DMD Fast Forward. Published on August 31, 2011 as doi:10.1124/dmd.111.040683

DMD #40683

Short Communication

# Correction for non-specific binding to various components of ultrafiltration apparatus and impact on estimating *in vivo* rat clearance for a congeneric series of 5-ethyl, 5-n-alkyl barbituric acids.

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DMD Fast Forward. Published on August 31, 2011 as DOI: 10.1124/dmd.111.040683
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**Estimating non-specific binding by ultrafiltration**

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Number of text pages: 10

Number of Tables: 4

Number of Figures: 0

Number of words in Abstract: 124

Number of words in Introduction: 404

Number of words in Discussion: 670

Abbreviations:

$Cl_{int}$, intrinsic clearance; fu, fraction unbound; $fu_{mic}$, fraction unbound to microsomes; $fu_{inc}$, fraction unbound in incubate; NADPH, nicotinamide-adenine dinucleotide phosphate; NSB, Non-specific binding; SRW, standard rat weight

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## Abstract

Accurately predicting *in vivo* metabolic clearance from *in vitro* liver microsomes or hepatocytes requires a good understanding of the factors contributing to the prediction. While much work has concentrated on deriving scaling factors and optimising the metabolic stability techniques for consistency and rigour it is only relatively recently that the importance of binding to microsomes and hepatocytes has been appreciated. Ultrafiltration is often used to estimate binding to plasma proteins and microsomes but the level of non-specific binding (NSB) to the ultrafiltration apparatus has not been adequately described. We derive an equation to correct for NSB and demonstrate that this can significantly affect the estimate of binding to microsomes and improve the accuracy of scaling to *in vivo* clearance for a series of barbiturates.

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## Introduction

In early drug research projects much emphasis is placed on being able to predict the human pharmacokinetics to help identify candidate drugs with appropriate characteristics. One of the key parameters to estimate is that of metabolic clearance. Generally this is achieved either via extrapolation using allometry (Lavé et al. 1999) or through translating an understanding of scaling from metabolic stability in pre-clinical species liver microsomes and hepatocytes to those in human (Carlile et al 1997, Riley et al 2005). In the latter case, there has often been the assumption that the concentration added to the microsome or hepatocyte incubation is the same as the unbound concentration in the incubation. More recently it has been shown that non-specific binding (NSB) to microsomes and hepatocytes can be significant and hence the unbound concentration is lower than the total concentration added, often resulting in a dramatic effect on the estimate of clearance (Obach 1999, McLure et al. 2000, Austin et al. 2002, Hallifax and Houston 2006). Consequently it is important to estimate NSB in these *in vitro* incubations where a variety of models have been used including equilibrium dialysis and ultrafiltration. Although equilibrium dialysis is perhaps considered the gold standard technique, a literature search on publications involving protein binding since 2000 suggests that both equilibrium dialysis and ultrafiltration are utilised to similar extents.

Ultrafiltration has commonly been used to estimate the percentage unbound in both plasma and microsomes, predominantly due to the rapidity of the technique. However, the technique assumes that there is no NSB to the ultrafiltration apparatus as this could substantially alter the estimate. While Dow suggested that if NSB using ultrafiltration exceeded 5% another technique should be used (Dow 2006), other workers have modified the technique to overcome solubility and NSB challenges (Taylor and Harker 2006) but some binding will

still occur. The purpose of the current work was to derive an equation that provides an estimate of the actual fraction of compound unbound in microsomal system, when using ultrafiltration. This equation was then applied to *in vitro* metabolic kinetic data derived from microsomal studies for a congeneric set of barbiturates and shown to provide a closer prediction of *in vivo* clearance than ignoring binding.

The barbiturates cover a range of physicochemical properties typical for drug molecules and with all homologues having a pKa of approximately 7.8 (Toon and Rowland 1983), they are essentially unionised at physiological plasma pH as indicated in Table 1.



