**Research Article**

**Intestinal In Vitro Transport Assay Combined with Physiologically Based Kinetic Modeling as a Tool to Predict Bile Acid Levels In Vivo**

Véronique M. P. de Bruijn, Willem te Kronnie, Ivonne M. C. M. Rietjens and Hans Bouwmeester

Division of Toxicology, Wageningen University and Research, Wageningen, The Netherlands

---

**Abstract**

Bile acid homeostasis is vital for numerous metabolic and immune functions in humans. The enterohepatic circulation of bile acids is extremely efficient, with ~95% of intestinal bile acids being reabsorbed. Disturbing intestinal bile acid uptake is expected to substantially affect intestinal and systemic bile acid levels. Here, we aimed to predict the effects of apical sodium-dependent bile acid transporter (ASBT)-inhibition on systemic plasma levels. For this, we combined in vitro Caco-2 cell transport assays with physiologically based (PBK) modeling. We used the selective ASBT-inhibitor odevixibat (ODE) as a model compound. Caco-2 cells grown on culture inserts were used to obtain transport kinetic parameters of glycocholic acid (GCA). The apparent Michaelis-Menten constant ($K_{m,app}$), apparent maximal intestinal transport rate ($V_{max,app}$), and ODE’s inhibitory constant ($K_i$) were determined for GCA. These kinetic parameters were incorporated into a PBK model and used to predict the ASBT inhibition effects on plasma bile acid levels. GCA is transported over Caco-2 cells in an active and sodium-dependent manner, indicating the presence of functional ASBT. ODE inhibited GCA transport dose-dependently. The PBK model predicted that oral doses of ODE reduced conjugated bile acid levels in plasma. Our simulations match in vivo data and provide a first proof-of-principle for the incorporation of active intestinal bile acid uptake in a bile acid PBK model. This approach could in future be of use to predict the effects of other ASBT-inhibitors on plasma and intestinal bile acid levels.

**Plain language summary**

Bile acids regulate digestion and immune functions. Too little bile acid reuptake in the gut is related to several diseases, including inflammatory bowel disease. This study investigates how reducing bile acid absorption affects bile acid levels in humans using the drug odevixibat (ODE) as an example. ODE reduces bile acid absorption by blocking the intestinal bile acid transporter protein in gut cells. The transport of a bile acid through a gut cell line commonly used to model the intestinal barrier was measured with and without ODE, and mathematical modeling was used to translate the laboratory results to whole-body effects. This combined approach accurately predicted the known effects of ODE on intestinal and bloodstream bile acid levels in humans. This novel approach could be used to predict the effects of other chemicals on intestinal bile acid absorption and intestinal and bloodstream bile acid levels instead of animal testing.

---

**1 Introduction**

Bile acids (BAs) have emerged as critical signaling molecules for energy, glucose and lipid metabolism, cell proliferation as well as regulation of the immune system (Jia et al., 2018; Fuchs and Trauner, 2022). BA homeostasis is primarily regulated by the gut-liver axis, where primary BAs are produced in the liver, metabolized to more hydrophobic secondary BAs by the intestinal microbiome, and taken up via the intestinal epithelium into the portal vein to be recirculated to the liver. The BA pool shapes the microbiome community by acting on bacterial cell membranes but also reduces intestinal membrane integrity by affecting the epithelial cells (Begley et al., 2005). High amounts of the secondary BA deoxycholic acid (DCA) reduced intestinal integrity in pig colonic crypts (Leschelle et al., 2002) and in rabbit small intestine (Fasano et al., 1990). Reduced intestinal integrity is related to diarrhea, bacterial overgrowth, and inflammatory bowel disease (Marasco et al., 2022; Miele et al., 2009). Furthermore, bacterial products are more likely to translocate to the liver and trigger an inflammatory response in the liver in case of reduced membrane integrity (Duan et al., 2022; Sabino et al., 2016). Disturbances of the BA pool predispose an individual to the development of liver disease, e.g., cholestasis (Gijbels and Vinken, 2019), metabolic dysfunction-associated fatty liver disease (MAFLD) (Mouzaki et al., 2016), and gastrointestinal carcinogenesis (Li et al., 2022). It has been shown that fecal concentrations of DCA and its conju-
gates increase with disease activity and fibrosis stage, an important hallmark of MAFLD (Smirnova et al., 2022). Hence, a well-balanced BA pool is essential for gut-liver axis homeostasis and human health.

BAs are de novo synthesized in the liver from cholesterol. Upon conjugation with glycine or taurine, BAs are actively secreted via the bile salt export pump (BSEP) to the bile canaliculi (Lin et al., 2023). Via the bile canaliculi, the conjugated BAs enter the intestinal lumen where they can be deconjugated and where ~95% of intestinal BAs are reabsorbed via both active and passive processes (Kullak-Ublick et al., 2004). Unconjugated BAs are passively absorbed along the whole length of the small intestine, while the majority of both conjugated and unconjugated BAs are actively reabsorbed from the ileum (Martinez-Augustin and Sanchez de Medina, 2008; Krag and Phillips, 1974; Li et al., 2018). The most effective reabsorption of BAs takes place in the terminal ileum and is mediated by the apical sodium-dependent bile acid transporter (ASBT), a member of the solute carrier (SLC) super-family encoded by the SLC10A2 gene (Dawson, 2011; Lin et al., 2023). Via ASBT, BAs are taken up into enterocytes where they bind to the bile acid binding protein (BABP) and are excreted to the basolateral side into the portal blood via the organic solute transporter (OST) α/β (Lu et al., 2022). Via the portal vein the BAs are transported to the liver where they are taken up by Na+-taurocholate co-transporting polypeptide (NTCP) and transporters from the organic anion transporting polypeptide (OATP) family (Chiang and Ferrell, 2022). BAs that escape ileal reabsorption are metabolized by the gut microbiome into a wide array of secondary BAs, followed by either absorption from the colon or fecal excretion (Jia et al., 2018). The remarkably efficient uptake of BAs from the intestinal lumen implies that this process is of crucial importance for BA homeostasis (Duan et al., 2022).

The observation that alterations in intestinal BA uptake potentially affect the onset and development of different types of liver disease has suggested ASBT as a target for drug development (Yang et al., 2020). For instance, odevixibat (ODE) has recently been approved for the treatment of progressive familial intrahepatic cholestasis, and trials for the treatment of other cholestatic diseases are ongoing (Deeks, 2021). ODE is a selective and reversible ASBT-inhibitor that reduces the BA levels in plasma/serum by reducing the reuptake of BAs in the ileum, while the fecal BA levels are increased (Graffinier et al., 2016). Individuals with progressive familial intrahepatic cholestasis typically have increased plasma BA levels, and a reduction is considered beneficial. Reduced bile acid absorption was observed in preclinical and clinical studies of inflammatory bowel disease, and was typically accompanied by decreased ASBT levels (Fitzpatrick and Jenabzadeh, 2020). This indicates that even though a reduction of ASBT-mediated BA absorption is beneficial for individuals suffering from cholestatic diseases, it might have an adverse effect on otherwise healthy individuals. Several xenobiotics, e.g., the mycoxin deoxynivalenol and the antibiotic tobramycin, are known to reduce intestinal BA uptake in vitro (Wang et al., 2022; Zhang et al., 2022). For an accurate human hazard assessment of chemicals, it is crucial to understand how xenobiotics alter BA homeostasis and can potentially affect host health.

