Effect of Temperature and Fat Content on the Binding of Hydrochlorothiazide and Chlorothiazide to Milk

PANAYOTIS E. MACHERAS*x, MICHAEL A. KOUPPARIS‡, AND SOPHIA G. ANTIMISIARIS*

Received April 28, 1987 from the *Departments of *Pharmacy and ‡Chemistry, University of Athens, Athens 10680, Greece.
Accepted for publication November 20, 1987.

Abstract □ The binding of hydrochlorothiazide and chlorothiazide to milk has been measured. Experiments were carried out at 5, 15, 25, and 37 °C on bovine milk samples with fat contents of 0.75, 1.70, and 3.50%, using a wide range of drug concentrations to mimic concentrations encountered when a drug–milk freeze-dried system is utilized. Binding experiments with a 2.6% solution of casein were also carried out at the same temperature and concentration range of drugs. The binding to milk and casein was found to be not dependent on the concentration of drugs. The fat content of milk had no significant effect on the binding of both drugs. Higher binding was observed at lower temperatures than at higher temperatures for both drugs examined. The binding of both drugs to casein at 37 °C agrees fairly well with the corresponding binding to all types of milk at 37 °C. The potential significance of the findings in respect to preparation and in vivo delivery of drugs from drug–milk formulations is discussed.

Recent studies[1,2] on drug–milk freeze-dried formulations substantiate the view that this delivery system is capable of enhancing the bioavailability of sparingly soluble drugs. It is therefore of interest to evaluate and explore the characteristics of the drug–milk system.

Since most of the drug is in solution when the drug–milk system is administered, concern is arising about the nature of drug in the milk. Previous studies[1,2] indicated that casein micelles promote the solubility of drugs. However, the binding of drugs to milk proteins and other milk components has not been quantitatively evaluated. From the data available, studies on drug–milk binding mainly deal with the drug excretion in human breast milk[3,4] or the distribution of drugs in the milk phases.[5,6] Accordingly, very low drug concentrations are encountered or utilized in these studies. For formulation purposes, however, the drug concentrations in the drug–milk system are much higher. The concentration range used is dependent on the dosage requirements and the value of the drug-to-milk volume ratio, and is limited by the solubility of drug in milk. Another factor of importance that may affect the overall binding is the type of milk utilized for the drug–milk system. The ability of a drug to distribute in the milk phases may depend on the fat content of the milk. Finally, protein binding and distribution phenomena are highly influenced by temperature. Under in vitro conditions, increased binding at lower temperatures has been reported for several drugs.[7–11] Considering the preparation, storage, and in vivo administration of the drug–milk system, one will probably find different degrees of binding at different temperatures during these stages.

Little is known about the effects of these and other factors on the free fractions of drugs in milk. We now present data on the effect of temperature and fat content on the binding of hydrochlorothiazide and chlorothiazide to milk. These two thiazide diuretics have structural similarity, but relatively different physicochemical properties.[12] In addition, both drugs show incomplete absorption,[12] which makes them candidates for a drug–milk freeze-dried preparation.

Experimental Section

Hydrochlorothiazide was kindly supplied by Ciba-Geigy, Athens, Greece. Chlorothiazide was obtained from Chropi, Piraeus, Greece. Both drugs were found to be 99% pure by high-performance liquid chromatography (HPLC). Soluble casein was obtained from BDH Chemicals, Ltd., Poole, UK. Sulfamethoxazole was from Sigma. All other chemicals were of analytical grade quality and used as received. Milk was provided by Landgenossenschaft, Ennstal, Stainach, Steiermark, Austria. The milk-fat content was 0.75, 1.50, and 3.50%. Skim milk was collected by centrifuging whole milk (0.75% in fat content) for 1 h at 3500 rpm and drawing off the skim milk layer (bottom) with a transfer pipette. Inter- and intrabatch variability of fat, protein, and lactose content of the various milk types utilized were estimated directly in undiluted samples by infrared absorption photometry (Multispec-N Infrared Milk Analyzer, York, UK). Samples yielded mean protein and lactose contents of 3.32 ± 0.1 and 4.82 ± 0.1%, respectively, for all types of milk examined. The fat content of skim milk was found to be 0.10 ± 0.08%; the estimates for the fat content of the other types of milk used in this study were in accord with that of the label of the manufacturing company.