## Materials and Methods

**Materials**. All chemicals were of analytical reagent grade unless otherwise stated. Acetonitrile (HPLC and far UV grade), methyl tertiary-butyl ether (MTBE), ethyl acetate, isohexane, methanol (HPLC grade), trifluoroacetic acid and orthoboric acid were supplied by Fisher Scientific UK (Loughborough, Leicestershire, UK), potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate (anhydrous) and sodium hydroxide (specified laboratory reagent) by Fisons Scientific Equipment (Loughborough, Leicestershire, UK), while double distilled water was produced in house using a Fistreem Cyclon distillation unit (Fisons Scientific Equipment, Loughborough, Leicestershire, UK). Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and phenobarbital were obtained from Sigma Chemical Company Ltd. (Poole, Dorset, UK). 5-n-pentyl-5-ethyl barbituric acid was a gift from Zambon Group spa (Bresso, Milan, Italy), with the remaining 5-n-alkyl-5-ethyl barbituric acids (n-hexyl, n-heptyl, n-octyl and n-nonyl) synthesised in the School of Pharmacy and Pharmaceutical Sciences (University of Manchester, Manchester, UK) and

purified to greater than 95% as estimated by micro-analysis (Toon and Rowland, 1983). The n-nonyl derivative was prepared as the sodium salt.

Phosphate buffer (0.1 M, pH 7.4) was prepared by adding potassium dihydrogen orthophosphate solution (13.6 g/l) to disodium hydrogen orthophosphate solution (3.58 g/l) until a pH of 7.4 was achieved. NADPH was freshly prepared at 20 mM in phosphate buffer.

Tissues were obtained from Wistar derived male rats (strain Alpk:AP$_f$SD) bred in house at AstraZeneca Pharmaceuticals (Alderley Park, Cheshire UK) and weighing between 200 and 320 g. The rats were terminated by cervical dislocation and the excised tissues were stored at -20°C for up to 6 months.

**Equipment**. The HPLC system used for the HPLC-UV assay consisted of a Perkin Elmer Series 200 HPLC pump and ISS200 autosampler (Beaconsfield, UK), Spectromonitor 3200 UV detector (LDC, Manchester, UK) and a Hypersil ODS 5 micron, 250 x 4.6 mm Hichrom Ltd. (Theale, Berkshire, UK). Chromatograms were recorded using PeakPro chromatography data system (Beckman Instruments (UK) Limited, High Wycombe, Buckinghamshire, UK). Centrifree ultrafiltration tubes (YMT membrane, 30,000 molecular weight cut off) were supplied by Amicon Ltd. (Stonehouse, Gloucestershire, UK).

**Non-specific binding to microsomes and ultrafiltration tubes.** Solutions of n-pentyl, n-hexyl, n-heptyl, n-octyl and n-nonyl barbituric acids were added individually to rat microsomes in phosphate buffer (0.1 M, pH 7.4, 0, 0.2, 0.5 and 1.0 mg microsomal protein/ml) to give final concentrations of 100 nmol/ml and 25 nmol/ml (n-nonyl analogue only) containing less than 3% acetonitrile (v/v). After incubation at 37°C for 30 min, aliquots (1 ml, in triplicate) of these solutions were added to Centrifree ultrafiltration tubes (excluding any air bubbles) and centrifuged (2,000 G) using a 33° fixed angle rotor for 30 min. Binding

DMD Fast Forward. Published on August 31, 2011 as DOI: 10.1124/dmd.111.040683
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to the Centrifree tube sample reservoir and collection cup was determined by addition of barbiturates in protein free phosphate buffer (0.5 ml, 0.1 M, pH 7.4 in triplicate) directly to the reservoir and cup, with samples (0.2 ml) taken after 20 min. Phosphate buffer (1.0 ml, 0.07 M, pH 5.4) was added to the buffer solutions from the sample reservoir, collection cup and ultrafiltrate with 10μl of phenobarbital (1.0mg/ml) added as an internal standard which were then extracted with MTBE (5 mL), the supernatant aspirated, evaporated to dryness under nitrogen at 40°C, the residue reconstituted in methanol:water (0.2 mL, 50:50 v/v) and analysed by HPLC-UV. Aliquots (50 μL) of all barbiturates were simultaneously analysed using a Hypersil ODS 5 micron (250 x 4.6 mm I.D.) column with a linear gradient operating of acetonitrile:water:trifluroacetic acid (15:85:0.1 to 90:10:0.1 v/v/v) at 1.0 ml/min, and the eluent monitored at 214 nm.

