Pharmaceutical Research, Vol. 7, No. 6, 1990

Report

Binding of Drugs in Milk: The Role of Casein in Milk Protein Binding

Thomas Stebler[1] and Theodor W. Guentert[1,2]

Received September 14, 1989; accepted December 14, 1989

Unbound fractions of ^{14}C-labeled diazepam and tenoxicam in skimmed milk of various species (man, horse, goat, cow, sheep, dog, rabbit) with different milk compositions were determined. Furthermore, the protein binding of five ^{14}C-labeled benzodiazepines differing in their lipophilicity (bromazepam, clonazepam, diazepam, flumazenil, and flunitrazepam) were measured in human milk and in artificially prepared solutions of individual milk proteins (lactoferrin, 2.4 g/liter; α-lactalbumin, 2.1 g/liter; albumin, 0.4 g/liter; and casein—2.1, 3.4, and 13.3 g/liter). The extent of binding was determined by equilibrium dialysis of protein solution against 1/15 *M* phosphate buffer, made isocryoscopic with lactose. The results showed that the casein fraction is a major binding component in milk for all tested drugs. The extent of binding of diazepam and tenoxicam in the milk of various species was independent of the whey protein concentration. In human milk the fraction of bromazepam, clonazepam, diazepam, and flunitrazepam bound to casein was higher than that bound to any other of the milk proteins tested. Albumin contributed little to the overall binding of these benzodiazepines, and lactoferrin and α-lactalbumin did not account for significant binding. The benzodiazepine antagonist flumazenil showed the lowest overall binding in milk and in casein solution. As the casein concentration is highest in colostral milk and drops during the course of lactation, it is expected that *M/P* ratios of drugs strongly bound to casein are higher during the first days postpartum than in later phases of lactation.

KEY WORDS: protein binding; milk; milk proteins; casein; tenoxicam; benzodiazepines.

INTRODUCTION

Passive diffusion is the most common mechanism by which drugs can pass from the blood stream into milk (1). Only the free, nonionized fraction of a drug may pass through lipoid membranes and the difference in its concentrations on each side of biological barriers determines the net substance flux. In milk, varying fat and protein contents may alter the amount of drug bound to milk components, thus influencing the active concentration gradient across the membrane. Fleishaker *et al.* (2) proposed an equation to predict the milk to plasma concentration ratio of a drug (steady-state conditions), taking these factors into account:

$$\frac{M}{P} = \frac{fu_p * f_p^{un}}{fu_m * f_m^{un} * (S/M)} \quad (1)$$

whereby fu_p/fu_m[3] is the fraction of drug unbound in plasma/milk, f_p^{un}/f_m^{un} the fraction of drug nonionized in plasma/milk, and *S/M* the skimmed to whole milk drug concentration ratio.

The protein and fat contents change over the lactation period and hence the fraction unbound is also expected to vary and to cause alterations in the *M/P* concentration ratio (3). Knowledge of the major binding components in milk might help to predict these alterations.

The binding proteins in milk are not yet known (3). Atkinson and Begg (4) showed that some drugs bind to albumin and lactoferrin. However, they could not correlate drug binding in milk with the extent of drug binding to whey proteins (lactoferrin, albumin, α-lactalbumin).

The present investigation was undertaken to clarify the question of major drug binding components in milk. We determined in the first step the unbound fractions of two different drugs in milk of various species with different milk compositions. The drugs chosen were the weak base diazepam and the weak acid tenoxicam. In the second step we investigated the bound fractions (fb_m) of several benzodiazepines in human milk and the extent of binding of the same compounds in solutions of the individual milk proteins.

MATERIALS AND METHODS

Collection and Characterization of Milk Samples

Pooled milk specimens of different animal species (horse and dog, two individuals; sheep, goat, and cow, one donor; rabbit, four donors) were obtained from Roche breeding facilities. For the comparative binding study in milk of various species, human milk from a single donor was used.

[1] F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4002 Basel, Switzerland.

[2] To whom correspondence should be addressed.

[3] Abbreviations used: [P], protein concentration; AAG, alpha-1-acid glycoprotein; dpm, disintegrations per minute; fm^{un}, fraction of drug nonionized in milk; fp^{un}, fraction of drug nonionized in plasma; fb_m, fraction of drug bound in milk; fu_m, fraction of drug unbound in milk; fu_p, fraction of drug unbound in plasma; *M/P*, milk-to-plasma drug concentration ratio; O/W, octanol/water partition coefficient; p.p., postpartum; r^2, coefficient of determination in nonlinear regression analysis; *S/M*, skimmed-to-whole milk drug concentration ratio.

