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Physico-chemical characterization and *in vitro*/*in vivo* evaluation of loratadine:dimethyl-β-cyclodextrin inclusion complexes

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ABSTRACT

A tricyclic, piperidine derivative of antihistamines, loratadine, which belongs in class II of the Biopharmaceutical Classification System, was investigated. It is an ionizable drug, whose solubility depends on the gastrointestinal pH, and the bioavailability is therefore very variable. Inclusion complexes were prepared by kneading method, containing loratadine (LOR) and dimethyl-β-cyclodextrin (DIMEB) in two different molar ratios in an attempt to achieve better dissolution and therefore the better bioavailability of loratadine. The formation and physicochemical properties of the inclusion complexes were investigated by means of dissolution tests, pH-dependent solubility studies, electrospray ionization mass spectrometry and diffusion-ordered ^{1}H NMR spectroscopy. The *in vivo* efficiency of the complexes was examined in rat animal experiments to confirm the better *in vitro* dissolution. The instrumental examinations proved the presence of total complexes in 1:1 ratio in both compositions. However, the *in vitro* pH-dependent solubility results, the *in vivo* blood levels and the greater pharmacological effect prove that excess DIMEB is needed to achieve the pH-independent and complete solubility of LOR, and therefore better and more consistent bioavailability.

1. Introduction

One of the prerequisites for successful oral drug therapy is sufficient intestinal absorption. The rate and extent of intestinal absorption are mainly dependent on the dissolution rate of the drug in the gastrointestinal fluids and the rate of transport across the intestinal membrane [1]. These two factors form the basis of the Biopharmaceutical Classification System (BCS) [2]. If the drug is an ionizable compound, the intestinal absorption depends on its pKa, too [3]. Poorly water-soluble drugs (BCS classes II and IV) are associated with slow drug absorption, leading eventually to inadequate and variable bioavailability (BA) [2,4].

Loratadine (LOR), chosen for the current studies, is a BCS class II drug [5], with dissolution- or solubility-limited absorption. It is a second-generation antihistamine. LOR is a weak base; its pKa value at 25 °C has been reported to be 4.85–6.00 [5–8]. The solubility of bases increases with decreasing pH at pH values less than the pKa [9]. However, according to the modified Hendersson–Hasselbach equation [10], bases are totally ionized at lower pH values, and at ~pH 7 and higher they are totally non-ionized, which is the form able to be absorbed. Thus, LOR will probably be absorbed from the intestines, in which it exhibits poor solubility.

The main objective of the present study was to investigate the stoichiometry of two inclusion complexes (kneaded products (KPs) 1:1 and 1:2) and the anti-inflammatory effects of LOR and these complexes *in vitro* (Parallel Artificial Membrane Permeability Assay – PAMPA model) and *in vivo* (rat). Our earlier studies [11] revealed that complexation with dimethyl-β-cyclodextrin (DIMEB) (at some compositions) resulted in pH-independent solubility for LOR; at the pH, where LOR is totally non-ionized and hence able to be absorbed, the complexed LOR dissolves about 10 times better than in the absence of DIMEB. The aim of this study was to confirm this observation *in vivo*. Many studies have been published on the pharmaceutical applications of cyclodextrins (CDs) and primarily on their ability to improve the solubility and dissolution rate of poorly soluble drugs [12,13].

Electrospray ionization mass spectrometry (ESI-MS) and diffusion-ordered ^{1}H NMR spectroscopy (DOSY) provide powerful techniques to analyse the stoichiometry of supramolecular complexes like CD inclusion complexes [14]. By the PAMPA model the characteristics of passive diffusion can be investigated. Therewith

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performing an *in vivo* experiment *in vitro*/*in vivo* correlation can be predicted.

2. Materials and methods

2.1. Materials

LOR was kindly provided by TEVA Ltd., Hungary. Heptakis-2,6-di-O-methyl-β-CD (DIMEB-50, 50% isomeric purity, DS∼14) was purchased from Cyclolab Ltd., Hungary. Compound 48/80 (N-methyl-4-methoxy-phenethylamine) was supplied by Sigma–Aldrich Logistic GmbH, Germany. Other chemical reagents were of analytical grade purity.

2.2. Preparation of the products

The KPs were prepared in two molar ratios (LOR:DIMEB = 1:1 and 1:2). The physical mixtures were suspended in the same mass of 50% ethanol, and the solvent was evaporated off at room temperature. After drying, the products were ground. All of the samples were sieved (100 μm) and stored at room temperature under normal conditions.

