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Report

Drug Binding and Solubility in Milk

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The binding and solubility of nitrofurantoin, piroxicam, indomethacin, prednisolone, diazepam, dicumarol, and griseofulvin in milk were determined at 15, 25, and 37°C in bovine milk samples with fat contents of 0.75 and 3.50%. Drug binding to milk components was independent of drug concentration over the drug concentration studied, and the fat content of milk strongly affected binding values of most of the listed drugs. Further, drug binding increased with decreasing temperatures for most of the drugs examined. The solubility of all drugs is greatly enhanced in milk compared to their aqueous solubility (pH 6.5 phosphate buffer). The high solubility cannot be accounted for solely on the basis of drug binding to milk components. An attempt is made to correlate the binding and solubility data with physicochemical properties of the drugs (log$P$, pK_a, aqueous solubility). The potential significance of these findings is discussed with regard to preparation and *in vivo* delivery of drugs from drug–milk formulations.

KEY WORDS: binding; solubility; drug; milk.

INTRODUCTION

The binding and solubility of drugs in milk are of interest because of the development of drug–milk freeze-dried formulations (1,2). These formulations have been found to enhance the bioavailability of sparingly soluble drugs. Two important factors determining drug bioavailability from such a formulation are binding to milk components and solubility in milk.

With chlorothiazide and hydrochlorothiazide as model drugs, it has been shown (3,4) that the binding (i) is independent of drug concentration and (ii) cannot fully account for the increased solubility (in comparison to the aqueous solubility) of the two thiazides in milk.

In this report we studied the relationship of milk binding, solubility, and physicochemical properties of drugs in order to derive guidelines for the design of drug–milk freeze-dried formulations. The selected drugs and their physicochemical properties (log$P$, pK_a, solubility) are listed in Table I (5–7). The results of this study are useful in the interpretation of *in vivo* performances of drug–milk formulations.

MATERIALS AND METHODS

Nitrofurantoin was obtained from Athenpharm (Athens); piroxicam was obtained from Chemica (Athens); dicumarol was obtained from Aldrich (Milwaukee, WI), while indomethacin, diazepam, griseofulvin, and prednisolone were purchased from Sigma (St. Louis, MO). All drugs were found to be >99% pure by HPLC or UV analytical methods. Milk was provided by Langenosseschaftt, Einstal, Stainach, Steiermark, Austria. Two milk types with fat contents of 0.75 and 3.50% were used. Inter- and intrabatch variabilities of fat, protein, and lactose content of the milk types utilized were found to be as described previously (3). A Perkin–Elmer lambda 6 spectrophotometer was utilized for the determination of nitrofurantoin, piroxicam, and dicumarol in samples.

The HPLC system consisted of a Waters Model U6K Universal injector, a Model 590 solvent delivery system, and a Model 481 LC variable-wavelength UV detector. A Lichrosorb 10 RP 18 reversed-phase column (25 cm × 4.6 mm) was utilized for the determination of griseofulvin, indomethacin, prednisolone, and diazepam.

Binding Studies

Drug binding to milk was determined by equilibrium dialysis against a 0.2 M phosphate buffer, pH 6.5 solution. The equilibrium dialysis was carried out at 15, 25, and 37°C for 4 to 5 hr using a Dianorm equilibrium dialyser with Teflon dialysis cells (type macro 2, Diachema AG Rushlikon, Zurich, Switzerland) for all drugs except dicumarol. At least three dialysis runs were carried out for each binding experiment. Stock solutions for griseofulvin, indomethacin, prednisolone, and diazepam were prepared in methanol; the stock solution of nitrofurantoin was prepared in dimethylformamide, while piroxicam and dicumarol were initially dissolved in 0.1 *M* NaOH. All stock solutions were diluted with a 0.2 *M* phosphate buffer to final concentrations ranging from 80 to 120 mg/liter for nitrofurantoin, 20 to 60 mg/liter for piroxicam, 100 to 300 mg/liter for indomethacin, 50 to 200 mg/liter for prednisolone, 20 to 40 mg/liter for diazepam, 8 to 10 mg/liter for griseofulvin, and 10 to 20 mg/liter for dicu-

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Table I. Physicochemical Properties of Drugs Utilized in the Present Study

