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Co-release of paclitaxel and encequidar from amorphous solid dispersions increase oral paclitaxel bioavailability in rats

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ABSTRACT

The oral bioavailability of paclitaxel is limited due to low solubility and high affinity for the P-glycoprotein (P-gp) efflux transporter. Here we hypothesized that maximizing the intestinal paclitaxel levels through apparent solubility enhancement and controlling the simultaneous release of both paclitaxel and the P-gp inhibitor encequidar from amorphous solid dispersions (ASDs) would increase the oral bioavailability of paclitaxel. ASDs of paclitaxel and encequidar in polyvinylpyrrolidone K30 (PVP-K30), hydroxypropylmethylcellulose 5 (HPMC-5), and hydroxypropylmethylcellulose 4 K (HPMC-4K) were hence prepared by freeze-drying. *In vitro* dissolution studies showed that both compounds were released fastest from PVP-K30, then from HPMC-5, and slowest from HPMC-4K ASDs. The dissolution of paclitaxel from all polymers resulted in stable concentration levels above the apparent solubility. The pharmacokinetics of paclitaxel after oral administration to male Sprague-Dawley rats was investigated with or without 1 mg/kg encequidar, as amorphous solids or polymer-based ASDs. The bioavailability of paclitaxel increased 3- to 4-fold when administered as polymer-based ASDs relative to solid amorphous paclitaxel. However, when amorphous paclitaxel was co-administered with encequidar, either as an amorphous powder or as a polymer-based ASD, the bioavailability increased 2- to 4-fold, respectively. Interestingly, a noticeable increase in paclitaxel bioavailability of 24-fold was observed when paclitaxel and encequidar were co-administered as HPMC-5-based ASDs. We, therefore, suggest that controlling the dissolution rate of paclitaxel and encequidar in order to obtain simultaneous and timed release from polymer-based ASDs is a strategy to increase oral paclitaxel bioavailability.

1. Introduction

Paclitaxel is a potent anticancer drug substance effective against a variety of cancers, which include breast, ovarian, non-small cell lung cancer, and colon cancer (Montana et al., 2011; Sandler et al., 2006; Sparano et al., 2008; Zhang et al., 2009). However, due to paclitaxel's poor oral absolute bioavailability (less than approximately 10 % in most species (Choi and Jo, 2004; He et al., 2022; Sparreboom et al., 1997; Zamek-Gliszczynski et al., 2012)), its therapeutic impact after oral administration is limited. Limited water solubility and dissolution, as well as a high affinity towards the multidrug efflux transporter P-glycoprotein (P-gp), contribute to poor oral bioavailability (Malingré et al., 2000; Peltier et al., 2006; Sparreboom et al., 1997).

Several studies have attempted to increase the oral bioavailability of paclitaxel through formulation strategies as nicely reviewed in detail by Nguyen et al. (2021). These strategies included amorphous solid dispersions (ASDs) (Miao et al., 2019), self-micro emulsifying drug delivery system (SMEDDS) containing various P-gp inhibitors (Al-Kandari et al., 2020), and microcapsules containing a P-gp inhibitor in an amorphous state (Kim et al., 2016). The ASD formulation strategy is, generally, used to increase the bioavailability of poorly soluble drugs by improving the dissolution rate and amount of drug dissolved (Van den Mooter, 2012),

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and the approach has also recently been used to control the release rate of a P-gp inhibitor and P-gp substrate to obtain increased intestinal absorption through inhibition of P-gp (Nielsen et al., 2023). Miao et al. (2019) formulated an ASD with paclitaxel, prepared with hydroxypropyl methylcellulose acetate succinate MF (HPMAS-MF) and Poloxamer 188 (F68), and evaluated the effect of supersaturation on the oral bioavailability of paclitaxel in Sprague-Dawley rats. The bioavailability of paclitaxel administered as an ASD compared to an aqueous solution of paclitaxel containing Cremophor® EL did not significantly increase the oral bioavailability of paclitaxel. The authors considered if the limited difference between the two formulations was either due to low levels of supersaturation from the dissolution of the ASD or due to inhibition of P-gp mediated efflux of paclitaxel caused by the presence of Cremophor® EL in the paclitaxel oral solution (Miao et al., 2019). Recently, Al-Kandari et al. (2020) compared the oral bioavailability of a paclitaxel suspension to the oral bioavailability of paclitaxel-loaded SMEDDS with or without 2 doses of 100 mg of the P-gp inhibitor cyclosporin A (CsA) (Al-Kandari et al., 2020), and showed that without CsA the bioavailability increased 4.5-fold whereas it was increased 7.8-fold with CsA. Kwak et al. (2010) investigated if encequidar, a third-generation P-gp inhibitor, could inhibit P-gp in the gastrointestinal tract to improve the oral bioavailability of paclitaxel in male Sprague-Dawley rats, and reported that the C_{max} and the AUC of paclitaxel increased approximately 10-fold, and the bioavailability increased from 3.4 % to 41.3 % after co-administration with encequidar (Kwak et al., 2010). Similarly, in male Sprague–Dawley rats (*mdr1a(-/-)*), the oral bioavailability of paclitaxel increased from 12 % in wild-type rats to 47 % in *mdr1a(-/-)* rats (Zamek-Gliszczynski et al., 2012). In another study, Kim et al. (2016) encapsulated encequidar mesylate salt into microcapsules by spray-drying, thereby, changing the solid state of encequidar mesylate salt to an amorphous one using hydrophilic polymers and solvents. Oral administration of a paclitaxel solution containing Cremophor® EL and ethanol with co-administration of encequidar mesylate salt powder or microcapsules to male Sprague-Dawley rats showed a 35- and 69-fold increase in oral bioavailability, respectively, relative to the paclitaxel solution alone (Kim et al., 2016). However, the intravenous (IV) use of Cremophor® EL is problematic as side effects such as anaphylactic hypersensitivity reactions and neutropenia have been observed (Gelderblom et al., 2001; Windebank et al., 1994). Recently, Nielsen et al. (2021) showed that the bioavailability of another P-gp substrate, etoposide, increased with co-administration of the P-gp inhibitor, zosuquidar (Nielsen et al., 2021). Moreover, a following paper showed that co-administration of etoposide and zosuquidar increased the etoposide bioavailability even further (approximately 1.8-fold) when administered as hydroxypropyl methylcellulose 5 (HPMC-5) based ASDs (Nielsen et al., 2023). Nielsen et al. (2023) showed that the simultaneous and controlled co-release of etoposide and zosuquidar from various ASDs, based on polyvinyl pyrrolidone K30 (PVP-K30), HPMC-5, or hydroxypropyl methylcellulose 4 K (HPMC-4K), was important for increasing the bioavailability of the P–gp substrate etoposide (Nielsen et al., 2023). HPMC-5 proved to be superior in terms of increasing the bioavailability of etoposide using zosuquidar. This raised the question if the bioavailability increasing effect of HPMC-5-based ASDs was specific to the mentioned etoposide-zosuquidar combination or could be applied to other combinations of P-gp substrates and inhibitors. To investigate this, we choose paclitaxel as a P-gp substrate and encequidar as a P-gp inhibitor because the ASD approach allows for making formulations without solubility enhancing surfactants or co-solvents. Therefore, we here hypothesized that maximizing the paclitaxel concentration by enhancing the apparent solubility and controlling the simultaneous dissolution of both paclitaxel and encequidar from ASDs could increase the oral bioavailability of paclitaxel. Thus, the present research aimed to increase the oral bioavailability of paclitaxel through the spatiotemporal release of paclitaxel and encequidar from polymer-based ASDs.

2. Materials and methods

2.1. Materials

Paclitaxel, free base, encequidar free base (ENC) for the *in vitro* study, and encequidar mesylate salt (ENC MS) for the *in vivo* study, were acquired from MedKoo Biosciences (Morrisville, NC, USA). Ultra-pure water was obtained from an in-house Milli Q water purification system (Merck Millipore, Burlington, MA, USA). Fasted State Simulated Intestinal Fluid V2 (FaSSIF-V2) powder was acquired from Biorelevant.com (London, UK) and FaSSIF V2 was prepared as prescribed by the manufacturer. Hydroxypropyl methylcellulose 5 (HPMC-5) was acquired from Norsk Medisinaldepot (NMD, Oslo, Norway), polyvinylpyrrolidone K30 (PVP-K30) was acquired from BASF Pharma (Ludwigshafen, Germany), and hydroxypropyl methylcellulose 4 K (HPMC-4K) was acquired from Fagron (Rotterdam, Netherlands). Tert-butanol was acquired from Sigma Aldrich (Darmstadt, Germany). Acetonitrile (HPLC graded), absolute ethanol, sodium chloride, acetic acid, and Tert-butanol were acquired from VWR Chemicals (Briare, France). Sodium hydroxide pellets and maleic acid were acquired from Merck (Darmstadt, Germany). Trifluoroacetic acid was acquired from Fisher Scientific (Loughborough, UK) and Alfa Aesar, Thermo Fischer (Kandel, Germany).