2.3. Diffusion-ordered spectroscopy (DOSY)

Diffusion coefficients (D) were estimated via DOSY NMR experiments. In the DOSY spectra, chemical shifts were located along the F2 axis and D values along the F1 axis. From the D values, it is possible to infer the size of the species and therefore the absolute stoichiometry of the supramolecular complexes.

The NMR spectra were recorded at 25 °C on a Bruker Avance DRX 400 MHz spectrometer. For 2D DOSY ^{1}H NMR, pulsed field-gradient spin-echo NMR measurements were performed by using the stimulated echo and longitudinal eddy current delay (LED) sequence [15]. A time of 1.5 ms was used for the dephasing/refocusing gradient pulse length (δ), and 100 ms for the diffusion delay (Δ). The gradient strength was changed quadratically from 5% to 95% of the maximum value (B-AFPA 10 A gradient amplifier), and the number of steps was 16. Each measurement was run with 32 scans and 16 K time domain points. For the processing, an exponential window function and single zero filling were applied. During the diffusion measurements, the fluctuation of the temperature was <0.1 K. Prior to the NMR scans, all the samples were equilibrated for 30 min. The data were analysed by using XWINNMR 2.5 software.

2.4. Electrospray ionization mass spectrometry (ESI-MS) studies

The compounds were characterized by MS, using a Finnigan MAT 95S sector field mass spectrometer equipped with an electrospray ion source. Positive-ion ESI-MS spectra were obtained. The solutions were prepared in a 1:1 mixture of acetonitrile/water. The solutions were infused directly into the mass spectrometer at a rate of 200 μl min^{-1}. Data were collected for approximately 100 scans. The scan range was 100–3000 m/z. The spectrometer was used at a resolution of ∼1000–1500.

2.5. In vitro dissolution study

The modified paddle method with the USP dissolution apparatus (Erweka Type DT, Germany) was used to examine 200 mg samples of pure LOR or products containing 200 mg of LOR in 100 ml of simulated intestinal medium (SIM) (pH 7.0 ± 0.1; 14.4 g of $Na_2HPO_4{\cdot}2H_2O$ and 7.1 g of KH_2PO_4 made up to 1000 ml with distilled water). The paddle was rotated at 100 rpm and sampling was performed up to 120 min (sample volume 5.0 ml). Aliquots were withdrawn at 5, 10, 15, 30, 60, 90 and 120 min and immediately filtered. At each sampling time, an equal volume of fresh medium was added, and the correction for the cumulative dilution was calculated. After filtration and dilution, the LOR contents of the samples were determined spectrophotometrically (λ_{SIM} = 248 nm).

2.6. Study of the effect of pH on the solubility

Seven buffer solutions were prepared with different pH values between 1.2 and 7.5 [16]. The defined daily dose of LOR is 10 mg, so 10 mg of LOR or a product containing 10 mg of LOR was examined in 900 ml of dissolution medium at 37 °C. The paddle was rotated at 100 rpm. After 2 h, the removed samples were filtered and the LOR concentrations were measured spectrophotometrically ($\lambda_{pH\ 1.2,\ 2.0,\ 2.5,\ 3.0}$ = 276 nm; $\lambda_{pH\ 4.5}$ = 244 nm; $\lambda_{pH\ 6.5,\ 7.5}$ = 248 nm).

2.7. PAMPA

PAMPA "sandwiches" were formed from a donor 96-well microtitre plate (Millipore MATRNPS 50) and a matching filter plate (Millipore Multiscreen®-IP, MAIPNTR 10) with an apparent porosity of 0.45 μm, coated with 5 μl of a 1% (w/v) n-dodecane solution of lecithin. The initial donor sample concentrations were about 150 μM. The plate sandwich was allowed to incubate at 25 ± 1 °C for 16 h without stirring, in an atmosphere saturated in humidity. Afterwards, sample concentrations in both the acceptor and donor wells were determined by HPLC. Effective permeability coefficients (P_e) were determined by taking into account the apparent filter porosity and sample mass balance.

The donor (150 μl) and acceptor (300 μl) compartments both contained pH 7.4 buffer solutions. The permeability rates were calculated by using the following equation:

$$\log P_e = \log\left\{C \cdot -\ln\left(1 - \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}}\right)\right\}$$

with

$$C = \frac{V_D \cdot V_A}{(V_D + V_A) \cdot A \cdot t}$$

where V_D, volume of the donor compartment (ml); V_A, volume of the acceptor compartment (ml); A, active surface area of the membrane (0.24 cm^2 in the case of the applied filter plate); t, incubation time for the assay (s); $[\text{drug}]_{\text{acceptor}}$, concentration of the compound in the acceptor compartment at the completion of the assay; $[\text{drug}]_{\text{donor}}$, concentration of the compound at theoretical equilibrium.