The binding of the drugs to milk proteins and other milk components was determined by equilibrium dialysis of the various milk samples against a phosphate buffer (0.2 M, pH 6.5) solution. The equilibrium dialysis was carried out at 5, 15, 25, and 37 °C for 4 h using a Dianorm equilibrium dialyzer with Teflon dialysis cells (type Macro 2, Diachema AG Rüshlikon, Zürich, Switzerland). Since caseins are the main proteins (i.e., 26 g·kg^{-1}) in cow's milk,[13] casein binding studies were also performed at the same range of temperatures in order to evaluate the extent of the interaction of the two drugs with casein and to hopefully correlate the results of the two groups of experiments. To this end, a solution of soluble casein (2.6%, w/w) in phosphate buffer (0.2 M, pH 6.5) was used to study the binding of drugs to casein. Samples were prepared as follows. Both drugs were initially dissolved in 0.01 M NaOH. This stock solution was diluted using phosphate buffer (0.2 M, pH 6.5), and the final drug concentration in the samples ranged from 50 to 500 μg/mL and from 50 to 400 μg/mL for hydrochlorothiazide and chlorothiazide, respectively. The drug samples (2 mL) in buffer were dialyzed against 2 mL of milk or casein solution using cellulose dialysis membranes (Diachema, type 10-14) with a declared molecular weight cutoff of 5000. The dialysis cells were rotated at 20 rpm in a thermostated water bath (Julabo SW1).

The binding of both drugs to the membranes and cells was checked and found to be negligible.

The "free" concentrations of hydrochlorothiazide and chlorothiazide were determined by high-performance liquid chromatography methods.[14] Each sample (1 mL) was vortexed with 0.4 mL of a sulfamethoxazole solution (250 μg/mL) or 0.2 mL of hydrochlorothiazide solution (200 μg/mL), which were used as internal standards for the measuring of hydrochlorothiazide and chlorothiazide, respectively. For both drugs, 20 μL of the vortexed solutions were injected in the chromatograph. The HPLC system consisted of a Waters model U6K universal injector, a model 590 solvent delivery system, a model 481 LC variable wavelength UV detector set at 280 nm for both drugs, and a Lichrosorb 10 RP18 reversed-phase column (25 cm

0022-3549/88/0400-0334$01.00/0

× 4.6 mm). The mobile phase consisted of 80% phosphate buffer (0.2 M, pH 6.5) and 20% acetonitrile. The flow rate was 2.5 mL/min.

Results and Discussion

For both drugs, the binding to milk was independent of drug concentrations over the ranges studied. Typical plots of the binding of hydrochlorothiazide and chlorothiazide to milk are shown in Figures 1 and 2, respectively. The binding of both drugs to casein was also not dependent on drug concentration. These results suggest a nonspecific binding to milk components, as well as to casein, for both drugs examined. An overall view of the binding of both drugs to milk and to casein as a function of temperature is shown in Figure 3. In all cases, chlorothiazide was bound less extensively than hydrochlorothiazide (Figure 3). This result probably mirrors the difference in the lipophilicity of two compounds (P values for chlorothiazide and hydrochlorothiazide are 0.54 and 0.84, respectively).[12]

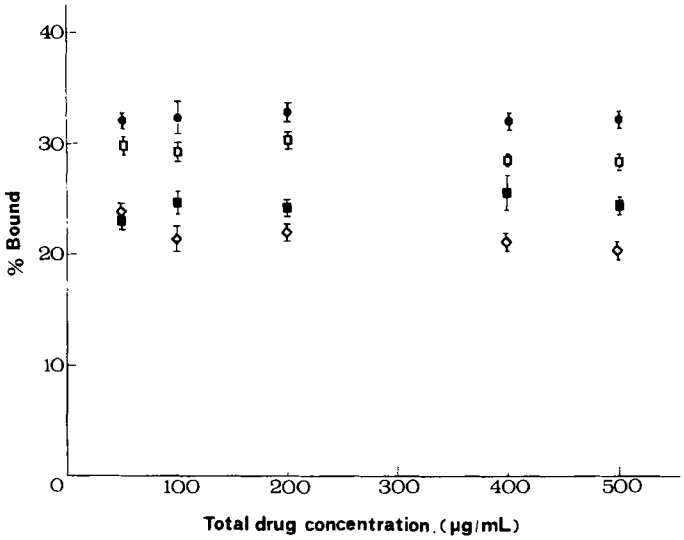

Figure 1—*Typical plots of the binding of hydrochlorothiazide to milk (fat content of milk: 3.50%). Key: (●) 5 °C; (□) 15 °C; (■) 25 °C; (◇) 37 °C. The same temperature effects occur for the other types of milk examined (not shown for the sake of clarity).*

