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The effect of Pro NanoLipospheres (PNL) formulation containing natural absorption enhancers on the oral bioavailability of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in a rat model

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ABSTRACT

The lipophilic phytocannabinoids cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) show therapeutic efficacy in various medical conditions. Both molecules are poorly water soluble and subjected to extensive first pass metabolism in the gastrointestinal tract, leading to a limited oral bioavailability of approximately 9%. We have developed an advanced lipid based Self-Emulsifying Drug Delivery System termed Advanced Pro-NanoLiposphere (PNL) pre-concentrate. The PNL is composed of lipid and emulsifying excipients of GRAS status and are known to increase solubility and reduce Phase I metabolism of lipophilic active compounds. Advanced PNLs are PNLs with an incorporated natural absorption enhancers. These molecules are natural alkaloids and phenolic compounds which were reported to inhibit certain phase I and phase II metabolism processes. Here we use piperine, curcumin and resveratrol to formulate the Advanced-PNL formulations. Consequently, we have explored the utility of these Advanced-PNLs on CBD and THC oral bioavailability. Oral administration of CBD-piperine-PNL resulted in 6-fold increase in AUC compared to CBD solution, proving to be the most effective of the screened formulations. The same trend was found in pharmacokinetic experiments of THC-piperine-PNL which resulted in a 9.3-fold increase in AUC as compared to THC solution. Our Piperine-PNL can be used as a platform for synchronized delivery of piperine and CBD or THC to the enterocyte site. This co-localization provides an increase in CBD and THC bioavailability by its effect at the pre-enterocyte and the enterocyte levels of the absorption process. The extra augmentation in the absorption of CBD and THC by incorporating piperine into PNL is attributed to the inhibition of Phase I and phase II metabolism by piperine in addition to the Phase I metabolism and P-gp inhibition by PNL. These novel results pave the way to utilize piperine-PNL delivery system for other poorly soluble, highly metabolized compounds that currently cannot be administered orally.

1. Introduction

Although therapeutic rationale for cannabis has been firmly established, an optimal oral dosage form for the delivery of cannabinoids is yet to be developed. This is due an extremely low oral bioavailability of the main cannabinoids delta-9-Tetrahydrocannabinol (THC) and Cannabidiol (CBD), stemming from their lipophilic characteristics, poor solubility and significant first pass effect (Zgair et al., 2016; Eisenberg et al., 2014; Grotenhermen, 2003). These poorly water soluble and highly metabolized compounds fall into Class II category of drugs according to both Biopharmaceutical Classification System (BCS) designed by Amidon et al. and Biopharmaceutical Drug Disposition Classification System (BDDCS) outlined by Wu and Benet (Amidon et al., 1995; Wu and Benet, 2005). Taking into consideration that the oral rout of administration is the most convenient and safe for patients but meets with absorption obstacles, we believe there is a place for an oral formulation that will improve the oral bioavailability of cannabinoids in terms of blood concentration, side effects and inter patient variability.

A potential approach used to enhance the oral bioavailability of lipophilic or BCS Class II drugs is their incorporation into lipid based formulations, specifically self nano-emulsifying drug delivery systems (SNEDDS) such as Pro NanoLipospheres (PNLs) (Elgart et al., 2012; Elgart et al., 2013).

PNLs are isotropic homogenous mixtures of an active lipophilic compound in a combination of natural or synthetic lipids, surfactants and co-solvents. The anhydrous liquid mixtures are commonly termed “pre-concentrates”. Upon gentle agitation in an aqueous phase, such as the upper GI lumen content, these pre-concentrates spontaneously form drug encapsulated O/W nano-emulsions with a particle diameter of

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200 nm or less. The lipophilic drug becomes entrapped in the lipid core of the nano-particles (Benito-Gallo et al., 2016; Mu et al., 2013; Domb, 1995; Domb and Masters, 1998).

Previous studies conducted by our group have indicated that PNLs can enhance the oral bioavailability of poorly water soluble compounds by multiple mechanisms which encompass not only enhanced solubility of the incorporated drug but also reduced intra-enterocyte metabolism by CYP3A4 enzymes and reduced P-gp efflux activity (Elgart et al., 2013).

In addition, in the past two decades several studies have indicated that alkaloids, flavonoids and several polyphenolic compounds often used as food supplements have potential to inhibit major drug-metabolizing enzymes of the CYP P450 family, inhibit P-gp efflux pumps and reduce phase II-metabolism by inhibiting UDP-glucuronyl-transferases, crowning these compounds as “absorption enhancers” (Bhardwaj et al., 2002; Valentine et al., 2006; Detampel et al., 2016; Patil et al., 2011; Wu et al., 2011; Shoba et al., 1998; Lambert et al., 2004; Jin and Han, 2004; Alexander et al., 2014; Suresh and Srinivasan, 2016). However, the reported potential of natural absorption enhancers was mainly observed in in-vitro studies. In-vivo studies usually show a less dominant effect on oral bioavailability. This could be attributed to the extremely poor water solubility of the absorption enhancers, which prevents their presence at the absorption site at required concentrations (Kesarwani and Gupta, 2013).

We rationalized that the PNL can be used as a platform for the delivery of THC and CBD together with an absorption enhancer to the enterocytes formulating an advanced PNL formulation. Thus, increasing solubility of the molecules and protecting them from first pass metabolism at the intestine.

In order to test our hypothesis, we reviewed published literature and selected three natural occurring potential absorption enhancers: curcumin, resveratrol and piperine. These compounds were chosen taking into consideration their non-toxicity to humans and animals, formulation feasibility in terms of water/lipid solubility characteristics, efficacy at relatively low concentration and potential to overcome absorption barriers.

1.1. Curcumin as an absorption enhancer

Curcumin is a polyphenolic compound being widely used as a food color and spice (Sharma et al., 2001). It has shown the potential to competitively inhibit P-gp efflux and to reduce intestinal and hepatic phase I and phase II metabolism mainly in in-vitro studies (Zhang and Lim, 2008; Mach et al., 2010; Bamba et al., 2011; Volak et al., 2008).

1.2. Resveratrol as an absorption enhancer

Resveratrol is a natural polyphenol found in grape skin and in red wine (Aumont et al., 2001). It was found to inhibit various CYPs responsible for Phase I oxidative metabolism of several compounds. Several in-vitro and a few in-vivo studies have reported that resveratrol has the potential to inhibit the activity of CYP3A4 (Regev-Shoshani et al., 2004; Choi et al., 2009; Hong et al., 2008).

