Prediction of drug concentrations in human skim milk from plasma protein binding and acid-base characteristics

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- 1 Protein binding in human skim milk of a series of seven drugs with diverse plasma protein binding and acid-base characteristics, was measured by ultrafiltration.
- 2 A mathematical relationship between plasma and skim milk unbound fractions was established using measured values from this study along with values from the literature.
- 3 The relationship enables prediction of unknown protein binding values of drugs in skim milk from known plasma protein binding values.
- 4 Knowledge of milk protein binding enables a more accurate assessment of total milk concentrations than is available from existing theory which is limited to prediction of unbound drug.

Keywords protein binding human milk drug distribution

Introduction

Natural breast feeding has many advantages for both the infant and mother (Jelliffe & Jelliffe, 1977 a,b; Vorherr, 1972). However, the ingestion of drugs via human milk is of some concern (Miller & Chopra, 1984) and has been the subject of extensive review (Anderson, 1977; American Academy of Pediatrics, 1983; O'Brien, 1974; Wilson, 1981). The extent of excretion of many drugs into human milk is still unknown.

The distribution of drugs between plasma and milk has been extensively studied in animals. These experiments demonstrate that the ratio of unbound drug concentrations in milk and plasma (Mu/Pu) can be predicted from milk and plasma pH, and drug pKa (Rasmussen, 1961). Therefore from unbound plasma concentration the likely unbound milk concentration of the drug can be predicted for a given drug. However, this does not allow prediction of *total* drug in milk, which will be affected by binding of drug to milk proteins and partitioning into milk lipid.

This study was designed to see if a predictable mathematical relationship exists between protein binding of drug in human milk and plasma. Because the protein binding of most drugs in plasma is known, while protein binding in milk

is often not known, a predictive relationship between the two would enable the calculation of the approximate protein binding of any drug in milk. The value of this is that when the relationship between milk concentration and plasma concentration for a given drug is not known, it can be estimated with more accuracy than is currently possible from the theory of distribution of free drug alone.

For the predictive relationship to have any real value, the curve should span the entire range of drugs so that the milk protein binding for an unknown drug can be derived from 'within' the curve, without the need for extrapolation. For this study drugs were therefore chosen to represent extreme ends of the spectrum of protein binding and acid-base characteristics.

The protein binding in human milk of this series of drugs was studied by ultrafiltration. Mature milk (> 15 days post-partum) was chosen because of relative stability of protein concentrations. In order to remove the possibility that partitioning within lipid might give a falsely high estimation of 'protein' binding, the lipid component was removed, thus producing 'skim' milk.

Abbreviations

In this study the following abbreviations will be used.

M:	Total concentration of drug in milk.
Mu:	Unbound (i.e. free) concentration of drug in milk.
M _{skim} :	Concentration of drug in skim milk.
P:	Total concentration of drug in plasma.
Pu:	Unbound concentration of drug in plasma.

Method

Chemicals

The following radiolabelled and non-radio-labelled drugs were used: [¹⁴C]-atenolol (ICI, England 4.6 mCi mmol⁻¹), atenolol (ICI, England), [¹⁴C]-diclofenac (Ciba-Geigy, Switzerland, 6.7 mCi mmol⁻¹), diclofenac (Ciba-Geigy, Switzerland), [³H]-digoxin (New England Nuclear, USA, 18 Ci mmol⁻¹), digoxin (Wellcome, England), ethosuximide (Parke-Davis, England), [³H]-prednisolone (Amersham, Australia, 67.4 Ci mmol⁻¹), prednisolone (Sigma, USA), naproxen (Sigma, USA), [³H]-naproxen (Syntex, USA, 18 Ci mmol⁻¹), [³H]-propranolol (New England Nuclear, USA, 18.5 Ci mmol⁻¹), and propranolol (ICI, England).

Drug assays

Ethosuximide concentrations in skim milk and milk ultrafiltrates were determined by gas liquid chromatography using the method of Sharman (1981). The remaining drug concentrations were determined by liquid scintillation counting of 100 µl of sample in toluene:triton X-100 (2:1) and 0.4% PPO (Omniflor, New England Nuclear, USA).

