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 (average diameter = 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 (pK_a) = 4.08], flunixin (296.2 g/mol; log P = 4.1; pK_a = 5.82), and thiabendazole (201.2 g/mol; log P = 2.92; pK_a = 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-hydroxy flunixin and 5-hydroxy thiabendazole, 31 ± 3.90 , 31 ± 1.3 , and $28 \pm 0.5\%$ of the content in milk was associated with casein micelles, respectively. Less than $\sim 5.0\%$ of the recovered hydrophobic API were found in the milk fat fraction, and the remaining $\sim 65\%$ were associated with the whey/serum fraction. A separate in vitro study showed that $66 \pm 6.4\%$ of meloxicam, $29 \pm 0.58\%$ of flunixin, $34 \pm 0.21\%$ of the metabolite 5-hydroxy flunixin, $50 \pm 4.5\%$ of thiabendazole, and $33 \pm 3.8\%$ 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 pro-

teins (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 phosphoserine 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 (Dalglish, 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 Brodtkorb, 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 D₂, 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 (Dalglish, 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 (Dalglish, 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 ($\log P = 2.92\text{--}4.1$) 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 (pK_a) values for the API in following equations:

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

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

The $\log P$ and pK_a values for the API were obtained from PubChem Database (<https://pubchem.ncbi.nlm.nih.gov>) and pK_a 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,

Table 1. Withdrawal times and physicochemical properties of hydrophobic API¹

Model hydrophobic API	Class of API	Molecular weight (Da)	Solubility (mg/L)	Log P ²	pK _a ³	Log D ^(4,5) (pH 6.8)	Withdrawal time ⁶ (h)
Meloxicam	Weakly acidic	351.4	7.15	3.43	4.08 (acidic)	0.71	96
Thiabendazole	Weakly basic	201.2	50	2.92	4.64 (basic)	2.92	96
5-Hydroxy thiabendazole (5OH-TBZ)	Weakly basic	217.2	—	2.1	—	2.06	96
Flunixin	Weakly acidic	296.2	0.85	4.1	5.82 (acidic)	3.08	36
5-Hydroxy flunixin (5OH-FLU)	Weakly acidic	312.2	—	3.7	—	1.93	36

¹API = active pharmaceutical ingredient.

²Log P-values were accessed from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) on January 26, 2015.

³pK_a values were accessed from Drug Bank (<https://www.drugbank.ca/>) on January 26, 2015.

⁴Log D values were calculated using log P and pK_a values (see above).

⁵Log D values were predicted using ChemAxon software (<https://www.chemaxon.com/>; ChemAxon, Budapest, Hungary).

⁶Withdrawal times are obtained from FARAD website (<http://www.farad.org/>), accessed on Aug. 20, 2014.

and stored at 4°C. The fat was removed from the raw milk by centrifugation at 20°C ($6,414 \times g$ for 20 min) followed by storage at −20°C until analysis. All the analyses were performed within 48 h of sample collection. Four different techniques were used to separate casein micelles from raw milk—ultracentrifugation, size exclusion chromatography, diafiltration/ultrafiltration, and isoelectric point precipitation of caseins—with the exception of size exclusion chromatography not used for the determination of flunixin and thiabendazole.

In Vitro Study. The whole raw milk obtained from cows in midlactation was fortified with 5 hydrophobic API (300 µg/L) with partition coefficient of log P = 3.43 for meloxicam, log P = 4.1 for flunixin and log P = 3.7 for its metabolite 5-hydroxyflunixin (**5-OH-FLU**), and log P = 2.92 for thiabendazole and log P = 2.1 for its metabolite 5-hydroxythiabendazole (**5-OH-TBZ**; Table 1). The concentration was arbitrarily set below the solubility limit of these API. Milk for fortification was obtained from the same cows used in the in vivo studies. The samples were spiked with the API and stirred at 25°C for 1 h.

Ultracentrifugation

The whole raw milk (20 mL/centrifuge tube) was subjected to ultracentrifugation ($100,000 \times g$ for 1 h; Sorvall MTX 150, S50-A 2036, Thermo-Fisher Scientific, Waltham, MA) at 20°C to separate 3 fractions: fat, whey (serum), and casein pellet. The fractions were weighed and stored at −20°C until analysis.