1.3. Piperine as an absorption enhancer

Piperine is an alkaloid which constitutes a major active component found in black paper. Different in-vitro and in-vivo experimental models have shown that piperine has the potential to reduce Phase I and Phase II metabolism, in the intestine and in the liver (Shoba et al., 1998; Lambert et al., 2004; Singh et al., 1986). Additionally, several studies have reported that piperine can inhibit major drug-metabolizing enzyme CYP3A4 and P-gp efflux pumps in-vivo and in-vitro (Bhardwaj et al., 2002; Jin and Han, 2004).

In order to achieve the ultimate PNL formulation we performed a screening of several components that serve as potential constitutes of the PNL. These formulations were evaluated for size and appearance. The optimized PNL formulation served as the basis for the development of the “Advanced PNL formulations”. These formulations were evaluated in-vitro and in-vivo. Tests encompassed size, zeta potential (ζ) and pharmacokinetic experiments using the freely moving rat model.

Of all the formulations screened, Piperine-PNL (P-PNL) proved to be a stable Advanced PNL formulation with the ability to increase oral bioavailability of the cannabinoids which are at the center of this paper.

2. Materials and methods

2.1. Chemicals

All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (Rehovot, Israel). THC and CBD were purchased from THC Pharm GmbH – The health concept, Germany. For the preparation of PNLs, tricaprine (CremerCOOR®; MCT C10-95) was purchased from CREMER Oleo Division, France, Hydrogenated castor oil (HCO 40) from BASF The Chemical Company, Germany. Tripalmitin and trimyristin were obtained from Dexcel® Pharma, Or-Akiva, Israel.

2.2. Preparation of PNL and Advanced-PNL

CBD-PNL and THC-PNL were prepared by pre-concentrate method. The final PNL composition was based on our preliminary formulation optimization studies and selected according to optimal solubilization capacity of the active ingredient and smallest particle size obtained upon dilution of the pre-concentrate in aqueous phase. Initially, an amphiphilic co-solvent and soy phospholipid (lecithin) at a ratio of a 4:1, respectively, were placed in a clean scintillation tube and heated to 40 °C till completely dissolved. Then, triglyceride, polyoxyl 40-hydroxy castor oil, Tween 20, and Span 80 were added at the ratio of 1:1:1:1; the mixture was gently stirred and heated to 40 °C until a homogenous solution was formed. Further, the active ingredient was added, forming the CBD-PNL pre-concentrate containing CBD 3% (w/w) or THC-PNL preconcentrate containing THC 3% (w/w). Pre-concentrates were gently stirred and heated to 40 °C until a homogenous solution was formed. Upon gentle agitation in aqueous phase, these pre-concentrates spontaneously formed drug encapsulated O/W nano-dispersion.

An absorption enhancer was incorporated into CBD-PNL pre-concentrate in order to form Advanced-PNL: CBD-Curcumin-PNL, CBD-Piperine-PNL and CBD-Resveratrol-PNL. Piperine was added to THC-PNL to form THC-Piperine-PNL. The amount of each absorption enhancer in each of the Advanced-PNLs was 2% (w/w). Blank PNL and Piperine-PNL were prepared by the same method, except that no active ingredient was added.

2.3. Characterization of PNL and of Advanced-PNL

2.3.1. Particle size and ζ potential determination

Particle size and ζ potential were determined using Zetasizer Nano ZS ZEN 3600 (Malvern Instruments Ltd., Malvern, UK). Prior to particle size and ζ potential determination 200 μL of the pre-concentrate were vortex-mixed in 1800 μL distilled water at 37 °C for 30s, forming a dilution in a ratio of 1:10 (v/v). The measurements were taken using Folded Capillary Cells (Malvern Instruments Ltd., Malvern, UK). Before the measurements were taken, the cells were flushed through with ethanol followed by de-ionized water to facilitate wetting and cleaning of the cell.

2.3.2. Drug load assessment

CBD-PNL and THC-PNL loading was assessed following 1:10 dilutions of the pre-concentrates prepared as described above. The obtained solution (400 μL) was placed into Nanosep® centrifugal devices (Pall Life Sciences, Ann Arbor, MI, US) with 30 K cut-off membranes and were centrifuged for 30 min. at 10,000 RPM at 25 °C. Additionally, CBD

and THC solutions (300 μg/mL) were placed inside similar centrifugal devices and centrifuged in the same manner to assess the non-specific adsorption of CBD and THC to the test-tubes and membrane surfaces. Duplicates were used for each concentration. The original solutions and the filtrates obtained were analyzed for CBD and THC concentrations using the analytical method described.

The non-specific adsorption (NSA) percent of CBD was calculated as:

$$100 - \frac{\text{CBD solution conc. after centrifugation}^{*}100}{\text{CBD solution conc. before centrifugation}}$$

CBD load percent was calculated as:

$$100 - \frac{\text{CBD solution conc. after centrifugation of CBD}-\text{PNL}^{*}100}{(\text{CBD solution conc. before centrifugation of CBD} - \text{PNL})^{*}\ (100\% - \text{NSA}\%)}$$

The NSA percent of THC and THC load percent were calculated using the same equation.

2.3.3. Transmission electron microscopy (TEM)

Size and morphology of the nano particles were analyzed by transmission electron microscopy (TEM) using a JEM-1400 *plus* (Jeol) microscope. The O/W nano emulsions were freshly prepared by vortex-mixing of the pre-concentrates in water pre-heated to 37 °C (1:10 v/v and 1:50 v/v) for 30 s. 5 μL of sample was placed on a formvar/carbon coated copper grids (200 mesh, EMS), mixed with 5 μL uranyl acetate (2%) for approximately 10 s. excesses of the sample and stain mix were gently absorbed using a wedge of filter paper. The grids were air-dried.

2.4. In-vivo studies

All surgical and experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of the Hebrew University Hadassah Medical School Jerusalem. Male Wistar rats (Harlan, Israel), 275–300 g in weight, were used for all surgical procedures. All animals were deprived of food but not water 12 h prior to the conducted experiments. Animals were anesthetized for the period of surgery by intra-peritoneal injection of 1 mL/kg of ketamine-xylazine solution (9:1, respectively). During the surgery, rats were placed on a heated surface and maintained at 37 °C (Harvard Apparatus Inc., Holliston, MA). An indwelling cannula was placed in the right jugular vein of each animal for systemic blood sampling, by a method described before (Hoffman and Levy, 1989). The cannula was tunneled beneath the skin and exteriorized at the dorsal part of the neck. After completion of the surgical procedure, the animals were transferred to individual cages to recover overnight (12–18 h). During this recovery period, food, but not water, was deprived. Throughout the experiments, free access to food was available 4 h post oral administration. Animals were randomly assigned to the different experimental groups.

