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The binding of orally dosed hydrophobic active pharmaceutical ingredients to casein micelles in milk

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ABSTRACT

Casein proteins (α_{S1}-, α_{S2}-, β- and κ-casein) account for 80% of the total protein content in bovine milk and form casein micelles ($\bar{D}_{11}$ = 130 nm, approximately 10^{15} micelles/mL). The affinity of native casein micelles with the 3 hydrophobic active pharmaceutical ingredients (API), meloxicam [351.4 g/mol; log P = 3.43; acid dissociation constant (pKa) = 4.08], flunixin (296.2 g/mol; log P = 4.1; pKa = 5.82), and thiabendazole (201.2 g/mol; log P = 2.92; pKa = 4.64), was evaluated in bovine milk collected from dosed Holstein cows. Native casein micelles were separated from raw bovine milk by mild techniques such as ultracentrifugation, diafiltration, isoelectric point precipitation (pH 4.6), and size exclusion chromatography. Acetonitrile extraction of hydrophobic API was then done, followed by quantification using HPLC-UV. For the API or metabolites meloxicam, 5-hyroxy flunixin and 5-hydroxy thiabendazole, 31 ± 3.90, 31 ± 1.3, and 28 ± 0.5% of the content in milk was associated with casein micelles, respectively. Less than ∼5.0% of the recovered hydrophobic API were found in the milk fat fraction, and the remaining ∼65% were associated with the whey/serum fraction. A separate in vitro study showed that 66 ± 6.4% of meloxicam, 29 ± 0.58% of flunixin, 34 ± 0.21% of the metabolite 5-hyroxy flunixin, 50 ± 4.5% of thiabendazole, and 33 ± 3.8% of metabolite 5-hydroxy thiabendazole was found partitioned into casein micelles. Our study supports the hypothesis that casein micelles are native carriers for hydrophobic compounds in bovine milk.

Key words: casein micelle, hydrophobic, meloxicam, flunixin, thiabendazole

INTRODUCTION

Bovine milk proteins are composed of 80% casein proteins (α_{S1}-, α_{S2}-, β-, and κ-CN) and 20% whey proteins (mainly β-LG, α-LA, and BSA). Casein proteins exist in the form of colloidal particles of ∼130 nm average diameter, known as casein micelles. Although controversy remains on the casein micelle nanostructure, it is known that calcium phosphate nanoclusters bind to phosphoseryl residues of α_S- and β-CN, forming the internal core of the micelles, and κ-CN predominantly exist at the surface, providing steric and electrostatic stability against micellar aggregation (Dalgleish, 2011; de Kruif et al., 2012). The agreed biological role of casein micelles is to transport calcium from mother to young, prevent calcification of the mammary gland, and provide AA for the growth and development of the neonate (Fox and Brodkorb, 2008; Holt et al., 2013). Environmental factors, including pH, solvent, and pressure, were explored extensively to dissociate and reassemble casein micelles to bind and deliver various hydrophobic (poorly water-soluble) nutraceuticals. These modified casein micelles were used to nonspecifically bind vitamin D_2, curcumin, and ritonavir, among others (Semo et al., 2007; Pan et al., 2014; Corzo-Martínez et al., 2015).

Whereas several reports demonstrated that the 2 major whey proteins (β-LG and α-LA) have binding sites for hydrophobic molecules (e.g., vitamin A, D, palmitic acid; Puyol et al., 1991; Forrest et al., 2005), the native binding properties of the casein micelles are not well characterized. The casein micelles have an open structure with serum filled channels and cavities accessible by small molecules (Dalgleish, 2011; Trejo et al., 2011). The reversible dissociation and association of β-CN on temperature fluctuations provides evidence for the accessibility of the internal core and potential for sites with affinity toward hydrophobic probes (Dalgleish, 2011; Atamer et al., 2017). A recent report demonstrated that native casein micelles isolated from raw milk exhibited a stronger binding affinity toward hydrophobic compounds (MW <900 Da, log P >4) versus hydrophilic compounds (Cheema et al., 2015). These hydrophobic compounds were identified as phospholipids, including sphingomyelins, phosphatidylcholines, and phosphatidylethanolamines. A separate study conducted on casein micelles from commercial

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ultrapasteurized milk showed that up to 40% vitamin A added to fortify milk eventually bound to casein micelles (Mohan et al., 2013).