Quantitative knowledge of the synthesis, absorption, distribution, metabolism, and excretion of BAs is paramount for understanding BA-associated pathologies. Here, physiologically based kinetic (PBK) modeling is used to integrate the physicochemical and biological properties of BAs and to predict the effects of drug interventions or xenobiotic exposure without the need for animal-derived data. PBK modeling thus contributes to the 3R principle (replacement, reduction and refinement) in chemical risk assessment.

Several kinetic models describing the processes involved in BA homeostasis have been developed previously. Early work describes the metabolism and circulation of several major BAs in the gastrointestinal tract and circulatory system (Hofmann et al., 1983; Molino et al., 1986). Active transport processes are increasingly being recognized as important modulators in BA homeostasis, but they were not included in this early work. For example, inhibition of BSEP can lead to toxic BA accumulation inside hepatocytes and cause cholestasis. The relevance of hepatic BSEP inhibition for cholestasis development has been recognized in the cholestasis adverse outcome pathway (AOP) (Vinken et al., 2013). Active transport processes were included in more recent modeling work (Sips et al., 2018; Voronova et al., 2020; Baier et al., 2019). Advances in computational power and biological understanding of the processes involved in BA homeostasis allowed for the development of more complex and dynamic models. The work of Voronova et al. (2020), for example, described the autoregulation of BA synthesis by farnesoid receptor X (FXR) (Voronova et al., 2020), and Sips et al. (2018) modelled the intestinal transit of BAs in detail.

Increased complexity of mathematical models typically comes with an increased number of required input parameters – parameters that cannot always be derived experimentally and pose the risk of overfitting. Therefore, we previously developed a data-driven PBK model describing BA homeostasis, i.e., a PBK model in which most parameters were derived experimentally. The model includes active BSEP-mediated hepatic canalicular BA efflux (de Bruijn et al., 2022a) but does not yet include transporter-mediated hepatic sinusoidal BA uptake or ASBT-mediated ileal BA absorption.

In the present study we aimed to extend the PBK model to also include ASBT-mediated ileal BA absorption and NTCP-mediated hepatic uptake and to predict the effects of ASBT-inhibition on systemic plasma levels. This provides mechanistic insights into the effects of ASBT and its inhibition on whole-body BA homeostasis, with a focus on the gut-liver axis. To this end, we employed PBK modeling and evaluated the effect of ODE-mediated ASBT-inhibition on plasma BA levels. The required kinetic parameters for ileal
absorption were derived from human Caco-2 cells grown in culture inserts. We focused on the glycine-conjugated forms of three major bile acids, i.e., glycocholic (GCA), glycodeoxycholic acid (GCDA), and a generic unconjugated BA (uBA). The taurine-conjugates were not explicitly considered, as the BA pool in human serum consists of only ~15% taurine-conjugates, compared to ~45% glycine-conjugates and ~40% unconjugated BAs (Bathena et al., 2013). GCA and GDCA were selected because their abundance in plasma showed the steepest decrease upon 7-day ODE-treatment in healthy individuals. GCDCDA is the most abundant BA in human plasma and was therefore also included. The model may serve as a quantitative tool to evaluate various potential modes-of-action in the framework of BA-associated diseases upon exposure to xenobiotics.

2 Material and methods

Cell culture
Human colon carcinoma Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Caco-2 cells (passage number 15-28) were grown at 37°C with 5% CO₂ in Modified Eagle’s Medium (MEM) GlutaMax™ supplemented with 20% (v/v) fetal calf serum, 0.86% (v/v) 50 mg/mL L-glutamine-penicillin-streptomycin solution, and 0.86% (v/v) 50 mg/mL pyruvate as culture medium (CM), all purchased at Gibco BRL (Breda, The Netherlands). Cells were subcultured twice a week at 50-60% confluence using 0.05% trypsin-EDTA (Gibco BRL).

Cell viability assessment
Caco-2 cells were seeded at 40,000 cells/well in a 96-well plate and allowed to attach to the plate for 24 h. The effect of ODE (99.85%, MedChemExpress, Monmouth Junction, NJ, USA) on the viability of Caco-2 cells 24 h post-seeding was evaluated using the WST-1 assay. Briefly, Caco-2 cells were exposed to 0-500 μM ODE for 24 h. ODE was dissolved in dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) and 200× diluted in CM. 0.5 μM potassium dichromate was used as a positive control. Subsequently, the cells were incubated with WST-1 reagent 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (Sigma-Aldrich, St. Louis, MO, USA) at 37°C. For this, WST-1 was added at 5% of the medium volume, and the absorbance was measured at 440 nm and 620 nm with a SpectraMax iD3 multi-mode microplate reader (Molecular Devices). Cell viability was expressed as percentage of the solvent control group.

Intestinal Caco-2 monolayer barrier integrity assessment
Caco-2 cells were seeded at 180,000 cells/cm² in 24-well polycarbonate membrane inserts with 0.4 μm pore size (Corning Costar, Schnelldorf, Germany) and maintained in culture for 19-21 days. The integrity of Caco-2 cells was assessed using transepithelial electrical resistance (TEER) and/or the Lucifer yellow assay. The TEER was measured with a MilliCell® ERS-2 epithelial voltohm meter (Millipore, Amsterdam, The Netherlands) pre and post transport experiments, and data obtained from inserts with TEER values > 300 Ω × cm² were considered acceptable.

For the Lucifer yellow assay, the exposure medium was removed by rinsing the monolayers with Hank’s balanced salt solution (Gibco BRL) supplemented with 10 mM HEPES (transport buffer), at pH 7.4. Then, 50 μM Lucifer yellow (Sigma-Aldrich) in DMSO (0.5% v/v) in transport buffer was added to the apical side of the culture insert. After 60 min, samples were taken basolaterally, and fluorescence was measured at 485/535 nm excitation/emission with a SpectraMax iD3 multi-mode microplate reader (Molecular Devices). Apparent permeability values (P_app) were calculated according to:

\[
P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}
\]

where \(P_{app}\) is expressed in cm/s, dQ/dt is the change in the basolateral Lucifer yellow concentration (nmol/s), A is the surface of the culture insert (cm²), and \(C_0\) is the initial apical Lucifer yellow concentration (nmol/cm²). Lucifer yellow Papp values < 4 × 10⁻⁶ cm/s were considered acceptable (Wang et al., 2008).

Transport assays
Caco-2 monolayers grown in culture inserts for 19-21 days were used. CM was removed and replaced with transport buffer. The cells were allowed to equilibrate for 30 min. Then, transport buffer was removed, the test solution was added apically, and samples were taken at the basolateral side after 0-180 min of incubation. The test solutions contained 0.5% DMSO (v/v). In the first set of experiments, the cells were incubated with 5 μM glycocholic acid hydrate (GCA) (Sigma-Aldrich, ≥ 97%), deoxycholic acid (DCA) (Sigma-Aldrich, ≥ 99%), glycodeoxycholic acid (GCDCDA) or glycodeloxycholic acid (GDCA) (Avanti, Birmingham, AL, USA, ≥ 99%), and samples were taken after 0, 30, 60, 90, 120, 150 and 180 min of incubation. These experiments were performed at 4°C and 37°C to distinguish between active and passive transport processes. Samples were taken from the apical and basolateral chambers, and after washing twice with ice-cold PBS, the membrane was cut out of the culture insert and transferred to an Eppendorf tube containing 65% (v/v) MeOH in MillIQ. The samples were ultrasonicated for 15 min (Bandelin Sonorex rk100), centrifuged for 15 min at 15,000 g, and the supernatant was transferred to LC-MS/MS vials and stored at -20°C until analysis by LC-MS/MS. The amounts detected in the cells, apical and basolateral buffer, were considered acceptable when > 80% of the initially added amount was recovered.

