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A Physiologically Based Pharmacokinetic (PBPK) Model Exploring the Blood-Milk Barrier in Lactating Species

-A case study with oxytetracycline administered to dairy cows and goats-

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Abbreviations: AUC: area under the time concentration curve; BLQ: below the lower limit of quantification; C_{max} : maximal concentration; CV: coefficient of variation; EW: extracellular water; GSA: global sensitivity analysis; IIV: inter-individual variability; IM: intramuscular; IMI: inter-milking interval; IV: intravenous; IW: intracellular water; LLOQ: lower limit of quantification; MAPE: mean absolute percentage error; MP: milk to plasma ratio; MRL: maximum residue limit; OTC: oxytetracycline; PBPK: physiologically based pharmacokinetic; PK: pharmacokinetic; PI: prediction interval; R^2 : coefficient of determination; RV: residual error; SAEM: stochastic approximation expectation-maximization; T_{max} : time of the maximal concentration; ULOQ: upper limit of quantification; VASC: vascular space; VPC: visual predictive check; WHO: World Health Organization; WP: withdrawal period.

35 **1. Introduction**

36 Understanding the passage of drugs into milk is important in order to manage residues
37 in foodstuffs of animal origin thus optimizing the withdrawal period for both food safety
38 (limiting risks for consumers), and for economic reasons such as milk being discarded where
39 drug residues are below the maximum residue limits (MRLs). To address these issues, it is
40 essential to accurately predict the concentration of a drug in milk over time.

41

42 After administration, drugs are absorbed, distributed, and eliminated, following
43 pharmacokinetic (PK) processes that depend on the physico-chemical properties of the
44 compound and the physiology of the animal. Their distribution in milk depends on the same
45 PK processes which are adapted to the particularities of the udder physiology and the
46 composition of the milk. Milk is an emulsion of fat globules in an aqueous medium
47 containing soluble proteins, casein, and minerals.

48

49 The distribution of a compound in the different milk fractions (*i.e.*, cream, curd, whey)
50 depends on factors specific to the compound, such as its lipophilicity or ionization, as well as
51 factors specific to the milk, such as its composition or volume (Hotham and Hotham, 2015;
52 Ozdemir, 2018; Whittem, 2012).

53

54 Physiologically based pharmacokinetic (PBPK) models integrate the physico-chemical
55 properties of drugs and the physiological parameters of species (Lin *et al.*, 2016). In
56 particular, these models make predicting compound concentrations in various fluids and
57 tissues after different dosing regimens possible. They can be of particular interest for
58 predicting the excretion of compounds into the milk of various species.

59

60 In recent years, several PBPK models have been developed for use in veterinary
61 medicine. Most of them are used to predict tissues residues and withdrawal periods in farm
62 animals for food safety assessment (Lautz *et al.*, 2019). Few PBPK models describing the
63 excretion of drugs into milk exist for dairy cows (Leavens *et al.*, 2014; Li *et al.*, 2018;
64 Woodward and Whittem, 2019). At present, there are no models for dairy goats or ewes.

65

66 Moreover, some essential factors affecting distribution in milk are not taken into
67 account in the currently published models. For instance, excretion into milk was modeled as a

68 simple, first order elimination from plasma (Leavens *et al*, 2014) and did not account for
69 udder size, composition, and perfusion, or variation in the volume of milk produced and
70 milking frequency (Abduljalil *et al*, 2021). Other models are more physiological and describe
71 the structure of the udder (Li *et al*, 2018; Woodward and Whitem, 2019), but they do not
72 consider permeability-limited passive diffusion and active transport within mammary cells
73 and they do not describe the distribution in the different milk fractions or the binding of
74 molecules to the different milk constituents.

75

76 Oxytetracycline (OTC) is a broad-spectrum antibiotic from the tetracycline family. It
77 is widely used in veterinary medicine to treat infectious diseases (Craigmill *et al*, 2000). OTC
78 PK is well-known in multiple veterinary species (Aktas and Yarsan, 2017; Craigmill, 2003;
79 Nouws *et al*, 1985b; Rule *et al*, 2001). Briefly, it has a good bioavailability in cattle after
80 intramuscular (IM) injection and, is distributed to most tissues and fluids such as milk. It is
81 mainly excreted unchanged in the urine by glomerular filtration and tubular secretion and, to a
82 lesser extent, it is excreted unchanged in bile (Nouws *et al*, 1985b). Its off-label use led to
83 various adverse effects at higher doses (in the kidneys and liver) as well as violative residues
84 in edible tissues (Riad *et al*, 2021). The published OTC PK studies have resulted in the
85 development of PBPK models of OTC in non-lactating food animal species such as cattle
86 (Achenbach, 1995), sheep (Craigmill, 2003), and goats (Riad *et al*, 2021) as well as in dogs
87 (Lin *et al*, 2015) and salmon (Law, 1999), for different formulations. To the knowledge of
88 the authors of this study, however, there is no PBPK model to predict OTC distribution in
89 milk.

90

91 The objective of this study has been to develop a PBPK model to describe the
92 excretion of OTC in cow and goat milk. To be able to complete this model at a later stage and
93 come up with a generic model that can be used for other drugs and/or species, the
94 development a detailed model for OTC was attempted. Therefore, the development of a
95 rather detailed PBPK model, which could be adapted to the data available for these different
96 compounds and species was chosen.

97

98 The generic PBPK model thus developed was then applied in a case study to predict
99 OTC concentrations in cow and goat milk.

100

101 **2. Materials and methods**

102 OTC plasma and milk concentrations in cows and goats were obtained experimentally
103 prior to the development of the PBPK model in order to calibrate and/or validate the model.
104 *In vitro* permeability studies, in a Parallel Artificial Membrane Permeability Assay (PAMPA),
105 were performed in order to calibrate the (transcellular) passive diffusion of OTC from plasma
106 to milk. In the following paragraphs, the experimental studies (properties of OTC, animal
107 study, *in vitro* experimentation, and analytical methods) will be presented first. Then, the
108 development of the PBPK model (the global structure, the udder, and milk model). And
109 finally, the calibration and validation of the model will be elaborated.

110

111 2.1. Experimental studies

112 2.1.1. Chemicals

113 (All chemicals used in analytical assays were of analytical grade)

114 OTC in hydrochloride form (Terramycine solution injectable®; Zoetis, Malakoff, France)
115 was used for the *in-vivo* experiments. Its octanol-water partition coefficient (logP) and its pKa
116 were determined from Chemicalize (“Chemicalize - Instant Cheminformatics Solutions,”
117 n.d.). The logP of OTC was estimated to be -4.54.

118

119 OTC has several chemical groups which, depending on the pH, may have an electric
120 charge. The two main groups of OTC that can have an electric charge in physiological pH
121 (~7) have an acid pKa of 7.25 and a basic pKa of 5.80, respectively. According to Rodgers
122 and Rowland’s publications (Rodgers *et al*, 2005; Rodgers and Rowland, 2006), OTC was
123 considered as a zwitterion of group 2 since it has no basic pKa ≥ 7 . The ionization ratios of
124 OTC in the different media were calculated from the equations reported by Rodgers and
125 Rowland (see Equation 1) (Rodgers *et al*, 2005; Rodgers and Rowland, 2006).

126

$$127 \text{ Ionisation ratio} = 1 + 10^{pK_{a_{Base}} - pH_i} + 10^{pH_i - pK_{a_{Acid}}} \quad (1)$$

127

128 where pH_i is the pH of the i^{th} media, and $pK_{a_{Base}}$ and $pK_{a_{Acid}}$, the basic and acid pKa of OTC,
129 respectively.

130

131 2.1.2. Animals

132 Six (6) Prim' Holstein cows along with five (5) Alpine goats and one (1) crossbred goat
133 were included in the study which had been approved by the Animal Research Ethics Committee
134 (APAPHIS n° 19369-2019022111373719). Each animal received a drug-free diet and water *ad*
135 *libitum*. All animals were free of clinical mastitis. Goats and cows were milked twice daily:
136 at 8h and 16h--meaning a milking interval of 8 hours during the day and of 16 hours during
137 the night--by a milking unit and a milking robot, respectively. Each animal received a single
138 dose of OTC conventional formulation at 10 mg.kg⁻¹ by intramuscular (IM) route in the neck,
139 at several injection points. Blood samples were taken from jugular vein by venipuncture at
140 0.5, 1, 2, 3, 4, 6, and 8-hours post-administration and then, twice daily (in the morning and
141 evening, at the same time as milking) until the 4th day after the administration. Within 30 min
142 after sampling, blood samples were centrifuged at 3500 rpm at room temperature for 10
143 minutes and plasma was collected. Milk samples (about 20 mL) mixed from all the teats (4
144 for cows, 2 for goats) were taken at each milking starting at 8 hours post-administration and
145 continued up to 5 days. The volumes of milk collected were measured at each milking. The
146 butyrous and protein rates were quantified once for each individual's milk at the beginning of
147 the experiment (see the demographic characteristics of the animals are presented in the
148 **Table S1 of the supplementary materials**).

149

150 Blank samples of blood and milk (without drug) were collected before administration. All
151 samples were frozen and stored at -20°C and protected from light until analysis.

152

153 2.1.3. PAMPA experiments

154 The PAMPA, as first described by Kansy *et al*, was used as an *in vitro* model of passive
155 transcellular permeation (Kansy *et al*, 1998). PAMPA was performed using the Corning®
156 Gentest™ Pre-coated PAMPA Plate System (Corning, NY 14831, USA). This plate system
157 consisted of pre-coated inserts of structured layers of phospholipids, composed of hexadecane
158 in hexane (5 % v/v). The PAMPA protocol published by Corning® (Chen *et al*, 2008), with
159 minor adaptations, was performed as follows: A stock solution of 10mM OTC in DMSO was
160 prepared and further diluted in PBS to a final concentration of 200 µM (100 mg/L). A
161 volume of 300 µL of solution was added to each well of the receiver plate and 200 µL of PBS

162 were added to the pre coated filter plate's wells. The plate was then incubated for four hours
163 at room temperature, without agitation. After the incubation period, two (2)-100 μ L samples
164 were collected from each well and stored at -80°C until analysis.

165

166 After analytical analysis of the samples, the apparent permeability of OTC over the
167 synthetic membrane was calculated using the following formula (Equation 2) (Shanler *et al*,
168 2021):

$$P_{app} = \frac{-\ln\left(\frac{1-C_A(t)}{C_{eq}}\right)}{A \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t} \quad (2)$$

$$\text{with } C_{eq} = \frac{C_D(t) \times V_D + C_A(t) \times V_A}{V_D + V_A}$$

169 where A is the filter area (0.3 cm^2), V_D is the donor well volume (0.3 mL), V_A is the acceptor
170 well volume (0.2 mL), t is the incubation time (in seconds), $C_A(t)$ is the compound
171 concentration in acceptor well at time t, $C_D(t)$ is the compound concentration in donor well at
172 time t, C_{eq} corresponds to the concentration at equilibrium.

173

174 2.1.4. Analytical Methods

175 Plasma samples were assayed by Shimadzu high-performance liquid chromatography
176 system (Shimadzu, SBM-20A, Marne la Vallée, France) coupled with an ultraviolet detector
177 (Shimadzu, SDP-20A). A volume of 100 μ L of plasma sample was mixed with 50 μ L of
178 internal standard solution: demeclocycline hydrochloride at 12 $\mu\text{g/ml}$ in trichloroacetic acid
179 10% and centrifuged for 10 min at 14000 rpm at about 7°C . Supernate was transferred into a
180 vial with a polypropylene insert and 20 μ L of the solution were injected into the
181 chromatographic system. The analytical column was a Luna Omega Polar C_{18} ($150 \times 2.1 \text{ mm}$,
182 $5 \mu\text{m}$), fitted with a Luna Omega C_{18} precolumn ($4 \times 2.0 \text{ mm}$) (Phenomenex, Torrance,
183 California, USA). The mobile phase was composed of KH_2PO_4 solution at 0.025 mol/L and
184 pH 3.0 (adjusted with phosphoric acid), and an acetonitrile solution (90:10 [vol/vol]) and was
185 delivered isocratically at 0.3 mL/min. Rinsing of the needle with 100 μ L of a
186 methanol/water/isopropanol/acetonitrile mixture (25:25:25:25) was done between each
187 injection and repeated rinsing of the system with ACN and HPLC grade water in gradient
188 mode was used at the end of the run. The UV detection was at 355 nm. The retention time

189 was 8 min for OTC and 14 min for the internal standard. The lower and upper limits of
190 quantification (LLOQ and ULOQ) of the method were 0.05 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$,
191 respectively. The trueness and precision were characterized at five concentration levels (0.05,
192 0.2, 6, 10, and 15 $\mu\text{g/ml}$) with a relative bias between -7.1% and 5.8% for goats; -5.1% and
193 10.2% for cows; a repeatability between 0.1% and 2.0% for goats; 0.6% and 3.1% for cows;
194 and an intermediary precision between 1.5% and 3.0% for goats; 1.0% and 5.4% for cows.

195

196 The analytical method used to assay PAMPA samples in PBS was a modified version of
197 the method in plasma. The same sample preparation method and chromatographic conditions
198 were used as for plasma. The difference being the mathematical function for calibration
199 which was quadratic instead of linear due to a slight flattening of the curve at high
200 concentrations.