**Stability in rat liver microsomes.** The barbiturates were incubated individually (each at 100 nmol/ml, containing less than 3% acetonitrile) with microsomal protein concentrations (0.2 to 1.5 mg/ml) for up to 1 h at 37°C to determine the metabolic linearity with time and protein concentration. NADPH was added immediately prior to incubation (at a final concentration of 2 mM) to each incubation. Intrinsic clearance was determined using Equation 2 with the AUC calculated at a range of microsomal protein concentrations as detailed in Table 4.

Aliquots of microsomal suspensions were removed from the incubations and added to an equal volume of methanol. The mixtures were vortexed for a few seconds, centrifuged (approximately 1000 G for 5 min) and an aliquot of the supernatant analysed by direct injection using HPLC-UV using the method describe above. Barbituric acid concentrations were estimated by reference to the parent peak height in unincubated (control) samples.

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**Equations**

The fraction unbound (fu), correcting for non-specific binding to the ultrafiltration apparatus under non-saturating conditions is described by Equation 1 (see Supplemental data for derivation):

$$fu = \frac{1}{1+\left[\frac{fu_{mem}fu_c}{fu_o} - \frac{1}{fu_R}\right]} \quad (1)$$

where $fu_{mem}$, $fu_c$ and $fu_R$ are the fraction not bound to the membrane, collection cup and sample reservoir, respectively and $fu_o$ is the observed ratio $Cu_c/C_{mic}$ with $Cu_c$ the measured concentration in the collection cup after filtration. For the experiments described in this paper, $fu = fu_{mic}$, where $fu_{mic}$ is the fraction unbound in microsomes.

Intrinsic clearance was estimated from substrate disappearance in the linearity experiments in an analogous manner to pharmacokinetic analysis (Houston, 1994; Houston and Carlile, 1997; Lavé, et al., 1997):

$$CL_{int} = \frac{Dose}{AUC.fu_{mic}} \quad (2)$$

where *Dose* is the amount of barbiturate incubated and *AUC* the total area under the barbiturate concentration time profile during the *in vitro* incubation. *AUC* was estimated by the linear trapezoidal rule up to the time of the last measurement with extrapolation to infinite time by adding $C_l/k$ (where $C_l$ is the concentration at the last sample time and $k$ is the slope of

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the terminal phase of the log concentration-time curve, determined by log-linear regression of the last few data points).

Intrinsic clearance was then scaled to quantify the full activity of the liver as measured *in vitro* using the methodology described by Houston (1994). The units of $CL_{int}$ (ml/min/mg protein) are converted to *in vivo* units (ml/min per SRW, where SRW is the standard rat weight = 250 g) by multiplying by the scaling factor of 610 mg protein per SRW. This scaling factor is a mean value derived from the product of microsomal protein mass per g of liver, approximately 61 mg (Smith et al. 2008), and liver weight, approximately 10 g.

The scaled intrinsic clearance can then be related to hepatic clearance ($CL_{h,b}$) using the venous equilibration model, where $Q_H$ is the hepatic blood flow, 72 ml/min/kg and $fu_b$ is the ratio of unbound plasma concentration to whole blood concentration of parent ($fu/R$; Table 1).

$$CL_{h,b} = \frac{Q_H . fu_b . CL_{\text{int}}}{Q_H + fu_b . CL_{\text{int}}} \qquad (3)$$

## Results

A set of barbituric acid derivatives were chosen to cover a range of plasma protein binding (*fu* ranges from 0.51 to 0.0093) to investigate non-specific binding to Centrifree ultrafiltration tubes and the effect of non-specific binding to microsomes and the consequent effect on scaling to *in vivo* clearance. In protein-free buffer the total non-specific binding to the Centrifree tubes ranged from 2.8 to 54.5% for the n-pentyl to n-nonyl barbituric acids respectively (Table 2). The high non-specific binding of the n-octyl and n-nonyl homologues is largely attributed to binding to the membrane (16.4 and 33.2% respectively), with a lower proportion binding to the reservoir and collection cup. In a separate experiment, the binding of the n-nonyl analogue was also determined at 25 and 100 nmol/ml where total binding and

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binding to the cap were similar at each concentration; 41 vs 36% (total) and 12.4 vs 11.4% (cup).

