

Active Transport of Nitrofurantoin Across the Mammary Epithelium *In Vivo*

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ABSTRACT

Nitrofurantoin is a commonly used urinary tract antibiotic that has been found at high concentrations in human milk. *In vivo* studies in rats were carried out to determine the mechanism by which this drug crosses the mammary epithelium. Lactating rats were gavage-fed with nitrofurantoin, and their milk and plasma levels of the antibiotic were measured at intervals up to 8 hr. The average milk-to-plasma (M/P) ratio, calculated from the areas under the milk and plasma curves, respectively, was 23 compared with a ratio predicted to be about 0.3 on the basis

of lipid partitioning and protein binding determinations. M/P ratios for two nitrofurantoin congeners were also calculated. The neutral compound furazolidone had a M/P ratio of about 1, as predicted, whereas the basic compound furaltadone had a M/P ratio of 3.49 compared with a predicted ratio of 1.4. These data suggest that nitrofurantoin and, to a lesser extent, furaltadone are actively transported across the mammary epithelium into milk.

Historically, a variety of medicinals have been evaluated for their propensity to accumulate in human milk. In general, lipophilic chemicals penetrate membrane barriers easily and are preferentially concentrated in the milk fat globules, thereby yielding a large M/P ratio. The partitioning of water-soluble chemicals that can pass through the plasma membrane of the alveolar cells is governed by the degree of ionization of the free chemical in the blood and the milk; the factors affecting this are the pH of the blood and milk and the pK of the chemical. The degree of binding by blood or milk proteins can modify the partitioning of all chemicals. The impact of these various determinants has been quantified, and pharmacokinetic models have been established for predicting the behavior of unstudied chemicals (Wilson *et al.*, 1980; Atkinson and Begg, 1988; Fleishaker *et al.*, 1987).

Although these principles successfully describe milk/plasma partitioning of many adequately studied chemicals, *in vivo* studies in a variety of species have identified some drugs that are found in milk at higher than predicted concentrations. Among these are aminopyrine (Banerjee *et al.*, 1967), N⁴-acetylated *p*-aminohippuric acid (Rasmussen, 1969a), N⁴-acetylated sulphanilamide (Rasmussen, 1969b), acyclovir (Lau *et al.*, 1987) and cimetidine (Oo, *et al.*, 1995b; Dostal, 1990; Somogyi and Gugler, 1979). It has been suggested that active transport mechanisms may be responsible for the unpredictable M/P ratios that are observed. Attempts

to observe these activities in *in vitro* model systems (Dostal, 1990) have not yet been successful.

Nitrofurantoin is a broad-spectrum antibiotic widely used in human medicine as a urinary tract antibiotic and in veterinary practice as an antibiotic and growth promoter. The current studies were motivated by preliminary results from a continuous breeding study with mice that revealed that chronic nitrofurantoin treatment of lactating mice resulted in decreased pup growth rate. Nitrofurantoin concentrations in the milk of these mice were greater than the theoretical M/P ratio of 0.5 that physicochemical principles would suggest. We therefore initiated a quantitative study in rats, where larger milk and plasma samples would be available to investigate this observation in more detail. The results demonstrate that transmammary transfer of nitrofurantoin into milk greatly exceeds the rates predicted on the basis of simple physicochemical partitioning.

Materials and Methods

Animals. Primiparous Sprague-Dawley rats were purchased with their natural litters of 2-day-old pups (Charles River Corp., Raleigh, NC) and were allowed *ad libitum* access to water and NIH-31 open formula diets (Zeigler Brothers, Inc., Gardners, PA.) until used at 10 days.

Chemicals

The following drugs and chemicals were used in this study: nitrofurantoin [N-(5-nitro-2-furfurylidene)-1-aminohydantoin]; furazoli-

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ABBREVIATIONS: M/P ratio, milk-to-plasma ratio; S/W ratio, skim-to-whole ratio.

done [3-(5-nitrofururylideneamino)-2-oxazolidinone; furaltadone (free base) [5-morpholinomethyl-3-(5-nitrofururylideneamino)-2-oxazolidinone], oxytocin, heparin, and methyl cellulose (4000 centipoise) (Sigma Chemical Co., St. Louis, MO), radiolabeled nitrofurantoin (^{14}C , methylene bridge-labeled; 97% radiopure; specific activity = 58.9 mCi/mMol) (Chemsyn Science Laboratories, Lexena, KA) and [^3H]-Mannitol Sp. Act. 30 Ci/mmol (New England Nuclear, Boston, MA), HPLC grade methanol, water, acetonitrile and sodium hydroxide (J. T. Baker, Phillipsburg, NJ) acetic acid (Mallinckrodt, St. Louis, MO) and Ketamine containing 1% xylazine (Fort Dodge Laboratories, Fort Dodge, IA).