201 Milk samples were assayed by LC-MS/MS. Two (2) g of milk sample were mixed with
202 1 mL of water (HPLC grade). A volume of 200 μL of internal standard solution,
203 demeclocyclin hydrochloride at 1 $\mu\text{g/ml}$ was added, and the mixture was vortexed and left for
204 at least 10 minutes away the light. Extraction was made by adding 10 mL of Mac
205 Ilvaine/EDTA buffer, vortexed during 30 seconds, mixed with a rotary stirrer at 100 rpm for
206 10 min, and centrifuged 10 min at 14000 g at 4°C. Deproteination was made by adding 1 mL
207 of TCA at 1 g/mL, vortexed, frozen for 15 min and centrifuged 5 min at 14000 g. Purification
208 was made by solid phase extraction (SPE) using Bond Elut C18 cartridges (200 mg/3cc -
209 Agilent Technologies) and elution by oxalic acid solution at 0.01 mol/L before filtration with
210 0.45 μm filter. Five (5) μL were injected into the chromatographic system composed of
211 Quadrupole tandem mass spectrometer: Type TSQ Vantage (Thermo Scientific) with data
212 acquisition station: Xcalibur version 2.2.SP1. The analytical column was a Kinetex®
213 Biphenyl C₁₈ (100 x 2,1 mm, 2.6 μm) (Phenomenex, Torrance, California, USA). The mobile
214 phase A was a formic acid solution at 0.2% in water, and mobile phase B was a formic acid
215 solution at 0.2% in methanol. The gradient for mobile phase B was set at 5%, 98%, and 5% at
216 0.01, 5, and 6.1 min, respectively, with a flow rate of 0.35 mL/min. Heated-electrospray
217 ionization (H-ESI) in positive mode was used for the detection of OTC. Ions were analyzed
218 in the multiple reaction monitoring, and the following transitions were inspected: 461.1 m/z
219 \rightarrow $[\text{M}+\text{H}]^+ > 426.16 m/z$ and 465.1 $m/z \rightarrow$ $[\text{M}+\text{H}]^+ > 448.10 m/z$ for OTC and IS, respectively.
220 The ion used for quantification is the one with the m/z of 426.16 for OTC and the one with
221 the m/z of 448.10 for IS. The retention time was 4.4 min for OTC and 4.8 min for the internal

222 standard. The lower and upper limits of quantification of the method were 20 µg/kg and 1000
223 µg/kg (0.2 to 10 times the MRL), respectively. The trueness and precision were characterized
224 at six (6) concentration levels (20, 40, 100, 300, 500, and 1000 µg/kg) with a relative bias
225 between -0.1% and 1.8% for goats; -3.2% and 3.7% for cows; a repeatability between 0.5%
226 and 1.7% for goats; 1.0% and 1.6% for cows; and an intermediary precision between 3.3%
227 and 4.8% for goats; 1.0% and 4.8% for cows.

228

229

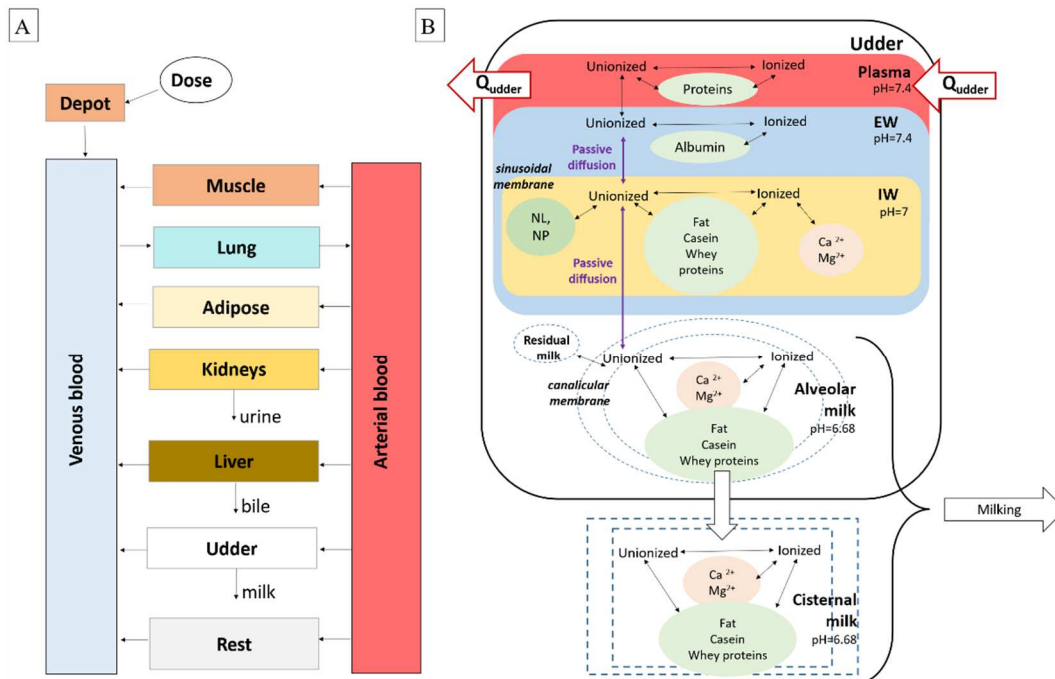


Figure 1: (A) Schematic diagram of the whole PBPK model to explore the blood-milk barrier in lactating species. Intramuscular (IM) administration was considered through the IM injection site compartment. The “rest” compartment was composed of remaining organs with no specific interest. (B) Schematic representation of the udder sub-compartments. The udder is described as a multi-compartmental, permeability-limited model, composed of an interstitial space consisting of extracellular water (EW), an intracellular water (IW) and the milk which is produced in the udder alveolar cells (alveolar milk) and stored in the cistern (cisternal milk). Passive diffusion between sub-compartments and binding to several proteins (*i.e.*, casein, whey proteins), lipids (NL for neutral lipids, NP for neutral phospholipids and ions (Ca^{2+} for calcium, Mg^{2+} for magnesium) were considered.

231

232 The PBPK model was composed of ten compartments corresponding to the main body
 233 organs and tissues (lung, adipose, muscle), blood compartments (arterial, venous), absorption
 234 compartment (IM injection site), excretory organs (kidneys, liver), tissue of interest (udder:
 235 see section 2.3), and a lumping compartment for the rest of the body (Figure 1A). All
 236 compartments interconnected by blood flows, with the exception of the IM injection site
 237 compartment, were defined by volume and blood flow rates obtained from published
 238 experimental studies with healthy animals for each species (**see Table S2 in supplementary**
 239 **materials**) (Lin *et al.*, 2020); (Li *et al.*, 2021); (Jackson and Cockcroft, 2002); (Upton, 2008);
 240 (Lin *et al.*, 2016); (Maltz *et al.*, 1984); (Ji *et al.*, 2017); (Lautz *et al.*, 2020); (Williams *et al.*,
 241 2007); (McCutcheon *et al.*, 1993); (Rodgers *et al.*, 2005). The distribution of OTC from the
 242 plasma to the tissues was considered as perfusion limited, consistent with previous PBPK
 243 models of OTC in cattle, sheep and goats (Craigmill, 2003; Riad *et al.*, 2021). A more
 244 detailed model was developed for the udder, taking into account the permeability data
 245 generated *in vitro* (see section 2.1.3).

246 The IM absorption was modelled by a single IM injection site compartment connected to
247 the venous blood compartment by an intramuscular absorption rate (k_{IM}) (Equation 3).

248

$$\frac{dA_{injection}}{dt} = -k_{IM} \times A_{injection} \quad (3)$$

249

250 with $A_{injection}$ the drug quantity in the IM injection site compartment calculated as

251 $A_{injection} = dose \times F_{IM}(\%)$ and F_{IM} as the intramuscular bioavailability. For each species
252 k_{IM} value was estimated by fitting the PBPK model with experimental plasma data from the
253 current study (see section 2.4), whereas F_{IM} values were obtained in the literature (Nouws *et*
254 *al*, 1985b; Rule *et al*, 2001) (see Table 1).

255

256 Drug partition between blood and plasma was based on the blood to plasma ratio (BP),
257 (see details in section 2.2.2 of the supplementary materials) and used to convert plasma
258 concentrations to blood concentrations.

259

260 Drug distribution was dependent on the tissue to plasma partition coefficient (k_{tissue})
261 for each tissue, defined as the ratio between total tissue drug concentration and total plasma
262 concentration (Yau *et al*, 2020). The k_{tissue} values were obtained in the literature from OTC
263 tissue and serum residues in sheep (Craigmill, 2003), with the exception of the lungs which
264 were estimated from residues data in cattle (Achenbach, 1995) (see details in section 2.2.3 of
265 the supplementary material) (see Table 1). For the lumping compartment, the partition
266 coefficient (k_{rest}) was calculated as the weighted mean of the partition coefficients of the
267 lumped organs (Equation 4) (Nestorov *et al*, 1998). As partition coefficients were missing for
268 these lumped tissues, the partition coefficient of the muscle was used for poorly perfused
269 tissues (brain and gastrointestinal tracts) and the partition coefficient of the kidneys was used
270 for richly perfused tissues (bones, carcass, heart, pancreas, spleen) (Lin *et al*, 2015).

271

$$k_{rest} = \frac{k_{muscle} \times V_{poorlyperfused} + k_{kidneys} \times V_{richlyperfused}}{V_{poorlyperfused} + V_{richlyperfused}} \quad (4)$$

272 where $V_{poorlyperfused} = V_{GItract} + V_{Intestines} + V_{Brain}$

273 and $V_{richlyperfused} = V_{Bones} + V_{Carcass} + V_{Heart} + V_{Pancreas} + V_{Spleen}$

274

275 With k_{muscle} and $k_{kidneys}$ as the tissue to plasma partition coefficient of the muscle and kidneys,
276 and V_{tissue} as the volume of tissue.

277

278 Distribution of drug in the non-eliminating organs is described using the following equation
279 (Equation 5):

$$\frac{dA_{tissue}}{dt} = Q_{tissue} \times \left(C_{art} - \frac{C_{tissue}}{k_{tissue}} \times BP \right) \quad (5)$$

280

281 with A_{tissue} the amount of drug in tissue, Q_{tissue} the tissue blood flow rate, C_{art} and C_{tissue} the
282 concentration of drug in arterial blood and tissue, respectively, and BP the blood to plasma
283 ratio and k_{tissue} the tissue to plasma partition coefficient.

284

285 OTC total plasma clearance values (CL_{plasma}) for both species was obtained by
286 averaging values reported in several articles for cattle (Craigmill *et al*, 2004; Nouws *et al*,
287 1985b), and goats (Aktas and Yarsan, 2017; Rule *et al*, 2001) (see Table 1). These clearances
288 were split between renal and hepatic clearances only based on the unchanged excreted
289 fraction in urine ($fe_{kidneys}$) reported in the literature (Nouws *et al*, 1985b) (see Table 1). The
290 excretion clearance in milk was considered independently of the systemic clearance (see
291 section 2.3). The differential equation describing the variation in the amount of OTC in the
292 eliminating organs (liver and kidney) is as follows (Equation 6):

293

$$\frac{dA_{tissue}}{dt} = Q_{tissue} \times \left(C_{art} - \frac{C_{tissue}}{k_{tissue}} \times BP \right) - Q_{tissue} \times ER_{tissue} \times C_{art} \quad (6)$$

294

295 with ER_{tissue} the extraction ratio of the corresponding tissue.

296 The extraction ratio of both organs were calculated based on CL_{plasma} and $fe_{kidneys}$ as follows
297 (Equations 7 and 8):

$$ER_{blood,kidneys} = \frac{CL_{plasma} \times fe_{kidneys}}{BP \times Q_{kidneys}} \quad (7)$$

$$ER_{blood,liver} = \frac{CL_{plasma} \times (1 - fe_{kidney})}{BP \times Q_{liver}} \quad (8)$$

299

300

301 2.3. Udder and Milk Model

302 The udder is described as a multi-compartment, permeability-limited model (Figure 1B).
 303 It is composed of a vascular space (VASC), an interstitial space consisting of extracellular
 304 water (EW), an intracellular water (IW) of mammary epithelial secretory cells, and 2
 305 compartments containing the milk. Between each milking, milk components were produced
 306 within the epithelial secretory cells (alveolar milk), then passed the canalicular membrane and
 307 reaching the cistern (cisternal milk) through the ducts.

308

309 This study hypothesized that only the unbound and unionized form of the drug diffused
 310 passively between EW and IW and between IW and alveolar milk. The fraction of ionized
 311 OTC in each sub-compartment was calculated based on the pH of these compartments and the
 312 pKas of OTC, and the diffusion rate between EW and IW and between IW and alveolar milk
 313 was estimated based on *in vitro* PAMPA results. In IW as in milk, OTC is presumed to be
 314 distributed between the fat and aqueous fractions and to bind to casein, whey proteins, ionized
 315 calcium, and magnesium.

316

317 2.3.1. Permeability

318 Passive diffusion, the main mechanism of OTC distribution into milk, was assessed by
 319 PAMPA experiments (Fujikawa *et al*, 2005) as detailed in section 2.1.3. This synthetic
 320 membrane permitted an estimation of the permeability (P_{app} in cm/s) (see Table 1)
 321 representing transcellular diffusion that could then be applied to sinusoidal and canalicular
 322 membranes. P_{app} was converted to passive milk clearance (CL_{diff} in L/h) according to the
 323 following equation (Badhan *et al*, 2014) (Equation 9):

324

$$CL_{diff} = P_{app} \times SA \times Tissue\ weight \quad (9)$$

325

326 where SA represented *in vivo* endothelial surface area (cm²/g) and tissue weight represented
327 the weight of the udder (g).

328

329 SA was not found in the literature for the udders of cows or goats; therefore, it was calculated
330 considering the cylindrical structure of capillary from geometric formula (Ng and
331 Hungerbühler, 2013) (see section 2.3.1 of the supplementary materials for calculation
332 details).

333

334 2.3.2. Extracellular water

335 In both the EW and the VASC, the pH was considered to be 7.4 (Rodgers *et al*, 2005) (see
336 Table 1), thus supposing instantaneous equilibrium in both spaces. The ratio between the
337 total EW concentration and the total blood concentration in the tissue vascular space ($K_{EW/B}$)
338 was used, allowing only one differential equation to determine the concentrations of OTC in
339 VASC and EW (Jamei *et al*, 2014).

340

341 In addition, OTC should bind to albumin in EW. Albumin concentration in EW was
342 found in the literature (Vestweber and Al-Ani, 1984) (see Table 1) and its affinity constant for
343 OTC was calculated based on the unbound fraction in plasma (see section 2.3.2.1 of the
344 supplementary materials, equation S20) (Rodgers and Rowland, 2006).

345

346 The variations of the amount of drug in the EW (A_{EW}) is described by the following
347 equation (Equation 10):

$$\frac{dA_{EW}}{dt} = Q_{udder} \times C_{art} - Q_{udder} \times \frac{Cu_{EW}}{fu_{EW}} \times \frac{1}{K_{EW/B}} + CL_{diff, sin} \times \left(\frac{Cu_{IW}}{X} - \frac{Cu_{EW}}{Y} \right) \quad (10)$$

348 with

$$349 \quad C_{u_{EW}} = \frac{A_{EW}}{V_{EW,eff}} \times f_{u_{EW}} ; \quad V_{EW,eff} = V_{EW} + \frac{V_{VASC}}{\frac{K_{EW}}{B}} ;$$

$$350 \quad V_{EW} = f_{EW} \times V_{udder} ; \quad V_{VASC} = f_{VASC} \times V_{udder}$$

351

352 where $f_{u_{EW}}$ is the unbound EW fraction, X and Y are the ionization ratios of OTC in IW and
 353 EW, respectively; $K_{EW/B}$ is the quotient of total EW concentration and the total blood
 354 concentration in the tissue vascular space (VASC); $CL_{diff,sin}$ is the clearance pertaining passive
 355 diffusion of drug across the sinusoidal membrane; $C_{u_{EW}}$ is the unbound concentration in EW;
 356 $C_{u_{IW}}$ is the unbound concentration in IW; f_{vasc} and f_{EW} are vascular and EW fractions, $V_{EW,eff}$
 357 is the volume of effective extracellular space which is a combination of EW and VASC; V_{EW}
 358 , V_{VASC} and V_{udder} are EW, vascular and udder volumes, respectively.