Milk bank

Samples of mature milk were obtained from five volunteers of the La Leche League (an organisation dedicated to the promotion of breast feeding). Each sample was freeze dried, then pooled and the protein content of the milk bank determined by a microprotein assay (Bradford, 1976) standardised with human albumin.

For the protein binding experiments, freeze dried milk was reconstituted with distilled water and the pH adjusted to 7.2 by bubbling carbon dioxide in nitrogen through the solution. Mean milk pH calculated from two large studies

(Ansell *et al.*, 1977; Harrison & Peat, 1972) was 7.2 which is comparable with the mean pH of the milk bank samples of 7.22. Skim milk was then prepared by removal of the lipid phase after centrifugation of the milk at 2000 g for 30 min, then vortexing to resuspend milk proteins. The remaining lipid content of the skim milk was determined by the Rose-Gottlieb method (Horwitz, 1980). Milk ultrafiltrate was prepared by ultrafiltration of human skim milk with a stirred ultrafiltration cell (Amicon model 8050, USA) through Diaflo YM10 43 mm membranes (Amicon, USA).

Milk drug concentrations

Total protein binding in milk was measured at three drug concentrations, which were calculated as follows from likely clinically relevant plasma concentrations:

1. Drugs with an established 'therapeutic range'; digoxin and ethosuximide;
 - (a) [Milk:plasma ratio (M/P) × [lower concentration of therapeutic range].
 - (b) (M/P) × [higher concentration of therapeutic range].
 - (c) (M/P) × [2 × higher concentration of therapeutic range].

Only one concentration (the lowest) was examined for naproxen as this was studied later to try and define more accurately the inflexion in the curve of the mathematical relationship between plasma unbound and milk unbound fractions.

2. For drugs without an established therapeutic range, atenolol, naproxen, prednisolone and propranolol, plasma concentrations at steady-state (C_{ss}) were calculated for an average 60 kg woman for the lowest and highest doses in clinical practice using known values of clearance, CL, and oral availability, F , ($C_{ss} = F \cdot \text{Dose rate} / CL$). The third concentration was twice the highest of these concentrations. The M/P ratio for diclofenac is not known and a value of 1 was arbitrarily assigned. The drug concentrations used are listed in Table 1.

Ultrafiltration

Free drug concentrations in skim milk were determined by ultrafiltration using MPS/1 devices (Amicon, USA) fitted with autoanalyser receiver cups (Techtron Corp, USA), with YM10 membranes (Amicon, USA, molecular weight cut-off 10,000), for all drugs studied except digoxin where YMT membranes (Amicon, USA, molecular weight cut-off 20,000) were used. YMT

Table 1 Protein binding in skim milk

Drug	Concentration	Correction factor (k')	% bound
Ethosuximide	35.4 mg l ⁻¹	1	0
Naproxen	280 µg l ⁻¹	1	13.2
Diclofenac	0.47 mg l ⁻¹	0.967	47.6
	0.68 mg l ⁻¹	0.957	43.0
	1.4 mg l ⁻¹	0.942	41.5
Atenolol	0.6 mg l ⁻¹	0.980	3.9
	2.4 mg l ⁻¹	0.970	7.4
	8.2 mg l ⁻¹	0.970	6.1
Propranolol	9 µg l ⁻¹	0.754	14.7
	100 µg l ⁻¹	0.745	18.3
	200 µg l ⁻¹	0.699	13.1
Digoxin	0.5 µg l ⁻¹	1	3.8
	1.3 µg l ⁻¹	1	5.9
	2.6 µg l ⁻¹	1	5.0
Prednisolone	1.1 µg l ⁻¹	0.910	10.4
	12.7 µg l ⁻¹	0.921	12.1
	317 µg l ⁻¹	0.936	10.1