Although fat-soluble compounds appearing in milk are believed to associate with the fat fraction, we hypothesized that the open structure of native casein micelles provides a better environment for the binding and transportation for hydrophobic molecules present in mammalian milk. Very few studies have been conducted to determine the distribution of hydrophobic active pharmaceutical ingredients (**API**; e.g., antibiotics, nonsteroidal anti-inflammatory drugs, anthelmintics) in cow milk components (Cerkvenik et al., 2004; Hakk et al., 2016; Shappell et al., 2017) , and no studies have examined the role of native casein micelles. Furthermore, the studies that have been completed were done by spiking pasteurized milk samples in vitro, resulting in a lack of information regarding in vivo distribution of the parent API and their major metabolites.

The main objective of the current study was to determine the binding ability of casein micelles in their native state using hydrophobic API appearing in bovine milk from dosed animals. As proof of concept, the partitioning of model hydrophobic API into native casein micelles was quantified using weakly acidic meloxicam, flunixin, and weakly basic thiabendazole. Meloxicam and flunixin are commonly used nonsteroidal anti-inflammatory API and are known to appear in milk of dosed cows (Jedziniak et al., 2009a; Kissell et al., 2012); thiabendazole is an anthelmintic API used for deworming affected cows (Su et al., 2003; Chen et al., 2010). Casein micelles are the major proteins in bovine milk and the building blocks of dairy foods, including cheese and yogurt. Understanding the role played by the casein micelles in binding hydrophobic API appearing in dosed animals would be helpful to limit consumer exposure. The binding ability of native casein micelles can potentially be used to protect and deliver hydrophobic nutraceuticals in dairy foods.

MATERIALS AND METHODS

This study was approved by The Pennsylvania State University (University Park, PA) Institutional Animal Care and Use Committees.

Criteria for Selection of API

The API were selected based on (1) approval for use in dairy animals; (2) withdrawal time in milk in API-dosed cows; (3) range of hydrophobicity (log P = 2.92–4.1) to understand the effect of hydrophobicity on the partition to casein micelles. The information regarding approved API and their withdrawal times in bovine milk was obtained from Food Animal Residue Avoidance Databank (2016).

The model API were selected based on their hydrophobicity, defined by log P values (P being partition in organic or aqueous phase); however, certain classes of API are ionizable at milk physiological pH 6.8, and the hydrophobicity of such API is better characterized by log D values instead of log P values. The log D at specific pH can be calculated by using the log P and acid dissociation constant (**pKa**) values for the API in following equations:

$$\log D_{acid} = \log P + \log[1/(1 + 10^{pH - pKa})], \quad [1]$$ and

$$\log D_{base} = \log P + \log[1/(1 + 10^{pKa - pH})], \quad [2]$$

The log P and pKa values for the API were obtained from PubChem Database (https://pubchem.ncbi.nlm.nih.gov) and pKa values were obtained from Drug Bank database (http://www.drugbank.ca/) and using appropriate software (ChemAxon, Budapest, Hungary).

Milk Sample Collection and Preparation

In Vivo Study. Three Holstein cows in midlactation were used for meloxicam, 3 separate cows were used for flunixin and 2 cows were used for thiabendazole in vivo treatments. The average BW of the cows treated with meloxicam, flunixin and thiabendazole was 665 ± 8, 572 ± 40, and 614 ± 55 kg, respectively. All the cows were fed on same TMR diet. Raw milk samples containing meloxicam residues were collected from cows (3 replications) from The Pennsylvania State University Research and Teaching Dairy Center (University Park) at 12-h intervals that received a single permissible oral dose of 1 mg/kg of BW of meloxicam (Table 1; Unichem Laboratories Ltd., Mumbai, India). Preliminary analysis conducted in our laboratory showed maximum concentration at 12 h for meloxicam, 36 h for flunixin, and 12 h for thiabendazole. Raw milk containing the metabolite 5-hydroxy flunixin was collected from Holstein cows (3 replications) treated with permissible intravenous doses of 2.2 mg/kg of BW of flunixin meglumine (Table 1; Banamine, Merck Animal Health, Madison, NJ) for 3 consecutive days. The milk was collected at 36 h after the last administered dose of flunixin. Raw milk samples containing metabolite 5-hydroxy thiabendazole were collected from Holstein cows (2 replications) 12 h after a single oral dose of 67 mg/kg of BW of thiabendazole (Table 1; Dorneyville Compounding Pharmacy, Allentown, PA). The raw milk samples were collected directly from each cow, transported on ice,