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Intestinal absorption characteristics of the low solubility thiocarboxanilide UC-781

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Abstract

The aim of this study was to determine the intestinal absorption characteristics of the antiviral agent UC-781 and to optimize the experimental conditions of the in vitro system for low solubility compounds. The absorption potential of UC-781 was studied with the Caco-2 system and with the rat intestinal perfusion technique. The low solubility of UC-781 required the use of solubility/dissolution rate enhancing agents (e.g. VitE-TPGS, Gelucire 44/14). The creation of sink conditions in the receiver compartment of the Caco-2 system was a prerequisite to reliably study the transport of this poorly soluble compound. After inclusion of VitE-TPGS in the acceptor solution, UC-781 could be characterized as a class II drug of the Biopharmaceutical Classification System (low solubility, high permeation across membranes). A significant concentration-dependent decrease in transport of UC-781 was observed upon increasing the concentration of VitE-TPGS in the apical compartment. This observation contrasts to the absorption enhancing properties of VitE-TPGS, and can probably be attributed to a decrease in the concentration of free UC-781 when using higher concentrations of the solubility/dissolution rate enhancing agents. The use of Gelucire 44/14 as a solubilizing agent resulted in a batch-dependent degradation of UC-781. The inclusion of the solubility/dissolution rate-enhancing agent VitE-TPGS did not result in absorption enhancement in the intestinal perfusion technique.

Keywords: HIV; UC-781; NNRTI; Caco-2; Vitamin E-TPGS; Gelucire

1. Introduction

The thiocarboxanilide UC-781 (Fig. 1) is a very potent and selective HIV type 1 (HIV-1) non-nucleoside reverse transcriptase inhibitor (NNRTI) with a favorable resistance spectrum. The EC_{50} (concentration required to inhibit 50% of the cytopathogenic effect induced by HIV-1) for UC-781 was 0.002 μg ml^{-1}, the IC_{50}-value (concentration required to inhibit the activity of the HIV-1 reverse transcriptase by 50%) was 0.02 μg ml^{-1} and the selectivity index (ratio of CC_{50}/EC_{50}, with CC_{50} the concentration required to reduce the

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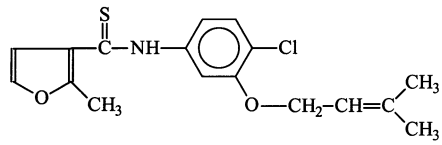

Fig. 1. Structure of UC-781.

viability of the host cells by 50%) was higher than 50 000 (De Clercq, 1999). These data indicate that UC-781 has superior antiviral properties compared with several other NNRTIs currently available (Balzarini and De Clercq, 1997; De Clercq, 1999). Besides strong antiviral activity, UC-781 has other beneficial properties: the chemical synthesis is relatively easy, the drug has a long intracellular half-life (ca. 5.5 days) and, when combined with other anti-HIV drugs, no antagonistic effects against HIV-1 replication were observed (Balzarini and De Clercq, 1997; Borkow et al., 1997). Recently, this drug has been described to restore the antiviral activity of azidothymidine (Retrovir®, AZT) against AZT-resistant HIV-1 (Borkow et al., 1999). UC-781 has also been proposed for use as a retrovirucidal chemical barrier to prevent the sexual transmission of HIV-1 (Borkow et al., 1997; Balzarini et al., 1998).

The availability of antiviral drugs that can be orally administered is mandatory to assure patients' compliance as well as for economic reasons. In the framework of developing an oral drug delivery system of UC-781, we determined the intestinal absorption characteristics of UC-781, by using an in vitro system (Caco-2) and an in situ system (rat intestinal perfusion technique). The effect of solubility/dissolution rate enhancing agents (Gelucire 44/14 and Vitamin E-D-α-tocopheryl polyethyleneglycol 1000 succinate; VitE-TPGS) was also determined. In addition, the necessity to create sink conditions to assess the transport characteristics for low solubility compounds in the Caco-2 system is discussed.

