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Investigating the effect of whey and casein proteins on drug solubility from a paediatric drug absorption perspective

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ABSTRACT

Considering the predominantly milk-based diet of neonates and infants and their immature gastrointestinal digestion, milk proteins may affect drug behaviour and absorption in this population. Using in vitro models, this study investigated the impact of the representative milk proteins, whey and casein, on the solubility and permeation of the lipophilic model drugs spironolactone, clopidogrel and ritonavir. Drug solubility experiments revealed that the presence of milk proteins increased drug solubility. Next, permeation studies demonstrated that the same milk proteins reduced drug permeation across an artificial membrane. These results highlight the importance of the solubility-permeability interplay and indicate the effect of these proteins may be considered during (paediatric) drug development. Lastly, the findings underscore the importance of considering milk protein-drug interactions to optimize drug delivery strategies during (paediatric) drug development and especially for the youngest and most vulnerable part of this population.

1. Introduction

The diet of neonates and infants is distinctly different from that of the adult population. These differences refer to both the frequency of feeding and the type of food. For children <6 months of age, the World Health Organization (WHO) advises feeding on demand, preferably with breast milk. After 6 months, breastfeeding should continue while adding (semi-)solid meals at a frequency of 2–3 times/day up to 8 months, and 3–4 times/day up to 24 months of age, supplemented with 1–2 nutritious snacks if required (World Health Organization, 2021). Based on these feeding recommendations, it is apparent that children below the age of two years are fed more frequently than adults, resulting in a continuous (semi-)fed state. Additionally, their diet is predominantly milk-based and generally presents a high protein and fat contribution (Batchelor et al., 2018).

Milk is the ideal food type to support neonate and infant growth. It is composed of 87 % water, 1 % protein, 4 % lipid, 1 % minerals and vitamins, and 7 % carbohydrates (Boquien, 2018). The protein composition of breastmilk changes during the first year of breastfeeding (Donovan, 2019). The initial breastmilk (colostrum) is composed of a 90/10 whey-to-casein ratio. It then slowly declines to a 65/35 ratio for transitional milk and reaches the 60/40 composition by one month after birth. This composition then remains stable throughout the first year (Donovan, 2019). Both casein and whey are a mixture of different proteins with human whey being composed of α-lactalbumin, secretory immunoglobulins A, lactoferrin, lysozyme, and osteopontin (Donovan, 2019). Human casein consists of αs1-casein, β-casein, αs2-casein, and κ-casein (Davies and Law, 1977; Donovan, 2019). While breastfeeding is strongly recommended, several infant formulae optimized to mimic breastmilk with regards to protein content and whey/casein ratio are available in case breastfeeding is either not possible or not preferred by the mother (Donovan, 2019).

The effect of food on drug absorption has been thoroughly studied in the adult population, resulting in the development of in vitro tools that are routinely used to anticipate the impact of food on oral drug product performance. These tools include, for example, solubility and dissolution tests in biomimetic media, or more advanced gastrointestinal in vitro models (e.g., TIM-1) (López Mármol et al., 2022). While the

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Table 1
Physicochemical properties of the investigated drugs.

	Molecular weight (g/mol)	S_0 in water (μg/mL)[1]	Acid/ Base	pKa[1]	Log P[1]
Clopidogrel	321.8	11.80	Base	4.77	4.03
Spironolactone	416.6	1.98	–	–	3.64
Ritonavir	720.9	1.26	Base	2.84	5.22

[1] Predicted data extracted from go.drugbank.com (*DrugBank Online | Database for Drug and Drug Target Info [WWW Document],* n.d.). Intrinsic solubility estimated using ALOGPS, Log P estimated using Chemaxon and pKa estimated using Chemaxon. S_0 = Intrinsic solubility.

prediction of food effects would be equally valuable for the paediatric population, there are still significant knowledge gaps regarding the gastrointestinal physiology in paediatric subjects, in both the fasted and fed state. Food effects cannot simply be extrapolated from the adult population due to the differences in physiology and food composition such as the high fat and protein content of milk (Batchelor et al., 2018).

These differences are reflected in the composition of intestinal contents, as evidenced by de Waal et al., who reported a high protein content (average 17.7 mg/mL) and total lipid concentration (average 3.8 mg/mL) in ileostomy fluids from neonates and infants. These values are similar to adult concentrations in the fed state (de Waal et al., 2023a). Additionally, de Waal et al. determined the pH, the osmolality, and the concentrations of bile salts, phospholipids, and cholesterol in enterostomy fluids. An observed lower ileal pH could be attributed to the timeframe of collection while osmolality and free fatty acid content was in line with an adult fed state. Lastly, the measured bile salts concentrations were much lower compared to adults. Considering these observations, it is apparent that adult simulated intestinal fluids and in vitro setups are not representative for neonates and infants. For example, the high observed protein concentrations are currently not taken into account and their effect on permeation and solubility is under investigated (Klumpp et al., 2019).

In addition to the differences in fluid composition, the secretion of digestive enzymes was also reported to be different than in adults (Gan et al., 2018; Hodgkinson et al., 2018). Protein digestion starts in the stomach where pepsinogen is secreted together with hydrochloric acid to acidify the gastric fluids. Stomach acid secretion is essential as it both activates the pepsinogen proenzyme to pepsin and provides an acidic environment for protein denaturation and consequently easier access for digestive enzymes. After the stomach, the chyme is transferred to the small intestine where the pancreas secretes trypsin and chymotrypsin into the lumen initiating a major part of protein digestion.