359

360 **(Details for calculations of X, Y, $f_{u_{EW}}$ and $K_{EW/B}$ were presented in section 2.3.2 of the**
 361 **supplementary materials)**

362

363 2.3.3. Intracellular water

364 In IW the pH was considered to be 7.0 (Rodgers *et al*, 2005) (see Table 1). Unionized
 365 OTC is assumed to bind to neutral lipids and phospholipids. The concentration of neutral
 366 lipids and phospholipids were fixed based on available information in the literature (Kinsella
 367 and McCarthy, 1968) (Larson, 1978). Their OTC affinity constants were calculated based on
 368 the drug lipophilicity (logP) and ionization rate in IW (X) following Rodgers and Rowland's
 369 equations (Rodgers *et al*, 2005; Rodgers and Rowland, 2006) (see section 2.3.2.2 of the
 370 **supplementary material**).

371

372 In addition, the IW compartment should contain the same components as milk, (*i.e.*, fat,
 373 casein, whey proteins [lactalbumin and lactoglobulin] and ionized calcium and magnesium).
 374 OTC should be distributed and bind to these components in IW as in milk.

375

376 The concentration of milk fat, casein, and whey proteins in IW were assumed to be
 377 identical to those in milk and are described in section 2.3.4.2. The concentration of ionized
 378 magnesium in IW was found in the literature (Martens and Stumpff, 2019). The

379 concentration of ionized calcium in IW was calculated based on the total concentration of
380 calcium in IW (Neville and Watters, 1983), and the free calcium fraction in IW that was
381 supposed to be the same as in milk (7%, see section 2.3.4.2). Calculations of their OTC
382 affinity constants are detailed in section 2.3.4.2.

383

384 The variation of the amount of drug in the IW (A_{IW}) was described by the following
385 equation (Equation 11):

386

$$\frac{dA_{IW}}{dt} = CL_{diff,sin} \times \left(\frac{Cu_{EW}}{Y} - \frac{Cu_{IW}}{X} \right) + CL_{diff,can} \times \left(\frac{Cu_{alveolar\ milk}}{Z} - \frac{Cu_{IW}}{X} \right) \quad (11)$$

387 with

$$388 \quad Cu_{IW} = \frac{A_{IW}}{V_{IW}} \times fu_{IW} ; V_{IW} = f_{IW} \times V_{udder}$$

389

390 where fu_{IW} is the unbound IW fraction; Z is the ionization ratio of OTC in milk; $CL_{diff,can}$ is
391 the passive diffusion of drug across the canalicular membrane; f_{IW} is the intracellular water
392 fraction; V_{IW} is the volume of udder intracellular water; and $Cu_{alveolar\ milk}$ is the unbound
393 concentration in alveolar milk space.

394 **(Details for the calculations of Z and fu_{IW} are presented in section 2.3.2.2 of the**
395 **supplementary materials)**

396

397 2.3.4. Milk

398 2.3.4.1. Production of milk

399 Milk is produced by the mammary epithelial secretory cells, exported into the alveolar
400 lumen, and transported by the ducts to the cistern where it is stored. The volume of milk
401 contained in the udder ($V_{milk,tot}$) is divided with 70% ($f_{alveolar}$) (see Table 1) in the alveolar
402 space (the alveolar lumen plus the ducts and the residual, see below) and 30% ($1-f_{alveolar}$) in
403 the cistern (Ayadi *et al*, 2003; Caja *et al*, 2004).

404

405 At each milking of the cows, the volume of milk collected ($V_{milk,obs}$) corresponded to 95%
406 ($1-f_{residual}$) of $V_{milk,tot}$ with 5% ($f_{residual}$) remaining in the alveolar space ($V_{residual}$) (Whittem,

407 2012), regardless of the value of inter-milking interval (IMI). In goats, $f_{residual}$ was considered
 408 to be 13% for an IMI of 16h and 19% for an IMI of 8h (Peaker and Blatchford, 1988).

409

410 In the current PBPK model, the volume of milk produced over a milking interval (V_{milk}
 411 (t)) used to compute the milk concentrations over time, is presumed to increase linearly over
 412 time until reaching the $V_{milk,obs}$ at the end of the milking interval (*i.e.*, when $t = IMI$). In this
 413 study, the volume of the available alveolar milk ($V_{alveolar}$ or milk volume of alveolar space and
 414 ducts, minus the residual milk) and the volume of the cisternal milk ($V_{cisternal}$) so that $V_{milk,tot}$,
 415 or total milk, was defined as the sum of alveolar, cisternal, and residual milk volumes (*e.g.*,
 416 $V_{milk,tot} = V_{alveolar} + V_{residual} + V_{cisternal}$).

417

418 Linear production of V_{milk} , $V_{alveolar}$, and $V_{cisternal}$ is described in the following equations
 419 (Equations 12-14):

420

$$V_{milk}(t) = (1 - f_{residual}) \times V_{residual} + t \times \left(\frac{V_{milk,obs} - (1 - f_{residual}) \times V_{residual}}{IMI} \right) \quad (12)$$

421

422 where $f_{residual}$ is the fraction of residual milk; $V_{residual}$ is the volume of milk remaining in the udder after
 423 the previous milking; t represents the time since the last milking; $V_{milk,obs}$ the volume of milk collected
 424 at each milking; and IMI the inter-milking interval (8 or 16h).

425 Thus,

426

$$427 \quad V_{milk,tot}(t) = \frac{V_{milk}(t)}{1 - f_{residual}}$$

428 Hence,

$$V_{alveolar}(t) = (f_{alveolar} - f_{residual}) \times V_{milk,tot}(t) \quad (13)$$

$$V_{cisternal}(t) = (1 - f_{alveolar}) \times V_{milk,tot}(t) \quad (14)$$

429 2.3.4.2. Drug distribution in udder and in milk

430 The pH of milk was inferred to be 6.68 for cattle and 6.65 for goats (Park *et al*, 2007) (see
431 Table 1). As described above, OTC in milk is distributed within the fats and aqueous phases
432 and bound to casein, whey, and ionized calcium and magnesium.

433

434 Milk concentrations of fat, casein, and whey proteins were calculated based on
435 experimentally measured butyrous and protein rates (see **Table S1 in supplementary**
436 **materials**). The concentration of fat (g/L) was determined by multiplying the butyrous rate
437 measured (g/kg) by the density of milk (kg/L) (Park *et al*, 2007). The total milk protein
438 concentration (g/L) was determined by multiplying the protein rate measured (g/kg) by the
439 density of the milk. The concentrations of casein and whey proteins were calculated
440 according to their respective proportions in the milk of each species (*i.e.*, 88/12% for cows
441 and 65/35% for goats) (Balthazar *et al*, 2017).

442

443 The percentage of free magnesium in milk (16%) was obtained in the literature (Oh and
444 Deeth, 2017) and multiplied by the total concentration of magnesium in milk of each species
445 (Balthazar *et al*, 2017) to obtain the ionized concentration of magnesium (see Table 1). The
446 percentage of free calcium in milk was estimated in cow's milk based on the ratio of free
447 calcium concentration (Neville and Watters, 1983) to total calcium concentration (Balthazar
448 *et al* . Due to missing values for free calcium concentration in other species, this percentage
449 in cattle was multiplied by the total calcium concentration in milk of each species (Balthazar
450 *et al*, 2017) to obtain the ionized calcium concentration (see Table 1).

451

452 Affinity constants for fat (k_{fat}) and casein (k_{casein}) were estimated from the measurement
453 of milk distribution of 15 drugs, published by Shappell and Lupton (Lupton *et al*, 2018;
454 Shappell *et al*, 2017). The k_{fat} and k_{casein} of each drug was calculated as the ratio of the
455 concentration in fat--or the concentration bound to casein--to the free concentration in milk
456 (total concentration in milk multiplied by the free fraction in milk). Then, a linear regression
457 was built between the logarithm of k_{fat} and the distribution coefficient (logD) of each drug
458 ($R^2 = 0.83$) (Equation 15) and another between the logarithm of k_{casein} and the distribution
459 coefficient of each drug ($R^2 = 0.80$) (Equation 16). Finally, the k_{fat} and k_{casein} of OTC in
460 milk and IW were calculated based on the logD of OTC using these equations.

461

$$\log(ka_{fat}) = 0.49 \times \log D - 3.112 \quad (15)$$

$$\log(ka_{casein}) = 0.29 \times \log D - 2.09 \quad (16)$$

with $\log D = \log P - \log X$ for IW

$\log D = \log P - \log Z$ for milk

462 The affinity constant of OTC for whey proteins (ka_{whey}) was found in the literature
463 (Shappell *et al*, 2017).

464

465 The affinity constants of OTC for calcium ($ka_{Ca^{2+}}$) and magnesium ($ka_{Mg^{2+}}$) were
466 estimated based on the results of Carlotti *et al* (Carlotti *et al*, 2012). Similar to fat and casein,
467 a linear regression was built between the logarithm of the OTC affinity constant for both
468 metal ions ($ka_{ion^{2+}}$ in L/mol) and the pH ($R^2 = 0.99$) (Equation 17). Thus, the $ka_{Mg^{2+}}$ and
469 $ka_{Ca^{2+}}$ were converted in L/g by dividing $ka_{ion^{2+}}$ by the molecular weight of each metal ion
470 and calculated as a function of the pH of IW or milk.

471

$$\log(ka_{ion^{2+}}) = 0.7085 \times \text{pH} - 2.0478 \quad (17)$$

472

473 where $ka_{ion^{2+}}$ is in L/mol and pH is the pH of IW (7.0) or milk (6.68 for cows, 6.65 for goats).

474 Hence, the unbound fraction in milk was defined in equation 18:

$$fu_{milk} = \frac{1}{(1+ka_{fat} \times [fat] + ka_{casein} \times [casein] + ka_{whey,prot} \times [whey,prot] + ka_{Mg^{2+}} \times [Mg^{2+}] + ka_{Ca^{2+}} \times [Ca^{2+}])} \quad (18)$$

475 (see calculation details in section 2.3.2.3 of the supplementary materials.)

476 The amount of drug in the alveolar ($A_{alveolar}$) and cisternal ($A_{cisternal}$) milk were described
477 by the following equations (Equations 19- 20):

$$\frac{dA_{alveolar\ milk}}{dt} = CL_{diff,can} \times \left(\frac{Cu_{IW}}{X} - \frac{Cu_{alveolar}}{Z} \right) - Q_{cistern} \times C_{alveolar} \quad (19)$$

$$\frac{dA_{cisternal\ milk}}{dt} = Q_{cistern} \times C_{alveolar} \quad (20)$$

478 with $Q_{cistern} = (1 - f_{alveolar}) \times \frac{V_{milk,obs}}{IMI}$; $Cu_{alveolar} = \frac{A_{alveolar}}{V_{alveolar}} \times fu_{milk}$

479

480 where $CL_{diff,can}$ is the passive diffusion clearance across the canalicular membrane; X and Z
 481 the ionization ratios in IW and alveolar milk; $Q_{cistern}$ the flow of milk from alveoli to cistern;
 482 $C_{alveolar}$ the OTC concentration in the alveolar milk; C_{uIW} and $C_{ualveolar}$ the OTC free
 483 concentrations in IW and alveolar milk; $f_{u,milk}$ the unbound fraction of OTC in milk; $f_{alveolar}$
 484 the fraction of alveolar milk; and $V_{alveolar}$ is the volume of alveolar milk.

485

486 Finally, the milk concentrations used for the PBPK model predictions over time (Equation 21)
 487 were calculated as the sum of alveolar and cisternal amounts of OTC divided by $V_{milk}(t)$, as
 488 defined in Equation 12.

489

$$C_{milk}(t) = \frac{A_{cisternal}(t) + A_{alveolar}(t)}{V_{milk}(t)} \quad (21)$$

490

491 Parameters generally used to describe and evaluate the passage of a molecule into milk,
 492 such as the milk to plasma ratio (M/P) (Equation 22), the milk clearance (CL_{milk}) (Equation
 493 23), and the percentage of the dose excreted in milk ($Dose_{percentage,milk}$) (Equation 24), were
 494 calculated.

$$M/P = \frac{AUC_{0-t,milk}}{AUC_{0-t,plasma}} \quad (22)$$

$$CL_{milk} = \frac{A_{excreted}}{AUC_{96h,plasma}} \quad (23)$$

$$Dose_{percentage,milk} = \frac{A_{excreted}}{Dose \times BW_i} \quad (24)$$

495 With AUC_{0-t} the area under the curve of milk or plasma total OTC concentrations over time
 496 (t); $A_{excreted}$ the total amount of OTC excreted in milk as predicted by the model; $Dose$ the
 497 weight-adjusted dose (10 mg/kg); and BW_i the body weight of animal "i".