In further experiments, GCA was used as model BA. Due to the similar physicochemical properties of the tested BAs, it was assumed that the inhibitor constant (Ki) was independent of the substrate used. As described in the Section “PBK model” below, data from literature was used to translate the experimentally obtained kinetic parameters for GCA to kinetic parameters for GCDCDA and GDCA. Cells were incubated at 37°C with 5 μM GCA and an ODE concentration range (0.0005-5 nM) to determine the half inhibitory concentration (IC₅₀) of ODE on GCA transport. Next, the cells were incubated under time-optimized conditions with a
concentration range of GCA to determine its kinetic parameters ($V_{\text{max,app}}$ and $K_{m,\text{app}}$).

As sodium binding drives the conformational changes in ASBT required for transport of its substrates (Al-Hilal et al., 2014), we performed transport assays in transport buffer without sodium to confirm the sodium-dependency of the transport and quantify the passive transport rate at different GCA concentrations. The active transport rate was determined by subtracting the passive transport rate from the total transport rate. The $K_i$ was determined by incubating the cells with a concentration range of GCA in the presence of an ODE concentration close to the IC$_{50}$. Albeit it is unlikely that ODE affected passive sodium-independent transport, this was not experimentally confirmed in the current work. We assumed that the passive transport rate was not altered by addition of ODE. All transport assays were performed in triplicate.

Reusing culture inserts

To overcome the limited availability of culture inserts, they were reused up to one time based on the protocol by Kato et al. (2021). Briefly, the cells were removed from the culture inserts after performing a transport assay with 0.25% (v/v) trypsin-EDTA and kept sterile. Culture inserts were incubated with trypsin-EDTA for 1 h at 37°C. Trypsin-EDTA was aspirated and replaced with fresh trypsin-EDTA solution, both apically and basolaterally. After 24 h, all trypsin-EDTA was removed and the culture inserts were washed twice with PBS, followed by two washes with sterile MilliQ water. Culture inserts were left to air dry and stored at 4°C until further use. We did not observe any significant differences in TEER values between new and recycled culture inserts. WST-1 showed similar cell viability (Fig. S1$^1$), and we did not detect any BAs in the basolateral chamber when we performed a blank transport assay without added BAs.

Bile acid profiling using LC-MS/MS

BA analysis was performed on a triple quadrupole LC-MS/MS system, model LCMS-8045 (Shimadzu Corporation, Japan), and based on our previous work (de Bruijn et al., 2022b; Wang et al., 2022; Zhang et al., 2022). GCA/GCDCA/GDCA/DCA were quantified. BAs in samples and standards were separated on a Kinetex C18 column (1.7 µm × 100 A × 50 mm × 2.1 mm, Phenomenex 00B-4475-AN, Utrecht, the Netherlands) using an ultra-high performance liquid chromatography (UHPLC) system (Shimadzu). The MS parameters, LODs and LOQs are provided in Table S1$^1$.

Results were considered statistically significant when $p < 0.05$. Data analysis

R version 4.1.0. was used for all data analysis and simulations.$^2$ R package tidyverse version 1.3.1$^3$ was used for data exploration and visualization. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s correction for multiple tests. Results were considered statistically significant when $p < 0.05$.

The appropriate model to estimate the IC$_{50}$ of ODE-mediated inhibition of GCA transport was selected by minimizing the log-Likelihood. The best fit was achieved by the three-parameter log-logistic function using the package drc version 3.0.1 (Ritz et al., 2015). The lower and upper limit were constrained to 0 and 100 (%), respectively, and the Hill slope was variable.

From the Lineweaver-Burk plot (Lineweaver and Burk, 1934), we derived that ODE inhibited GCA transport in a competitive nature. Previous research showed that in vitro permeability is affected by unstirred water layers in the vicinity of the culture insert and the culture insert itself, which is jointly referred to as the aqueous boundary layer (ABL). The ABL acts as a permeability barrier in transport experiments and was previously shown to bias kinetic estimates (Balakrishnan et al., 2007). Therefore, taking into account ABL resistance, the transport rate could be described accounting ABL resistance, the transport rate could be described incorporating the aqueous boundary layer (ABL).

\[ V_{\text{app}} = \frac{P_{\text{ABL}} \times V_{\max,\text{app}}}{K_{m,\text{app}} (1 + \frac{[\text{ODE}] \times F_{\text{sub}}}{K_i} + [\text{GCA}] \times F_{\text{sub}})} + \frac{[\text{GCA}] \times F_{\text{sub}}}{K_{m,\text{app}} (1 + \frac{[\text{ODE}] \times F_{\text{sub}}}{K_i} + [\text{GCA}] \times F_{\text{sub}})} \]

(Eq. 2)

References

1. doi:10.14573/altex.2302011s1
2. https://www.r-project.org/
3. https://cran.r-project.org/web/packages/tidyverse/index.html
where $V_{\text{app}}$ is the active apparent GCA transport rate from the apical to basolateral chamber in nmol min$^{-1}$ cm$^{-2}$, $P_{\text{ABL}}$ the permeability across the ABL in cm min$^{-1}$, $V_{\text{max,app}}$ the maximal apparent active GCA transport rate in nmol min$^{-1}$ cm$^{-2}$, [GCA] the concentration of GCA in nmol cm$^{-3}$, $F_{\text{ub}}$ the fraction unbound in vitro, $K_{\text{m,app}}$ the apparent Michaelis-Menten constant in nmol cm$^{-3}$, [ODE] the concentration of ODE in nmol/L, and $K_v$ the inhibitor constant in nmol/L. $P_{\text{ABL}}$ was set to $4.2 \times 10^3$ cm min$^{-1}$ (Balakrishnan et al., 2007). Fraction unbound in vitro was set to 1, i.e., the nominal concentration equaled the free concentration. This was justified as the transport buffer did not contain any protein (Gilbert-Sandoval et al., 2020). The parameters were optimized using the nls function in R$^2$.

**PBK model**

To describe the synthesis, absorption, distribution, metabolism, and excretion of BAs, four PBK submodels were constructed; a GCDCA, a GCA, a GDCDA and an unconjugated BA (uBA) model. Combined, these BAs represent ~85% of the BA pool in human serum (Bathena et al., 2013). The PBK submodels were based on our previous work (de Bruijn et al., 2022a). Modifications were made to expand the model to four BAs instead of one lumped BA pool, to describe intestinal transit and absorption in more detail, and the liver as a permeability-limited model.