With regards to the paediatric population, Hodgkinson et al. described pepsin concentrations in 1 to 3 year old children to be 10–30 % of the adult values (Hodgkinson et al., 2018). Together with the increased gastric pH in infants after a milk-based meal, this is assumed to slow down the digestion of proteins compared to adults (Gan et al., 2018). In fact, an in vitro digestion experiment mimicking paediatric conditions has shown approx. 40 % undigested casein after 1 h (Hodgkinson et al., 2018), whereas in an in vitro setup simulating the adult population, only 6.15 % of micellar casein remained undigested after 1 h (Zhang et al., 2023). Consequently, it is expected that the digestion of food proteins will be slower in infants and more undigested and large protein digestion products will remain in the paediatric gastrointestinal tract.

In a follow up study, de Waal et al. tested the solubility of clopidogrel, spironolactone, tacrolimus, domperidone, and ibuprofen in ileostomy fluids (de Waal et al., 2023b). No or poor correlations were found between solubility and the compositional parameters of ileostomy fluids. One exception was the protein content, where a weak to moderate correlation was found with the solubility of the different drugs (de Waal et al., 2023b). Hence, the comparatively high protein concentrations might contribute to the (apparent) solubilization of poorly soluble drugs and thus play a relevant role in oral drug absorption.

Table 2
Composition of the different media used for solubility and permeation studies. All proteins were dissolved in a 5-fold concentrated FaSSIF buffer pH 6.5.

Media name	Whey (mg/ mL)	Casein (mg/ mL)	BS (mM)	PL (mM)
Whey 5 mg/mL	5	0	0	0
Whey 10 mg/mL	10	0	0	0
Whey 20 mg/mL	20	0	0	0
Casein 5 mg/mL	0	5	0	0
Casein 10 mg/mL	0	10	0	0
Casein 20 mg/mL	0	20	0	0
Whey + Casein (60/40) 5 mg/ mL	3	2	0	0
Whey + Casein (60/40) 10 mg/mL	6	4	0	0
Whey + Casein (60/40) 20 mg/mL	12	8	0	0
Whey 5 mg/mL + 1 mM BS + 0.25 mM PL	5	0	1	0.25
Whey 10 mg/mL + 1 mM BS + 0.25 mM PL	10	0	1	0.25
Whey 20 mg/mL + 1 mM BS + 0.25 mM PL	20	0	1	0.25
Casein 5 mg/mL + 1 mM BS + 0.25 mM PL	0	5	1	0.25
Casein 10 mg/mL + 1 mM BS + 0.25 mM PL	0	10	1	0.25
Casein 20 mg/mL + 1 mM BS + 0.25 mM PL	0	20	1	0.25
Whey + casein (60/40) 5 mg/ mL + 1 mM BS + 0.25 mM PL	3	2	1	0.25
Whey + casein (60/40) 10 mg/ mL + 1 mM BS + 0.25 mM PL	6	4	1	0.25
Whey + casein (60/40) 20 mg/ mL + 1 mM BS + 0.25 mM PL	12	8	1	0.25
1 mM BS + 0.25 mM PL	0	0	1	0.25
3 mM BS + 0.75 mM PL (mFaSSIF)	0	0	3	0.75
15 mM BS + 3.75 mM PL (mFeSSIF)	0	0	15	3.75
5-fold concentrated FaSSIF buffer	0	0	0	0

BS: bile salts; PL: phospholipids.

The current study aims at investigating the impact of milk proteins on drug solubility in an in vitro setup. Additionally, the effect on the fraction of drug unbound and available for absorption will be studied using equilibrium dialysis. Spironolactone, clopidogrel, and ritonavir were chosen as model compounds. A summary of their characteristics can be found in Table 1. Taking into account the predominantly milk-based diet in children <2 years of age, whey proteins and casein were selected as representative proteins.

2. Materials and methods

2.1. Chemicals

Clopidogrel bisulphate, spironolactone, methanol (MeOH) and acetonitrile (ACN) high-performance liquid chromatography (HPLC) grade were bought from ThermoFisher Scientific (Waltham, MA). ACN and formic acid (FA) LC/MS grade were acquired from Biosolve (Valkenswaard, NL). Ritonavir, sodium and potassium dihydrogen phosphate (NaH_2PO_4 and KH_2PO_4), tris aminomethane, whey from bovine milk and casein from bovine milk were procured from Sigma-Aldrich (St. Louis, MO). Acetic acid was purchased from Chem-Lab analytical (Zedelgem, B). All drugs used for solubility and permeability experiments had a purity above 95 %. Fasted and fed state simulated intestinal fluid powder (3F powder) was obtained from Biorelevant (London, UK).

Purified water was produced using a Purelab® Flex water system from Veolia (Paris, F). Regenerated cellulose membranes with a molecular weight cut-off at 2 kDa were bought from Orange Scientific (Braine-l'Alleud, B).

2.2. Media preparation

The composition of the different protein-containing media is summarized in Table 2. The range of protein concentrations from 5 to 20 mg/mL was selected to include the average protein concentration of 17.7 mg/mL measured in ileostomy fluids from neonates and infants (de Waal et al., 2023a) as well as the reported protein concentrations in human breast-milk and in infant formula (11.1–20.6 mg/mL) (Donovan, 2019). Although slightly different from their human equivalent, bovine whey and casein were selected considering their availability and their usage in infant formula (Bakshi et al., 2023). As a solvent for all media, a 5-fold concentrated fasted state simulated intestinal fluid (FaSSIF) buffer at pH 6.5 containing 17.19 g/L sodium phosphate monobasic anhydrous, 2.1 g/L sodium hydroxide and 30.93 g/L sodium chloride was used to prevent a change in pH during solubility testing.