The microsomal binding of n-pentyl to n-nonyl homologues ($fu_{mic}$) was calculated using Equation 1. Except for the n-pentyl homologue, which was largely unbound to microsomes, $fu_{mic}$ decreased with increasing protein concentration (Table 3). For example, $fu_{mic}$ of the n-nonyl homologue decreased from 0.45 to 0.18 for microsomal concentrations of 0.2 and 1 mg/ml, respectively. If the correction for non-specific binding to the apparatus were not applied, the $fu_{mic}$ values for the n-nonyl homologue would be 0.21, 0.14 and 0.09 for microsomal concentrations of 0.2, 0.5 and 1.0 mg/ml respectively.

The intrinsic clearances of the barbiturates determined in rat liver microsomes were scaled to predicted *in vivo* blood clearances using Equation 3 as shown in Table 4 both with and without correcting $CL_{int}$ for $fu_{mic}$; i.e. $fu_{mic}$ = 1 in Equation 2. There was a noticeable difference in the predicted hepatic clearance only for the n-octyl and n-nonyl analogues with the $fu_{mic}$ corrected predictions being closer to the *in vivo* $CL_{h,b}$ results (Toon and Rowland 1983, 28.4 vs 32.6 and 32.3 vs 31.3 ml/min/kg for the n-octyl and n-nonyl analogues respectively).

## Discussion.

To accurately predict *in vivo* clearance from *in vitro* incubations, one of the initial assumptions often used is that the drug concentrations added to the incubation can be considered as essentially unbound and free to interact with metabolic enzymes. However, more recently the importance of binding to microsomal proteins has been shown to effect the unbound concentration and consequently the *in vivo* clearance prediction. Ultrafiltration is commonly used to determine protein binding although the aspect of NSB to the various components of the apparatus is often not fully considered and may appreciably alter the

unbound drug concentration and therefore binding estimate. Depending on the relative binding affinity, drug concentration and protein concentrations, it may be that NSB could be a more significant factor than binding to protein in the prediction of clearance for some drugs. The results for the lipophilic barbiturates in this study indicate that they bind extensively to the ultrafiltration tube reservoir, membrane and collection cup, each of which has an effect on the microsomal protein binding estimate. Consequently, using ultrafiltration can significantly overestimate protein binding if the non-specific binding to the ultrafiltration tubes is not taken into account. In the case of the n-nonyl derivative, there was an approximate 2-fold difference in protein binding estimates across a range of microsomal protein concentrations. Alternative approaches have been adopted to circumvent non-specific binding. For example, studies have looked to modify the experimental conditions by addition of Tween 80 or benzalkonium chloride to reduce binding to the membrane (Lee et al. 2003) or by addition of retentate from control plasma to test compound filtrate to reduce non-specific binding (Taylor and Harker 2006). However, these techniques may still not account for binding to all of the ultrafiltration device and could lead to erroneous results, hence determining all the non-specific binding parameters described in Equation 1 is likely to give a more accurate assessment of binding.

The assumption made in Equation 1 is that the system is operating under non-saturating conditions. This was found to apply to the most hydrophobic and highly bound analogue, the n-nonyl barbituric acid, for which the total binding and binding to the collection cap were found to be essentially the same for a four-fold change in concentration (25 to 100 nmol/ml). Accordingly, it is reasonable to assume that non-saturating conditions also apply to the other analogues.

Microsomal binding is often infrequently determined for *in vitro* metabolic studies but it can be an important parameter when scaling to *in vivo* clearances. Chiba (1990) used the binding of imipramine and desipramine to rat liver microsomes ($fu_{mic}$ = 0.453 and 0.409 respectively) to successfully predict steady-state plasma concentrations. Igari (1982) reported similar bindings for hexobarbital and thiopental to microsomes ($fu_{mic}$ = 0.82 and 0.99 respectively) as found currently with similarly lipophilic homologues, n-pentyl and n-hexyl barbituric acids ($fu_{mic}$ = 0.95 to 0.73). The effect of physicochemical parameters on microsomal binding from equilibrium dialysis ($fu_{inc}$) was demonstrated by Austin et al. (2002) resulting in an empirical relationship with LogP/D that predicted the microsomal binding of the barbiturates from this study with $fu_{mic}/fu_{inc}$ ratios of between 0.9 and 2.2. Hallifax and Houston (2006) refined the model to a non-linear approach, which was more accurate than the Austin model for calculating $fu_{mic}$ for the n-pentyl to n-heptyl barbituric acids ($fu_{mic}/fu_{inc}$ ratios of between 0.9 and 1.2), but tended to underestimate the binding of the more lipophilic n-octyl and n-nonyl homologues ($fu_{mic}/fu_{inc}$ ratios of between 0.5 and 0.6).