Pooled human milk from four different donors in their early days of lactation, with a surplus of milk production, was used for all other experiments.

Aliquots of milk samples were defatted by centrifugation (30 min at 3000*g*) for protein concentration determinations and for binding experiments. The remaining milk fat in the skimmed milk was quantitated with the Gerber butyrometer (partition, 0–1%) (5). The pH was measured aerobically with a Metrohm 632 (Mettler, Switzerland).

The total milk protein concentration in skimmed milk was determined with a commercial protein determination kit (Sigma, USA; No. 609-A) making use of a combined Biuret/Lowry reaction (6). Whey protein concentration in skimmed milk was measured by precipitation of the casein with acetate buffer pH 4.6 (7) and subsequent protein concentration determination, using the Sigma kit mentioned above. The difference between total protein concentration and whey concentration provided an estimate of the casein concentration.

Albumin in human skimmed milk was determined by means of LC-Partigen plates (Behringwerke, FRG, No. OTCN 03), using the protein standard provided by Behringwerke (OTFO 03). Alpha-1-acid glycoprotein (AAG) was also determined by means of LC-Partigen plates (Behringwerke, FRG, No. OSLI 02).

The concentrations of the main protein components in skimmed milk samples were additionally quantitated by SDS-PAGE with a gel concentration of 12% (Mini Protean II, Bio-Rad, USA) and densitometry (scanning densitometer Model 1650, Bio-Rad, USA).

Preparation of Pure Protein Solutions

Human lactoferrin, albumin, casein, and bovine α-lactalbumin (Sigma, USA; Nos. L-3639, A-8763, C-5415, L-5385) were dissolved in buffer, pH 6.70 [1/15 *M* phosphate buffer, made isocryoscopic with lactose monohydrate (Ph. Eur.)]. Single concentrations were prepared for lactoferrin (2.4 g/L), albumin (0.4 g/L), and α-lactalbumin (2.1 g/L), and three different concentrations for casein (2.1, 3.4, and 13.3 g/L).

Model Compounds

^{14}C-Labeled tenoxicam (40 μCi/mg) and diazepam (35 μCi/mg) (F. Hoffmann-La Roche Ltd., Switzerland) were chosen for the protein binding experiments in milk of various species. Their radiochemical purity was greater than 96% as determined by thin-layer chromatography. They were added to the milk samples of various species to achieve a concentration of tenoxicam of 0.04 mg/L and of diazepam of 0.08 mg/L.

For the binding experiments involving individual milk components, five ^{14}C-labeled benzodiazepines were studied: bromazepam (37 μCi/mg), clonazepam (35 μCi/mg), diazepam (35 μCi/mg), flumazenil (50 μCi/mg), and flunitrazepam (69 μCi/mg) (F. Hoffmann-La Roche Ltd., Switzerland). The radiochemical purity of each of the chosen drugs was again greater than 96%. For the determination of fu_m, these drugs were added to the protein solutions or to skimmed milk to achieve concentrations usually found in milk under clinical conditions [tenoxicam, 0.04 mg/L (unpublished); flunitrazepam, 0.002 mg/L (8); and diazepam, 0.08 mg/L (8); or, in the absence of such information (bromazepam, clonazepam, and flumazenil), at the same concentrations as diazepam].

Determination of *S/M* Drug Concentration Ratios

The *S/M* drug concentration ratios were determined as described by Fleishaker *et al.* (2). In this procedure spiked human whole milk is incubated in a shaking water bath at 37°C for 1 hr. A sample is taken for drug concentration measurement. The milk is then centrifuged to separate skim milk and fat, and drug concentration is determined in an aliquot of the skimmed milk. The *S/M* drug concentration ratio can be calculated as the ratio of skimmed-to-whole milk drug concentrations.

Protein Binding Determination

Protein binding was determined by equilibrium dialysis, using a dialysis membrane with a cutoff of 12,000 (Union Carbide, USA) in Plexiglas dialysis cells (Technilab, USA). Before use, the membrane was soaked in distilled water (10 min), in absolute alcohol (15 min), and finally, in buffer (30 min).

Aliquots of 800 μl of skimmed milk or pure protein solution were dialyzed with 800 μl of buffer solution (1/15 *M* phosphate buffer, pH adjusted to milk pH, made isocryoscopic with lactose monohydrate). The dialysis cells were rotated (8 rounds/min) in a Dianorm apparatus (Diachema, Switzerland) in a water bath at 37°C for 5 hr. Each determination was carried out in duplicate. No binding to the dialysis membrane occurred. In all determinations the volume shift was below 11%. Precision (coefficient of variation) of fu_m determinations as calculated in 52 duplicated dialysis experiments was always below 8%.