membranes were used for digoxin because a high non-specific binding value (23%) was seen with YM10 membranes, probably due to a sieve effect because of the relatively high molecular weight of digoxin (see Discussion). The ultrafiltration units were pre-warmed, and centrifuged in an angle head rotor (angle 34°) for 15 min at 500 g, (at 37° C), to yield 400 µl of ultrafiltrate. Any losses due to non-specific binding to the ultrafiltration system and membrane, and/or sieve effect of the membrane were determined by measuring apparent non-specific binding of drug in phosphate buffer or milk ultrafiltrate using identical ultrafiltration conditions. Initially, apparent non-specific binding of ethosuximide and atenolol was measured in phosphate buffer (pH 7.2) (Weast, 1981). However, the apparent non-specific binding of propranolol and prednisolone was found to be greater in buffer than in pH adjusted milk ultrafiltrate. The remaining apparent non-specific binding values were therefore all measured in milk ultrafiltrate. Since the apparent non-specific binding for ethosuximide (0%) and atenolol (2%) in phosphate buffer was negligible, repeating the studies in milk ultrafiltrate was not thought necessary. All experiments were measured in sets of at least 4. Ultrafiltration conditions were adjusted to be as close as possible to physiological conditions, and therefore all measurements were made at 37° C.

The retention of total milk proteins by the YM10 membranes was assessed in milk ultrafiltrate by a microprotein assay (Bradford, 1976) standardised with human albumin.

Calculation of skim milk protein binding

Apparent non-specific drug binding values were

first determined as follows:

Apparent non-specific binding = 1 -

$$\frac{C_{\text{ultrafiltrate}}}{C_{\text{original solution}}} \quad (1)$$

The mean ultrafiltrate and original concentrations were compared statistically, and if significantly different, a correction factor (*k'*) was calculated:

$$k' = 1 - \text{apparent non-specific binding} \quad (2)$$

The skim milk protein binding was then determined by:

Skim milk protein binding = 1 -

$$\frac{C_{\text{ultrafiltrate}}}{C_{\text{skim milk}} \times k'} \quad (3)$$

The effect of the residual milk lipid content in skim milk on the measured protein binding values was negligible for all the drugs studied except propranolol (milk lipid: ultrafiltrate partition coefficient = 26.9) for which the final protein binding result was corrected to allow for the lipid contribution.

To determine significant protein binding, the original concentration multiplied by the correction factor (*k'*) was compared with the ultrafiltrate concentration using Student's *t*-test at a 95% confidence level. The groups were first compared for homogeneity of variance using the *F*-ratio test at a 95% confidence level. For those cases where a difference in variances was significant, the degrees of freedom were transformed (Bailey, 1959).

Establishment of a relationship between plasma and milk protein binding

The measured milk protein binding values at the

lowest concentration, expressed as fractions unbound, (i.e. $1 - \text{percentage protein bound}/100$), and known literature values (Table 2) were plotted (see Figure 1) against plasma unbound fractions to obtain a mathematical relationship which might enable interpolative prediction of milk protein binding from known plasma protein binding values. Where variable protein binding was quoted in the literature, mean values were used. Warfarin binding in milk is pH dependent (Syverson & Ratjke, 1985) and the protein binding at pH 7.2 (23%) was therefore calculated from the original results.

The data were fitted to various mathematical models using MKMODEL, version 3.03, (Holford, 1985). Final choice of the most appropriate model was based upon: (1) obtaining the minimum Schwartz criterion (Schwartz, 1978), and (2) visual evaluation of plots of unweighted residuals vs plasma unbound fractions and predicted milk unbound fractions to obtain a model

with a relatively random and homogeneous scatter of residuals about the respective axes.

Results

The protein binding in human skim milk and the correction factors (k') are shown in Table 1. The 'model of best fit' relating the milk unbound fraction to the plasma unbound fraction is an equation corresponding to the Hill equation (Hill, 1910), which describes the association of oxygen with haemoglobin; $Y = (Y_{\max} \cdot X^n)/(K^n + X^n)$, where Y is the milk unbound fraction, $Y_{\max}$ the maximum possible milk unbound fraction (theoretically 1), K the plasma unbound fraction at which milk unbound fraction is half maximum, X the plasma unbound fraction and 'n' the Hill coefficient, a parameter determining the steepness of the curve. In accordance with theory, $Y_{\max}$ was assigned a value of 1.

