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SHORT COMMUNICATIONS

Investigation of the mechanisms of the extensive excretion of cimetidine into rat milk

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Cimetidine is one of the most commonly prescribed H_2-receptor antagonists for the treatment of peptic ulcers. The Henderson–Hasselbach equation predicts that for this water-soluble base, with a pK of 7.1, and pH levels of 6.8 and 7.4 in rat milk and plasma, respectively, the milk/plasma ratio (M/P) should be 2.0. However, in a previous study we found the M/P for cimetidine in lactating rats to be 24–31.* This result was consistent with a study showing M/P ratios of 4–12 in a human mother following oral doses of cimetidine [1]. The elevated M/P for cimetidine in rats was not due to differences in the rates of elimination from milk and plasma since the M/P was greater than 2.0 even at times preceding the plasma peak, and the ratio of the peak concentrations in milk and plasma was also greater than 2.0.* In addition to lipid solubility, ionization, and pharmacokinetic considerations, drug excretion into breast milk is also influenced by protein binding [2–4]. Cimetidine is only minimally bound to plasma proteins (18–26% [5]), but the extent of protein binding in milk has not been determined. In addition, Somogyi and Gugler [1] suggested that "active transport" of cimetidine by the mammary gland could be responsible for the high M/P for cimetidine. Therefore, the following studies were done to investigate the role of protein binding, active transport by the mammary gland, and pharmacological effects of cimetidine in producing the high M/P for cimetidine in rats.

Materials and methods

Cimetidine, *N''*-cyano-*N'*-{2-[(5-methylimidazol-4-yl)methylthio]ethyl}guanidine, was obtained from the Sigma Chemical Co. (St Louis, MO) and was >99% pure as determined by HPLC and infrared spectroscopy. [*N*-methyl-^{3}H]Cimetidine was obtained from the Amersham Corp. (Arlington Heights, IL) (sp. act. 10.1 Ci/mmol) and was 93–98% radiochemically pure as determined by thin-layer chromatography. Insulin (bovine pancreas) and corticosterone were obtained from the Sigma Chemical Co. Rat prolactin was a gift from Dr Salvatore Raiti, the National Hormone and Pituitary Program, and the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK).

Sprague–Dawley (CD) rats with natural litters were obtained from the Charles River Breeding Laboratories (Raleigh, NC). Rats were housed under controlled conditions (22 ± 2°, 50 ± 20% humidity) with 12-hr light/dark cycles in clear polycarbonate cages with chipped hardwood bedding. The NIH-30 diet [6] and deionized water were provided *ad lib*. Milk was collected manually from lactating rats anesthetized with 100 mg/kg ketamine and 32 mg/kg xylazine and given 1 I.U. oxytocin. Blood was collected by cardiac puncture using heparinized plastic syringes, and radioactivity was determined in 0.5-ml aliquots of plasma. Milk fractions were prepared by centrifuging whole rat milk at 100,000 *g* for 1 hr at 4°. The three phases [top fat globule layer, whey (infranatant), and casein pellet] were separated, the fat and casein layers were digested in NCS tissue solubilizer (Amersham Corp.), and the radioactivity in each phase was determined. To produce skim milk, 1 ml of whole milk was centrifuged for 15 min at 2000 *g*, 4°, and the lower skim milk fraction was collected.

Protein binding was determined by ultrafiltration using the MPS-1 Micropartition System (Amicon Corp., Danvers, MA) with YMT membranes (30,000 mol. wt cutoff) for serum and YM-10 membranes (10,000 mol. wt cutoff) for skim milk and whey. YM-10 membranes have been shown previously to retain >95% of milk proteins [7]. Samples (0.5 to 0.8 ml) were incubated at 37° for 30 min with 10^6 dpm [^{3}H]cimetidine, 50–75 μl 50 mM HEPES† (pH 7.4 for serum, pH 6.8 for milk fractions), and cimetidine at various concentrations. Two aliquots were removed for counting, and the remaining sample was transferred into MPS-1 devices and capped. The samples were further incubated in a prewarmed angle head rotor for 30 min and centrifuged at 1800 *g* for 20–30 min to produce a clear ultrafiltrate.