2.8. In vivo experiments

This study was approved by the Committee on Animal Research at the University of Szeged (IV./01758-6/2008). A colony of inbred 150 ± 5 g male Wistar rats (Charles River Laboratories, Germany) was used, fed commercial rodent pellets and tap water. The animals were housed in groups of 5 at a controlled room temperature (22 ± 1 °C) and maintained under an alternating 12 h light/12 h dark cycle (light on at 6:00 am).

The following materials were tested in this study: LOR, DIMEB, KP 1:1 and KP 1:2 (LOR:DIMEB). The test substances were given orally in suspension in 0.25% methylcellulose in a dose of 10 mg kg^{-1} (1.00 ml kg^{-1}).

The animals were divided into five groups, with 6 rats per group. Group I received the vehicle and served as a control. To study the influence of DIMEB on the oedema, the Group II animals received DIMEB. LOR, KP 1:1 and KP 1:2 were administered to the other three groups, respectively.

In these experiments, at first the animals were treated orally by the above mentioned suspensions. 1 h later the histamine liberator compound 48/80 in physiological solution (10 μg/0.1 ml) was administered subplantarly to elicit the inflammatory reaction [17,18]. In light isoflurane narcosis the intensity of the arising inflammatory reaction was measured after 30 min with the use of a plethysmometer (Ugo Basile, Harvard Apparatus, Germany) on the basis of the volume difference between the right hind leg treated with compound 48/80 and the left hind leg treated with vehicle (physiological saline). Immediately after measuring the extent of the oedema, blood samples were taken from rats by cardiac puncture and collected in tubes containing sodium ethylenediaminetetraacetate. Then these samples were centrifuged and the obtained plasmas were frozen until the measurement of LOR-content (see below).

A high-performance liquid chromatographic (HPLC) method has been developed for quantitative analyses of LOR in *in vitro* (PAMPA model) and *in vivo* samples.

HPLC measurements were performed with a JASCO PU-1580 binary pump (JASCO Inc., Japan) and a programmable variable UV–visible detector. LOR was chromatographed on a 100 mm × 4.6 mm i.d., 3 μm particle, Phenomenex Luna C8(2), 100 Å analytical column under reversed-phase conditions at 30 °C, protected with a SecurityGuard Cartridge (4.0 mm × 2.0 mm) precolumn. The degassed mobile phase was a 47:42:11 (v/v) mixture of acetonitrile, purified water and a phosphate buffer solution (0.5 M, pH 3.0 ± 0.1, adjusted by the addition of 85% orthophosphoric acid). The flow rate was 1.0 ml min^{-1} and the analyte was monitored at 250 nm.

Calibration plots were constructed by analysis of working solutions (concentrations of 5, 10, 25, 50 and 75 μg ml^{-1} (*in vitro*) and 5, 10, 50, 75 and 100 ng ml^{-1} (*in vivo*)) of LOR in the mobile phase and plotting concentration against peak-area response for each injection. The calibration curves were linear throughout the whole range tested and described by the equations $y = 20.318x - 23{,}775$ ($R^2 = 0.9912$) and $y = 1083.4x + 31.777$ ($R^2 = 0.9957$) for the *in vitro* and *in vivo* measurements, respectively. Unknown samples were quantified by reference to these calibration plots. Inter-day precision was calculated from results on the calibration sample of 5 μg ml^{-1} analysed on 20 consecutive days ($n = 5$). The mean amount found was 4.98 μg ml^{-1} and the RSD value was 2.47%. The limits of detection (LOD) and quantification (LOQ) were determined on the basis of the S.D. of the response (*y*-intercept) and the slope of the calibration plot. LOD and LOQ for LOR were 0.004 and 0.013 μg ml^{-1}, respectively.

500 μl of mobile phase was added to 500 μl of plasma, and this mixture was then centrifuged at 17,000 rpm for 15 min. The clear supernatant was next collected and filtered through a 0.22 μm membrane filter (Millipore). From this solution, a 100 μl aliquot was injected for HPLC analysis.

Statistical analyses were performed with Prism 4.0 software (GraphPad, San Diego, CA, USA). Differences in paw oedema between the treatment and control groups were determined by one-way analysis of variance (ANOVA) with the Newman–Keuls *post hoc* test. The criterion for statistical significance was taken as $p < 0.05$. An experimental group contained 6 rats. All values are expressed as mean ± S.E.

