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Biopharmaceutical classification of desloratadine – not all drugs are classified the easy way

KATJA BERGINC[1]
NADICA SIBINOVSKA[1,2]
SIMON ŽAKELJ[2]
JURIJ TRONTELJ[2]
IGOR LEGEN[1]

[1] *Lek d.d., 1526 Ljubljana, Slovenia*

[2] *University of Ljubljana, Faculty of Pharmacy, Chair of Biopharmaceutics and Pharmacokinetics, SI- 1000 Ljubljana Slovenia*

The biopharmaceutical classification of drugs was designed as a basis for bio-waivers – a mechanism with the double ethical benefit of delivering new drug formulations to the market with less human testing and lower cost. However, many drugs defy simple classification because *in vitro* permeability and stability assessment can be challenging as shown in this study for desloratadine. Literature shows that desloratadine is highly soluble, while data on luminal stability and permeability are circumstantial. Combined with borderline bioavailability and not really known fraction of absorbed dose, desloratadine was found to be a good example for showing the innovative *in vitro* approaches necessary to unambiguously classify desloratadine according to Biopharmaceutical Classification System (BCS) guideline. Presented study undoubtedly confirmed that desloratadine solubility is high and dissolution is very rapid for immediate release reference tablets. We have demonstrated desloratadine stability under legally required conditions and also in more physiologically relevant media. High *in vitro* desloratadine permeability was confirmed using Caco-2 and Parallel Artificial Membrane Permeability Assay (PAMPA). Well-established *in vitro* model with rat intestinal tissue could not be used due to reasons elaborated in this paper.

Keywords: desloratadine, BCS-based bio-waiver, luminal stability, permeability, dissolution

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A bio-waiver may be granted for immediate release formulations containing Biopharmaceutical Drug Classification 1 and 3 drug candidates, which enable (very) rapid drug release and behave as stable, oral solutions in the gut.

Compared to other marketed antihistamines, desloratadine lacks several adverse effects, is less prone to drug-drug interactions, and exerts superior potency and selectivity for H_1-receptors (1). Following oral administration, the mean time to reach maximum plasma concentrations and elimination half-life are approximately 3 and 27 hours, respectively. Neither food nor grapefruit juice affect its rate (C_{max}) nor extent (*AUC*) of absorption (2, 3). After

* Correspondence; e-mail: katja.berginc@sandoz.com

absorption, desloratadine is subjected to extensive hepatic metabolism to active metabolite 3-hydroxydesloratadine, which is subsequently inactivated by glucuronidation (4). Given that desloratadine is extensively metabolized, only 8 % of total plasma AUC corresponds to desloratadine, which is in line with very low amounts of unchanged drug detected in urine (< 2 %) and feces (< 7 %) (2, 5). The main plasma species are thus desloratadine metabolites (5). Desloratadine is also a P-glycoprotein (P-gp) substrate (5, 6); however, linear pharmacokinetics over a dosage range of 5–20 mg has been established in healthy volunteers (4), which suggest that the intestinal absorption is not limited by the active transport. The recommended dose is one 5 mg tablet once daily. Available literature suggests that desloratadine can be classified as BCS 1 substance (4, 7, 8), which would make formulations eligible for waiving *in vivo* bioequivalence human studies if several conditions would be fulfilled based on the guidelines (9, 10). Namely, the formulations have to be immediate release solid oral dosage forms without excipients that can affect the rate or the extent of absorption (9, 10). For BCS 1 compounds, the formulation must enable fast dissolution over the pH range 1.2, 4.5, and 6.8 (≥ 85 % dissolved at 30 min). The drug must be highly soluble (*i.e.* the highest dose has to dissolve in 250 mL or less of aqueous media over pH range 1–6.8), and highly permeable (fraction of dose absorbed (F_{abs}) in humans ≥ 85 %). The applicant must further demonstrate drug stability in the intestinal environment by showing > 95 % recovery after 1 h incubation in simulated gastric fluid (SGF) and 3 h incubation in simulated intestinal fluid (SIF), both at 37 °C. The eligibility for waiving *in vivo* human study also depends on the drug's therapeutic index (9), which is not an issue for desloratadine, because it is not a drug with narrow-therapeutic range.