Size Exclusion Chromatography

The raw skim milk was subjected to size exclusion chromatography to separate casein micelles using a gel filtration column (Superdex 200 prep grade, GE

Life Sciences, Piscataway, NJ) connected to an AKTA fast-performance liquid chromatography unit (GE Life Sciences) using a previously reported method (Cheema et al., 2015). Briefly, protein-free serum from the same animal was used as the mobile phase in size exclusion chromatography to ensure a native environment for the casein micelles. The protein-free serum was prepared by tangential flow cross-filtration of skim milk using a regenerated cellulose membrane with a 3-kDa molecular weight cut off (PLBC Prep scale TFF Cartridge, Millipore, Billerica, MA). The protein-free serum was preserved in 0.07% NaN₃ to prevent microbial growth and stored at 4°C until size exclusion chromatography. Two milliliters of raw skim milk was loaded onto the column and proteins were eluted using a flow rate of 1.0 mL/min for 2 column volumes (240 mL) and collected in 30-mL fractions. The elution of proteins was monitored using a UV absorbance detector set at 280 nm. A total of 4 replications were done for each sample. The casein micelle fractions were collected, freeze-dried, and stored at −20°C for the further analysis of the API by HPLC.

Diafiltration

Fifty milliliters of raw skim milk was subjected to diafiltration using a composite regenerated cellulose membrane with a 300-kDa molecular weight cut off (Pellicon XL cassette, Millipore) connected to a peristaltic pump to obtain a casein micelle concentrate. Protein-free serum was used to wash the casein micelles in their native environment. Diafiltration was performed by subjecting the raw skim milk to 6 cycles of removing and replacing the protein-free serum to ensure the removal of low-molecular-weight residues in the serum phase of skim milk and retain those associated with the casein micelles. After exhaustive diafiltration, the

concentrated casein micelles (retentate) were collected and stored at -20°C for further analysis of API residue concentration.

Isoelectric Point Precipitation

The casein micelles were precipitated at the isoelectric point by adjusting the pH of 20 mL of raw skim milk to 4.6 using HCl. The precipitates were washed and centrifuged at $1,000 \times g$ for 3 min at 20°C for 6 replicates. The casein micelle precipitates were stored at -20°C for further API residue analysis.

Standards and Chemicals

Standards of meloxicam, flunixin, thiabendazole, and 5-OH-TBZ were purchased from Sigma-Aldrich (St. Louis, MO) and standard 5-OH-FLU was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Acetonitrile (HPLC grade), MeOH (HPLC grade), acetic acid (HPLC grade), ammonium acetate ($\text{CH}_3\text{COONH}_4$, HPLC grade), and NaCl were purchased from VWR international (Bridgeport, NJ).

Standard Solutions and Calibrations

Stock-standard solutions with a $1,000 \mu\text{g/mL}$ concentration of meloxicam, 5-OH-FLU, and 5-OH-TBZ were prepared by dissolving 10 mg of analytical standard in 10 mL of methanol. The intermediate-standard solutions ($100 \mu\text{g/mL}$), were prepared by diluting suitable aliquots of the stock-standard solutions, which were further diluted to prepare working-standard solutions ($10 \mu\text{g/mL}$). The blank milk samples were fortified with suitable amounts of working-standard solutions ($10 \mu\text{g/mL}$), to prepare calibration standards of 10, 25, 50, 100, 150, 300, and $600 \mu\text{g/L}$ for all 3 API.

Sample Extraction for Hydrophobic API

The casein micelle preparations from 4 different separation protocols were thawed and reconstituted by adding appropriate amount of water and extracted using methods by Jedziniak et al. (2009b). Briefly, the whole raw milk, skim raw milk, fat, whey (serum), and casein proteins were subjected to extractions for API using 20 mL of acetonitrile and 4 g of sodium chloride, followed by vigorous vortexing for 1 min and centrifugation ($3,200 \times g$, 20 min, -5°C). The supernatant was transferred to a glass test tube and evaporated to dryness using a continuous nitrogen stream at 40°C . The dry residue was reconstituted using $250 \mu\text{L}$ of 0.05 *M* ammonium acetate (pH 5.0):acetonitrile:methanol

(1+1+1; vol), transferred to HPLC vials, centrifuged ($16,000 \times g$, 5 min, 4°C), and $50 \mu\text{L}$ was injected to the HPLC column.