498

499 Table 1: Parameter values used in the PBPK Model.

500
 501 Parameters in g/kg in the literature were converted to g/L using udder density (1.06 kg/L)
 502 (Upton, 2008) or milk density (1.031 kg/L for cows, 1.034 kg/L for goats) (Park *et al*, 2007).
 503 One value per row means that the same value was used for both species. The parameters
 504 identified by an asterisk were evaluated in the global sensitivity analysis (see section 2.6).
 505

Parameter	Abbreviation	Cow	Goat
Species physiology			
Alveolar fraction *	f_{alveoli}		0.7 ¹
Blood cell pH *	pH _{BC}		7.22 ²
Plasma pH *	pH _P		7.4 ²
Udder extracellular water pH *	pH _{EW}		7.4 ²
Udder intracellular water pH *	pH _{IW}		7.0 ²
Milk pH *	pH _{milk}	6.68 ³	6.65 ³
Udder vascular fraction*	f_{VASC}		0.12 ⁴
Udder extracellular water fraction *	f_{EW}		0.44 ⁵
Udder intracellular water fraction *	f_{IW}		0.24 ⁵
Udder neutral lipid fraction *	f_{NL}		0.0120 ^a
Udder neutral phospholipid fraction *	f_{NP}		0.0094 ^a
Plasma albumin (g/L) *	alb _P	30.81 ⁶	31.80 ⁷
Udder extracellular albumin (g/L) *	alb _{EW}		14.52 ⁸
Udder intracellular calcium (g/L) *	Ca ²⁺ _{IW}		0.0114 ^{9,10}
Udder intracellular magnesium (g/L) *	Mg ²⁺ _{IW}		0.024 ¹¹
Milk calcium (g/L) *	Ca ²⁺ _{MILK}	0.08 ^{9,10}	0.0931 ^{9,10}
Milk magnesium (g/L) *	Mg ²⁺ _{MILK}	0.018 ^{10,12}	0.024 ^{10,12}
Molecule parameters			
Compound type		Zwitterion of group 2 ¹³	
pKa of acid function *	pKa _{Acid}		7.25 ¹⁴
pKa of basic function *	pKa _{Base}		5.80 ¹⁴
Partition coefficient between octanol and water *	logP		-4.54 ¹⁴
Fraction unbound in plasma *	f_{up}	0.81 ¹⁵	0.80 ^b
Intramuscular bioavailability *	F_{IM}	0.88 ¹⁶	0.92 ¹⁷
Intramuscular absorption rate (1/h) *	k_{IM}	0.17 ^c	0.45 ^c
Total plasma clearance (L/h/kg) *	CL _{plasma}	0.09 ^d	0.14 ^e
Dose fraction excreted unchanged in urine	$f_{\text{e}_{\text{kidneys}}}$		0.85 ¹⁸
Blood to plasma ratio	BP	0.79 ^f	0.82 ^f
Partition coefficient	Muscle *	k_{muscle}	0.90 ¹⁹
	Lung *	k_{lung}	2.30 ²⁰
	Kidneys *	k_{kidneys}	6.62 ¹⁹
	Liver *	k_{liver}	1.87 ¹⁹
	Adipose *	k_{fat}	0.09 ¹⁹
	Lumping	k_{rest}	1.41 ^g
Udder permeability			
Capillary radius (mm) *	r		0.0025 ²¹
Udder blood flow fraction to lobules *	$f_{\text{VASC}_{\text{Lobules}}}$		0.98 ²²
PAMPA permeability (cm/s) *	Papp		1.4 * 10 ^{-7h}

506
 507 ^a Calculation based on lipid repartition of Kinsella *et al* (Kinsella and McCarthy, 1968) and total phospholipid concentration
 508 of Bruce *et al* (Larson, 1978).

509 ^b This value is the average of the unbound fraction in cows and sheep from Ziv *et al* (Ziv and Sulman, 1972).

510 ^c These values were estimated by fitting the PBPK model with experimental plasma data (see section 2.2).

511 ^dThis value is the average between plasmatic clearance of the empirical model of Craigmill *et al* (Craigmill *et al*, 2004) and
512 Nouws *et al* (Nouws *et al*, 1985b).
513 ^e This value is the average between plasmatic clearance from Aktas *et al* (Aktas and Yarsan, 2017) and Rule *et al* (Rule *et al*,
514 2001).
515 ^f See calculation details in Supplementary Material.
516 ^g See equation 4 in the text.
517 ^h Experimental data (see section 2.1.2).
518 Sources: ¹(Ayadi *et al*, 2003; Caja *et al*, 2004); ²(Rodgers *et al.*, 2005); ³(Park *et al.*, 2007); ⁴(Snipes and Lengemann, 1972);
519 ⁵(Konar *et al*, 1972); ⁶(Bobbo *et al*, 2017); ⁷(Alberghina *et al.*, 2010); ⁸(Vestweber and Al-Ani, 1984); ⁹(Neville and Watters,
520 1983); ¹⁰(Balthazar *et al*, 2017); ¹¹(Martens and Stumpff, 2019); ¹²(Oh and Deeth, 2017); ¹³(Rodgers and Rowland, 2006);
521 ¹⁴(“Chemicalize - Instant Cheminformatics Solutions,” n.d.); ¹⁵(Ziv and Sulman, 1972); ¹⁶(Nouws *et al*, 1985a); ¹⁷(Rule *et al.*,
522 2001); ¹⁸(Nouws *et al*, 1985b); ¹⁹(Craigmill, 2003); ²⁰(Achenbach, 1995); ²¹(Prosser, 1996); ²²(Thompson, 1980).

523

524 2.4. Model calibration

525 Individual plasma concentrations measured in the current study (study A, see Table 2)
526 were used to calibrate the rate of OTC absorption after IM administration (k_{IM}). The PBPK
527 model with all parameters fixed (except k_{IM}) was used (see Table 1) and only k_{IM} and its
528 standard deviation were estimated using the Stochastic Approximation Expectation-
529 Maximization (SAEM) algorithm method, for each species. For cows, four (4) concentrations
530 in milk and 8 concentrations in plasma were reported as below the LOQ. For goats, sixteen
531 (16) concentrations in milk and nineteen (19) concentrations in plasma were reported as
532 below the LLOQ. Data below the LOQ were treated as left-censored observations using the
533 M4 method (*i.e.*, the likelihood that they were between zero (0) and the LLOQ) was
534 calculated) (Beal, 2001).

535

536 The residual errors (RV) of the PBPK model were set to the value of the intermediate
537 precision of the analytical method (see section 2.1.4) in plasma and milk for each species,
538 assuming that the errors due to model misspecification or other unknown sources were
539 negligible.

540

541 Table 2: Experimental studies of OTC in cows and goats used for calibration and validation
 542 of the PBPK Model

Modelling purpose	Study ID	Route	Dose (mg/kg)	Species (n)	BW (kg)	Matrix	IMI	V _{milk}	TB, TP
Calibration (estimation of k_{IM}) ¹	A	IM	10 (SD)	Cow (6), Goat (6)	568	P	8h, 16h	Yes ^a	Yes ^b
Internal evaluation ¹	A	IM	10 (SD)	Cow (6), Goat (6)	568	M	8h, 16h	Yes ^a	Yes ^b
External evaluation ²	B	IV	5 (SD)	Cow (19)	603	P, M	NA	NA	NA
External evaluation ³	C	IM	10 (5 doses, OD)	Cow (20)	577	M	12h	Yes ^a	NA
External evaluation ³	D	IV	10 (SD)	Cow (4)	643	M	12h	Yes ^a	NA
External evaluation ³	E	IV + IM	10 (SD) for IV + 10 (4 days, OD) for IM	Cow (4)	634	M	12h	Yes ^a	NA
External evaluation ⁴	F	IM	20 (SD)	Goat (5)	56	P, M	24h	NA	NA
External evaluation ⁴	G	IV	20 (SD)	Goat (5)	56	P, M	24h	NA	NA

543
 544 ^a At each milking; ^b Once per animal before OTC administration
 545 Note: IM: intramuscular; k_{IM} : intramuscular absorption rate; IV: Intravenous; SD: single dose; OD: once daily; P: plasma;
 546 M: milk; NA: not available; IMI: inter-milking interval; V_{milk}: Volume of milk measured, TB: butyrous rate; TP: protein
 547 rate. Sources: ¹Current study; ²(Nouws *et al*, 1985a); ³Unpublished study; ⁴(Rule *et al.*, 2001)
 548
 549

550 2.5. Establishment of the Population PBPK (popPBPK) Model

551 Monte Carlo simulations were performed to generate a virtual population of cows and
 552 goats (n=1000 each) taking into account the inter-individual variability (IIV) and uncertainties
 553 of the parameters from the PBPK model. A log-normal distribution was assumed for all
 554 parameters because of their asymmetric distribution and their strictly positive values
 555 (Fenneteau *et al*, 2009), and was assumed to represent the IIV (for physiological parameters)

556 and uncertainty (for physico-chemical parameters). Coefficients of variation (CV) were
557 found in the literature based on previous experimental data or set at 30% for physiological and
558 20% for drug-related parameters with no experimental values, (see **Table S3 for cow and S4**
559 **for goat in the section 2.4 of the supplementary materials**) (Clewell and Clewell, 2008;
560 Henri *et al*, 2017a; Li *et al*, 2019a).

561

562 The code was written to ensure that the sum of the volume fractions or that of the blood
563 flow fractions did not exceed 1, (Fisher *et al*, 2020) (see **code in section 4. of the**
564 **supplementary materials**).

565

566 2.6. Model Evaluation: Validation and Sensitivity Analysis

567 The evaluation of the popPBPK model was carried out by graphical goodness-of-fit
568 (GOF) and statistical criteria, with an internal validation comparing the model predictions to
569 the experimental data generated in the study (study A), and an external validation comparing
570 the predictions to data from the literature and unpublished data (studies B to G, see Table 2).
571 If predictions fell within a factor of 2 of the experimental data, the popPBPK model was
572 considered as acceptable by the World Health Organization (WHO) (WHO *et al*, 2010). The
573 mean absolute percentage error (MAPE) and the determination coefficient (R^2), based on
574 linear regression of model observations *vs* simulations, were also calculated to assess GOF
575 and model performance (Cheng *et al*, 2016). The popPBPK model was considered as valid if
576 the MAPE value was lower than 50% and the R^2 was at least 0.75 (Li *et al*, 2018) (Li *et al*,
577 2019b). Visual Predictive Checks (VPC) were generated by performing Monte Carlo
578 simulations (n= 1000 animals), considering both IIV and RV. The milk and plasma
579 predictions interval (PI) (5th and 95th percentiles) were plotted against the experimental data.

580

581 The external validation was conducted using average concentrations (and the standard
582 deviation if available) from external and independent datasets for each species treated with a
583 conventional OTC formulation with different dosing regimens (dose and route of
584 administration) (studies B to G see Table 2). Plasma and milk data found in the literature
585 were extracted from selected studies using WebPlotDigitizer (version 4.4, year 2020,
586 California USA, <https://automeris.io/WebPlotDigitizer>).

587

588 For study B, the IMI was not specified in the article and was set at eight (8) and sixteen
589 (16) hours, as in the current study. When some parameters were not specified, such as
590 volume of milk produced per milking (for studies B, F and G), and fat, casein, and protein
591 levels (for all external data sets), they were simulated (as explained below).

592

593 In order to simulate milk volumes of the 1000 animals, a linear mixed effects model
594 (SAEM algorithm with Monolix software) was fit to to the experimentally measured milk
595 volumes (study A, see Table 2) (performed separately for cows than for goats). This model
596 included an intercept, an effect of milking interval, and inter- and intra- animal variability.
597 From this model different volumes of milk for each animal (n=1000) at each milking could be
598 simulated.

599

600 To simulate the milk fat, casein and whey proteins levels and the body weights of the
601 1000 animals, data (mean and dispersion value) was used from the literature ((Balthazar *et al*,
602 2017; Leitner *et al*, 2004) for milk composition; (Nouws *et al*, 1985a; Rule *et al*, 2001) and
603 unpublished study for body weight) to draw different values for each simulated animal
604 (n=1000) of each species. The nominal doses administered (in mg) were adjusted to match
605 the simulated weights for each animal of each species.

606

607 The ability of the model to predict concentrations of OTC in the milk of cows and goats,
608 with other doses and routes of administration, was assessed by comparing the external dataset
609 with the 90% PI from the VPC.

610

611 A global sensitivity analysis (GSA), using the “*extended-FAST*” method (Saltelli and
612 Bolado, 1998), was performed to assess the influence of sixty-six (66) input parameters on
613 milk model predictions (see **parameters with an asterisk in Table 1 and Table S2 in
614 supplementary materials**). A uniform law was considered and each parameter was changed
615 by $\pm 10\%$ of the median value, simultaneously (Peters, 2012). With the GSA, input parameter
616 influence could be divided into main effects and total effects where the difference between
617 main and total effects (additional effects) represented parameter interactions. Because total
618 effects are the most robust indicators of PBPK parameter influences (Jarrett *et al*, 2016), they
619 are the only ones discussed here. The outputs considered were the area under the curve of

620 OTC total concentration in milk between time 0 hours and 96 hours (AUC_{96h}) and OTC total
621 concentration in milk at 96 hours (C_{96h}). Total effects above the typical threshold value of 0.1
622 indicated significantly sensitive parameters (Jarrett *et al*, 2016; Li *et al*, 2017; Peters, 2012).

623

624 Moreover, variations of a factor 10 of the nominal value of the Papp, which can vary far
625 more than 10% between drugs, have been explored (see section 3.3).

626

627 2.7. Model Application

628 An estimation of the withdrawal period (WP) in milk was performed using the popPBPK
629 model, based on results of the Monte Carlo simulations ($n=1000$) with the IIV but without the
630 RV, with the standard dosing regimen specified in the SPC of the Terramycin® speciality
631 (*i.e.*, 10 mg/kg/day for four (4) days or 5 mg/kg/day for three (3) days by IM route). The time
632 (rounded up to the next whole day) for which the 99th percentile is equal to the MRL (100
633 $\mu\text{g}/\text{kg}$ for milk) since the last injection was defined as the (predicted) WP in milk. Volume of
634 milk and rate of milk fat, casein, and whey proteins, of each animal were drawn as explained
635 above (see section 2.6). Body weights were simulated based on mean and dispersion values
636 from the literature (Lautz *et al*, 2020).

637

638 2.8. Software

639 Monolix (version 2020R1. Antony, France: Lixoft SAS, 2020) was used to develop the
640 popPBPK model and Simulx (version 2020R1. Antony, France: Lixoft SAS, 2020) to run all
641 simulations. **(See the model code available in Supplementary Materials)**. Graphs were
642 generated using GraphPad Prism (version 9.1.0 for Windows, GraphPad Software, San Diego,
643 California USA, www.graphpad.com). GSA was performed with Rstudio (v 1.4.1106,
644 RStudio Team, 2009-2021, PBC, Boston, MA, <http://www.rstudio.com/>) and the sensitivity
645 package (v 1.26.0; Iooss 2021; <https://CRAN.R-project.org/package=sensitivity>).

646

647

648 3. **Results**

649 3.1. Model Calibration

650 The observed plasma data BLQ (18%) were used to estimate only the IM absorption rates
651 (k_{IM}) for each species with a very good confidence (RSE < 15%), given a typical value of
652 0.17 1/h and 0.45 1/h for cows and goats, respectively (**see section 3.1.1 of the**
653 **supplementary materials**). The associated IIV values (used for the popPBPK model
654 application, see below) were equal to 22% for cows and 30% for goats, with rather good
655 precision (RSE < 30%).