Time Course Sampling of Nitrofurantoin in Milk and Plasma of Lactating Rats

Primiparous Sprague-Dawley rats with their natural litters were allowed *ad libitum* access to NIH-31 diets. On lactation day 10, dams were gavaged with nitrofurantoin (50 mg/kg b.wt.) in a 0.5% methylcellulose vehicle. At predetermined times, pups were removed from dams. Three hours later, milk and blood samples were collected from anesthetized animals. Blood obtained by heart puncture was collected *via* syringe into heparinized glass tubes; milk was collected in glass tubes after i.p. injection of oxytocin (0.05 U/g b.wt.). All samples were then stored on ice in the dark. Each animal was sampled only once, and 3 to 11 animals were studied at each time-point.

HPLC Methods

Extractions. Solid-phase extractions of milk and plasma were performed under low light conditions. Milk and plasma were thawed at 37°C for 15 min. All volumetric transfers were carried out with a positive displacement pipet fitted with glass capillaries. A 0.5-ml aliquot of milk or plasma was spiked with 0.025 ml of furazolidone as an internal standard (100 $\mu\text{g}/\text{ml}$). For furazolidone quantitation, nitrofurantoin was used as an internal standard. The mixture was passed over a Sep-pak C18 cartridge prewashed with 3 ml of acetonitrile followed by 9 ml of water. The sample container was washed with 0.7 ml of water, and the wash fluid was passed through the cartridge. The remaining water was displaced from the cartridge with air, and the compounds were eluted from the cartridge with 2.5 ml of acetonitrile. The acetonitrile eluate was dried under a stream of nitrogen at 40°C to 45°C. Furaltadone extraction was performed by using a Sep-pak C18 cartridge prewashed with 2 ml methanol and 8 ml of 0.01 M potassium monophosphate, pH 3.75. Furazolidone was used as the internal standard. The sample was acidified with 1 drop of concentrated phosphoric acid and loaded on the cartridge. The cartridge was washed with 0.75 ml of 0.01 M potassium monophosphate, pH 3.75, and compounds were eluted with 2.5 ml of methanol and dried.

Nitrofurantoin and furazolidone. The dried acetonitrile residue was dissolved in 0.5 ml of HPLC mobile phase (70% sodium acetate, 0.012 M, pH 5.0, and 30% methanol; (Aufrere, *et al.*, 1977). The solution was centrifuged 5 min at 13,000 $\times g$ in a microfuge. Milk lipids were further removed from the supernatant by passage through a 0.45- μm filter. The clarified supernatant (15 μl) was injected on a 5- μm Hypersil ODS (C18) column and eluted isocratically with 70% 0.012 M sodium acetate, pH 5.0, and 30% methanol. The eluate was monitored at 368 nm, 0.1 absorbance units full scale. Run time was 11 min, nitrofurantoin eluting at 6.7 min and furazolidone at 8.8 min.

Furaltadone. The dried methanol residue was dissolved in 0.5 ml of 70% 0.01 M potassium monophosphate, pH 3.75, and 30% methanol and clarified by centrifugation. The supernatant (15 μl) was injected onto Adsorbosphere phenyl, 4.1 \times 300-mm column, and eluted at a flow rate of 1 ml/min isocratically with 70% 0.01 M potassium phosphate, pH 3.75, and monitored at 368 nm. Run time was 20 min, furazolidone eluting at 10 min and furaltadone at 14.5 min. Recoveries from milk and plasma for all three compounds

exceeded 83%. Standard curves for all three compounds were linear over the range used (0.5–100 $\mu\text{g}/\text{ml}$; $R^2 > 0.995$).