2.4.1. CBD relative bioavailability studies

For bioavailability studies, CBD-PNL and CBD-Advanced-PNLs (containing curcumin, resveratrol or piperine) were freshly prepared 30 min before each experiment, by vortex-mixing of the pre-concentrates in water pre-heated to 37 °C (1:10 v/v) for 30 s forming O/W nano-dispersions. The obtained CBD concentration was 3 mg/mL CBD-PNL and the Advanced CBD-PNLs (CBD-Curcumin-PNL, CBD-Resveratrol-PNL and CBD-Piperine-PNL), were administered to the animals by oral gavage (n = 6). The administered dose of CBD was 15 mg/kg. In the CBD-PNL bioavailability studies the control group received CBD solution in a vehicle composed of propylene glycol:ethanol:water (4.5:4.5:1 v/v) at a concentration of 3 mg/mL (n = 6). In the CBD-Piperine-PNL bioavailability studies the control group received CBD + piperine solution in the same vehicle (propylene glycol:ethanol:water, 4.5:4.5:1 v/v) at a concentration of 3 mg/mL CBD and 2 mg/mL piperine. In the CBD-Piperine-PNL bioavailability studies the administered dose of CBD was 15 mg/kg and the administered dose of piperine was 10 mg/kg. Additionally, CBD was given intravenously in the solution described above (propylene glycol:ethanol:water, 4.5:4.5:1 v/v), prepared at a concentration of 2 mg/mL and in a dose of 200 μg/kg.

2.4.2. THC relative oral bioavailability studies

For bioavailability studies, dispersed THC-PNL and THC-Piperine-PNL were freshly prepared 30 min before each experiment, by vortex-mixing of the pre-concentrate in water (1:10 v/v) pre-heated to 37 °C for 30 s. The obtained THC concentration was 3 mg/mL. Dispersed THC-PNL and THC-Piperine-PNL were administered to the animals by oral gavage (n = 6). The administered dose of THC was 20 mg/kg. In the THC-PNL bioavailability studies the control group received 20 mg/kg THC dissolved in propylene glycol:ethanol (1:1 v/v) to obtain THC concentration of 3 mg/mL (n = 6). In the THC-Piperine-PNL bioavailability studies the control group received THC + piperine solution in a vehicle composed of propylene glycol:ethanol (1:1 v/v) at a concentration of 3 mg/mL THC and 2 mg/mL piperine. In the THC-Piperine-PNL bioavailability studies the administered dose of THC was 20 mg/kg and the administered dose of piperine was 12 mg/kg. Additionally, THC was given intravenously in the solution described above (propylene glycol:ethanol, 1:1 v/v), prepared at a concentration of 2 mg/mL and in a dose of 400 μg/kg.

For both substances, in IV studies, systemic blood samples (0.35 mL) were taken at 5 min pre-dose, 0.33, 0.66,1, 1.5, 2, 3, 4 and 6 h post dose. In PO studies, systemic blood samples (0.35 mL) were taken at 0, 0.33, 0.66, 1, 1.5, 2, 4 and 6 h post dose. To prevent dehydration, equal volumes of physiological solution were administrated following each withdrawal of blood sample. Plasma samples were separated by centrifugation (4*g*, 7 min, 4 °C) and stored at − 20 °C pending analysis.

2.5. Analytical methods

Plasma aliquots of 150 μL were spiked with 20 μL of internal standard cannabigerol (CBG; 1 μg/mL). ACN (200 μL) was added to each test tube (tubes A) and vortex-mixed for 2 min. The extraction of THC, CBD and CBG was performed by ethyl acetate (3 mL) that was added to each test-tube A, followed by 2 min vortex-mixing. After centrifugation at 4000 rpm for 10 min, the ethyl acetate organic layer was transferred to fresh glass test tubes (tubes B) and evaporated to dryness (Vacuum Evaporation System, Labconco, Kansas City, MO). Then, tubes B were reconstituted in 80 mL of 20/80 (v/v) water/acetonitrile. Injection volume was 10 μL. THC and CBD amount was determined using a high-performance liquid chromatography (HPLC) system (Waters 2695 Separation Module) with a mass-spectrometer (Waters Micro-mass ZQ, Waters Corporation, Milford, MA). The HPLC-MS conditions were as follows: XTerra MS C18 Column 3.5 μm 2.1 × 100 mm column (Waters®, Milford, MA), an isocratic mobile phase, of 20:80 (v/v) 2 mM ammonium acetate/acetonitrile, with flow rate of 0.2 mL/min at 35 °C. Retention time for THC and CBD were 4.6 and 2.5 min respectively. The detection masses (*m/z*) were 313.2 for THC and CBD and 315.2 for CBG.

2.6. PK analysis

Noncompartmental PK analysis was performed using WinNonlin® (version 5.2, Pharsight, Mountain View, CA.)

2.7. Statistical analysis

All values are expressed as mean ± standard error mean (SEM) if not stated otherwise. To determine statistically significant differences among the experimental groups, *t*-test or one-way ANOVA, followed by Tukey's test, was used. A p value < 0.05 was termed significant.

Table 1
Formulations screened in PNL development.

	% (w/w)	F1	F2	F3	F4	F5	F6	F7	F8
CBD/THC	3	X	X	X	X	X	X	X	X
Tween 20	12.5	X	X	X	X	X	X	X	X
Span 80	12.5	X	X	X	X	X	X	X	X
Lecithin	9	X	X	X	X	X	X	X	X
HCO 40	12.5	X	X	X	X	X	X	X	X
Ethyl lactate	36	X	X	X	X				
NMP	36					X	X	X	X
Tricaprin	12.5	X				X			
Trilaurin	12.5		X				X		
Trimyristin	12.5			X				X	
Tripalmitin	12.5				X				X
Visual inspection		Visually clear dispersion	Milky-white dispersion	Milky-white dispersion	Milky-white dispersion	Milky-white dispersion	Milky-white dispersion	Milky-white dispersion	Milky-white dispersion

Table 2
Particle size, zeta potential and polydispersity index (PDI) of various PNLs and Advanced-PNLs obtained by 1:10 v/v dilution in aqueous phase.
(Data presented as mean, n = 3.) *N/A - not available.