Transport studies. The right abdominal-inguinal mammary gland and both kidneys were quickly removed from carbon dioxide-anesthetized rats on days 10–15 of lactation and placed in cold, modified Krebs buffer containing 25 mM HEPES, 5.6 mM dextrose, 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4, pH 7.4. Slices of mammary gland (1 mm) and kidney (0.5 mm) were prepared on a McIlwain tissue chopper and placed in 5 ml of one of three media: the modified Krebs buffer, Medium 199 (Gibco Laboratories, Grand Island, NY), or potassium-lactose (K-lactose) buffer [8]. Slices were preincubated with or without inhibitors and hormones at 37° in a shaking water bath for 60 min with one change of buffer at 30 min. Incubations with Medium 199 or potassium-lactose buffer were carried out under 95% O_2:5% CO_2. To start the reaction, 0.11 μCi [^{3}H]cimetidine (1 μM cimetidine) was added in 20 μl of 25 mM HEPES. At various times, 2–3 slices were removed from each flask, blotted on filter paper, and weighed. Tissue slices were digested in 1 ml NCS at 50°, and the radioactivity content was determined. Aliquots (200 μl) of the incubation medium were removed for counting at each time point.

Representative slices of kidney and mammary gland were weighed immediately after slicing and dried at 100° to constant weight to determine the percent tissue water (%TW). The extracellular water (%ECW = percent of total tissue water which is extracellular) was determined by incubating slices with 0.2 μCi [^{3}H]inulin for 60 min, which gave steady-state levels of [^{3}H]inulin in the tissue (data not shown). The percent intracellular water (%ICW) was calculated using the following equation: 100% − %ECW = %ICW. The concentration of [^{3}H]cimetidine in the intracellular water was then determined by the following

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† HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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equation

$$\text{dpm/ml ICW} = \frac{(\text{dpm/g wet wt} \times \%\text{TW}) - (\text{dpm/ml medium} \times \%\text{ECW})}{\%\text{ICW}}.$$

Finally, the slice/medium ratio (S/M), representing the intracellular uptake of [^{3}H]cimetidine, was determined as follows:

$$\text{S/M} = \frac{\text{dpm/ml ICW}}{\text{dpm/ml medium}}.$$

Results and discussion

The physicochemical properties of a drug (i.e. lipid solubility, the degree of ionization, and the extent of protein binding) are reported to be the most important determinants of the amount of drug secreted into the milk [3, 4, 9]. Cimetidine is a weak base with low lipid solubility, log octanol:water = 0.40 [10]. In the present study, a skim/whole milk concentration ratio of 1.14 ± 0.01 (mean ± SE, N = 8) for [^{3}H]cimetidine equilibrated with rat milk *in vitro* was consistent with the low lipid solubility of cimetidine. A further demonstration of the minimal affinity of cimetidine for milk fat is indicated by the small amount of [^{3}H]cimetidine present in the fat globule layer when whole rat milk was equilibrated with [^{3}H]cimetidine *in vitro* or *in vivo* (Table 1). Since [^{3}H]cimetidine was primarily distributed in the aqueous whey fraction (62–72%) and in the casein pellet, both of which are protein rich, the extent of protein binding in milk, as well as serum, was determined. Consistent with the literature [5], plasma protein binding of cimetidine was very low (6–8%; Table 2). In addition, only small amounts of cimetidine were protein bound in skim milk (whole milk with the fat removed; 22%) and milk whey (6%). To predict the M/P for drugs, Fleishaker *et al.* [4] have derived an equation which takes into account the pH partitioning, the extent of protein binding in milk and plasma, and the fat solubility of the drug. Substituting in their equation the skim/whole milk ratio of 1.14 and the plasma and skim milk protein binding of 7.9% and 23%, respectively (Table 2), a theoretical M/P of 2.06 was obtained (assuming pH 6.9 in milk and pH 7.6 in plasma, as determined in control rats). This ratio was virtually the same as the ratio predicted by pH partitioning alone (2.0), and was obviously much lower than the ratio observed *in vivo* (i.e. 24–31). To obtain a M/P of 24, the Fleishaker equation predicts that milk protein binding would have to be >95%, whereas actual milk protein binding was much less. These results suggest that high protein binding in milk does not account for the high M/P observed *in vivo*. Since the physicochemical properties of cimetidine did not account for the high M/P for cimetidine, mammary slices were incubated *in vitro* with [^{3}H]cimetidine to determine if an active transport system for cimetidine was present in the mammary gland. Cimetidine has been shown to be secreted by the organic ion transport systems in renal proximal tubules [11–14]; therefore, kidney slices were incubated under the same conditions as a positive control. The total tissue water contents of rat mammary gland and kidney slices were 74.1 ± 0.7 and 79.2 ± 0.7% of wet wt respectively (mean ± SE, N = 6–8). The %ECW was 76.7 ± 0.8% in mammary gland slices and 22.0 ± 2.4% in kidney slices. Therefore, the intracellular fluid as a fraction of tissue wet weight was 0.17 ± 0.01 mg/g wet weight for mammary gland and 0.62 ± 0.02 mg/g wet weight for kidney. These values agree well with values obtained in bovine and mouse mammary tissue [15, 16] and in rat kidney slices [17]. The low S/M ratios for cimetidine in mammary slices incubated in Krebs buffer were not affected by the presence of the metabolic inhibitors, cyanide or arsenic, in the incubation medium, or by the combination of these inhibitors in the absence of glucose (Table 3). However, kidney slices incubated under the same conditions had S/M ratios of 8–9, and the ratios were reduced significantly ($P < 0.05$) by the organic cation tetraethylammonium (38%), which has been shown to compete with cimetidine uptake in renal brush border and basolateral membrane vesicles [13]. Cimetidine uptake into kidney slices was also inhibited by cyanide (31%), arsenic (32%), and cyanide plus arsenic in the absence of glucose (44%). Since there was no evidence of active uptake into the mammary slices incubated in the Krebs buffer, slices were also incubated in Medium 199 which has been used to demonstrate cationic amino acid transport in bovine mammary tissue [16] and in potassium-lactose medium, which has an ionic composition similar to milk and has been shown to maintain normal intracellular ion concentrations in isolated mammary cells [8]. Attempting to optimize the incubation conditions further, the hormones insulin, corticosterone, and prolactin were added at concentrations previously used to culture mammary epithelial cells [18]. However, even with the addition of these hormones, there was no evidence of accumulation of [^{3}H]cimetidine against a concentration gradient in the mammary slices.

Because the intracellular space in the mammary gland was only 17% of the total wet tissue weight, it would be more difficult to detect an increase in the intracellular [^{3}H]cimetidine concentration in this tissue than in the kidney in which 62% of the wet weight is intracellular fluid. Furthermore, if the transport of cimetidine was equally efficient into and out of the mammary cell, accumulation within the mammary slices might not be detectable. It is possible that investigations using intact mammary gland preparations under the normal hormonal conditions of lactation may allow the demonstration of active transport.

Table 1. Distribution of [^{3}H]cimetidine in rat milk fractions

Milk fraction	Percent of total radioactivity	
	In vitro	*In vivo*
Fat globule	3.5 ± 1.2	6.5, 5.3
Whey	72.1 ± 1.1	62, 64
Casein	24.4 ± 1.1	31, 31

In vitro values are from whole milk equilibrated with 0.04 μCi/ml of [^{3}H]cimetidine and 20 μg/ml cimetidine for 1 hr at 37° and are means ± SE, N = 8. *In vivo* values are from individual rats (day 17 of lactation) given 10 μCi of [^{3}H]cimetidine (9 mg/kg) 4 hr prior to milking. The total radioactivity ranged from 98,000–186,000 dpm in the *in vitro* samples, and was 64,000 and 85,000 dpm in the *in vivo* samples.