2.2. Paclitaxel and encequidar solubility studies

Solubility studies were performed to determine the equilibrium solubility, i.e. the free concentration of solute measured after solvent-solid incubation for 72 h, for pure compounds in FaSSIF-V2, pH 6.50. These were used to compare to the apparent solubility measured for the meta stable systems measured with the ASD formulations. Therefore, 3.94 mg paclitaxel and 3.00 mg encequidar were weighed in two separate 10 mL glass vials and covered with aluminium foil. 4.5 mL FaSSIF-V2, pH 6.50, was added and the vials were placed inside a Grant-bio-orbital shaker-incubator ES-20 (Biosan, Riga, Latvia) at 37 °C with a shaking of 250 rpm for at least 72 h. After 72 h there was still an excess amount of the two compounds in the glass vials. Hereafter, 1.5 mL was sampled with a 3 mL PP Soft-Ject syringe (Henke Sass Wolf, Tuttlingen, Germany) and discarded; this was done twice to saturate the syringe. Afterwards, another 1.5 mL was sampled and a 13 mm 0.45 μm Spartan filter (regenerated cellulose, Whatman plc, Maidstone, UK) was applied and the first 500 μL was used to saturate the filter and was hereafter discarded. The last approximately 1000 μL was filtered, collected, and immediately diluted 1:1 with 2 % (v/v) acetic acid in acetonitrile in three separated HPLC vials (n = 3). The diluted samples were analysed by HPLC-UV as described below (Section 2.3). All glassware, equipment, and FaSSIF-V2 were pre-heated to 37 °C before use.

The solubility of paclitaxel in 70 % (v/v) *tert*-butanol in ultra-pure water, was determined by adding an excess amount of paclitaxel in a 15 mL falcon tube and adding 70 % (v/v) *tert*-butanol in ultra-pure water until a clear solution was obtained. This method was also used for the investigation of the solubility of encequidar in 100 % (v/v) acetonitrile (ACN). The solubility of encequidar mesylate salt in 50 % (v/v) ACN in ultra-pure water was determined by adding an excess amount of encequidar mesylate salt in a 5 mL tube and adding 50 % (v/v) ACN in ultra-pure water until a clear yellow solution, without particles, was obtained. The solubility studies in organic solvents were done to determine the

possible amount of paclitaxel, encequidar as the free base, and the mesylate salt that could be used in the solutions to prepare ASDs (Section 2.4).

2.3. Quantification of paclitaxel and encequidar using HPLC-UV

An Ultimate 3000 HPLC-UV (Thermo Fisher Scientific, Waltham, MA, USA) or a Waters 2695 HPLC system connected to a Waters 2487 Dual λ Absorbance Detector (Waters Corporation, MA, USA) was used to quantify paclitaxel and encequidar in the equilibrium solubility studies in FaSSIF-V2, pH 6.50 and the dissolution studies. The mobile phase consisted of ACN (A) and ultra-pure water containing 0.1 % trifluoroacetic acid (B). A reversed phase column (Nova-Pak® C18, 4 μm, 3.9 × 150 mm, Waters, Milford, MA, USA, or ACE 5 C18, 5 μm, 4.6 × 250 mm, Avantor delivered by VWR, Biowest, Radnor, PA, USA) was applied and maintained at 25 °C. The flow was constant at 0.8 mL/min with an isocratic elution of 65 % A and 35 % B for a run time of 8 min. Samples of 20 μL, maintained at 4 °C, were injected and paclitaxel and encequidar were detected at 245 nm and 275 nm (254 nm for ACE 5 C18 column), respectively. Paclitaxel and encequidar had retention times of 2.2 and 2.5 min, respectively (Nova-Pak® C18 column), and 5.0 and 6.2 min, respectively (ACE 5 C18 column). Calibration curves of paclitaxel were made by preparing samples from a 7.51 mg/mL stock solution in ethanol and ranged from 0.15 μg/mL to 19.53 μg/mL prepared in the mobile phase. Linear regression of concentration versus peak area was performed in GraphPad Prism 8.4.3. The lower limit of quantification (LOQ) was 0.157 μg/mL (Nova-Pak® C18 column) and 3.112 μg/mL (ACE 5 C18 column). Calibration curves of encequidar were made by preparing samples from a 0.508 mg/mL stock solution in DMSO and ranged from 0.039 μg/mL to 5.075 μg/mL prepared in the mobile phase. Linear regression of concentration versus peak area was performed in GraphPad Prism 8.4.3. The LOQ was 0.045 μg/mL (Nova-Pak® C18 column) and 0.639 μg/mL (ACE 5 C18 column). Calibration curves prepared for the dissolution and solubility studies to quantify the paclitaxel and encequidar concentrations are shown in Supplementary Material (Fig. **S1**).

2.4. Preparation of amorphous solid dispersions and amorphous controls

ASDs for dissolution studies were prepared by freeze-drying solutions of paclitaxel or encequidar with either PVP-K30, HPMC-5, or HPMC-4K. The concentrations in the final solutions for freeze-drying were 2.11 mg/mL, 0.04 mg/mL, and 40 mg/mL for paclitaxel, encequidar, and polymer, respectively. Solutions were prepared by overnight stirring with a magnet to ensure complete dissolution. The solutions of paclitaxel and polymers were prepared by dissolving the components together in 70 % (v/v) *tert*-butanol in ultra-pure water. These solutions were freeze-dried as they were. It proved difficult to obtain adequate solubility of encequidar in the 70 % (v/v) *tert*-butanol in ultra-pure water. Hence, the concentration of encequidar was lowered and ACN was titrated into samples containing encequidar until complete dissolution was obtained. Solutions of encequidar were prepared in 100 % ACN and added to the solutions of polymer the day after dissolving the polymers, making the final solvent a 63:27:10 (v/v/v) mixture of *tert*-butanol: ultra-pure water: ACN.

The resulting solutions with either paclitaxel or encequidar were clear or clear light yellow, respectively. These were then transferred to 24-well plates (Corning, NY, USA) by adding 1 mL solution to each well. The plates were placed in a −80 °C freezer for at least 2.5 h. The frozen solutions were afterwards freeze-dried in an Alpha 1–2 LD_{plus} freeze-dryer (Martin Christ, Osterode, Germany). The main drying was performed over 24 h (0.011 mbar, −60 °C). The ASDs were afterwards placed in a desiccator for at least 4 h before further handling. ASDs with HPMC-5 or HPMC-4K were solid, porous, and light, while PVP–based ASDs were more brittle, though still porous. PVP–based ASDs were broken apart with a spatula into a powder, while HPMC-5- and HPMC-4K-based ASDs were blended, separately, in an electric coffee mill (Bistro, Bodum, Triengen, Switzerland) to obtain a powder for the dissolution study.

Amorphous controls of paclitaxel and encequidar mesylate salt, used in the *in vivo* studies, were prepared by freeze-drying in a similar method to the ASDs described above by dissolving approximately 2 mg/mL paclitaxel in 70 % (v/v) *tert*-butanol in ultra-pure water. Amorphous encequidar mesylate salt was prepared by adding 4 mL of a 2 mg/mL encequidar mesylate salt solution in 50 % (v/v) ACN in ultra-pure water to 6 mL 70 % (v/v) *tert*-butanol in ultra-pure water, resulting in a concentration of 0.8 mg/mL encequidar mesylate salt. The solutions were transferred to centrifugation tubes (Sarstedt, Germany) and frozen with either liquid nitrogen or placed in a −80 °C freezer for at least 2.5 h before freeze-drying. The solutions were freeze-dried in a Heto Drywinner freeze-dryer (Model DW 1,0–110, Alleroed, Denmark). The main drying was performed over a period of at least 24 h at –110 °C and with a pressure of $6 \bullet 10^{-2}$ mbar. The freeze-dried products were placed in a desiccator for at least 4 h before further handling.

ASDs for *in vivo* studies were prepared by freeze-drying solution of paclitaxel or encequidar mesylate salt with either HPMC-5 or PVP-K30. The concentrations in the final solutions for freeze-drying were 2.09 ± 0.11 mg/mL, 1.20 mg/mL, and 40 mg/mL for paclitaxel, encequidar mesylate salt, and polymer, respectively. Solutions were prepared by overnight stirring with a magnet to ensure complete dissolution. The solutions of paclitaxel and polymers were prepared by dissolving the components together in 70 % (v/v) *tert*-butanol in ultra-pure water. These solutions were freeze-dried as they were. For the ASDs with encequidar mesylate salt, solutions of encequidar mesylate salt were prepared in 50 % (v/v) ACN in ultra-pure water and added to the solution of polymer the day after dissolving the polymers, making the final solvent a 56:24:20 (v/v/v) mixture of *tert*-butanol: ultra-pure water: ACN. The solutions were transferred to centrifugation tubes (Sarstedt, Germany) and placed in a −80 °C freezer for at least 2.5 h upon freeze-drying. The freeze-drying procedure was performed as for the amorphous controls. HPMC-5 and PVP-K30-based ASDs were blended, separately, in an electric coffee mill (Bistro, Bodum, Triengen, Switzerland) to obtain a fine powder for the *in vivo* studies.

2.4.1. Solid-state characterization

X-ray powder diffraction (XRPD) was carried out on a PANalytical X'PERT Pro (Malvern PANalytical Ltd, UK) with a Cu Kα source, a voltage of 45 kV, and a current of 40 mA. Scanning was performed at a diffraction angle (2θ) from 5 to 90° with a run time of 10.01 min.

Differential scanning calorimetry (DSC) was done using a DSC822e (Mettler-Toledo, Ohio, USA). 3 mg to 5 mg sample was put into 40 μL aluminium pans and sealed with a punctured lid. Samples were heated from 25 to 300 °C with a heating rate of 10 °C/min under nitrogen purge gas with a flow of 50 mL/min. The DSC was calibrated daily with an indium standard. Data were analysed using the STARE software (Mettler-Toledo, Ohio, USA).