Based on the information for the reference product Aerius® (a 5 mg desloratadine immediate release tablet), desloratadine solubility is high; reported solubility values in the literature are 39.7 mg mL^{-1} in 0.1 mol^{-1} HCl, 0.1 mg mL^{-1} in water, and 1.5 mg mL^{-1} in phosphate buffer pH 7.4 (11). Desloratadine permeability could also be designated as "high", because the absorbed fraction of oral dose is more than 90 % (*i.e.* almost complete) in laboratory animals and humans. Although mass balance studies suggest high permeability (*i.e.* F_{abs} > 90 %) (11, 12), approximately half of the 87.1 % of recovered metabolites were found in the urine and half in feces (13). This could raise the question regarding the origin of fecal metabolites, since they could be formed in the gut lumen prior to systemic absorption, making the estimation of a fraction of desloratadine absorbed much less reliable for the assessment of desloratadine permeability. The data on desloratadine´s permeability and gastrointestinal stability are thus still ambiguous and do not allow a simple BCS classification. In cases when *in vivo* data are inconclusive, they should be complemented by carefully selected *in vitro* measurements and models. Therefore, the aim of the present study was to show BCS classification of desloratadine by following the guidelines for waiving *in vivo* bioequivalence human studies and providing the necessary complementary *in vitro* results (9, 10).

EXPERIMENTAL

Chemicals

Acetonitrile, HPLC gradient grade and methanol (Ultra) HPLC gradient grade were purchased from JT Baker. Metoprolol tartrate and Lucifer Yellow (Sigma Aldrich Chemie,

Germany) and desloratadine USP reference standard were used. 85 % ortho-phosphoric acid and 25 % ammonia solution were purchased from Merck, Germany.

Caco-2 cells were obtained from National Collection of Type Cultures, Public Health England. Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), sodium pyruvate, non-essential amino acids solution, Hank's balanced salt solution (HBSS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), TrypLE™ Select were purchased from Life technologies (UK). Cell culture plate assemblies were purchased from Merck Millipore (IRL) and cell culture flasks were from Sarstedt (Germany).

Caco-2 culture

Caco-2 cells (ECACC, 09042001, Lot No. 09E012, passage number 47, STR verification: 09/06/2009, PCR based mycoplasma detection: 12/06/2009, routine mycoplasma detection with MycoAlert™ Mycoplasma Detection Kit (Lonza)) were maintained between passages 51–57 at 37 °C, 5 % CO_2 and 95 % relative humidity. Cells were grown in DMEM supplemented with 10 % FBS, 1 % sodium pyruvate and 1 % non-essential amino acids. After reaching approximately 90 % confluence in 75 cm^2 cell culture flasks, they were detached with TrypLE™ Select and plated onto polyethylene terephthalate (PET) membranes (1 μm pores, 0.7 cm^2, Millipore) in 24-well cell culture plates at a density of 1×10^5 cells cm^{-2}. Cell culture medium was replaced by fresh medium 24 h post-seeding and then every 2–3 days (400 μL apical, 800 μL basolateral wells). It was also replaced 24 h before the permeability assay.

Desloratadine solubility

Reported desloratadine pK_a values are 4.4 and 10.0 (14). By using the shake flask method, saturated desloratadine solubilities were determined at 37 °C after 24 h incubation in water, 0.1 mol L^{-1} HCl, acetate buffer pH 4.5, and in phosphate buffer pH 6.8. The experiments were carried out in triplicate; average values and accompanied RSD values (expressed in %) are presented in Table I.

Dissolution of reference samples

Dissolution studies for 5 mg immediate release reference tablets (Merck, Clarinex, US market) were performed with Apparatus 1 at 100 rpm in 500 mL of 0.1 mol L^{-1} HCl, acetate

Table I. Equilibrium desloratadine solubility at 37 °C in different media. An average equilibrium solubility and RSD values (expressed in %) of three measurements are presented in parentheses

Media	Equilibrium solubility (mg mL^{-1}) and RSD	Volume of media needed to dissolve 5 mg dose (mL)
0.1 mol L^{-1} HCl	40.49 (0.4 %)	0.123
Acetate buffer pH 4.5	10.65 (1.4 %)	0.469
Sodium phosphate buffer pH 6.8	2.46 (1.2 %)	2.03
Water	0.92 (3.4 %)	5.43

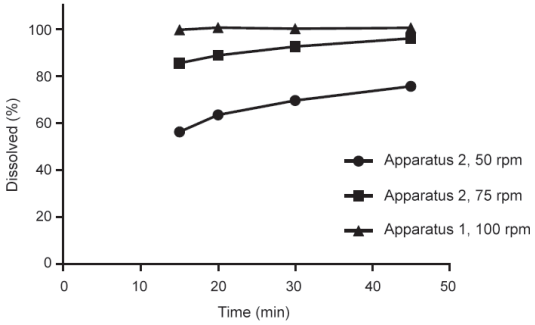

Fig. 1. The impact of dissolution apparatus selection and agitation speed on desloratadine release in sodium phosphate buffer pH 6.8. Average data is shown (6 tablets were analyzed for each condition).

buffer pH 4.5, and in phosphate buffer pH 6.8 (9, 10). Apparatus 2 was also considered but it was not used due to coning (Fig. 1). Samples were analysed by UV quantification at 283 nm wavelength. Six tablets per sample were analyzed.