3. Results and discussion

3.1. ESI-MS results

ESI-MS is the most promising tool for the characterization of different kinds of host–guest complexes in the gas phase [19]. It can provide evidence of complexation and stoichiometry on the basis of the molecular weights of all vaporized species. The study of host–guest interactions in the gas phase allows the detection of specific interactions not necessarily present in solution, thereby giving a complementary picture of the intrinsic phenomena responsible for molecular recognition. Also, there are interpretation ambiguities as concerns the ESI-MS spectra of supramolecular assemblies, e.g. deciding whether the species present in the mass spectra correspond to those present in solution, or they rather result from processes occurring under high-vacuum conditions. Moreover, it is not clear whether the molecular ions observed are real inclusion complexes or only ion-dipole external adducts, i.e. "false positives" [20]. In our particular case, however, a comparison can easily be made with the aid of results obtained from independent solution-phase techniques.

The hydroxyl groups of CDs are not easily protonated or deprotonated; ESI-MS analyses are usually carried out in the presence of salts in order to enhance detection.

The positive ESI spectra of KP 1:1 and KP 1:2 are reported in Fig. 1a and b, respectively.

An interesting feature of the ESI-MS binding of the analytes with the CDs is the reproducible loss of water. This water loss is presumed to arise from displacement of water from the CD cavity.

Host–guest complexes formed in solution are also stable for characterization by ESI in the gas phase.

The ESI of DIMEB with positive ion detection leads to a series of protonated molecules, $[M+H]^+$, at *m*/*z* values depending on the number of methyl groups in each individual sugar unit of the CD derivative. The mass spectra reflect the average distribution of the methyl groups of DIMEB. It should be noted that the ion peak at *m*/*z* 1331 corresponds to 14 *O*-methyl groups (i.e. heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose to a first approximation) [21].

As expected, the spectrum essentially involves peaks due to singly charged ions of pure LOR [at *m*/*z* 383 as $(LOR)H^+$] and pure DIMEB [at *m*/*z* 1331 as $(DIMEB)H^+$]. A new signal corresponding to the inclusion complex as a singly charged ion $(DIMEB + LOR)H^+$ is observed at *m*/*z* 1730.

When the mixtures with different molar ratios (1:1 or 1:2) of LOR and DIMEB were analysed by ESI-MS, only the 1:1 complex was found in all the mass spectra, which suggests that the DIMEB inclusion complex in the gas phase has a certain stoichiometry.

3.2. DOSY results

The *D* value observed in the NMR experiment (fast-exchange condition) is the weighted average of those of the bound and the free guest. The rationale behind the extraction of the bound fraction from diffusion NMR measurements is simple. The host and guest have their own *D* values in the free state, reflecting their molecular weight and shape. The guest molecules are significantly smaller than CD, and the *D* values of the bound guests were taken to be equal to that of CD; it was assumed that, for the binding of a small guest molecule to a large host molecule, the *D* value of the host is not greatly perturbed and that of the host–guest complex can be assumed to be the same as that of the non-complexed host molecule. In the case of a weak or negligible association, the *D* values of the host and the guest will remain unchanged. For any other case, assuming fast exchange on the NMR time scale, the observed (measured) *D* values are weighted averages of the free and bound *D* values [22].

In the 2D DOSY spectra (Fig. 2), the F2 dimension shows the chemical shift and F1 stands for log *D*. Groups belonging to the same molecule will therefore appear in almost the same F1 row.

In the molecule of DIMEB, there are $=CH_2$ groups and $-O-CH_3$ groups, which have chemical shifts (δ) in the range 1–5 ppm. In the molecule of LOR, there are aromatic protons which have higher δ values, at about 7–8 ppm. In the DOSY spectrum of KP 1:1, log *D*