The whey protein stock solution was prepared by adding an excess of bovine whey powder to the 5-fold concentrated FaSSIF buffer, sonicating the suspension for 1 h, and subsequent centrifugation for 2 h at 21 °C and 4500 *g* (Eppendorf 5804R benchtop centrifuge, Eppendorf, Germany). The protein concentration in the clear supernatant was determined as described below. For the casein stock solution, an approximate 100 mg/mL casein solution was prepared in accordance with the protocol published by Yao et al. (Yao et al., 2019). Briefly, 10 g of casein from bovine milk was added to 85 g of milli-Q water and 5 g of NaOH aqueous solution (5 wt%). To allow the casein to fully dissolve, the solution was gently stirred at 50 °C for 45 min. The stock solution was also centrifuged (20,238 *g* at 21 °C for 10 min) and the protein content of the clear supernatant was determined. To prepare the final media, the stock solutions containing whey proteins or casein were diluted with 5-fold concentrated FaSSIF buffer to reach the concentrations described in Table 2.

To investigate the additive effect of bile salts and phospholipids on the solubilization properties of casein and whey, the same media were prepared with the addition of 1 mM of bile salt and 0.25 mM of phospholipids (Table 2). The bile salt concentrations are based on the data published by de Waal et al. and correspond to the maximum measured total bile salts concentration in ileostomy fluids from neonates and infants (not accounting for one outlier). Phospholipids were kept in the same bile salts:phospholipids-ratio as for the standard adult FaSSIF and fed state simulated intestinal fluids (FeSSIF) media. To spike with bile salts and phospholipids, a stock solution containing 100 mM of taurocholic acid and 25 mM of lecithin using 3F powder was prepared in the 5-fold concentrated FaSSIF buffer and added to the protein media to obtain the target concentrations.

Lastly, as a reference, solubility was assessed in protein-free media, i.e., 5-fold concentrated FaSSIF buffer, 1 mM of bile salts + 0.25 mM phospholipids medium, FaSSIF V1 and FeSSIF V1. To mitigate pH dependent effects, the same 5-fold concentrated FaSSIF buffer was used to make the 1 mM bile salts + 0.25 mM phospholipids, FaSSIF and FeSSIF media instead of the standard FaSSIF and FeSSIF buffer (Table 2). Consequently, they will be referred to as modified FaSSIF (mFaSSIF) and modified FeSSIF (mFeSSIF), respectively.

Table 3
Summary of the HPLC methods used for the analysis of the different drugs. MP = mobile phase. Clopidogrel, spironolactone and ritonavir were run using an isocratic HPLC analysis.

Drug	Column[1]	MP A	MP B	Elution (%A-%B)	Detection
Clopidogrel	1	ACN	25 mM NaH_2PO_4 buffer pH 2.2	70–30	UV: 250 nm
Spironolactone	1	ACN	H_2O	50–50	UV: 237 nm
Ritonavir	2	MeOH	25 mM Acetic acid buffer pH 3.5	80–20	UV: 241 nm

[1] Column 1 = Zorbax XBD eclipse C18 (150 x 4.6 mm, 5 µm), column 2 = Novapak C18 column under radial compression (4 µm, 8x100mm, Waters, Milfort, MA, U.S.A.).

2.3. Protein content determination

Protein content in the different media was determined in accordance with the method published by de Waal et al. (de Waal et al., 2023a). In short, total protein concentration was determined using the tryptophan fluorescence assay. Assuming an average tryptophan of 1.17 % of the total protein content (de Waal et al., 2023a; Wiśniewski and Gaugaz, 2015), 2 µL sample was added to 200 µL of 8 M urea in 100 mM tris aminomethane buffer pH 8.5. Both samples and calibration curve were measured using fluorescence with an excitation wavelength of 295 nm and emission wavelength of 350 nm on a Tecan infinite m200 pro plate reader (Männedorf, Switzerland).

2.4. Apparent solubility measurement

The apparent solubility of spironolactone, clopidogrel bisulphate and ritonavir was determined in the media presented in Table 2 in triplicate. In line with the commercial products, clopidogrel bisulphate was selected rather than clopidogrel free base. All solubility testing was performed at 37 °C using 500 µL of each medium and an excess of solid drug. Samples were shaken for 24 h at 175 rpm. After 24 h, samples were centrifuged for 30 min at 20,000 g and 37 °C.

For sample analysis, 100 µL samples were taken from the clear supernatants. For clopidogrel analysis, 700 µL of ice cold MeOH were added, vortexed for 1 min and subsequently centrifuged for 20 min at 20,000 g and 4 °C. The clear supernatant was then diluted 100-fold in 50:50 *v*/v ACN:H_2O. For ritonavir, a similar sample preparation was performed by adding 900 µL of ice cold MeOH containing 1 % of FA, vortexing for 1 min and subsequent centrifugation for 20 min at 20,000 g and 4 °C. The clear supernatant was then diluted 2-fold by adding an equal volume of water. Lastly, for spironolactone, 1 µL of FA was added to 100 µL of supernatant. Next, an extraction using 700 µL of diethyl ether was performed by vortex mixing for 1 min and centrifuging the sample for 20 min at 20,000 g and 4 °C. The clear ether supernatant was transferred to an empty test tube. Next, the ether was evaporated, and the residue was redissolved in 1 mL of 20:80 v/v ACN:H_2O.