Table 2 Drug protein binding (PB) in plasma and skim milk. This combines the data from this study (*) with literature values for other drugs

Drug	Acid/Base	Plasma PB (%)		Milk PB (%)	
Ethosuximide *	Acid	0	[1]	0	
Phenobarbitone	Acid	43	[2]	3-10	[3]
Phenytoin	Acid	93	[4]	10	[3]
Naproxen *	Acid	97.3-99.6	[5]	13.2	
Warfarin	Acid	98.8	[6]	20-50†	[3]
Diclofenac *	Acid	99.9	[7]	48	
Atenolol *	Base	3	[8]	4.6	
Paracetamol	Base	19.8	[9]	2.6	[9]
Theophylline	Base	54	[10]	6.3	[10]
Disopyramide	Base	62-82	[11]	4-9	[3]
Propranolol *	Base	91	[12]	14.8	
Diazepam	Base	98.8	[13]	17-24	[3]
Digoxin *	Neutral	23	[14]	5.3	
Prednisolone	Neutral	81-96	[15]	10.2	

Footnote: † pH dependent. A value of 23% was calculated for pH 7.2.

- [1] Goodman Gilman *et al.* (1985)
- [2] Melten *et al.* (1985)
- [3] Syverson & Ratjke (1985)
- [4] Lunde *et al.* (1970)
- [5] Runkel *et al.* (1974)
- [6] Serlin *et al.* (1979)
- [7] Wagner & Sulc (1979)
- [8] Barber *et al.* (1978)
- [9] Bitzen *et al.* (1981)
- [10] Yurchak & Jusko (1976)
- [11] Haughey & Kraft (1984)
- [12] Evans & Shand (1973)
- [13] Greenblatt *et al.* (1980)
- [14] Lukas & De Martino (1969)
- [15] Steele *et al.* (1982)

Figure 1 Relationship of plasma and milk unbound fractions.

The model parameter estimates derived in these experiments were:

$$K = 6.94 \times 10^{-4}$$

$$n = 0.448$$

The actual expression therefore is:

$$Y = \frac{X^{0.448}}{(6.94 \times 10^{-4})^{0.448} + X^{0.448}} \quad (4)$$

From this expression the milk unbound fraction, and hence the milk protein binding can be derived for any drug whose plasma protein binding is known.

The Amicon YM10 membranes retained 99.7–99.8% of total milk proteins, and residual lipid content in skim milk was 0.06%.

Discussion

The model gives a mathematical relationship enabling the prediction of unknown milk unbound fraction values (and hence protein binding) from known plasma unbound fraction values for any given drug.

Intuitively the predictive model should have two limiting values, corresponding to 100% and 0% protein binding respectively, (i.e. the milk unbound fractions have to be within the values of 0 and 1). The 'model of best fit' had the maximum value for the Schwartz criterion of the models tested and a relatively random and homogeneous scatter of residuals. Models rejected on the basis of the Schwartz criterion include:

$$(i) \quad Y = \frac{X}{K + X}$$

$$(ii) \quad Y = 1 - \left(\frac{K_1}{X}\right)$$

$$(iii) \quad Y = 1 - e^{-K_2 X}$$

where the variables are as described above for the model of best fit, and K_1 and K_2 are slope factors.

The accuracy of the model can be assessed by comparison of milk unbound fractions predicted by the model, with actual measured values. As can be seen in Table 3 there was less than 2% error in the prediction of 8 out of 14 milk unbound fractions (and hence milk protein binding). The maximum error observed was 8.0% (naproxen). The model therefore closely fits the experimental data.

In order to understand how this model improves our ability to predict concentrations in human milk, the basic mechanisms involved in the equilibrium between drug in plasma and milk must be addressed.

Animal experiments have shown that for unbound drug, the milk: plasma ratio (Mu/Pu) can be predicted on the basis of the milk pH and drug pKa (Rasmussen, 1961). The Mu/Pu ratio is independent of milk volume (Rasmussen, 1961), and plasma drug concentration (Miller *et al.*, 1967), consistent with passive diffusion of unionised free drug between plasma and milk (Rasmussen, 1961; Schanker, 1962; Wilson, 1981).