Milk Fractionation

Rat milk was fractionated into a lipid cake, whey and a casein pellet by centrifugation in a swinging bucket rotor (100,000 $\times g$) for 1 hr at 4°C in a polyallomer tube. After centrifugation, the tube was pierced at the lipid/whey interface with an 18-gauge hypodermic needle with the needle bevel facing the whey, and the whey was withdrawn. The tube was cut with a razor blade at the lipid cake and at the casein pellet area, and both tube portions were placed in separate glass tubes. The casein pellet was suspended in 0.01 M sodium hydroxide, and the lipid and whey in water. The final volume of each solution was equal to the starting milk volume. Sonication was used to resuspend the casein and lipid fractions. Volumes of each fraction were passed over an appropriate Sep-pak cartridge and assayed by HPLC.

For ultrafiltration studies, skim milk (produced by removing the lipid cake obtained after a 15-min centrifugation at 1500 $\times g$) and plasma were placed in the top of a Centricon 10 filter and centrifuged at 1500 $\times g$ in an angle-head rotor until sufficient filtrate was obtained for assay. The amount of drug was quantified in starting material, retentate, and filtrate after extraction and assay by the appropriate HPLC method.

Results

Figure 1 shows the concentration of nitrofurantoin in the milk and plasma of lactating rats at various time-points after the administration of 50 mg/kg nitrofurantoin. The drug appeared rapidly in both plasma and milk, reaching measurable levels in the first sample at 7.5 min after dosing and maximal plasma levels at 15 min. The peak value in the plasma was observed at 15 min, that of the milk at 30 min. At all time-points, the concentration of nitrofurantoin in milk exceeded that in plasma, and the computed M/P ratio, obtained by integrating the curves in figure 1, was 23.1. Analogous determinations of plasma and milk values for two structurally related analogs, furazolidone and furaltadone, yielded M/P ratios of 1 and 3.49 (table 1; milk and plasma concentrations not shown).

In an effort to evaluate the M/P ratio after a protracted

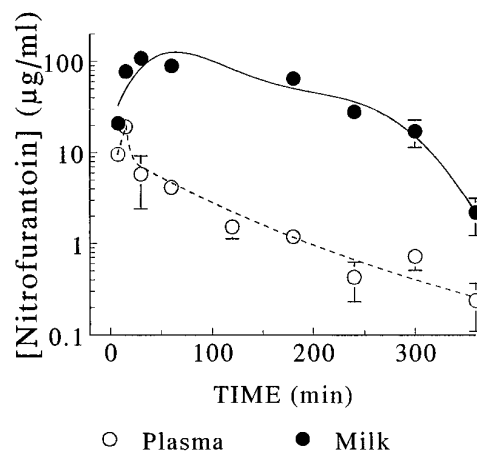
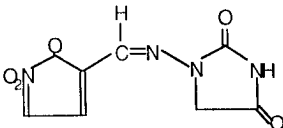
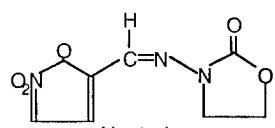
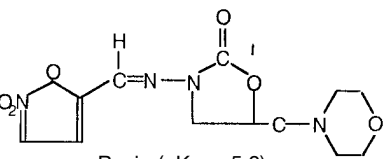


Fig. 1. Time course of nitrofurantoin concentrations in milk and plasma of 10-day lactating rats gavage-fed with nitrofurantoin (50 mg/kg b.wt.) at zero time. At appropriate times, milk and blood samples were collected from anesthetized animals. Pups were removed from dams 3 hr before milk collection to allow milk to accumulate. Each animal was sampled only once. Nitrofurantoin was extracted from milk or plasma and quantitated by HPLC (see "Materials and Methods" for details).

TABLE 1
 Chemical structures, milk and plasma fractionation data and milk/plasma partitioning ratios for three nitrofurans