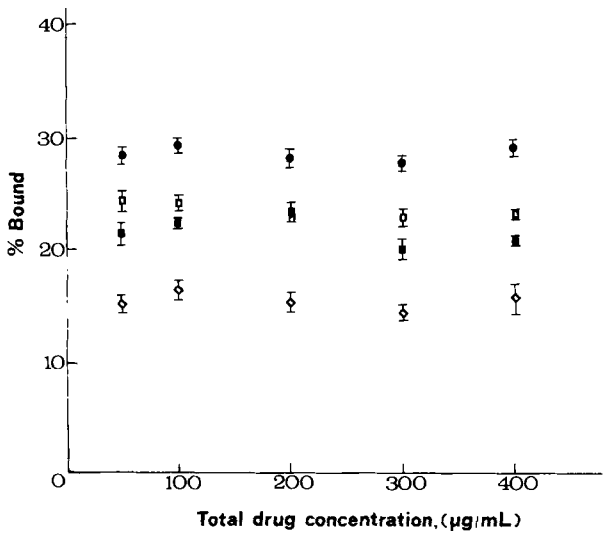

Figure 2—*Typical plots of the binding of chlorothiazide to milk (fat content of milk: 3.50%). Key: (●) 5 °C; (□) 15 °C; (■) 25 °C; (◇) 37 °C. The same temperature effects occur for the other types of milk examined (not shown for the sake of clarity).*

Temperature was found to profoundly affect the binding of both drugs to milk. Thus, as seen in Figure 3, the percent bound of both drugs to milk decreases progressively by increasing the temperature from 5 to 37 °C. With the exception of skim milk, these temperature effects on binding occur for all types of milk utilized. A sudden drop in the percent bound of both drugs to skim milk was observed between 15 and 25 °C (Figure 3). Interestingly though, the estimates of binding of both drugs to skim milk and casein at 25 and 37 °C were found to be almost identical. It should be mentioned also that similar estimates of binding were found for both drugs to all types of milk and casein at 37 °C. These observations are indicative of the fact that the binding of both drugs to milk at 37 °C is predominantly due to casein binding, while at lower temperatures, the binding to other milk components contributes to an overall higher binding of drugs to milk (Figure 3).

In view of the results presented in Figure 3, it is clear that the fat content of the three types of milk utilized does not have a significant effect on the binding of both drugs to milk. It seems likely, therefore, that the presence of a small amount of fat (in the range of fat contents studied) in the milk samples is adequate for a certain and appreciable participation of fat partitioning to the binding. An attempt was made to calculate how much of the thiazides binding to milk is due to protein binding and to lipid distribution. The results presented in Figure 3 reveal that the chlorothiazide binding to casein mimics the binding of this drug to skim milk at all temperatures studied. For hydrochlorothiazide, the same phenomenon was observed at 25 and 37 °C. Although this difference cannot be explained with any degree of certainty, it might be due to the interaction of less hydrophilic hydrochlorothiazide with residual fat in skim milk at 5 and 15 °C (fat content of skim milk was 0.10 ± 0.08%). Such a hypothesis has also been postulated for diazepam and phenytoin in relevant studies.[5,6]

If this is a valid consideration for the hydrochlorothiazide data at 5 and 15 °C, then the partial contribution of protein binding and fat partitioning to the total binding in milk can be calculated at all temperatures (Table I). The data of Table I reveal that the distribution of both drugs to fat was responsible for a considerable part of the bound drug at 5, 15, and 25 °C. However, the extent of binding to proteins is much more extensive at these temperatures. The results in Table I also indicate that the binding of both drugs to milk at 37 °C can be attributed to protein binding exclusively.