2.5. Paclitaxel and encequidar dissolution studies

The presented dissolution study was performed and designed based on a release study performed by Nielsen et al. (2023). A modified dissolution-like setup was applied in a Grant-bio-orbital shaker-incubator ES-20 (Biosan, Riga, Latvia) with a temperature set at 37 °C and a shaking set to 160 rpm. 100 mL beakers (50 × 70 mm), three beakers per

Table 1
Overview of *in vitro* dissolution setups, crystalline control, and applied amorphous solid dispersions of paclitaxel and encequidar in vessels containing 80 mL FaSSIF-V2 pH 6.50 at 37 °C.

#	Applied setups	Paclitaxel added in dissolution vessel (mg)	Encequidar added in dissolution vessel (mg)	Times equilibrium solubility of paclitaxel	Times equilibrium solubility of encequidar
Drug release from pure crystalline materials or two separate ASDs containing either paclitaxel or encequidar					
1	Crystalline control	2.03 ± 0.88	0.51 ± 0.42	21.36 ± 9.22	54.45 ± 44.00
2	Paclitaxel in HPMC-5 + encequidar in HPMC-5 Paclitaxel in PVP-K30 + encequidar in PVP-K30 Paclitaxel in HPMC-4K + encequidar in HPMC-4K	0.96 ± 0.02	0.014 ± 0.001	10.14 ± 0.16	1.48 ± 0.06
3	Paclitaxel in HPMC-5 + encequidar in HPMC-5	0.50 ± 0.01	0.014 ± 0.002	5.24 ± 0.15	1.53 ± 0.20
4	Paclitaxel in HPMC-5 + encequidar in HPMC-5	1.47 ± 0.01	0.014 ± 0.001	15.42 ± 0.09	1.51 ± 0.13
5	Paclitaxel in HPMC-5 + encequidar in PVP-K30	0.97 ± 0.02	0.015 ± 0.001	10.21 ± 0.19	1.59 ± 0.09
6	Paclitaxel in PVP-K30 + encequidar in HPMC-5	0.94 ± 0.01	0.015 ± 0.001	9.92 ± 0.08	1.60 ± 0.07

dissolution study to perform triplicates, were used as dissolution vessels, covered in aluminium foil, and a USP type 1 basket was used as a sinker. Inside the basket, the ASDs or crystalline drugs were weighed and placed with the open end downwards on the bottom of the beaker. The dissolution study was started by adding 80 mL pre-warmed (37 °C) FaSSIF-V2, pH 6.50, in the beakers inside the shaker-incubator. Sampling was at 1, 5, 10, 15, 20, 30, 45 min, 1, 2, 3, 4, 5, and 6 h by aspirating 0.5 mL media with a 3 mL PP Soft-Ject syringe (Henke Sass Wolf, Tuttlingen, Germany) fitted with a 10 μm porous polyethylene prefilter (Quality Lab Accessories, Telford, PA, USA). The samples were immediately filtered through a 13 mm 0.45 μm Spartan filter (regenerated cellulose, Whatman plc, Maidstone, UK). The first 200 μL was discarded and the rest was collected, 200 μL of the collected sample was diluted 1:1 in 2 % (v/v) acetic acid in ACN. The samples were then analysed by HPLC-UV as described above (Section 2.3).

Six different setups were applied (Table 1) to investigate the dissolution from two separate ASDs containing paclitaxel or encequidar, or the dissolution of a crystalline mixture of paclitaxel and encequidar in the same basket. To investigate the effect on apparent solubility enhancement of paclitaxel, experiments were designed with doses corresponding to between 5-times and 15-times the equilibrium solubility of paclitaxel in FaSSIF-V2, pH 6.50 at 37 °C, while a dose corresponding to 1.5-times the equilibrium solubility was chosen for encequidar based on a previous study (Nielsen et al., 2023). Furthermore, different polymers, HPMC-5, PVP-K30, and HPMC-4K, were tested to investigate the dissolution rate of paclitaxel and encequidar. The combination of the two different polymers HPMC-5 and PVP-K30 was also tested to investigate the dissolution rate of paclitaxel and encequidar at 10-times the equilibrium solubility of paclitaxel and 1.5-times the equilibrium solubility of encequidar. Moreover, the combination of the two polymers was tested to investigate if the polymers would impact the dissolution of each other and if paclitaxel in either HPMC-5 or PVP-K30 had an impact on encequidar in either HPMC-5 or PVP-K30. For the crystalline control, it was aimed to weigh out 1.5-times the equilibrium solubility of encequidar and 10-times the equilibrium solubility of paclitaxel, however, since the amount that should be weighed was low, 0.95 mg crystalline paclitaxel and 0.01 mg crystalline encequidar, it was not possible to weigh out this specific low amount and instead the lowest amount possible was weighed.

2.6. *In vivo absorption of paclitaxel in Sprague-Dawley Rats*

All experiments involving the use of animals have been conducted in accordance with the European Directive of 2010 (2010/63/EU) on the protection of animals used for scientific purposes and the Belgian and Flemish Region implementing legislation and were conducted in and have been approved by the ethics committee on Animal Experiments of the Research Center of Janssen Research & Development, a division of Janssen Pharmaceutica NV, located in Beerse, Belgium which is accredited by AAALAC (https://www.aaalac.org/) since 2004.

2.6.1. *Animals*

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were housed under a controlled environment in a building at a temperature of 22 ± 2 °C, relative humidity of 55 ± 10 %, and a 12-hour light/dark cycle. Food was deprived ~16 h prior to dosing, and access to food was returned at 4 h post-dosing. At dosing, the animals weighed between 255.4 g and 309.5 g and were randomly assigned into eleven groups of six animals.

2.6.2. *Design of dosing and Sampling, and Bioanalysis*

All groups received a dose of 10 mg/kg paclitaxel orally, either as a crystalline drug, amorphous drug, or as HPMC-5- or PVP-K30-based ASDs. For seven groups, 1 mg/kg encequidar was co-administered as an amorphous drug or as HPMC-5- or PVP-K30-based ASDs. Encequidar mesylate salt was chosen instead of the encequidar free base for the *in vivo* studies based on the poor solubility of the encequidar free base, and thereby the amount present in the formulated ASDs, 0.4 mg encequidar free base versus 12 mg encequidar mesylate salt. An overview of how the formulations were combined is shown in Table 2. The formulations were suspended in 15 mL FaSSIF-V2, pH 6.50, and administered in 5 mL/kg. When paclitaxel was co-administered with encequidar as ASDs, two separate ASDs were physically mixed before suspension, one for paclitaxel and one for encequidar.

Table 2
Overview of the formulations used for the *in vivo* pharmacokinetic study.

Group number	Formulation/-s
1	Crystalline paclitaxel
2	Paclitaxel in HPMC-5
3	Paclitaxel in HPMC-5 + encequidar mesylate salt in HPMC-5
4	Paclitaxel in HPMC-5 + encequidar mesylate salt in PVP-K30
5	Paclitaxel in PVP-K30
6	Paclitaxel in PVP-K30 + encequidar mesylate salt in PVP-K30
7	Paclitaxel in PVP-K30 + encequidar mesylate salt in HPMC-5
8	Amorphous paclitaxel
9	Amorphous paclitaxel + Amorphous encequidar mesylate salt
10	Amorphous paclitaxel + encequidar mesylate salt in PVP-K30
11	Amorphous paclitaxel + encequidar mesylate salt in HPMC-5

Previous studies have demonstrated that zosuquidar, a P-gp inhibitor, at a dose of 0.63 mg/kg, increased the oral bioavailability of the P-gp substrate etoposide 2.5-fold (Nielsen et al., 2021; Nielsen et al., 2023). The odd number came from a dose-escalation study; however, such a study is not available for encequidar. Therefore, in the present study, the inhibitor, encequidar, should increase absorption but not fully saturate P-gp, in order to see potential differences between the apparent solubility enhancement of paclitaxel and inhibition by encequidar on the overall absorption of paclitaxel. Thus, the dose of encequidar, 1 mg/kg, in the present study was chosen since zosuquidar and encequidar both are third-generation P-gp inhibitors, and have somewhat comparable affinity towards P-gp, 5–10 nM (Nielsen et al., 2021) and 53 nM (Kwak et al., 2010) in Caco-2 intestinal cells, respectively, and both showed poor oral absorption in male Sprague-Dawley rats, 4.21 % (Nielsen et al., 2021) and 6.25 % (Kwak et al., 2010), respectively.