	Plasma		Milk		Ratio of Skim Milk to Whole Milk	Predicted Milk/Plasma Ratio	Observed Milk/Plasma Ratio	Ratio of Observed to Predicted
	Un-ionized	Unbound	Un-ionized	Unbound				
<p>Nitrofurantoin</p>  <p>Acidic ($pK_a = 7.2$) Solubility $H_2O = 190 \mu g/ml$</p>	0.39	0.43	0.61	0.74	1.19	0.31	23.1	~75
<p>Furazolidone</p>  <p>Neutral Solubility $H_2O = 40 \mu g/ml$</p>	0	0.56	0	0.48	1.21	0.97	1	~1
<p>Furaltadone</p>  <p>Basic ($pK_a = 5.0$) Solubility $H_2O = 753 \mu g/ml$</p>	1	0.86	1	0.56	1.11	1.4	3.49	~2.5

exposure to nitrofurantoin, we fed lactating dams with their natural litters diets containing 2500 ppm nitrofurantoin. Single-time determinations of milk and plasma drug values yielded M/P ratios of 44.6 ± 4.1 (S.E.M.; $n = 5$) after 7 days of feeding and 41.2 ± 5.0 (S.E.M.; $n = 6$) after 14 days. Thus both acute experiments and chronic dosing gave a high M/P ratio for nitrofurantoin in lactating rats.

To examine nitrofurantoin partitioning into the milk fat, we measured the nitrofurantoin concentration in whole milk 3 hr after dosing. The milk was then centrifuged to remove the cream layer, and the nitrofurantoin concentration was measured in the resulting skim milk. The mean concentrations in whole and skim milk were 24.0 ± 0.8 (S.E.M.) and 28.4 ± 0.7 (S.E.M.) $\mu g/ml$, an increase of 18%. Calculating the nitrofurantoin concentration in the nonfat portion of the whole milk using the mean milk fat concentration in the milk of the rats in our laboratory (11.9%), we obtained $26.9 \pm 0.9 \mu g/ml$, a value not significantly different from the concentration actually measured in the skim fraction. Thus all the nitrofurantoin can be accounted for by the skim milk fraction, and accumulation in the milk fat globule cannot account for the observed M/P ratio.

To determine whether nitrofurantoin accumulation in the milk could be attributed to binding to milk proteins, the skim milk from the experiment described in the previous paragraph was subjected to ultrafiltration through a 5-kD molecular weight cutoff filter. The nitrofurantoin concentration in the filtrate was $21.0 \pm 0.1 \mu g/ml$, which indicated that at least 74% of the nitrofurantoin in the milk was free. When plasma was treated similarly, only 43% of the nitrofurantoin

was unbound (table 1). Similar experiments for furazolidone and furaltadone gave the results shown in table 1.

Finally, a difference in pH between milk and plasma can lead to M/P ratios different from unity. The proportion of ionized compound was calculated from the Henderson-Hasselbach equation:

$$\frac{[A^-]}{[HA]} = 10^{pH-pK} \quad (1)$$

where A^- and HA are the ionized and un-ionized forms of the compound. The resulting values for the three compounds are also given in table 1.

To obtain the predicted M/P ratios, we used the equation of Fleishaker and McNamara, (Fleishaker *et al.*, 1987):

$$\frac{M}{P} = \frac{f_p^{free} \times f_p^{un}}{f_m^{free} \times f_m^{un} \times S/W} \quad (2)$$

In equation (2), f_p^{free} and f_m^{free} represent the mean observed fraction of unbound chemical in plasma and milk, respectively, and f_p^{un} and f_m^{un} are the un-ionized chemical in plasma and milk. S/W is the ratio of skim milk to whole milk for the compound. The predicted values based on a plasma pH of 7.4 and a milk pH of 6.8 were 0.31, 1, and 1.4 for nitrofurantoin, furazolidone and furaltadone, respectively. Standard physicochemical principles can therefore explain the concentration of furazolidone in rat milk. However, furaltadone and nitrofurantoin are both accumulated in milk at a concentration higher than predicted. In the case of nitrofurantoin, the ratio

of observed M/P to predicted M/P is about 75, a result that suggests the presence of an active transporter for the drug.