For hydrochlorothiazide, the binding to milk is enhanced at lower temperatures, while the binding of this drug to

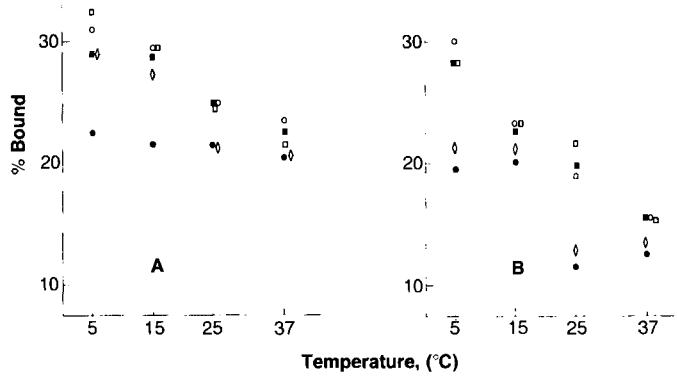

Figure 3—*Binding of hydrochlorothiazide* (A) *and chlorothiazide* (B) *to milk and casein as a function of temperature. Each experimental point is the average of 8–10 experiments in the range studied. Standard deviations of the percent bound (not shown for the sake of clarity) ranged from 0.6 to 2.1. Key: (●) casein; (◇) skim milk; (■) milk of 0.75% in fat; (○) milk of 1.70% in fat; (□) milk of 3.5% in fat.*

Table I—Contribution (mean values ± SD) of Protein Binding and Fat Partitioning to the Overall Binding in Milk of Hydrochlorothiazide and Chlorothiazide at Various Temperatures

Temperature, °C	Drug[a]	Percent Total Bound[b]	Percent Fat Partitioned[c]	Percent Protein Bound[d]	Percent Bound to Casein[e]
5	A	30.8 ± 1.7	8.3 ± 2.7[f]	22.5 ± 0.8[f]	22.5 ± 0.8
	B	28.9 ± 1.0	7.2 ± 2.6	21.8 ± 1.6	19.4 ± 0.9
15	A	29.1 ± 0.6	7.6 ± 1.0[f]	21.5 ± 0.9[f]	21.5 ± 0.9
	B	23.1 ± 0.4	2.0 ± 0.8	21.1 ± 0.4	20.1 ± 1.3
25	A	24.8 ± 0.2	3.3 ± 0.7	21.5 ± 0.9	21.4 ± 3.2
	B	20.2 ± 1.4	7.3 ± 2.9	12.9 ± 1.5	11.5 ± 1.0
37	A	22.6 ± 1.0	1.8 ± 1.9	20.8 ± 0.9	20.3 ± 2.3
	B	15.5 ± 0.3	1.8 ± 1.9	13.7 ± 1.6	12.8 ± 0.9

[a] A denotes hydrochlorothiazide and B denotes chlorothiazide. [b] Average of percent total bound to milk for the three fat contents utilized (i.e., 0.75, 1.50, and 3.50%). [c] Estimated as a difference between percent total bound and percent bound to skim milk. [d] Based on the binding with skim milk. [e] Quoted for comparative purposes. [f] Values of percent bound to casein were used instead of percent bound to skim milk.

casein remains constant over the temperature range studied. This is an additional indication of the participation of the lipid distribution to the milk binding at lower temperatures. The temperature-dependent character of the participation of lipid distribution in milk binding can be probably linked with the physical changes of lipids which take place with temperature. Thus, most of the triglycerides[13] of milk are in the solid state at 5 °C, while liquid fat becomes predominant as temperature increases up to the melting point of ~37 °C. In parallel, fat globules in milk are agglutinated at lower temperatures by the precipitation of cryoglobulins onto the fat globules.[13] As far as the temperature-dependent chlorothiazide binding to casein is concerned, it can be linked with the considerable changes of casein micelles at lower temperatures.[13] Hydrophobic interaction becomes much weaker since β-casein dissociates from the micelles.[13] On the other hand, hydrochlorothiazide–casein binding is not dependent on temperature; this may be explained by the fact that hydrochlorothiazide is totally un-ionized at pH 6.5 and simultaneously less hydrophilic than chlorothiazide.

The data obtained in this study indicate that temperature has a marked influence on the binding of hydrochlorothiazide and chlorothiazide to milk. The percent bound of the two thiazides at the temperatures studied ranged from 15 to 32%. Although quantitative generalizations for other drugs cannot be made, it is anticipated that large variations in the percent bound at various temperatures should be observed. This prediction can be based on the variability found for the binding of drugs in human breast milk excretion studies,[3,4] taking into account, however, the lower concentrations as well as the fixed temperature encountered in the excretion studies.