656

657 3.2. Simulations from the popPBPK Model

658 One thousand (1000) virtual individuals of each species with specific body weight,
659 volume, and composition of milk produced were simulated (**see the distribution and range**
660 **of all parameters of these virtual populations are presented in section 2.4. of the**
661 **supplementary materials**).

662

663 3.3. Model Evaluation: Validation and Sensitivity Analysis

664 For internal validation using the observed concentrations from the current study (study A,
665 see Table 2), the VPC showed that most of the observed data (95%) in both plasma and milk
666 were within the 90% PI for both species (Figure 2 for cows and Figure 3 for goats). The BLQ
667 data were also well predicted.

668

669 In cow's milk (Figure 2B and D), 73% of typical predictions were within a factor of 2 of
670 the experimental data. The R^2 and the MAPE were 0.92 and 29%, respectively, which met the
671 validation criteria in cow's milk (Figure 2F).

672

673 Similarly, to the cow model, 67% of the typical predictions in goat's milk were within 2-
674 fold of the experimental data (Figure 3B and D). The R^2 and MAPE of 0.99 and 12%, met the
675 validation criteria in goat's milk (Figure 3F). The internal validation of our popPBPK model,
676 based on this experimental data, was therefore considered successful.

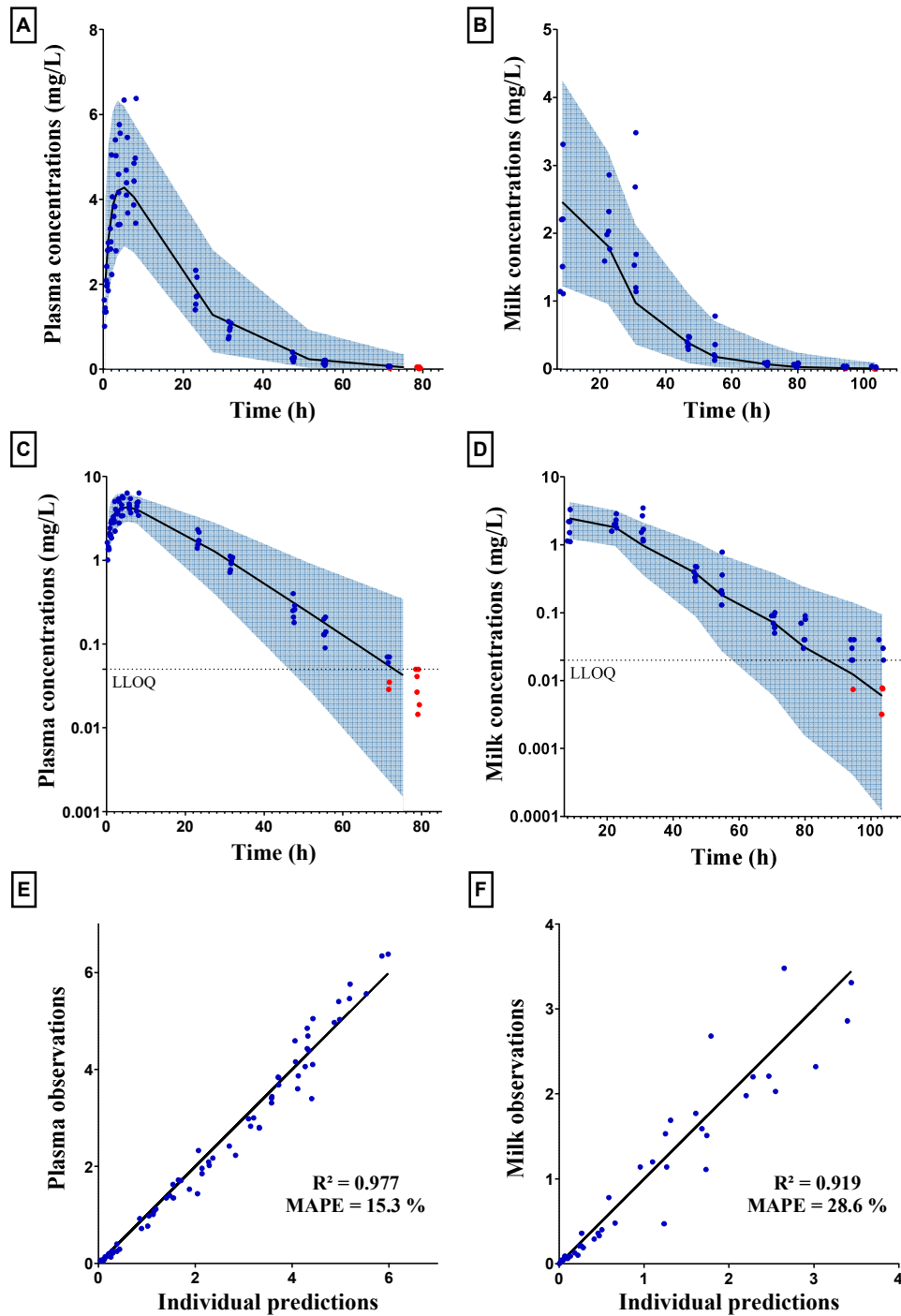


Figure 2: Visual predictive check of OTC concentrations in cow plasma (A, C) and milk (B, D) using the popPBPK model. The median is represented by a solid black line and the 90% prediction interval is represented by the area in blue. The experimental data (individual data points) from the current study are represented with circular points. The dotted line represents the lower limit of quantification (LLOQ, 0.05 $\mu\text{g/ml}$ for plasma, 0.02 $\mu\text{g/ml}$ for milk). Data below the LLOQ are marked in red and estimated by the model. The bottom graphs illustrate the result of a regression analysis between model predictions and measured OTC concentrations in plasma (E) and in milk (F). The coefficient of determination (R^2) and mean absolute percentage error (MAPE) are shown in the graphs.

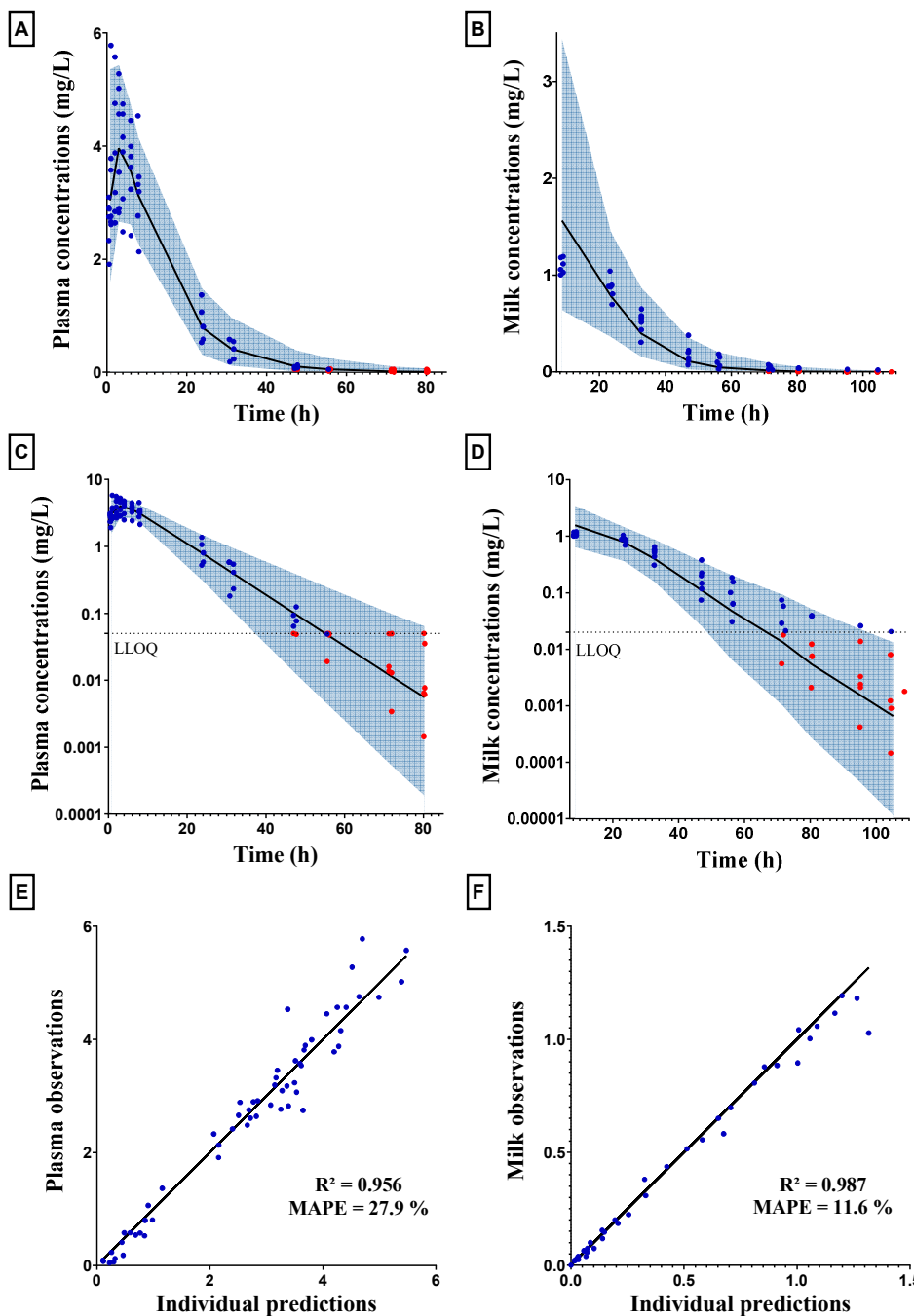


Figure 3: Visual predictive check of OTC concentrations in goat plasma (A, C) and milk (B, D) using the popPBPK model. The median is represented by a solid black line and the 90% prediction interval is represented by the area in blue. The experimental data from the current study are represented by circular points. The dotted line represents the lower limit of quantification (LLOQ, 0.05 $\mu\text{g/ml}$ for plasma, 0.02 $\mu\text{g/ml}$ for milk). Data below the LLOQ are marked in red and estimated by the model. The bottom graphs represent the result of a regression analysis between model predictions and measured OTC concentrations in plasma (E) and in milk (F). The coefficient of determination (R^2) and mean absolute percentage error (MAPE) are shown in the panel.

678

679

680 For the external validation in cows, (see **Figures S3 and S4 of the supplementary**
 681 **materials**), after IV of 5 mg/kg (study B, see Table 2), the model underestimated the initial
 682 plasma concentration (the typical predictions for the first 0.6 hours were 33-47% of the

683 observed mean values), but concentrations measured at late time points were well predicted
684 (within a factor of 2 after one-hour post administration). In milk, 86% of the predictions were
685 within a factor of 2 of the observed data. After five (5) IM administrations of 10 mg/kg/day
686 (study C), the model well predicted (within a factor of 2) milk concentrations up to 168 hour
687 and under-estimated the final time points (predictions of the final time points were lower than
688 37% of the observed data). Overall, 78% of the milk predictions were within a factor of 2 of
689 the observed data. After IV administration of 10 mg/kg (study D), the model underestimated
690 the initial plasma concentrations, predicted milk concentrations well for up to ninety-six (96)
691 hours post-administration, but under-estimated the final time points. The LLOQ of the
692 unpublished data was 0.01 µg/mL, and 3, 4, 2, and 4 animals were BLQ at 108 hours, 120
693 hours, 132 hours, and 144 hours after administration, respectively. After one IV and four IM
694 injections of 10 mg/kg (study E), 72% of the predictions were within a factor of 2 of the
695 observed data.

696

697 For the external validation in goats, (see **Figure S5 A-B in the section 3.2 of the**
698 **supplementary materials**), after IM administration of 20 mg/kg (study F), the model slightly
699 underestimated the final time points in plasma (but still within a factor of 2). In milk, 60% of
700 model predictions were within a factor of 2 of the observed data. After IV administration of
701 20 mg/kg (see **Figure S5 C-D in the section 3.2 of the supplementary materials**) (study G),
702 the model overpredicted the late plasma concentrations. In milk, 88% of predictions were
703 within a factor of 2 of the observed data.

704

705 Overall, based on external data on OTC concentrations in cow or goat milk from the
706 literature or unpublished dataset, the popPBPK model was considered to be valid.

707

708 A GSA was carried out for sixty-six (66) parameters of the PBPK model, (**identified by**
709 **an asterisk in Table 1 and Table S2 in supplementary materials**). The results are
710 presented in Figure 4 for AUC_{96h} and C_{96h} in cow and goat milk. Three parameters having a
711 sensitivity coefficient (total effect) higher than 0.1 were considered to have a significant
712 impact on AUC_{96h} for both species. Seven (7) parameters for cows and height parameters for
713 goats were considered to have a significant impact on C_{96h}.

714

715 The predicted AUC_{96h} in milk was sensitive to the acid pK_a of OTC (0.42 for cows, 0.48
 716 for goats), the pH of EW (0.39 for cows, 0.42 for goats) and the pH of milk (total effect of
 717 0.15 for both species). The predicted C_{96h} in milk was also sensitive to these three parameters
 718 but also to the pH of IW (0.77 for cows, 0.80 for goats), the total plasma clearance (CL_{plasma} ,
 719 coefficient of 0.23 for cows, 0.34 for goats), the partition coefficient in muscle (k_{muscle} ,
 720 coefficient of 0.21 for cows, 0.56 for goats), and in kidneys (k_{kidney} , coefficient 0.10 for cows
 721 and of 0.54 for goats), and to the F_{IM} (0.13) for goats.