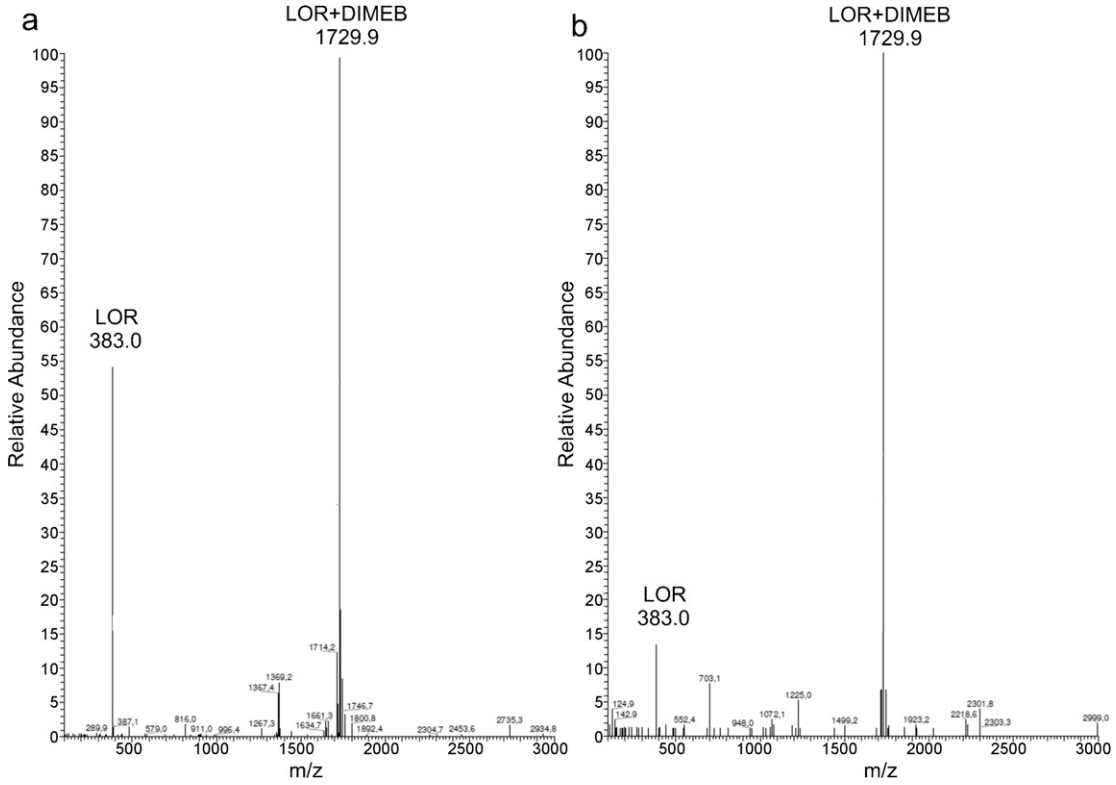

Fig. 1. ESI-MS results on KP 1:1 (a) and KP 1:2 (b).

is the same for every chemical shift, which is possible only when LOR is complexed in DIMEB, when they compose a unit. If LOR is not complexed, it should diffuse more quickly due to its small molecular weight; it should have a smaller *D* value.

The *D* value measured for the complex indicates that it is best formulated as the 1:1 complex. For perfect spheres, theory predicts that increasing the molecular weight *n*-fold should lead to a *D* value decreased by a factor of $n_{-1/3}$. Unfortunately, in the absence of well-defined ^{1}H NMR spectra, X-ray crystallographic results or ESI-MS data, assignment of absolute stoichiometry to these aggregates is speculative [23].

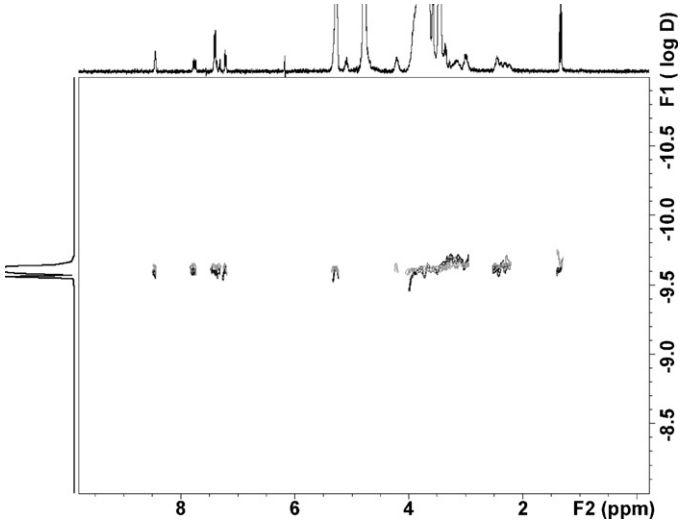

Fig. 2. Representative DOSY spectra of KP 1:1 (grey) and KP 1:2 (black).

3.3. In vitro dissolution study

According to the pKa value, the solubility of LOR depends on the pH:LOR can undergo protonation on the N of the pyridine ring in acidic media, forming salts with good solubility. It therefore exhibits good dissolution in acidic media, but dissolves poorly in alkaline media (e.g. SIM).

The rate of dissolution was improved for all of the products, but the extent of the increase depended on the molar ratio (see Fig. 3). For the KP 1:1 composition, the dissolution of LOR did not reach in 100%: only 70% of the LOR dissolved during 2 h. For KP 1:2, the whole of the investigated samples dissolved in the first 15 min, i.e. the dissolution in SIM was as good as in acidic media, which means that the same good dissolution would be attained at the extreme pH values of the gastrointestinal tract on the use of this DIMEB product. Accordingly, the rate-limiting step of absorption would not be the dissolution: the passage through the membrane would be regulated by the permeability. As LOR has good permeability, the application of LOR complexed with a CD such as DIMEB in 1:2 molar ratio would lead to a greater quantity of drug being absorbed, so that better BA would be obtained.