Formulation	Size (diameter, nm)	Zeta potential (mV)	PDI
THC-PNL	30	− 12	0.23
CBD-PNL	26	− 13	0.25
CBD-piperine-PNL	30	− 15	0.23
CBD-resveratrol-PNL	65	− 10	0.5
CBD-curcumin-PNL	N/A*	N/A*	N/A*

3. Results

3.1. In-vitro studies

3.1.1. Development of cannabinoid-PNLs and cannabinoid advanced PNLs

During the development process, a variety of materials and their combinations were tested. First, we tested the dissolution of CBD and THC in different organic solvents (*N*-methyl pyrolidine, DMSO, Propylene glycol, ethyl lactate and a combination of these solvents) each time with one or two of the following emulsifiers: tween 20, tween 80, SPAN 20, SPAN 65, SPAN 80, Polyoxyl 40 Hydrogenated Castor oil (HCO 40), and/or the following triglycerides: tricaprin, trilaurin trimyristin with the phospholipid lecithin. Formulations were screened and those that formed homogeneous, visually clear and stable pre-concentrates with no precipitation of both THC and CBD were selected.

8 final formulations are presented in Table 1 as an example of end-point development products. All formulations successfully dissolved the active molecules, however, upon suspension in water, only formulation attributed as F 1 was visually clear. The rest of the formulations (with a different triglyceride or co-solvent) formed milky white dispersions with particle size above 200 nm. Since formulations forming particles in size > 100 nm are too large to be candidates for oral drug delivery, these formulations were rejected. On the other hand, after introduction to the water phase, formulation 1 (with THC or CBD) formed particles of < 50 nm in size. These two formulations were stable; no precipitation of THC or CBD was evident. Therefore, these formulations were the basis for further developing Advanced-PNLs.

Incorporation of curcumin, piperine and resveratrol into the final PNL formulation formed pre-concentrates in which the absorption enhancer was fully dissolved. This led us to add the absorption enhancer to a CBD (3% w/w) containing PNL formulation, to form an Advanced-PNL pre-concentrate; Following introduction of pre-concentrates to the water phase no precipitation of both the active ingredient (CBD) and of the absorption enhancer was observed (10 min after dilution for curcumin and up to two months for piperine and resveratrol), enabling pre-clinical trials.

3.1.2. In-vitro characterization of cannabinoid-PNLs and cannabinoid advanced PNL

PNLs and Advanced-PNLs pre-concentrates were dispersed in water (1:10, v/v) prior to their administration to animals. Thus, PNLs and Advanced-PNLs were characterized following this dilution. For examining particle size and shape in the TEM, a more diluted nano dispersion (1:50 v/v) was also tested.

Resveratrol-CBD-PNL and piperine-CBD-PNL formed clear solutions which enabled size and zeta potential tests. Since Curcumin-CBD-PNL diluted in water had limited physical stability, the time frame was not practical for conducting characterization studies of this formulation. Nevertheless, this window of time was sufficient to perform pre-clinical studies. Mean particle diameter, zeta (ζ) potential and polydispersity index (PDI) of the developed PNLs and Advanced-PNLs are presented in Table 2. PNL formulations with piperine as an absorption enhancer resulted in a more homogeneous solution with a smaller particle size. This finding led us to incorporate the piperine into the THC-PNL formulation creating an advanced PNL for this cannabinoid as well.

CBD-PNL and THC-PNL were assessed for drug load. The dispersion of these pre-concentrates in aqueous phase resulted in high drug load, both for CBD and THC, of > 99%. These results indicate that the amount of free drug, i.e. CBD and THC not incorporated into these delivery systems was negligible.

Particles size and morphology were further confirmed via the TEM. Samples were tested in two dilutions, 1:10 v/v which is the dilution in all experiments conducted in this paper and 1:50 in order to achieve a more technically suitable sample for the microscope. Fig. 1 (A, B, C) represents the particles captured in the TEM. Although the size of the particles is larger than seen via Zetasizer, we could outline spherical particles. In 1:50 dilution of the pre-concentrate, nano particles are of 50–100 nm.

3.2. In-vivo studies

3.2.1. The effect of PNL and Advanced-PNLs on the PK profile of CBD

Plasma concentration time profiles for CBD and the different CBD-Advanced-PNL formulations following their oral administration to rats are depicted in Figs. 2–4. Oral administration of CBD-curcumin-PNL resulted in significantly lower AUC and Cmax values as compared to the oral administration of CBD-PNL to rats (Fig. 2). Moreover, oral administration of CBD-curcumin-PNL resulted in a similar oral bioavailability as compared to the administration of CBD alone (Fig. 2).

Incorporation of resveratrol to the PNL resulted in an increased AUC and Cmax of CBD compared to CBD alone. However, when compared to the CBD-PNL administration, AUC and Cmax were lower, proving no added value for the examined absorption enhancer (Fig. 3).

CBD-piperine-PNL oral administration resulted in significantly increased AUC and Cmax values as compared to the administration of CBD-PNL and CBD alone. The CBD-piperine-PNL formulation enabled