Briefly, each submodel consisted of separate compartments representing gall bladder, intestine, blood, rapidly perfused tissue, slowly perfused tissue, adipose tissue, and extra- and intracellular water in liver. Figure 1 displays the conceptual model. In PBK models, organs are typically described using perfusion-limited models. This modeling approach assumes that compounds diffuse passively into the organ’s water and are instantly homogeneously distributed throughout the organ (Rietjens et al., 2011). For transporter-mediated uptake, however, permeability and not perfusion is the rate-limiting step. Therefore, perfusion-limited models cannot be used, and a permeability-limited model should be used to simulate transporter-mediated uptake (Jamei et al., 2014). In these models, the respective organ is divided into an extracellular and intracellular water compartment. In the present BA model, the liver compartment was modelled in this way to enable description of the NTCP-mediated active transport from the extracellular to the intracellular liver water compartment and of the BSEP-mediated active transport from the intracellular water compartment to the bile. The extracellular water compartment is in instantaneous equilibrium with the blood in the vascular space and serves as a barrier compartment for entry into the intracellular tissue compartment. As sinusoidal efflux is low under normal physiological conditions (Dawson et al., 2009), this process was not included to limit model complexity. The extracellular water:plasma partition coefficients were calculated by a quantitative property-property relationship (Peyret et al., 2010). The BA fraction available for transport and metabolism in intracellular and extracellular water was set to 1, since transcellular trafficking of BAs is highly efficient and effectively mediated by bile acid-binding proteins (BABPs) (Toke, 2022). Tissue:plasma partition coefficients for the BAs were calculated by a method described in literature (Rodgers and Rowland, 2006) and obtained via the QIVIVE toolbox (Punt et al., 2020). Recent work showed that the method of Lobell and Sivarajah (2003) to describe the $F_{\text{ub}}$ resulted in the most accurate model predictions (Punt et al., 2022), hence this method was used here. As DCA is the most abundant uBA in human serum, DCA’s physicochemical properties were selected to describe uBAs. The physicochemical properties of the BAs used to calculate the tissue:plasma partition coefficients are described in Table 1.

The enterohepatic circulation was included as a circulation of BA between the liver, gall bladder, and intestine. BAs are de novo synthesized in the liver and excreted via the intestinal compartments into the feces. Hepatic BA efflux was simulated to be actively transported from the liver directly into the intestine or to the gallbladder by BSEP following Michaelis-Menten kinetics. The BSEP-mediated effluxes of BAs were described by:

$$V_{\text{BSEP}} = \frac{V_{\text{max,BSEP}} \times [\text{CVL}]}{K_{\text{m,BSEP}} + [\text{CVL}]}$$

(Eq. 3)

where $V_{\text{BSEP}}$ is the BSEP-mediated efflux of BAs from the liver in $\mu$mol/min, $V_{\text{max,BSEP}}$ is the maximum rate of BSEP-mediated BA efflux from the liver in $\mu$mol/entire liver/h, [CVL] is the BA concentration in the venous blood leaving the liver in $\mu$mol/L and assumed to be in equilibrium with the intrahepatic concentration, and $K_{\text{m,BSEP}}$ is the Michaelis-Menten constant in $\mu$mol/L for BSEP-mediated BA efflux.

The $V_{\text{max,app}}$ and $K_{\text{m,app}}$ for BSEP-mediated transport of GCDCA and GCA were taken from Kis et al. (2009) as obtained in a vesicular transport assay in a baculovirus-infected SF9 system and scaled to the in vivo situation as described previously (de Bruijn et al., 2022a). The authors showed that the $V_{\text{max,app}}$ values in the vesicular transport assay increased upon addition of physiological levels of cholesterol; hence, these values were used in the current PBK model. Kis et al. (2009) investigated GCA and GCDCA, but not GDCDA. Therefore, the kinetic data for BSEP-mediated GDCA transport were extracted from a study with transfected HEK293 cells reported by Notenboom et al. (2018). The $V_{\text{max,app}}$ of BSEP-mediated transport as obtained by Notenboom et al. (2018) was initially measured in pmol min$^{-1}$ mg$^{-1}$ protein. To make the measurements consistent with GCA and GCDCA, the $V_{\text{max,app}}$ values for GDCA were converted to $\mu$mol/min/mg BSEP using GCDCA mediated transport, which was measured in both the study by Notenboom et al. (2018) and Kis et al. (2009), as a reference. Consequently, the GDCA simulations were performed with $V_{\text{max,app,GDCDA}} = V_{\text{max,app,GCDCA[Sf9 vesicles]}} / V_{\text{max,app,GCDCA[HEK293 vesicles]]}} \times V_{\text{max,app,GDCDA[HEK293 vesicles]}} = 8.4 \mu$mol min$^{-1}$ mg$^{-1}$ BSEP.

The intestine was divided into three compartments: 1) the upper intestine (duodenum + jejunum), where BAs are passively absorbed; 2) the lower intestine (ileum), where BAs are absorbed by a carrier-mediated process; and 3) the colon, where BAs are rapidly deconjugated and subsequently passively absorbed. Transit times were taken from the modified gastrointestinal transit absorption (GITA) model introduced by Kimura and Higaki (2002), different transit times were used during fasting (17:30-8:00) and fed state. During daytime, three meals and gall bladder contractions were simulated, i.e., at 8:00, 12:00 and 16:00. The parameters describing
different BAs. We used the following equation to scale the relative $V_{\text{max,BA},\text{rel}}$ to the in vivo $V_{\text{max,BA},\text{in vivo}}$:

$$V_{\text{max,BA},\text{in vivo}} = V_{\text{max,BA},\text{rel}} \times \frac{V_{\text{max,GCA},\text{in vivo}}}{V_{\text{max,GCA},\text{rel}}}$$  \hspace{1cm} (Eq. 5)

where $V_{\text{max,BA},\text{in vivo}}$ is the in vivo maximal ASBT-mediated transport rate for the BA of interest, $V_{\text{max,BA},\text{rel}}$ the maximal ASBT-mediated transport rate for the BA of interest relative to TCA, $V_{\text{max,GCA},\text{in vivo}}$ the maximal ASBT-mediated GCA transport rate obtained from our in vitro studies and scaled to the in vivo situation, and $V_{\text{max,GCA},\text{rel}}$ the maximal ASBT-mediated transport rate of GCA relative to TCA. For GCA and GCDCA, $K_{\text{m,app}}$ values from Balakrishnan et al. (2006) were incorporated into the PBK model without any further scaling. For GCA, we averaged our experimentally derived $K_{\text{m,app}}$ and the $K_{\text{m,app}}$ values from Balakrishnan et al. (2006). The effects of the different values for $K_{\text{m,app}}$ on the outcomes of the model calculations were also analyzed.

BAs escaping ileal absorption enter the colon, where they are rapidly and instantly deconjugated by the gut microbiome (Zhang et al., 2022). Upon reabsorption and entering the liver, these uBAs are rapidly and instantly conjugated (Falany et al., 1994). This is supported by the fact that only 0.3% of hepatic BAs exist in their unconjugated form (Garcia-Canaveras et al., 2012). Consequently, the intestinal deconjugation and hepatic conjugation rates were set to 1000 h$^{-1}$.  