Drug Concentration Measurements

Drug concentrations were measured after dialysis in both buffer and milk phases by liquid scintillation counting, using Bruno-Christian scintillation cocktail (9) and a Betamatic II scintillation counter (Kontron, Switzerland). Measured disintegrations per minute (dpm) were automatically corrected for quenching by an external standard. The fu_m was calculated by dividing dpm measured in buffer by dpm measured in milk. The fraction bound in milk, fb_m, was calculated by $1 - fu_m$.

Data Analysis

To estimate which protein fraction of the milk (whey or casein) determines the fb_m of diazepam and tenoxicam, the goodness of fit of the following model was used:

$$fu_m = \frac{1}{1 + b * [P]} \quad (2)$$

whereby b is a binding constant (influenced by number of binding sites per mole of protein and drug–protein association constant) and [P] is the protein concentration. This model was fitted by nonlinear fitting procedures to fractional protein (total protein, casein, or whey) concentrations in the

milk of various species and to resulting fu_m values of a model drug. The coefficient of determination (r^2) was used to select the model best describing the extent of milk binding. Additionally a multiple regression analysis was performed.

To estimate the contribution of binding to single milk proteins for overall binding in whole human skimmed milk, a stepwise multiple linear regression was performed. All statistical calculations were done using the computer program RS/1 (Bolt Beranek and Newman, USA) and accepting a significance level α of 0.05 to indicate differences between groups.

RESULTS

Binding Study in Milk of Various Species

The pH values of skimmed milk from different species were as follows: human, 6.42; horse, 6.85; sheep, 7.20; goat, 6.60; cow, 6.61; dog, 6.20; and rabbit, 6.92.

The remaining fat content in skimmed milk samples was in all cases below 0.02%.

A large range of fu_m of diazepam and tenoxicam in milk from different species was found, paralleling the wide range of protein concentrations (Table I). Curvilinear relationships were found between fu_m of both drugs investigated and fractional protein concentrations (total protein, casein or whey) in milk samples. When the model [Eq. (2)] was fitted to these data, Fisher's F test showed significance of the regressions. The r^2 showed the highest value in the model, where [P] equals the casein (tenoxicam; $b = 0.274$) (Fig. 1) or total protein (diazepam; $b = 0.820$) concentration (Fig. 2). The multiple regression analysis confirmed these findings.

Binding of Benzodiazepines to Pure Milk Protein Solutions and in Human Milk

The pH in the skimmed human milk used for binding experiments with five different benzodiazepines was 6.66, and the remaining milk fat less than 0.01%.

The concentrations of individual milk proteins in artificially prepared pure protein solutions were chosen such that they were similar to those determined in the whole human skimmed milk used (lactoferrin in whole skimmed milk, 2.5 g/L; albumin, 0.4 g/L; casein, 2.3 g/L; α-lactalbumin, 2.1 g/L). The AAG concentration in skimmed milk was below 0.18 g/L.

Casein strongly bound all five benzodiazepines (Table II), and the drug fraction bound in the 2.1 g/L casein solution for bromazepam, clonazepam, diazepam, and flunitrazepam was larger than that bound to any other of the milk proteins tested. When fb_m values of these benzodiazepines were compared between whole skimmed milk and the individual proteins, casein proved to be the most important binding protein.

Albumin, at the very low concentration encountered in human milk, contributed only to a minor degree to the overall binding. Lactoferrin and α-lactalbumin did not account for significant binding of bromazepam, clonazepam, diazepam, and flunitrazepam.

Flumazenil showed a higher binding to lactoferrin than to casein or albumin at the concentration chosen. However, the overall binding of this antagonistic benzodiazepine in milk is very low. Fractions of the five benzodiazepines bound to casein and to skimmed milk were linearly related ($P < 0.05$). A stepwise linear regression analysis confirmed that the binding to casein contributed most to binding in whole skimmed milk.