2. Materials and methods

2.1. Materials

UC-781 (*N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furan-carbothioamide) was supplied by Uniroyal Chemical Ltd (Guelph, Ont., Canada). VitE-TPGS was kindly provided by Eastman Chemical Company (Kingsport, TN) and Gelucire 44/14 was obtained from Gattefossé (Saint-Priest, France). Methanol (BDH, Poole, England) and *n*-hexane (Biosolve, Valkenswaard, Netherlands) were HPLC grade. Sodium fluorescein was obtained from UCB (Drogenbos, Belgium). All chemicals used for culturing the Caco-2 cells were purchased from Gibco Inc (Life Technologies, Belgium). Cell culture medium (CM) consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acids solution and 100 IU ml^{-1} penicillin–streptomycin. Transport medium (TM) consisted of Hanks' Balanced Salt solution (HBSS) containing 25 mM D-(+)-glucose (Sigma Chemical, St. Louis, MO) and 10 mM Hepes. pH was adjusted to 7.4 at 37 °C with sodium hydroxide (0.05 N) (BDH, Poole, England).

2.2. Cell culture

Caco-2 cells were from Bio-Whitakker (Walkersville, MD). Caco-2 cells were grown in 75 cm^2 culture flasks at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1–7. Cells were negative for Mycoplasma infection.

2.3. Transport experiments

For transport experiments, Caco-2 cells were plated at a density of 40 000 cells cm^{-2} on Costar® Transwell membrane inserts (0.2 μm pore diameter, 12 mm diameter; Corning Inc, NY). Confluence was reached within 3–4 days after seeding and the monolayers were used for the experiments 21–25 days post-seeding. Cell passages between 85 and 99 were used in the experiments. Transepithelial Electrical Resistance-values (TEER-values) were measured with an End Ohm Voltohmmeter (WPI, Aston, England). Only monolayers with TEER-values higher than 350 Ω cm^2 were used. As an additional control of the integrity of the monolayers, the flux of

sodium fluorescein (1 mg ml^{-1}) across the Caco-2 monolayers was assessed at the end of the experiment. Concentrations of sodium fluorescein were determined with a Uvikon 810P spectrophotometer (Kontron Instruments, Watford, England) at 490 nm. Typical sodium fluorescein fluxes were lower than 0.6% h^{-1}.

2.4. HPLC analysis

Concentrations of UC-781 were determined using an HPLC-UV system. (Waters Associated Inc, Milford, MA). UV signals were monitored at 297 nm and the obtained peaks integrated using a Digital personal computer running Waters Milennium chromatography software (Waters Associated Inc, Milford, MA). The column used was a Novapak RP18 (4 μm) operated under radial compression (Waters RCM 8 × 10, Waters Associated Inc, Milford, MA). The flow rate amounted to 1.5 ml min^{-1}, and the mobile phase consisted of 80% MeOH and 20% H_2O (purified by a Maxima system, Elga Ltd, High Wycombe Bucks, England). The volume injected was 50 μl. The retention time of UC-781 under these conditions was 8 min. Samples from the Caco-2 system were analyzed by direct injection into the HPLC-system. In presence of a biological matrix, samples were extracted according to the procedure described in the next paragraph.

2.5. Extraction of UC-781

Concentrations of UC-781 in blood (1 ml), CM (1 ml) or dialysis samples (2 ml) were determined after extraction with 8 ml n-hexane. After vortex mixing for 2 min, the mixture was centrifuged at 4500 rpm for 5 min. Six ml of the supernatant was transferred into a new test tube and replaced by 6 ml of fresh *n*-hexane. The extraction procedure was repeated, and the pooled hexane-layers were evaporated to dryness. The residue was dissolved in 500 μl of methanol, of which 100 μl were injected into the HPLC system.