The importance of microsomal binding when scaling *in vitro* data to predict *in vivo* clearance was apparent in the current study. If $fu_{mic}$ had not been determined and was assumed to be 1, the calculated $CL_{h,b}$ values for the n-octyl and n-nonyl homologues, from substrate disappearance, would have underpredicted the *in vivo* values by 63 and 60% compared to an accuracy of 13 and 3% respectively when including binding. Also, $fu_{mic}$ varies with protein concentration and consequently this factor may help to account for any differences that may exist between metabolic rates at different protein concentrations.

## Conclusion.

For compounds that bind non-specifically to the various components of the ultrafiltration device, the protein binding value that ignores this binding can be misleading and a correction

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factor needs to be taken into account. We have defined an equation to allow for this correction factor under non-saturating conditions which can simply be determined by measuring the non-specific binding to the ultrafiltration apparatus. In the case of the barbiturates investigated where non-specific binding was high, this correction factor and microsomal binding had a dramatic effect on the prediction of hepatic clearance, bringing the predictions much closer to *in vivo* values.

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**Authorship contributions:**

Participated in research design: Peter Ballard, Malcolm Rowland.

Conducted experiments: Peter Ballard.

Contributed new reagents or analytic tools: Peter Ballard and Malcolm Rowland.

Performed data analysis: Peter Ballard.

Wrote or contributed to the writing of the manuscript: Peter Ballard and Malcolm Rowland.

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**Footnote.**

Peter Ballard would like to thank AstraZeneca for funding this research and Brett Litton for providing the Figure in the supplementary information.

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**Table 1**

Physicochemical and binding data[a] for a series of 5-n-alkyl-5-ethyl barbituric acids

| Barbituric acid homologue | Molecular weight. | pKa | LogP | fu[b] | R[c] |
|---|---|---|---|---|---|
| n-pentyl | 226.2 | 8.00 | 2.20 | 0.51 [d] | 1.56 [d] |
| n-hexyl | 240.2 | 7.74 | 3.08 | 0.19 | 1.00 |
| n-heptyl | 254.2 | 7.78 | 3.64 | 0.061 | 0.98 |
| n-octyl | 268.2 | 7.78 | 3.85 | 0.026 | 1.08 |
| n-nonyl | 282.2 | 7.82 | 4.13 | 0.0093 | 1.05 |

[a] Toon and Rowland 1983; [b] fraction unbound in rat plasma; [c] whole blood-to-plasma concentration ratio; [d] values for i-pentyl derivative as equivalent values not available for n-pentyl derivative.

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**Table 2**

Binding of barbituric acid homologues (mean %, ± SE, n = 3) to the Centrifree ultrafiltration sample reservoir, membrane, collection cup and in total from protein-free media.

| | n-pentyl | n-hexyl | n-heptyl | n-octyl | n-nonyl |
|---|---|---|---|---|---|
| Total | 2.8 (± 0.84) | 4.1 (± 2.4) | 7.3 (± 0.76) | 22.9 (± 2.4) | 54.5 (± 14) |
| Reservoir | NT | NT | NT | 6.3 (± 2.8) | 20.3 (± 1.8) |
| Membrane [a] | NT | NT | NT | 16.4 | 33.2 |
| Cup | NS | NS | NS | 1.6 (± 0.55) | 14.5 (± 2.9) |

NT - not tested as total binding <10%, NS - not significantly different from zero,

[a] determined by difference from the mean values of binding to other surfaces (see Supplementary Data).