3.4. Effect of pH on solubility

The defined daily dose of LOR is 10 mg. The solubility of LOR has been reported to decrease with increasing pH [5]. As can be seen in Fig. 4, in the acidic range (to pH ~ 3) both the 1:1 and 1:2 compositions allow total dissolution of the applied dose. However, with increasing pH, DIMEB is not able to dissolve the whole quantity in the case of the 1:1 product, and the BA will therefore not be sufficient: only about 70% of the investigated amount of the 1:1 product dissolved at higher pH. In contrast, virtually the whole quantity of LOR dissolved from KP 1:2 at each pH value, proving the solubility of LOR is independent of the pH. This clearly suggests an oppor-

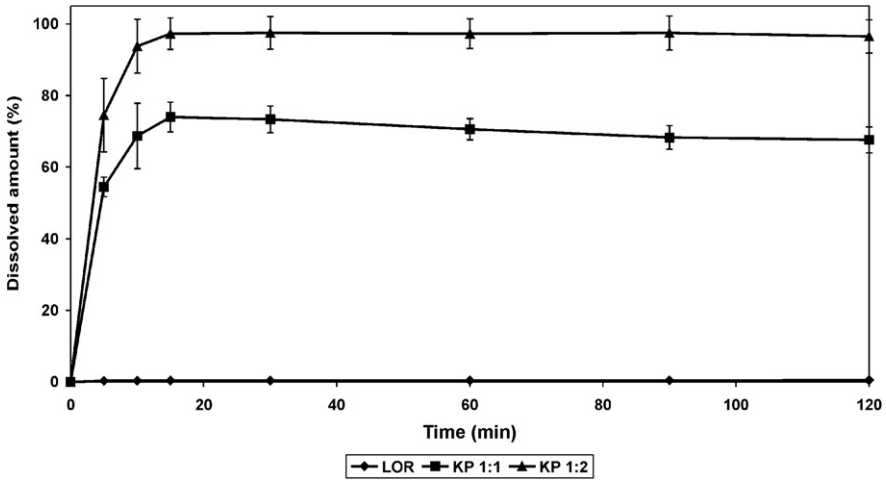

Fig. 3. Dissolution of LOR, KP 1:1 and KP 1:2 in SIM.

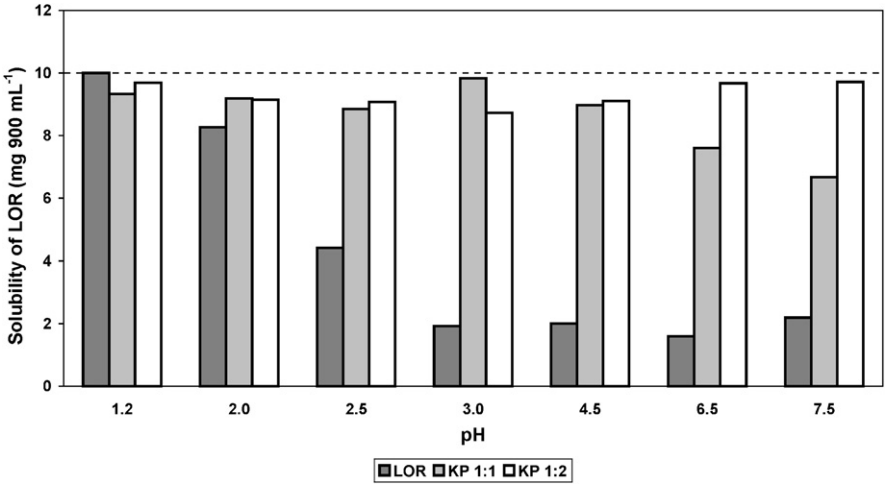

Fig. 4. Comparative solubility data of LOR, KP 1:1 and KP 1:2.

tunity to ensure consistent dissolution for LOR, thereby achieving better and more uniform BA.

3.5. PAMPA

LOR is a highly variable drug from the aspect of BA: its absorption depends greatly on whether the patient has eaten or not, and what was eaten. In the fasted state, different enzymes and natural solubilizers (e.g. bile acids) are released, which help to dissolve the drug. All forms of food have their own acidic (e.g. meats, cheese, egg, alcohol, mustard, sweeties, etc.), basic (e.g. potato, carrot, onion, mushroom or coconut) or natural characteristics, which influence the pH in the GI tract, and hence the solubility of this ionizable API. The pH of the stomach is known to differ significantly among individuals and is within the range of 1–3 or even higher (1–5) under fasting conditions. If undissolved LOR is emptied from the stomach, the absorption rate will be dramatically lower as compared with that in individuals where complete dissolution occurs in the stomach [5].