2.5. Permeation measurement

Permeation was measured using an artificial membrane setup (HTD 96b dialysis setup from HTDialysis, LLC, Gales Ferry, CT, US). The setup consisted of a donor and an acceptor compartment separated by a regenerated cellulose membrane with a molecular weight cut-off of 2 kDA. This membrane should prevent the permeation of colloidal structures while allowing the unbound drug to permeate freely (Riethorst et al., 2018). Before use, membranes were hydrated according to the manufacturer's instructions. The acceptor medium was 0.2 % D-α-tocopheryl polyethylene glycol succinate (TPGS) in Hanks' Balanced Salt Solution (HBSS) at pH 7.4. The osmolality of the acceptor solution was adjusted with glucose to match the osmolality of the donor solution. Osmolality was measured using a freeze-point depression osmometer (Advanced Instruments 3250, Norwood, MA, USA). In the donor compartment, the protein media without bile salts as described in Table-2 were used. Based on the determined aqueous solubility, donor compartments were spiked with 30 µM of spironolactone and clopidogrel and 2 µM of ritonavir from a stock solution ensuring ≤1 % final concentration of stock

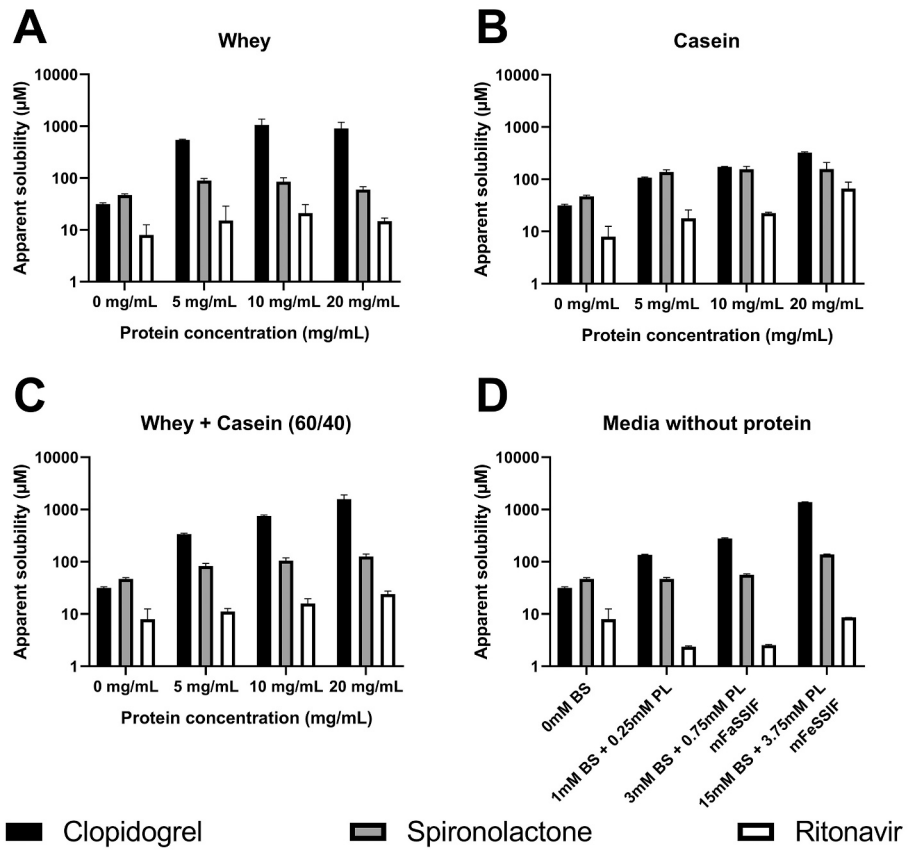

Fig. 1. Apparent solubility of clopidogrel, spironolactone, and ritonavir in aqueous buffer containing milk proteins (A-C), or bile salts and phospholipids (D). All experiments were performed in triplicate. Black, grey and white bars indicate the mean solubility of clopidogrel, spironolactone and ritonavir, respectively. Error bars indicate the standard deviation. BS: bile salts; PL: phospholipids.

solution solvent. To start the experiment, 150 µL of the donor and acceptor medium were added to the dialysis setup. A volume of 10 µL was removed from the acceptor compartment after 4 h and immediately diluted. Spironolactone, clopidogrel and ritonavir samples were 10-fold diluted in 20:80 ACN:H_2O *v*/v, 50:50 ACN:H_2O v/v and 50:50 MeOH:H_2O v/v, respectively.

2.6. High-performance liquid chromatography analysis

HPLC analysis of clopidogrel and spironolactone was performed as described by de Waal et al. (de Waal et al., 2023b). For ritonavir, a separate unpublished method was used. HPLC analysis was performed using isocratic methods at 1 mL/min, 1.3 mL/min and 1 mL/min for clopidogrel, spironolactone and ritonavir, respectively, using a VWR Hitachi Chromaster HPLC system (VWR, Radnor (PA), USA). The calibration line covered a range of 0.10–0.200 µM, 0.04–20 µM and 0.02–200 µM for clopidogrel, spironolactone and ritonavir respectively. A summary of the used HPLC methods is available in Table 3. The analysis methods of clopidogrel, ritonavir (protein precipitation) and spironolactone (ether extraction) were validated according to ICH M10 standards (European Medicines Agency, 2019).