Discussion

In full lactation, the mammary epithelium is a tight epithelium (Peaker, 1983) whose junctional complexes preclude the transfer of substances between the interstitial fluid and the milk space. To be transferred to milk, therefore, a chemical must permeate the basolateral membrane of the mammary epithelial cells, diffuse across the aqueous interior of the cell and permeate the apical cell membrane into the milk space. Because they readily cross plasma membranes, lipid-soluble compounds such as steroid hormones are readily transferred into milk. Further, because they tend to have high oil-water partition coefficients, they dissolve in the lipid of the milk fat globule and remain in the milk (Toddywalla *et al.*, 1995). Nonpolar, water-soluble molecules may also cross the plasma membranes of the mammary epithelial cell. These are thought to be distributed in milk according to well-established principles of chemical equilibria: the equilibrium concentration in milk for several drugs has been shown to depend on the pH of the milk (between 6.5 and 7.0 depending on the species (Neville *et al.*, 1995), on the relative binding to plasma and milk proteins and on the pK of the drug (Oo *et al.*, 1995a); (Fleishaker and McNamara, 1988a; Fleishaker and McNamara, 1988b; Fleishaker *et al.*, 1987). Although the theory makes the possibly unwarranted assumptions that the ionized form of the drug does not cross the mammary epithelium and that, therefore, the potential difference across the mammary epithelium (Berga, 1984; Peaker, 1983) has no effect on the equilibrium concentration in the milk, the agreement between prediction and experiment for these several drugs suggests that it is often valid.

The actual concentration of a chemical achieved in milk depends on the rate of transfer across the epithelium and the half-life of the substance in the plasma. Chemicals with a short plasma half-life may not have time to equilibrate across the mammary epithelium, and the milk concentrations may thus be low compared with plasma concentrations. It was important to take these considerations into account in designing an experiment to examine accumulation of nitrofurantoin in rat milk.

By examining the concentrations of nitrofurantoin in milk and plasma after gavage dosing in rats that were milked and bled only once, we found that the rat mammary epithelium maintains a M/P ratio of about 23 over a considerable time. Negligible nitrofurantoin was found in the milk fat. Taking the pH and protein binding in plasma and milk into account, the ratio of observed M/P to predicted M/P for free nitrofurantoin is about 75. This is incontrovertible *in vivo* evidence that nitrofurantoin is actively transported across the rat mammary epithelium. Although careful studies conducted in women are not available, there is evidence that the M/P ratio is greater than 1 in humans as well (Pons *et al.*, 1990; Varsano, 1973).

Definitive evidence for active transport of a drug into human milk has been obtained in only one study, in which the M/P ratio for cimetidine was 5.5 (Oo *et al.*, 1995b). This drug had previously been found to be accumulated in rat milk to a M/P ratio of 30 (Dostal, 1990). It is not clear whether the ratio found in this study for rat milk represents a particularly

active system in the rat or whether an appropriate time study would reveal a higher M/P ratio in women. Nitrofurantoin is an inexpensive antibiotic that is often used in developing countries where women cannot easily give up breast feeding. Because it is mutagenic and carcinogenic in animal models (NTP, 1989), additional studies in women are urgently needed.

There is currently no evidence on the mechanism of transport. In the kidney, nitrofurantoin is excreted by the renal organic anion transporter (Møller and Sheikh, 1983). This transporter has not been identified in the mammary epithelium, but if it is present, it is likely to show broad specificity and to be present in the basal membrane, where it would function by increasing the activity of nitrofurantoin in the cells (Pritchard and Miller, 1992). The fact that a derivative of *p*-aminohippuric acid, a model substrate for this transporter (Rasmussen, 1969a), has been found at concentrations higher than predicted by passive transport would be consistent with the presence of a organic anion transporter in the basal membrane of the mammary alveolar cell. Another drug transporter, *p*-glycoprotein or the multidrug-resistance transporter, has been shown to be present in mammary cells both normal and neoplastic (van der Valk *et al.*, 1990). This transporter has been implicated mainly in the transport of cationic drugs out of cells (Thalhammer *et al.*, 1994; Lampidis *et al.*, 1989) and is present on the luminal membrane of normal epithelial cells (Stewart and Gorman, 1991) and the bile canalicular membrane of hepatocytes (Thalhammer *et al.*, 1994). Although a transporter that operated to extrude nitrofurantoin across the apical membrane could also produce the results observed in these rats, nitrofurantoin bears a net negative charge at pH 7.4 and therefore seems less likely to be a substrate for the multidrug-resistance transporter. *In vitro* studies will be necessary to determine whether either of these candidate transporters is, in fact, responsible for the observed transport of nitrofurantoin. Fortunately, we have identified a mammary cell line that transports nitrofurantoin against a concentration gradient *in vitro* (Toddywalla *et al.*, 1997). Studies are currently in progress to characterize the transport properties of this cell line in some detail.

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