Table 2. Protein binding of [^{3}H]cimetidine in rat serum and milk

	Cimetidine (μg/ml)	% Protein bound
Serum	0.8	7.9 ± 0.9
	4.0	6.6 ± 0.5
Skim milk	15	23.1 ± 0.7
	115	20.9 ± 0.8
Milk whey	15	6.9 ± 1.6
	115	5.0 ± 1.1

Cimetidine was added to serum and milk fractions at concentrations equivalent to those found in rat serum and milk 4 hr after the fourth daily dose of 18 or 180 mg/kg/day cimetidine, and was equilibrated along with [^{3}H]cimetidine for 1 hr. Values are means ± SE, N = 4–6.

Table 3. Uptake of [^{3}H]cimetidine into rat mammary gland and kidney slices *in vitro*

	S/M ratio	
	15 min	60 min
Mammary gland		
Krebs buffer	1.49 ± 0.04	1.62 ± 0.04
+ cyanide (0.01 M)	1.57 ± 0.04	1.69 ± 0.03
+ arsenic (0.1 M)	1.33 ± 0.06	1.58 ± 0.04
+ cyanide, arsenic without glucose	1.43 ± 0.05	1.67 ± 0.04
Medium 199	1.61	1.96
+ insulin, corticosterone, and prolactin	1.15	1.58
Potassium-Lactose medium	1.58	2.26
+ insulin, corticosterone, and prolactin	1.14	1.56
Kidney		
Krebs buffer	8.25 ± 0.49	9.00 ± 0.30
+ tetraethylammonium bromide (0.01 M)	5.22 ± 0.16*	5.56 ± 0.15*
+ cyanide (0.01 M)	5.99 ± 0.39*	6.19 ± 0.18*
+ arsenic (0.1 M)	5.59 ± 0.49*	6.13 ± 0.32*
+ cyanide, arsenic without glucose	4.83 ± 0.46*	5.04 ± 0.15*

Mammary slices (1 μm) or kidney slices (0.5 μm) were incubated with [^{3}H]cimetidine in medium ± inhibitors, ± hormones, and the intracellular concentration in the slice relative to the concentration in the medium (S/M) was determined. The concentrations of hormones were insulin, 5 μg/ml, corticosterone, 5 μg/ml, and prolactin, 10 μg/ml. Values are the means ± SE for N = 5 rats. Individual values are the average of duplicate incubations from one rat.

* Significantly different from the corresponding control; $P < 0.05$ (unpaired *t*-test).

Alternatively, cimetidine transport may be observable across intact monolayers of cultured mammary cells. These and other mechanisms remain to be investigated.

To investigate the possibility that a pharmacological effect of cimetidine was involved in producing the elevated M/P for cimetidine, a dose of 20–22 μCi/kg [^{3}H]cimetidine with no added unlabeled cimetidine was given. This dose of [^{3}H]cimetidine resulted in a cimetidine dose of only 0.22 μg/kg which presumably would have no pharmacological effects. The M/P 4 hr after dosing was 26.7 ± 3.8 (mean ± SE, N = 4) compared with a ratio of 25.9 ± 2.7 (N = 7) in rats which were given the pharmacological dose, 9 mg/kg cimetidine. This suggests that other properties of cimetidine, such as its ability to bind to androgen receptors [19], its antihistamine actions, or its hyperprolactinemic effect [20, 21], are not responsible for the efficient excretion of cimetidine into milk. Unpublished results from our laboratory showed no elevation in plasma prolactin levels 20 or 45 min after an oral dose of 9 mg/kg cimetidine in lactating rats, but an increase occurred 20 but not 45 min after a dose of 90 mg/kg. These results give further evidence against a role of prolactin in producing the high M/P for cimetidine. However, it is still possible that high levels of prolactin, or other hormones such as glucocorticoids or insulin which are normally associated with lactation, may be involved in the excretion of high concentrations of cimetidine in milk.