2.6. Dialysis experiments

Dialysis experiments were performed with a Spectrapor 7 dialysis membrane (MWCO 1000) (Spectrum Laboratories, Rancho Dominguez, CA). The membrane was filled with 750 μl of a solution of UC-781 (100 μM) in VitE-TPGS (0.1–1%) to which 2% FBS was added. The membranes were sealed and put in a beaker filled with 15 ml of 0.1% VitE-TPGS, containing 2% FBS. The beakers were put on a 3D-rocking platform (Gyro-Rocker®, Stuart Scientific, Staffordshire, England) with a speed of 80 rocks per min. Samples of 2 ml were taken of the receiver compartment at the beginning of the experiment and after 24 h. The samples were extracted and further analyzed as described in the previous paragraphs.

2.7. In situ perfusion

2.7.1. In situ rat intestinal perfusion: experimental set-up

In situ perfusion experiments were performed based on a previously described method (Annaert et al., 2000). Male Wistar rats (± 300 g) were used. After anaesthetizing the animals with sodium pentobarbital (60 mg kg^{-1}), the right jugular vein was cannulated with a heparinized (50 IU ml^{-1}) polyethylene cannula (1.02 mm o.d.; Portex, Kent, England) for blood supply from a donor rat during the perfusion experiment. A laparotomy was performed and the small intestine was exposed. The mesenteric vein draining the last part of the ileum (20–25 cm) was cannulated using a heparinized (50 IU ml^{-1}) catheter (Insyte-W® 0.7 × 19 mm, Beckton Dickinson, Salt Lake City, UT). The cannula was secured with a few drops of cyanoacrylate adhesive. A mesenteric-jugular shunt was constructed to avoid blood loss during further preparation of the perfusion experiment. A segment of the ileum (10–18 cm) was isolated by inserting two glass cannulas (4 mm o.d., 3 mm i.d.) at the proximal and distal end of the segment. Polyethylene tubing (6.5 mm o.d., 3.1 mm i.d.) was connected to the inlet cannula. The intestinal content was removed by perfusing the segment with pre-warmed TM (38 °C) at a flow rate of 1–3 ml min^{-1}. In contrast with the TM used for Caco-2 transport studies, no glucose was supplemented to the HBSS buffer. The perfusion pump (HVL Tris, Brussels, Belgium) was placed between the reser-

voir and the inlet cannula. A three-way valve placed immediately before the inlet cannula allowed sampling from the perfusion medium.

2.7.2. Single pass in situ intestinal perfusion studies with UC-781

Before the start of the experiment, the intestinal segment was perfused with TM in the presence or absence of VitE-TPGS (0–1% w/v) for 15 min. The flow rate of the perfusate amounted to 1 ml min^{-1}. At the beginning of the perfusion with the antiviral compound, the mesenteric-jugular shunt was opened and donor blood supply initiated via the jugular vein at a rate of 0.5 ml min^{-1}. The intestinal segment was perfused with UC-781 (100 μmol l^{-1}) in the presence of various concentrations of VitE-TPGS (0–1% w/v). Blood from the mesenteric vein was collected in heparinized tubes over 5 min time intervals for 30 min. Concentrations of UC-781 were determined in total blood using HPLC with UV detection.

2.8. Calculations

Results of the transport experiments with the Caco-2 monolayers are expressed as the percentage of the amount initially added to the donor side. Values are expressed as mean ± S.D. ($n = 3$).

Results of the in situ perfusion experiments with UC-781 are expressed as absolute amounts appearing in the mesenteric blood, corrected for time and length of the perfused segment. Values are expressed as mean ± S.E.M. ($n \geq 3$).

3. Results and discussion

Preliminary experiments with UC-781 in the Caco-2 system had shown that, irrespective of the presence of solubility/dissolution rate enhancing agents in the apical compartment, absorptive transport was very low if TM was used in the basolateral compartment. Modulation of the transport of UC-781 by a P-glycoprotein (P-gp) related efflux mechanism as a possible explanation for this low transport was excluded since no polarity in transport nor any effect of co-administration of verapamil (a known P-gp inhibitor) on the transport of UC-781 was observed (data not shown).