Table 3 Predicted milk unbound fractions (*f_u*) from the model compared with measured values, i.e. accuracy of the model

Drug	Measured milk <i>f_u</i>	Predicted Milk <i>f_u</i>	(Observed–) (Predicted)	% error
Diclofenac	0.52	0.54	–0.02	3.8
Warfarin	0.77	0.78	–0.01	1.3
Diazepam	0.79	0.78	0.01	1.3
Propranolol	0.85	0.90	–0.05	5.9
Naproxen	0.87	0.80	0.07	8.0
Phenytoin	0.90	0.89	0.01	1.1
Prednisolone	0.90	0.90	0	0
Disopyramide	0.93	0.94	–0.01	1.1
Phenobarbitone	0.93	0.95	–0.02	2.2
Paracetamol	0.93	0.96	–0.03	3.2
Theophylline	0.94	0.95	–0.01	1.1
Digoxin	0.95	0.96	–0.01	1.1
Atenolol	0.95	0.96	–0.01	1.1
Ethosuximide	1	0.96	0.04	4.0

Milk has a lower pH than plasma, and steady state distribution of unbound drug between milk and plasma (*Mu/Pu*) may be predicted using a rearrangement of the Henderson-Hasselbach equation (Wilson, 1981):

$$\text{For acidic drugs: } Mu/Pu = \frac{1 + 10(pH_m - pK_a)}{1 + 10(pH_p - pK_a)} \quad (5)$$

$$\text{For basic drugs: } Mu/Pu = \frac{1 + 10(pK_a - pH_m)}{1 + 10(pK_a - pH_p)} \quad (6)$$

where *pH_m* = pH of milk, *pH_p* = pH of plasma. Equations (5) and (6) predict that the *Mu/Pu* ratio will be < 1 for acidic drugs and > 1 for basic drugs. For neutral drugs the *Mu/Pu* ratio would be expected to equal one.

Conceptually this may be understood on the assumption that only the unionised drug may diffuse across the lipid membrane, and a basic drug will be more ionised in milk and hence 'trapped' in the milk compartment.

Considering the case of a basic drug of *pK_a* 7.4:

Plasma (pH = 7.4)		Milk (pH = 7.2)
50 (Unionised)	$\xrightleftharpoons{\quad}$	50 (Unionised)
50 (Ionised)	$\xrightleftharpoons{\quad}$	79 (Ionised)
100		129

$$Mu/Pu = 1.29$$

This ratio, however, may be very different from the ratio of drug in skim milk to plasma (*M_{skim}/P*), or whole milk to plasma (*M/P*), because of protein

and lipid binding of drug. This can be seen as follows. If the drug above with *Mu/Pu* of 1.29 is 90% protein bound in plasma and 10% in skim milk the skim milk/plasma would be:

(Plasma, pH = 7.4)		(Milk, pH = 7.2)
900 $\rightleftharpoons$ 100	$\xrightleftharpoons{\quad}$	129 $\rightleftharpoons$ 12.9
90% Bound		90% Unbound
10% Unbound		10% Bound

$$M_{\text{skim}}/P = [129 + (129 \times 0.1/0.9)]/[100 + (100 \times 0.9/0.1)] = 0.143$$

It is clear therefore that *Mu/Pu* ratios may not give a true idea of likely amounts of total drug in milk.

The *M_{skim}/P* ratio for a drug may be calculated using the following equation:

$$\frac{M_{\text{skim}}}{P} = \frac{Mu}{Pu} \times \frac{\text{plasma } f_u}{\text{milk } f_u}$$

where *Mu/Pu* is calculated from equations 5 and 6 for acids and bases respectively and has the value of one for neutral compounds. The plasma unbound fraction (plasma *f_u*) is calculated from (1 – fraction PB) and this value is used in equation 5 to calculate the unknown milk unbound fraction, (milk *f_u*).