Blood sampling was performed at 15, 30 min, 1, 2, 3, 4, 6, and 8 h. Samples were taken by tail vein puncture with a 23G needle followed by sample collection into Micro haematocrit tubes 32–64 μL EDTA (Vitrex Medicals, Herlev, Denmark). Plasma was obtained after centrifugation at 1900 g for 10 min at 4 °C and was transferred into 10 μL End-to-End pipettes (Vitrex REF. 174313). The plasma samples were stored at −20 °C until further bioanalysis. Quantification of paclitaxel was achieved using a qualified LC-MS/MS method. The End-to-End pipettes were washed out with 10 parts of 5 % BSA in PBS pH 7.5 prior to further sample preparation. All samples were subjected to protein precipitation, followed by LC-MS analysis. Calibration standards for paclitaxel and quality control samples to cover the calibration range were prepared in rat plasma. Calibration standards, quality control samples, and study samples were processed at the same time. A linear regression model with 1/x2 weighing was used, and peak area ratios of the analyte to its internal standard were plotted against the analyte concentrations. The sample concentrations were calculated by interpolation from the standard curve. The lower limit of quantification (LLOQ) obtained for paclitaxel was 1.00 ng/mL in plasma. Bioanalysis was carried out on a Triple Quad 6500+ mass spectrometer (Sciex, Danaher Corporation, DC, USA), coupled to an UPLC system (Shimadzu, Kyoto, Japan). Sample extracts were injected into an Acquity UPLC BEH C18 1.7 μm, 2.1x50 mm column (Waters, Milford, MA, USA). Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B consisted of ACN. The gradient started with a gradient from 45 % to 70 % B in 1.5 min, with a step gradient of 95 % B which was held for 0.7 min before re-equilibration to the starting conditions for 1.3 min. The flow rate was set at 0.50 mL/min and the column temperature was held at 50 °C. A mixture of 2-propanol: ACN: water: formic acid (40:40:20:0.1, v/v/v/v) was used as a rinsing solution for the autosampler. The MS was operated in the positive ion mode using electrospray ionization and the parameters were optimized for the quantification of paclitaxel (MRM transition m/z 876.3 > 308) and the stable isotope-labelled internal standard paclitaxel-C13 (MRM transition m/z 882.3 > 314).

2.7. Data analysis

The pharmacokinetic parameters were first calculated individually for each animal and then combined for graphing and statistical analysis. The area under the curve from 0 to 8 h ($AUC_{0\text{-}8h}$) was calculated in GraphPad Prism 8.4.3 with the area under the curve analysis. The elimination rate constant (k_{el}) was calculated by linear regression of the last linear part of the ln (plasma concentration) versus time profile, where k_{el} was minus slope. The AUC from 0 to infinity (∞) was calculated:

$$AUC_{0-\infty h} = AUC_{0-8h} + \left(\frac{C_{pt}}{k_{el}}\right) \quad (1)$$

where C_{pt} is the plasma concentration at the last quantifiable time point for each animal in each group. The t_{max} and C_{max} values were first determined individually for each animal in each group using GraphPad Prism 8.4.3 and then combined for statistical analysis. The plasma half-life ($t_{½}$) was calculated:

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k_{el}} \quad (2)$$

The relative bioavailability was calculated as a fraction (3). Where $AUC_{0\text{-}\infty h}X$ is the area under the curve from 0 h to infinity for the chosen group number:

$$Relativebioavailability = \frac{AUC_{0-\infty h}X}{AUC_{0-\infty h}Amorphouspaclitaxel} \quad (3)$$

GraphPad Prism 8.4.3 was used for statistical analysis of all pharmacokinetic parameters.

2.8. Statistics

The statistical analysis was done using GraphPad Prism 8.4.3, where n represents the number of replicates or animals. Results in the present paper are represented as mean values ± SEM (standard error of the mean) unless stated otherwise.

3. Results

3.1. Solubility of paclitaxel and encequidar

The equilibrium solubility of paclitaxel and encequidar in FaSSIF-V2, pH 6.50, at 37 °C, indicated that the solubility of paclitaxel was higher than encequidar, 0.968 ± 0.145 μg/mL and 0.072 ± 0.001 μg/mL, receptively. These solubilities were used to assess the possibility of enhancing the apparent solubility as shown later. In order to prepare organic solvent-based solutions for the preparation of the ASDs, the solubility of paclitaxel in 70 % (v/v) *tert*-butanol in ultra-pure water was also measured and was determined to be higher than the equilibrium solubility of paclitaxel in FaSSIF-V2, pH 6.50, 3.03 mg/mL versus 0.968 ± 0.145 μg/mL. Moreover, the results indicated that the solubility of encequidar in 100 % (v/v) ACN was lower than the solubility of encequidar mesylate salt in 50 % (v/v) ACN in ultra-pure water, 0.49 mg/mL versus 18.88 mg/mL. All solutions for preparations of ASDs were visually inspected for appearance to ensure all compounds were in solution.

3.2. Solid state and visual characterization of the ASDs

Paclitaxel, encequidar free base, and encequidar mesylate salt, as received from the supplier, showed well-defined peaks in their X-ray Powder Diffraction (XRPD) diffractograms. The diffractograms of all ASDs showed clear halos without any peaks. Furthermore, the diffractogram of the amorphous paclitaxel and amorphous encequidar mesylate salt showed clear halos with no peaks. See Supplementary Material for XRPD diffractograms (Figs. **S2-S4**).

The differential scanning calorimetry (DSC) thermograms of crystalline paclitaxel and encequidar showed a sharp endothermic peak around 225 °C and 135 °C, respectively, which was not seen in the DSC thermograms of the HPMC-5- and PVP-K30-based ASDs. Furthermore, the DSC thermograms of paclitaxel and encequidar showed an exothermic peak around 240 °C and 235 °C, respectively (Fig. 1).

XRPD of the ASDs and amorphous paclitaxel and encequidar mesylate salt formulated for the *in vivo* study were re-investigated a day after the *in vivo* study and all diffractograms showed clear halos with no peaks. See Supplementary Material for XRPD diffractograms (Fig. **S4**).

The prepared HPMC-5-based ASDs were light and soft, and the HPMC-4K-based ASDs were dense and hard. The PVP-K30-based ASDs were so brittle that a spatula was used to obtain a light and fine powder. For the *in vivo* study the amorphous paclitaxel and amorphous encequidar mesylate salt were fine white and yellow powders, respectively. The prepared HPMC-5-based and the PVP-K30-based ASDs for the *in vivo*

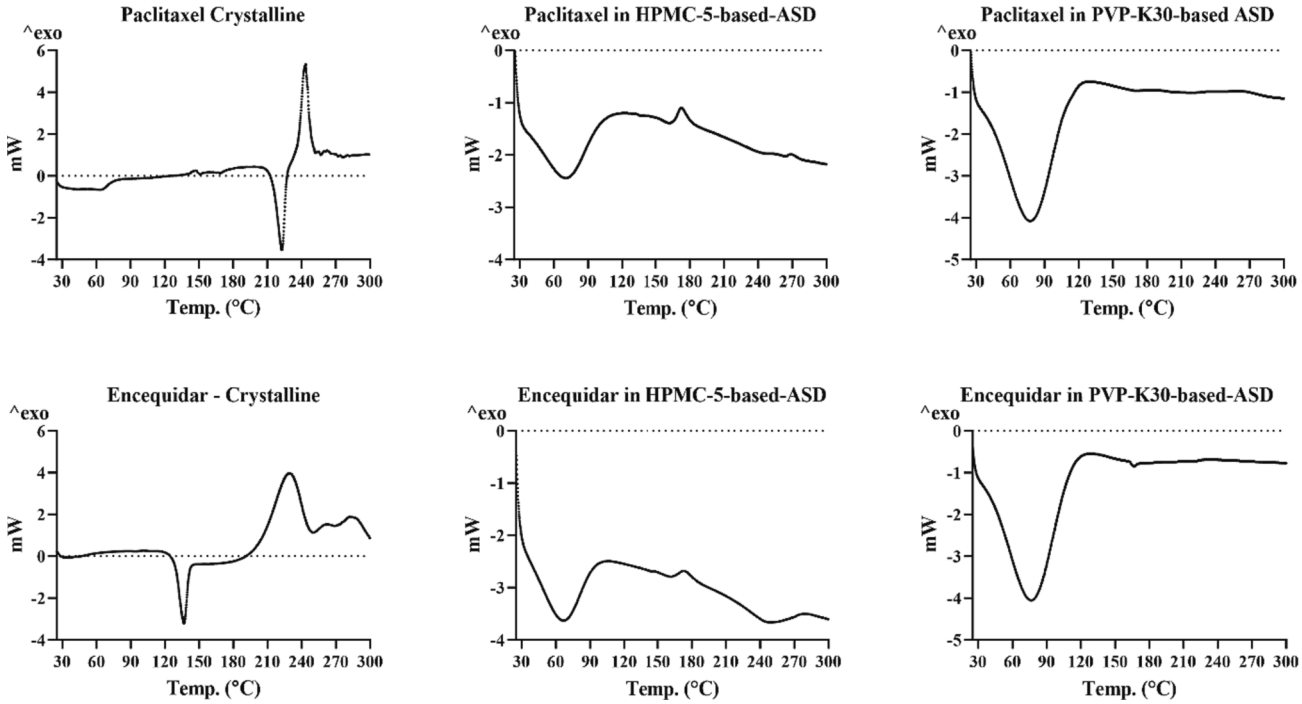

Fig. 1. The differential scanning calorimetric profiles of crystalline paclitaxel and encequidar, HPMC-5-based and PVP-K30-based ASDs of paclitaxel and encequidar. The total heat flow response in mW is shown on the y-axis, where exothermic events are up (over 0), and the endothermic events are down (under 0). The increasing temperature in Celsius is given on the x-axis.

