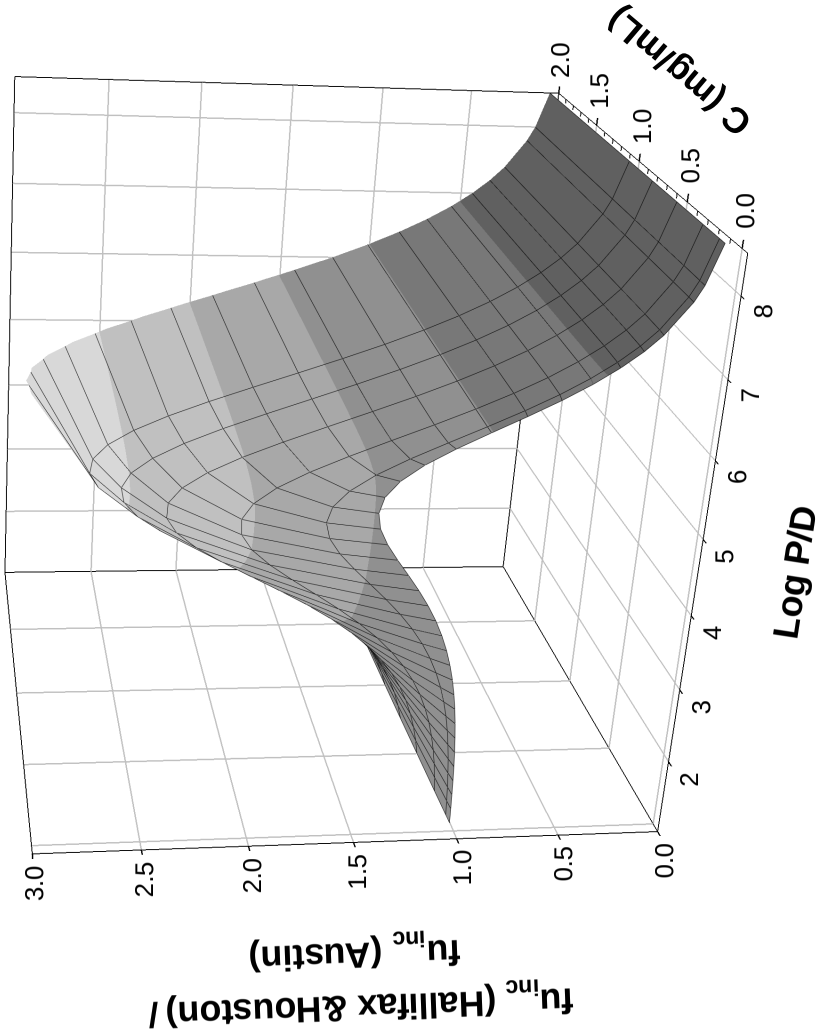

Figure 2

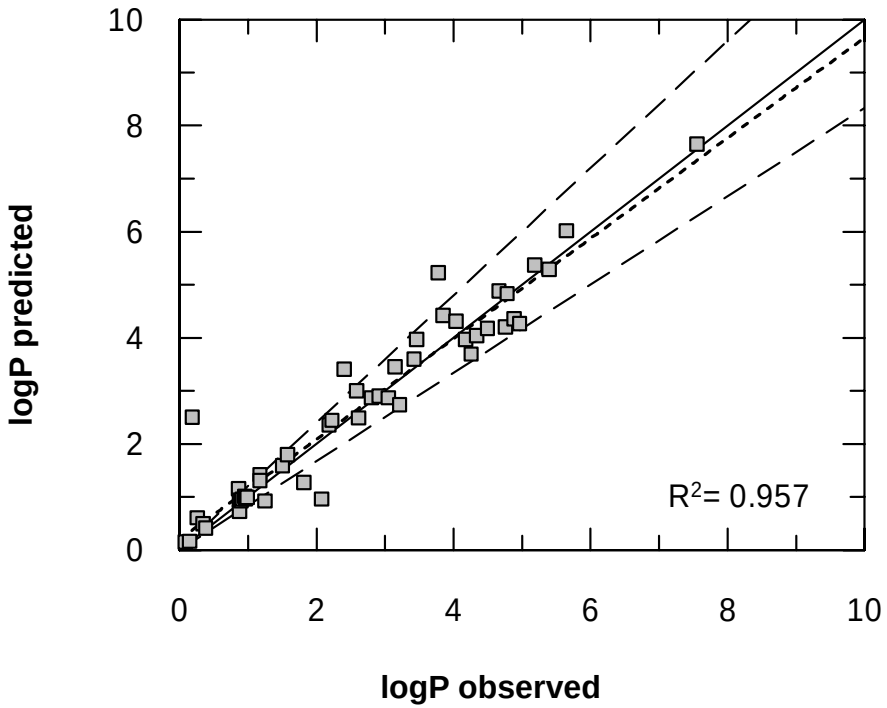

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Figure 3 A

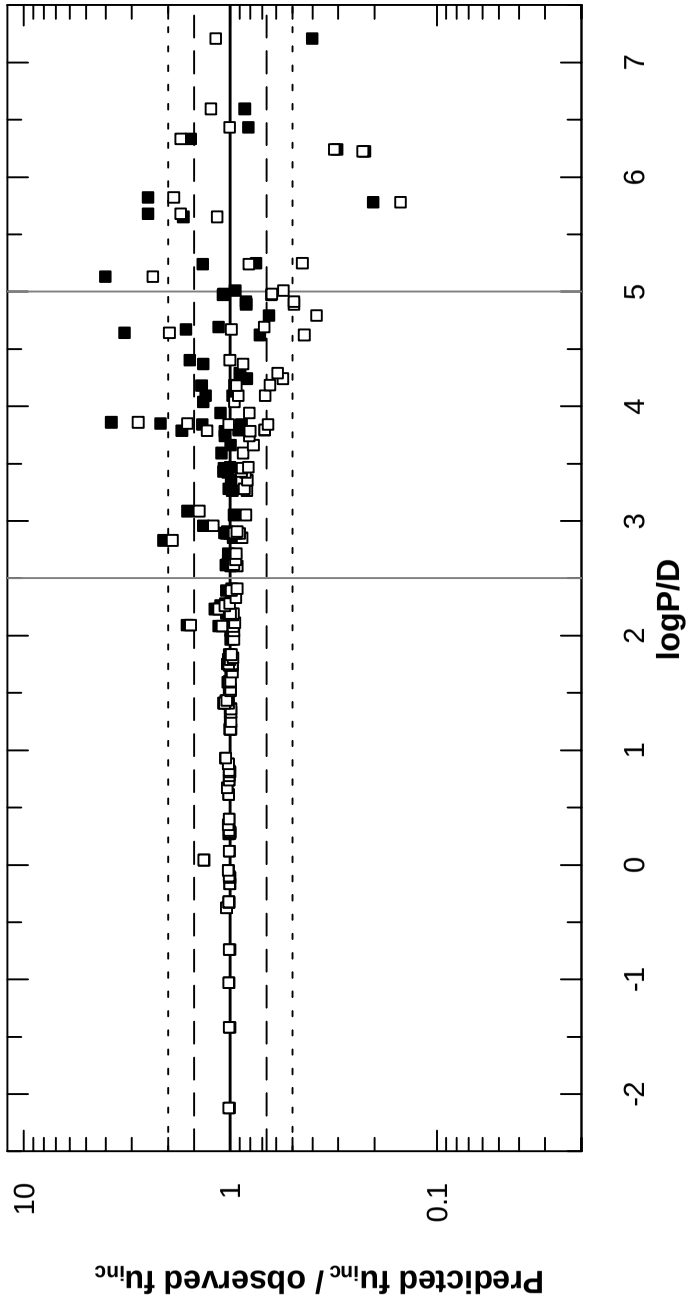