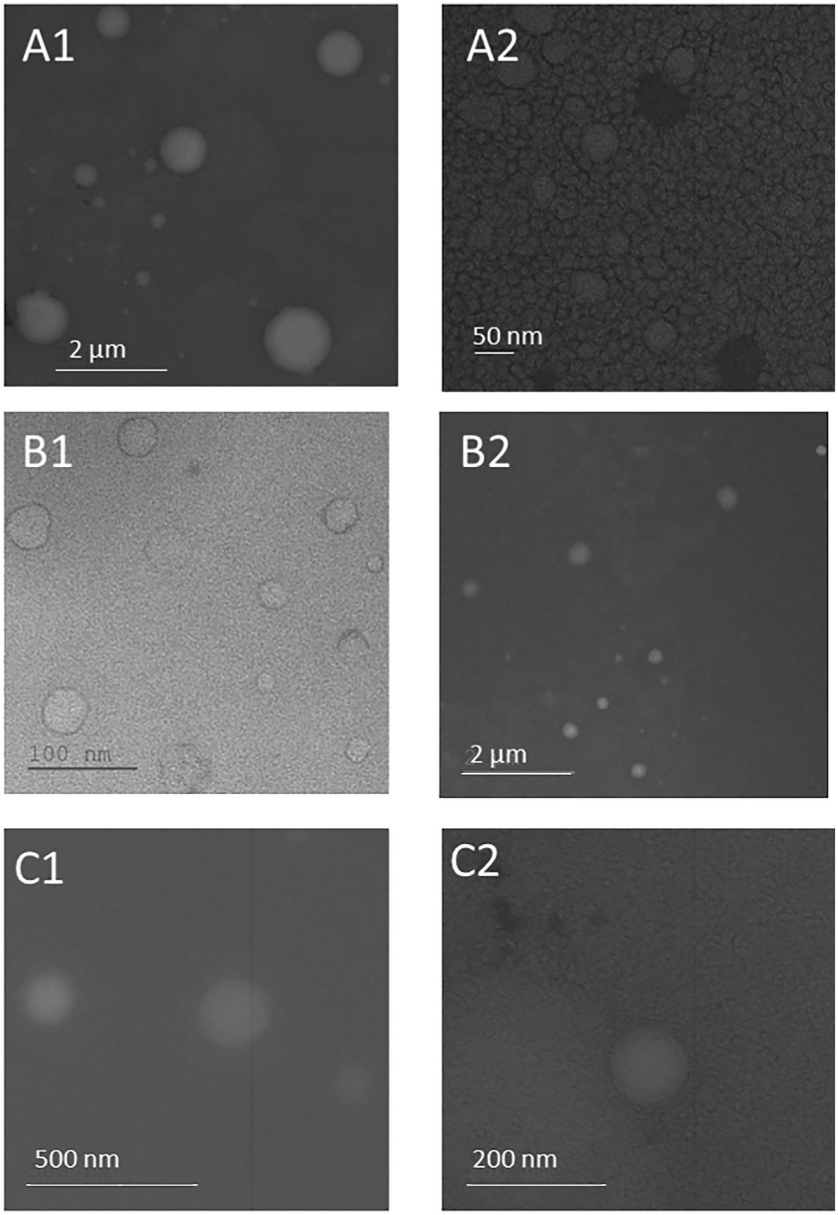

Fig. 1. TEM analysis of PNL dispersion: blank PNL A1 (1:10 v/v) and A2 (1:50 v/v), CBD-piperine-PNL B1 (1:10 v/v) and B2 (1:50 v/v), THC-piperine-PNL (1:10 v/v) and (1:50 v/v)

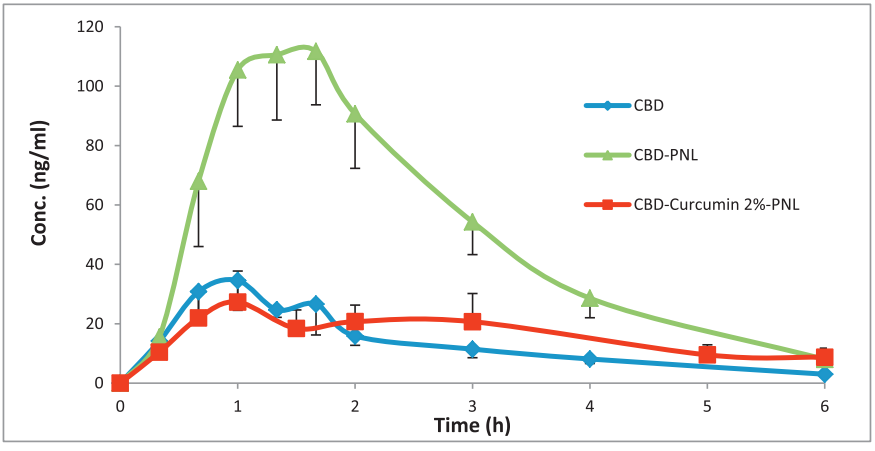

Fig. 2. Plasma CBD concentration vs. time plot (mean ± SEM) following PO administration of CBD, CBD-PNL and CBD-Curcumin-PNL. CBD dose 15 mg/kg. Curcumin dose of 10 mg/kg (n = 6 for each group)

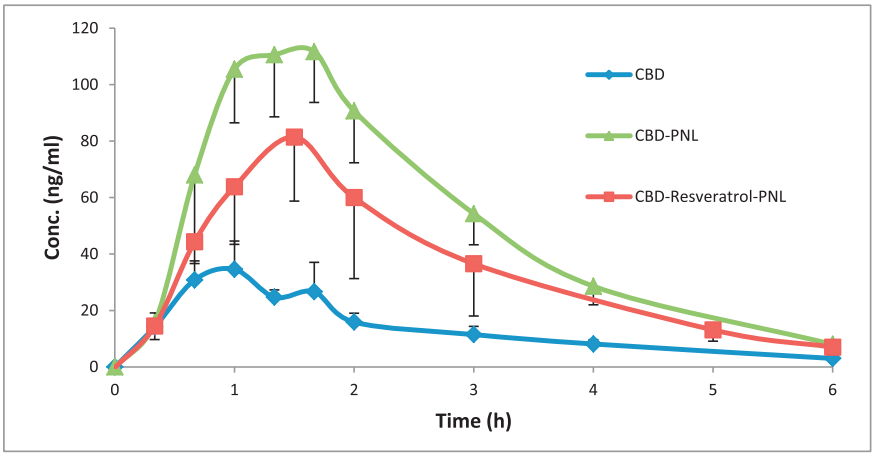

Fig. 3. Plasma CBD concentration vs. time plot (mean ± SEM) following PO administration of CBD, CBD-PNL and CBD-resveratrol-PNL. CBD dose 15 mg/kg. Resveratrol administered at a dose of 10 mg/kg (n = 6 for each group)

an additional 2-fold increase in bioavailability (Fig. 4, Table 3). These study results, which identified piperine as the leading absorption enhancer, encouraged us to investigate the effect of piperine on THC oral bioavailability.

3.2.2. The effect of PNL and Advanced-PNLs on the PK profile of THC

In order to investigate the effect of piperine on THC oral bioavailability, THC was incorporated with piperine into the PNL system, as developed for CBD, to form THC-Piperine-PNL. Thereafter, this THC-Advanced delivery system was characterized in-vitro using the same method as described for CBD-Advanced-PNLs. The incorporation of THC into piperine-PNL resulted in a clear and homogeneous pre-concentrate. Upon dilution of this pre-concentrate with water phase (1:10 v/v, respectively), a visually clear and stable nano-dispersion with particles of 40 nm in size was formed. Similarly to CBD-piperine-PNL, THC-Piperine-PNL exhibited a high drug load of > 99%. THC-piperine-PNL oral administration resulted in significantly increased AUC and Cmax values as compared to the administration of THC-PNL and THC alone, demonstrating an additional, nearly 1.5-fold increase, in the oral bioavailability of THC compared to the administration of THC-PNL (Fig. 5 and Table 4).

3.2.3. CBD and THC IV administration vs. CBD-PNL and THC-PNL PO administration

Plasma concentration time profiles represented in a semi-logarithmic plot (Fig. 6) indicate that the terminal elimination phase obtained following PO administration of CBD-PNL is the same as the one obtained following CBD IV administration. Similar results were evident in the case of THC (Fig. 7). These results indicate that the incorporation of THC and CBD into PNL does not affect the terminal elimination processes of these compounds.

4. Discussion

Intestinal absorption and bioavailability following oral drug administration is dictated by several factors such as drug solubility in the gastro-intestinal (GI) milieu, permeability of the drug through the enterocyte membrane, activity of efflux transporters and metabolizing enzymes (Porter et al., 2007).