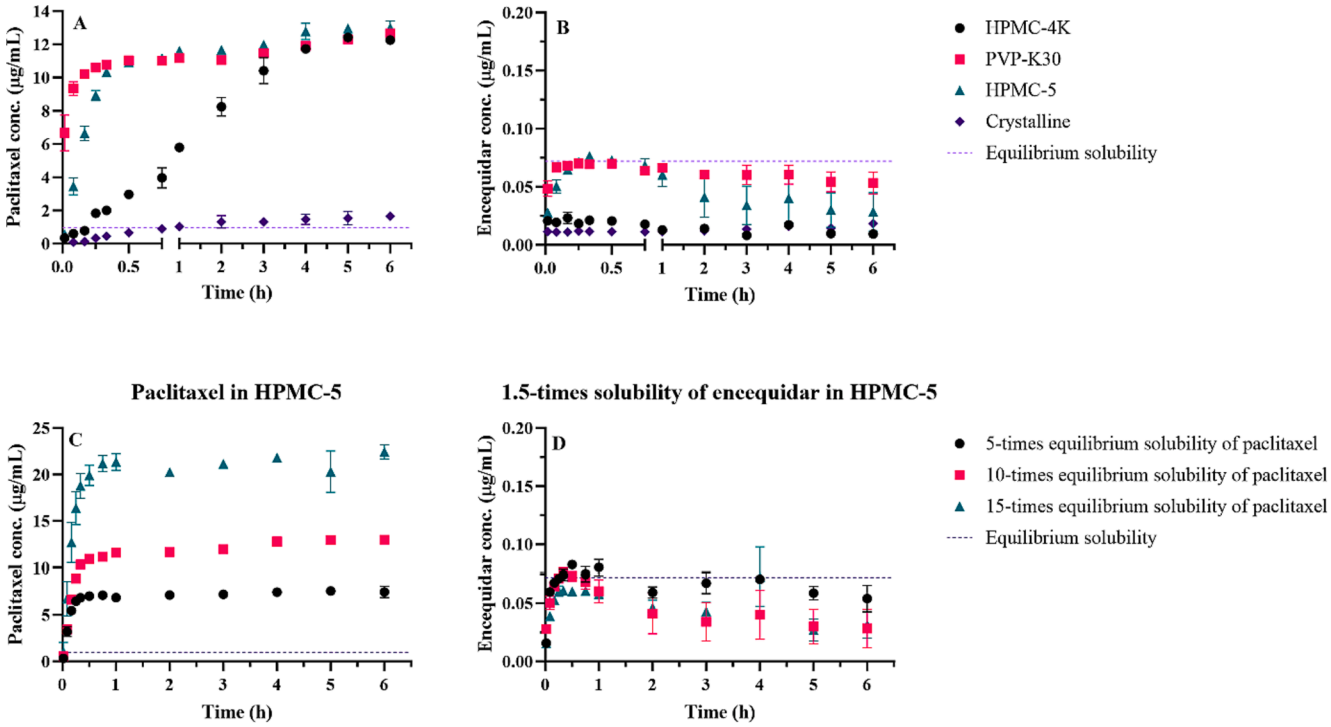

Fig. 2. Dissolution profiles of PVP-K30, HPMC-5, and HPMC-4K-based amorphous solid dispersions (ASDs) with paclitaxel and encequidar, and crystalline paclitaxel and encequidar in 80 mL FaSSIF-V2 pH 6.50 at 37 °C. (A) Dissolution of paclitaxel in the three ASDs all containing 12.00 ± 0.25 µg/mL paclitaxel, 10-times the equilibrium solubility, and dissolution of 25.38 ± 11.00 µg/mL crystalline paclitaxel, all in the same vessel with the respective encequidar formulation or crystalline form (B). (B) Dissolution of encequidar in the three ASDs all containing 0.18 ± 0.01 µg/mL encequidar, 1.5-times the equilibrium solubility, and dissolution of 6.38 ± 5.25 µg/mL crystalline encequidar, all in the same vessel with the respective paclitaxel formulation or crystalline form (A). (C) Dissolution of paclitaxel in HPMC-5-based ASDs at 5-times equilibrium solubility, 6.25 ± 0.13 µg/mL, 10-times the equilibrium solubility, 12.00 ± 0.25 µg/mL, and 15-times the equilibrium solubility, 18.38 ± 0.13 µg/mL. (D) Dissolution of encequidar in HPMC-5-based ASDs at 1.5-times equilibrium solubility, 0.18 ± 0.03 µg/mL. The amount (µg/mL) of paclitaxel and encequidar, on the left y-axis, plotted as a function of the sampling time point in hours (h). Data points are reported as mean values from three replicates (mean ± SEM, n = 3). SEMs smaller than the symbol size are not shown.

study were cloudy and fine powders, respectively. The colours of the ASDs of paclitaxel and encequidar mesylate salt were white and yellow, respectively. All ASDs and amorphous controls for both the dissolution studies and *in vivo* study were stored at −18 °C and protected from light.

3.3. Apparent solubility enhancement of paclitaxel and encequidar from separate ASDs

The dissolution rate and apparent solubility enhancement of paclitaxel and encequidar from separate ASDs combined in one dissolution vessel were investigated and compared with physical mixtures of pure crystalline paclitaxel and encequidar. The dissolution from crystalline paclitaxel indicated that the equilibrium solubility in FaSSIF-V2, pH 6.50, was reached within 1 h and increased slightly above the equilibrium solubility during the 6-hour dissolution study (Fig. 2.**A**). Moreover, the dissolution of crystalline paclitaxel resulted in a concentration similar to the equilibrium solubility after 2 h having a mean concentration of approximately 1.70 μg/mL. In contrast, the dissolution of crystalline encequidar did not reach the equilibrium solubility. Throughout the dissolution study, the concentration of encequidar was measured to be between 0.011 and 0.018 μg/mL (Fig. 2.**B**).

All three polymers facilitated enhancement of the apparent solubility of paclitaxel to approximately 12 μg/mL, which is consistent with 10-times the equilibrium solubility of paclitaxel. The apparent solubility enhancement of paclitaxel was reached with different dissolution profiles showing that the dissolution was fastest from PVP-K30, then HPMC-5, and a much slower dissolution was observed from HPMC-4K (Fig. 2.**A**). The same order of dissolution rate could be observed for encequidar (Fig. 2.**B**). The PVP-K30-based ASD with encequidar reached a plateau after approximately 10 min with a mean concentration of 0.070 μg/mL. In contrast, the HPMC-5-based ASD reached a plateau after approximately 20 min with a mean concentration of 0.076 μg/mL. The HPMC-4K-based ASD reached a mean concentration of 0.021 μg/mL after approximately 20 min. Furthermore, the dissolution profiles indicated that the dissolution of encequidar decreased faster and reached a lower mean concentration at the end of the experiment when formulated as an ASD with HPMC-5, than when formulated with PVP-K30. The encequidar mean concentrations reached in the formulated ASDs were all over 1.5-times the reached mean concentration of crystalline encequidar (Fig. 2.**B**), moreover the ASDs formulated with PVP-K30 and HPMC-5 reached a mean concentration consistent with the equilibrium solubility of encequidar, however, the results were not consistent with 1.5-times the equilibrium solubility of encequidar.

The apparent solubility enhancement of paclitaxel was studied with an HPMC-5-based ASD using doses corresponding to 5-times, 10-times, and 15-times the equilibrium solubility of paclitaxel in FaSSIF–V2, pH 6.50. All three scenarios facilitated apparent solubility enhancement of paclitaxel, which in all investigated cases was reached after 30 min, with concentration maxima of approximately 7 μg/mL, 12 μg/mL, and 22 μg/mL, respectively (Fig. 2.**C**). The encequidar dose was kept constant at 1.5-times the equilibrium solubility. The dissolution of encequidar from an HPMC-5-based ASD indicated that the highest concentration was reached within 20 min, having a mean concentration of 0.07 μg/mL reaching the equilibrium solubility, and then the concentration tended to decrease (Fig. 2.**D**). The mean concentration reached was under 1.5-times the equilibrium solubility of encequidar, however, over 1.5-times the reached mean concentration of crystalline encequidar in Fig. 2.**B**.

3.4. Dissolution of paclitaxel and encequidar from separated ASDs formulated with different polymers

When combining paclitaxel in the HPMC-5-based ASD with encequidar in the PVP-K30-based ASD (Fig. 3.**A**), the enhancement of the apparent solubility of paclitaxel plateaued after 30 min and reached a mean saturated concentration of approximately 11.14 μg/mL, which was consistent with the dissolution time and concentration when paclitaxel and encequidar were both released from the HPMC-5–based ASDs (Fig. 2.**A**). The maximal concentration of encequidar was reached after approximately 5 min with a mean concentration of 0.070 μg/mL, having a 5 min faster dissolution than when both paclitaxel and encequidar were released from a PVP-K30-based ASD, however, the maximum concentration released was consistent with what was seen before (Fig. 2.**B**). The encequidar concentration decreased slowly after reaching the observed maximal concentration, being consistent with what was seen in Fig. 2.**B** of a faster decrease in encequidar concentration when HPMC-5 was present.