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**Table 3**

Calculated unbound fraction of barbiturates in rat microsomal preparations (mean $fu_{mic}$, ± SE, n = 3).

| Microsomal concentration (mg/ml) | n-pentyl | n-hexyl | n-heptyl | n-octyl | n-nonyl |
|---|---|---|---|---|---|
| 0.2 | 0.95 (± 0.006) | 0.90 (± 0.012) | 0.83 (± 0.010) | 0.77 (± 0.033) | 0.45 (± 0.063) |
| 0.5 | 0.90 (± 0.001) | 0.81(± 0.005) | 0.64 (± 0.006) | 0.40 (± 0.005) | 0.27 (± 0.009) |
| 1.0 | 0.95 (± 0.041) | 0.73 (± 0.008) | 0.49 (± 0.001) | 0.25 (± 0.012) | 0.18 (± 0.008) |

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**Table 4**

Barbiturate metabolic parameters estimated from substrate disappearance in microsomal studies, scaled to hepatic blood clearance ($CL_{h,b}$) both with and without a correction for $fu_{mic}$, compared to *in vivo* clearance ($CL_{h,b}$).

| Barbiturate homologue | Microsomal protein conc (mg/ml) | $fu_{mic}$ | $Cl_{int}$ | $CL_{h,b}$ | $CL_{h,b}$ $fu_{mic}$ = 1 | $CL_{h,b}$, mean | $CL_{h,b}$ mean $fu_{mic}$ = 1 | *In vivo* non-renal CL[a] |
|---|---|---|---|---|---|---|---|---|
| n-pentyl | 0.2 | 0.95 | 22 | 14.7 | 14.1 | 15.0 | 14.4 | 5.6 |
| n-pentyl | 1 | 0.95 | 23 | 15.3 | 14.7 | | | |
| n-hexyl | 0.2 | 0.9 | 141 | 35.2 | 33.3 | 39.0 | 35.3 | 24.4 |
| n-hexyl | 0.5 | 0.81 | 158 | 40.9 | 35.3 | | | |
| n-hexyl | 1 | 0.73 | 175 | 41.0 | 37.2 | | | |
| n-heptyl | 0.2 | 0.83 | 188 | 24.5 | 21.6 | 32.4 | 24.7 | 26.9 |
| n-heptyl | 0.5 | 0.64 | 235 | 32.8 | 25.1 | | | |
| n-heptyl | 1 | 0.49 | 267 | 39.9 | 27.3 | | | |
| n-octyl | 0.5 | 0.39 | 250 | 24.1 | 11.8 | 28.4 | 12.1 | 32.6 |
| n-octyl | 1 | 0.25 | 264 | 32.6 | 12.4 | | | |
| n-octyl | 1.5 | - | 255 | | 12.0 | | | |
| n-nonyl | 0.2 | 0.4 | 582 | 24.5 | 12.3 | 32.3 | 12.4 | 31.3 |
| n-nonyl | 0.5 | 0.25 | 615 | 33.6 | 12.9 | | | |
| n-nonyl | 1 | 0.17 | 558 | 38.7 | 11.9 | | | |

$Cl_{int}$ (µl/min/mg), $CL_{h,b}$ (ml/min/kg), [a] Toon and Rowland 1983

**Supplemental data:**

# Correction for non-specific binding to various components of ultrafiltration apparatus and impact on estimating *in vivo* rat clearance for a congeneric series of 5-ethyl, 5-n-alkyl barbituric acids.

**Drug Metabolism and Disposition**

Peter Ballard and Malcolm Rowland

## Derivation of an equation to correct for non-specific binding to components of the ultrafiltration apparatus.

Consider the scheme depicting various parts of the ultracentrifuge apparatus (Supplemental Figure 1). Assume that the drug concentration is below the association constant of the protein and let $\theta = {}^{C_b}/_{Cu}$ , where $C_b$ and $Cu$ are the bound and unbound concentrations, respectively. Now consider the events in each part of the apparatus.

1. ***Addition of protein-free drug solution to the top of the ultrafiltration reservoir, without filtration.***

Then the mass balance can be expressed as:

$$Total = V_M . C_{tot} = V_M . Cu_R + A_R \qquad \textbf{Equation A1}$$

where $V_M$ is the volume of the added solution, $C_{tot}$ and $Cu_R$ are the total and unbound concentrations of compound in the reservoir, and $A_R$ is the amount adsorbed onto the walls of the reservoir.