Drug	pK_a[a]	log$P$[a]	Solubility (mg/liter)[b]
Nitrofurantoin	7.2	−0.36	124
Piroxicam	6.3	0.255	84
Indomethacin	4.5	3.08	286
Prednisolone	—	1.42	208
Diazepam	3.3	2.86	31
Dicumarol	5.4	2.07	18
Griseofulvin	—	2.18	8

[a] Data taken from Refs. 5, 6, and 7.
[b] Solubility values obtained at 25°C in pH 6.5 buffer.

marol. The drug samples (2.0 ml) in buffer were dialyzed against 2.0 ml of milk using dialysis membranes (Diachema, type 10–14) with a declared molecular cutoff of 5000. The dialysis cells were rotated at 20 rpm in a thermostated water bath (Julabo SW1). The binding of the drugs to the membranes and cells was checked and found to be negligible for all drugs except diazepam; the percentage of diazepam bound to membranes and cells was 4.9 ± 0.2% in the concentration range utilized in the binding experiments. The estimates of diazepam binding were appropriately corrected.

Analytical Procedures

Nitrofurantoin. The free concentration of nitrofurantoin was determined by measuring the absorbance of the buffer dialysates at 368 nm. Each milk type was also dialyzed against buffer to provide a blank for any interfering substance which may have entered the dialysate from the milk compartment.

Piroxicam. The free concentration of piroxicam was determined by measuring the absorbance of the buffer dialysates at 339 nm. Absorbance values were appropriately corrected using the absorbance data from blank experiments.

Indomethacin. The free concentration of indomethacin was determined by a slightly modified HPLC method (8); 1.00 ml of the buffer dialysates was vortexed with 200 μl of a phenylbutazone solution (200 μg/ml), used as internal standard, and 20 μl of the vortexed solutions was injected into the chromatograph. The mobile phase consisted of 70% methanol and 30% acetic acid (0.05%, v/v). The UV detector was set at 254 nm. The flow rate was 3 ml/min.

Prednisolone. The determination of prednisolone free concentration in the buffer dialysates was performed by an HPLC method (9). Each sample (1.00 ml) was vortexed with 100 μl of a griseofulvin solution (600 μg/ml), used as internal standard, and 20 μl of the vortexed solutions was injected into the chromatograph. The mobile phase consisted of 45% acetonitrile and 55% water. The flow rate was 3.5 ml/min. The UV detector was set at 254 nm.

Diazepam. The free concentration of diazepam at equilibrium was determined by an HPLC method (10). Each sample (1.00 ml) was vortexed with 100 μl of a griseofulvin solution (300 μg/ml) and 20 μl of the vortexed solutions was injected into the chromatograph. The mobile phase, flow rate, and drug monitoring were identical to these used for prednisolone determination.

Griseofulvin. The free concentration of griseofulvin in the buffer dialysates was determined by an HPLC method (11). Each sample (1.00 ml) was vortexed with 200 μl of a diazepam solution (300 μg/ml) used as internal standard, and 20 μl of the vortexed solutions was injected into the chromatograph. The mobile phase and flow rate were identical to these used for the prednisolone determination. The UV detector was set at 290 nm.

Dicumarol. Due to the extensive binding of dicumarol to milk, the conventional equilibrium dialysis technique resulted in a minute free concentration in the dialysate. To overcome this difficulty, a modified equilibrium dialysis method (12) utilizing transit of drug from a large volume (20 ml) of dialysate to a small volume (0.5 ml) of milk was employed. The free concentration of dicumarol was determined by measuring the absorbance of samples at 314 nm.

Solubility Studies

The solubility of drugs in milk was studied using the equilibrium dialysis apparatus as described previously (4). Studies were performed at 15, 25, and 37°C, using equilibration times ranging from 6 to 14 hr. At least four runs were carried out for each solubility experiment. The analytical procedure for the determination of drugs in the milk samples consisted of two steps. The milk samples were initially dialyzed utilizing the flow-injection dialysis technique described previously (4,13,14). The concentration of drugs in the dialysates was determined by the analytical methods used in the binding studies. Drug milk samples were diluted with 0.2 *M* buffer, pH 6.5, to provide an adequate volume during the dynamic dialysis for the circulating donor solution. Due to the low free concentration of dicumarol in the milk samples, these samples were diluted with a solution of 0.01 *M* NaOH. This treatment caused disruption of casein micelles and release of all dicumarol into the solution. The dialysis time ranged, depending on the drug, from 5 to 10 min. Water was used as the carrier solution for all drugs except dicumarol, where 0.01 *M* NaOH was used.