Does the model derived in this study provide a more accurate assessment of whole milk *M/P* ratios than that provided by unbound *Mu/Pu* ratios? In Table 4, unbound drug (*Mu/Pu*), unbound and protein bound drug (*M_{skim}/P*) and total drug (*M/P*) ratios are compared. It can be

Table 4 A comparison of predicted unbound milk: plasma ratios with predicted skim milk: plasma ratios and whole milk: plasma ratios

Drug	pKa	Mu/Pu	M _{skim} /P	M/P	
Atenolol	9.6	1.58	1.60	1.67	[1]
				2.32*	[2]
				4.5*	[3]
Propranolol	9.45	1.57	0.17	0.43*	[4]
				0.36*	[5]
Digoxin	N/A	1.00	0.8	0.85	[6]
				0.75	[7]
				0.62	[8]
				0.55*	[9]
Prednisolone	N/A	1.00	0.12	0.13*	[10]
Ethosuximide	9.3	0.995	0.995	0.86	[11]
				0.78	[12]
Naproxen	4.15	0.63	0.01	c.a. 0.01	[13]
Diclofenac	4.0	0.63	0.001	—	

Footnote: *Derived from AUC values.

References

[1] Holt <i>et al.</i> (1982)	[6] Loughan (1978)
[2] Kulas <i>et al.</i> (1984)	[7] Levy <i>et al.</i> (1977)
[3] Liedholm <i>et al.</i> (1981)	[8] Rheinhardt <i>et al.</i> (1982)
[4] Bauer <i>et al.</i> (1979)	[9] Chan <i>et al.</i> (1978)
[5] Levitan <i>et al.</i> (1973)	[10] Ost <i>et al.</i> (1985)

seen that predicted M_{skim}/P values were more accurate for all drugs except for ethosuximide which is not bound to proteins in either milk or plasma. Some deviations between predicted M_{skim}/P values and measured M/P values would be expected because the latter includes the contribution of drug partitioned into the lipid phase. For drugs which are relatively lipid soluble such as propranolol, an underprediction of the M/P ratio would be expected. Further studies are underway to try and quantify this contribution.

Because human milk contains less protein than plasma (10.3 g l^{-1} in this study), vs 74.6 g l^{-1} (Lentner, 1984), the fraction protein bound might be expected to be lower in milk than plasma. This is supported by both our results and other studies which are listed in Table 2. Protein binding measured at higher concentrations for atenolol, digoxin, ethosuximide, prednisolone and propranolol was not significantly different from that measured at the lowest concentration. However the protein binding of diclofenac at higher concentrations, 43 and 41% respectively, was significantly lower than the 48% bound at the lowest concentration, suggestive of saturable protein binding.

Apparent non-specific binding is due to a combination of binding to the membrane and ultra-filtration apparatus components, and a 'sieve effect'. The sieve effect is a term used to describe the phenomenon caused by solvent molecules passing through the membrane more readily than drug molecules, resulting in a higher apparent binding value. Initial results indicated that digoxin had a significant apparent non-specific binding value (23.4%, $P = 0.006$) when the YM10 (molecular weight cut-off 10,000) membrane was used. Digoxin has a relatively high molecular weight of 781 (Windholz, 1983) and it has been shown that a sieve effect can be appreciable for drugs with a molecular weight higher than 500 (Kurz *et al.*, 1978). A change of membrane to the YMT (MW cut-off 20,000) resulted in no significant apparent non-specific binding. The apparent non-specific binding values obtained were not significant for ethosuximide (0%) digoxin (0%) and naproxen (0%), and were low for atenolol (2%) and diclofenac (4.5%). However, higher non-specific binding values were obtained for prednisolone (8%) and propranolol (25.9%). High apparent non-specific binding values (75–100%) have been previously reported

for verapamil, prazosin, nifedipine (Zhirkov & Piotrovskii, 1984), theophylline and paracetamol (Tasker & Nakatsu, 1982), which limits the usefulness of the technique for some drugs. The apparent non-specific binding was allowed for by a correction factor (k') which enabled true unbound concentrations in the ultrafiltrate to be estimated (Melten et al., 1985).

The maximum possible error involved in the calculations of the protein binding from equations 1 and 3 is high. However, repeat determinations of binding on three different days, and subsequent calculation of the associated confidence interval at a 95% level, gives a better idea of the practical errors involved. The 95% confidence intervals

were all within $\pm 4\%$, except for propranolol which was 7%. This is probably predictable in view of the greater non-specific binding which was found for propranolol (25.9%).

This study has helped clarify the role of protein binding in determining the amount of drug which equilibrates into human milk.

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