Chromatographic Conditions

The quantitative analysis of the API extracted from milk samples and fractions was performed using an HPLC system (pump model LC-10 ADvp, Shimadzu, Columbia, MD) equipped with a degasser, autosampler, and UV-Vis diode array detector, controlled by LC Solutions Workstation software (Shimadzu). The chromatographic separations were performed using a reversed-phase C18 column (Supercosil LC-18, $25 \text{ cm} \times 4.6 \text{ mm}$, $5 \mu\text{m}$, Supelco Inc., Bellefonte, PA). The column was conditioned with a mobile phase consisting of 90% 0.05 *M* ammonium acetate (solvent A), with pH adjusted to 5.0 using acetic acid and 10% acetonitrile (solvent B) at flow rate of 1.2 mL/min . The gradient elution was applied for 25 min with 10% of solvent B held for 3 min, increased to 40% at 10 min, increased to 60% at 12 min, held at 60% for 3 min, and reduced to 10% at 22 min and, for next 3 min, the column was re-equilibrated with 10% solvent B. The detector was programmed to monitor the elution of the API at an absorbance wavelength of 365 nm for meloxicam (elution time 12.4 min), 290 nm for flunixin (elution time 14.2 min) and metabolite 5-OH-FLU (marker metabolite; elution time 13.1 min), and 292 nm for thiabendazole (elution time 11.8 min) and 318 nm for 5-OH-TBZ (major metabolite; elution time 9.2 min).

Statistical Analysis

The statistical differences were evaluated by ANOVA followed by Tukey's honest significant difference test ($P < 0.05$). Data were analyzed as completely randomized designs using SAS (version 9.4, SAS institute, Inc. Cary, NC). Average values were calculated using 3 replications (cows) each for meloxicam and flunixin, and 2 replications (cows) for thiabendazole.

RESULTS AND DISCUSSION

In Vitro Association

The HPLC analysis of fractions of whole raw milk showed that $66 \pm 6.4\%$ of meloxicam, $29 \pm 0.58\%$ of flunixin, and $50 \pm 4.5\%$ thiabendazole added to milk associated with native casein micelles. The percentages were calculated corresponding to the total concentration of the API present in whole raw milk. The partition into the whey fraction of raw milk was $21 \pm 8.0\%$

for meloxicam, $54 \pm 2.1\%$ for flunixin, and $29 \pm 3.1\%$ for thiabendazole. Contrary to expected strong binding to the milk fat globules, the fat fraction of raw bovine milk showed little association with the 3 hydrophobic API tested in the current experiment. The content of the hydrophobic active ingredients found to be associated with the fat fraction of fortified raw milk was 11 ± 0.9 , 13 ± 0.15 , and $18 \pm 2.9\%$ for meloxicam, flunixin, and thiabendazole, respectively.

The results for flunixin and thiabendazole were compared with their metabolites, 5-OH-FLU and 5-OH-TBZ, fortified in raw whole milk. Both 5-OH-FLU and 5-OH-TBZ are the major marker metabolites for in vivo studies; hence, an in vitro study by fortifying raw whole milk with the metabolites was also conducted. The percentage of 5-OH-FLU found associated with fat, whey, and casein micelles, was 11 ± 1.1 , 53 ± 2.4 , and $34 \pm 0.2\%$, respectively. Although the 5-OH-FLU was expected to be less hydrophobic ($\log D = 1.93$ at pH 6.8) as compared with flunixin, the percentages associated with the milk components followed similar trends as flunixin. The partition of 5-OH-TBZ into fat, whey, and casein micelles was 2.7 ± 0.14 , 62 ± 0.04 , and $35 \pm 5.4\%$, respectively. Although the majority of thiabendazole was found in casein proteins (50%), its hydroxylated metabolite was mainly concentrated in the whey (serum) fraction. The distribution of 5-OH-TBZ into whey aqueous phase can be attributed to either binding to whey proteins or enhanced aqueous solubility of the metabolite in its free form due to the presence of hydroxyl groups.

Based on their pK_a values, certain active ingredients undergo ionization at the physiological pH of milk (6.6 to 6.8). Meloxicam and flunixin are weakly acidic API with pK_a values of 4.08 and 5.82, respectively, and exist as negatively charged species at pH 6.8. Thiabendazole is a weakly basic API, with pK_a 4.64 (basic), and is not ionized at pH 6.8. The distribution coefficients ($\log D$) calculated using equations [1] and [2] at pH 6.8 provided information on the hydrophobicity of the active ingredients at milk's native pH. Despite different $\log D$ values for meloxicam (0.71) and thiabendazole (2.92; Table 1), similar distribution trends were found for both API, with their majority concentration partitioning into casein proteins, followed by the whey and fat fractions. In spite of the strong hydrophobicity of flunixin ($\log D = 3.08$), only $\sim 30\%$ flunixin was found associated with the casein micelles with majority showing up in the whey (serum) fraction. Whereas the hydroxylated metabolites of flunixin and thiabendazole are slightly hydrophilic compared with their parent compounds, no significant differences were found between the distribu-

tion of flunixin and its metabolite 5-OH-FLU; however, thiabendazole showed significantly different distribution as compared with its metabolite. The differences in partitioning among the 3 active ingredients could be attributed to the differences in functional groups involved in interactions with the milk components. However, the effect of differences in chemical structure was found only in the in vitro studies. Also, in the in vitro studies, the milk was fractionated after 1 h of stirring at room temperature ($\sim 25^\circ\text{C}$), which can affect the distribution equilibrium of the API among the milk fractions. This is further discussed in terms of compositional changes of casein micelles induced by different temperatures in comparison to the in vivo study below.