Fig. 1: Conceptual model
cBA, conjugated BA; uBA, unconjugated BA; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; DCA, deoxycholic acid
Hepatic uptake of conjugated BAs from the extracellular water into the intracellular water was simulated as a permeability limited NTCP-mediated process. uBAs are taken up via passive diffusion (Notenboom et al., 2018). In this study, it was assumed that the diffusion of uBAs from the extracellular water to the intracellular water occurs at a rate equivalent to the hepatic blood flow. The \( K_{m,app} \), \( V_{max,app} \) and scaling factor for \textit{in vitro-in vivo} scaling for the NTCP-mediated transport were obtained from a study with NTCP-transfected HEK293 cells (Notenboom et al., 2018). These authors determined the scaling factor by comparing the NTCP abundance in NTCP-transfected HEK293 cells and human liver tissue. The hepatic BA uptake rate is described by:

\[
\frac{d}{dt} \text{AL}_{\text{IW,upt}} = \frac{V_{\max,\text{NTCP}} \times [\text{CVL}_{\text{EW}}]}{K_{m,\text{NTCP}} + [\text{CVL}_{\text{EW}}]} \tag{Eq. 6}
\]

where \( \frac{d}{dt} \text{AL}_{\text{IW,upt}} \) is the BA uptake rate into the intracellular water in \( \mu \text{mol/entire liver/h} \), \( \text{CVL}_{\text{EW}} \) the effluent concentration of the extracellular water, \( V_{\max,\text{NTCP}} \) is the maximal NTCP-mediated uptake rate in \( \mu \text{mol/entire liver/h} \), and \( K_{m,\text{NTCP}} \) the Michaelis-Menten constant in \( \mu \text{mol/L} \) for NTCP-mediated uptake. The \( V_{\max,\text{NTCP}} \) in pmol \( \text{min}^{-1} \times 10^{-6} \) hepatocytes was extrapolated to units appropriate for the PBK model, i.e., \( \mu \text{mol entire liver}^{-1} \times \text{h}^{-1} \), by multiplying the \( V_{\max,\text{NTCP}} \) with the hepatocellularity (10^6 hepatocytes/g liver) (Barter et al., 2007), weight of the liver in g (Soars et al., 2002), 60 to convert minutes to hours and 10^{-6} to convert pmol to \( \mu \text{mol} \). OATP-transporters were not explicitly considered, because rat Ntcp was proven to be responsible for more than 80% of conjugated bile acid (TCA) uptake (Kouzuki et al., 1998) and we assumed a comparable ratio in human hepatocytes. Instead, \( V_{\max,\text{NTCP}} \) was divided by 0.8 to account for the additional effects of OATP-mediated transport. As an up to 18-fold difference in GCDCA affinity for NTCP was reported in literature (Notenboom et al., 2018; Jani et al., 2018), GCDCA simulations were ran with the \( K_{m} \) as reported by Notenboom et al. (2018), an 18-fold reduced \( K_{m} \) and the average of these two values.

Lastly, given that the inhibition of ASBT by ODE was shown to be competitive in nature, the effects of ODE on the plasma BA concentrations was simulated by adjusting the \( K_{m} \) of ASBT-mediated uptake with a modulation factor of 1 \( + \frac{[\text{ODE}]}{K_{i}} \) \text{Fab} as described in Eq. 2. Where ODE is the total concentration of ODE in the ileum, \( \text{Fab} \) is the fraction unbound in the ileum \textit{in vivo}, and \( K_{i} \) is the inhibitory constant as derived from our experiments. As ODE is poorly absorbed, no absorption was simulated and the full dose was assumed to be equally distributed over 9 L of gastrointestinal fluid (Deeks, 2021; Hendriksen et al., 2003). \( F_{ab} \) in the ileum was set equal to the \( F_{ab} \) in plasma, i.e., 0.3% (EMA, 2021).

The differential model equations were encoded and solved using the package \textit{RxODE} version 1.1.5. The model code and input parameters are available in the supplementary files in .docx and .xlsx format, respectively.

\textbf{Sensitivity analysis}

To assess the influence of the parameters on the model outcome, a local sensitivity analysis was performed for the \( C_{\text{max}} \) of BAs using the package \textit{FME} version 1.3.6.2 (Soetaert and Petzoldt, 2010). The sensitivity analysis for \( T_{\text{max}} \) was performed manually. Based on the method reported by Evans and Andersen (2000), the sensitivity coefficients (SCs) for the model parameters were calculated as follows:

\[
SC = \frac{C - C'}{P - P'} \times \frac{P'}{C'} \tag{Eq. 7}
\]

where \( C \) indicates the initial value of the model output, \( C' \) indicates the modified value of the model output resulting from an increase in the parameter value. \( P \) indicates the initial parameter value, and \( P' \) indicates the modified parameter value after a 5% increase of its value, keeping all other parameters at their original value.

\section{3 Results}

\subsection{3.1 Bile acids cross Caco-2 monolayers via passive and active transport}

19-21-day-old Caco-2 monolayers grown in culture inserts were apically exposed to 500 pmol of four BAs to optimize the incubation time for obtaining kinetic parameters and study the mode of transport. We observed apical-to-basolateral transport of GCDCA, GCA, GDCA, and DCA. Transport of GCA was observed apically exposed to 500 pmol of four BAs to optimize the incubation time for obtaining kinetic parameters and study the mode of transport. We observed apical-to-basolateral transport of GCDCA, GCA, GDCA, and DCA. Transport of GCA was observed at 37°C (> 300 pmol), no transport was detected at 4°C. Comparable amounts of GCDCA and GDCA were transported at 37°C, while for these two conjugated BAs limited transport at 4°C was observed especially at 180 min, albeit so low that it could not be quantified (< LOQ) (Fig. 2). DCA trans-

\[\text{https://nlmixrdevelopment.github.io/RxODE/}\]

4 \[\text{doi:10.14573/altex.2302011s2}\]

Tab. 1: Physicochemical properties of the model BAs used to calculated the blood:tissue partition coefficients

<table>
<thead>
<tr>
<th></th>
<th>GCDCA</th>
<th>GCA</th>
<th>GDCA</th>
<th>DCA</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{pKa} )</td>
<td>3.77</td>
<td>3.77</td>
<td>4.6</td>
<td>4.65</td>
<td>Law et al., 2014; Schwarz et al., 1996</td>
</tr>
<tr>
<td>( \log P )</td>
<td>2.12</td>
<td>1.65</td>
<td>2.25</td>
<td>3.5</td>
<td>Roda et al., 1990</td>
</tr>
<tr>
<td>( \text{MW} )</td>
<td>449.62</td>
<td>465.6</td>
<td>449.62</td>
<td>392.57</td>
<td></td>
</tr>
<tr>
<td>Fraction unbound</td>
<td>0.061</td>
<td>0.088</td>
<td>0.055</td>
<td>0.02</td>
<td>Calculated; Lobell and Sivarajah, 2003</td>
</tr>
</tbody>
</table>
Port was observed at both 4°C and 37°C without a significant effect of temperature on the total amount transported after 180 min. For all four BAs a linear relationship between time and amount of BAs transported was observed at 37°C (Pearson’s correlation coefficient 0.79-0.94, p < 0.05).

3.2 ODE inhibits GCA transport dose-dependently
To assess the effect of the known ASBT-inhibitor ODE on the GCA apical-to-basolateral transport over Caco-2 cells, 500 pmol GCA and a concentration range of ODE were added to the apical chamber of a culture insert with a confluent monolayer of Caco-2 cells, and the basolateral GCA was measured. The results from the WST-1 assay confirmed that ODE concentrations up to 500 µM did not affect cell viability (Fig. S2A\(^1\)). Combined exposure to increasing concentrations of ODE and 500 pmol GCA did not have statistically significant effects on TEER values (Fig. S2B\(^1\)). We observed that ODE inhibited GCA apical-to-basolateral transport in a dose-dependent manner (Fig. 3), and the IC\(_{50}\) value was estimated to be 0.04 nM ODE.

3.3 ODE inhibits GCA transport in a competitive manner
Finally, the Caco-2 transport assay was used to obtain kinetic parameters for the PBK model. Our results showed that the GCA

Fig. 2: Bile acid transport across a Caco-2 monolayer measured during 180 min at 37°C (orange circles) or 4°C (blue triangles) 500 pmol GCA/GCDCA/ GDCA/DCA were added to the apical chamber at t = 0, and samples were taken from the basolateral chamber at different timepoints. Data are expressed as mean ± SD, N = 3. LOD/LOQ are reported in Table S1\(^1\). GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; DCA, deoxycholic acid. Significance compared to t = 0 min was assessed with a one-way ANOVA followed by post hoc tests using Bonferroni’s correction. Statistically significant differences in the basolateral amount of bile acids compared to t = 0 are indicated with *.