In summary, the present studies demonstrated that the extensive excretion of cimetidine into rat milk is not due to physicochemical properties such as high protein binding in milk, lipid solubility, or its distribution at ionic equilibrium. Although there was no evidence of active transport of cimetidine in mammary slices *in vitro*, further experiments performed under *in vivo* conditions or in cell cultures may reveal an active uptake process for cimetidine in the mammary gland. Thus, the mechanism of the high M/P for cimetidine is still not clear.

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Occurrence of 'natural' diazepam in human brain

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There is accumulating evidence that benzodiazepines (BZD), especially diazepam (D) and desmethyldiazepam, are naturally present in tissues of different animal species [1–3] as well as in plant products [2–4]. 'BZD-like' immunoreactivity has been detected even in human brain samples stored in paraffin since 1940 [1]. However in this experiment the material was not identified by specific chemical analysis. As the source of the BZD detected in the different biological samples has not been clarified, it might be speculated that it is of synthetic origin [5]. Therefore we have examined human brain material which has been kept in storage since at least 1959, a time prior to the first laboratory synthesis of these compounds. BZD were quantified by a specific gas chromatography (GC)/mass spectrometry (MS) technique in different parts of the stored brains.

Materials and methods

Slices of cerebrum, cerebellum and brainstem from three different human brains, which were stored in 10% formaldehyde solution, were examined.

Brain No. 1 was from a 31-year-old subject, whose death was due to an amyotrophic lateral sclerosis in February 1955. Brain No. 2 was obtained from a 27-year-old male schizophrenic patient whose death in July 1958 was attributable to an acute circulatory collapse. Brain No. 3 was taken from a 36-year-old male epileptic patient who died of pulmonary embolism in February 1959.

Slices of different areas of brains No. 1 and 2 were stored separately in formaldehyde solution. The individual tissue slices were homogenized thoroughly in this solution using an ultraturrax mixer for 1 min prior to extraction. Tissues of brain No. 3 were stored together in formaldehyde solution and were separated prior to analysis. Slices of cerebellum and brainstem were removed from the solution and homogenized in distilled water. One section of the cerebrum was homogenized in distilled water, whereas the remainder was homogenized in the original formaldehyde solution. In addition, the formaldehyde solution was analysed separately to examine whether BZD had been extracted during storage. A sample from each part of each brain (7.5 to 10 g wet weight tissue) was analysed at least in duplicate.

Deuterated D (d_5-D, Sigma Chemical Co., Poole, U.K., 5 ng) was added to the homogenized samples as the internal standard. The biological samples were extracted and subsequently purified by HPLC according to the method described in detail recently [3]. The HPLC fraction, collected at a retention time corresponding to that analysed for D was quantified by GC/MS in negative ion chemical ionization mode (for details see Ref. 3). Concentrations of D were calculated from the peak height ratios of D to d_5-D recorded from the tracings in the selected ion monitoring (see Fig. 1).

To test the stability of D under the influence of formaldehyde, a 10% formaldehyde solution was spiked with aliquots of 100 ng D and stored at 80° for one week to simulate storage at 10°–15° for over 30 years.

Results and discussion

All brain material examined by GC/MS contained D. The concentrations observed ranged from 0.15 to 0.34 ng/g wet weight tissue (see Table 1).

Controls of 20 ml distilled water were processed through the whole work-up procedure and contained maximally 0.005 ng/ml D. The original solution in which tissues of brain No. 3 were stored contained a concentration of