RESULTS AND DISCUSSION

Binding Studies

For all drugs examined the binding to both types of milk was found to be independent of drug concentration over the concentration range studied. This result agrees with the nonspecific binding of hydrochlorothiazide and chlorothiazide to milk (3). The mean percentage of binding to milk is given in Tables II and III.

Nitrofurantoin, piroxicam, and indomethacin showed increased binding to both milk types at lower temperatures. The binding of dicumarol increased with temperature in both milk types, while prednisolone exhibited higher binding with elevated temperature only in milk with the higher fat content. Griseofulvin and diazepam exhibited relatively constant binding over the temperature range studied in both milk types.

While the varying effect of temperature on the binding of each drug examined cannot be explained, the extent of drug binding correlates with lipophilicity, as shown in Fig. 1. Similar results were obtained at 15°C (Fig. 1) and at 25 and

Table II. Percentage Bound of Drugs to Milk with 0.75% Fat[a]

Drug	Temperature (°C) 15	25	37
Hydrochlorothiazide[b]	28.4 (1.1)	24.9 (0.7)	22.5 (1.4)
Chlorothiazide[b]	22.7 (2.1)	19.9 (1.0)	15.6 (1.0)
Nitrofurantoin	15.2 (1.0)	11.6 (1.3)	12.9 (2.4)
Piroxicam	28.9 (3.3)	25.4 (2.1)	23.6 (0.7)
Indomethacin	61.3 (1.1)	59.3 (1.0)	54.6 (2.1)
Prednisolone	42.3 (6.5)	39.1 (4.4)	36.9 (2.7)
Diazepam	65.8 (1.6)	69.2 (0.5)	65.5 (1.2)
Dicumarol	91.4 (1.7)	93.9 (1.0)	94.8 (0.6)
Griseofulvin	56.3 (0.8)	58.1 (1.6)	53.8 (0.9)

[a] SD in parentheses.
[b] Data taken from Ref. 3.

37°C (not shown). All plots demonstrate a linear relationship between the logarithm of the ratio bound/free concentration, log(*B*/*F*), and log(*P*) of the drugs at all temperatures in both milk types examined. Linear correlations between log(*B*/*F*) and log*P* have been reported in the literature (15,16). In all plots the dicumarol data deviate significantly from the regression line, showing higher binding than that anticipated on the basis of its log(*P*) value. Further, the highest deviation was observed at 37°C in parallel with the increased binding of dicumarol at higher temperature.

Since thermodynamic analysis of binding results provides information concerning the interactions which take place or the transfer process, the changes in enthalpy (ΔH) were calculated from the slope of the Van't Hoff plots (plots of log K_d^m versus $1/T$):

$$\log K_d^m = \frac{\Delta S}{2.303R} - \frac{\Delta H}{2.303R} \cdot \frac{1}{T} \quad (1)$$

where k_d^m is the molal distribution coefficient (17), which had to be used in this case due to the complex nature of milk. The values for K_d^m were obtained from Eq. (2):

$$K_d^m = \left(\frac{C_0 - C_w}{C_w}\right) \cdot \left(\frac{w_1}{w_2}\right) \quad (2)$$

where C_o is the initial buffer concentration (before equilibrium), C_w is the final buffer concentration (after equilibrium), w_1 is the weight of the aqueous phase, and w_2 is the weight of the nonaqueous phase of the sample. The ratio w_1/w_2, obtained by lyophilizing samples, was 19.5 and 15.2 for the 0.75 and 3.50% fat-content milk, respectively.

Table III. Percentage Bound of Drugs to Milk with 3.5% Fat[a]

Drug	Temperature (°C) 15	25	37
Hydrochlorothiazide[b]	29.5 (0.9)	24.5 (1.2)	21.6 (1.1)
Chlorothiazide[b]	23.4 (0.8)	21.7 (1.2)	15.1 (0.8)
Nitrofurantoin	10.4 (0.6)	10.1 (2.7)	9.1 (0.3)
Piroxicam	32.0 (1.7)	30.7 (2.8)	27.5 (2.8)
Indomethacin	70.2 (0.3)	63.9 (2.0)	65.6 (0.7)
Prednisolone	35.8 (3.9)	36.1 (5.3)	40.9 (2.3)
Diazepam	79.0 (0.9)	83.3 (4.8)	78.5 (4.8)
Dicumarol	84.8 (4.3)	92.3 (1.5)	94.6 (0.9)
Griseofulvin	61.7 (2.3)	65.5 (2.4)	64.3 (0.4)

[a] SD in parentheses.
[b] Data taken from Ref. 3.