In contrast, when combining paclitaxel in the PVP-K30-based ASD with encequidar in the HPMC-5-based ASD (Fig. 3.**B**), it was seen that the apparent solubility enhancement of paclitaxel did not reach a plateau since the concentration of paclitaxel kept increasing. In the same dissolution profile, encequidar reached a maximal concentration of 0.073 μg/mL after approximately 10 min, having an approximately 5

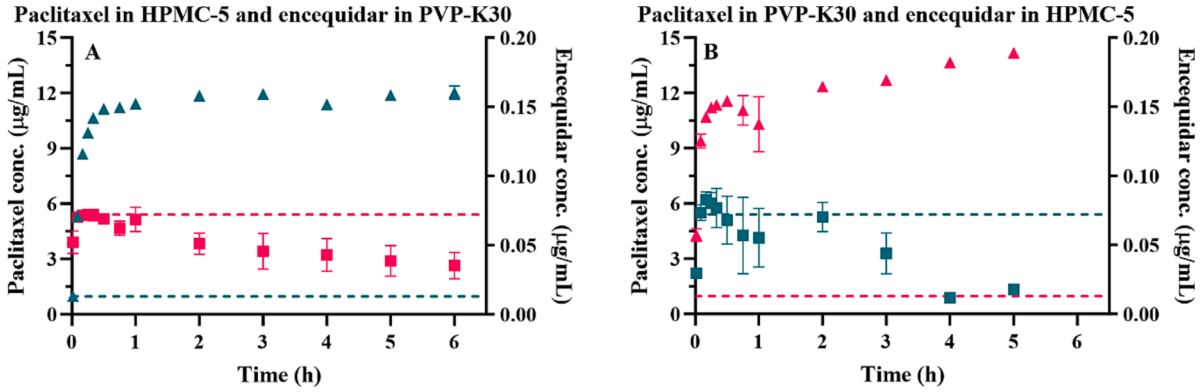

Fig. 3. Dissolution profile of paclitaxel (10-times solubility) in either a PVP-K30- or HPMC-5-based amorphous solid dispersion (ASD) with encequidar (1.5-times solubility) in either a PVP-K30- or HPMC-5-based ASD, in FaSSIF-V2 pH 6.50 at 37 °C. (A) Dissolution from an HPMC-5-based ASD (green) containing paclitaxel (triangles), and a PVP-K30-based ASD (pink) containing encequidar (squares). (B) Dissolution from a PVP-K30-based ASD (pink) containing paclitaxel (triangles), and an HPMC-5-based ASD (green) containing encequidar (squares). An experimental error occurred so there is missing a data point for the last sampling time in (B). The dotted lines indicate the equilibrium solubility of paclitaxel, (A) green and (B) pink, and encequidar (A) pink and (B) green, in FaSSIF-V2, pH 6.50 at 37 °C. Data points are reported as mean values from three replicates (mean ± SEM, n = 3). SEMs smaller than the symbol size are not shown.

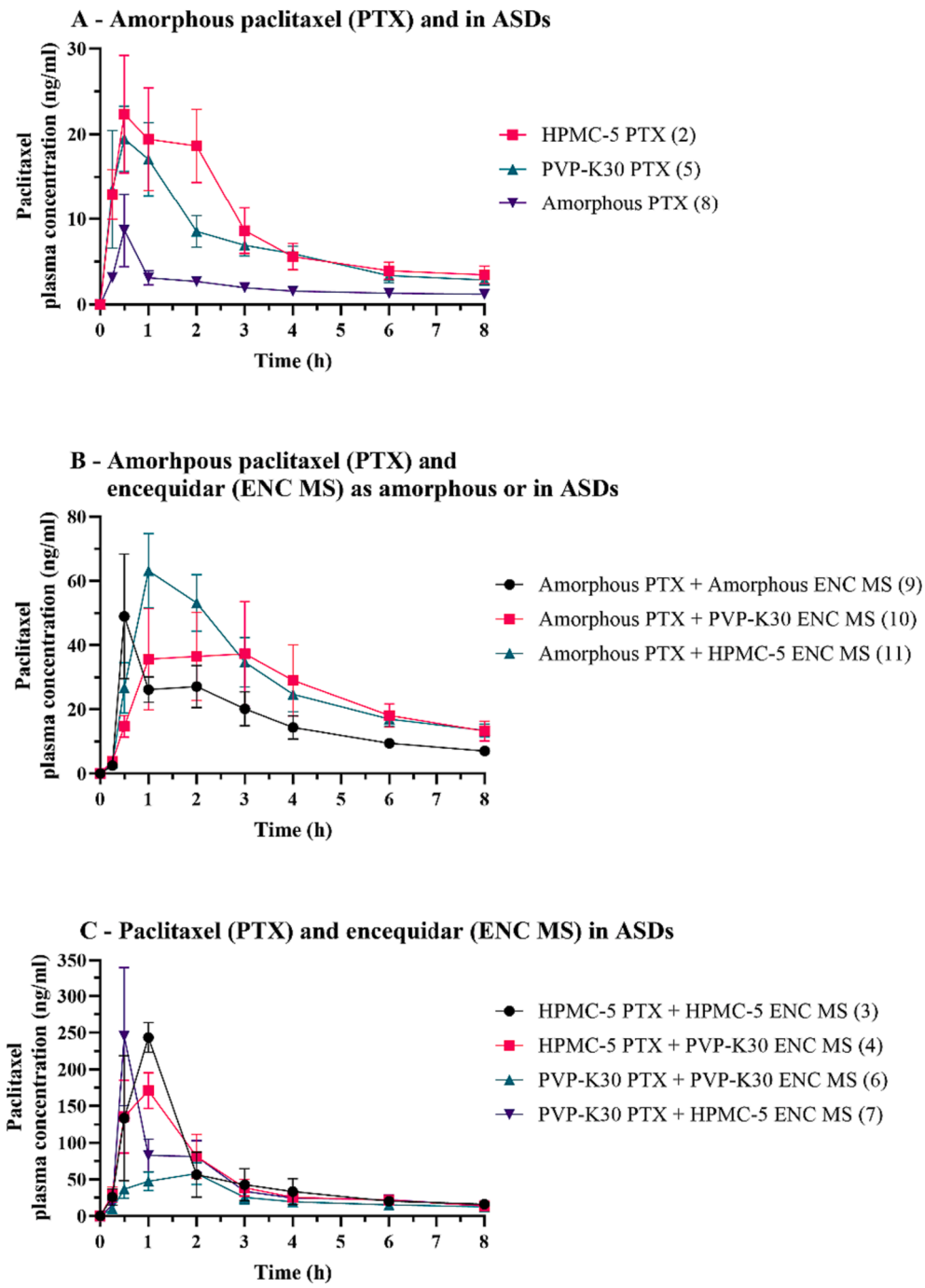

Fig. 4. Pharmacokinetic profiles of paclitaxel (PTX) after oral administration to fasted male Sprague Dawley rats. (A) Pharmacokinetic profiles after oral administration of 10 mg/kg paclitaxel administered as a crystalline drug, amorphous drug, or as amorphous solid dispersions (ASDs) in PVP-K30 or HPMC-5. (B) Pharmacokinetic profiles after oral administration of 10 mg/kg paclitaxel co-administered with 1 mg/kg encequidar mesylate salt administered as amorphous drugs, or encequidar mesylate salt administered as an ASD in PVP-K30 or HPMC-5. (C) Pharmacokinetic profiles after oral administration of 10 mg/kg paclitaxel co-administered with 1 mg/kg encequidar mesylate salt administered as ASDs in PVP-K30 or HPMC-5. Straight connecting lines for illustrative purposes. Data are shown as mean ± SEM, n = 4 -6. SEMs smaller than symbol size are not shown.

min faster dissolution than seen before when released from HPMC-5 (Fig. 2.**B**). Furthermore, it was observed that the encequidar concentration in the dissolution vessel decreased rapidly after 3 h of the dissolution, which was consistent with previous findings of encequidar concentration decreasing faster when HPMC-5 was present in the dissolution vessel (Fig. 2.**B**). However, the released encequidar concentration decreased faster when paclitaxel was formulated with PVP-K30 and encequidar with HPMC-5 (Fig. 3.**B**) than when paclitaxel was formulated with HPMC-5 and encequidar with PVP-K30 (Fig. 3.**A**).

3.5. Co-administration of paclitaxel and encequidar as polymer-based ASDs increase oral absorption of paclitaxel in vivo

The oral absorption of paclitaxel after oral administration of crystalline paclitaxel could not be quantified as the paclitaxel concentration in plasma was below the detection limit. In contrast, administration of amorphous paclitaxel resulted in a measurable bioavailability (Table 3). Due to the low solubility of paclitaxel, intravenous administration was omitted to avoid using solubility-enhancing excipients, which may interfere with P-gp. Oral administration of paclitaxel as PVP-K30- or HPMC-5-based ASDs further increased the oral absorption of paclitaxel as evidenced by the increased AUC and hence relative bioavailability (Fig. 4.**A,** Table 3). The C_{max} increased when paclitaxel was formulated as an ASD, where the highest C_{max} was seen for the HPMC-5-based ASD of paclitaxel (Fig. 4.**A** and Table 3). In addition, the AUC for the three formulations tended to be higher in the HPMC-5-based ASD, than the amorphous paclitaxel and the PVP-K30-based ASD indicating higher oral absorption. The relative bioavailability was calculated relative to the amorphous paclitaxel (Table 3). For formulations containing only paclitaxel (Fig. 4.**A**), the HPMC-5 and PVP-K30-based ASD increased the relative bioavailability of 3- and 4-fold, receptively (Table 3), with the HPMC-5 ASD having a slower absorption based on the longer t_{max} value (Table 3), hereby also a higher relative bioavailability, which was consistent with the dissolution profiles in Fig. 2.**A**.