3. Results and discussion

3.1. Effect of proteins on drug solubility

The apparent solubility of clopidogrel, spironolactone, and ritonavir as a function of protein concentration and type is summarized in Fig. 1 and supplementary table 1. In general, the solubility of the three compounds increased with the addition of whey and/or casein (Fig. 1A, B, and C, supplementary table 1). Based on the goodness-of-fit measures (r^2) of the corresponding linear regressions, this increase in solubility was more or less linear in solutions of mixed whey/casein (60/40, Supplementary table 1). In contrast, the trend was less consistent with the individual proteins. For whey, no specific trend was observed (Fig. 1A, Supplementary table 1). For casein, the increase in solubility was linear for clopidogrel and ritonavir (Fig. 1B, Supplementary table 1), but less clear for spironolactone (Fig. 1B, Supplementary table 1).

When comparing the apparent solubility in the presence of protein to the solubility in plain buffer, the most pronounced effect was seen for clopidogrel, with an increase in solubility ranging from 3.4- to 50.3-fold, depending on medium composition. For spironolactone and ritonavir, the protein-based increase in solubility ranged between 1.3- and 3.3-fold, and between 1.4- and 8.3-fold, respectively (Fig. 1, supplementary table 1).

A second interesting aspect is the difference in effect of the protein type on the apparent solubility of the selected drugs (Fig. 1, supplementary table 1). For spironolactone and ritonavir, casein exhibited the most pronounced effect on their apparent solubility (on average 3.2-fold for spironolactone and 4.4-fold for ritonavir). In contrast, the apparent solubility of clopidogrel was most influenced by increasing levels of whey proteins (on average 26.5-fold).

While the effect of proteins on drug solubility is poorly investigated, more extensive research was done in the context of proteins used as excipients in oral formulations. For example, Dezhampanah et al. and Madan et al. showed the positive effect of casein on the solubility of dipyridamole and celecoxib when using casein micelles as a drug delivery system (Dezhampanah et al., 2018; Madan et al., 2020). Furthermore, Mishra et al. investigated the effect of whey proteins as stabilizers in amorphous solid dispersions for indomethacin, carvedilol,

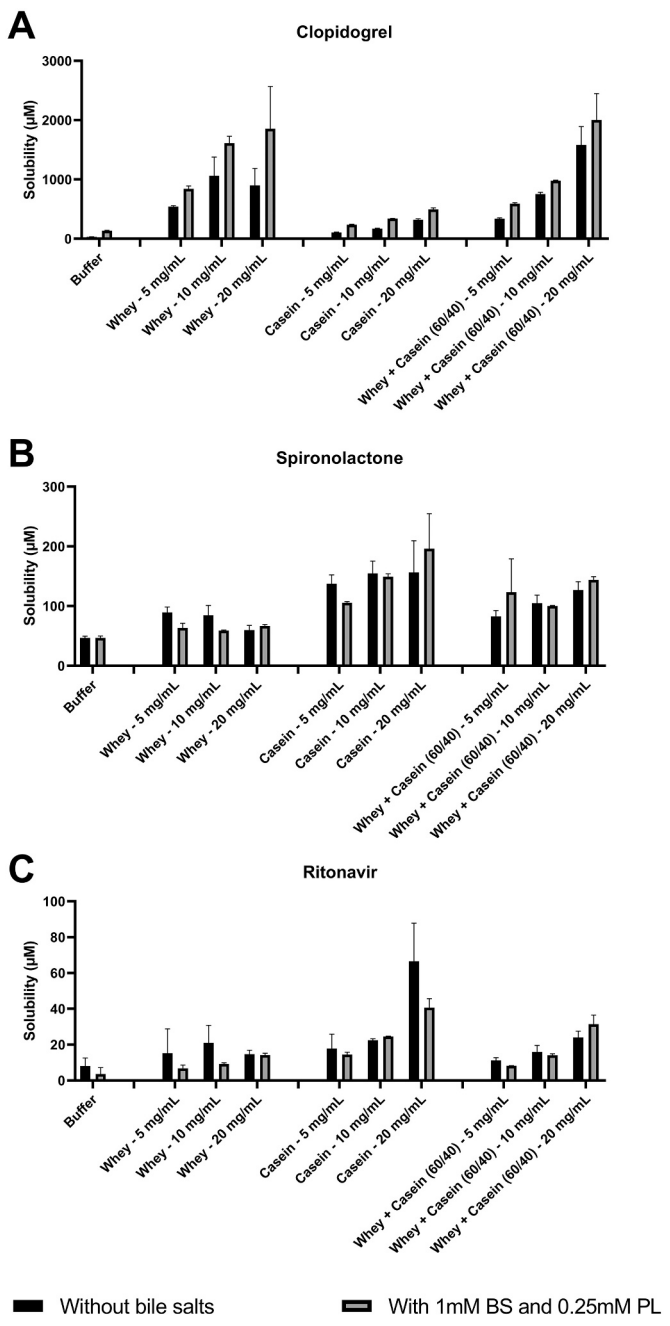

Fig. 2. Additive effect of bile salts (1 mM) and phospholipids (0.25 mM) on the drug solubility of clopidogrel (A), spironolactone (B) and ritonavir (C). All experiments were performed in triplicate. Black, grey and white bars indicate the mean solubility of spironolactone, clopidogrel and ritonavir with (grey bars) or without (black bars) bile salts (BS) and phospholipids (PL). Error bars indicate the standard deviation.

and furosemide. When assessing the excipient effect of whey by adding low concentrations of whey proteins ($\leq$ 3 mg/mL) to their crystalline powder during a solubility experiment, they also observed an increase in solubility. These reported results are in line with the increase in apparent solubility observed in the present study.