Table I. Protein Concentrations and Free Fractions of Diazepam and Tenoxicam in Milk of Various Species

	Milk protein conc. (g/L)			Free fraction of	
Species	Total	Casein	Whey	Diazepam	Tenoxicam
Man	16.1	6.3	9.8	0.405	0.880
Horse	22.4	12.1	10.3	0.405	0.800
Goat	24.5	20.6	3.9	0.309	0.605
Cow	24.5	20.6	4.0	0.307	0.697
Sheep	35.5	20.1	15.4	0.320	0.684
Dog	77.4	48.2	29.2	0.070	0.347
Rabbit	102.8	72.3	30.5	0.111	0.306

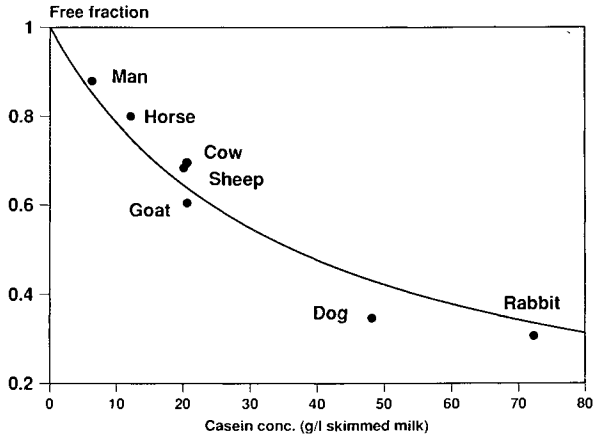

Fig. 1. Relationship between free fractions of tenoxicam in skimmed milk of various species and casein concentration in skimmed milk. (●) Observed values; (——) fit of binding model [see text; Eq. (2)]. The correlation is statistically significant ($P < 0.001$).

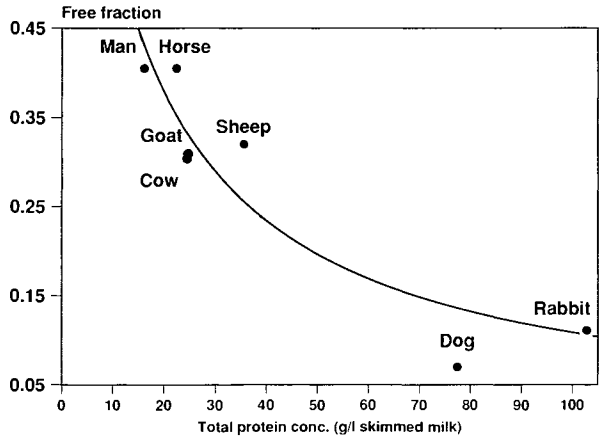

Fig. 2. Relationship between free fractions of diazepam in skimmed milk of various species and total milk protein concentration. (●) Observed values; (——) fit of binding model [see text; Eq. (2)]. The correlation is statistically significant ($P < 0.001$).

Table II. Binding of Benzodiazepines to Major Milk Proteins

Drug	Bound fraction (%) in Skimmed milk	Albumin	Lactoferrin	Lactalbumin	Casein 2.1 g/L	Casein 3.4 g/L	Casein 13.3 g/L
Diazepam	45.7	12.1	2.2	4.3	22.3	29.9	63.3
Clonazepam	28.9	1.9	0.4	0.9	16.3	21.0	55.6
Flunitrazepam	23.4	3.1	0.3	1.5	8.0	14.4	37.5
Bromazepam	12.4	1.0	2.9	0.1	5.3	15.5	38.0
Flumazenil	5.8	0.5	1.8	0.5	0.5	2.3	11.9

The binding of each of the five benzodiazepines showed, at least in the range of the concentrations tested, a linear relationship between casein concentration (2.1, 3.4, and 13.3 g/L) and extent of binding to this protein. Linear regression analysis revealed statistical significance for clonazepam, diazepam, and flumazenil.

The fraction bound in skimmed milk of the tested benzodiazepines was related to the lipophilicity of the drug. The higher the lipophilicity, the higher the binding in skimmed milk and to casein (Tables II and III). The apparent relationship between binding to milk and plasma proteins and the octanol/water (O/W) partition coefficient of the five chosen drugs is shown in Fig. 3.

DISCUSSION

The five benzodiazepines were chosen according to their physicochemical properties [O/W partition coefficient and pK_a (10)] and extent of plasma protein binding (11). They covered a broad range of values in all of these parameters (Table III).

The use of skimmed milk for binding experiments allowed exclusion of partition phenomena between milk and milk fat.

The results of this study showed that the casein fraction is a major binding partner in milk for all tested drugs. When comparing the goodness of fit of the binding model [Eq. (2)], the binding in skimmed milk for diazepam and tenoxicam did not correlate with the whey protein concentration. The casein (tenoxicam) or the total protein (diazepam) concentration mainly determined the extent of drug binding in skimmed milk.