Amorphous paclitaxel co-administered with amorphous encequidar mesylate salt (encequidar MS) increased the relative bioavailability 7-fold, relative to the relative bioavailability of amorphous paclitaxel (Table 3, Fig. 4.**A** and **.B**). In contrast, when encequidar MS was formulated as PVP-K30- or HPMC-5-based ASDs the relative bioavailability increased 12- and 14-fold, respectively (Table 3). Amorphous paclitaxel co-administered with encequidar MS formulated as a PVP-K30-based ASD decreased the C_{max} relative to amorphous paclitaxel co-administered with amorphous encequidar MS (Table 3, Fig. 4.**B**), while the opposite was observed for the HPMC-5-based ASD. For the formulations shown in Fig. 4.**A** and **4.B** similar t_{max} values were reached. Co-administration of amorphous paclitaxel with encequidar MS increased the AUC approximately 9-fold, whereas the co-administration of encequidar MS as PVP-K30- or HPMC-5-based ASDs showed an increase of approximately 13- and 15-fold, respectively (Table 3).

From Fig. 4.**C** it is evident that the pharmacokinetic profile of paclitaxel depended on the polymer chosen for the ASD. When paclitaxel and encequidar MS were both formulated in HPMC-5 the highest AUC was observed with a t_{max} around an hour. When paclitaxel was in a PVP-K30-based ASD and encequidar MS in an HPMC-5-based ASD the C_{max} was similar but occurred at an earlier t_{max}, with a slightly lower AUC. C_{max} was then lowered when paclitaxel was in an HPMC-5-based ASD and encequidar MS in a PVP-K30-based ASD, with a similar t_{max} and lower AUC. The lowest AUC, $C_{max,}$ and longest t_{max} values were observed when both paclitaxel and encequidar MS were formulated as ASDs in PVP-K30. This suggested that the presence of HPMC-5 in the intestine, regardless of which ASD it was administered as had an increasing effect on the absorption of paclitaxel under P–gp-inhibited conditions, i.e., when paclitaxel was co-administered with encequidar MS. In contrast, if the ASDs were solely made using PVP-K30 a lower increase in bioavailability was observed, yet still better than the amorphous paclitaxel and encequidar MS alone.

Table 3
Pharmacokinetic parameters of paclitaxel (PTX) in the absence or presence of encequidar mesylate salt (ENC MS), as crystalline paclitaxel, amorphous drugs, or as ASDs in PVP-K30 or HPMC-5. ND = non detected.

Group nr.:	1	8	2	5	3	4	6	7	9	10	11
Formulation:	Crystalline PTX	Amorphous PTX	PTX in HPMC-5	PTX in PVP-K30	PTX in HPMC-5	PTX in HPMC-5	PTX in PVP-K30	PTX in PVP-K30	Amorphous PTX	Amorphous PTX	Amorphous PTX
					ENC MS in HPMC-5	ENC MS in PVP-K30	ENC MS in PVP-K30	ENC MS in HPMC-5	Amorphous ENC MS	ENC MS in PVP-K30	ENC MS in HPMC-5
$AUC_{0\text{-}8h}$ (ng/mL·h)	ND	14.7 ± 3.10	72.7 ± 14.3	57.2 ± 10.3	416 ± 110	391 ± 72.4	207 ± 56.4	373 ± 76.9	134 ± 20.3	200 ± 60.9	230 ± 38.1
$AUC_{0\text{-}\infty}$ (ng/mL·h)	ND	21.3 ± 2.13	87.1 ± 17.9	70.2 ± 13.3	514 ± 130	465 ± 82.3	337 ± 70.5	459 ± 86.3	163 ± 22.6	260 ± 69.7	308 ± 41.5
Relative Bioavailability (%)	ND	100	408	329	2410	2180	1580	2150	762	1220	1440
C_{max} (ng/mL)	ND	5.45 ± 1.31	27.8 ± 3.11	23.3 ± 6.04	243 ± 20.1	193 ± 26.6	61.9 ± 13.4	256 ± 90.0	60.0 ± 14.9	51.9 ± 19.5	46.0 ± 13.6
t_{max} (min)	ND	30[19;53]	60[45;120]	30[26;38]	60[60;60]	60[38;105]	120[105;120]	45[30;120]	60[30;150]	60[60;135]	60[60;90]
k_{el}	ND	0.24 ± 0.07	0.23 ± 0.01	0.24 ± 0.02	0.16 ± 0.02	0.19 ± 0.02	0.12 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	0.15 ± 0.03	0.17±0.02
$t_{½}$ (h)	ND	2.75 ± 0.65	3.00 ± 0.12	2.95 ± 0.23	4.91 ± 0.80	3.79 ± 0.39	6.63 ± 1.00	4.031 ± 0.32	4.17 ± 0.72	5.81 ± 1.15	4.17 ± 0.49

Pharmacokinetic parameters of paclitaxel (PTX) after oral administration of 10 mg/kg paclitaxel to fasted male Sprague Dawley rats (group nr. 1, 8, 2, and 5). Group nr. 3, 4, 6, 7, 9, 10, and 11 also received 1 mg/kg encequidar mesylate salt (ENC MS). Paclitaxel and encequidar mesylate salt were administered as suspensions and were suspended in 5 mL/kg FaSSIF-V2, pH 6.50, prior to administration. For groups receiving both paclitaxel and encequidar, formulations were given as physical mixtures of two ASDs before being suspended. Area under the curve from 0 to 8 h ($AUC_{0\text{-}8h}$) and area under the curve from 0 to infinity ($AUC_{0\text{-}\infty}$), maximal plasma concentration (C_{max}), the elimination rate constant (k_{el}), and plasma half-life ($t_{½}$) are given as mean ± SEM, n = 4–6, t_{max} is given as median [Q1; Q3], and the relative bioavailability relative to amorphous paclitaxel is given in percent, as single values, n = 4–6.

4. Discussion

At present, products for oral administration of paclitaxel are not available due to low bioavailability, and hence products for IV administration are used (Taxol®, Genetaxyl). Administration by IV also presents challenges due to substances like Cremophor® EL or polysorbate 80, serving as a surfactant, along with macrogols or ethanol as solubility enhancers, and PEG-300 as a water-miscible vehicle, are required in IV formulations due to paclitaxel's limited solubility. The IV formulation strategy can result in adverse effects, such as neutropenia and anaphylactic hypersensitivity (Dorr, 1994; Gelderblom et al., 2001; Jeong and Choi, 2007; Liu et al., 1997; Malingré et al., 2001; Rowinsky et al., 1993; Ta-Chung et al., 2005). An alternative formulation on the marked is Abraxane®, also known as *nab*-paclitaxel, which is a formulation without co-solvents or solubility-enhancing excipients, employing 130-nanometer albumin-bound (*nab*™) technology (Gradishar, 2006). The approach of this technology utilizes the inherent characteristics of albumin to bind paclitaxel reversibly, facilitating paclitaxel's transport across endothelial cells and increasing concentrations in tumour areas (Gradishar, 2006). However, it would be more convenient if an oral formulation without surfactants or co-solvents could be developed resulting in sufficient oral bioavailability of paclitaxel. Therefore, we have here investigated if ASDs formulated with paclitaxel and the P-gp inhibitor encequidar, using PVP-K30, HPMC-5, or HPMC-4K polymers could provide a sufficiently high oral bioavailability through timely release of paclitaxel and encequidar in the small intestine, thus providing increased solubility and at the same time inhibition of P-gp mediated efflux.