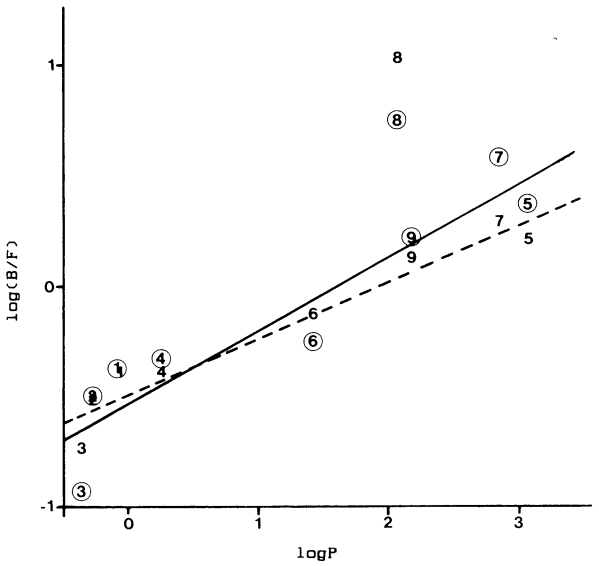

Fig. 1. Plots of log(*B*/*F*) as a function of log*P* for the binding of drugs to milk at 15°C. For the 0.75% fat-content milk data points are indicated with numbers and the regression line with a dashed (– – –) line. For the 3.5% fat-content milk, data points are indicated with circled numbers and the regression line with a solid (——) line. (1) Hydrochlorothiazide; (2) chlorothiazide; (3) nitrofurantoin; (4) piroxicam; (5) indomethacin; (6) prednisolone; (7) diazepam; (8) dicumarol; (9) griseofulvin.

Although as systems become more complicated, the use of Van't Hoff operators to obtain ΔH data becomes less valid (18,19), the correlation coefficients of the log(*B*/*F*) versus log*P* correlations were significantly increased, from 0.74–0.78 to 0.98–0.99, when multiple regression analysis was performed using ΔH as a second independent variable for the binding data of milk with 0.75% fat. For the binding data of the 3.5% fat milk, the correlation coefficients were significantly increased, from 0.83–0.88 to 0.98–0.99, when the percentage of ionized drug at pH 6.5 was used as a third independent variable. The significance tests for additional independent variables in multiple regression (20) were taken into account for the increases in the coefficients of multiple determination. These observations indicate that the lipophilicity of drugs is not the sole parameter controlling the distribution of drugs between milk phases. Other factors, such as the mechanism(s) involved and the ionized fraction of drug at pH 6.5, also affect drug distribution. Therefore, the deviation of the dicumarol values in all plots of log(*B*/*F*) versus log*P* may result from differences in the mechanism of transfer of dicumarol between the milk phases.

Solubility Studies

Drug solubility as a function of temperature is shown in Fig. 2. The solubility of drugs is remarkably higher in milk than in buffer at all temperatures studied. However, the greatest enhancement of drug solubility in milk is noted at