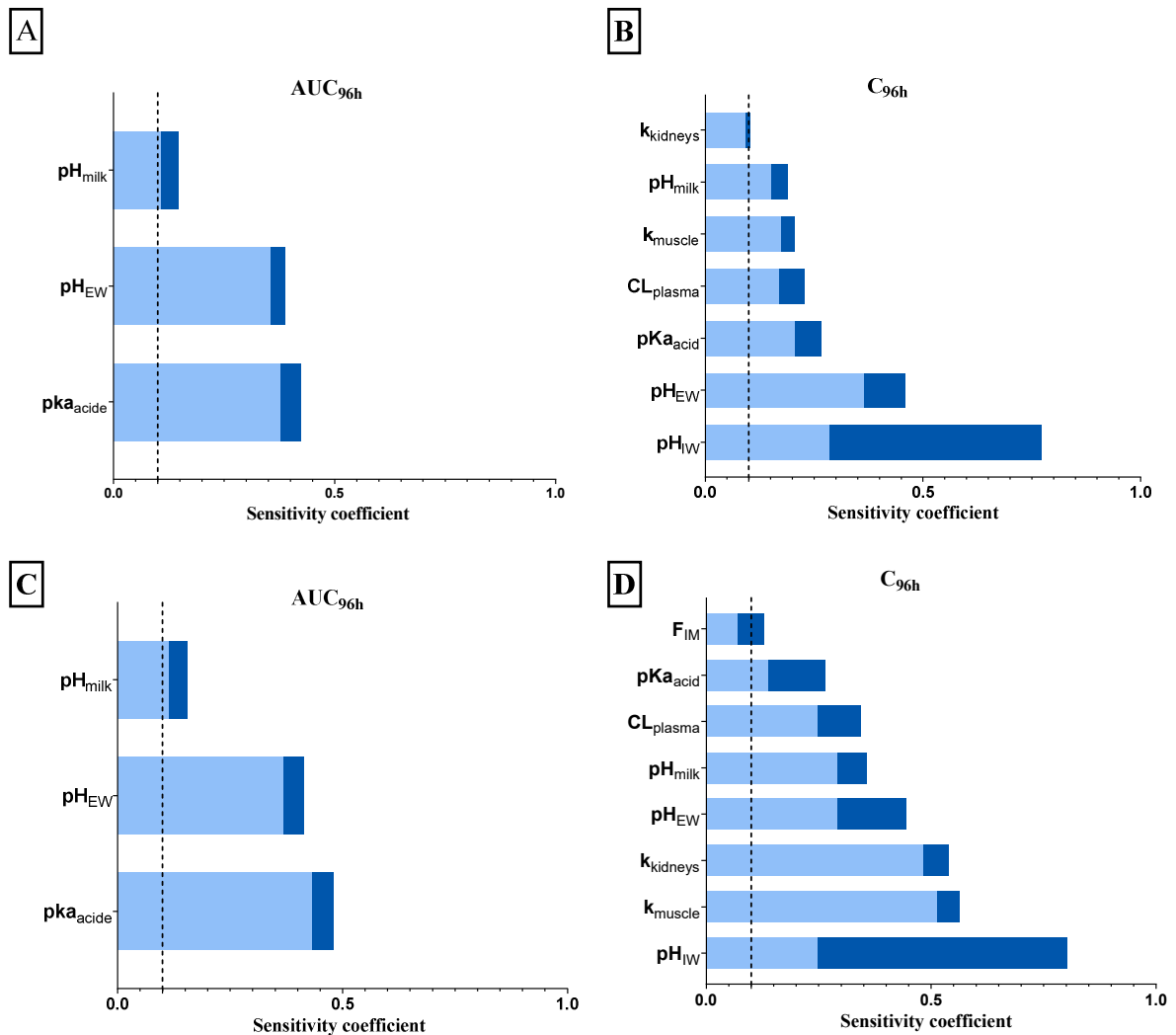


Figure 4: Global sensitivity analysis of the PBPK model of OTC in cow milk (upper panel) and in goat milk (lower panel). The sensitivity coefficient was estimated after a + 10% variation of the sixty-six (66) input parameters on OTC area under the curve (AUC) between 0 and 96h (A, C), and concentration at 96h in milk (B, D). The main effects are presented in light blue and total effects in dark blue. The dotted lines represent the threshold of 0.1 for determining the impact of a parameter.

722

723 To illustrate the influence of the milk pH, a change of 10% of its initial value in cows
 724 (6.68), resulted in a variation of the AUC of its initial value (68 mg/L.h) ranging from -18%

725 (56 mg/L.h) when the pH is lower (6.01) to +84% (125 mg/L.h) when the pH is higher (7.35)
726 (see Figure 5A).

727

728 To illustrate the influence of the OTC acid pKa, a 10% change in its initial value (7.25),
729 resulted in a variation of the AUC of its initial value (68 mg/L.h) ranging from -60% (28
730 mg/L.h) when the acid pKa is lower (6.53) to +67% (113 mg/L.h) when the pH is higher
731 (7.98) (see Figure 5B).

732

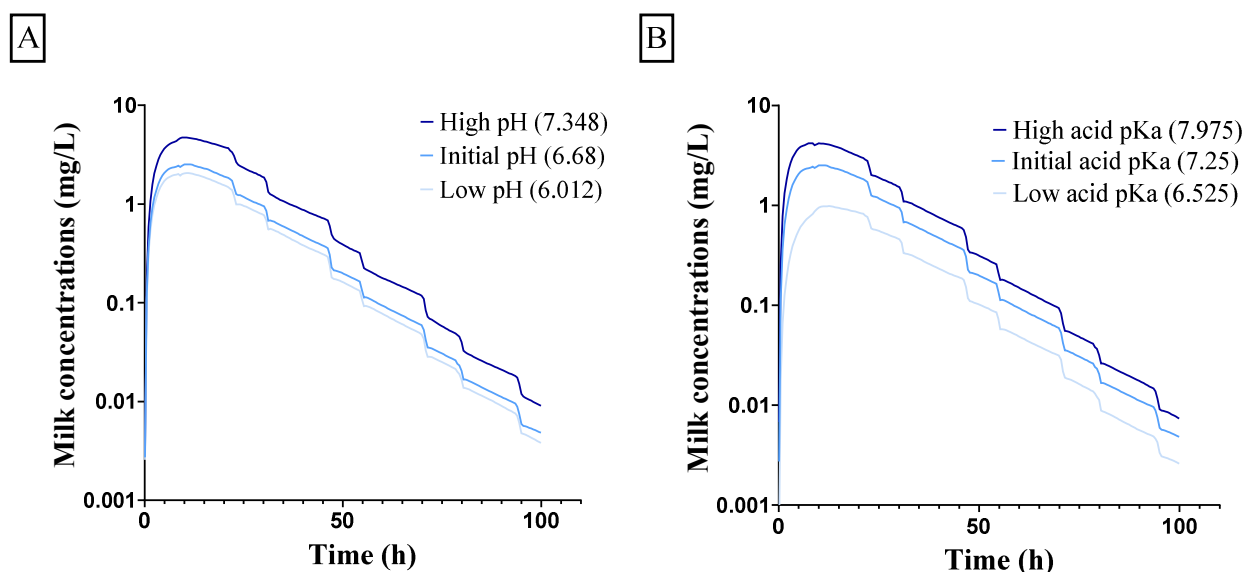


Figure 5: PBPK model predictions of OTC in cow milk with a variation of $\pm 10\%$ of milk pH (A) and acid pKa of OTC (B).

733

734 The impact of permeability (P_{app}) on OTC excretion into milk with hypothetical lower or
735 higher permeability was also evaluated. The concentration profiles in cow's milk are
736 presented for four (4) different P_{app} values in Figure 6. Thus, when P_{app} increases from 1.4×10^{-8} cm/s (molecules with very low permeability) to 1.4×10^{-5} cm/s (molecules with high
737 permeability), the model predicts that AUC_{96h} increases by 626% (from 17 h.mg/L to 103
738 h.mg/L). Furthermore, in this case T_{max} in milk decreases from 19 hours to 6 hours and the
739 maximum concentration (C_{max}) increases from 0.4 mg/L to 4 mg/L. However, between a
740 P_{app} of 10^{-5} (AUC_{96h} of 103 h.mg/L) and 10^{-6} cm/s (AUC of 99 h.mg/L), the impact on AUC
741 was negligible.

742

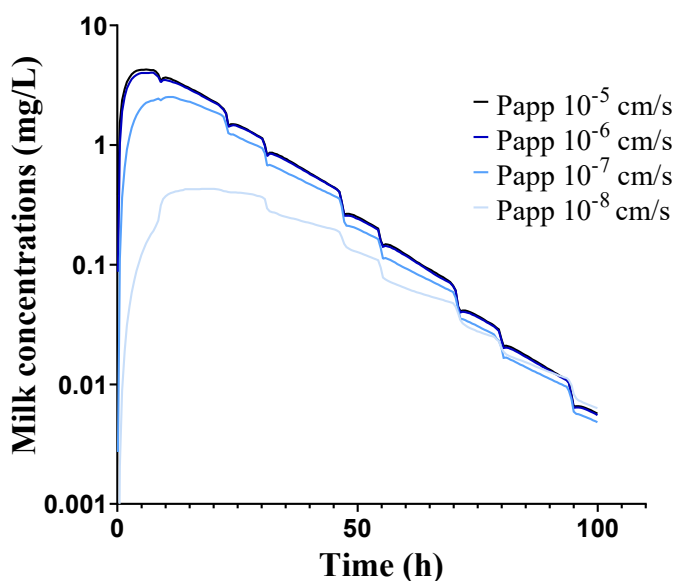


Figure 6: PBPK model predictions of OTC in cow milk with a variation of P_{app} from 10^{-8} to 10^{-5} cm/s.

743

744 3.4. Drug distribution in udder and milk

745 The total and free OTC concentrations, over time, within the plasma, udder sub-
 746 compartments, and the milk as predicted by the popPBPK model are presented in
 747 supplementary materials in Figure S6 A, C for cows and Figure S7 A, C for goats. Free
 748 concentrations were higher in plasma and EW (which were superimposed) than in IW and
 749 milk. In comparison, total concentrations were higher in IW but still lower than those in
 750 plasma and EW. The elimination slopes were parallel in all sub-compartments. In total
 751 concentrations, the T_{max} in plasma and EW were equivalent (5 hours for cows, 3 hours for
 752 goats), whereas that of IW (7.5 hours for cows, 6 hours for goats) and milk (11 hours for
 753 cows, 8 hours for goats) were delayed.

754

755 Proportions of free or bound OTC to each component are detailed on Figure S6 B for
 756 cows and Figure S7 B for goats in supplementary materials. In plasma and EW, the model
 757 predicts that OTC is mainly in unbound form (about 81% and 90% respectively) and weakly
 758 bound to albumin. In IW, the model predicts that OTC is more strongly bound (the unbound
 759 fraction is about 47%) and that binding is mainly to magnesium (Mg^{2+}). In milk the model
 760 predicts that OTC is approximately 60% bound: primarily to Ca^{2+} (~40%) and Mg^{2+}
 761 (~15%).

762

763 Proportions of ionized or unionized OTC in each sub-compartment are presented in
764 Figures S6 D for cows and S7 D for goats in supplementary materials. The fraction of ionized
765 OTC was predicted to be 60% in plasma and EW (pH=7.4), 39% in IW (pH=7.0), and 29% in
766 milk (pH=6.68 for cows, 6.65 for goats), highlighting again the strong influence of pH on
767 OTC distribution.

768

769 Based on the results of the popPBPK model, the ratio of M/P (Eq 22, calculated
770 between 0 hours and 96 hours), was 0.7 for cows and 0.5 for goats. The CL_{milk} was estimated
771 as 0.44 L/h ($7.76 \cdot 10^{-4}$ L/h/kg) and 0.051 L/h ($7.67 \cdot 10^{-4}$ L/h/kg) for cows and goats,
772 respectively.

773

774 Overall, 0.75% of the dose was excreted in the milk for cows, whereas it was 0.50 % for
775 goats.

776

777 3.5. Model Application

778 In milk, the WP after IM injections of 10 mg/kg/day for four (4) days and 5 mg/kg/day
779 for three (3) days were, predicted to be five (5) and four (4) days respectively for cow milk,
780 whereas it was predicted to be four (4) and three (3) days for goat milk (Figure 7).

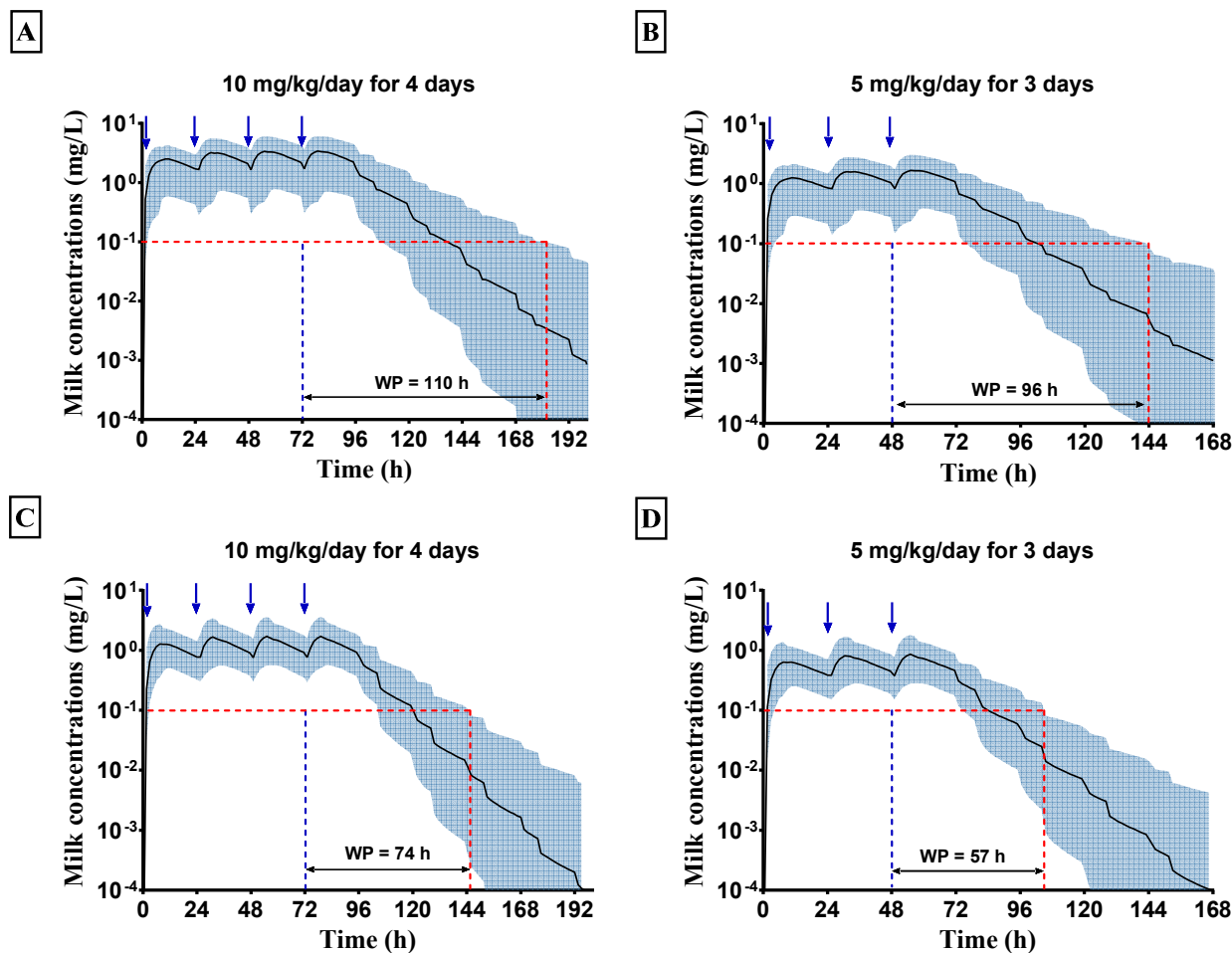


Figure 7: Simulation of OTC administration in 10 mg/kg/day for four (4) days (A,C) and 5 mg/kg/day for three (3) days (B,D) by IM route in cow (A,B) and goat milk (C,D). The maximum residue limit (MRL) is represented by red dotted horizontal lines and the time when the 99th percentile is equal to the MRL by red dotted vertical lines. WP is calculated by the difference between this time and the time of the last injection: seventy-two (72) hours or forty-eight (48) hours.