Absolute oral bioavailability of THC was reported to be 6%–10%. The molecule's absorption is not only variable but is also influenced by the vehicle of administration (Ohlsson et al., 1982). Several factors may account for the low oral bioavailability of THC, including poor water solubility, variable absorption, degradation of the drug in the stomach, and significant first-pass metabolism by CYPs to an equipotent 11-OH-THC metabolite and further metabolism to inactive THC-COOH. This first-pass metabolism process is mediated mainly by CYP3A4 and CYP2C9 (Bornheim et al., 1992; Watanabe et al., 2007). Phase II metabolism forms THC-COO-glucuronide as the major metabolic end product that is eliminated in the urine (Pallante et al., 1978). It is important to note that the conjugation reaction involves THC metabolite only, whereas there is no evidence for direct Phase II metabolism of the parent compound. In addition, it was demonstrated that P-gp mediates direct THC excretion from the enterocytes into intestinal lumen thus limiting its oral uptake (Bonhomme-Faivre et al., 2008).

CBD has similar oral absorption and bioavailability to THC ranging

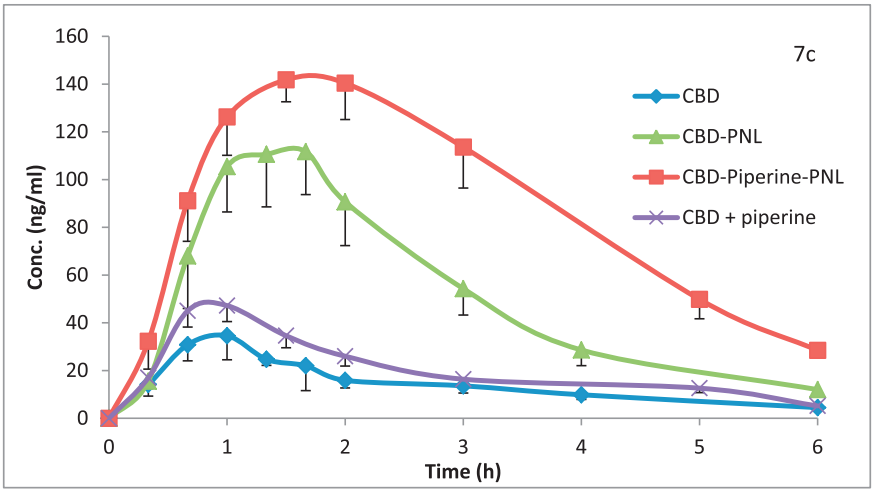

Fig. 4. Plasma CBD concentration vs. time plot (mean ± SEM) following PO administration of CBD, CBD-PNL, CBD-Piperine-PNL and CBD with piperine in solution. CBD dose 15 mg/kg piperine was administered at a dose of 10 mg/kg (n = 6 for each group).

Table 3
AUC and Cmax values (mean ± SEM) obtained following PO administration of CBD, dispersed CBD-PNL and dispersed CBD-Advanced-PNLs, i.e. CBD-Piperine-PNL, CBD-Curcumin-PNL and CBD-Resveratrol-PNL. CBD dose.

	CBD	CBD-PNL	CBD-Piperine-PNL	CBD-Curcumin-PNL	CBD-Resveratrol-PNL
Cmax (ng/mL)	39 ± 8	137 ± 43(*)	170 ± 13(*✗)	63 ± 15	96 ± 50
Tmax (h)	1.07 ± 0.12	1.11 ± 0.16	1.67 ± 0.19	1.56 ± 0.42	1.90 ± 0.33
AUC (h∗ng/mL)	90 ± 21	300 ± 95 (*)	570 ± 23 (*✗)	168 ± 41	202 ± 89
k_{el} (h^{-1})	0.39 ± 0.17	0.42 ± 0.15	0.39 ± 0.05	0.44 ± 0.09	0.33 ± 0.10

(*) A significant difference ($p < 0.05$) from CBD corresponding values was found.
(✗) A significant difference ($p < 0.05$) from CBD-PNL corresponding values was found.

between 9 and 13% in average (Ohlsson et al., 1986). The low oral bioavailability is attributed to poor water solubility and extended first-pass metabolism. It is reported that CBD undergoes monohydroxylation at C-7 forming the 7-OH metabolite, mediated by CYP3A4 and CYP2C19 (Jiang et al., 2011). Unlike THC or any other cannabinoid, another prominent metabolic route of CBD is direct glucuronidation of the parent compound, leading to the formation of an O-glucuronide (Harvey and Mechoulam, 1990). The oxidative Phase I metabolism in both molecules, P-gp efflux in the case of THC and direct phase II metabolism in the case of CBD, together with poor water solubility properties result in extremely low oral bioavailability.

The utilization of lipid based drug delivery systems such as PNL have shown potential to enhance the oral bioavailability of BCS/BDDCS Class 2 compounds. In this paper, we rely on this experience to achieve increased oral bioavailability of THC and CBD, which fall into this category of molecules. The developed lipid based drug delivery systems presented in this paper have the added value of an incorporated absorption enhancer. We define these formulations as Advanced-PNLs.

In the development of Advanced-PNLs, we have incorporated three absorption enhancers into an optimized PNL system leading to three Advanced-PNL formulations with the model drug being CBD: CBD-Curcumin-PNL, CBD-Resveratrol-PNL and CBD-Piperine-PNL. Throughout the development process, three key parameters needed to be met: PNLs had to fully dissolve the selected absorption enhancers, clear and transparent dispersions upon dissolution of the Advanced pre-concentrates with water had to be formed and finally the dispersed Advanced pre-concentrates had to be stable, i.e. with no evidence for the precipitation of the absorption enhancers or CBD.

Our characterization studies focused on clarity of the dispersed formulations since it proves that the drugs are completely in their solubilized state and that the incorporation of poorly water-soluble molecules in the PNL increases their apparent solubility. In addition, transparent formulations are an indication for the small particle size formed upon contact with water. Bekerman et al. demonstrated that there is an inverse correlation between the particle size of the studied SNEDDS and the oral bioavailability of the incorporated cyclosporine

Table 4
AUC and Cmax values (mean ± SEM) obtained following PO administration of THC, dispersed THC-PNL and dispersed THC-Piperine-PNL. THC dose 20 mg/kg, Piperine dose 10 mg/kg (n = 6 for each group).