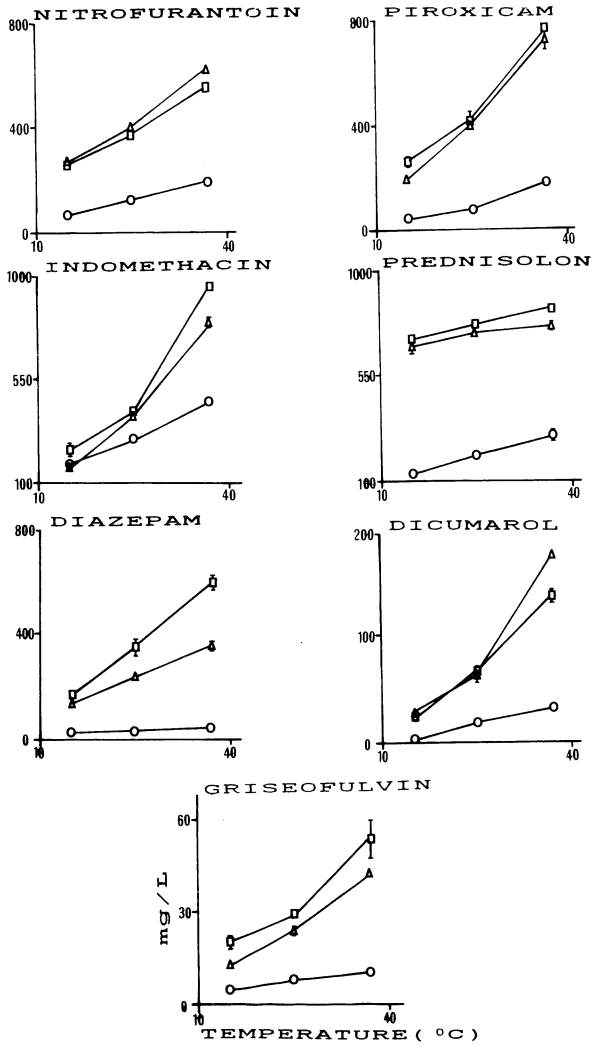

Fig. 2. Plots of aqueous and milk solubility of drugs as a function of temperature. The lettering of axes in all plots is identical to that used for griseofulvin at the bottom of the figure. (○) Solubility in pH 6.5 buffer; (△) solubility in 0.75% fat-content milk; (□) solubility in 3.5% fat-content milk. Not shown SDs are overlapped by the size of symbols.

37°C. This observation is in agreement with the results obtained with chlorothiazide and hydrochlorothiazide (4). Further, the solubility in milk increased with drug lipophilicity. Since drug binding to milk at 37°C is either lower than or similar to that observed at 15 and 25°C (Tables II and III), while milk solubility was higher at 37°C, the high drug solubilities in milk cannot be accounted for solely by drug binding to milk components. Assuming that the concentration-independent binding can be extrapolated to the solubility region, a predicted milk solubility (PMS) can be obtained from Eq. (3), which interprets any increase in milk solubility solely on the basis of drug binding to milk components.

$$\begin{aligned} \text{PMS} &= \text{solubility in buffer, pH 6.5,} \\ &\quad + [(\text{solubility in buffer, pH 6.5}) \\ &\quad \times (\text{fraction bound})] \\ &= (\text{solubility in buffer, pH 6.5}) \\ &\quad \times (1 + \text{fraction bound}) \end{aligned} \tag{3}$$

The values for the fraction bound needed for Eq. (3) were derived from the data listed in Tables II and III. A comparison of the estimates of PMSs with the experimentally found milk solubilities is given in Table IV. With the exception of indomethacin, all the experimentally determined milk solubilities are higher than the predicted milk solubilities at all temperatures examined. For indomethacin, experimental and predicted solubilities at 25°C are statistically equivalent, while higher solubilities than those predicted were found at 37°C in both milk types. At 15°C the milk solubility of indomethacin is equal to that in buffer and lower than the theoretically predicted milk solubility. This result applies only to the less lipophilic type of milk since the predicted and experimental solubility values in milk with 3.50% fat are equivalent. The unique behavior of indomethacin may be linked to its ionization characteristics. It is the only drug from these examined with a low pK_a 4.5 which implies complete ionization at pH 6.5. Apparently, the reduced hydrophobic drug–milk interactions at lower temperatures (21) result in equal solubility of indomethacin in buffer and 0.75% fat milk at 15°C. However, at higher temperatures micelles are formed (above 25°C), and the solubility of indomethacin in milk greatly exceeds the buffer solubility. When analyzing the relationship between aqueous and milk solubility, it was found that the milk solubility increases with the aqueous solubility in a nonlinear fashion. Typical plots, using the solubilities at 37°C, are presented in Fig. 3. Plots of a similar pattern were obtained at 15 and 25°C. The nonlinear increase in milk solubility with water solubility was found to be more consistent (less scattering of data points) at 37°C than at 15 and 25°C. This result should not be attributed to reduced variability of the experimental values at this temperature. Rather, it is probably due to the hydrophobic character of milk at 37°C, which enhances intrinsic drug solubility. In contrast, at lower temperatures the physical changes (21) (liquid fat progressively solidifies, fat globules are agglutinated, and casein dissociates from the micelles) cause a dynamic "transit" milk structure accompanied by a diminution of its hydrophobicity. These changes introduce additional factors regulating drug solubility in milk and contribute to greater variability of the solubility data.