As LOR is a BCS class II drug, it readily passes through the intestinal wall, with a permeability value of 2.7×10^{-3} to 4.8×10^{-5} cm min^{-1} [5]. The PAMPA model represents only passive diffusion, and deceptive results can be observed if other mechanisms also play a role in the absorption of the API. LOR is a substrate of P-gp, which hinders its absorption, and thus the BA of this molecule. Fig. 5 indicates that LOR has higher permeability than the DIMEB-containing products.

The results demonstrate that, if passive diffusion were the main force of absorption, DIMEB would hinder this process. It is possible that the diffusion through this artificial membrane is bidirectional, and therefore the absorbed LOR can back-diffuse to the donor side and become recomplexed by DIMEB. Another explanation is that the stability of the complex is too high, and the association–dissociation balance is shifted to association. There is

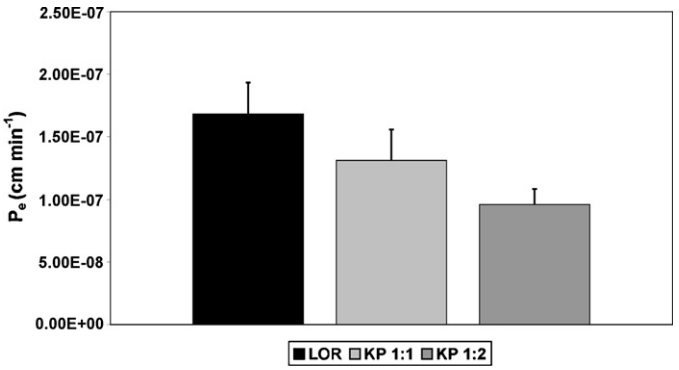

Fig. 5. Calculated effective permeability values of LOR, KP 1:1 and KP 1:2.

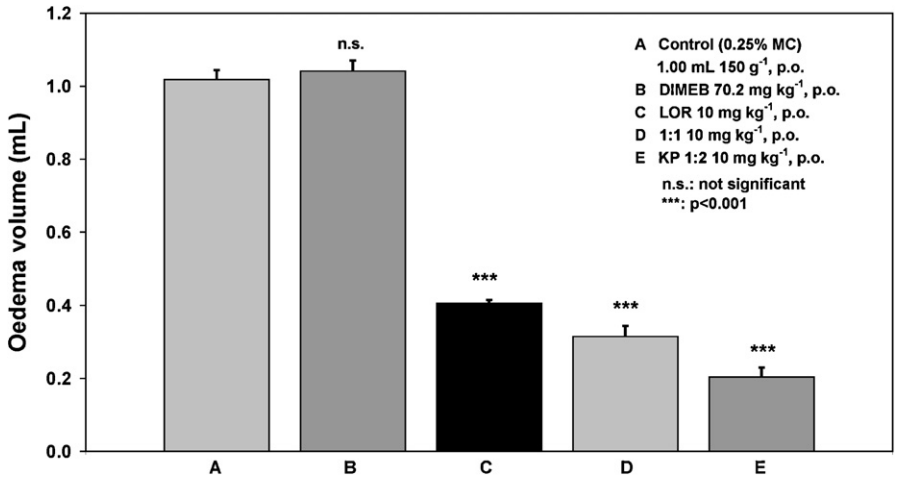

Fig. 6. Oedema volumes in the treated and the control groups (significance evaluations involve comparisons with LOR).

an observable relationship between the amount of DIMEB and the calculated permeability. The higher the concentration of DIMEB, the lower the permeability is. This confirms the theory that the association–dissociation balance is shifted to association, and the back-diffused LOR is recomplexed by free DIMEB. The statistical analysis reveals that the differences between the P_e values of LOR and KP 1:1, LOR and KP 1:2, and KP 1:1 and KP 1:2 are significant ($p < 0.05$).

The permeability of LOR increases with increasing pH: at acidic pH, LOR is in a protonated form, which cannot pass through the membrane, but at basic pH it is not protonated (non-ionized), and can be absorbed. This tendency also holds for KP 1:2, but the permeability is always lower at each pH value, which corresponds to the above discussion.

3.6. In vivo experiments

The main effect of the oedema inducer compound 48/80 is the release of histamine from the mastocytes, and hence the effect of the antihistamine LOR can be well examined with this model [17,18].