The overall in vitro study results showed that the majority of meloxicam and thiabendazole partitioned into casein proteins, whereas the majority of flunixin, 5-OH-FLU, and 5-OH-TBZ were found in the whey (serum phase) of milk, with less than 20% of all API found associated with the fat fraction. The whey (serum) fraction is the aqueous phase of milk containing whey proteins (e.g., β -LG and α -LA), which possess binding affinities toward hydrophobic compounds (Livney, 2010; Kimpel and Schmitt, 2015). The API appearing in the serum phase either preferably bound to the whey proteins or remained suspended in free form in the aqueous phase. The binding affinities exclusively toward whey proteins were not investigated in the scope of the current study. Previously reported in vitro studies conducted using thiabendazole showed 47% of the API partitioning into casein curds and 54% into whey serum, of which only 12% was associated with whey proteins (Shappell et al., 2017). The favorable retention of thiabendazole by the casein curds could be explained by the binding of the API to casein micelles but also by the API physical entrapment in the gel matrix.

In Vivo Association

The in vivo study was done to determine the distribution of the low-molecular-weight hydrophobic API under physiological conditions. The raw milk obtained from the cows dosed with 3 API (i.e., meloxicam, flunixin, and thiabendazole) was subjected to ultracentrifugation to separate the fat, whey (serum), and casein protein fractions. In whole milk collected from the dosed cows, $281.7 \pm 3.0 \mu\text{g/L}$ of meloxicam was recovered after 12 h of dosing. Further analysis of milk components obtained by ultracentrifugation showed less than 5% of meloxicam associated with fat with the majority showing up in whey ($52 \pm 0.76\%$) and casein micelles ($31 \pm 3.9\%$; Figures 1 and 2). Analysis of casein micelles

obtained by diafiltration and size exclusion chromatography also showed a similar percentage of meloxicam association to casein micelles (26 ± 1.5 and $27 \pm 3.3\%$, respectively). The casein proteins isolated using isoelectric precipitation (pH = 4.6) showed significantly higher association with meloxicam ($54 \pm 0.02\%$) as compared with other 3 methods (Figure 1). This can be attributed to increased concentration of unionized meloxicam at pH 4.6 due to protonation (pK_a 4.08), resulting in increase of hydrophobic interactions with casein proteins. Meloxicam is a hydrophobic API with log P value of 3.43 and was expected to show preferred partition into the fat fraction of milk. However, meloxicam is a weakly acidic API with pK_a value of 4.08, resulting in reduced lipophilicity of the API, as log P value of 3.4 decreased to log D value of 0.71 due to ionization of the API at pH 6.8 (Table 1). The interactions of ionized meloxicam with the casein micelles were attributed to the combination of electrostatic and hydrophobic interactions with the charged and hydrophobic domains present in the internal structure of the casein micelles. The ~52% recovery from whey was either due to the negatively charged free form of meloxicam in aqueous phase or the association to whey proteins known to bind hydrophobic probes. Further separation of whey proteins from serum would be needed to investigate the complete partition of API into whey proteins. The strong difference found in the partition of meloxicam to casein proteins in the *in vitro* versus *in vivo* studies (~66 vs. ~30%, respectively; Figure 2) was partially due to the equilibration time taken by the API to partition into casein micelles of the raw bovine milk. The distribution of API in milk components depends

Figure 1. *In vivo* distribution of meloxicam (MLX) in bovine casein micelles. UC = ultracentrifugation; SEC = size exclusion chromatography; DF = diafiltration; IEP = isoelectric point precipitation. All values are mean $\pm$ SE with $n = 3$; means followed by same letter (a,b) are not significantly different using Tukey ($\alpha = 0.05$). API = active pharmaceutical ingredients.

on the *de novo* synthesis of fat globules and proteins into the ducts of the mammary glands. However, the mechanism of loading of hydrophobic API inside the mammary gland *in vivo* versus *in vitro* casein micelles is not known.