Testing desloratadine luminal stability

Desloratadine stability in SGF, SGF with pepsin (SGF_{pep}) and in SIF was tested. Buffers were prepared according to USP. Desloratadine stock solution (1 mg mL^{-1} concentration) was prepared in buffer pH 7.4 used for Parallel Artificial Membrane Permeability Assay (PAMPA) and 5 mL were added to 245 mL of SGF, SGF with pepsin, and SIF and were left at 100 rpm in dissolution vessel at 37 °C. Samples were withdrawn after 1 h (for SGF, SGF_{pep}) and 3 h (for SIF), respectively, filtered through 0.45 μm PVDF and analyzed on UPLC. The test was carried out in duplicate and average values are reported in the result section.

PAMPA experiments

Gentest™ pre-coated PAMPA plates were used according to manufacturer instructions. Prior to use, the PAMPA plate was warmed to room temperature. Buffers for PAMPA solutions were McIlvaine buffers (pH 5.6, 6.8, and 7.4), prepared from 0.2 mol L^{-1} $Na_2PO_4 \times 2H_2O$ and 0.1 Mol L^{-1} citric acid in defined volumetric ratios (15). Donor solution contained desloratadine (0.05 mg mL^{-1}) and metoprolol (0.02 mg mL^{-1}), and were added on donor (bottom) side. Different pH values of donor solutions were tested. The permeation of both drugs into McIlvaine acceptor buffer pH 7.4 was determined after 3 h incubation at 37 °C by UPLC. Permeability was calculated according to Eq. (*1*) and Eq. (*2*).

$$P_{app} = \frac{-ln\left[1 - \frac{A_A(t)}{A_{eq}}\right]}{S \times \left[\frac{1}{V_D} + \frac{1}{V_A}\right] \times t} \quad (1)$$

$$A = \frac{A_D(t) \times V_D + A_A(t) \times V_A}{V_D + V_A} \quad (2)$$

where P_{app} is apparent permeability coefficient (cm s^{-1}), A_{eq} is concentration in the donor well plate at the beginning of the experiment, $A_D(t)$ is the concentration of the donor well sample after 3 h incubation period, $A_A(t)$ is the concentration of the acceptor well sample after 3 h incubation, V_D is the donor well volume, V_A is the acceptor well volume, S is filter area, and t is incubation time.

In vitro *permeability through Caco-2 experiment*

Caco-2 cells were seeded and cultured on porous membranes as described above. After three weeks, the cell culture medium was removed. Caco-2 monolayers were rinsed three times with buffer solution (HBSS with 0.01 mol L^{-1} HEPES), pre-warmed to 37 °C and transepithelial electrical resistance (TEER) was determined. Only monolayers exhibiting TEER values ≥ 800 W cm^2 were used.

Desloratadine, metoprolol and Lucifer Yellow solutions were prepared in pH 7.4 buffer (HBSS with 0.01 mol L^{-1} HEPES). Donor concentrations used were 5 mg desloratadine/250 mL (≈ 65 mM), 100 mg metoprolol/250 mL (≈ 1.4 mmol L^{-1} concentration) and 20 mmol L^{-1} Lucifer Yellow.

The permeability of each drug substance was tested in both directions; apical to basolateral (AP-BL) and basolateral to apical (BL-AP). For AP to BL direction, 400 μL of test solutions were added to the apical side of monolayers and 800 μL of buffer solution to each acceptor well. When tested BL-AP direction, 800 μL of test solutions and 400 μL of buffer solution were added to the basolateral and apical wells, respectively. Samples were withdrawn from the receiver wells at 30, 60, 90, 120 and 180 min and from the donor wells at 0, 90 and 180 min. The volume withdrawn was replaced with fresh buffer or donor solution. Following sample collection, the TEER values were determined and then the monolayers were rinsed with buffer and incubated the second time with 20 μM Lucifer Yellow solution for 60 min. Fluorescence for 100 μL aliquots withdrawn from the receiver wells was read using the Infinite M1000 reader (Tecan, Switzerland) (Ex: 430 nm, Em: 540 nm).

For the assessment of desloratadine and metoprolol permeability, only those Caco-2 cell monolayers that retained acceptable monolayer integrity were used; TEER at the end of permeability tests ≥ 500 W cm^2 and Lucifer Yellow permeability ≤ 10.0×10^{-7} cm s^{-1}.