3.2. Effect of bile salts on drug solubility

The solubility enhancing effect of bile salts and phospholipids on the model drugs was also investigated in the present study. Above the CMC, bile salts and phospholipids form micelles which can help solubilize lipophilic compounds. However, the CMC for taurocholic acid, which is the bile salt used in 3F powder, is between 3 and 11 mM, which is above the 1 mM bile salt concentration representative for paediatric ileostomy fluids (Sigma-Aldrich, n.d.). In vivo, however, micelles are not formed by a single type of bile salt but by a variety of bile salts, phospholipids, and digestion products. These structures are also referred to as mixed micelles. Additionally, for both the whey and casein proteins, it is described in literature that they can form mixed micelles with bile salts and phospholipids (Anema, 2021; Donato and Guyomarc'h, 2009; Madan et al., 2020). It was hypothesized that, although bile salts were present in intestinal samples at concentrations below the CMC (de Waal et al., 2023a), they could, together with the phospholipids, contribute to the formation of mixed micelles with the whey and casein proteins. This, in turn, might have an additive effect on drug solubility.

To focus solely on the micelle effect, the same buffer composition and pH were used to prepare media with increasing levels of bile salts and phospholipids. A medium containing 1 mM bile salts and 0.25 mM phospholipids was selected to represent the measured bile salt concentrations in paediatric ileostomy fluids (de Waal et al., 2023a). The other two media (mFaSSIF and mFeSSIF) contain higher concentrations of bile salts and phospholipids and are, as such, more representative for the fasted and fed state intestinal fluids of adults (Fig. 1D, supplementary table 1). In these protein-free media, spironolactone, clopidogrel and ritonavir showed a similar linear increase in apparent solubility with increasing bile salt and phospholipid concentrations, as expected for these lipophilic compounds (Fig. 1D, Supplementary table 1). Again, clopidogrel showed the most pronounced increase in apparent solubility upon addition of bile salts and phospholipids compared to spironolactone and ritonavir. The apparent solubility of spironolactone and ritonavir was not increased in the medium containing 1 mM of bile salts and 0.25 mM of phospholipids compared to the pure buffer, while a solubility increase for clopidogrel was observed already at the lowest bile salt and phospholipid concentrations.

In Fig. 2, the effect of including 1 mM bile salts and 0.25 mM phospholipids in the protein media on the apparent drug solubility is visualized. For clopidogrel, the addition of bile salts consistently increased the solubility of the drug (Fig. 2A, supplementary table 1). For spironolactone, the additive effect was variable depending on the protein type (Fig. 2B, supplementary table 1). For ritonavir, no consistent additive effect of bile salts on the solubility was observed (Fig. 2C, supplementary table 1). In general, however, the increase in apparent solubility in the presence of a low concentration of bile salts and phospholipids was limited compared to the protein-mediated increase in apparent solubility.

Lastly, it should be noted that, due to the 5-fold concentrated FaSSIF buffer, the pH remained fairly stable in all media over the 24 h equilibration time, thus ruling out a pH dependent influence on the observed solubilization effects. For clopidogrel, an average pH decrease of 0.15 $\pm$ 0.02 was observed, which is likely due to the use of the bisulphate salt. As this decrease was consistent across the different media, it is not expected to significantly affect the interpretation of the solubility results. For spironolactone and ritonavir, the pH was even more stable with an average pH decrease of 0.02 $\pm$ 0.02 and 0.03 $\pm$ 0.02, respectively.

3.3. Effect of proteins on drug permeation

Fig. 3 visualizes the amount of spironolactone and clopidogrel that permeated during a 4 h incubation across a regenerated cellulose membrane, starting from donor solutions consisting of the different protein media. Drug concentrations in the donor were 30 µM for spironolactone and clopidogrel. For ritonavir, a significantly lower donor concentration had to be used (2 µM) due to the low aqueous solubility, which resulted in no permeation detected in any of the conditions after 4 h (LOD 200 nM).

As seen in Fig. 3, the presence of protein reduced the permeation of both clopidogrel and spironolactone in a concentration dependent