Let $Kp_R = {}^{A_R}/_{Cu_R}$

Then $$V_M.C_{tot} = V_M.Cu_R + Kp_R.Cu_R$$ **Equation A2**

Therefore $$fu_R = \frac{Cu_R}{C_{tot}} = \frac{V_M}{V_M + Kp_R}$$ **Equation A3**

2. ***Addition of microsomal protein solution into the top reservoir of the ultrafiltration tube without filtration.***

If non-specific binding to the reservoir is absent, then it follows that the mass balance is:

$$V_M.C_{mic} = V_M.C_{b,mic} + V_M.Cu_{mic}$$ **Equation A4**

where $C_{mic}$, $C_{b,mic}$ and $Cu_{mic}$ are the total, microsomal bound and unbound drug concentrations, so that:

$$V_M.C_{mic} = V_M.\theta_{mic}.Cu_{mic} + V_M.Cu_{mic}$$ **Equation A5**

Therefore, rearranging gives: $$fu_{mic} = \frac{Cu_{mic}}{C_{mic}} = \frac{1}{1+\theta_{mic}}$$ **Equation A6**

If the drug also binds non-specifically to the reservoir then the mass balance becomes:

$$V_M.C_{mic} = V_M.C'_{b,mic} + V_M.Cu'_{mic} + A'_R$$ **Equation A7**

where the apostrophe denotes the situation where there is binding to the reservoir.

Now $\theta_{mic} = \frac{C'_{b,mic}}{Cu'_{mic}}$ and $A_R = Kp_R.Cu'_{mic}$

Therefore $$V_M.C_{mic} = \left(V_M.\theta_{mic} + V_M + Kp_R\right).Cu'_{mic}$$ **Equation A8**

Rearranging gives:

$$C_{mic} = \left[\theta_{mic} + \frac{1}{fu_R}\right].Cu'_{mic} \qquad \textbf{Equation A9}$$

So that

$$fu'_{mic} = \frac{Cu'_{mic}}{C_{mic}} = \frac{1}{\theta_{mic} + \frac{1}{fu_R}} \qquad \textbf{Equation A10}$$

However, we actually require an expression for $fu_{mic}$

where

$$\theta_{mic} = \frac{1}{fu'_{mic}} - \frac{1}{fu_R} \qquad \textbf{Equation A11}$$

which when substituted into Equation 10 and rearranging gives:

$$fu_{mic} = \frac{1}{1 + \left(\frac{1}{fu'_{mic}} - \frac{1}{fu_R}\right)} \qquad \textbf{Equation A12}$$

Therefore, to calculate $fu_{mic}$, $Cu'_{mic}$ has to be estimated by relating it to the concentration measured after filtration (i.e. accounting for losses to membrane and collection cup).

3. ***Events after filtration.***

Consider first the loss on the membrane as unbound drug passes through it. Drug concentration in the ultrafiltrate ($Cu_{UF}$) is then related to $Cu'_{mic}$ via Equation A13.

$$Cu_{UF} = Cu'_{mic}(1 - f_{mem}) \qquad \textbf{Equation A13}$$

where $f_{mem}$ is the fraction of the filtered drug that remains on the membrane.

However, it is not possible to measure $Cu_{UF}$ directly, but the unbound concentration in the collection cup after any binding to the cup ($Cu_C$) is related to $Cu_{UF}$ via the mass balance described in Equation A14.

$$V_{UF}.Cu_{UF} = V_{UF}.Cu_C + A_C \qquad \textbf{Equation A14}$$

where $V_{UF}$ is the volume of ultrafiltrate and $A_C$ is the amount of drug adsorbed to the collection cup.

Rearranging Equation A14 in an analogous manner to Equation  and Equation  gives:

$$fu_c = \frac{Cu_C}{Cu_{UF}} = \left( \frac{V_{UF}}{V_{UF} + Kp_c} \right) \qquad \textbf{Equation A15}$$

where $fu_C$ is the fraction of drug unbound to the collection cup and $Kp_c = {A_c}/{Cu_c}$

Therefore, substituting Equation A15 into Equation A13 gives:

$$Cu'_{mic} = \frac{Cu_C}{fu_{mem}.\, fu_C} \qquad \textbf{Equation A16}$$

where, in this case, $Cu_c$ is the unbound fraction of drug in the collection cup after filtration. The fraction unbound to collection cup can be determined by adding protein-free media directly to the cup through Equation A17, i.e. by assuming $Cu_{UF} = C_{tot}$.