	Cmax (ng/mL)	Tmax (h)	AUC (h∗ng/mL)	k_{el} (h^{-1})
THC	5.5 ± 1	1.93 ± 0.49	10 ± 2	0.40 ± 0.14
THC-PNL	27 ± 4(*)	1.90 ± 0.33	63 ± 11(*)	0.52 ± 0.06
THC-Piperine-PNL	32 ± 4(*)	0.67 ± 0.19	93 ± 10(*✗)	0.38 ± 0.08

(*) A significant difference ($p < 0.05$) from THC corresponding values was found.
(✗) A significant difference ($p < 0.05$) from THC-PNL corresponding values was found.

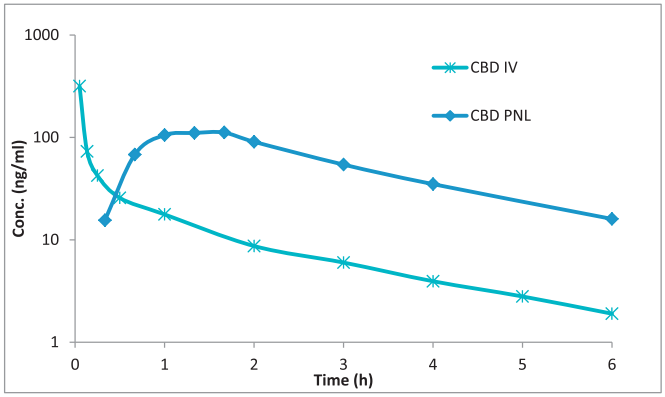

Fig. 6. Semi-logarithmic plot of plasma concentration vs. time profiles in rats for 200 µg/kg CBD following IV bolus administration and for 15 mg/kg CBD-PNL following oral administration

(Bekerman et al., 2004). The idea that might stand behind this correlation derives from the structure of the enterocyte lining the villus of the small intestine. The apical side of the enterocytes consists of protrusions called microvilli which form the brush border of the gut wall. The inter-villous space as reported by Brown et al. is between 50 and

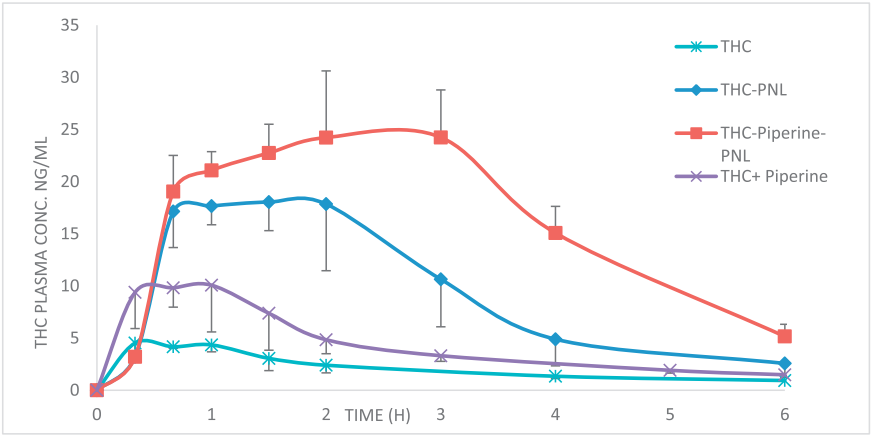

Fig. 5. Plasma THC concentration vs. time plot (mean ± SEM) following PO administration of THC, THC-PNL, THC-Piperine-PNL and THC with piperine in solution. THC dose 20 mg/kg, piperine dose 10 mg/kg (n = 6 for each group).

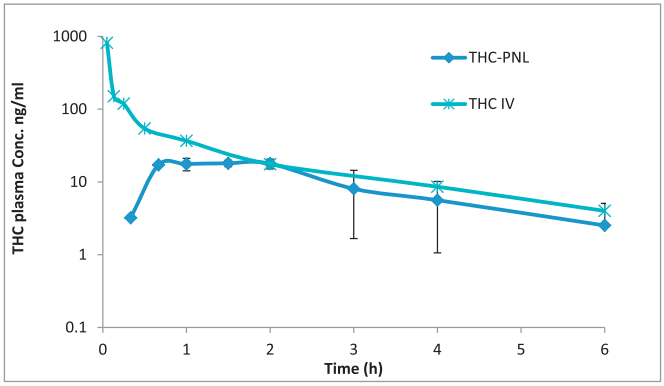

Fig. 7. Semi-logarithmic plot of plasma concentration vs. time profiles in rats for 400 μg/kg THC following IV bolus administration and for 20 mg/kg THC-PNL following oral administration.

250 nm (Brown, 1962). Our group hypothesizes that the nano particles formed by PNL pre-concentrates or Advanced PNLs manage to readily enter the inter-villus space, thus gaining accesses to additional surface area available for absorption as opposed to larger particles (e.g. liposomes or micron sized particles) (Elgart et al., 2013).

In addition to the parameter of size it is also paramount to refer to the homogeneity of the formulation. The uniformity is expressed via the polydispersity index (PDI).

CBD-Resveratrol-PNL and CBD-Piperine-PNL formed O/W nanoemulsions with particles of < 70 nm in size (Table 2). The nano emulsions formed were uniform as indicated by the low values of PDI (< 0.5, Table 2). CBD-piperine-PNL formulation had similar size and PDI values as the CBD-PNL and THC PNL formulations. Moreover, the obtained ζ potential for theses formulations was high enough to maintain stability throughout their passage in the GI tract (Table 2). The introduction of CBD-Curcumin-PNL into water phase resulted in precipitation of curcumin after a period of 10 min. Thus, the size of the particles formed could not be determined. However, it provided a sufficient time window to perform pre-clinical studies. The dilution of CBD-Curcumin-PNL with water was performed immediately before its administration to rats and prior to the precipitation of curcumin. Evaluation of size via TEM resulted in larger particles than seen via zetasizer. The zetasizer provides us with a distribution of size, and finally an average size of the particles in the dispersion. We believe that this difference between the devices is the reason for the discrepancy we see.

As indicated by our pre-clinical studies, single oral administration of CBD-Curcumin-PNL to rats resulted in significantly decreased AUC and Cmax values in comparison to CBD-PNL (Fig. 2 and Table 3). Indicating that the addition of curcumin into PNL resulted in significantly decreased oral bioavailability of the incorporated cannabinoid CBD compered to CBD-PNL. These results can be attributed to the fact that the CBD-Curcumin-PNL formulation was not stable enough following the introduction of CBD-Curcumin-PNL to water phase in-vitro. The same effect most probably occurred in-vivo. Additionally, our study results indicated that the precipitation of curcumin disturbs the formed particles in the nanoemulsion, which in turn promoted the precipitation of CBD as well. Moreover, these results point out that unfavorable in-vitro physico-chemical characteristics of PNLs have a direct impact on its in-vivo performance.