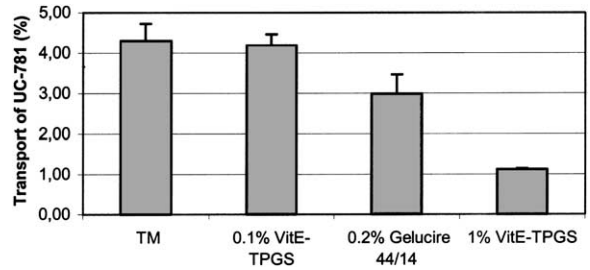

Fig. 2. Influence of the presence of solubility/dissolution rate enhancing agents (Gelucire 44/14 and VitE-TPGS) in the apical medium on the cumulative absorptive transport of UC-781 after 2 h of incubation. The basolateral compartment contained CM. Results are expressed as the percentage of the total amount of UC-781 initially administered (100 μmol l^{-1}) ± S.D. ($n = 3$).

Due to the very low aqueous solubility of UC-781 (0.2 mg l^{-1}), saturation in the basolateral compartment may offset transepithelial transport. Indeed, when establishing sink conditions by completely refreshing the fluid at the basolateral compartment at certain timepoints, a much higher transport of the drug was obtained. Therefore, the influence of the experimental conditions as well as the presence of solubility/dissolution rate enhancing agents (VitE-TPGS, Gelucire 44/14, CM) on the transport of UC-781 across Caco-2 monolayers and rat intestinal mucosa was systematically investigated. The use of VitE-TPGS and Gelucire 44/14 up to concentrations of 1% did not affect the integrity of the Caco-2 monolayers as was shown by the TEER-values in comparison to those of the control.

In the first set of experiments, different apical conditions were used (Fig. 2), while the basolateral condition was kept constant (CM). On the apical side, UC-781 (100 μmol l^{-1}) was added as a suspension in TM or dissolved in 0.2% Gelucire 44/14, 0.1% VitE-TPGS or 1% VitE-TPGS. The results show that transport increased when the concentration of the solubility/dissolu-

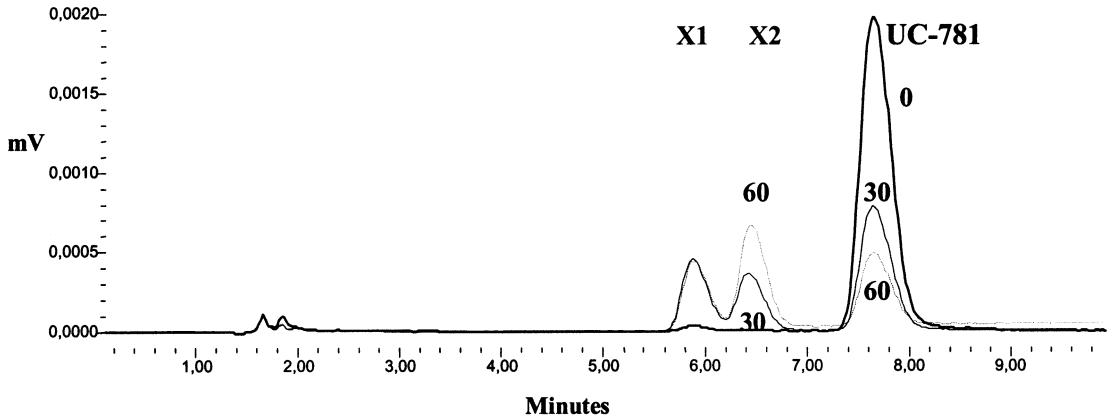

Fig. 3. Chromatograms of UC-781 (2 μM) in TM containing Gelucire 44/14 (1%) incubated at 37 °C for 0, 30 and 60 min.