$$fu_c = \frac{Cu_c}{C_{tot}} \qquad \textbf{Equation A17}$$

Now, the fraction of drug bound to membrane can be estimated from a control filtration containing no protein in the supernatant since, under these conditions $Cu'_{mic} = Cu_R$. Therefore, substituting into Equation A16 gives:

$$fu_{mem} = \frac{Cu_c}{Cu_R.\, fu_c} \qquad \textbf{Equation A18}$$

Consequently, $Cu_c$ can be determined in an ultrafiltrate containing protein in the sample reservoir and therefore, by assuming that the fraction of non-specific binding to sample reservoir, membrane and collection cup does not alter with barbiturate or microsomal concentration, $fu_{mic}$ can be estimated by rearrangement of Equations A10, A12 and A16 to give:

$$fu_{mic} = \frac{1}{1 + \left[ \frac{C_{mic}.\, fu_{mem}.\, fu_c}{Cu_c} - \frac{1}{fu_R} \right]} \qquad \textbf{Equation A19}$$

or $$fu_{mic} = \frac{1}{1+\left[\frac{fu_{mem}.fu_c}{fu_o} - \frac{1}{fu_R}\right]}$$ **Equation A20**

where $fu_o$ is the observed ratio $Cu_c/C_{mic}$.

**Supplemental data:**

# Correction for non-specific binding to various components of ultrafiltration apparatus and impact on estimating *in vivo* rat clearance for a congeneric series of 5-ethyl, 5-n-alkyl barbituric acids.

**Drug Metabolism and Disposition**

Peter Ballard and Malcolm Rowland

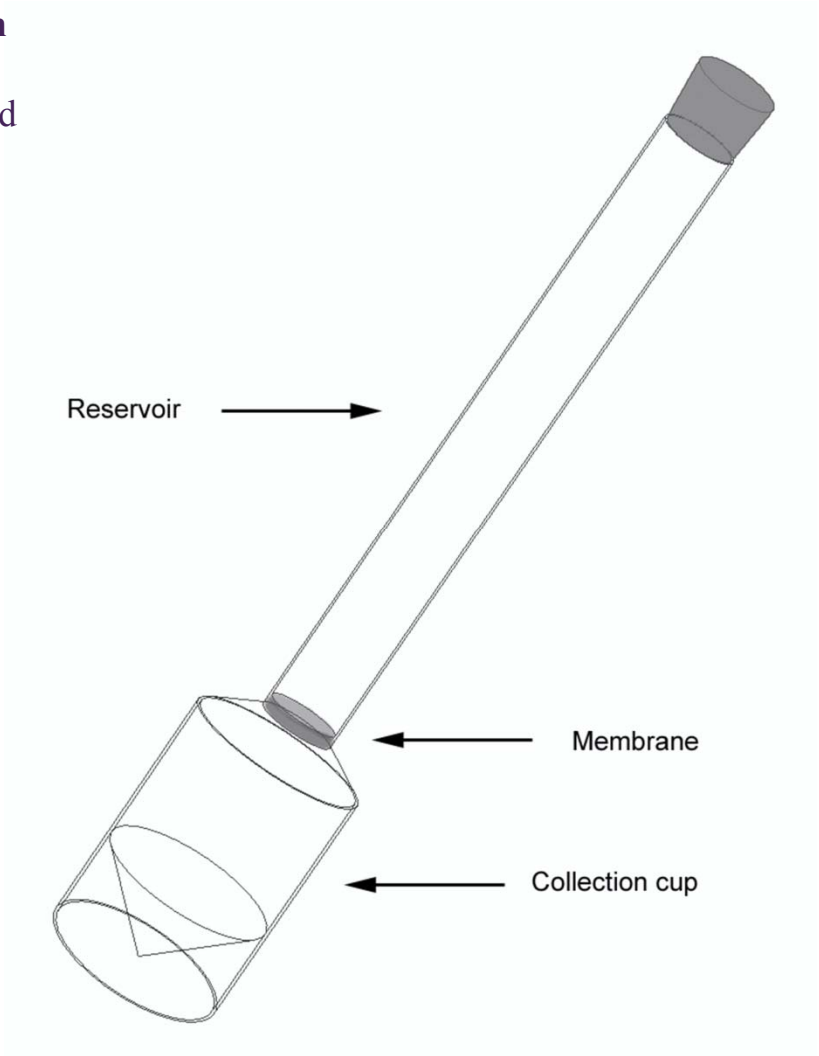


Figure 1. Schematic representation of an ultrafiltration apparatus, indicating sample reservoir, membrane and collection cup.