The raw milk collected from cows dosed with flunixin and thiabendazole showed the presence of metabolites 5-OH-FLU and 5-OH-TBZ as major markers. Flunixin is a weakly acidic hydrophobic API with log D value of 3.08. The metabolite 5-OH-FLU is slightly less hydrophobic than flunixin, due to the presence of hydroxy group on the aromatic ring, with log D value of 1.93 at pH 6.8. Despite the differences in extent of hydrophobicity between flunixin and its metabolites, the evaluation of partitioning of hydrophobic metabolites in various fractions of raw milk would be an important step in understanding the interactions between hydrophobic compounds and native casein micelles. Whole raw milk from dosed cows contained 135 ± 0.4 $\mu\text{g/L}$ of 5-OH-FLU, with 31.4 ± 1.3 and $32 \pm 1.8\%$ associated with the casein micelle fraction isolated by ultracentrifugation and diafiltration, respectively (Figure 3). In comparison, a higher percent of 5-OH-FLU was found in casein micelles when separated by isoelectric precipitation ($52 \pm 3.6\%$). This increase was attributed to the protonation of the carboxylate group on 5-OH-FLU (pK_a 5.82) at acidic pH 4.6, resulting in an increase in hydrophobicity and enhancement of the interaction with hydrophobic domains of casein proteins. No significant differences were found in the distribution of

Figure 2. *In vivo* and *in vitro* distribution of meloxicam in bovine milk fractions. All values are mean $\pm$ SE with $n = 3$; means followed by the same uppercase letter (A–C) are not significant across the fat, whey, and casein fractions using Tukey ($\alpha = 0.05$). Means followed by the same lowercase letter (a,b) are not significantly different within each fraction.

Figure 3. In vivo distribution of 5-hydroxy flunixin (5-OH-FLU) in bovine casein micelles. UC = ultracentrifugation; DF = diafiltration; IEP = isoelectric point precipitation. All values are mean $\pm$ SE with $n = 3$; means followed by the same letter (a,b) are not significantly different using Tukey ($\alpha = 0.05$).

flunixin and 5-OH-FLU in casein proteins and whey fraction in the in vivo versus in vitro studies (Figure 4), and both in vivo and in vitro studies showed a similar percent partition of flunixin and 5-OH-FLU into casein micelles (~ 29 – 30%). Significant differences were found in distribution of the API in fat fraction with >5 and 11 to 18% associated with the fat fraction in the in vivo and in vitro studies, respectively. The majority of the flunixin and 5-OH-FLU API was found to be associated with the whey (serum) fraction, except for casein

Figure 4. In vivo and in vitro distribution of flunixin (FLU) and 5-hydroxy flunixin (5-OH-FLU) in bovine milk fractions. All values are mean $\pm$ SE with $n = 3$; means followed by the same uppercase letter (A–C) are not significantly different across the fat, whey, and casein fractions using Tukey ($\alpha = 0.05$). Means followed by the same lowercase letter (a,b) are not significantly different within each fraction. API = active pharmaceutical ingredients.

micelles separated by isoelectric precipitation, probably due to the presence of negatively charged carboxylate groups enhancing solubility in aqueous phase and binding to whey proteins in serum. The goal of the current study was to understand the role of native casein micelles in binding hydrophobic compounds. The binding of hydrophobic probes to whey proteins was considered outside the scope of the present study and not tested.

The hydrophobic metabolite 5-OH-TBZ was the major marker for thiabendazole in the in vivo study, and parent compound thiabendazole was not detected. Although thiabendazole and 5-OH-TBZ possess comparable hydrophobicity in terms of log D values (i.e., 2.92 and 2.1 at milk pH 6.8, respectively; Table 1), the in vitro analysis for thiabendazole showed higher percentage ($50 \pm 4.5\%$; mean $\pm$ SE) associated with casein micelles as compared with the in vivo distribution of its metabolite 5-OH-TBZ ($28 \pm 0.5\%$). The 3 methods to isolate the casein micelles from milk showed similar results in the binding of 5-OH-TBZ to casein micelles (i.e., $28 \pm 0.5\%$ for ultracentrifugation, $31 \pm 3.2\%$ for diafiltration, and $27 \pm 3.9\%$ for isoelectric precipitation; Figure 5). The in vivo results showed that the majority of 5-OH-TBZ ($55 \pm 2.0\%$) partitioned into whey fraction (bound or free), which was similar to the distribution observed in the in vitro study, with $60 \pm 2.80\%$ of 5-OH-TBZ partitioning to the whey fraction; however, the in vitro distribution of thiabendazole showed only $29 \pm 3.0\%$ partitioning to the whey fraction (Figure 6). Both in vitro and in vivo studies showed similar distribution of 5-OH-TBZ associated with casein proteins ($\sim 30\%$), whereas the majority of thiabendazole partitioned to the casein proteins ($50 \pm 4.5\%$) during the in vitro studies. Less than 5% of metabolite 5-OH-TBZ was found to be associated with the fat fraction in both in vitro and in vivo analysis, whereas the in vitro analysis showed that $18 \pm 2.9\%$ of thiabendazole associated with the fat fraction. Overall, similar trends were found for the metabolite 5-OH-TBZ in both in vivo and in vitro, whereas thiabendazole significantly differed in its distribution among fractions ($P < 0.05$). Although no information is available on partitioning of thiabendazole to milk when API is dosed to lactating cows, similar results were found for the related anthelmintic albendazole, with 30% of the albendazole metabolite dosed to cows found associated with rennet casein curds and 54% found associated with the whey fraction (Fletouris et al., 1998).