All plots (similar to these of Fig. 3) reveal that the rel-

Table IV. Comparison of the Predicted Milk Solubility (PMS) with the Experimentally Found Milk Solubility (EFMS) for All Drugs[a]

Drug	(PMS)/(EFMS) * 100		
	15°C	25°C	37°C
Nitrofurantoin	28.3–28.1	34.7–36.9	35.0–37.7
Piroxicam	27.0–20.8	26.2–26.4	31.4–30.7
Indomethacin	176.0–125.8[b]	117.1[b]–114.7[b]	87.5–77.9
Prednisolone	27.7–25.6	39.2–36.6	53.1–49.7
Diazepam	30.9–27.0	21.9–16.2	21.1–13.7
Dicumarol	16.8–19.4	54.2–50.9	36.0–45.8
Griseofulvin	56.1–35.6	52.6–45.4	37.4–31.4

[a] The values on the left-hand side and the right-hand side correspond to 0.75 and 3.5% fat-content milk, respectively, at the specified temperature.

[b] Nonsignificant difference at $P = 0.05$ level using paired t test.

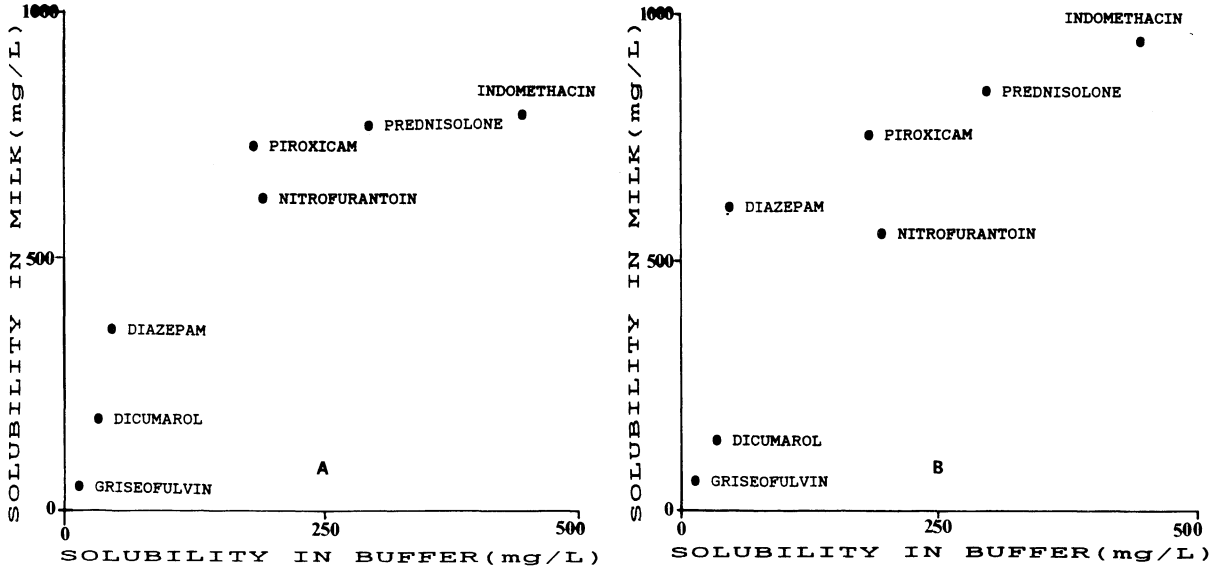

Fig. 3. Typical plots of the relationship between aqueous and milk solubility at 37°C in two milk types: (A) with 0.75% fat content; (B) with 3.5% fat content.

ative increase in milk solubility is higher for the sparingly water-soluble drugs. Milk solubility levels off as the aqueous solubility approaches ~100, ~200, and ~300 mg/liter at 15, 25, and 37°C, respectively. However, incorporation of the thiazide plots (3) reveals that the more water-soluble hydrochlorothiazide deviates significantly from the plateau values of milk solubility observed at various temperatures. The present data are insufficient to establish the behavior of drugs with water solubilities higher than 900 mg/liter at 37°C; however, the less water-soluble drugs (<100 mg/liter) are of greater interest as milk–drug formulations. For poorly water-soluble drugs, a dramatic solubility increase in milk was observed. The high drug solubility in milk could account for the high bioavailability of dicumarol–milk (1) and nitrofurantoin–milk (2) freeze-dried formulations. Our results can be used as a basis for the proper design of drug–milk formulations. Moreover, the established relationship between aqueous and milk solubility (Fig. 3) can be employed to derive an estimate of the drug solubility in milk once its aqueous solubility is known.

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