The apparent permeability coefficients (P_{app}) were calculated using the following formula:

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

where (mmol s^{-1} or mg s^{-1}) is the total amount of investigated drug substance transported to the acceptor side per unit time, is the exposed surface area of the cell monolayer (0.7 cm^2), (mol L^{-1} or mg mL^{-1}) is the average initial concentration of drug substance in donor wells.

Calculation of efflux ratio (*ER*) was performed according to the following formula:

$$ER = \frac{P_{app(B-A)}}{P_{app(A-B)}}$$

where is the apparent permeability coefficient of drug substance in BL to AP direction and is the apparent permeability coefficient of the same drug substance in AP to BL direction.

In vitro *permeability through rat jejunum*

The experiments conform to the law for the protection of animals (Republic of Slovenia) and are registered at the Veterinary Administration of the Republic of Slovenia. Jejunum from male Sprague-Dawley rats (250–320 g) was obtained after 18 h without food and water ad libidum followed by decapitation. It was prepared and mounted in Easy Mount side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA) as previously described (16). Ringer buffer of pH 7.0 and 7.4 were used on donor/mucosal and acceptor/serosal sides, respectively. Throughout the experiments, Ringer solutions on both sides of the jejunum were continuously bubbled with carbogen (95:5, O_2/CO_2). To sustain tissue viability, 10 mM D-glucose and 4 % human serum were present on the serosal side, and 10 mmol L^{-1} D-mannitol on the mucosal side to assure adequate osmolarity. During a 2 h experiment, tissue was incubated at 37 °C and acceptor solutions were sampled every 20 min. The withdrawn volume was replaced by fresh 10 mmol L^{-1} D-glucose Ringer buffer pH 7.4 with 4 % human serum. The withdrawn acceptor samples were used to determine the permeability of desloratadine.

Tissue viability and integrity during the experiment were controlled by monitoring the trans-tissue potential difference, the short circuit current and trans-tissue electrical resistance with a multichannel voltage-current clamp. At the end of the permeability experiment, a decrease of trans-tissue potential difference after the addition of 100 μL of 625 mmol L^{-1} D-glucose had been recorded and used to assess tissue viability and adequacy of active transporters. The permeability of FITC dextran was used for the determination of paracellular tight-junction integrity (17).

Desloratadine retention in rat intestinal rings

Intestinal jejunum was obtained as mentioned in "*In vitro* permeability through rat intestine". Intestinal rings were weighed (720 mg wet weight of intestinal rings was used) and incubated at 37 °C in 7.5 mL of carbonated Ringer buffer pH 7.4, supplemented with 10 mmol L^{-1} D-glucose and desloratadine (0.02 mg mL^{-1}). Samples were withdrawn after 1 and 2 h, and at the end, the tissue was homogenized and desloratadine entrapped by the tissue was extracted during protein precipitation with ice-cold acetonitrile (tissue/acetonitrile = 1:3) for 24 h. After centrifugation at 4 °C and 10000×*g* for 10 min the amount of desloratadine in the supernatant was determined by HPLC.

UPLC method – quantification of desloratadine and metoprolol for Caco-2 in vitro *experiment*

For the quantification of desloratadine and metoprolol in the acceptor and donor solution obtained in Caco-2 permeability experiments, a Kinetex C18 (2.6 mm × 50 mm) column at 40 °C was used. Gradient method was used with the organic component of the mobile phase increasing from 4 % at 0–1 min to 45 % at 3.5 min with the end of analysis at 4 min. 10 μL of samples were injected and the absorbance was detected at 220 nm. Samples in the autosampler were kept at 10 °C.

HPLC method

For desloratadine quantification after the uptake into intestinal rings and extraction from the tissue, the Agilent 1100 system (degasser, binary pump, well-plate sampler, column

thermostat and DAD) was used with the Waters XTerra MS C18 column (3.5 μm particles; 4.6 × 100 mm) at 45 °C. The flow of the mobile phase (77 % of 0.5 % ammonium phosphate pH = 3.0 and 23 % of acetonitrile) was 1.5 mL min^{-1}. The detection was made at 276 nm. The autosampler temperature was 10 °C and the injected sample volume was 10 μL.