Fig. 3: Odevixibat (ODE)-dependent inhibition of glycocholic acid (GCA) transport across Caco-2 monolayers Values are normalized to the control. N = 3.
transport rate saturated with increasing GCA concentrations. A relatively small amount of GCA (~16% of the total GCA transport) was transported in the absence of sodium, i.e., via passive processes. The active transport rate was determined by subtracting the passive transport rate from the total transport rate (Fig. 4A). The nature of ODE inhibition of GCA transport was evaluated using a Lineweaver-Burk plot (Fig. 4B). For this the reciprocal of the GCA concentration (1/GCA) was plotted versus the reciprocal of the reaction speed (1/V), either with or without 0.08 nM ODE. ANOVA demonstrated that the slope, but not the intercept, was significantly different between the two curves. Hence, incubation with ODE increased the $K_{m,\text{app}}$ but did not affect $V_{\text{max,app}}$, indicating that ODE inhibits GCA transport in a competitive manner. Consequently, the following parameters were obtained for active GCA transport from Eq. 2: $V_{\text{max,app}} = 71.5 \text{ pmol min}^{-1} \text{ cm}^{-2}$, $K_{m,\text{app}} = 22.5 \text{ µM}$, $K_i = 0.02 \text{ nM}$.

### 3.4 Postprandial bile acid kinetics are readily described by the PBK model

The parameters for active GCA transport obtained using the Caco-2 transport assay were incorporated into the PBK model using the total ileal surface and an empirical scalar for in vitro-in vivo scaling, as described in Eq. 4. The PBK model describes the synthesis, absorption, distribution, metabolism, and excretion of BAs for a healthy individual. For the first set of predictions, meals were simulated at 8:00, 12:00 and 16:00. This meal regimen was in accordance with the regimen used in the study of Hepner and Demers (1977) of which the data were used to validate our predictions. A second in vivo dataset available for model evaluation describes the postprandial BA kinetics for 8 hours after one meal (Lamaziere et al., 2020). The observed and predicted plasma BA-time profiles are displayed in Figure 5. GCA and uBA postprandial kinetics were predicted within 2-fold of both in vivo data sets, GDCA was predicted within 2-fold of the data obtained by Hepner and Demers (1977), but the model overpredicted the other in vivo data set 25-fold (Lamaziere et al., 2020), which also underlines the interstudy and/or interindividual differences. Three scenarios with different $K_m$ values for NTCP-mediated GCDCA transport were simulated (Fig. 6). $K_m$ values of 10, 0.6 and 5.3 µM, derived from Notenboom et al. (2018), Jani et al. (2018) and the average of these two studies, respectively, were used to run the simulations. The GCDCA $C_{\text{max}}$ decreased with a decreasing $K_m$. The lowest $K_m$ value resulted in only a minor increase in plasma GCDCA levels compared to the fasting state. The best fit to the in vivo data was obtained when the $K_m$ values of the two studies were averaged. For further simulations, the averaged $K_m$ value reported was used for GCDCA, given its better fit to the in vivo data. We used the average of our experimentally derived ABL-corrected $K_{m,\text{app}}$ and the $K_{m,\text{app}}$ reported in literature for ASBT-mediated GCA transport. The influence of different $K_m$ values on the postprandial GCA kinetics is depicted in Figure S3.

### 3.5 ODE lowers the simulated plasma levels of conjugated bile acids

The effects of ODE-administration on the plasma and colonic BA levels were simulated using the perfusion-limited liver model and the two different sets of kinetic parameters to describe BSEP-mediated hepatic GDCA efflux. The simulated change
Fig. 5: Observed and predicted postprandial bile acid kinetics
Black solid line = prediction. (A) Meals were simulated at 8:00, 12:00 and 16:00. Orange circles = in vivo data obtained from Hepner and Demers (1977). (B) A single meal was simulated at 8:00. Blue circles = in vivo data obtained from Lamaziere (2020). GCA, glycocholic acid; GDCA, glyco-deoxycholic acid; uBA, unconjugated bile acids.

Fig. 6: Observed and predicted postprandial GCDCA kinetics using different kinetic parameters for NTCP affinity
Black solid line = prediction. Left column: Meals were simulated at 8:00, 12:00 and 16:00. Orange circles = in vivo data obtained from Hepner and Demers (1977). Right column: Meal was simulated at 8:00. Blue circles = in vivo data obtained from Lamaziere (2020). (A) $K_m = 10 \, \mu M$ (Notenboom et al., 2018), (B) $K_m = 5.3 \, \mu M$ (average Notenboom et al., 2018 and Jani et al., 2018), (C) $K_m = 0.568 \, \mu M$ (Jani et al., 2018). GCDCA, glycochenodeoxycholic acid.
in total plasma BAs was within the 95% confidence interval (CI) observed in a previous phase I clinical trial (Graffner et al., 2016). The current PBK model only simulated the concentrations of GCA, GCDCA, GDCA, and uBA, while tauro-conjugates also circulate through the human body. Tauro-conjugates represent ~15% of the BAs in human plasma and to correct for the presence of tauro-conjugates, the predicted BA concentrations in Figure 7A-C were divided by 0.85 (black dashed line) to present a better estimate of the whole BA pool. The plasma levels of conjugated BAs (GCA, GCDCA and GDCA) were predicted by the PBK model to decrease ODE dose-dependently, while the uBA plasma levels showed a slight increase (Fig. 7B). The uBA levels in the colon were predicted to increase with increasing ODE dosages (Fig. 7C).

### 3.6 Sensitivity analysis

To assess the influence of the parameters on the $C_{\text{max}}$ and $T_{\text{max}}$, a local sensitivity analysis was performed. Figure 8A shows that parameters related to the active transport of GCA over the intestine and from extracellular to intracellular water and their scaling have a strong influence on the GCA $C_{\text{max}}$ (absolute normalized sensitivity coefficient $> 0.5$). Not only the maximal ASBT-mediated intestinal uptake rate ($V_{\text{maxASBTc}_{\text{GCA}}}$), but also the kinetic parameters describing NTCP-mediated hepatic uptake ($V_{\text{maxNTCPc}_{\text{GCA}}}$ and $K_{\text{mNTCP}_{\text{GCA}}}$) strongly influence the model outcome. ES_all and surface_all are used for the scaling of $V_{\text{maxASBTc}_{\text{GCA}}}$, and Hep_all_GCA is included in the scaling of $V_{\text{maxNTCPc}_{\text{GCA}}}$, but also in the BSEP-mediated hepatic efflux. Furthermore, the ileal transit time in the fed state ($kti_{\text{fed}}$), blood:plasma (BP_all) ratio, body weight (BW_all), and the factor used to incorporate OATP-mediated transport (SF_OATP) negatively impact GCA $C_{\text{max}}$. The portal blood flow ($QPVc_{\text{all}}$) has a positive influence on the GCA $C_{\text{max}}$. The sensitivity analyses for GCA, GCDCA, and GDCA are roughly similar (Fig. S4), while uBA $C_{\text{max}}$ is not strongly influenced by transporter-mediated processes or their scaling factors. Even though parameters related to BSEP-mediated active hepatic efflux do not have a strong influence on plasma BA $C_{\text{max}}$, they were shown to strongly influence the maximal intracellular concentrations of GCA, GCDCA, and GDCA in the liver (Fig. S5). Figure 8B shows that the gall bladder emptying time ($get_{\text{all}}$) and body weight (BW) have the strongest delaying influence on the GCA $T_{\text{max}}$, while the cardiac output ($QC_{\text{all}}$), the fraction of the arterial blood flow to the liver ($QA/Vc_{\text{all}}$), and ileal transit time in fed state ($kti_{\text{fed}}$) have the strongest accelerating influence on GCA $T_{\text{max}}$. The sensitivity analyses for the other BAs consistently identified intestinal transit time as an important parameter for $T_{\text{max}}$ (Fig. S6). It should be noted that the normalized sensitivity coefficients for $T_{\text{max}}$ are around one order of magnitude smaller than those for $C_{\text{max}}$.