Although hydrophobic API are expected to partition into the fat fraction of whole milk, the results obtained indicate that hydrophobic API tend to partition preferably to casein proteins or serum (bound or free) as compared with fat. Lower partitioning into fat fraction for thiabendazole is in agreement with a recent study

Figure 5. In vivo distribution of 5-hydroxy thiabendazole (5-OH-TBZ) in bovine casein micelles. UC = ultracentrifugation; DF = diafiltration; IEP = isoelectric point precipitation. All values are mean $\pm$ SE with $n = 2$; means with a letter (a) are not significantly different using Tukey ($\alpha = 0.05$).

where whole milk was spiked with thiabendazole and the majority of the API partitioned into the skim milk fraction versus the fat fraction (Hakk et al., 2016). The observed lower partition to casein proteins was partially due to the presence of the metabolite 5-OH-TBZ, which is slightly less hydrophobic ($\log D = 2.1$) than thiabendazole ($\log D = 2.92$). The hydroxylated hydrophobic API, including flunixin and thiabendazole, leads to enhanced solubility in aqueous serum of the milk and, thus, reduces the partition to hydrophobic sites, including those available in casein micelles. However, the binding to whey proteins was not determined in the

Figure 6. In vivo and in vitro distribution of thiabendazole (TBZ) and 5-hydroxy thiabendazole (5-OH-TBZ) in bovine milk fractions. All values are mean $\pm$ SE with $n = 2$; means followed by the same uppercase letter (A–C) are not significantly different across the fat, whey, and casein fractions, using Tukey ($\alpha = 0.05$). Means followed by the same lowercase letter (a,b) are not significantly different within each fraction. API = active pharmaceutical ingredients.

current study, so the true partition among proteins is not known.

Differences in trends between meloxicam and thiabendazole in vivo versus in vitro could be due to differences in the mechanism by which the API are loaded into the casein micelles in milk based on their interactions with electrostatic and hydrophobic domains. β -Casein is located in the internal core of bovine casein micelles, and is known to improve the loading of hydrophobic probes into reassembled casein micelles by providing available domains (Sahu et al., 2008). The quenching of fluorescent Trp residues showed that hydrophobic interactions were dominant in the binding of low molecular probes, and stronger interaction with β - versus α _S-CN were reported for the hydrophobic curcumin and polyphenols (Bourassa et al., 2013).

Several studies focused on the protective effect resulting from hydrophobic nutraceuticals and pharmaceuticals binding to reassembled β -CN protein micelles (Shapira et al., 2010a,b; Bachar et al., 2012; Turovsky et al., 2015). The role of β -CN in the binding properties of native casein micelles is further supported by a previous study, where human milk was spiked with lipid-soluble API and most partitioning occurred in the casein proteins (Stebler and Guentert, 1990). In human milk, β -CN accounts for $\sim 70\%$ of all casein content, with α _S-CN present in trace amounts and β -LG absent (Farrell et al., 2006).

β -Caseins are known to reversibly dissociate from the casein micelles at low temperatures and migrate back to the colloidal phase on increasing temperature due to hydrophobic association with the micelles. As the in vivo API distribution studies were done at 37°C whereas in vitro studies were done at 25°C , β -CN migration out of micelles may have rendered hydrophobic sites available for interactions with API, as observed for meloxicam ($\sim 66\%$ binding to the casein fraction) and thiabendazole ($\sim 50\%$ binding to the casein fraction). A similar phenomenon was reported, where the removal of β -CN by cooling enhanced the interactions between casein micelles and the hydrophobic curcumin (Yazdi et al., 2014). Based on the previous studies, β -CN is hypothesized to play a role in providing binding sites for association of hydrophobic API with casein micelles.