781

782

783 4. Discussion

784 4.1. PBPK model objectives

785 A generic popPBPK model was developed, detailed in terms of udder physiology and
 786 milk composition, in order to exploit it to various compounds and species. To illustrate its
 787 usefulness, the model was used to predict the kinetic of OTC in cow and goat milk.

788

789 This model predicted the OTC concentrations measured in plasma and milk from cows
 790 and goats well. In this study, the M/P ratio for the cows was 0.7, which is slightly lower than
 791 the previously published value (1.2 to 2), but consistent with the observed value (0.7
 792 calculated with a non-compartmental analysis from the observed data) (Nouws *et al*, 1985a).
 793 For goats, it was 0.5 (no value in the literature for comparison was found). The predicted

794 percentage of the dose excreted in milk (0.8% for cows and 0.5 % for goats) was equal to that
795 observed with the experimental data.

796

797 The model predictions compared to external data obtained in cows with different
798 dosing regimens were also satisfactory. However, the predicted milk concentrations for more
799 than ninety-six (96) hours after the last administration occasionally underestimated the
800 average concentrations measured (of that found in unpublished studies C, D, E). As these
801 concentrations were well below the quantification limits, this potential bias is perhaps
802 analytical. On the other hand, since the measured plasma concentrations are not available
803 with these external data sets, it cannot be known whether this bias is due to poor plasma
804 predictions or only to poor milk predictions. During the external validation, an
805 underestimation of the initial plasma concentrations by this study model was also observed.
806 This bias may be due to choosing to use a flow limited model for the systemic portion. This
807 choice may have been valid for IM administration, but may not be valid for IV administration.
808 Indeed, during an IM administration, the initial distribution phase may be masked by the
809 absorption phase. It would therefore be useful, in order to develop a generic PBPK model, to
810 take into account a model with limited permeability for the systemic portion. This would
811 allow better extrapolation between different types of administration or formulations. Note
812 that if this PBPK model is selected for another formulation than the one used for this study
813 data on the release of the active ingredient as well as the absorption rate from the injection site
814 is available. Either these data are available in the literature, or they have to be determined
815 experimentally, as has been done here.

816

817 In goats, the model correctly predicted external concentrations of OTC in milk after IV
818 and IM of 20 mg/kg (Rule *et al*, 2001), with a slight overestimation of late concentrations
819 after IV administration, because of poor predictions of plasma concentrations.

820

821 4.2. Comparison to other PBPK model

822 4.2.1. PBPK model of OTC in non-lactating species

823 A few PBPK models are available for OTC in non-lactating species. The model of OTC
824 in sheep (Craigmill, 2003) and that of cattle (Achenbach, 1995) allowed the tissue partition
825 coefficients to be obtained for the current PBPK model. The model of Lin *et al* of OTC in
826 dogs (Lin *et al*, 2015) considered some tissues as permeability limited (*i.e.*, fat, muscle, and

827 slowly perfused tissues), whereas the models of Craigmill and of Riad (Riad *et al*, 2021)
828 considered all tissues as perfusion limited. For the current model, a perfusion-limited model
829 structure was also chosen for tissue distribution, except in the udder, because it adequately
830 predicted the OTC plasma concentrations while avoiding the addition of uncertainties of
831 limited permeability parameters.

832

833 Some existing models use a two-compartments model (with two different rates) to
834 describe the IM absorption of a long-acting formulation of OTC (Achenbach, 1995; Lin *et al*,
835 2015; Riad *et al*, 2021). In the current model, a simple first order absorption was sufficient to
836 correctly predict the plasma kinetic of the conventional formulation of OTC. As the IM
837 absorption rate (k_{IM}) was not available for the target formulation and species, it was estimated
838 with the current plasma data. This was the only parameter that was estimated in the model,
839 with good confidence.

840

841 4.2.2. PBPK model of the udder

842 This new model is the first PBPK model to use a permeability-limited sub-model for
843 the udder, distinguishing the VASC, EW, and IW spaces of the udder. Compared to
844 perfusion-limited models, it allows describing the mechanisms by which slowly equilibrated
845 drugs are distributed into the udder and reach the milk.

846

847 The udder IW acted as a deep compartment (see discussion below) in a more
848 physiological manner than the empirical retention compartment mimicking the hypothetical
849 drug adsorption on the alveolar epithelium described by Woodward *et al* (Woodward and
850 Whittem, 2019). Moreover, the low permeability (Papp) of OTC impacted the rate of release
851 into milk.

852

853 In the current model, exchanges between milk (alveolar space) and the IW of the udder
854 were considered, mimicking reabsorption into the systemic circulation (Ziv and Sulman,
855 1975), as discussed by Li *et al* (Li *et al*, 2018).

856

857 Similarly to the model by Woodward *et al* (Woodward and Whittem, 2019), the
858 current model considered milk production in the alveoli, transport via the ducts, storage in the
859 cistern, and residual volume remaining in the ducts after milking. This studies model,

860 however, did not take into account a delay before filling the cistern, but considered a passage
861 of milk from the alveolar space to the cistern at a rate equal to the average rate of increase of
862 the milk volume in the cistern during the milking intervals.

863

864 In order to be able to predict milk concentrations over time, and not only at the time of
865 milking, it was assumed a linear increase in the volume of milk produced during the milking
866 intervals. The Hill equation, described in several papers (Li *et al*, 2018; Whittem, 2012;
867 Woodward and Whittem, 2019), mimicking the nonlinear increase in milk volume, was not
868 used because it would have required the estimation of parameters from the volumes of milk
869 measured and it was preferred to use these experimental values directly. Furthermore, the
870 experimental data obtained here showed that milking volume was almost proportional to IMI,
871 which justified the linear increase in volume.

872

873 4.3. Factors influencing passage into the milk

874 In this model the many physiological and physico-chemical factors were taken into
875 account, such as binding to milk components or passive diffusion, that can influence the
876 passage into milk. In the case of the passage of OTC into cow and goat milk, the global
877 sensitivity analysis (GSA) showed that the most influential factors were the ionization (pH
878 and pKa) on AUC_{96h} and the systemic exposure (CL_{plasma} and k_{tissue}) on C_{96h}. The rest of the
879 sixty-six (66) parameters tested in the sensitivity analysis (**see parameters with an asterisk**
880 **in Table 1 and Table S2 in supplementary materials**) had less impact on the predictions of
881 AUC_{96h} and C_{96h} of OTC in milk.

882

883 In addition to the factors identified by the GSA, influence of two other factors is
884 discussed: binding in the whole udder ($ka_{Ca^{2+}}$, $ka_{Mg^{2+}}$...) (see section 4.3.3) and OTC
885 permeability (Papp) (see 4.3.4) on the passage of OTC in milk.

886

887

888 4.3.1. Systemic circulation

889 In the current model, the exchange between plasma, IW, and milk acts by passive
890 diffusion of the unbound and unionized form of OTC. Therefore, the same AUC_{96h} of the
891 unbound and unionized form of OTC in these 3 compartments, highlighting the strong impact
892 of plasma PK on the passage into milk, was observed. In such a PBPK models, it is therefore
893 important to have a reliable model for the systemic PK to adequately describe the passage of a
894 compound over the blood-milk barrier. That's why the estimation of k_{IM} from was chosen for
895 this experimental animal study.

896

897 A whole PBPK model, including systemic PK was chosen because of the available PK
898 models in cows and goats (*i.e.*, known CL_{plasma} , $f_{e_{kidneys}}$, F_{IM} , and f_{up}) and the available PBPK
899 models, allowed the partition coefficients for each tissue to be obtained. In addition, having a
900 complete PBPK model, which is more generalizable, will allow easier future cross-species
901 and cross-molecule extrapolations. Alternatively, a classical compartmental model of the
902 plasma data could have been developed in order to have a reliable plasma PK model, and
903 established a minimal PBPK model including only the udder part as Woodward *et al*
904 (Woodward and Whitem, 2019). This could be interesting for species where physiological
905 data are lacking.

906

907 Plasma clearance had a significant impact on the C_{96h} of OTC in milk. The impact of
908 CL_{plasma} on the terminal elimination slope in plasma and milk explains its influence on the
909 predicted residual concentrations in milk. In this study model, CL_{plasma} was obtained from the
910 literature and split between renal and hepatic clearances based on published data of
911 unchanged excreted fraction in urine (Nouws *et al*, 1985b). This elimination model is not the
912 most mechanistic one and for future cross-species extrapolations, it will have to be adapted
913 with more physiological data of hepatic or renal elimination (*e.g.* glomerular filtration and
914 tubular secretion), using also more mechanistic PBPK model for such pathway (Mevius *et al*,
915 1986; Viel, 2018).

916

917 Among the partition coefficients, the k_{muscle} and the $k_{kidneys}$ significantly affected C_{96h} in
918 the GSA (see Figure 4). As these parameters were used in the calculation of the lumping
919 compartment partition coefficient (k_{rest} , see Equation 4), and since the lumping compartment
920 was the largest compartment in terms of volume (about 40% of the total weight), it is not

921 surprising that these partition coefficients were the most influential on systemic PK,
922 especially on the terminal slope, and therefore milk exposure. As observed in GSA, the
923 impact of k_{kidneys} is greater for goats than for cows. This is probably due to the fact that the
924 volume of the richly perfused tissue is greater in goats (8% of BW vs 4% for cows).

925

926 Several methods can be used to predict the k_{tissue} if *in vivo* data are missing from the
927 literature. For comparison, cow k_{tissue} were calculated using the equations of Rodgers and
928 Rowland (RR) (Rodgers *et al*, 2005) (see **Table S6 in section 3.3 of the supplementary**
929 **materials**). Only the k_{tissue} of fat calculated by RR is equivalent to that found in the literature,
930 while the others were lower (by 15% for kidneys to 52% for liver). The much lower systemic
931 distribution predicted using RR k_{tissue} led to much lower plasma and milk elimination half-
932 lives (4.5h vs 9.1h in milk and 4.2h vs 7.9h in plasma) (see **Figure S8 in the section 3.3 of**
933 **the supplementary materials**). The k_{tissue} obtained from literature allowed a better
934 description of the experimental data, even if the predictions of RR were quite good,
935 confirming that when possible, experimental data should be preferred to predictions from
936 theoretical equations (Yau *et al*, 2020).

937

938 4.3.2. Ionization

939 The current PBPK model accounts for the ionization of OTC in each udder and milk
940 sub-compartments. As observed in GSA, the acid pKa of OTC, as well as the pH of the udder
941 and milk sub-compartments, had a significant impact on the AUC_{96h} and C_{96h} of OTC (see
942 Figure 4).

943

944 Only the unionized form can passively diffuse across physiological membranes, but
945 equilibria are re-established once the membranes are passed depending on the pH of the
946 medium. As the acid pKa of OTC (7.25) is close to the plasma (7.4), IW (7.0) and milk (6.7)
947 pH values, small variations in pKa or pH led to large variations in ionized fractions. Thus,
948 due to the lower pH in milk than in plasma, the ionized fraction was lower in milk (29%
949 instead of 60%). Since the non-ionized concentration of OTC is the same in the different
950 compartments (notably plasma and milk), and the ionized concentration is lower in milk than
951 in plasma, the whole free concentration in milk is predicted to be lower than that in plasma
952 (see **Figure S6 and S7 in supplementary materials**).

953

954 The pKa values of OTC were based on an empirical calculation (Szegezdi and
955 Csizmadia, 2007, 2004) and were comparable to values reported in the literature (Carlotti *et*
956 *al*, 2012; Stephens *et al*, 1956; Tongaree *et al*, 1999). However, as evidenced by these
957 publications, for zwitterions (with multiple chemical groups), it is difficult to determine with
958 certainty to which groups these pKa are assigned. This difficulty is probably related to the
959 determination method and conditions (temperature, solvent) used (Reijenga *et al*, 2013).

960

961 Regarding cells and tissues, due to missing values in the literature for ruminants, pH
962 values were taken from other species (Rodgers *et al*, 2005; Waddell and Bates, 1969). These
963 have been shown to be equivalent for animal cells and tissues from different species (for
964 healthy and un-anesthetized animals). Regarding the milk, the pH is also similar between
965 species. However, it can change over time, *e.g.* colostrum has a lower pH (6.3) than milk at
966 the end of lactation (McIntyre *et al*, 1952), and according to certain physio-pathological
967 conditions, *e.g.* when the animal suffers from mastitis the pH is higher (7.4) (Ziv *et al*, 1974).
968 Hence, to refine the PBPK model it would be useful to include the physiological variation of
969 milk pH over the different lactation and (patho)physiological stages.

970

971 4.3.3. Binding

972 Our PBPK model has the advantage of accounting for the distribution of OTC in milk
973 fat and aqueous phase, its binding to casein, whey protein, ionized magnesium, and calcium.
974 Because the composition of milk varies between species (Balthazar *et al*, 2017; Park *et al*,
975 2007) and that affinities differ between molecules, it is important to account for all major milk
976 components for future cross-species and cross-molecule extrapolations. The binding of OTC
977 to ionized cations was taken into consideration as this is a known characteristic of
978 tetracyclines (as for fluoroquinolones) (Ross and Riley, 1993) and since Ca²⁺ and Mg²⁺
979 concentrations are high in milk. Somatic cell binding was neglected, because even in case of
980 serious mastitis (5 000 000 cells/ml), the volume of somatic cells represents less than 0.2% of
981 the milk volume (Campbell and Marshall, 2016). Furthermore, to the knowledge of the
982 authors of this study, no article mentions the binding of OTC to white blood cells.