The free fraction of diazepam in dog milk was smaller than expected based on the used model (Fig. 2). An electrophoretic characterisation of milk of all species included in our study indicated that dog milk differs qualitatively and quantitatively from all other species with respect to protein composition. This may explain the observed higher binding.

In the study with the five benzodiazepines a linear relationship between binding in milk and binding in casein solution was found ($P < 0.05$). The drug with the lowest binding in milk (flumazenil) also exhibited the lowest binding to casein, whereas the high fraction bound in milk (e.g., diazepam) was also reflected in a high binding to casein.

By adding the drug binding to the individual protein fractions tested (casein, albumin, lactoferrin, and α-lactalbumin), 55 to 89% of the observed binding in milk could be accounted for (75% bromazepam, 67% clonazepam, 89% diazepam, 57% flumazenil, and 55% flunitrazepam). This analysis assumes the absence of influence of one protein on the binding behavior of other proteins (e.g., absence of cooperativity or displacement), which may be incorrect. However, 92% of the total milk protein concentration was accounted for, and the AAG concentration (below 0.18 g/L) cannot account for the observed high drug binding in milk. Alterations of the proteins in their binding behavior during isolation and purification processes are also possible.

The pH of milk can be measured aerobically or anaerobically. Differences in pH values by using the two different methods have been reported (12). However, according to the pK_a values of the chosen benzodiazepines, small pH alterations have essentially no effect on the unionized fraction of these drugs.

Table III. Physicochemical Properties of Model Compounds

Drug	pK_a	Plasma protein binding (%)	O/W part. coeff.	S/M
Diazepam	3.30	97	646	0.11
Clonazepam	1.57	82	309	0.33
Flunitrazepam	1.87	80	115	0.26
Bromazepam	2.80	70	35	0.70
Flumazenil	1.70	40	14	0.88
Tenoxicam	5.40	98	<1	ND[a]

[a] Not determined.

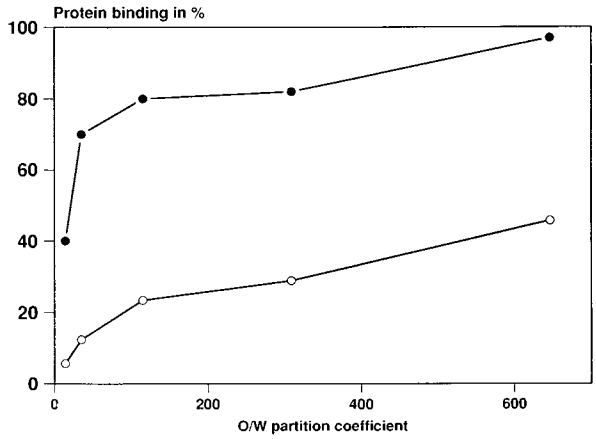

Fig. 3. Relationship between O/W partition coefficient and percentage plasma and milk binding of five benzodiazepines. (●) Binding to plasma proteins; values taken from Ref. 11. (○) Binding to skimmed milk; values from the present study.

Atkinson and Begg (4) determined the binding of flunitrazepam and other drugs in milk protein solutions. The weak base flunitrazepam did not bind to individual whey proteins. In our study flunitrazepam also did not significantly bind to whey proteins; however, it did bind to components of skimmed milk (fb_m, 23%) and to casein in solution (Table II).

Drayer (13) described the dependence of the plasma protein binding of various benzodiazepines on the O/W partition coefficient. Our investigations confirm these findings and allow extension of the results to compounds with lower O/W partition coefficients than used by that author. No linear relationship between extent of binding and lipophilicity can be seen. Differences in O/W partition coefficient between drugs with low lipophilicity (O/W < 100) show a more dramatic effect on protein binding than with drugs of higher lipophilicity. Our results show that this is true not only for binding to albumin in plasma but also for binding to skimmed milk (Fig. 3).

In conclusion, the casein fraction in milk mainly determined the extent of binding in skimmed milk and was found to be the major binding partner for bromazepam, clonazepam, diazepam, and flunitrazepam. Further, the binding to milk and to casein solutions correlated with the lipophilicity of the drug. These findings not only have practical consequences for the design of studies on drug excretion into milk but also may be of clinical relevance. The casein concentration in milk drops during the first days postpartum (p.p.) from 21 to 5 g/L (14). A change from higher *M/P* ratios in the first days p.p. to lower ratios in the later lactation period is therefore expected for drugs where casein is a major binding partner, provided that no alterations in other determinants of drug excretion into milk occur.

ACKNOWLEDGMENT

We thank Mrs. Monika Huber for providing us with milk of various animal species.

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