CONCLUSIONS

The in vivo studies showed that ~ 31 , 34 , and 28% of hydrophobic API meloxicam, 5-OH-FLU, and 5-OH-TBZ, respectively, associated with casein micelles, whereas less than 5% of the API were found partitioned into the fat fraction of raw bovine milk and $\sim 50\%$ partitioned to the whey (serum) fraction (either free or bound to whey proteins). The in vitro study results for

meloxicam were different ($P < 0.05$) as compared with in vivo results with ~66% of the API found associated with the casein micelles. The partitioning of flunixin and 5-OH-FLU followed similar trends in the in vivo versus in vitro studies. The partitioning of 5-OH-TBZ into casein proteins in vivo and in vitro showed similar trends with 28 and 35%, respectively, whereas 50% of thiabendazole was found associated with the casein proteins. Overall, ~30% of the hydrophobic API found in milk from dosed lactating cows was associated with casein micelles. Although no linear relationship was found between increased API hydrophobicity (log D) and association to casein micelles, the percent partition of API to casein micelles exceeded that of the fat fraction for all 3 API. The thorough understanding of binding properties of native casein micelles is useful in applications for increasing the net solubility of nutraceuticals in complex food systems or the masking of therapeutic compounds in liquid food systems. Furthermore, the association of hydrophobic API to casein proteins should be accounted when evaluating API residues in bovine milk. This study demonstrates that the open structure and available sites for interaction allow the native casein micelles to incorporate low-molecular-weight hydrophobic compounds. It appears that the transport of hydrophobic molecules from mother to young is a biological role of casein micelles in milk beyond transporting calcium.

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REFERENCES

- Atamer, Z., A. E. Post, T. Schubert, A. Holder, R. M. Boom, and J. Hinrichs. 2017. Bovine β -casein: Isolation, properties and functionality. A review. *Int. Dairy J.* 66:115–125.
- Bachar, M., A. Mandelbaum, I. Portnaya, H. Perlstein, S. Even-Chen, Y. Barenholz, and D. Danino. 2012. Development and characterization of a novel drug nanocarrier for oral delivery, based on self-assembled β -casein micelles. *J. Control. Release* 160:164–171.
- Bourassa, P., J. Bariyanga, and H. A. Tajmir-Riahi. 2013. Binding sites of resveratrol, genistein, and curcumin with milk α - and β -caseins. *J. Phys. Chem. B* 117:1287–1295.
- Cerkvenik, V., B. Perko, I. Rogelj, D. Doganoc, V. Skubic, W. Beek, and H. Keukens. 2004. Fate of ivermectin residues in ewes' milk and derived products. *J. Dairy Res.* 71:39–45.
- Cheema, M., M. S. Mohan, S. R. Campagna, J. L. Jurat-Fuentes, and F. M. Harte. 2015. The association of low-molecular-weight hydrophobic compounds with native casein micelles in bovine milk. *J. Dairy Sci.* 98:5155–5163.
- Chen, D., Y. Tao, Z. Liu, Z. Liu, L. Huang, Y. Wang, Y. Pan, D. Peng, M. Dai, and Z. Yuan. 2010. Development of a high-performance liquid chromatography method to monitor the residues of benzimidazoles in bovine milk. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 878:2928–2932.
- Corzo-Martínez, M., M. Mohan, J. Dunlap, and F. Harte. 2015. Effect of ultra-high pressure homogenization on the interaction between bovine casein micelles and ritonavir. *Pharm. Res.* 32:1055–1071.
- Dalgleish, D. G. 2011. On the structural models of bovine casein micelles—Review and possible improvements. *Soft Matter* 7:2265–2272.
- de Kruif, C. G., T. Huppertz, V. S. Urban, and A. V. Petukhov. 2012. Casein micelles and their internal structure. *Adv. Colloid Interface Sci.* 171–172:36–52.
- Farrell, H. M., E. L. Malin, E. M. Brown, and P. X. Qi. 2006. Casein micelle structure: What can be learned from milk synthesis and structural biology? *Curr. Opin. Colloid Interface Sci.* 11:135–147.
- Fletouris, D. J., N. Botsoglou, I. Psomas, and A. Mantis. 1998. Albendazole-related drug residues in milk and their fate during cheese-making, ripening, and storage. *J. Food Prot.* 61:1484–1488.
- Food Animal Residue Avoidance Databank. 2016. Component of Food Animal Residue Avoidance & Depletion Program. Accessed Aug. 20, 2014. <http://www.farad.org/wdilookup/>.
- Forrest, S. A., R. Y. Yada, and D. Rousseau. 2005. Interactions of vitamin D3 with bovine β -lactoglobulin A and β -casein. *J. Agric. Food Chem.* 53:8003–8009.
- Fox, P. F., and A. Brodtkorb. 2008. The casein micelle: Historical aspects, current concepts and significance. *Int. Dairy J.* 18:677–684.
- Hakk, H., N. W. Shappell, S. J. Lupton, W. L. Shelver, W. Fanaselle, D. Oryang, C. Y. Yeung, K. Hoelzer, Y. Ma, D. Gaalswyk, R. Pouillot, and J. M. Van Doren. 2016. Distribution of animal drugs between skim milk and milk fat fractions in spiked whole milk: understanding the potential impact on commercial milk products. *J. Agric. Food Chem.* 64:326–335.
- Holt, C., J. A. Carver, H. Ecroyd, and D. C. Thorn. 2013. Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. *J. Dairy Sci.* 96:6127–6146.
- Jedziniak, P., T. Szprengier-Juszkiewicz, and M. Olejnik. 2009a. In-house reference materials: 5-hydroxyflunixin and meloxicam in cow milk—Preparation and evaluation. *Anal. Chim. Acta* 637:346–350.
- Jedziniak, P., T. Szprengier-Juszkiewicz, and M. Olejnik. 2009b. Multi-residue screening method for the determination of non-steroidal anti-inflammatory drug residues in cow's milk with HPLC-UV and its application to meloxicam residue depletion study. *Bull. Vet. Inst. Pulawy* 53:731–739.
- Kimpel, F., and J. J. Schmitt. 2015. Review: Milk proteins as nano-carrier systems for hydrophobic nutraceuticals. *J. Food Sci.* 80:R2361–R2366.
- Kissell, L. W., G. W. Smith, T. L. Leavens, R. E. Baynes, H. Wu, and J. E. Riviere. 2012. Plasma pharmacokinetics and milk residues of flunixin and 5-hydroxy flunixin following different routes of administration in dairy cattle. *J. Dairy Sci.* 95:7151–7157.
- Livney, Y. D. 2010. Milk proteins as vehicles for bioactives. *Curr. Opin. Colloid Interface Sci.* 15:73–83.
- Mohan, M. S., J. L. Jurat-Fuentes, and F. Harte. 2013. Binding of vitamin A by casein micelles in commercial skim milk. *J. Dairy Sci.* 96:790–798.
- Pan, K., Y. Luo, Y. Gan, S. J. Baek, and Q. Zhong. 2014. pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter* 10:6820–6830.