Sample analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS)

The permeability samples were prepared by protein precipitation using a threefold volume of acetonitrile and brief 10 min centrifugation at 15000×*g*. The samples (1.0 μL) were injected onto the Agilent 1290 Infinity liquid chromatograph coupled with the 6460 Triple Quadrupole detector (Agilent Technologies, Santa Clara, USA) equipped with the Jet-Stream™ electrospray. The chromatographic separation was performed on the Kinetex column C18 50 × 2.1 mm, with 2.6 μm particles (Phenomenex, Torrance, USA) maintained at 40 °C and using a linear gradient elution with a flow rate of 0.65 mL min^{-1} of the mobile phase consisting of 0.1 % formic acid in water (mobile phase A) and 100 % acetonitrile (mobile phase B) with the following gradient steps (min, % B): (0.0, 2), (0.25, 2), (0.5, 15), (1.00, 35), (1.25, 45), (1.50–1.80, 55), (2.00–3.00, 2). The total run time including re-equilibration was 3.0 min. The mass spectrometer was operated in positive mode using the following ion source parameters: gas flow (and temperature): 5 L min^{-1} (275 °C), nebulizer pressure: 45 psi, sheath gas flow (and temperature): 11 L min^{-1} (320 °C) and the capillary voltage was 4000 V. The fragmentor voltage was 181 V. Both quadrupoles were set at the widest mass resolution (2.5 amu) with the following *m*/*z* transitions: 311.1 → 259.1 at 17 eV and 311.1 → 243.1 at 53 eV for quantifier and qualifier ion of desloratadine, respectively. The qualifier/quantifier ratio was 37 %. Haloperidol was used as an internal standard with the final concentration in each sample of 45 μg L^{-1} and quantitated with the *m*/*z* transition 376 →165 at 24 eV collision energy with unit resolution. The method was checked for selectivity (Fig.

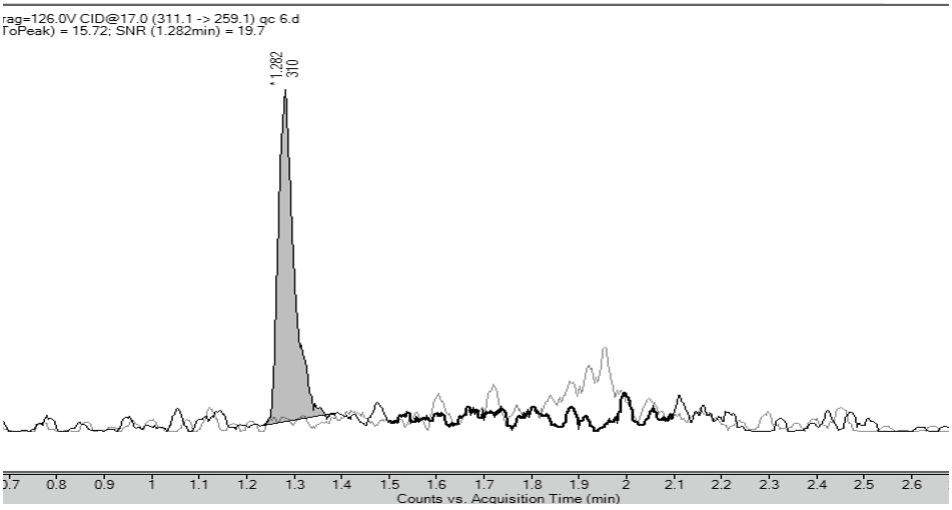

Fig. 2. A typical chromatogram of desloratadine obtained with liquid chromatography with tandem mass spectrometry (LC-MS/MS).

2), working range, accuracy, precision, and matrix effect. The linear range was confirmed over the concentration interval between 0.325 and 8000 μg L^{-1}; however, due to the observed lower-than-anticipated test sample concentrations, the calibration range was narrowed down to 0.325–2000 μg L^{-1} with good accuracy (bias < 10 %) and injection precision (< 10 % RSD) using the weighted ($1/c^2$) linear regression. The limit of quantitation was demonstrated to be 0.325 μg L^{-1} based on accuracy, precision and signal-to-noise ratio criterion (area, peak-to-peak) (Fig. 2). The signal abundance was checked after each 10 test samples and the uniformity and stability of the obtained responses indicated absence of any significant matrix buildup from the tissue incubation medium causing signal deterioration as demonstrated by RSD values from test samples below 7.2 %. Furthermore, the signal drop due to the presence of matrix background from tissue incubation medium was only 4 % compared to the neat standard solution (determined in 8 parallels).

RESULTS AND DISCUSSION

Equilibrium desloratadine solubility

Equilibrium solubility results (Table I) show pH-dependent solubility. Regardless of the media used for solubility determinations, the entire desloratadine 5 mg dose was completely dissolved in 250 mL, demonstrating desloratadine as a highly soluble compound.