### 4 Discussion

The aim of this work was to predict the effects of ASBT-inhibition on systemic plasma levels and by that obtain mechanistic insights into whole-body BA homeostasis, with a focus on the gut-liver axis. To this end, we obtained kinetic parameters for the active intestinal GCA transport across Caco-2 monolayers and incorporated these into a PBK model describing the synthesis, absorption, distribution, metabolism, and excretion of BAs. The current study
hepatic BA uptake, BSEP-mediated hepatic BA efflux and ASBT-mediated ileal BA uptake.

In the current study it was shown that Caco-2 cells transport BAs via active transport and passive diffusion processes. Although no differences were observed in the total transport of GCA, GCDCA, GDCA, and DCA, notable differences in passive diffusion were observed between the BAs tested upon addition of 500 pmol BA to the apical chamber, especially between the conjugated and unconjugated BAs. DCA was translocated primarily via passive diffusion, while for the conjugated BAs GCDCA and GDCA a very small amount (< LOQ) and for GCA no detectable amount was transported via passive diffusion. In line with literature, the passive BA diffusion rate followed the BA’s lipophilicity: DCA > GCDCA ≈ GDCA > GCA (Aldini et al., 1996).

Caco-2 cells are a robust intestinal cell model with apical brush borders, tight junctions, and expression of several clinically relevant transporters, including transporters of the ATP binding cassette (ABC), multidrug resistance protein (MRP), and solute carrier (SLC) family (Olander et al., 2016; Antunes et al., 2013). Our results confirm that GCA transport over Caco-2 cells is active and sodium-dependent, indicating the presence of a functional ASBT transporter. We trust that the Caco-2 cells performed ASBT-mediated BA transport, because a) GCA transport was completely inhibited upon addition of ≥ 0.5 nM of the selective ASBT inhibitor ODE at a low (5 µM) GCA concentration and b) our $K_{m,app}$ shows that Caco-2 cells transported GCA in an active and sodium-dependent manner, indicating that ASBT functionality is maintained in Caco-2 cells. Subsequently, Caco-2 cells were applied to obtain kinetic parameters for ASBT-mediated GCA transport over the intestinal epithelium. These parameters were incorporated into the PBK model after in vitro to in vivo extrapolation of Caco-2-derived kinetic parameters, surface = cylindrical surface of ileum, $OPVc = \text{fraction of blood flow through the portal vein}$, $BP = \text{blood:plasma ratio}$, Hep = hepatocellularity, $SF_{OATP} = \text{scaling factor to adjust hepatic uptake for OATP-mediated uptake}$, $Vmax_{NTCPc} = \text{maximal NTCP-mediated GCA hepatic uptake rate}$, $BW = \text{body weight}$, get = gall bladder ejection time, $k_{ti,\text{fed}} = \text{ileal transit time in fed state}$, $QAVc = \text{fraction of blood flow to liver through the arterial vein}$, $QC = \text{cardiac output}$, GCA indicates a parameter specifically for the GCA submodel, _all indicates a parameter that is shared for all BA submodels.
is within a 2-fold range of the $K_{m,\text{app}}$ value obtained from ASBT transfected MDCK cells reported in literature (Balakrishnan et al., 2006). Although ASBT gene expression was confirmed for Caco-2 cells (Wang et al., 2022; van der Mark et al., 2014), ASBT protein expression was not conclusively demonstrated. ASBT protein expression could not be detected by LC-MS/MS (Bruck et al., 2017; Olander et al., 2016), but Western blot analysis by van der Mark et al. (2014) revealed that ASBT expression is low in Caco-2 cells compared to the human ileum. Differences in the detection limit of the analytical techniques, Caco-2 cell clones and/or culture conditions provide plausible explanations for the inconsistent results for ASBT protein expression in Caco-2 cells (Sambuy et al., 2005). We consider the sodium-dependency and ODE-mediated inhibition of GCA transport strong indicators of the presence of functional ASBT in Caco-2 monolayers. In the current study, an empirical scalar of 2.8 was employed to correct for differences between the in vitro and in vivo situation, such as differences in ASBT expression and/or activity and other chemical, physical, or biological differences. However, for optimal scientific validity, a mechanistic justification for the scaling factor is desirable, enabling its application to other substances. The mechanistic justification of the scaling factor could be based on the establishment of a relative activity factor (RAF), in which transport of a probe substrate in vitro is compared against the in vivo situation. RAFs have been proven relatively successful for the prediction of metabolic conversions, but their applicability for transporter-mediated processes remains to be verified (Kumar et al., 2021).

The Caco-2 cells were cultured in medium containing fetal calf serum (FCS). FCS contains an undefined cocktail of growth factors, hormones, and vitamins and is typically used to ensure growth and proliferation in cell cultures. In order to contribute to the 3R (reduce, refine, replace) principles, increasing attention goes to the development of animal-free chemically-defined alternatives to FCS. Several synthetic supplements have been tested but did not allow various cell types to proliferate and differentiate properly (van der Valk et al., 2018). Human platelet lysate seems a promising substitute for FCS. Caco-2 cell viability was slightly enhanced in cells grown with this lysate compared to FCS, and the cells differentiated to cells of the enterocytic lineage (Wanes et al., 2021). Yet, functional similarity to the human intestinal epithelium remains to be demonstrated.

The kinetic parameters obtained in the Caco-2 transport assay were incorporated into a PBK model describing the synthesis, distribution, metabolism, and excretion of BAs. Our simulations for GCA, GDCDA, and uBA predicted at least one dataset of observed BA concentrations within 2-fold. GDCDA postprandial kinetics were simulated with three different $K_m$ values for NTCP-mediated transport, where the lowest value (0.6 µM, Jani et al., 2018) gave a close fit with the in vivo data, while the highest value (10 µM, Notenboom et al., 2018) resulted in a ~5-fold overprediction. The large discrepancy between the two experimental datasets can be attributed to intersystem (transfected CHO versus HEK293 cells), biological, and/or chemical differences. Given its large influence on the postprandial kinetics, it is crucial to establish reliable kinetic parameters for NTCP-mediated transport and understand the reasons for these discrepancies. The predicted BA concentrations were still within the range of $C_{\text{max}}$ values reported in literature. Up to 22-fold differences in plasma BA $C_{\text{max}}$ between individuals have been reported, which could be due to biological interindividual differences (Baier et al., 2019; Lamaziere et al., 2020; Steiner et al., 2011; Fiamoncini et al., 2016), while interstudy differences between individuals may also be due to technical differences. Due to these differences, a PBK model predicted $C_{\text{max}}$ may be more than 2-fold different from data reported for individual volunteers in literature.