An important issue which is relevant to these considerations is the overall effect of milk binding on the solubility of drugs in milk at various temperatures. This has not been studied in the present investigation and remains to be explored since it has an obvious impact on the bioavailability of drugs administered as drug–milk formulations.[1,2]

Conclusively, the following remarks concerning the potential significance of the findings with respect to the preparation and in vivo performance of drug–milk formulation systems can be pointed out. Since the binding of drugs to milk is lower at 37 °C than at ambient temperatures, the "availability" of free drug is enhanced at physiological temperatures. In the present study, the milk fat content did not affect the binding of thiazides; however, current research[15] in this topic shows that the milk fat content affects the binding of lipophilic drugs to milk. Thus, the utilization of a certain type of milk for the preparation of the freeze-dried drug–milk formulations should be based on studies with each particular drug. This, in conjunction with the effect of milk on drug solubility, can be important for formulation purposes as well as for rationalizing the effect of milk on the bioavailability of drugs. Considerable attention[16–20] has recently been paid to the latter topic. The present work provides additional information about the factors which may be involved in the dissolution and absorption of drugs in the presence of milk or milk components.

In summary, studies such as this are valuable in elucidating the biopharmaceutical aspects of drug–milk formulations[1,2] or the effect of milk on drug absorption.

References and Notes

1. Macheras, P.; Reppas, C. *J. Pharm. Sci.* **1986**, *75*, 692.
2. Macheras, P.; Reppas, C. *J. Pharm. Sci.* **1986**, *75*, 1145.
3. Wilson, J. T.; Brown, R. D.; Cherek, D. R.; Dailey, J. W.; Hilman, B.; Jose, P. C.; Manno, B. R.; Monno, J. E.; Redetzki, H. M.; Stewart, J. J. *Clin. Pharmacokinet.* **1980**, *5*, 1.
4. Lewis, P. F.; Hurden, E. L. *Drugs and Pregnancy; Human teratogenesis and related problems*; Hawkins, D. F., Ed.; Churchill Livingstone: Edinbourgh, 1983; pp 204–228.
5. Syversen, G. B.; Ratkje, S. K. *J. Pharm. Sci.* **1985**, *74*, 1071.
6. Fleishaker, J. C.; Desai, N.; McNamara, P. J. *J. Pharm. Sci.* **1987**, *76*, 189.
7. Lunde, P. K. M.; Rane, A.; Yaffe, S. J.; Lund, L.; Sjöqvist, F. *Clin. Pharmacol. Ther.* **1970**, *11*, 846.
8. Shaw, L. M.; Fields, L.; Maycock, R. *Clin. Pharmacol. Ther.* **1982**, *32*, 490.
9. David, B. M.; Tjokrosetio, R.; Illet, K. F. *Ther. Drug. Monit.* **1983**, *5*, 81.
10. Brors, O.; Sager, G.; Sandness, D.; Jacobsen, S. *Br. J. Clin. Pharmacol.* **1983**, *15*, 393.
11. Melten, J. W.; Wittebroad, J.; Wemer, J.; Faber, D. B. *J. Pharm. Pharmacol.* **1986**, *38*, 643.
12. Dressman, J. B.; Amidon, G. L.; Fleisher, D. *J. Pharm. Sci.* **1985**, *74*, 588.
13. Walstra, P.; Jennes, R.; Badings, H. T. *Dairy Chemistry and Physics*, John Wiley and Sons: New York, 1984; pp 106, 89, 264, 234.
14. Barbhaiya, R. H.; Phillips, T. A.; Welling, P. G. *J. Pharm. Sci.* **1981**, *70*, 291.
15. Macheras, P.; Koupparis, M.; Antimisiaris, S., unpublished results.
16. Rosen, A; Macheras, P. *J. Pharm. Pharmacol.* **1984**, *36*, 723.
17. Rosen, A.; Macheras, P. *J. Pharm. Pharmacol.* **1985**, *37*, 154.
18. Macheras, P.; Koupparis, M.; Tsaprounis, C. *Int. J. Pharm.* **1986**, *33*, 125.
19. Macheras, P.; Koupparis, M.; Apostolelli, E. *Int. J. Pharm.* **1987**, *36*, 73.
20. Macheras, P.; Reppas, C. *Int. J. Pharm.* **1987**, *37*, 103.