Dissolution of desloratadine immediate release formulations

To assess whether an immediate release formulation complies with the guidelines pertaining to drug release, dissolution in 500 mL of various dissolution media (0.1 mol L^{-1} HCl, acetate buffer pH 4.5, or sodium phosphate buffer pH 6.8) should be performed using either Apparatus 2 at 50 rpm or Apparatus 1 at 100 rpm. Preliminary evaluations indicated that desloratadine release is obscured by coning (Fig. 1). Namely, increasing agitation speed improved desloratadine recoveries at 45 min considerably; recoveries increased from 75.7 % released at 50 rpm to 96.1 % released at 75 rpm, or even 100.6 % released in Apparatus 1 at 100 rpm. Therefore, to avoid coning and assure rugged dissolution method, Apparatus 1 and 100 rpm was used to evaluate the release as per guideline.

Table II. The average of desloratadine released in 500 mL of media using Apparatus 1, 100 rpm[a]

Time (min)	0.1 mol L^{-1} HCl	Acetate buffer pH 4.5	Phosphate buffer pH 6.8
10	100.4	102.5	103.3
15	101.0	102.1	102.6
20	104.7	102.0	102.5
30	101.0	103.0	102.6
45	100.3	102.3	102.8

[a] Each test was performed on 6 units.

Reference formulation was therefore analyzed in 500 mL using Apparatus 1, at 100 rpm. Regardless of the media used formulation showed very fast desloratadine release (Table II).

Intraluminal stability

It had been previously published that the absorption of desloratadine is almost complete, with F_{abs} > 90 % (11, 12). Ramnathan *et al.* (12) reported the recovery of 88 % of ^{14}C-desloratadine dose based on the metabolite recovery; 47 % metabolites were recovered in feces and 41 % in urine (13). Mass balance studies do not show whether the metabolites detected in the feces are formed before, during or after the intestinal absorption and thus fail to provide conclusive evidence of desloratadine high intestinal permeability (13).

To reliably classify desloratadine as highly permeable, one needs to show that the fecal part of metabolites was not formed from the parent drug in the gut lumen, but rather after the absorption (18). Therefore, potential instability or degradation in the gut lumen due to enzymatic and/or hydrolytic degradation should be excluded.

Desloratadine recoveries were analyzed according to the guidelines (9) and they were all well above the 95 % threshold value (102.6 % in SIF, 99.6 % in SGF and 101.1 % in SGF with pepsin). Based on these results, desloratadine can be considered stable in the intestinal environment at 37 °C for the designated incubation time.

Desloratadine stability was additionally evaluated in more complex, physiologically relevant media to further strengthen the conclusion of desloratadine luminal stability (see "Desloratadine retention in rat intestinal rings").

PAMPA permeability

Metoprolol has been widely accepted as a high permeability marker with complete absorption from the intestine (17). If the permeability of concomitantly investigated compound is similar or higher than that of metoprolol, one can safely assume that tested drug also belongs to a high permeability class (19). Based on Table III, desloratadine has pH-dependent permeability with the highest permeability found at higher pH values, which

Table III. The impact of incubation media pH on average PAMPA permeabilities of desloratadine compared to high-permeability standard metoprolol[a]

DONOR pH	ACCEPTOR pH	Average DESLORATADINE permeability ($\times 10^{-6}$ cm s^{-1})	Average METOPROLOL permeability ($\times 10^{-6}$ cm s^{-1})
5.5	7.4	1.13 (n = 8; RSD 32 %)	1.09 (n = 8; RSD 31 %)
6.8	7.4	7.81 (n = 8; RSD 17 %)	4.60 (n = 8; RSD 25 %)
7.4	7.4	16.6 (n = 8; RSD 9.7 %)	9.09 (n = 8; RSD 22 %)
5.5	5.5	5.74 (n = 7; RSD 27 %)	2.22 (n = 7; RSD 27 %)
6.8	6.8	14.9 (n = 8; RSD 8.0 %)	7.53 (n = 8; RSD 11 %)

[a] The number of measurements (n) for each condition and relative standard deviation (RSD, expressed in %) are shown in parentheses.

is in line with its protonation under various pH conditions. Regardless of pH, desloratadine permeabilities are significantly higher than those of metoprolol, implying that desloratadine belongs to a highly permeable class.

Although PAMPA membranes have not gained legal recognition as broad as other available models (9, 10), they have been used successfully for determining rank-order permeability of different compounds (20). Because of PAMPA membrane limitations (*i.e.* they can only be used to assess the contribution of passive diffusion to compound's permeability), the assessment of desloratadine permeability was continued using more complex *in vitro* models such as rat intestine and Caco-2 cell monolayers, which are known to express functional P-gp (17).