The incorporation of resveratrol and piperine formed homogeneous Advanced-PNL pre-concentrates forming clear and stable O/W nano emulsions. However, the administration of these absorption enhancers in Advanced-PNLs had different effect on the oral bioavailability of CBD. While the relative oral bioavailability of CBD-resveratrol-PNL was not significantly different as compared to CBD-PNL (Fig. 3, Table 3), single oral administration to rats of CBD-piperine-PNL resulted in approximately 2-fold increase in the AUC compared to CBD-PNL and a 6-fold increase in comparison to CBD solution (Fig. 4 and Table 3). Both resveratrol and piperine have shown potential to inhibit Phase I and/or Phase II metabolism mainly in in-vitro studies. However, only piperine enhanced the oral bioavailability of CBD in-vivo as indicated by our studies. Thus, we conclude that piperine is not a trivial absorption enhancer.

Interestingly, when administering piperine with CBD without its incorporation into the PNL delivery system, no effect on CBD's oral bioavailability is evident (Fig. 4). We assume that the poor water solubility of piperine prevents its presence at appropriate concentrations required to surpass the intestinal absorption barriers. On the other hand, the rather high lipophilic nature of piperine (log p 2.25) enables its incorporation into the PNL lipid core. This further emphasizes the great value in PNLs utilization. Furthermore, this result corroborates our hypothesis that the incorporation of an absorption enhancer into the PNL lipid core can provide an oral synchronous delivery system. Such simultaneous administration can deliver sufficient amounts of the absorption enhancer and the incorporated drug at the enterocyte luminal surface.

Because THC and CBD have somewhat different metabolic routs in terms of Phase II metabolism we were interested to investigate how the utilization of piperine-PNL would impact the oral bioavailability of THC. We have incorporated THC into Piperine-PNL to develop THC-Piperine-PNL. Fig. 5 and Table 4 indicate that single oral administration of THC-Piperine-PNL to rats resulted in a 1.47-fold increase in the AUC as compared to THC-PNL and a 9.3-fold increase in AUC as compared to THC solution. We believe that the addition of piperine into THC-PNL further enhances its oral bioavailability by promoting additional inhibition of Phase I metabolism and reduction of P-gp efflux. Oral co-administration of THC with piperine in a solution (without its incorporation into PNL) had no effect on THC's bioavailability. This observation corroborates the findings obtained in our similar studies using CBD.

Upon comparing the linear terminal slopes obtained following IV administration of CBD ($0.57 \pm 0.04\ h^{-1}$) and PO administration of CBD-PNL ($0.42 \pm 0.15\ h^{-1}$) as well as when comparing linear terminal slopes of THC in IV administration ($0.40 \pm 0.01\ h^{-1}$) and THC-PNL in the PO rout ($0.52 \pm 0.06\ h^{-1}$) we receive approximately parallel slopes for each compound (Figs. 6–7). When a compound is administered via the IV rout, the terminal slop obtained following the representation of drug plasma concentration vs. time on a semi-logarithmic plot reflects drug's elimination rate. Parallel linear terminal slopes for PO and IV plots lead to the understanding that the slopes represent drugs' elimination rate (i.e. they are controlled by drug clearance and extent of distribution) and a flip-flop phenomenon where the terminal half-life of the orally administered drug represents the absorption rather than elimination rate, can be ruled out. It is important to note that in the IV studies THC and CBD were administered as free compounds without the presence of PNL, enabling isolation of the PNL effect on the PK parameters of these cannabinoids following their oral administration. Thus, this indicates, that the main difference in the PK profile of THC and CBD upon their incorporation into PNLs is associated with increased absorption phase since the elimination is not affected. Furthermore, the parallel slopes obtained from this comparison, lead to the understanding that the effect of PNL can be attributed to decreased intestinal rather than hepatic metabolism. To our knowledge, the contribution of the intestine to the first-pass metabolism of the cannabinoids which is the focus of this article, has not been evaluated yet (Huestis, 2005). While for many years, the liver has been regarded as the main metabolic organ responsible for the low bioavailability attributed to a myriad of drugs, these results place the intestine at the center stage of drug metabolism and thus, proper pharmaceutical formulations can affect this preceding metabolic stage.

5. Conclusions

Results of our research indicate that contrary to the prevailing view, intestinal rather than hepatic Phase I and Phase II metabolism processes have a prominent contribution to the first-pass effect of poorly water soluble and highly metabolized compounds. Study results indicate that effective inhibition of intestinal metabolism by the utilization of absorption enhancers is achieved only if it reaches the enterocyte surface in its solubilized state. The low intrinsic solubility of natural compounds with absorption enhancing properties prevents their presence at the appropriate concentrations to facilitate inhibition of the metabolism processes in the enterocyte. Thus, our research has shown that PNLs enable the simultaneous delivery of the poorly water soluble absorption enhancer and the poorly water soluble drug such as THC and CBD, to the enterocyte surface in their solubilized state. This emphasizes the added value of PNLs as they exert two actions in a synchronized manner: inhibition of first-pass intestinal metabolism and delivery of the poorly water soluble absorption enhancer and the incorporated drug to the enterocyte surface in its solubilized state. These two actions result in additional increase in the oral bioavailability of the incorporated compound into Advanced-PNLs as compared to PNLs. Our results reveal that piperine is the most potent absorption enhancer as compared to curcumin and resveratrol administered at the same dose. The enhanced oral bioavailability of poorly water soluble and highly metabolized drugs incorporated into PNLs and Advanced-PNLs is accomplished by the modification of an absorption phase by these delivery systems. As the incorporation of such drugs into PNLs and Advanced-PNLs does not alter their elimination phase, the enhanced oral bioavailability can be attributed to the inhibition of intestinal rather than hepatic first-pass metabolism processes. Furthermore, additional increase in the AUC of CBD proves that piperine-PNL has effect not only on Phase I but also Phase II metabolism. In order to confirm this mechanism of action, we have to select a model molecule that exclusively undergoes Phase II, thus isolating (and highlighting) the effect of the formulation on this metabolic pathway. PNLs and Advanced-PNLs are effective biopharmaceutical approaches that can be utilized to avoid intestinal first-pass effect i.e. P-gp efflux, Phase I and Phase II intestinal metabolism of poorly water soluble and highly metabolized compounds. The pharmaceutical methodology for enhancing oral bioavailability, in contrary to chemical modification approach, presents considerable advantages. Because PNLs are composed of components of GRAS status approval to conduct clinical studies can be granted more easily. Overall, the advantages of Advanced-PNLs as vehicles for delivery of cannabinoids demonstrate the potential these formulations have in developing the status of cannabis as legalized medication with appropriate standardization that will meet patients' needs.

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