Our simulations reflect a consistently delayed time to reach $C_{\text{max}}$ (T$_{\text{max}}$) compared to the in vivo datasets. The sensitivity analysis revealed that a prolonged gall bladder ejection time had the strongest delaying effect on T$_{\text{max}}$. In the current model the gall bladder ejection time was set to 90 minutes, which was derived from a study using scintigraphy measurements in healthy individuals. In this study, the volunteers were administered an isotope and the isotope amount in the gallbladder was quantified at selected timepoints using a gamma camera (Jazrawi et al., 1995). The accuracy of the predicted T$_{\text{max}}$ might be improved by describing gall bladder motility in more detail, for example by using a normalized Rayleigh function, which shows a transient increase and subsequent decrease of gall bladder emptying rate over time (Sips et al., 2018). Given that the effect of gall bladder ejection time on T$_{\text{max}}$ was relatively small (NSC < 0.1), this approach was not applied in the current work.

The data from our transport assays indicated that ODE is a competitive inhibitor of GCA transport, with an estimated IC$_{50}$ of 0.04 nM, which is about 2-fold lower than in literature (0.1 nM) (EMA, 2021). We predicted a decrease in plasma BAs, and especially the conjugated BAs (GCA, GCDCA and GDCDA), upon treatment with a single oral dose of ODE. The decrease in total BAs was in line with literature. In vivo data in healthy individuals on day 7 of treatment with ODE showed a similar change in BA profile, i.e., a decrease in plasma conjugated BAs and a slight increase in the unconjugated BAs DCA and CDCA (Graffner et al., 2016). The authors also observed an increase in fecal unconjugated BAs on day 7 of ODE treatment. Likewise, we simulated an increased amount of colonic unconjugated BAs, which can be directly translated into increased fecal amounts due to reduced intestinal ASBT-mediated uptake of these conjugated BAs. Treatment with 3 mg ODE is shown to significantly reduce plasma fibroblast growth factor 19 (FGF-19) levels in healthy individuals. This results in a reduced inhibition of hepatic BA synthesis, which is reflected by increased plasma levels of the BA precursor C4 (Graffner et al., 2016). It is important to note that the PBK model developed included only a basic adaptive response, which does not allow for dynamic changes in synthesis rate, microbiome composition, or intestinal membrane integrity. Therefore, we only modeled the effects of ODE upon a single administration. Quantitatively capturing dynamic changes in a computational model requires many assumptions and fitting of parameters, which has been done previously (Voronova et al., 2020). A major strength of our PBK model is that most input parameters are derived experimentally, but accordingly the adaptive response was not modelled in detail. Nevertheless, the current model was proven useful for the prediction of short-term effects in healthy individuals.
In the current work, we report an apparent Michaelis-Menten constant or $K_{\text{m,app}}$ because the value relates to the overall process of transport across the Caco-2 monolayer, including not only apical influx but also intracellular transport by bile acid binding protein (BABP) and basolateral efflux by organic solute transporter (OST) $\alpha/\beta$. The $K_{\text{m,app}}$ can differ between in vitro and in vivo test systems. For example, for taurocholic acid (TCA), the typical model BA for transport experiments, $K_{\text{m,app}}$ values for intestinal uptake ranging from 4.4 to 600 µM have been reported using transfected cell lines/oocytes, human precision cut intestinal slices, and a human perfusion study in vivo (Balakrishnan et al., 2006; Zhu et al., 2021; Li et al., 2018; Krag and Phillips, 1974). Besides biological differences in, e.g., OST$\alpha/\beta$ or BABP activity, the physical hydrodynamic barrier between the bulk solution and the surface with the transporter differs depending on the test system used. In vitro the restricted liquid flow in the vicinity of a culture insert will create a zone where the diffusional movement of molecules exceeds the convection. The formed layer is referred to as the unstirred water layer or ABL. Besides, the culture insert itself will affect the compound’s permeability (Korjamo et al., 2009). Balakrishnan et al. (2007) studied the effect of the ABL in culture inserts and derived an equation that can be used to correct the affinity of influx transporters for the ABL. This equation was employed in this work (Eq. 2) and resulted in a $K_{\text{m,app}}$ of 23 µM versus 39 µM when the resistance by the ABL was not considered. The intestinal peristaltic movements can be expected to drastically reduce the ABL in vivo, resulting in lower $K_{\text{m,app}}$ values. It has been concluded previously that the ABL does not play a clinically significant role in the intestinal absorption of drugs in vivo, and hence, $K_{\text{m,app}}$ values derived from static in vitro models should be corrected for the ABL before the values can be extrapolated to the in vivo situation (Korjamo et al., 2009). Where in vitro the ABL hampers a molecule's transport to a culture insert, in vivo a molecule first has to migrate through the mucus layer before it reaches the intestinal epithelium. Caco-2 cells represent cells of the enterocyte lineage and do not fully represent the cell types present in the human ileal epithelium. Especially the lack of mucin-producing goblet cells and thus a mucus layer could affect the $K_{\text{m,app}}$. The HT29-MTX cell line has emerged as a cell line that forms goblet cells and can be used to complement Caco-2 cells. Co-culturing HT29-MTX cells with Caco-2 cells results in similar gene expression profiles to gastrointestinal tissue, and a mucus layer on top of the epithelium (Pontier et al., 2001). Yet, coculturing HT29-MTX and Caco-2 cells did not modulate permeability compared to Caco-2 cells alone, and ergo does not provide an improved $K_{\text{m,app}}$ that takes into account the mucus effects (McCright et al., 2022; Lock et al., 2018). Due to the absence of functional differences in permeability between the coculture and monoculture system, we decided to use the well-characterized and robust Caco-2 monoculture system.

The current results underline the importance of active ileal and hepatic transport processes for accurate predictions of systemic plasma BA levels. We demonstrate that Caco-2 cells can be used to quantitatively study ODE’s inhibitory effects on GCA transport. By incorporating the obtained kinetic parameters in a PBK model we were able to accurately predict the changes in plasma BA levels upon a single oral dose of ODE. The current model can serve as a quantitative tool to predict alterations in plasma BA levels upon xenobiotic exposure.

References


Lipid Res 53, 25.149


Li, M., Vokral, I., Evers, B. et al. (2018). Human and rat precision-cut intestinal slices as ex vivo models to study bile acid uptake by the apical sodium-dependent bile acid transporter. Eur J Pharm Sci 121, 65-73. doi:10.1016/j.ejps.2018.05.005


Sabino, J., Vieira-Silva, S., Machiels, K. et al. (2016). Primary sclerosing cholangitis is characterised by intestinal dysbiosis independent from IBD. Gut 65, 1681-1689. doi:10.1136/gutjnl-2015-311004


Smirnova, E., Muthiah, M. D., Narayan, N. et al. (2022). Metabolic reprogramming of the intestinal microbiome with functional bile acid changes underlie the development of NAFLD. *Hepatology* 76, 1811-1824. doi:10.1002/hep.32568


Wang, J., Bakker, W., Zheng, W. et al. (2022). Exposure to the mycotoxin deoxynivalenol reduces the transport of conjugated bile acids by intestinal Caco-2 cells. *Arch Toxicol* 96, 1473-1482. doi:10.1007/s00204-022-03256-8


Conflict of interest
The authors do not report a conflict of interest.

Data availability
The model code can be found in supplementary file 1. The input parameters are provided in .xlsx format in supplementary file 2. In vitro transport data are available upon request from the corresponding author.

Acknowledgements
We would like to thank Wouter Bakker for his help with the LC-MS/MS measurements.