The formulated ASDs containing paclitaxel and encequidar were shown to be amorphous and physically stable from their production and after the *in vitro* and *in vivo* studies were completed. It turned out to be an advantage to prepare solutions for ASD production with the mesylate salt of encequidar instead of the free base due to the higher solubility of the mesylate salt in the organic solvents used. Higher concentrations of encequidar in the solutions for freeze-drying enabled higher drug-polymer ratios, resulting in less total amounts of ASD required for the *in vivo* administration. The dissolution of paclitaxel from all prepared ASDs showed apparent solubility enhancement after approximately 30 min, which lasted throughout the experiment, thereby confirming the hypothesis of polymer-based ASDs being able to enhance the apparent solubility of paclitaxel during the dissolution. Similar observations were also made by Nielsen et al. (2023) with etoposide and zosuquidar using comparable formulations with the same polymers. The dissolution rate of paclitaxel was faster than the dissolution rate of etoposide from the same polymer-based ASD reported by Nielsen et al. (2023). A likely explanation for this could be a difference in the experimental setup, where Nielsen et al. (2023) formulated the ASDs as wafers in B24 wells, which resulted in a more dense polymer matrix, that could withhold the release of etoposide for a longer period (Nielsen et al., 2023). The ranked order of paclitaxel dissolution rate, corresponded with the previous finding by Nielsen et al. (2023), herewith confirming that by preparing ASDs in the different polymers the release rate of paclitaxel can be controlled. The dissolution of encequidar from the prepared ASDs with PVP-K30 and HPMC-5 reached the measured equilibrium solubility, however, the crystalline encequidar and the encequidar ASD prepared with HPMC-4K did not reach the measured equilibrium solubility within the timeframe of the experiments. After these maxima, dissolution profiles from ASDs indicated a continuous decline in encequidar concentrations. The ASDs prepared with HPMC-5- and PVP-K30 appeared to reach similar levels of maximum concentrations, however, the HPMC-4K reached approximately 3.5-fold lower maxima concentration than the other ASDs. It was therefore concluded that HPMC-4K was not a suitable polymer choice for these formulations. Similar to what was reported for zosuquidar, enhancement of the apparent solubility of encequidar could not be reached using ASDs of PVP-K30, HPMC-5, or HPMC-4K, suggesting that future research should attempt strategies to increase the solubility of such compounds. The dissolution rates of encequidar and paclitaxel from the different polymers followed the same order as the molecular weight of the polymers, with PVP-K30 having the lowest molecular weight and HPMC-4K the highest, supporting the hypothesis of controlling the release of encequidar and paclitaxel when formulated as ASDs. Higher molecular weight polymers in ASDs create a more viscous diffusion layer, leading to delayed drug release in aqueous environments, indicating a polymer-controlled release (Vaka et al., 2014). The dissolution of paclitaxel indicated that HPMC-5 was better at maintaining a steady paclitaxel apparent solubility enhancement, surpassing the performance of both PVP-K30 and HPMC-4K. In the terminology of the spring-parachute analogy, HPMC-5 demonstrated superior parachute capabilities and PVP-K30 demonstrated better spring capabilities, possibly due to a lower molecular weight and being a more water-soluble polymer (Guzmán et al., 2007; He and Ho, 2015; Xie and Taylor, 2016). However, if PVP-based ASDs show less effective inhibition of crystallization and a more rapid dissolution rate, the possibility of recrystallization of the amorphous or dissolved drug could occur during release (Augustijns and Brewster, 2012; Knopp et al., 2016). This could be an explanation for the lower levels of paclitaxel apparent solubility enhancement observed from the PVP-K30-based ASD compared to HPMC-5-based ASD. The dissolution profiles of paclitaxel and encequidar were not affected by co-formulation with an ASD prepared with a different polymer, as seen in the dissolution studies on materials made from mixing PVP-K30- and HPMC-5-based ASDs. This showed that the ASDs could be mixed without affecting the overall dissolution rate, enabling the possibility of investigating the effect on bioavailability when adjusting the release rate of both paclitaxel and encequidar independently.

The paclitaxel absorption after oral administration of crystalline paclitaxel in Sprague-Dawley rats could not be estimated since the concentrations in plasma samples were lower than the LLOQ (1 ng/mL). Previously, studies have documented a low absolute oral bioavailability of paclitaxel in male Sprague-Dawley rats. The reported bioavailability values include 1.7 % when dosed with 25 mg/kg (Choi and Jo, 2004), 1.68 % dosed with 20 mg/kg (Yang et al., 2015), 3.4 % dosed with 20 mg/kg (Kwak et al., 2010), or 12 % (Zamek-Gliszczynski et al., 2012). In the latter reference, paclitaxel was dosed orally at 5 mg/kg or IV at 1 mg/kg, both in a vehicle of 50 % Cremophor® EL in ethanol. Due to the low solubility of paclitaxel and the fact that Cremophor® EL is a P-gp inhibitor (Shono et al., 2004), we chose not to investigate the IV pharmacokinetic profile of paclitaxel, hence we have here reported the relative bioavailability relative to paclitaxel administered as an amorphous powder. Nevertheless, considering the IV data reported by Zamek-Gliszczynski et al. (2012), an absolute bioavailability of approximately 1 % is roughly estimated for the oral amorphous paclitaxel dosing, consistent with the very low paclitaxel plasma concentrations measured. Interestingly, when paclitaxel was administered as PVP-K30– or HPMC-5-based ASDs the relative bioavailability increased. These results may be due to the combination of an effect of the polymers on reaching and maintaining high concentrations of paclitaxel, as well as

on the transit of paclitaxel in the intestine.

Co-administrating encequidar with paclitaxel both formulated as ASDs, led to a significant increase in paclitaxel bioavailability. This supports the hypothesis of enhancing paclitaxel bioavailability by optimizing the apparent solubility enhancement of paclitaxel and controlling the simultaneous release of both paclitaxel and encequidar from the ASDs. These results support a prior study that identified limited paclitaxel bioavailability due to the involvement of P–gp in the intestine (Sparreboom et al., 1997). Interestingly, when paclitaxel and encequidar were formulated in ASDs, the presence of HPMC-5 was the key to the increased absorption observed, since the combinations containing HPMC-5 (dosing group 3, 4, and 7) had similar relative bioavailability, whereas dosing group 6, where ASDs of paclitaxel and encequidar were based on PVP-K30, showed lower bioavailability, similar to dosing groups 10 and 11, where paclitaxel was not in an ASD. Even though the relative bioavailability is similar for dosing groups 3, 4, and 7, the formulation having paclitaxel in PVP-K30 had a shorter t_{max}, yet the presence of HPMC-5 in the encequidar formulation could act as a parachute for paclitaxel. In dosing group 6, only containing PVP-K30, PVP-K30 likely dissolves fast acting as a spring but with a poor ability to act as a parachute. This was consistent with PVP-K30 being a better spring and HPMC-5 a better parachute in the spring-parachute terminology (Guzmán et al., 2007; He and Ho, 2015; Xie and Taylor, 2016). Following this logic, dosing group 11 should have had a relative bioavailability as dosing group 7, as both groups were administered ASDs containing HPMC-5, however, this was not observed. A possible explanation could be that the wetting and dissolution step was better facilitated from an ASD based on hydrophilic polymers than from an amorphous powder. That the paclitaxel bioavailability was similar after dosing with PVP-K30 and HPMC-5 combinations corresponded to what was previously observed for etoposide in the study by Nielsen et al. (2023). However, for paclitaxel, it could be observed that the paclitaxel bioavailability was highest when HPMC-5 was present in either the paclitaxel or encequidar ASD. When considering the IV data reported by Zamek-Gliszczynski et al. (2012), it is likely possible that the absolute bioavailability of paclitaxel could be around 30 % when administered with encequidar, both formulated as HPMC-5-based ASDs (Zamek-Gliszczynski et al., 2012).

There are examples of slightly related formulation strategies to increase oral paclitaxel bioavailability in the literature. Tablets containing solid dispersion granules (SDG-T) of amorphous paclitaxel, PVP-K30, sodium lauryl sulphate, and polysorbate 80, where the tablets were dosed orally to beagle dogs with the co-administration of HM30181 (encequidar) (Shanmugam et al., 2015). Shanmugam et al. (2015) reported a 1.3-fold increase in the relative bioavailability of paclitaxel from SDG-T in comparison to Oraxol^{TM} solution co-administered with encequidar, at a dose of 60 mg paclitaxel (Shanmugam et al., 2015). Solid dispersions (SDs) have also been prepared of paclitaxel formulated with various copolymers, and polymeric micelles of paclitaxel formulated with Soluplus, an amphiphilic P-gp inhibiting polymer (Choi et al., 2019). Both the prepared SDs and polymeric micelles were mixed with D-α-tocopheryl polyethylene glycol 1000 succinate, a semi-solid P-gp inhibiting surfactant. Choi et al. (2019) dosed male Sprague-Dawley rats orally with paclitaxel formulated as an SD with PVP/VA S-630, an SD without co-polymer, and a polymeric micelle. They reported a relative bioavailability increase of 6.7-, 3.6-, and 3.7–fold, relative to pure paclitaxel, respectively (Choi et al., 2019). In the present research, the co-administration of paclitaxel and encequidar as HPMC-5-based ASDs increased the relative bioavailability of paclitaxel approximately 24- and 6-fold, relative to amorphous paclitaxel and paclitaxel as an HPMC-5-based ASD, respectively. It, therefore, seems that the formulation approach chosen in the present research showed a higher increase in paclitaxel bioavailability, roughly estimated to be around 30 %, and therefore is an improved formulation approach compared to what has been shown before for oral dosing of paclitaxel without co-solvents or surfactants.

5. Conclusion

The present research clearly demonstrated that formulating paclitaxel ASDs and co-dosing these with encequidar ASDs increased the oral absorption of paclitaxel significantly, thus supporting the hypothesis of the study. The findings confirm that polymers, such as PVP-K30, HPMC-5, or HPMC-4K act solubility enhancing on a P-gp substrate, paclitaxel, but not on the P-gp inhibitor, encequidar, thereby producing stable enhancement apparent solubilities of paclitaxel. This resulted in increased oral absorption after co-administration to rats. Especially the presence of HPMC-5 was essential for the increased absorption as similar increases in absorption were observed regardless of whether HMPC-5 was presented in the paclitaxel or encequidar ASD. This suggests that when comparing PVP-K30 and HPMC-5, the presence of HPMC-5 in the intestinal lumen was more important for overall AUC than the dissolution rate of paclitaxel and encequidar from the formulation. Our study offers a practical example of enhancing oral bioavailability for anticancer drugs, as shown by the increased absorption of paclitaxel with encequidar in ASDs. This approach may provide a foundation for future research aimed at improving the effectiveness and patient accessibility of oral chemotherapy treatments.

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CRediT authorship contribution statement

Emilie Fynbo Petersen: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bjarke Strøm Larsen:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Rasmus Blaaholm Nielsen:** Writing – review & editing, Formal analysis, Conceptualization. **Ils Pijpers:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Dries Versweyveld:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **René Holm:** . **Ingunn Tho:** . **Jan Snoeys:** Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carsten Uhd Nielsen:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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