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Figure 3 B

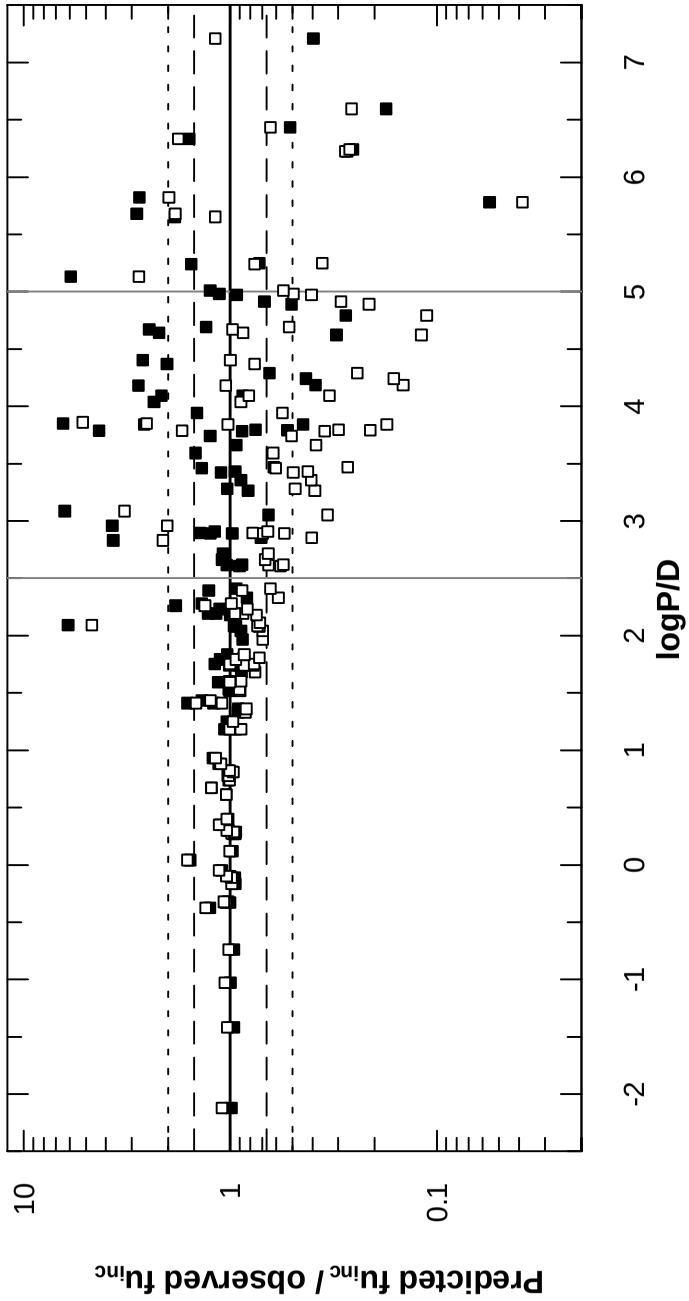

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Figure 4