tion rate enhancing agent decreased (1% VitE-TPGS < 0.1% VitE-TPGS). A possible explanation for this observation could be an inclusion of UC-781 in the micelles, formed by VitE-TPGS (CMC = 0.02% at 25 °C). Since UC-781 is a lipophilic compound, it can concentrate in the micelles formed by the solubility/dissolution rate enhancing agents, thus decreasing the concentration of free UC-781 that is able to cross the monolayer. Another important observation is the degradation of UC-781 in the presence of Gelucire 44/14. When UC-781 was incubated in TM containing 1% Gelucire 44/14, it was rapidly degraded (half-life, 100 min at 25 °C and 37 min at 37 °C) and this decomposition resulted in two (presently unknown) degradation products (retention time, 5.8 and 6.4 min, respectively). Example chromatograms of UC-781 and its degradation products are shown in Fig. 3. Although there are indications that this degradation is batch-dependent, Gelucire 44/14 was not used in further experiments.

In a second set of experiments different concentrations of VitE-TPGS (0.05–0.5%) were added to the basolateral compartment, while the apical conditions were kept constant. Transport was initiated by adding 100 μM UC-781 in CM to the apical side of the monolayer. To ensure sink conditions, the inserts were placed into new wells filled with 1.5 ml of fresh medium after 40, 80 and 120 min. Fig. 4 clearly shows an enhancement in absorptive transport of UC-781 upon increasing the concentration of VitE-TPGS in the basolateral compartment. Again, this could be explained by the micellar inclusion of UC-781: as soon as UC-781 crosses the monolayer, it concentrates in the micelles formed by VitE-TPGS, thus maintaining the concentration gradient as a driving force for transport of the free compound.

In a third set of experiments, the influence of various concentrations of VitE-TPGS (0–1%) in CM on the apical compartment of the monolayers was determined. The basolateral compartment contained VitE-TPGS 0.2%. Fig. 5 shows that transport of UC-781 decreases upon increasing the concentration of the solubilizing agent in the apical compartment. This observation can also be

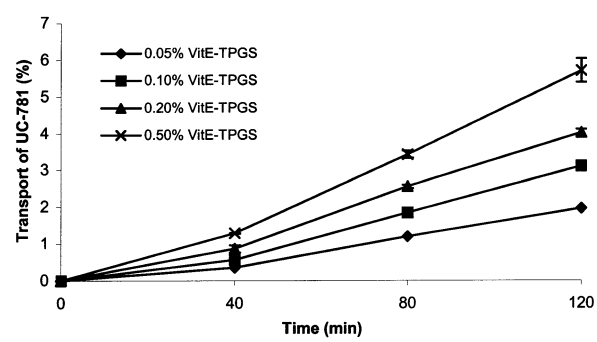

Fig. 4. Influence of the concentration of VitE-TPGS (0.05–0.5%) in the basolateral compartment on the cumulative absorptive transport of UC-781 after 2 h of incubation. The apical compartment contained UC-781 (100 μM) in CM. Results are expressed as the percentage of the total amount of UC-781 initially administered ± S.D. ($n = 3$).