Permeability measurements in side-by-side diffusion chambers with rat jejunum

The reliability and abundance of experience with this validated in-house model (17) made it a natural first choice for the *in vitro* classification of desloratadine permeability as well as for the evaluation of the mechanism of desloratadine intestinal absorption and, if relevant – elimination. Unfortunately, after following the experimental design described in the section "*In vitro* permeability through rat jejunum", at the end of the *in vitro* permeability experiment, we could not detect desloratadine in the acceptor solutions with UPLC method described in the appropriate section of this paper. Extremely low recoveries were established by LC-MS/MS, which were at levels inadequate for the calculation of permeability coefficients. We confirmed that no significant metabolic processes converting desloratadine to its known metabolites took place in the rat small intestinal tissue *in vitro.* The experiment with intestinal rings described hereafter clarified the reasons for the inability of the Sweetana-Grass-type experiment to measure the permeability of desloratadine.

Desloratadine retention in rat intestinal rings

To exclude desloratadine loss owing to instability in carbonated Ringer buffer or adsorption to plastics of side-by-side diffusion chambers for *in vitro* permeability experiments, desloratadine recoveries at 37 °C after 2 h incubation in carbonated Ringer buffer pH 7.4 supplemented with 10 mM D-glucose were compared to the unexposed standard solution with the same starting donor concentration (5 mg/250 mL, solution kept at room temperature in the glassware).

Table IV. Desloratadine recoveries after 1 and 2 h incubation in carbonated Ringer buffer pH 7.4 supplemented with 10 mmol L^{-1} D-glucose compared to desloratadine standard solution (*), which were kept at room temperature and it was not carbonated

	1 h	2 h
Unexposed standard solution in glassware (*), room temperature	100 %	99.1 %
Carbonated standard solution in glassware, 37 °C	100.4 %	101.3 %
Carbonated standard solution in side-by-side diffusion chambers, 37 °C	90.5 %	90.5 %
Carbonated standard solution incubated with intestinal rings, 37 °C	38.9 %	34.0 %

The recoveries of desloratadine incubated with intestinal tissue were also assessed and tissue extraction was performed (Table IV). Data showed that desloratadine is stable in physiologically relevant Ringer buffer at 37 °C for the time needed to perform permeability tests. Approximately 10 % loss of drug was noted during the incubation of solution in side-by-side diffusion cells, indicating minor drug adsorption to the plastic surface. However, this loss was too small to explain our inability to quantify desloratadine after *in vitro* permeability test in the acceptor solution. On the other hand, a significant decrease of desloratadine concentration was noted, when the standard solution was incubated with intestinal rings, indicating a high affinity of desloratadine for the tissue. Extraction of intestinal rings after 2 h incubation proved our hypothesis, since approximately 50 % of the initial donor desloratadine dose (5 mg/ 250 mL) was retained by the tissue.

In vitro *permeability of desloratadine through Caco-2 monolayers*

Permeability of desloratadine was determined through Caco-2 cell monolayers concomitantly with a highly permeable marker metoprolol. Only those cell inserts, which retained suitable monolayer integrity until the end of experiments, were considered as assessed by TEER values and Lucifer Yellow permeability. The average TEER values before the start of *in vitro* permeability test were 1338 W cm^2 (AP-BL direction) and 1202 W cm^2 (BL-AP direction), respectively. After the experiments, average TEER values were 706 W cm^2 (AP-BL direction) and 847 W cm^2 (BL-AP direction), while average Lucifer Yellow permeabilities were 4.87×10^{-7} cm s^{-1} (AP-BL direction) and 4.73×10^{-7} cm s^{-1} (BL-AP direction), respectively.

Desloratadine permeability in AP-BL direction (9.54×10^{-6} cm s^{-1}) was similar to the metoprolol permeability (9.43×10^{-6} cm s^{-1}) in the same direction; therefore, desloratadine can be considered highly permeable. Although desloratadine permeability in BL-AP direction was slightly higher (16.7×10^{-6} cm s^{-1}) than the one in the opposite direction, the efflux ratio (*i.e.* the ratio between average BL-AP and AP-BL permeability) was approximately 1.7. BCS based guidelines (10) suggest that all efflux ratios below 2 are not considered relevant and the drug is not subjected to significant active transport *in vivo*. In the case of desloratadine, the *in vivo* pharmacokinetic data states linear pharmacokinetics over a dose range of 5–20 mg. This means that potential active transport in the intestine does not have any significant role in limiting desloratadine absorption. This is in line with our permeability data, since the efflux ratio is well below 2 even when measured by an *in vitro* model, which is known to over-express P-gp efflux transporter (21).