- Puyol, P., M. D. Perez, J. Manuel, and M. Calyo. 1991. Interaction bovine β -lactoglobulin in and other bovine human whey proteins with retinol and fatty acids. *Agric. Biol. Chem.* 55:2515–2520.
- Sahu, A., N. Kasoju, and U. Bora. 2008. Fluorescence study of the curcumin-casein micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules* 9:2905–2912.
- Semo, E., E. Kesselman, D. Danino, and Y.D. Livney. 2007. Casein micelle as a natural nano-capsular vehicle for nutraceuticals. *Food Hydrocoll.* 21:936–942.
- Shapira, A., Y. G. Assaraf, D. Epstein, and Y. D. Livney. 2010a. Beta-casein nanoparticles as an oral delivery system for chemotherapeutic drugs: Impact of drug structure and properties on co-assembly. *Pharm. Res.* 27:2175–2186.
- Shapira, A., Y. G. Assaraf, and Y. D. Livney. 2010b. Beta-casein nanovehicles for oral delivery of chemotherapeutic drugs. *Nanomedicine* 6:119–126.
- Shappell, N. W., W. L. Shelver, S. J. Lupton, W. Fanaselle, J. M. Van Doren, and H. Hakk. 2017. Distribution of animal drugs among curd, whey, and milk protein fractions in spiked skim milk and whey. *J. Agric. Food Chem.* 65:938–949.
- Stebler, T., and T. w. Guentert. 1990. Binding of drugs in milk: The role of casein in milk protein binding. *Pharm. Res.* 7:633–637.
- Su, S. C., C. L. Chang, P. C. Chang, and S. S. Chou. 2003. Simultaneous determination of albendazole, thiabendazole, mebendazole and their metabolites in livestock by high performance liquid chromatography. *J. Food Drug Anal.* 11:307–319.
- Trejo, R., T. Dokland, J. Jurat-Fuentes, and F. Harte. 2011. Cryo-transmission electron tomography of native casein micelles from bovine milk. *J. Dairy Sci.* 94:5770–5775.
- Turovsky, T., R. Khalfin, S. Kababya, A. Schmidt, Y. Barenholz, and D. Danino. 2015. Celecoxib encapsulation in β -Casein micelles: Structure, interactions, and conformation. *Langmuir* 31:7183–7192.
- Yazdi, S. Y., M. Corredig, and D. G. Dalgleish. 2014. Studying the structure of β -casein-depleted bovine casein micelles using electron microscopy and fluorescent polyphenols. *Food Hydrocoll.* 42:171–177.