LOR, DIMEB and the inclusion complexes were investigated to establish to what extent they can reduce induced oedema. The volumes of the oedema induced in the right back paws are presented in Fig. 6. It was found that DIMEB did not influence the extent of oedema relative to the control group, and thus the effects of the DIMEB-containing products can only be due to the LOR in the products. Rats pretreated with LOR, KP 1:1 and KP 1:2 significantly decreased the compound 48/80-induced oedema. The results of the Newman–Keuls multiple comparison test are presented in Table 1. It is evident that the abilities of LOR, KP 1:1 and KP 1:2 to decrease oedema differ significantly; the complexation of LOR with DIMEB resulted in better BA.

We also measured the blood concentrations by HPLC. The data shown in Fig. 7 relate to the effects on oedema, except that the blood concentrations of LOR and KP 1:1 do not differ significantly, which demonstrates that KP 1:1 does not reach the desired BA. Excess DIMEB (as in KP 1:2) is needed to obtain pH-dependent solubility and more consistent and greater BA.

LOR was well separated from the biological background under the described chromatographic conditions, with mean RT = 7.50 min. No interference with plasma matrix constituents was observed. The mobile phase used guaranteed good repeatability of the retention times. The LOR concentration in samples from the animals is about a few ng ml^{-1}, and thus the analytical wavelength was selected according to the maximum LOR absorbance with respect to a stable baseline.

P-gp recognizes many compounds as substrates, and tends to have high affinity for hydrophobic and positively charged compounds at physiological pH. DIMEB appears not to be a substrate of P-gp because it is a hydrophilic and electrically neutral cyclic oligosaccharide with a relatively high molecular weight. Furthermore, DIMEB should not compete with P-gp substrates due to its lack of cell permeability. Thus, DIMEB must have an alternative inhibitory effect on P-gp activity, differing from the P-gp inhibitors that suppress the efflux by their surface activity. Some results suggested that DIMEB has a direct inhibitory effect on the P-gp level on the cell surface. DIMEB did not exhibit cytotoxicity on Caco-2 cells and had no effect on the paracellular and transcellular transport [24].

Hence, one part of the DIMEB will affect P-gp, and the other part remains for the recomplexation. This can be another explanation why there is a need for excess DIMEB to achieve much better BA. These two mechanisms probably play a role in the enhanced

Table 1
Results of the Newman–Keuls multiple comparison test.

Comparisons	Mean difference	Q	p value
Control vs. DIMEB	−2.333	1.097	>0.05 ns.
Control vs. LOR	−60.83	30.08	<0.001***
Control vs. KP 1:1	−70.33	33.08	<0.001***
Control vs. KP 1:2	−81.43	35.98	<0.001***
LOR vs. KP 1:1	−9.50	4.015	<0.01**
LOR vs. KP 1:2	−20.60	8.273	<0.001***
KP 1:1 vs. KP 1:2	−11.10	4.311	<0.01**

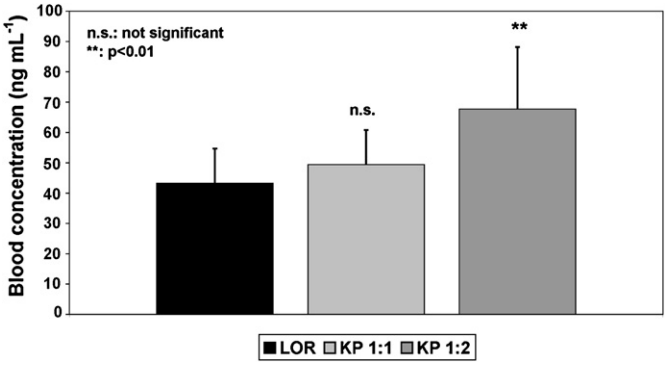

Fig. 7. Blood concentrations of LOR after oral administration of LOR and products (significance evaluations involve comparisons with LOR).

absorption, and therefore the greater BA. So these observations also suggested that the increase in the AUC value of LOR by DIMEB may be caused not only by the solubilizing activity of DIMEB, but also by inhibition of the efflux in the GI tract.

4. Conclusion

We have already demonstrated that DIMEB is able to make the solubility of LOR independent of the pH. It has now been established based on the ESI-MS and ^{1}H NMR DOSY results that the stoichiometry of the complex is 1:1. However, it is not enough to obtain pH-independence. For the 1:2 product, the solubility and the dissolution rate of LOR is higher and independent of pH against the KP 1:1 product. This is also indicated by the *in vivo* experiments, so better and more consistent BA can be achieved due to complexation in the appropriate molar ratio. The kneading method is one of the simplest complexation techniques; it can easily be scaled up and is often used in preclinical formulations.

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