For ethical and economic reasons the BCS based biowaiver guidelines (9, 10) allow waiving the *in vivo* bioequivalence studies, if the *in vitro* surrogate tests demonstrate that two immediate-release (IR) drug products exert rapid dissolution under all luminal conditions. Biowaiver approach is eligible only for those BCS1 and BCS3 compounds that are known to be stable inside the gastrointestinal milieu and do not belong to narrow therapeutic index drugs (10). While the demonstration of high solubility, sufficient intestinal drug stability and rapid dissolution of IR formulation is a straightforward approach with well-defined *in vitro* conditions, the assessment of permeability may be a daunting undertake. The major agencies agree that a high permeability classification is justified when the fraction of the drug absorbed in humans exceeds 85 % as can be shown with bioavailability data and/or mass balance studies. Several methodologies are considered for providing sup-

porting information (*in vivo* intestinal perfusion in humans, *in vivo* or *in situ* intestinal perfusion studies on animals, *in vitro* permeation studies through excised human or animal intestinal segments, and *in vitro* permeation studies on cultured cells), if the permeability model used is adequately characterized to show its suitability for the intended purpose. Since data on human intestinal permeability is limited (22), using other available models to assess drug permeability seems inevitable. As the case of desloratadine was laid down in the "Introduction", the available data indicates that this drug could be a BCS 1 substance. While only one reference (11) mentions ≥ 90 % of absorbed dose, other data are inconclusive. Namely, a mass balance study, which reported at least 88 % of drug absorbed, is based on metabolite recovery in urine and feces. The recovery of desloratadine determined for the fecal part, unfortunately, could point to drug instability in the gastrointestinal tract prior to the intestinal absorption.

Therefore, to check whether desloratadine could indeed be a BCS 1 candidate, *in vitro* evaluations according to BCS based biowaiver guidelines were performed (9, 10) and supported with additional testing to verify their validity. The high solubility of 5 mg desloratadine dose was demonstrated, as well as very fast dissolution of the reference IR tablet. Further, desloratadine incubation in SGF with and without pepsin, and in SIF indicated ≥ 95 % recovery, which confirms adequate desloratadine luminal stability. This disambiguates the *in vivo* human metabolite recovery data, which was probably a critical issue of biowaiver applicability to desloratadine. Namely, the metabolites from the fecal fraction are indeed formed after the drug has been absorbed from the intestine, therefore the fraction of desloratadine dose absorbed must be at least 88 % (13). Desloratadine stability in this paper was additionally shown in more physiologically relevant media (*i.e.* carbonated Ringer buffer pH 7.0 supplemented with glucose at 37 °C for 2 h) because during our initial attempt to determine desloratadine intestinal permeability using a validated permeability model, *i.e.* excised rat jejunum mounted in side-by-side diffusion cells (17), turned out to be an unsuccessful attempt. Namely, no drug could be quantified in the acceptor solution, although the measurements with PAMPA membranes indicated high desloratadine passive permeability comparable to that of metoprolol, which is a known high permeability standard (9). The in-depth investigation has shown an extensive desloratadine retention by the tissue (*i.e.* more than half of the administered dose), when rat intestinal segments were used. Desloratadine permeability was therefore measured through Caco-2 cell monolayers. This is also a suggested model in the BCS biowaiver guidelines (9), but it is structurally less complex, which was an advantage because of less biological material available for nonspecific binding. This cell line originates from human colorectal adenocarcinoma cells and resembles native human enterocytes to some, limited extent (23). Because Caco-2 monolayer constitutes only a single-cell-thick barrier compared to the full-thickness intestinal wall of the rat jejunum, the unspecific retention of desloratadine by Caco-2 cells was indeed lower. It was low enough to enable reliable desloratadine quantification in the acceptor solution and the determination of apparent permeability coefficients. By using this model, we were able to determine desloratadine permeability compared to the highly permeable internal standard metoprolol. This data was further supported by a non-biological *in vitro* assay with artificial membranes – the PAMPA test. It is also of relevance that the intestinal active transport of desloratadine, which can only be established *in vitro,* was shown to be low and therefore of no relevance for desloratadine biopharmaceutical classification. With all these information now available, combined with the solubility, dissolution and *in* vivo

absorption data known previously and confirmed within this paper one should conclude that desloratadine is a good biowaiver candidate.

CONCLUSIONS

While the ethical and economical reasoning for expanding the number biowaivers are obvious, the complexity of drug absorption too often does not allow a simple justification of a biowaiver based solely on available *in vivo* pharmacokinetic data. As aimed, the presented paper shows the usefulness of well-established *in vitro* methods for biopharmaceutical drug classification in cases when the *in vivo* data alone are inconclusive. While setbacks are possible, as it was also demonstrated within the presented example, they can be overcome by a rational choice of methods. We believe that the classification of desloratadine as a BCS 1 class drug which is chemically and metabolically stable in the gut lumen has been confirmed. It would, therefore, be ethically preferable to register new.

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