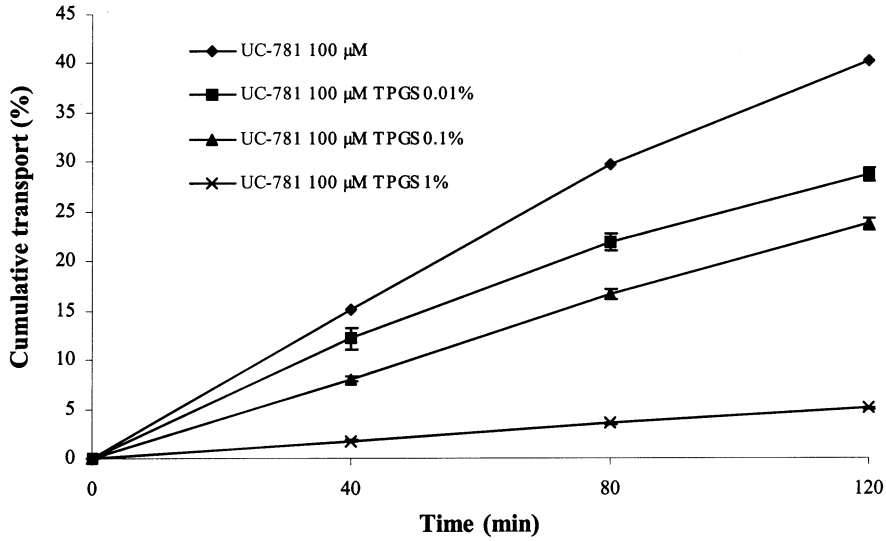

Fig. 5. Influence of the concentration of VitE-TPGS (0–1%) in the apical compartment on the cumulative absorptive transport of UC-781 after 2 h of incubation. The medium in the basolateral compartment contained 0.2% VitE-TPGS in TM. Results are expressed as the percentage of the total amount of UC-781 initially administered (100 μM) ± S.D. ($n = 3$).

explained by a decrease in free concentration as a consequence of the incapsulation of UC-781 in micelles.

More conclusive evidence to support the hypothesis that the presence of micelles seriously affects the transport of UC-781, was obtained by dialysis experiments. The release of UC-781 from solutions of UC-781 (100 μM) in VitE-TPGS (0.1–1%) through a dialysis membrane was studied. After 24 h, approximately, 1.3% of the initial concentration of UC-781 could be observed in the receiver compartment when a solution of UC-781 in 0.1% VitE-TPGS was used inside the dialysis membrane, whereas only 0.02% of the initial concentration of UC-781 was detected when a solution of UC-781 in 1% VitE-TPGS was used inside the membrane. These data clearly show incapsulation of UC-781 in VitE-TPGS micelles.

Finally, experiments were performed to evaluate the effect of VitE-TPGS using the in situ perfusion technique. The results of these experiments are summarized in Table 1. Although the variability of these results is very high, it could be stated that the presence of the dissolution/solubility enhancer did not result in an increase in epithelial transport. These results suggest that the absorptive transport of UC-781 is not determined by the total drug concentration in solution, but rather by the concentration of free compound. Since the concentrations of VitE-TPGS used are above its CMC-value, UC-781 will be incapsulated in the micelles. This incapsulation results in a reduced concentration of free UC-781 and, hence, does not cause an enhancement in absorption of the drug.

4. Conclusion

Our results show that UC-781 belongs to class II of the Biopharmaceutical Classification System (low solubility, high permeation across membranes) (Amidon et al., 1995). The creation of sink conditions is a prerequisite to perform reliable transport experiments of low-solubility compounds. Compared with the use of serum to create sink conditions (Aungst et al., 2000), the use of solubility/dissolution rate enhancing agents, e.g. VitE-TPGS, offers the advantage that no sample preparation is required. Furthermore, it is shown that the efficiency of UC-781 to cross biological membranes is not dependent on the

Table 1
In situ perfusion results

	Suspension ($n = 4$) (100 μmol l^{-1})	0.2% VitE-TPGS ($n = 3$) (100 μM)	1% VitE-TPGS ($n = 5$) (100 μM)
Average amount of UC-781 in blood (in pmol cm^{-1} min^{-1})	11.0 ± 6.0	12.6 ± 2.0	14.3 ± 2.0

Absorption is expressed as the average amount of UC-781 appearing in the blood (in pmol cm^{-1} min^{-1}) $\pm$ S.E.M. ($n \geq 3$).

total concentration of UC-781 in solution, but on the concentration of free UC-781. This is shown by an absence of absorption enhancement by the presence of solubility/dissolution rate enhancing agents and by the results obtained in the in situ perfusion technique. The lack of stability of UC-781 in solutions of Gelucire 44/14 precludes this additive as an efficient solubility enhancer for this antiviral drug.

Further studies on the identification of the degradation products of UC-781 and the batch-limited degradation of UC-781 in the presence of Gelucire 44/14 still need to be performed. In addition, the influence of media, which are more representative for the in vivo situation, on transport characteristics of low-solubility drugs requires further investigation.

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