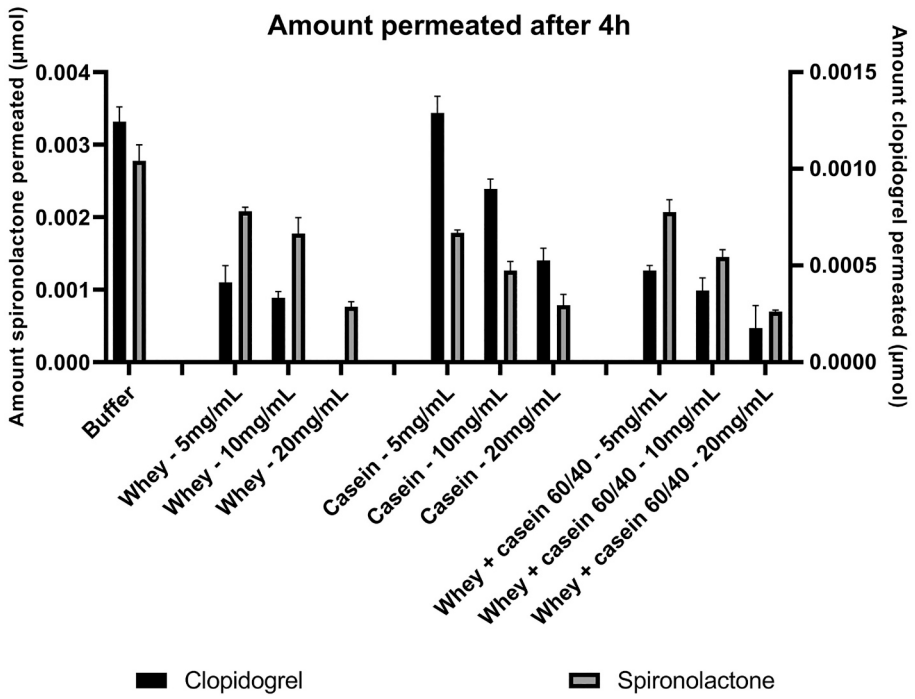

Fig. 3. Amount of spironolactone and clopidogrel permeating across a regenerated cellulose membrane after a 4 h incubation with compound solutions (30 µM) in different media containing milk proteins. All experiments were performed in triplicate. Black and grey bars indicate the mean solubility of clopidogrel and spironolactone respectively. Error bars indicate the standard deviation.

manner with a higher protein concentration translating into a lower amount permeated. In the condition with 20 mg/mL whey, no measurable amount of clopidogrel was detected after 4 h of permeation.

To compare the effects of proteins on solubility on one hand and permeation on the other hand, relative effects were calculated by normalizing the solubility and the amount permeated measured in the protein-containing media to the respective values measured in plain buffer (Fig. 4A and C). With the exception of clopidogrel and spironolactone in the presence of whey proteins, an inverse correlation between the relative effects on the apparent drug solubility and the amount permeated was observed (Fig. 4). These results suggest that, while proteins may have a positive effect on the apparent solubility, they also reduce the concentration of drug molecules that are free in solution and thus available for permeation. Hence, the solubilizing effect may not necessarily translate into a positive effect on the overall rate and extent of permeation. This is further highlighted in Fig. 4B and C where the normalized solubility and permeation are set out in a scatterplot. For clopidogrel a negative, logarithmic decrease in permeation with an increase in solubility is apparent when considering all proteins in different concentrations. For spironolactone, this correlation is linear except for whey proteins where an inverse correlation is present.

For other solubility enhancing strategies, the interplay between solubility and permeability was reviewed by Dahan et al. (Dahan and Miller, 2012). In their review, Dahan et al. highlight that an increase in apparent solubility based on complexation and micellar solubilization might have a trade-off with decreased permeability. These observations are in line with the current results for proteins where both effects (complexation and micellar solubilization) might contribute to the solubility enhancing effects. As previously discussed, both whey and casein are known to be able to form colloidal structures possibly resulting in a solubility enhancing effect due to micellar solubilization. Additionally, as proteins are large molecular structures with possible hydrophilic and hydrophobic pockets, a solubility increase due to complexation is also reasonable.

To assess the impact of (food) proteins on drug absorption, an in vivo study comparing the different conditions would be ideal. To our knowledge, very few studies, mostly focusing on nutrients, have been published so far that address the protein effect on absorption. Furthermore, Iddir et al. conducted a human cross-over study which showed a positive effect of easily digestible whey protein isolates on carotenoids uptake (Iddir et al., 2021). Carotenoids are lipophilic compounds similar to the poorly soluble and often lipophilic BCS class 2 and 4 drugs (Castenmiller and West, 1998; Iddir et al., 2021). Iddir et al. hypothesized that the digestion of whey to amphiphilic peptides could result in the formation of mixed micelles which have a positive effect on the solubilization and thus facilitate the absorption of the carotenoids (Iddir et al., 2021). While at first sight this result may appear contradictory to the permeation data presented here, one needs to take into account the complex interplay between drug dissolution, potential binding to protein (and other) colloids, and permeation in the gastrointestinal tract. For a poorly soluble compound, the overall absorption rate is often limited by the solubilization rate from the solid phase. The positive effect of proteins on solubilization may accelerate this otherwise slow process and outweigh a possible negative effect of proteins on drug permeation. In contrast, for molecules with comparatively fast dissolution properties, the negative impact of proteins on the free fraction dissolved may become relevant and thus result in an overall reduced absorption rate. The latter situation is more similar to the condition simulated in the permeation test in this study, where the drug was entirely dissolved in the donor phase, and thus, increasing protein concentrations directly reduced the concentration of molecularly dissolved drug. As a consequence, a pronounced impact on the overall permeation rate was observed.

In addition, it should not be overlooked that the in vivo situation is highly dynamic. In this respect, the gastrointestinal digestion of proteins will, most likely, substantially impact their role in drug solubilization and permeation. For instance, this digestion may reduce the solubility enhancing effect of proteins, but also cause the release of bound drug molecules thus favouring absorption. Since protein digestion has been reported to be slower in young children (Gan et al., 2018; Hodgkinson et al., 2018), the impact of proteins on drug absorption might be more pronounced in this population. To substantiate these hypotheses, more