983

984 The affinities of OTC for the milk components (*i.e.*, fat, casein, calcium, and
985 magnesium) were calculated using linear regressions constructed from experimental data
986 available in the literature for several drugs (Carlotti *et al*, 2012; Lupton *et al*, 2018; Shappell

987 *et al*, 2017). However, in case of extrapolation to other molecules, these data may not be
988 available. Recently, Abduljalil *et al* (Abduljalil *et al*, 2021) proposed an approach to calculate
989 the free fraction in milk ($f_{u,milk}$) from the lipophilicity ($\log P$) of a molecule. However, this
990 approach would appear to have some limitations, as for OTC, which has a low $\log P$ (-4.54),
991 the $f_{u,milk}$ value estimated using the formula of Abduljalil *et al* is higher than 1 (1.26 for both
992 species).

993

994 According to this study model, OTC was predicted to be bound more in milk (~60%)
995 than in plasma (~20%). OTC was predicted to be bound mainly to ionized calcium, ionized
996 magnesium and lightly to whey proteins, so it was mainly within the skimmed milk. These
997 findings could be interesting concerning milk by-products.

998

999 This PBPK model also takes into account the binding of OTC to different cellular
1000 components in the udder (*i.e.*, lipids and phospholipids). The partition coefficient of OTC
1001 between udder and plasma (k_{udder} , ratio of udder and plasma concentrations) was calculated to
1002 be 0.75 for cows and 0.77 for goats. It is of the same order of magnitude as those of other
1003 tissues (*i.e.*, muscle, liver, the rest) (see Table 1). As mentioned above, the binding in the
1004 udder IW resulted in drug retention and IW acted as a deep compartment. The slow release of
1005 the drug from the IW sub-compartment partly explains the delay of T_{max} in milk compared to
1006 plasma. This compartment could thus be useful for extrapolation to other molecules,
1007 especially those with strong IW binding.

1008

1009 4.3.4. Passive diffusion

1010 The current PBPK model accounts for passive diffusion (CL_{diff}) between the udder and
1011 milk, calculated from *in vitro* permeability (P_{app}). P_{app} is a parameter that varies greatly
1012 between molecules (*e.g.* caffeine with a P_{app} of 10^{-5} cm/s; norfloxacin with a P_{app} of 10^{-7}
1013 cm/s (Chen *et al*, 2008) and amoxicillin with a P_{app} of 10^{-8} cm/s in an internal unpublished
1014 study). A lower value of P_{app} (10^{-8} cm/s) slows down the passage into milk (C_{max} of 0.43
1015 mg/L at 19h vs 2.52 mg/L at 12h for a P_{app} of 10^{-7} cm/s) whereas a higher value of P_{app} (10^{-5}
1016 cm/s) increases the passage in the first hours after administration (C_{max} of 4.27 mg/L at 6h)
1017 (Figure 6). The low permeability of OTC ($P_{app} = 1.4 \times 10^{-7}$ cm/s) was responsible for the
1018 delay in appearance in milk (see Figure S6 and S7 in supplementary materials), but also for
1019 the slow release from the IW compartment that led to an increased elimination half-life in

1020 milk (7.9h). By simulating other values of Papp for the OTC, similar elimination half-life
1021 values for Papp of 10^{-5} cm/s and 10^{-6} cm/s (7.4 hours) and a value 30% higher for Papp of 10^{-8}
1022 cm/s (9.8 hours) would have been obtained. Therefore, permeability might be of importance
1023 for generalizing the PBPK model to other molecules.

1024

1025 4.4. Differences between cows and goats

1026 As explained above, this generic PBPK model is intended to be used in cross-species
1027 extrapolation. It was able to satisfactorily predict milk concentrations of OTC for both cows
1028 and goats, but the predictions did not differ much between the two species. As the milk pH is
1029 almost identical between the two species (6.68 for cows and 6.65 for goats), the rate of
1030 ionization of OTC is identical in the milk of cows and goats. A key difference between cow
1031 and goat milk is the concentration of whey proteins (Balthazar *et al*, 2017), but as OTC has a
1032 low affinity for these proteins this difference has little impact on its binding in milk. OTC
1033 binds mainly to ionized calcium, which is in similar concentrations in both species (0.08 g/L
1034 for cows, 0.09 g/L for goats). Therefore, we did not predict any difference in the binding of
1035 OTC in milk between the two species. Finally, the main differences in milk excretion
1036 between the two species were explained by the differences in the plasma profile of OTC.

1037

1038 For other molecules with stronger affinities for milk components that vary between
1039 species, such as fat and protein, greater differences in milk concentrations could be observed.

1040

1041 4.5. Model application to estimate withdrawal period in milk

1042 For the estimation of WP in milk, a literature was performed for estimates of inter-
1043 individual variabilities (IIV) for the different parameters of the PBPK model. For some
1044 parameters the variability took into account the differences between animals of the same
1045 breed, for others it took into account the differences between breeds of the same species. For
1046 the few parameters without available experimental data, the fixed CV of 30% represents a
1047 moderate level of variability (Covington *et al.*, 2007), while for chemical parameters, a CV of
1048 20% represents the uncertainty of these parameters values. These CV values are well
1049 accepted in the field of PBPK modelling and are often used in recently published papers
1050 (Clewell and Clewell, 2008; Henri *et al*, 2017a; Li *et al*, 2017, 2019a; Riad *et al*, 2021).

1051

1052 For cows, the model-predicted WPs (rounded up to the next full day) were slightly
1053 higher than the labelled milk discard time for both standard dosing regimens, *i.e.*, five (5)
1054 days vs four (4) days after four (4) repeated IM injections of OTC 10 mg/kg and four (4) days
1055 vs three (3) days after three (3) repeated IM injections OTC 5 mg/kg. These WPs are highly
1056 dependent on the prediction intervals, which were based on the IIV and uncertainty of the
1057 model parameters. The latter may have been overestimated and resulted in slightly higher
1058 estimated WPs.

1059

1060 For goats, predicted WPs were consistent with the labelled milk discard time for both
1061 standard dosing regimens, *i.e.*, four (4) days after four (4) repeated IM injections of OTC 10
1062 mg/kg and three (3) days after three (3) repeated IM injections OTC 5 mg/kg). Because total
1063 plasma clearance is higher in goats, plasma concentrations are lower and the time that
1064 residues fall below the established MRL is reduced compared to that of cows. This
1065 observation was also reported by Riad *et al* (Riad *et al*, 2021). In addition, for goats, the
1066 labelled WP of OTC have been fixed at the WP of cows (EMA, 2016) and do not take into
1067 account the physiology and particularities of the species. Thus, an experimental study would
1068 be necessary to confirm those predicted WP in goat milk.

1069

1070 Overall, the popPBPK model described in this study could be used as a tool to anticipate
1071 residue depletion studies. Such a model does not completely replace animal studies but it can
1072 help to design such studies.

1073

1074 4.6. PBPK model limitations

1075 Although this generic model accurately predicted OTC concentrations in milk of both
1076 species, it has some limitations.

1077

1078 First, passive diffusion is the only mechanism of drug distribution in milk that was
1079 considered in the current model. Indeed, although OTC is known to be a substrate of PgP
1080 (Schrickx and Fink-Gremmels, 2007), the latter is under expressed in the udder during
1081 lactation and does not play a role in transporting the molecule into milk (Yagdiran *et al*,
1082 2016). However, other transporters that are highly expressed in the udder or whose
1083 expression increases during lactation (García-Lino *et al*, 2019) may have an important role in

1084 the exchange of drugs (and nutrients) between plasma and milk (Mahnke *et al*, 2016;
1085 Ozdemir, 2018). Further studies will be conducted with other drugs to investigate the role of
1086 active transport of molecules in milk. An influx and/or efflux clearance could then be added
1087 to equations 8, 9, and 18.

1088

1089 Thus, the current model considers drug binding to milk components but does not vary
1090 the milk components concentrations over time, as observed in reality (Ciappesoni *et al*, 2011;
1091 Coulon *et al*, 1991). Further studies are needed to add variation over time of these parameters
1092 to this model. Similarly, changes in milk pH over time may be added to the model.

1093

1094 On the other hand, to build this population model variabilities and uncertainties on
1095 parameters (Balthazar *et al*, 2017; Craigmill *et al*, 2004, 2000; Lautz *et al*, 2020; Li *et al*,
1096 2021; Lin *et al*, 2020, 2016; Nouws *et al*, 1985b; Upton, 2008) were applied. The
1097 physiological data for cows and goats were mainly derived from two recent reviews of the
1098 literature (Li *et al*, 2021; Lin *et al*, 2020). These two reviews are compiled based on a number
1099 of original experimental studies and represent the most accurate physiological parameter data
1100 for these species in the literature. Thus, uncertainty has been substantially decreased for
1101 many physiological parameters, but some are still missing. On the other hand, the
1102 correlations between the different physiological values are not described and considering
1103 independent variabilities may lead to an overestimation of the overall variability. The choice
1104 of the random effects model is critical for the determination of WP with population PBPK
1105 models. However, there is no real consensus on how to predict WP from popPBPK models.
1106 Some authors consider that the 99th percentile of the PI obtained by a population model is a
1107 good approximation of the 95th percentile of the tolerance interval, which is the criterion used
1108 by regulatory agencies, and can therefore be used to predict a WP (Chevance, 2017; Henri *et al*,
1109 2017b; Viel, 2018). In this study case, the internal and external validations that were
1110 carried out give an indication that the choice of the random model was reasonable. However,
1111 the choice was arbitrary and since it incorporates both IIV and parameter uncertainty it is
1112 difficult to determine whether the resulting interval was a prediction interval or a confidence
1113 interval.

1114

1115 4.7. Perspectives

1116 In conclusion, the popPBPK model successfully predicted OTC concentrations in cow
1117 and goat milk after different doses and routes of administration. This is the first generic
1118 PBPK model to consider the udder as a permeability-limited compartment, accounting for
1119 vascular, EW, IW, alveolar, and cisternal spaces, as well as passive diffusion across
1120 physiological membranes, pH, ionization, and the composition of milk.

1121
1122 By integrating inter-individual variability in the physiological values into the model,
1123 and uncertainty in the parameters used, the model allowed for a suitable estimation of the
1124 WPs of OTC for cows and goats for different dosing regimens.

1125
1126 In the future, this model could be extrapolated to different drugs and chemicals, such as
1127 pesticides, with different affinities and physico-chemical properties, and to different species,
1128 such as sheep and humans, with a different milk composition and milk production.

1129

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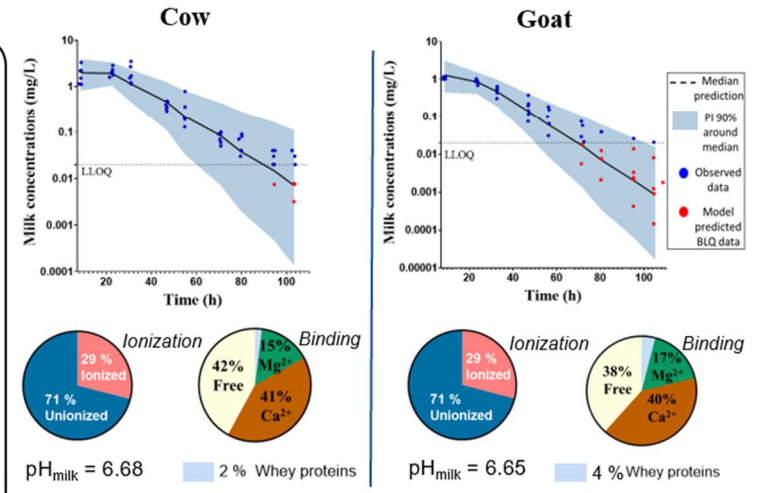
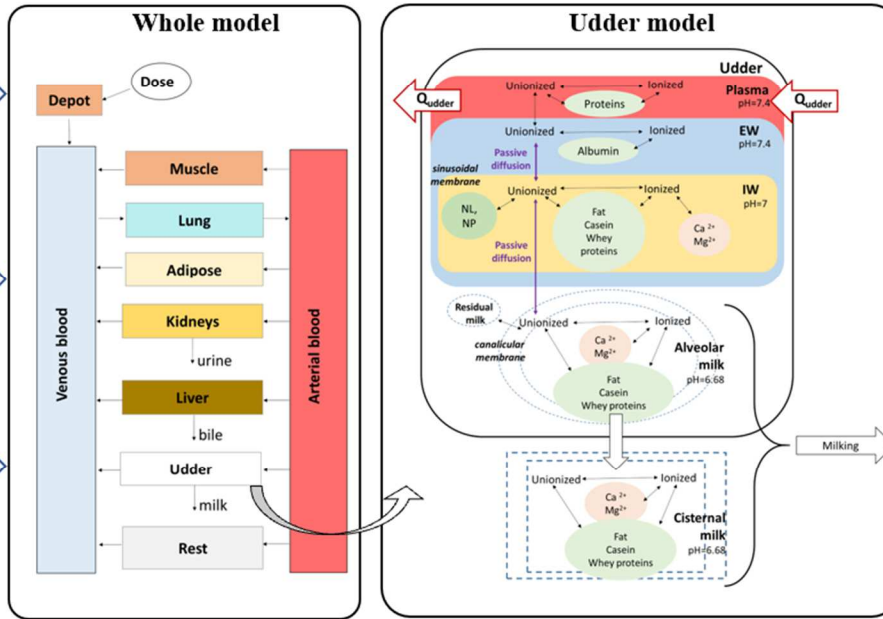
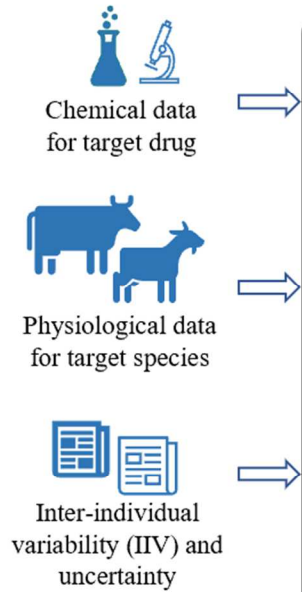
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Literature review

Physiologically based pharmacokinetic (PBPK) model

Predictions of OTC in milk of cow and goat



CONCLUSION: The PBPK model successfully predicted OTC concentrations in cow and goat milk after different doses and routes of administration. It could be extrapolated to xenobiotics with different affinities and physicochemical properties and to others lactating species with different composition and milk production.