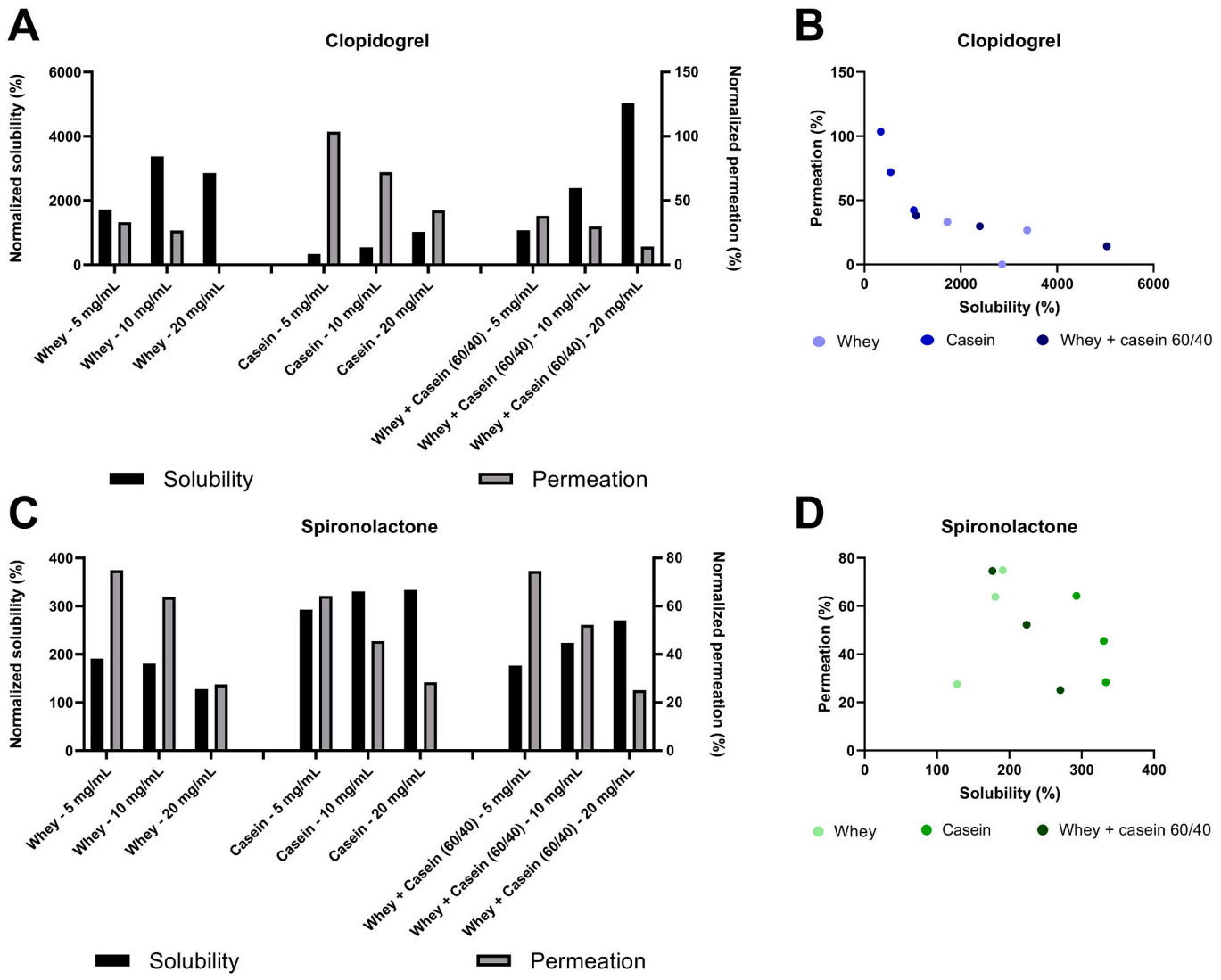

Fig. 4. Visualization of the normalized solubility and normalized permeation for clopidogrel and spironolactone. (A) and (C) visualize the individual mean permeation and solubility which were normalized to the solubility in or permeation from a buffer solution. B and D visualize the mean buffer normalized permeation as a function of the mean buffer normalized solubility in a scatterplot.

research is required.

4. Conclusion

This study investigated the effect of milk proteins on the apparent solubility and the permeation of drug molecules. While the data from solubility assays showed a significant positive effect of milk proteins on the apparent drug solubility, permeation data revealed a reduced permeation rate, which points at a lower free drug concentration in solution in the presence of proteins. These findings indicate the importance of considering the impact of proteins on the rate and extent of in vivo drug absorption. Particular attention may be required in the context of drug absorption in neonates and infants due to their heavily milk-based diet, immature digestion, and continuous semi-fed state. A larger fraction of undigested proteins is likely present in the gastrointestinal tract of the very young, which may affect drug release and absorption in a relevant manner. Of note, proteins are currently not considered in standard biorelevant media, which are routinely used to assess the impact of gastrointestinal media on drug substance and drug product solubility and dissolution.

The mechanistic understanding of the interaction between the proteins and the drugs requires further exploration. Additionally, parallel effects such as the sticking of the proteins or compound to the membrane, a change in permeability of the membrane, or other unknown factors may occur. Hence, the results from the current study highlight the need for further research in order to ensure safe and effective use of oral drugs in the paediatric population.

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CRediT authorship contribution statement

Matthias Van der Veken: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joachim Brouwers:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Neil Parrott:** Writing –

review & editing, Visualization, Supervision, Methodology, Conceptualization. **Patrick Augustijns:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Cordula Stillhart:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Matthias Van der Veken reports financial support was provided by F Hoffmann-La Roche Ltd. C.S. and N.P. are employees of F. Hoffmann-La Roche Ltd., Basel, Switzerland. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpx.2024.100290.

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